ACTA
UNIVERSITATIS PALACKIANAE
OLOMUCENSIS

FACULTAS RERUM NATURALIUM
CHEMICA 43S
SUPPLEMENT
2004

ACTA
UNIVERSITATIS PALACKIANAE
OLOMUCENSIS

FACULTAS RERUM NATURALIUM
2004

CHEMICA 43S

SUPPLEMENT
COLLECTED REPORTS
OF THE NATURAL SCIENCE FACULTY,
PALACKÝ UNIVERSITY OLOMOUC,
CZECH REPUBLIC

PALACKÝ UNIVERSITY OLOMOUC
OLOMOUC 2004

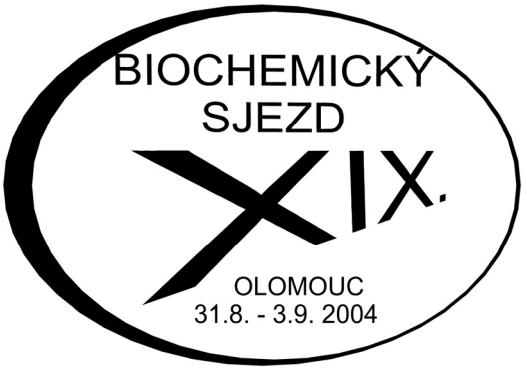
This volume has been accepted to be printed on July 15, 2004.

The authors are responsible for the content and for the translation of the papers.

ISBN 80-244-0882-1

ISSN 0232-0061

ACTA UNIVERSITATIS PALACKIANAE OLOMUCENSIS
FACULTAS RERUM NATURALIUM 2004 CHEMICA 43S



PROCEEDINGS OF THE XIX. MEETING OF CZECH AND SLOVAK SOCIETIES FOR BIOCHEMISTRY AND MOLECULAR BIOLOGY

Olomouc, August 31 – September 3, 2004

Edited by:

Ivo Frébort
Department of Biochemistry, Faculty of Science
Palacký University in Olomouc

Daniela Walterová
Institute of Medical Chemistry and Biochemistry, Faculty of Medicine
Palacký University in Olomouc

ACTA UNIVERSITATIS PALACKIANAE OLOMUCENSIS
FACULTAS RERUM NATURALIUM 2004 CHEMICA 43S

PROCEEDINGS OF THE XIX. MEETING OF CZECH AND SLOVAK SOCIETIES FOR BIOCHEMISTRY AND MOLECULAR BIOLOGY

Olomouc, August 31 – September 3, 2004

Contents page

ACTA UNIVERSITATIS PALACKIANAE OLOMUCENSIS
FACULTAS RERUM NATURALIUM 2004 CHEMICA 43S

PROCEEDINGS OF THE XIX. MEETING OF CZECH AND SLOVAK SOCIETIES FOR BIOCHEMISTRY AND MOLECULAR BIOLOGY

Olomouc, August 31 – September 3, 2004

PLENARY LECTURES

PL01

BIOGENESIS OF MITOCHONDRIA: PATHWAYS AND MACHINERIES INVOLVED IN THE IMPORT OF PROTEINS

Walter Neupert*

Institute of Physiological Chemistry, University of Munich, Butenandtstr. 5, Bldg. B, D-81377 Munich, Germany

** E-mail: neupert@bio.med.uni-muenchen.de*

The vast majority of mitochondrial proteins are encoded by nuclear genes and synthesized by ribosomes in the cytosol. They are imported into the mitochondria and sorted to the various mitochondrial subcompartments. So far, four protein translocases were identified which mediate these reactions: The TOM complex of the outer membrane is the entry gate for virtually all mitochondrial proteins. The TIM23 complex facilitates the translocation of proteins across the inner membrane into the matrix and in the insertion of the majority of inner membrane proteins. The TIM22 complex mediates insertion of certain subclasses of polytopic inner membrane proteins. The OXA1 complex facilitates the insertion of inner membrane proteins from the matrix side; mitochondrially encoded proteins are substrates for this translocase (or "insertase") as well as nuclear encoded proteins which are first imported across both mitochondrial membranes. In addition, we recently identified a protein complex in the outer membrane, the TOB complex, which is essential for the biogenesis of beta-barrel proteins.

Two of the molecular machines will be discussed in some detail, TIM23 and the newly discovered TOB complex.

The TIM23 preprotein translocase consists of a protein conducting channel in the inner membrane and of a protein import motor. This motor is associated with the import channel at the matrix face. It drives the vectorial movement of unfolded polypeptide chains and is powered by ATP hydrolysis. Three components of this motor have been identified during the past years, mitochondrial Hsp70, Tim44 which links mtHsp70 to the channel and Mge1, a nucleotide exchange factor. We have recently identified two new components, Tim14 and Tim16, which are integral parts of the motor. Tim14 represents a J-domain protein and stimulates the ATPase activity of mtHsp70. Tim16 is also a J-domain related protein but lacks the classical HPD motif. The interaction of these new components with Tim44 and mtHsp70 has been analyzed. The results provide novel insights into the function of the import motor.

The TOB complex of the outer membrane of mitochondria functions in the insertion of beta-barrel proteins into this membrane. The TOB complex interacts with precursors of e.g. porin and Tom40 after they have been recognized by the TOM complex. The TOB complex functions in the insertion of the precursors, most likely from the intermembrane space side. The main and essential constituent of the TOB complex is Tob55, itself a beta-barrel membrane protein. Tob55 forms an oligomeric assembly which has pore-like structure. In electrophysiological measurements Tob55 shows discrete high conductance upon insertion into lipid bilayers. In yeast two further proteins are present in the TOB complex, Mas37 and Tob38. Both proteins are required for the function of the TOB complex, in particular Tob38. In the absence of Tob38 mitochondria are greatly defective in the import of beta-barrel membrane

proteins. Tob55 is related in amino acid sequence to the Omp85 protein of Gram-negative bacteria which was proposed to have a function in the insertion of β-barrel proteins into the outer membrane. We propose that the biogenesis of beta-barrel membrane proteins follows a pathway conserved during evolution of mitochondria from α-proteobacterial ancestors.

PL02

PROTEOMICS: CLINICAL PERSPECTIVES

Jean-Daniel Tissot* and Philippe Schneider

Service régional vaudois de transfusion sanguine, Rue du Bugnon 27, 1005 Lausanne, Switzerland

** E-mail: Jean-Daniel.Tissot@chiv.hospvd.ch*

The main progresses in proteomics have been made possible through the developments of mass spectrometry (MS). Thanks to bioinformatics, a number of proteomic databases containing plasma as well as normal and/or pathological cell proteins are now available on the Internet (http://www.expasy.org). In our laboratory, we have used a combination of high-resolution two-dimensional gel electrophoresis (2-DE), image analysis and MS to study different biological samples.

We have characterized various cryoproteins from plasma/serum samples, and have identified a new protein associated with IgM, CD5L (Spα). This protein is a member of the scavenger receptor cysteine-rich superfamily of proteins. This finding may have implications in the understanding of the homeostasis of IgM antibodies.

Using purified populations of human lymphocytes we have identified a novel protein that we named swiprosin 1, which appears to be preferentially associated with CD8+ lymphocytes. Swiprosin 1 contains two EF-hand domains, and therefore may have a role in calcium signalling.

We have found that the treatment of plasma with methylene blue and light (an approach used in transfusion medicine for virus inactivation) induced biochemical modifications of a restricted number of plasmatic proteins such as fibrinogen, apolipoprotein A-I or transthyretin. Removal of methylene blue by filtration after irradiation was not responsible for additional protein alterations.

Human cutaneous cell cultures *in vitro* have been developed for the treatment of dermatological lesions and burns. Fetal skin cells open new horizons for therapeutic usage because no frank rejection occurs, and grafts on large surfaces are possible due to the rapid expansion properties of these cells. Using liquid chromatography-tandem MS analyses, we have observed post-translational modifications of various proteins such as triosephosphate isomerase or cyclophilin A occurring after cell culture. These were due to modifications of cysteine residues to cysteic acid, probably reflecting either an oxidative stress related to cell culture, or, alternatively, maturation, differentiation and aging of the cells.

Finally, in order to try to identify new potential markers of premature rupture of the membranes (PROM), responsible for many pre-term deliveries, we have done proteomic studies on samples collected from women at terms (pairs of maternal serum and amniotic fluid) as well as on samples of amniotic fluid collected at the 17th week of gestation. Two protein fragments, agrin and perlecan, were identified as being new potential biological markers for PROM diagnosis.

Taken together, our experience of combining 2-DE, image analysis, and MS, clearly shows that proteomics can be used successfully in laboratories oriented to clinical investigations.

PL03 – Josef V. Koštíř Award Lecture

PRIMARY CULTURES OF HUMAN HEPATOCYTES AS A MODEL FOR THE STUDY OF BIOLOGICAL ACTIVITY OF NATURAL COMPOUNDS

Zdeněk Dvořák*

Institute of Medical Chemistry and Biochemistry, Faculty of Medicine, Palacký University, Hněvotínská 3, 77515 Olomouc, Czech Republic

** E-mail: moulin@email.cz*

The biological effects of natural substances are the object of multidisciplinary interest from time immemorial. Following the boom of synthesis of new compounds, drugs in particular, the attention is drawn again to substances of natural origin. These substances are nowadays used in both prevention and therapy for variety of diseases. The knowledge on biotransformation of a particular compound is an indispensable pre-requisite for the approval of the drug in human therapy, with regard to drug absorption, organ distribution, metabolism and elimination (ADME) but also with regard to adverse effects of the drug such as interaction with biomacromolecules, intrinsic toxicity etc. Primary cultures of human hepatocytes are considered to be one of the most suitable models for the studies mentioned above.

This presentation demonstrates the use of primary cultures of human hepatocytes (PCHH) as a model for the study of biological activity of natural substances. As the first group of studied compounds, we selected flavonolignans from silymarin, a fraction from *Silybum marianum* seeds. The cytotoxicity of studied flavonolignans together with their cytoprotectivity against the cellular damage by model toxins were evaluated using the PCHH model. All compounds tested exhibited dose-dependent cytoprotectivity against oxidative damage to the cells by carbon tetrachloride and allyl alcohol, while they were ineffective against drug-induced damage by paracetamol and metabolic damage by D-galactosamine. The feasible mechanism of the protection observed could be free radicals quenching by tested flavonolignans. Furthermore, the effects of tested flavonolignans on specific activities of cytochrome P450 were studied in isolated human liver microsomes, a subcellular fraction, which consists of endoplasmatic reticulum fragments where P450 enzymes are anchored. The inhibition of cytochrome P450 isoforms CYP2D6, 2E1 and 3A4 by silybine, silychristine and silydianine was observed, where IC_{50} values were in micromolar range. In the second group of natural substances, tropolone alkaloids from *Colchicum autumnale*, the effect on drug metabolizing enzymes was investigated. The effect of colchicine and its derivatives on the expression, regulation and activity of cytochrome P450 was tested in the PCHH model. Tropolone alkaloids showing no antimitotic activity (colchicein) did not affect CYP1A2, 2C9, 2C19, 2E1, and 3A4 enzymes expression and activity in the PCHH model. On the contrary, potent antimitotic agent colchicine strongly inhibited both basal and inducible expression of CYP2B6, 2C8/9 and 3A4 enzymes at the level of protein and mRNA contents. Additionally, it was demonstrated that the suppression of particular CYPs was due to the disruption of the regulatory cascade cytochrome P450 – pregnane X receptor/constitutive androstane receptor – glucocorticoid receptor (GR). The transcriptional inhibition of GR activity

and consequently the down-regulation of receptors PXR and CAR and the target P450s suppression were observed as the result of microtubules depolymeration in PCHH. In two distinct models i.e. PCHH and human embryonic kidney cells (HEK293) transiently transfected with a green fluorescent protein – GR chimera construct, the inhibition of hormone-dependent nucleo-cytoplasmic trafficking of GR by microtubules disarray was confirmed. The results reached further suggest, that GR inactivation by microtubule disrupting compounds is mediated by c-Jun N-terminal kinase, which is concomitantly activated by these compounds.

In conclusion, the experimental model of primary cultures of human hepatocytes proved its worth in studies of cytotoxicity, cytoprotectivity, enzyme kinetics, cell signalization, and interactions in biological systems when investigating activity of substances of plant origin.

Acknowledgement

This research was supported by grant MSM 151100003 from the Ministry of Education, Youth and Sports of the Czech Republic, and by grant 303/04/P074 from the Grant Agency of the Czech Republic.

ACTA UNIVERSITATIS PALACKIANAE OLOMUCENSIS
FACULTAS RERUM NATURALIUM 2004 CHEMICA 43S

PROCEEDINGS OF THE XIX. MEETING OF CZECH AND SLOVAK SOCIETIES FOR BIOCHEMISTRY AND MOLECULAR BIOLOGY

Olomouc, August 31 – September 3, 2004

SECTION 1: PROTEOMICS AND ENZYMOLOGY

1L01

FOURIER TRANSFORM ION CYCLOTRON RESONANCE MASS SPECTROMETRY (FTICR): NEW POSSIBILITIES FOR PROTEOMICS

Vladimír Havlíček*

Laboratory of Molecular Structure Characterization, Institute of Microbiology, Academy of Sciences, Vídeňská 1083, 14220 Praha 4, Czech Republic

** E-mail: vlhavlic@biomed.cas.cz*

Mass spectrometry is one of the most widely used technologies applied for solving proteomics tasks. The general versatility and power of this analytical technique is documented by an extreme sensitivity, specificity, speed and ability to handle complicated sample mixtures. In proteomics nowadays, the two major peptide and protein characterization strategies based on mass spectrometry are the bottom-up and top-down approaches. The first one is based on tandem mass spectral analysis of peptides derived from enzymatic digestion of large proteins, the latter one on the analysis of intact protein ions. The top-down methodology has been possible mostly due to the specific features of FTICR and advantages of its combination with electrospray ionization (ESI).

Mass analyzing principle of FTICR is based on precise measuring the frequency of the signal of macromolecular ions rotating in high magnetic field. The technique has an ultrahigh resolving power (> 1 000 000 at m/z 1000), high precision (about 0.1-1 ppm) and high sensitivity (attomoles on peptides ionized by ESI). The analytical armory is supported by large arsenal of external ionization techniques (EI, CI, SIMS, FAB, MALDI, ESI, ICP, glow discharge) and ion fragmentation techniques such as electron capture dissociation (ECD), sustained off-resonance irradiation (SORI), infrared multiphoton dissociation (IRMPD), etc.

Quite low number of installations by 2004 (only about 450 worldwide) is caused by significant necessary initial investment and expensive subsequent cost of operation. Despite this fact, the first installation in the Czech Republic underwent in summer 2004 at Prague Institute of Microbiology. During the talk, basic principles of FTICR will be outlined and evaluated and applications of this technology in proteomics will be reported.

Acknowledgement

Grant Agency of the Czech Republic (203/02/1417).

1L02

MASS SPECTROMETRY-BASED STRATEGIES FOR PROTEIN IDENTIFICATION AND CHARACTERIZATION

Petr Halada[a,*], Petr Man[a], Jindřich Volc[b] and Vladimír Havlíček[a]

[a]*Laboratory of Molecular Structure Characterization and* [b]*Laboratory of Fungal Biology, Institute of Microbiology, Academy of Sciences, Vídeňská 1083, 14220 Praha 4, Czech Republic*

** E-mail: halada@biomed.cas.cz*

In the late 80's the introduction of two new ionization techniques – matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) moved mass spectrometry (MS) from an analytical method applicable only to small volatile compounds to the field of large biomolecules. Both ionization techniques allow the recording of mass spectra of biological samples with superior accuracy, sensitivity, speed, and mass range. After this breakthrough mass spectrometry has become an indispensable tool in protein studies on all levels, ranging from proteome analysis to studies of protein higher order structure and protein interaction.

The talk will describe the basic principles of ionization and mass analysis of peptides and proteins as well as MS-based methodologies used in protein characterization including peptide mass mapping and peptide sequencing. Database searching approaches employed for protein identification using mass spectrometric data will be briefly mentioned. Examples on molecular weight determination, protein identification, and characterization of post-translational and chemical modifications will be shown.

1L03

THERMOSTABLE TRYPSIN DERIVATIVES FOR ENHANCED IN-GEL DIGESTION IN HIGH THROUGHPUT PROTEOMICS

Marek Šebela[a,*], Jan Havliš[b], Taťána Drhlíková[a], Henrik Thomas[c], Natalie Schmalz[c] and Andrej Shevchenko[c]

[a]*Department of Biochemistry, Faculty of Science, Palacký University, Šlechtitelů 11, CZ-783 71 Olomouc, Czech Republic*
[b]*Department of Analytical Chemistry, Faculty of Science, Masaryk University, CZ-611 37 Brno, Czech Republic*
[c]*Mass Spectrometry Group, Max Planck Institute of Molecular Cell Biology and Genetics, Pfotenhauerstrasse 108, D-01307 Dresden, Germany*

** E-mail: sebela@prfholnt.upol.cz*

Proteomics is the emerging scientific discipline focused on understanding of molecular processes in the cell through a large-scale study of proteins existing in a specific biological context (proteome). Identification of proteins after electrophoretic separations in polyacrylamide gels is typically achieved by their enzymatic in-gel digestion and analyzing the recovered peptides by mass spectrometry. However, a poor yield of the digestion products has been recognized as a serious bottleneck in further progress in high throughput proteomics. Hence accelerated digestion of protein would represent a major advance towards the cost-effective "real-time" flexible characterization of proteomes.

Here we report a study on development of autolysis-resistant bovine trypsin (BT) conjugates showing high catalytic activity at elevated temperatures. BT was modified by coupling of various sugars or sugar-moiety-containing compounds to primary amino groups (lysine residues) in the enzyme molecule. For doing that, an established protocol based on the sugar activation by periodate oxidation before coupling was used. The following BT conjugates were prepared: ATP-modified BT (ATP-BT), β-cyclodextrin-modified BT (BCD-BT), raffinose-modified BT (RAF-BT), raffinose-modified BT with additionally modified arginine residues (by the reaction with 2,3-butanedione, RAFR-BT) and stachyose-modified BT (STA-BT). These conjugates were characterized by the determination of molecular mass, sugar content and the amount of reacted primary amino groups. For RAF-BT and RAFR-BT, amino acid analysis was also performed. The catalytic activity and thermostability of the studied conjugates were evaluated by measurements with an artificial trypsin substrate (Nα-benzoyl-DL-arginine-4-nitroanilide). All the prepared BT conjugates were tested in the proteomic procedure of peptide mass fingerprinting by MALDI TOF mass spectrometry using various proteins as substrates for in-gel digestion. Temperatures for the digestions were set between 37 and 65 ^{o}C, incubation times were between 0.5 – 12 h. Autolysis was evaluated by simple incubating of the conjugates in the digestion buffer (ammonium bicarbonate) at 55 ^{o}C followed with MALDI TOF mass spectrometry. The obtained results were compared with those provided by unmodified BT and a commercially available methylated porcine trypsin (MET-PT). Each of the studied trypsin conjugates showed an increased

thermostability in comparison with those of unmodified BT or MET-PT, and provided legible peptide mass fingerprint spectra. Moreover, BCD-BT, RAF-BT, RAFR-BT and STA-BT showed enhanced resistance to autolysis in the mass spectrometry experiments. Instead, ATP-BT provided a strong autolytic background. The digestion power of the conjugates expressed as a yield of registered peptide peaks was 5 to 10 fold lower in comparison with that of MET-PT, probably due to hindered binding of protein substrates at the active site. From the conjugates resistant to autolysis, RAF-BT was found to be the best choice for the ongoing research.

Acknowledgement

This work was supported by the grant ME 664 from the Ministry of Education, Youth and Sports, Czech Republic and by the joint project CZE 02/003 within a bilateral scientific co-operation (granted by the Czech Ministry of Education, Youth and Sports and German Bundesministerium für Bildung und Forschung).

1L04

THE USE OF 2-D ELECTROPHORESIS AND 2-D LIQUID PHASE SEPARATION FOR INTERLYSATE COMPARISON OF CANCER CELLS TREATED BY CYCLIN-DEPENDENT KINASE INHIBITOR

Zdeňka Ellederová[a], Helena Skalníková[a], Petr Halada[b], Petr Man[b], Petr Džubák[c], Marian Hajdúch[c], Michal Kubelka[a], Jiří Moos[d] and Hana Kovářová[a,*]

[a]*Laboratory of Reproduction Physiology, Institute of Animal Physiology and Genetics, Academy of Science, Rumburská 89, 27721 Liběchov, Czech Republic*
[b]*Laboratory of Molecular Structure Characterization, Institute of Microbiology, Academy of Sciences, Vídeňská 1083, 14220 Praha 4, Czech Republic*
[c]*Laboratory of Experimental Medicine, Clinic of Pediatrics, Faculty of Medicine, Palacký University, Puškinova 6, 775 20 Olomouc, Czech Republic*
[d]*Immunotech, Radiová 1, 10227 Praha 10, Czech Republic*

** E-mail: kovarova@iapg.cas.cz*

The methods for analyzing biological samples alternative to the classical proteomics approach, e.g. 2-D electrophoresis coupled to mass spectrometry have been developed recently. The new PF 2D protein fractionation system represents 2-D liquid phase separation technique that resolves the proteins by isoelectric point in the 1st dimension and by hydrophobicity in the 2nd dimension. All components are collected into 96-well plate and in liquid phase are ideal for identification by mass spectrometry.

In the present work, the 2-D gel electrophoresis/mass spectrometry as well as 2-D liquid separation PF 2D/mass spectrometry were used for interlysate comparison between cultured T-lymphoblastic leukemia CEM cells and the same cell line treated by

cyclin-dependent kinase inhibitor, bohemine. Several unique proteins were identified and shown to be up- or down-regulated between treated and non-treated malignant cells. The 2-D liquid separation PF 2D approach and corresponding protein identification is valuable for proteomic analyses and provides the results complementary to classical 2-D gel based methods.

1L05

NEW APPROACH IN PROTEOMICS OF GLUTEN: USING CHYMOTRYPSIN AND MALDI HYBRID MULTISTAGE MASS SPECTROMETER

Jiří Šalplachta[a,b,*], Josef Chmelík[a] and Günter Allmaier[c]

[a]*Institute of Analytical Chemistry of the Academy of Sciences, Veveří 97, 61142 Brno, Czech Republic*
[b]*Institute of Chemistry and Biochemistry, Faculty of Agronomy, Mendel University of Forestry and Agronomy, Brno, Czech Republic*
[c]*Institute of Chemical Technologies and Analysis, Vienna University of Technology, Vienna, Austria*

** E-mail: salplachta@iach.cz*

The knowledge of protein composition of the cereals is helpful for characterization of cereal varieties that is of importance to food industry. Furthermore, these cereal proteins, which are frequently present in common diet, are toxic to patients with coeliac disease (gluten-sensitive enteropathy). Gluten is alcohol-soluble fraction of wheat proteins classically subdivided into the monomeric gliadins (wheat prolamins) and the polymeric glutenins consisting of low- and high-molecular-weight subunits. Gliadins are monomeric proteins with intra-chain disulfide bonds, while the low- and high-molecular-weight glutenin subunits form polymers stabilized by inter-chain disulfide bonds. Gluten proteins are main storage proteins of wheat grains. Both gliadins and glutenins play a key role in dought formation and viscoelastic properties of the gluten. Gliadins have a great importance in grain germination as a source of nitrogen.

In recent years, proteomics has become a powerful method for characterization, identification and determination of peptide sequence of gluten proteins. Mass spectra of gliadins and low-molecular-weight glutenin subunits show characteristic pattern of proteins in the 30-40 kDa range while the high-molecular-weight glutenin subunits range in molecular mass from 65-90 kDa. As the mass spectrometric analyses of intact proteins are not enough sensitive the proteomic identification of gluten proteins in ethanolic extract from wheat flour is described in this study. Proteins in ethanolic extract were separated via 1-D gel electrophoresis and subjected to in-gel digestion with the chymotrypsin which was found as proper proteolytic enzyme for this purpose.

Identification based on peptide mass fingerprinting is not sufficient due to extensive homology of these proteins, therefore, collision-induced dissociation of selected peptides using a MALDI hybrid multistage mass spectrometer was used for unambiguous identification of some gluten proteins.

Acknowledgement

This work was supported by the grant QD 1023 from NAZV, Czech Republic.

1L06

DETERMINATION OF THE THREE-DIMENSIONAL STRUCTURE OF PROTEINS USING CHEMICAL CROSS-LINKING AND HIGH RESOLUTION MASS SPECTROMETRY

Petr Novák[a,b]*, Malin M. Young[b], Joseph S. Schoeniger[b] and Gary H. Kruppa[b]

[a]*Laboratory of Molecular Structure Characterization, Institute of Microbiology, Academy of Sciences, Vídeňská 1083, 14220 Praha 4, Czech Republic*
[b]*Sandia National Laboratories, Livermore, CA-94550, USA*

* *E-mail: halada@biomed.cas.cz*

Modern structural methods have been successful in elucidating a wide range of protein structures, and have yielded invaluable insight into biological mechanisms, the classification of protein folds, and the dynamics of proteins. However, structural genomics efforts and individual structure determination efforts share the same modest net success rates (10-20% starting from selected clones)[1]. Furthermore, proteins that can be successfully solved by X-ray and NMR methods tend to have few domains, low molecular weight, and are shorn of flexible regions and post-translational modifications. Certain classes of proteins are poorly represented in the PDB, because of their insolubility and difficulty to crystallize (http://blanco.biomol.uci.edu/ Membrane_Proteins_ xtal.html). From this point of view, there is a clear need for new structural methods that can be applied to larger proteins/complexes, can tolerate heterogeneity, and yield both structural measurements and information on conformational changes occurring on physiological time scales. Recently, a method combining the use of chemical cross-linking and mass spectrometry to identify the inter-residue cross-links, and the use of these cross-links to constrain structural models of proteins, referred to as MS3-D, has been reported[2]. Subsequent studies indicate that this approach appears to have promise for defining large sets of inter-atomic distance constraints in proteins and a structural model for a protein could be determined based on these constraints using threading and homology methods [2-4].

Despite the initial report indicating the potential of MS3-D, the number of distance constraints using this method has been limited. Several publications have indicated that the cross-linking chemistry itself may be difficult to optimize. The method is further complicated by the difficulty of using proteolytic digestion and HPLC-MS to localize the cross-links. We have developed a whole protein approach (top down) using Fourier Transform Mass Spectrometry to localize the cross-links using tandem mass spectrometry rather than proteolytic digestions. This method also facilitates optimization of the cross-linking chemistries. Our initial results have elucidated the complexity of cross-linking chemistry and enabled us to find optimal conditions for several types of reagents. We have clearly shown for small proteins that the top-down method can be used to localize the cross-links without the need for proteolytic digestion and HPLC. Furthermore MS3-D efforts have been hampered by the limited diversity of commercially available crosslinkers; previous studies have employed crosslinking of lysine residues. Our improved ability to analyze the results of crosslinking has enabled us to demonstrate crosslinking with a wider set of reagents, allowing access to an increased number of distance constraints.

References

1. Bourne P. E., Allerston C. K., Krebs W., Li W., Shindyalov I. N., Godzik A., Friedberg I., Liu T., Wild D., Hwang S., Ghahramani Z., Chen L. and Westbrook J.: *Pac. Symp. Biocomput.* **9**, 375 (2004)
2. Young M. M., Tang N., Hempel J. C., Oshiro C. M., Taylor E. W., Kuntz I. D., Gibson B. W. and Dollinger G.: *Proc. Natl. Acad. Sci. USA* **97**, 5802 (2000)
3. Novák P., Young M. M., Schoeniger J. S. and Kruppa G. H.: *Eur. J. Mass Spectrom.* **9**, 623 (2003)
4. Taverner T., Hall N. E., O'Hair R. A. and Simpson R. J.: *J. Biol. Chem.* **277**, 46487 (2002)

Acknowledgement

Presented work was supported by the Laboratory Directed Research and Development program at Sandia National Laboratories, which is a multi-program laboratory operated by Sandia Corporation, a Lockheed Martin Company for the United States Department of Energy under contract DE-AC04-94AL85000.

1L07

BIOCHEMISTRY AND PHYSIOLOGICAL FUNCTION OF CYTOKININ DEHYDROGENASE IN PLANTS

Ivo Frébort[a,]*, Petr Galuszka[a], Jitka Frébortová[b], Hana Popelková[a] and Jiří Drábek[a]

[a]*Division of Molecular Biology, Department of Biochemistry, Faculty of Science, Palacký University, Šlechtitelů 11, CZ-78371 Olomouc, Czech Republic*
[b]*Laboratory of Growth Regulators, Palacký University/Institute of Experimental Botany, Academy of Science, Šlechtitelů 11, CZ-78371 Olomouc, Czech Republic*

** E-mail: frebort@prfholnt.upol.cz*

Degradation of growth hormones cytokinins in plants is controlled by flavoprotein cytokinin dehydrogenase (EC 1.5.99.12)[1]. This enzyme is encoded in individual species by multiple genes that show mutual evolutionary relations. Best studied gene family of *Arabidopsis thaliana* contains seven genes that appear to have different cellular and tissue targeting, thus achieving distinct functions during the development [1,2].

The enzyme is a typical flavoprotein with covalently bound FAD in the catalytic centre that is buried in the hydrophobic pocket of the protein. Results of site-directed mutagenesis reveal C-terminus as an important domain for functional conformation of the enzyme. The cytokinin cleavage proceeds by "concerted covalent catalysis" via a ternary complex involving covalently bound FAD cofactor and a quinonic electron acceptor[3]. The enzyme is capable of using DCPIP and some *p*-quinones as electron acceptors, while oxygen is almost ineffective. Natural electron acceptor of the enzyme is not yet known. Experimental data support the theory that the electron acceptor is generated *in vivo* from plant phenolics by other enzymatic systems such as peroxidase and laccase/catechol oxidase. Histochemical localizations in maize show co-staining of cytokinin dehydrogenase and laccase, suggesting possible cooperation of these enzymes in cytokinin degradation.

Structure-function relationships of the cytokinin cleavage by cytokinin dehydrogenase and its possible link to the metabolism of plant phenolics will be discussed.

References

1. Popelková H., Galuszka P., Frébortová J., Bilyeu K. D. and Frébort I.: In *Recent Research Developments in Proteins, Vol. 2*, (Pandalai S. G., ed.), Transworld Research Network, Kerala, India 2004, p. 63
2. Schmülling, T.: *J. Plant Growth Regul.* **21**, 40 (2002)
3. Frébortová J., Fraaije M. W., Galuszka P., Šebela M., Peč P., Hrbáč J., Bilyeu K. D., English J. T. and Frébort I.: *Biochem. J.* **380**, 121 (2004)

Acknowledgement

This work was supported by the grant 522/03/0979 from the Grant Agency, Czech Republic.

1L08

EFFECT OF ENDOGENOUS CYTOKININ LEVELS ON THE *ARABIDOPSIS THALIANA* PROTEOME

Pavlína Váňová[a,b,*], François Chevalier[b], Gabriela Böhmová[a], Hana Konečná[a], Zbyněk Zdráhal[a], Michel Rossignol[b] and Břetislav Brzobohatý[a]

[a]*Department of Functional Genomics and Proteomics, Faculty of Science, Masaryk University, Kotlářská 2, 611 37, Brno, Czech Republic*
[b]*Unité de Protéomique, INRA, 2 Place Viala, 34060, Montpellier Cedex 1, France*

** E-mail: vanova@sci.muni.cz*

The plant hormone cytokinin plays a crucial role in plant development from germination to senescence. Nevertheless, the exact mechanisms of its influence on these several developmental processes remain unexplained. We report an attempt to reveal more information about the mode of action of cytokinin in the model plant – *Arabidopsis thaliana.* For this purpose a classical proteome-based approach using two-dimensional electrophoretic separation followed by protein digestion and identification by mass spectrometry was established.

Total proteins were extracted from whole *Arabidopsis* plantlets of following genotypes: wild-type ColO, *amp1* mutant accumulating six-fold higher level of endogenous cytokinin[1] and pOp-*ipt*::LhGR transgenic line in which the production of endogenous cytokinin is inducible by dexamethasone[2]. All plants were grown for 15 days under long-day conditions (16 h light/8 h dark) on MS media, in parallel with and without 2.5 μM dexamethasone.

Image analysis of two-dimensional gels in triplicates using Progenesis Workstation software and subsequent statistical evaluation revealed both qualitative and quantitative changes in protein expression between plants with elevated cytokinin levels and controls. Out of these differently expressed protein spots so far 21 significant ones were in-gel digested with trypsine and the resulting peptides were subjected to MALDI-TOF MS analysis. Proteins corresponding to the acquired mass spectra were identified in 17 of the analysed spots.

Elucidating the relationship of the identified proteins to cytokinin metabolism and action will be a longer-term task. A study of the kinetics of the proteome changes after dexamethasone treatment in pOp-*ipt*::LhGR plants represents the immediate challenge.

References

1. Chaudhury A. M., Letham S., Craig S. and Dennis E. S.: *Plant J.* **4**, 907 (1993)
2. Moore I., Galweiler L., Grosskopf D., Schell J. and Palme K.: *Proc. Natl. Acad. Sci. USA* **95**, 376 (1998)

Acknowledgement

This project was supported by the Ministry of Education of the Czech Republic (MSM 143100008) and by the scholarship IN-CO Czech 2002-2004 within the Leonardo da Vinci programme.

1L09

CATALYTIC MECHANISM OF THE HALOALKANE DEHALOGENASE LinB FROM *SPHINGOMONAS PAUCIMOBILIS* UT26

Zbyněk Prokop[a,*], Marta Monincová[a], Radka Chaloupková[a], Martin Klvaňa[a], Yuji Nagata[b], Dick B. Janssen[c] and Jiři Damborský

[a]*National Centre for Biomolecular Research, Masaryk University, Kotlářská 2, 611 37 Brno, Czech Republic*
[b]*Department of Life Sciences, Graduate School of Life Sciences, Tohoku University, Sendai, 980-8577, Japan*
[c]*Department of Biotechnology, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands*

** E-mail: zbynek@chemi.muni.cz*

Haloalkane dehalogenases (EC 3.8.1.5) make up important class of enzymes that are able to cleave carbon-halogen bonds in halogenated aliphatic compounds. There is a growing interest in these enzymes due to their potential use in bioremediation, as industrial biocatalysts, or as biosensors. Structurally, haloalkane dehalogenases belong to the alfa/beta-hydrolase fold superfamily[1]. A central beta-sheet, flanked on both sides by alfa-helices, forms the hydrophobic core of the main domain that carries the catalytic triad Asp-His-Asp/Glu. The second domain, consisting solely of alfa-helices, lies like a cap on a top of the main domain. Residues on the interface of the two domains form the active site. A reaction mechanism of haloalkane dehalogenase has been proposed on basis of X-ray crystallography[2], and kinetic[3,4] studies with haloalkane dehalogenase of *Xantobacter autotrophicus* GJ10 (DhlA). Catalysis proceeds by the nucleophilic attack of the carboxylate oxygen of an aspartate group on the carbon atom of the substrate, yielding a covalent alkyl-enzyme intermediate. The alkyl-enzyme intermediate is subsequently hydrolyzed by a water molecule that is activated by a histidine, catalytic acid (Asp or Glu) stabilises the charge developed on the imidazole ring of the histidine. To obtain insight in the kinetic mechanism of another haloalkane dehalogenase[5] from *Sphingomonas paucimobilis* UT26 (LinB), we studied the steady-state and pre-steady-state kinetics of the conversion of the substrates 1-chlorohexane, chlorocyclohexane and bromocyclohexane. Proposed minimal kinetic mechanism of LinB consisted of three main steps: (i) substrate binding, (ii) cleavage of a carbon-halogen bond with simultaneous formation of an alkyl-enzyme intermediate and (iii) hydrolysis of the alkyl-enzyme intermediate[6]. Release of products, halide and alcohol, is a fast process that was not included in reaction mechanism as a distinct step. Comparison of the kinetic mechanism of LinB with that of DhlA and DhaA from *Rhodococcus rhodochrous* NCIMB 13064 shows considerable similarity in the overall mechanisms. The binding of substrate and the following cleavage of the carbon-halogen bond were found to be the fastest step in the catalytic cycle of haloalkane dehalogenases. The main difference is in the rate-limiting step, which is hydrolysis of the alkyl-enzyme intermediate in LinB, halide release in DhlA, and liberation of an alcohol in DhaA. Our study indicates that

extrapolation of rate-limiting steps from one family member to another can be misleading even for evolutionary closely related proteins.

References

1. Ollis D. L., Cheah E., Cygler M., Dijkstra B., Frolow F., Franken S. M., Harel M., Remington S. J., Silman I., Schrag J., Sussman J. L., Verschueren K. H. G. and Goldman A.: *Prot. Engineering* **5**, 197 (1992)
2. Verschueren K. H. G., Seljee F., Rozeboom H. J., Kalk K. H. and Dijkstra B. W.: *Nature* **363**, 693 (1993)
3. Schanstra J. P. and Janssen D. B.: *Biochemistry* **35**, 5624 (1996)
4. Schanstra J. P., Kingma J. and Janssen D. B.: *J. Biol. Chem.* **271**, 14747 (1996)
5. Nagata Y., Miyauchi K., Damborský J., Manová K., Ansorgová A. and Takagi M.: *Appl. Environ. Microbiol.* **63**, 3707 (1997)
6. Prokop Z., Monincová M., Chaloupková R., Klvaňa M., Janssen D.B., Nagata Y. and Damborský J.: *J. Biol. Chem.* **278**, 45094 (2003)

Acknowledgement

This work was supported by Grant from the Czech Ministry of Education LN00A016. Zbyněk Prokop acknowledges the scholarship from the Federation of European Microbiological Societies and NATO Travel Fellowship.

1L10

HALOALKANE DEHALOGENASES FROM PARASITIC BACTERIUM *MYCOBACTERIUM BOVIS* 5032/66

Andrea Jesenská[a,*], Iva Těšínská[a], Marta Monincová[a], Yuji Nagata[b] and Jiří Damborský[a]

[a]*National Centre for Biomolecular Research, Faculty of Science, Masaryk University, Kotlářská 2, 61137 Brno, Czech Republic*
[b]*Department of Environmental Life Sciences, Tohoku University, Sendai, 980-8577, Japan*

** E-mail: andreaj@chemi.muni.cz*

Halogenated organic compounds constitute one of the largest groups of environmental pollutants as a result of their widespread use in industry and agriculture. Haloalkane dehalogenases are bacterial enzymes capable of carbon-halogen bond cleavage in halogenated compounds using a hydrolytic mechanism. The enzymes act on a broad range of halogenated aliphatic compounds. Haloalkane dehalogenases have been isolated especially from bacteria colonizing environments contaminated with

halogenated compounds. Genome sequencing project indicated the presence of the genes rv2296 and rv2579 sequentially similar to haloalkane dehalogenases in the genome of human parasite *Mycobacterium tuberculosis* H37Rv[1]. Genetically closely related *Mycobacterium bovis* 5032/66 izolated from the cattle was used to clone the genes of these putative mycobacterial dehalogenases.

Gene rv2296 was amplified by PCR reaction using specific primers and inserted in-frame to *Escherichia coli* expression plasmid pCR T7/NT- TOPO. The resulting recombinant plasmid was transformed into *E. coli* OneShot TOP10F competent cells. Transformant carrying a vector with gene rv2296 in correct orientation was used to express Rv2296 protein. Expression was performed in bacterial cells *E. coli* BL21(DE3)LysS. Analysis of the *E. coli* induced with isopropyl-β-D-thiogalactopyranoside revealed that the protein is accumulated in the insoluble inclusion bodies. Solubility of the protein was enhanced by modification of cells growth conditions (lower temperature and lower concentration of the inducer) and using different conditions for preparation of the crude extract. Rv2296 was obtained in pure form and its CD spectrum was compared to those of other haloalkane dehalogenases. Purified protein did not show any dehalogenating activity using GC-MS analysis.

Gene rv2579 was amplified by PCR reaction with specific primers based on the N- and C-terminal dehalogenase sequence with introduced hexahistidyl tail to C-terminus of the protein. PCR amplicon of the gene rv2579 was cloned in the pUC18 vector and sequenced. The gene was cloned to the expression vector pAQN for the production of an active haloalkane dehalogenase in *E. coli* BL21. Expressed protein was purified to homogeneity by metal affinity chromatography and tested for its biochemical properties. The highest activity of protein Rv2579 was observed at 45°C. Interestingly, protein displayed two pH optima at pH 5.5 and pH 8.5. The effect of storage temperature and the effect of stabilizing additives on the protein were also studied. The presence of 10% glycerol positively influenced long-term stability of enzyme at -60 ^{o}C, whereas 1 mM EDTA showed the positive effect at 4 ^{o}C. Steady-state kinetic parameters towards 31 selected halogenated substrates were determined and compared to haloalkane dehalogenase LinB showing 82% of sequence identity with Rv2579.

References

1. Jesenská A., Sedláček I. and Damborský J.: *Appl. Environ. Microbiol*. **66**, 219 (2000)

1L11

THE EFFECTS OF TERMINAL ELECTRON ACCEPTORS AND SODIUM AZIDE ON THE *PARACOCCUS DENITRIFICANS* PROTEIN COMPOSITION

Pavel Bouchal[a,*], Petra Přecechtělová[a], Zbyněk Zdráhal[b] and Igor Kučera[a]

[a]*Department of Biochemistry and* [b]*Laboratory of Functional Genomics and Proteomics, Faculty of Science, Masaryk University*

** E-mail: bouchal@chemi.muni.cz*

Paracoccus denitrificans is a non-fermentative, facultatively autotrophic soil bacterium often studied in the field of bioenergetics, particularly due to resemblance of its aerobic respiratory chain to that of mitochondria. Also an aspect of a great nutritional adaptability was discovered, related to the ability of exploiting various electron donors and electron acceptors for maintenance and growth. To discuss mechanisms underlying regulation of gene expression by growth conditions, a high-throughput proteome mapping is one of the most effective tools to-date.

For 2-D electrophoretic analysis of whole-cell extract and its membrane fraction, isoelectric focusation with immobilized pH gradients in the first dimension and SDS-PAGE in the second dimension were used. Using this approach, more than 800 protein spots of total cell extract and membrane fraction were detected in the range pI 3-10 and M_r 15-100 kDa.

We compared complex protein composition of whole cells of *P. denitrificans* cultivated under aerobic and various anaerobic conditions (with nitrate, nitrite and nitrous oxide). We also investigated the effect of respiratory inhibitor azide on proteomic profiles of the cells grown aerobically and anaerobically with NO_2^-. The similar approach was applied also on membrane fractions of cells (grown on O_2, O_2+N_3^-, NO_3^-).

The most distinct proteomic profile matches the growth on nitrous oxide; the probable reasons are discussed. The significant changes in protein composition of total cell lysates were caused also by azide.

About 50 protein spots have been submitted to the analysis by peptide mass fingerprinting using MALDI-TOF MS. However, the genome of *P. denitrificans* has not been completely sequenced yet and, currently information about only 98+126 proteins is available from SwissProt/TrEMBL database. Due to this fact, we were able to identify only 8 proteins up to-date. Among them, nitrite reductase and succinate dehydrogenase are key enzymes in *P. denitrificans* metabolism. Nitrite reductase is the second of four enzymes catalyzing the denitrification pathway in *P. denitrificans*, where NO_3^- is reduced via NO_2^-, NO, N_2O to molecular nitrogen under anaerobic conditions. Succinate dehydrogenase, fumarate-producing enzyme in citrate cycle, was previously found to be very close to the bovine heart mitochondrial enzyme, having four subunits (64.9, 28.9, 13.4 and 12.5 kDa). Our ID corresponds with the 28.9 kDa FeS subunit.

We also compared expression data of nitrite reductase obtained by proteome analysis with the measurement of nitrite reductase enzyme activity. These data were in a good agreement.

The quantitative data and protein maps are kept in a database in PDQUEST format. The web-accessible proteome 2-D database has been established at http://www.mpiib-berlin.mpg.de/2D-PAGE.

Acknowledgement

This work was supported by the grant 203/01/1589 from the Grant Agency, Czech Republic.

1L12

AMPEROMETRIC FLOW-INJECTION DETERMINATION OF PHENOLIC COMPOUNDS AT SELF-ASSEMBLED MONOLAYER-BASED TYROSINASE, LACCASE AND PEROXIDASE BIOSENSORS

Renáta Solná* and Petr Skládal

Department of Biochemistry, Masaryk University, Kotlářská 2, 61137 Brno, Czech Republic

** E-mail: R.Solna@email.cz*

Phenol and its derivatives are an important class of environmental pollutants introduced to the environment as a result of their industrial applications (i.e. petroleum, pharmaceutical, pulp and paper industry); some of them exhibit fairly high acute toxicity. Multienzymatic biosensors represent a promising tool for simple characterisation and rapid analysis of many compounds, which can function directly as substrates for enzymes selected for the sensing array.

The screen-printed four-electrode sensor based on immobilisation of tyrosinase (mushroom), laccase (*Coriolus hirsutus*) and peroxidase (horseradish) in the same array were developed for monitoring of phenols. The enzymes were immobilised onto self-assembled monolayer (4-mercapto-1-butanol) formed surface on the gold via covalent attachment by epichlorohydrin coupling. The amperometric detection of several substituted phenolic compounds was carried out using a single line flow-injection system. Hydrogen peroxide served as co-substrate for peroxidase to measure phenols.

The limits of detection for phenols in aqueous solutions were in the micromolar range. One assay was completed in less than 5 min. The compatibility of the above mentioned enzyme array enabled to apply the developed multichannel biosensors for evaluation in real samples; industrial wastewaters and surface waters.

1P01

MUTATION ANALYSIS OF THE ACTIVE SITE OF THE HUMAN RECOMBINANT ORNITHINE AMINOTRANSFERASE

Michaela Marková[a,*], Blanka Králová[a] and Robert A. John[b]

[a]*Department of Biochemistry and Microbiology, Institute of Chemical Technology, Technická 3, 166 28 Praha 6, Czech Republic*
[b]*School of Biosciences of Wales, Cardiff CF1 3US, United Kingdom*

** Email: markovai@vscht.cz*

Ornithine aminotransferase (OAT) catalyzes the transfer of the amino group from L-ornithine to α-ketoglutarate, forming L-glutamate and glutamate-5-semialdehyde. It is found in bacteria, fungi, plants and higher animals including man. The structure of the human enzyme, both free[1] and with irreversibly-bound substrate analogues[2], has been solved by X-ray crystallography. There are many similarities in structure, particularly at the active site, with other members of the alpha family of pyridoxal-5'-phosphate-dependent enzymes. This is especially true for GABA-aminotransferase, the active site of which contains only two significant differences from that of OAT[3,4]. Unlike majority of aminotransferases, both OAT and GABA-AT catalyze the transfer of amino group from carbon atom that does not bear carboxyl group. However, both enzymes are using α-ketoglutarate to complete the transamination cycle and they are able to transfer of the amino group from glutamate.

To explain the part played by the amino acid residues from active site, we used mutation analysis. Two sets of mutants were prepared, the first one based on comparison of active sites of GABA-AT and OAT and the second based on comparison of OAT and aminotransferases catalyzing transfer of amino group exclusively from carbon bearing carboxyl group.The specific activity towards L-ornithine and GABA and rate constants for single steps of the reactions of mutants were compared with those of the wild type OAT.

References

1. Shen B. W., Hennig M., Hohenester E., Jansonius J. N. and Schirmer T.: *J. Mol. Biol.* **277**, 81 (1999)
2. Shah S. A., Shen B. W. and Brünger A. T.: *Structure* **5**, 1067 (1997)
3. Storici P., Capitani G., Schirmer T. and Jansonius J. N.: *J. Mol. Biol.* **285**, 297 (1999)
4. Storici P., Capitani G., De Biase D., Moser M., John R. A., Jansonius J. N. and Schirmer T.: *Biochemistry* **38**, 8628 (1999)

Acknowledgement

This work was performed as part of project 204/02/0843 supported by the Grant Agency of the Czech Republic and research grant MSM 223300006 from the Ministry of Education, Youth and Sports, Czech Republic.

1P02

EXPRESSION, PURIFICATION AND STRUCTURAL ANALYSIS OF CALEOSIN FROM *ARABIDOPSIS THALIANA*

Zita Purkrtová[a,*], Sabine d'Andrea[a], Blanka Králová[b] and Thierry Chardot[a]

[a]*Laboratoire de Chimie Biologique, CNRS-INRA-INAPG UMR 2585, 78850 Thiverval-Grignon, France*
[b]*Department of Biochemistry and Microbiology, Institute of Chemical Technology, Technická 3, 166 28 Praha 6, Czech Republic*

** E-mail: zipur@seznam.cz*

Caleosins are a recently described class of plant EF-hand proteins that are embedded in seed oil bodies. These small proteins are composed of three regions, hydrophilic N-terminal region, central hydrophobic domain and C-terminal hydrophilic region, which make them amphiphatic. Up to date, caleosins from 5 higher plants were described. According to sequence alignment, one caleosin gene was found in genome of rice, sesame and Theobrama cacao; 2 caleosin genes were found in *Brassica napus* genome. Analysis of *Arabidopsis thaliana* genome shows seven caleosin genes, one being expressed in seed oil bodies.

The function of caleosin remains mysterious but it is supposed that caleosin are involved in lipid trafficking process during biogenesis, maturation and mobilization of lipid bodies. The conserved structural features of caleosin, calcium binding and phosphorylation domains indicate that caleosin function is presumably modulated by calcium binding and phosphorylation. The presence of a 36 residues hydrophobic sequence, along with a proline knot motif is typical of seeds oil bodies' integral proteins.

Caleosin (At4g26740) from *A. thaliana* was expressed as N-terminal histidine tagged protein in *E. coli* BL21(DE3) and purified to almost electrophoretic homogeneity using IMAC chromatography. In order to better understand its emulsifying properties, its interfacial behavior (water/oil interface) was studied using pendant drop method. Study of solution structure and size of caleosin was carried out using laser dynamic light scattering. Effect of calcium on these properties was investigated.

Acknowledgement

During her stay in Grignon Zita Purkrtová was recipient of an Erasmus-Mundus scholarship.

1P03

IS IT POSSIBLE TO STUDY CONFORMATION OF AN UNPURIFIED PROTEIN?

Radovan Hynek, Milan Kodíček*, Martin Strohalm and Jiří Šantrůček

Department of Biochemistry and Microbiology, Institute of Chemical Technology, Technická 5, 166 28 Praha 6, Czech Republic

** E-mail: kodicekm@vscht.cz*

During the last period enormous progress in protein conformation studies has been made thanks to the methods of X-ray crystalography as well as of NMR spectroscopy. These two methods are able to provide information about localization of individual atoms in three-dimensional structure of the protein. In addition to these "absolute" methods, different quicker, cheaper and technically less demanding methods (circular dichroism, fluorimetry etc.) are available; they usually give sensitive information about changes in protein conformation and facilitate to compare structures of functionally analogical proteins.

Chemical modifications of proteins have already been successfully performed in order to obtain information about localization of certain amino acid residues in protein globule. These methods work on a simple principle: 1) To a native protein of known covalent structure a specific agent, which modifies a single (or limited number) amino acid side chain, is added. 2) The excess of the reagent and low-molecular-weight by-products are removed. 3) The protein is specifically cleaved (usually by trypsin or chymotrypsin) and the peptides produced are characterized by mass spectrometry. 4) As for each modification the characteristic shift of molecular mass is well defined (e.g. 45 Da for nitro-group, 151 Da for Koshland reagent), it is possible to detect those peptides that have been modified; they contain amino acids that are localized on the surface of the native protein globule.

All the methods mentioned above can give reasonable results only if the protein is pure and native; in the case of crystalographic and spectroscopical methods, they must be prepared in amounts of milligrams. We have developed a method which allows getting certain information about conformation of a protein which is still in a complex mixture, in its natural environment in its native state and is available in microgram or even nanogram amount. The method is based on the chemical modifications and mass spectrometry described in previous paragraph. The modification itself (items 1 and 2) is carried out in the protein mixture, then the protein of interest is separated by SDS-PAGE; in this stage of the procedure, denaturation caused by sodium dodecylsulphate does not affect the results of conformational analysis. The following step, enzymatic cleavage, can be performed in gel (standard "in-gel digest" known from proteomic procedures) and the surface localized amino acids are detected as described.

A model mixture of soluble proteins was modified by tetranitromethane (specific for tyrosine) and by 2-hydroxy-5-nitrobenzyl bromide (Koshland reagent, specific for tryptophan). The proteins were separated by SDS-PAGE and cleaved in-gel by trypsin. The following MALDI-TOF mass spectrometry analysis gave the same results as the

analogical procedure applied on pure proteins in solution. We consider the presented method highly promising because it opens new possibilities for studying conformation of small amounts of proteins in their native environment.

Acknowledgement

This research is supported by grant 203/02/0922 from the Grant Agency of the Czech Republic.

1P04

TYROSINE RESIDUES MODIFICATION STUDIED BY MALDI-TOF MASS SPECTROMETRY

Jiří Šantrůček*, Martin Strohalm, Radovan Hynek and Milan Kodíček

Department of Biochemistry and Microbiology, Institute of Chemical Technology, Technická 5, 166 28 Praha 6, Czech Republic

** E-mail: santrucj@vscht.cz*

Residue specific reactivity is of great current interest in structural biology as it provides information about the solvent accessibility and/or reactivity of a residue that can be used to probe protein structure and interactions. Although NMR and X-ray crystallography can give very detailed information about the solvent accesibility and environment of a specific residue, both methods are time-consuming and require miligrams of proteins. The development of mass spectrometry in protein science provides a sensitive and quick methodology for the measurement of molecular weights of peptide fragments, which allows one to identify the modified sites in the protein using micrograms of protein.

In presented work tyrosine residues of three model proteins with known spatial structure (horse heart cytochrome *c*, chicken egg white lysozyme and human serum albumin) were modified with two reagents specific for tyrosine: tetranitromethane and iodine. Modified proteins were specifically digested with proteases and mass of resulting peptide fragments determined using MALDI-TOF (Matrix Assisted Laser Desorption/Ionisation Time Of Flight) mass spectrometer. For modified peptide identification, Bruker Biotools software was used to match the molecular mass of peptides against the protein amino acid sequences.

Our results show that there are only small differences in the extent of tyrosine residues modification by tetranitromethane and iodine. However, data about solvent accessibility obtained by chemical modifications of reactive residues are not identical with those obtained by NMR and X-ray crystallography. These interesting discrepancies

can be caused by local molecular dynamics and/or by specific chemical structure of the residue´s surrounding.

Acknowledgement

This work was supported by grant 203/02/0922 from the Grant Agency of the Czech Republic.

1P05

ANALYSIS OF TRYPTOPHAN SURFACE ACCESSIBILITY BY MALDI-TOF MASS SPECTROMETRY

Martin Strohalm* and Milan Kodíček

Department of Biochemistry and Microbiology, Institute of Chemical Technology, Technická 5, 166 28 Praha 6, Czech Republic

** E-mail: martin.strohalm@vscht.cz*

Side chains of surface accessible amino acids play very important role in biological activities of proteins. They can act as a part of enzyme‘s active center or participate on many intermolecular interactions. Information about the surface accessibility of selected amino acids can contribute to understanding the protein structure and function. The main interest of this work is to find out whether a specific chemical modification of tryptophan side chain in a molecule of a native protein could reflect some relevant information about the accessibility and subsequently, information about the protein conformation. The reaction with 2-hydroxy-5-nitrobenzyl bromide (HNB) as a common and highly specific covalent modification of tryptophan seems to be very useful for this purposes[1,2].

This method is based on the side chain modification followed by the analysis of the tryptic digest by MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight) mass spectrometry. The procedure was tested on model proteins with known spatial structure. Our experiments indicate that reactivity of tryptophan side chain is very complex and is not affected by its surface accessibility only, but also by its microenvironment formed by other side chains. Reagent concentration also plays significant role since the modification seems to influence a fine protein structure and its molecular dynamics; lower concentrations give more reliable conformational information. For all above mentioned factors positive reaction only can be interpreted as surface accessible amino acid.

References

1. Koshland Jr. D. E. , Karkhanis Y. D. and Latham H. G.: *J. Am. Chem. Soc.* **86**, 1448 (1964)
2. Strohalm M., Kodíček M. and Pechar M.: *Biochem. Biophys. Res. Commun.* **312**, 811 (2003)

Acknowledgement

This work was supported by the grants 203/02/0922 from the Grant Agency of the Czech Republic and MSM 223300006 from the Ministry of Education, Youth and Sports, Czech Republic.

1P06

HISTOCHEMICAL LOCALIZATION OF ACTIVE OXYGEN SPECIES AND STUDY OF ANTIOXIDANT ENZYMES INVOLVED IN DEFENCE MECHANISMS OF *LACTUCA* SPP. TO *BREMIA LACTUCAE*

Michaela Sedlářová[a,]*, Lenka Luhová[b] and Aleš Lebeda[a]

[a]*Department of Botany and* [b]*Department of Biochemistry, Palacký University, Šlechtitelů 11, 783 71 Olomouc, Czech Republic*

** E-mail: sedlarova@prfholnt.upol.cz*

Pathogen infection as well as action of other stress inductors are followed by excess production of active oxygen species (AOS) in host plants[1]. Plants employ several defence mechanisms which operate either by preventing the formation of AOS or by scavenging them once they are formed. Plants have evolved non-enzymatic and enzymatic protection mechanisms that efficiently scavenge AOS. Enzymatic antioxidant system includes superoxide dismutase, peroxidases, catalase, monodehydroascorbate reductase, glutathion reductase, etc. Mechanisms of plant resistance to oomycete pathogens (including race-specificity but even non-host and field resistance) are mostly associated with hypersensitive reaction (HR)[2]. This form of programmed cell death follows perception of pathogen *Avr* genes products and is conditioned by oxidative burst in limited number of cells at the site of attempted infection[3]. AOS are discussed to operate both in signalling during the phase of recognition and irreversible membrane damage initiating HR (peroxidation of lipids), destruction of proteins and DNA, enzyme inhibition[4].

Six genotypes of *Lactuca* spp. as follows: *L. sativa* (UCDM2, Mariska), *L. serriola* (LSE/18, PIVT 1309), *L. saligna* (CGN 05271) and *L. virosa* (NVRS 10.001602) inoculated by *Bremia lactucae* race NL16 were subjected to AOS histochemical detection and time-course studies (6 to 96 hours after inoculation) of antioxidant enzymes such as catalase, peroxidase (POX) and superoxide dismutase (SOD). Genotypes UCDM2 (*L. sativa*) and LSE/18 (*L. serriola*) are susceptible to *B. lactucae*

race NL16, others express resistance differing in defence mechanisms[5]. Differences were found among and within studied *Lactuca* spp. genotypes in timing and extent of oxidative changes after challenge by *B. lactucae.* A dark blue insoluble precipitate of NBT[6] caused by production of $O_2^{\cdot -}$ (supposed signaling role) was not observed at any studied model interaction of *Lactuca* spp. - *B. lactucae*. H_2O_2 was visualized by reaction with 3,3´-diaminobenzidine (DAB), control experiments were performed in the presence of ascorbate (scavenger of H_2O_2)[7]. Visualisation of POX activity was performed by vacuum infiltration of DAB and H_2O_2 solution into leaves[8]. Steady weak signal of POX and H_2O_2 was characteristic for all *L. sativa* and *L. serriola* genotypes. Only in resistant genotypes stronger accumulation of H_2O_2 occurred at 48-96 hai (hours-after-inoculation). Biochemical assays of POX[9] revealed weak activities with enhancement at 6 hai and slight steady increase from 36 hai and almost no changes of catalase activity[10] in both *L. sativa* genotypes and compatible LSE/18 (*L. serriola*). In resistant PIVT 1309 the activity of POX culminated at 24-36 hai (correlation with histological data) and catalase culminated at 6 hai. In *L. saligna* an intensive signal for H_2O_2 was detected early after inoculation (6-12 hai), POX signal in infected leaves increased only at 18 hai with gradation from 24 to 48 hai, when the activity of enzyme was the highest. Catalase activity slightly increased until 18 hai. Prompt formation and extensive spread of hydrogen peroxide characterize resistance of *L. virosa* (consistent with phenotypic expression by HR). Timing of H_2O_2 accumulation and POX activation correlated, being enhanced early after contact with pathogen (6-12 hai), decreased at 18 hai to rise slightly again at 30-36 hai (attending pathogen growth retardation by HR) and subside at 48 hai. Catalase showed tendency to increase until 36 hai and to decrease later on.

In general, incompatible interactions proved biphasic oxidative burst, whereas monophasic was found in compatible ones. The absolute values of enzyme activities in resistant wild *Lactuca* spp. were approximately twice higher when compared to others. Superoxide was not assessed histochemically in none of the studied genotypes of *Lactuca* spp. Failure to detect its accumulation might indicate that either $O_2^{\cdot -}$ is not an intermediate or it is rapidly dismutated.

References

1. Grant J. J. and Loake G. J.: *Plant Physiol.* **124**, 21 (2000)
2. Kamoun S., Huitema, E. and Vleeshouwers V. G. A. A.: *Trends Plant Sci.* **4**, 196 (1999)
3. Greenberg J. T.: *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **48**, 525 (1997)
4. Neill S., Desikan R. and Hancock J.: *Curr. Opin. Plant Biol.* **5**, 388 (2002)
5. Lebeda A. and Sedlářová M.: In: *Proceedings of Eucarpia Leafy Vegetables Conference* 2003, CGN Wageningen (The Netherlands), 55 (2003)
6. May M. J., Hammond-Kosack K. E. and Jones J. D. G.: *Plant Physiol.* **110**, 1367 (1996)
7. Thordal-Christensen H., Zhang Z., Wei Y. and Collinge D. B.: *Plant J.* **11**, 1187 (1997)
8. Alcázar M. D., Egea C., Espín A. and Candela M. E.: *Physiol. Plant.* **94**, 737 (1995)
9. Angelini R., Manes F. and Federico R.: *Planta* **182**, 89 (1990)
10. Góth L.: *Clin. Chim. Acta* **196**, 143 (1991)

Acknowledgement

The work was supported by the grants 522/02/D011 from the Grant Agency and MSM 153100010 from the Ministry of Education, Youth and Sports, Czech Republic.

1P07

RESTRICTION-MODIFICATION SYSTEM RCPSBI FROM *RHODOBACTER CAPSULATUS* SB1003

Hynek Strnad[a,*], Věra Jenčová[a], Zdeněk Chodora[a] and Václav Pačes[a]

[a]*Department of Biochemistry and Microbiology, Institute of Chemical Technology Prague, Technická 3, 166 28 Prague 6, Czech Republic*

[b]*Institute of Molecular Genetics, Academy of Science, Czech Republic*

** E-mail: strnad@img.cas.cz*

All bacteria are known to harbor restriction-modification systems as a defensive mechanism against inappropriate invasion of foreign DNA. Methylases typically alter specific nucleotides by addition of a methyl group, while restriction enzymes cut DNA that does not have correct methylation pattern of the species.

Rhodobacter capsulatus belongs to the group of purple nonsulfur bacteria and is broadly used model organism in studies of fotosynthesis and nitrogen fixation. Three different clusters of genes encoding type I (RcpSBI, RcpSBII) and type III (RcpSBIII) DNA restriction - modification systems were found in complete nucleotide sequence of this bacteria. Here we describe genetic and biochemical analysis of restriction-modification system RcpSBI. This cluster comprises of three genes for: DNA sequence specificity subunit (HsdS), modification subunit (HsdM) and restriction subunit (HsdR).

Expression vectors carrying hsdS, *hsd*M, *hsd*R genes and their combinations were constructed. The hsdS and hsdM genes were expressed together in *E. coli* BL21 (DE3) and produce active RcpSBI methylase complex (MTase) which consist of one HsdS subunit (44.2 kDa) and two HsdM subunits (54.3 kDa). Proteins were isolated and purified by using ammonium sulphate precipitation, affinity chromatography on heparine and cibacron sepharose. The isoelectric point was determined to be 5.9. The HsdS:HsdM stoichiometry of MTase complex was verified as 1:2. By using radioactive substrate SAM and different DNA templates the recognition sequence was determined as AGAN7RTAG. New software developed specifically for the sequence determination from measured data, was used. Based on the knowledge of the recognition sequence preliminary kinetic data for DNA methylation was measured. The results were compared with the data known for MTases from other organisms. Endonuclease activity of RcpSBI restrictase (ENase) was not measured due to insolubility of HsdR subunit after cell lysis.

1P08

CHARACTERIZATION OF *ARABIDOPSIS* CYTOKININ DEHYDROGENASE EXPRESSED IN YEAST *SACCHAROMYCES CEREVISIAE* AND TOBACCO PLANTS

Jitka Frébortová[a,*], Hana Popelková[b], Petr Galuszka[b], Tomáš Werner[c], Jiří Drábek[b], Thomas Schmülling[c] and Ivo Frébort[b]

[a]*Laboratory of Growth Regulators, Palacký University/Institute of Experimental Botany, Academy of Sciences, Czech Republic, Šlechtitelů 11, CZ-78371 Olomouc, Czech Republic*
[b]*Division of Molecular Biology, Department of Biochemistry, Palacký University, Šlechtitelů 11, CZ-783 71 Olomouc, Czech Republic*
[c]*Institute of Biology-Applied Genetics, Free University of Berlin, Albrecht-Thaer-Weg 6, D-14195 Berlin, Germany*

** E-mail: frebortova@prfholnt.upol.cz*

Cytokinin dehydrogenase (CKX, EC 1.5.99.12) controls cytokinin levels in plants by oxidative cleavage of their side chain. The amount of cytokinin dehydrogenase in plants is generally very low and its isolation and purification is thus challenging. The cloning of CKX gene from maize[1,2] opened a new opportunity for study of CKX and cytokinin function in plants. In *Arabidopsis thaliana*, seven distinct genes coding for seven proteins (AtCKX1 to AtCKX7) were found. In present study we aim to prepare yeast strains for production of recombinant AtCKX2 and to characterize CKX enzymes in tobacco plants overexpressing individual enzymes from *Arabidopsis thaliana*.

AtCKX2 gene was cloned into pYES2 and pDR197 yeast expression plasmids or integrated into yeast genome using vectors YIplac211 and pYESX. The *Saccharomyces cerevisiae* strain 23344c (MATα ura3) was used. CKX was secreted into the media in all tested yeast systems, the constitutive and inducible promoter driven expression showed comparable enzymatic activity. SDS-PAGE electrophoresis of concentrated yeast media did not show major differences in protein composition produced by individual yeast strains, though some differences in relative amount of proteins could be observed. The highest specific CKX activity was obtained with pYESX system.

For characterization of individual CKXs, leaf extracts of wild-type and transgenic *Nicotiana tabaccum* L. cv. Samsun NN overexpressing *Arabidopsis* CKX genes AtCKX1 to AtCKX4[3], AtCKX6 and AtCKX7 genes, were used. The adult transgenic tobacco plants were tested for activity using various electron acceptors and substrates at various pH. Only AtCKX2 and AtCKX4 were found to be highly active proteins using N^6-(2-isopentenyl)adenine and its riboside as substrates and 2,6-dichlorophenol indophenol (DCPIP) or 2,3-dimethoxy-5-methyl-1,4-benzoquinone (Q_0) as electron acceptors, while activities of other CKXs were low. Furthermore, with these proteins no activity was observed without any artificial electron acceptor. The results indicate that the AtCKX1, AtCKX3, AtCKX6, and AtCKX7 enzymes probably use electron acceptors other than oxygen, DCPIP and Q_0. The AtCKX2 and AtCKX4 proteins were

further characterized with respect to the organ distribution of activity, pH optima and substrate specificity.

References

1. Morris R. O., Bilyeu K. D., Laskey J. G. and Cheikh N. N.: *Biochem. Biophys. Res. Commun.* **255**, 328 (1999)
2. Houba-Herin N., Pethe C., d'Alayer J. and Laloue M.: *Plant J.* **17**, 615 (1999)
3. Werner T., Motyka V., Strnad M. and Schmülling T: *Plant Biol.* **98**, 10487 (2001)

1P09

DEVELOPMENT OF HIGHLY EFFICIENT ENZYME REACTOR FOR DEGRADATION OF AZODYES IN THE WASTEWATER AFTER TEXTILE DYEING PROCESS

Jana Ježová[a,]*, Romana Šuláková[b], Radim Hrdina[b] and Zuzana Bílková[c]

[a]*Department of Analytical Chemistry,* [b]*Department of Technology of Organic Compounds and* [c]*Department of Biological and Biochemical Sciences, University of Pardubice, nám. Čs. legií 565, 53210 Pardubice, Czech Republic*

** E-mail: jana.jezova@upce.cz*

Laccases (*p*-diphenol:O_2 oxidoreductases) are multicopper enzymes catalysing the oxidation of *p*-diphenols with the concomitant reduction of molecular oxygen to water. Laccases are glycoproteins, usually monomeric, heterogeneous in their biochemical properties and molecular structures[1].

Soluble laccase was already used for the degradation of azodyes immediately after dyeing process. This way of degradation is not convenient in the textile industry due to the contamination and low stability of free form of enzyme molecules in the wastewater. The immobilization of laccase molecules on the appropriate supports is one of possible solution how to eliminate this disadvantage. Enzyme immobilization provides many advantages as: controlled product formation, enhanced enzyme activity, simplified and efficient processing, enzyme reusability[2] and high storage stability.

The choice of the matrix (chemical and magnetic properties, particle size and distribution, porosity) is a key factor influencing quality of the affinity reactor and the scope of final applications and/or process automation. The binding activity of reactors and economic feasibility of this process depends upon a combination of the immobilization efficiency and activity of the immobilized enzyme. Recently, magnetic spherical particles are gaining an increasing attention for highly efficient and gentle separation during the immobilization procedure.

Laccase was immobilized on various types of nonmagnetic and magnetic supports. Appropriate method of immobilization was chosen with regards to the operational and storage stability of immobilized enzyme, long-term efficiency and the economic availability of prepared reactor. Our aim is to optimise conditions of immobilization process on biopolymer cellulose, which is gaining an increasing attention in large-scale application not only due to cost. The main advantages of this support are the no toxicity, biodegradability and the price availability. Major objective of this research was to prepare stable and highly active laccase reactor even under extreme conditions as high concentration of salts, detergents, extreme level of pH, temperature.

References

1. Record E., Punt P. J., Chamkha M., Labat M., van den Hondel C. A. M. J. J. and Asther M.: *Eur. J. Biochem.* **269**, 602 (2002)
2. Bílková Z., Slováková M., Horák D., Lenfeld J. and Churáček J.: *J. Chromatogr. B* **770**, 177 (2002)

Acknowledgement

This work was supported by the grants 203/02/0023 from the Grant Agency and MSM 253100002 from the Ministry of Education, Youth and Sports, Czech Republic.

1P10

PROTEOMIC DETECTION OF SOYBEAN PROTEINS IN MEAT PRODUCTS

Zdeněk Tůma[a,*], Zbyněk Zdráhal[b] and Josef Chmelík[c]

[a]*Faculty of Chemistry, Brno University of Technology, Purkyňova 118, 612 00 Brno, Czech Republic*
[b]*Faculty of Science, Masaryk University, Kotlářská 2, 611 37 Brno, Czech Republic*
[c]*Institute of Analytical Chemistry, Academy of Sciences of the Czech Republic, Veveří 97, 61142 Brno*

** E-mail: tuma@iach.cz*

Soybean proteins (*Glycine max*) are widely used in animal and human nutrition. They have found many applications in many different food products. The use of soybean proteins as meat extenders has spread significantly due to their good nutritional and functional properties. Together with these, health and economical reasons are the major causes for the addition of soybean proteins to meat products. Despite the good technological and nutrition properties of soybean proteins, there are many countries in which the addition of these proteins is forbidden or in which the addition of soybean proteins is allowed up to a certain extent.

Electrophoretic, immunologic, and chromatographic methods are the most widely used for detection of soybean proteins in meat products. Soy isoflavonoids as markers of soybean addition can be analysed by using capillary zone electrophoresis. It is also possible to use PCR for detection of soy DNA in meat products. Soybean proteins in meat products can be detected and quantified by polyacrylamide gel electrophoresis (PAGE-SDS).

Proteomics is a powerful tool for identification of proteins separated by one or two-dimensional gel electrophoresis. We chose 1D polyacrylamide gel electrophoresis (PAGE-SDS) as a separation method with Coomassie blue staining.

Standard mixtures (beef meat, pork meat and soy isolate/concentrate) were prepared. Proteins extracted from these mixtures were separated by PAGE-SDS at the same time with meat protein extract and soy isolate/concentrate. Proteins from the molecular mass range between 31 – 35 kDa, 52 – 56 kDa and 68 kDa were selected for peptide mass mapping, because they correspond to molecular masses of soybean storage proteins (glycinin and conglycinin).

Selected protein spots from the lanes of both meat samples and soy isolate/concentrate were excised from the gel, digested by trypsin, and peptides were analysed by MALDI-TOF mass spectrometry. Alpha and alpha´-subunit of conglycinine was identified in the soy isolate. Several proteins (bovine albumin, porcine albumin and transferrin and bovine L-lactate dehydrogenase) were identified in the meat samples.

Acknowledgement

This work was supported by the grant 203/02/0023 from the Grant Agency of the Czech Republic.

1P11

THE CHARACTERIZATION OF THE BARLEY PROTEINS AND GLYCOPROTEINS BY USING SEPARATION METHODS AND MASS SPECTROMETRY

Markéta Štikařovská[a,*], Ivana Márová[a], Martina Marchetti[b], Günter Allmaier[b] and Josef Chmelík[c]

[a]*Faculty of Chemistry, Brno University of Technology, Purkyňova 118, 612 00 Brno, Czech Republic*
[b]*Institute of Chemical Technologies and Analysis, Vienna University of Technology, Getreidemarkt 9/164, A-1060, Vienna, Austria*
[c]*Institute of Analytical Chemistry, Academy of Sciences of the Czech Republic, Veveří 97, 61142 Brno*

** E-mail: stikarovska@fch.vutbr.cz*

This work deals with the combination of separation methods with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) for the characterization of the barley proteins. Barley (*Hordeum vulgare*) proteins were extracted from the flour with plain deionized water and the obtained total aqueous extract was subjected to separation on the affinity column with immobilized Concanavalin A. In this way the fraction of the glycoproteins (high-mannose and hybrid types N-glycoproteins) that have affinity for this kind of lectin were enriched. The individual proteins (glycoproteins) contained in the non-bounded fraction as well as bounded fraction were then separated by using gel electrophoreses (SDS-PAGE; Bis-TRIS gels; Coommasie staining). In the non-bounded fraction two proteins were selected and identified on the basis of peptide mass fingerprinting. AXIMA LNR (Kratos Analytical Shimadzu Biotech, Manchester, UK) was employed as the mass spectrometer and ProFound and MASCOT were used as the tools for peptide search. The first non-bounded protein was identified as "Protein z-type serpin" (P40076). It is a protein with 400 AA length and molecular mass of 43220 Da. The second protein was determined as the β-amylase. In the bound fraction several intensive bands were detected and a lot of minor bands with molecular masses between 10-100 kDa.

Acknowledgement

This work was supported by the grant FRVŠ G4/1684/2004.

1P12

INTERACTION OF PENT-2-YNE-1,5-DIAMINE WITH PLANT COPPER AMINE OXIDASES

Marek Šebela[a,*], Zbyněk Lamplot[a], Michal Maloň[b], René Lenobel[b], Karel Lemr[c], Jan Havliš[d], Pavel Peč[a] and Lawrence M. Sayre[e]

[a]*Department of Biochemistry, Faculty of Science, Palacký University, Šlechtitelů 11, CZ-783 71 Olomouc, Czech Republic*
[b]*Laboratory of Growth Regulators, Faculty of Science, Palacký University, Šlechtitelů 11, CZ-783 71 Olomouc, Czech Republic*
[c]*Department of Analytical Chemistry, Faculty of Science, Palacký University, Tř. Svobody 8, CZ-771 46 Olomouc, Czech Republic*
[d]*Department of Analytical Chemistry, Faculty of Science, Masaryk University, CZ-61137 Brno, Czech Republic*
[e]*Department of Chemistry, Case Western University, Cleveland, Ohio, OH 44106, USA*

** E-mail: sebela@prfholnt.upol.cz*

Plant copper-containing amine oxidases (EC 1.4.3.6) catalyze the oxidative deamination of biogenic amines, which are known as cellular regulators, to form the corresponding aldehydes, ammonia and hydrogen peroxide. The enzymes are dimeric proteins containing both cupric ion and topaquinone as cofactors in each of the two subunits. They have been localized in the cell wall. Thus their physiological role, except for that in the catabolism of amines, may be seen in connection with the production of hydrogen peroxide that is utilized in peroxidase-mediated reactions in this cell compartment[1]. Here we report a study on the reaction of grass pea (*Lathyrus sativus*, GPAO) and sainfoin (*Onobrychis viciifolia*, OVAO) amine oxidases with pent-2-yne-1,5-diamine (DAPY). DAPY reacted with the topaquinone cofactor and was only weakly oxidized. Prolonged incubations, however, resulted in irreversible inhibition of the enzymes. For GPAO and OVAO, the rates of inactivation of 0.1-0.3 min^{-1} were determined, the apparent K_I values (half-maximal inactivation) were of 10^{-5} M. DAPY was found to be a mechanism-based inhibitor of the enzymes, since the substrate cadaverine significantly prevented from the irreversible inhibition by primary DAPY oxidation product – an unsaturated aldehyde. When GPAO or OVAO solutions were incubated with DAPY for a longer time, they turned yellow-brown (λ_{max} = 310-325 nm depending on the working buffer). After dialysis, the color intensity was substantially decreased indicating the formation of a low-molecular-weight secondary product. To find its chemical structure, several analytical methods were involved. The compound provided positive reactions with ninhydrin, 2-aminobenzaldehyde and Kovac´s reagents suggesting a nitrogen-containing heterocyclic structure. When excited at 310 nm, it emitted fluorescence with a maximum at 445 nm similarly to pyrrole- and dihydropyridine-derived models. The secondary product was finally isolated using chromatographic methods. The compound did not irreversibly inhibit GPAO and its exact molecular weight was found to be 177.1377 by ESI Q TOF MS. Mass

spectrometry analyses (ESI Q TOF MS, MALDI PSD TOF MS) yielded fragment mass patterns consistent with the structure of a dihydropyridine derivative of DAPY and this was also confirmed by NMR experiments revealing *N*-(2,3-dihydropyridinyl)-pent-2-yne-1,5-diamine.

References

1. Šebela M., Frébort I., Petřivalský M. and Peč P.: In *Studies in Natural Products Chemistry, Vol. 26 - Bioactive Natural Products, Part G* (Atta-ur-Rahman, ed.), Elsevier, Amsterdam 2002, p. 1259

Acknowledgement

This work was supported by the grants MSM 153100010, MSM 153100013 and 14BI3 (Czech-Italian cooperation, a joint grant with the Ministry of Foreign Affairs, Italy) from the Ministry of Education, Youth and Sports, Czech Republic.

1P13

REACTIONS OF PLANT AMINE OXIDASES WITH N^6-AMINOALKYL DERIVATIVES OF ADENINE

Zbyněk Lamplot[a,*], Marek Šebela[a], Michal Maloň[b], Petr Fryčák[c], Silvia Longu[d], Alessandra Padiglia[d], Rosaria Medda[d], Giovanni Floris[d] and Pavel Peč[a]

[a]*Department of Biochemistry, Faculty of Science, Palacký University, Šlechtitelů 11, CZ-783 71 Olomouc, Czech Republic*
[b]*Laboratory of Growth Regulators, Faculty of Science, Palacký University, CZ-783 71 Olomouc, Czech Republic*
[c]*Department of Analytical Chemistry, Faculty of Science, Palacký University, CZ-771 46 Olomouc, Czech Republic*
[d]*Department of Applied Sciences in Biosystems, University of Cagliari, Città Universitaria, I-09042 Monserrato (CA), Italy*

** E-mail: lamplot@email.cz*

Plant copper-containing amine oxidases (CAOs, EC 1.4.3.6) catalyze the oxidation of various amines. Once there was a hypothesis that the enzymes could oxidize cytokinins - plant hormones derived from adenine[1], however, it has been shown that they rather cause inhibition[2]. Here we report a study on the reactions of three plant CAOs from grass pea (*Lathyrus sativus*), lentil (*Lens esculenta*) and a Mediterranean shrub (*Euphorbia characias*) with synthetic *N*^{6}-aminoalkyl derivatives of adenine representing combined analogues of cytokinins and polyamines. The following compounds were prepared using an established method: *N*^{6}-(3-aminopropyl)adenine, APAD; *N*^{6}-(4-

aminobutyl)adenine, ABAD; N^6-(4-amino-*trans*-but-2-enyl)adenine, ATBAD; N^6-(4-amino-*cis*-but-2-enyl)adenine, ACBAD and N^6-(4-aminobut-2-ynyl)adenine, ABYAD. First, reaction rates of enzymatic oxidation were measured to decide between substrate and inhibitory properties. Two of the studied adenine derivatives were found to be substrates (ABAD, ATBAD) and the corresponding K_m values and reaction stoichiometry were measured. Surprisingly, ATBAD oxidation product caused inhibition of the enzymes and this was reflected in an unusual stoichiometry obtained 0.3 mol oxygen consumed in oxidation of 1 mol substrate. Absorption spectra recorded after the additions of ABAD or ATBAD to the enzymes clearly confirmed the interaction of the compounds with the cofactor topaquinone. Anaerobic absorption spectra of APAD, ACBAD and ABYAD reactions revealed a similar kind of initial interaction, although the compounds finally inhibited the enzymes. Kinetic measurements allowed determining both type and strength of those inhibitions. APAD and ACBAD seem to be reversible competitive inhibitors of all three enzymes. Instead, ABYAD fulfils criteria of a mechanism based inactivator.

References

1. Hare P. D. and van Staden J.: *Physiol. Plant*. **91**, 128 (1994)
2. Galuszka P. Šebela M., Luhová, L., Zajoncová L., Frébort I., Strnad M. and Peč P.: *J. Enzym. Inhib*. **13**, 457 (1998)

Acknowledgement

This work was supported by the grant MSM 153100010 from the Ministry of Education, Youth and Sports, Czech Republic and by the joint project 14BI3 within a bilateral scientific co-operation (granted by Czech Ministry of Education, Youth and Sports and Italian Ministry of Foreign Affairs).

1P14

ISOLATION AND PURIFICATION OF MICROBIAL RIBONUCLEASE INHIBITOR

Bronislava Uhnáková*, Vladena Hlinková, Juraj Gašperík and Jozef Ševčík

Institute of Molecular Biology, Slovak Academy of Sciences, Dúbravská cesta 21, 84551 Bratislava, Slovak Republic,

** E-mail: umikaqua@savba.sk*

Structures of the enzyme-inhibitor complexes have been extensively studied in the aim to understand protein-protein interaction and recognition. The structure of the complex formed by RNase Sa from *Streptomyces aureofaciens* strain BMK and wild-

type inhibitor barstar from *Bacillus amyloliquefaciens* strain H2 was solved in our laboratory[1]. Barstar (M_r ~ 10,212), specific inhibitor of barnase, a ribonuclease from *B. amyloliquefaciens* H2, contains two cysteines at position 40 and 82, which do not form disulfide bond[2]. Barstar forms tight one-to-one non-covalent complex with RNase Sa (K_D ~ 10^{-10} M) and sterically blocks its active site. The complex is stabilized by electrostatic interactions, hydrogen bonds and van der Waals contacts.

Streptomyces aureofaciens strain R8/26 produces two intracellular polypeptide inhibitors of a ribonuclease[3]. The function of inhibitors is to prevent ribonuclease from being active prior its secretion. In spite of low identity of amino-acid sequences of barstar, SaI14 and SaI20 which is only about 13 %, streptomycete inhibitors inhibit also barnase with K_D ~ 10^{-10} M. The genes of both ribonuclease inhibitors SaI14 and SaI20 were cloned into pET 28a vector and expressed in *E. coli* B834. Wild-type inhibitor SaI14 (M_r ~ 14,034) contains two cysteines at position 41 and 70 and in solution forms polymers via intermolecular disulfide bonds. The SaI20 (Mr ~ 11,815) contains only one cysteine at position 29. It was shown, that in solution it forms dimers via intermolecular disulfide bond. The dimers of SaI20 inhibitor are inactive. To prevent dimerization of SaI20, Cys29Ala mutant was prepared. The modified gene of SaI20 was cloned and expressed in the same system as the gene of the wild-type SaI20. Production of mutated inhibitor during 20 hours at 24°C was induced by adding IPTG to the concentration of 0.1 mM. The protein was purified using precipitations by streptomycin sulfate and ammonium sulfate followed by gel filtration on Sephadex G50 and ion-exchange chromatography on Mono Q column, yielding approximately 30 mg of purified protein from 9.0 g of cells. SDS-polyacrylamide and native gel electrophoresis confirmed purity of the inhibitor. Crystallization of modified SaI20 inhibitor, as well as its complex with RNase Sa is under way.

References

1. Hartley R. W.: *J. Mol. Biol.* **202**, 913 (1988)
2. Ševčík J., Urbániková Ľ., Dauter Z. and Wilson K. S: *Acta Cryst.* **D54**, 954 (1998)
3. Krajčíková D., Hartley R. W. and Ševčík J.: *J. Bacteriol.* **180**, 1582 (1998)

1P15

STUDY OF THE REACTIVE OXYGEN SPECIES AND ANTIOXIDANT ENZYMES DURING *OIDIUM NEOLYCOPERSICI* INFECTION ON *LYCOPERSICON* SPP.

Kateřina Mlíčková[a,]*, Lenka Luhová[a], Pavel Peč[a] and Aleš Lebeda[b]

[a]*Department of Biochemistry and* [b]*Department of Botany, Faculty of Science, Palacký University, Šlechtitelů 11, 78 371 Olomouc, Czech Republic*

** E-mail: katrynka@email.cz*

Oidium neolycopersici L. Kiss (tomato powdery mildew) is a biotrophic parasitic fungus occurring on greenhouse tomato crops in Europe and in the New World[1]. Extensive investigations have failed to detect resistance among existing tomato (*Lycopersicon esculentum* L.) cultivars[1] and this has led to the search for resistance among wild *Lycopersicon* species[2]. However, very little information exists on resistance mechanisms in *Lycopersicon* spp. where some physiological and biochemical aspects play an important role[3]. For example production of reactive oxygen species (ROS: H_2O_2, $O_2^{\cdot-}$, $OH^{\cdot}$) and low molecular antimicrobial compounds (phenols, chinones, alkaloids) was followed by hypersensitive reaction of challenged plant cells[4]. The role of catalase (EC 1.11.1.6.) in the protection of plant cells against toxic H_2O_2 and the participation of various peroxidases (EC 1.11.1.7.) in stress-related physiological processes has been demonstrated[5].

In this study, we report on some aspects of defence responses of three *Lycopersicon* spp. (*L. esculentum* cv. Amateur – susceptible control, *L. hirsutum* (LA 2128) – resistant, and *L. chmielewskii* (LA 2663) – moderately resistant) to *O. neolycopersici* from the viewpoint of ROS production and function of antioxidant enzymes. Experiments were focused to quantification of H_2O_2 and phenolic compounds, lignification, extent of cell death (hypersensitive response) and changes in activity of various antioxidant enzymes (peroxidases, catalase) during *O. neolycopersici* infection. Modified method using KI demonstrated intensive H_2O_2 production in resistant *Lycopersicon* accessions in two phases, early after inoculation (4-8 hpi) and again 24-120 hpi, suggesting its signalling role in plant-pathogen recognition and HR. In susceptible *L. esculentum* cv. Amateur, very low concentrations of H_2O_2 were detected. These results well correspond with previous histochemical experiments[6]. Cytosolic, membrane- and ionic-bound peroxidase activity was measured by a modified method with guaiacol and syringaldazine. There was a relationship between production of H_2O_2 and the activity of guaiacol peroxidase (in resistant accessions increased at 8-12 hpi and again at 48-120 hpi). The activity of cytosolic enzymes was two folds higher than the activity of membrane- and ionic-bound enzymes. In late phases of infection, substantial increase of membrane-bound guaiacol peroxidase activity was recorded. Syringaldazine peroxidase increased at 48-120 hpi and its activity was half compared with the activity of guaiacol peroxidase. Considerable increase in ascorbate peroxidase activity, measured using modified method with ascorbate, was observed at 48 hpi in both resistant accessions. The assay of lipid

peroxidase activity, according modified method using thiobarbituric acid, revealed increase in enzyme activity in all investigated *Lycopersicon* accessions. However, the activity of this enzyme was lower in susceptible control than in resistant accessions. Catalase was detected by assay of H_2O_2 based on the formation of its stable complex with ammonium molybdenate. The increase in catalase activity during pathogenesis was characteristic for resistant accessions (*L. hirsutum* - 3 times, *L. chmielewskii* - 10 times increase at 120 hpi). The substantial increase in peroxidase and catalase activity at 120 hpi is probably linked to intensive H_2O_2 production and the cell death observed at this time in the tissue of *L. chmielewskii* and *L. hirsutum*. Production of phenolic compounds was quantified using Folin-Ciocalteau assay and no changes in accumulation of cell wall bound phenols were observed during 120 hpi. On the opposite, the level of free cytosolic phenols increased at 4-8 hpi and again at 24-120 hpi in all studied *Lycopersicon* spp.

The results of this study indicate to reciprocal relation among intensity of H_2O_2 and phenolic compounds formation, cell death, changes of activities of antioxidant enzymes, their timing and resistance level of studied *Lycopersicon* accessions.

References

1. Mieslerová B. and Lebeda A.: *J. Plant Dis. Protect.* **106**, 140 (1999)
2. Lebeda A. and Mieslerová B.: *J. Plant Dis. Protect.* **109**, 129 (2002)
3. Lebeda A., Mieslerová B., Luhová L. and Mlíčková K.: *Plant Protect. Sci.* **38** (Special Issue 1), 141 (2002)
4. Levine A., Tenhaken R., Dixon R. and Lamb C.: *Cell* **79**, 583 (1994)
5. Mehdy M. C.: Plant Physiol. 105, 467 (1994)
6. Mlíčková K., Sedlářová M., Luhová L., Peč P. and Lebeda A.: *Biologické Listy* **68**, 212 (2003)

Acknowledgement

This work was supported by the grant MSM 153100010 from the Ministry of Education, Youth and Sports of the Czech Republic.

1P16

IS THE DEGRADATION OF CYTOKININS IN PLANTS ASSISTED BY PHENOLICS?

Ivo Frébort[a,*], Petr Galuszka[a], Jitka Frébortová[b], Hana Popelková[a], Jiří Drábek[a] and Kristin D. Bilyeu[c]

[a]*Division of Molecular Biology, Department of Biochemistry, Faculty of Science, Palacký University, Šlechtitelů 11, CZ-78371 Olomouc, Czech Republic*
[b]*Laboratory of Growth Regulators, Palacký University/Institute of Experimental Botany, Academy of Sciences, Šlechtitelů 11, CZ-78371 Olomouc, Czech Republic*
[c]*USDA/ARS, University of Missouri, 210 Waters Hall, Columbia, MO 65211, USA*

** E-mail: frebort@prfholnt.upol.cz*

Degradation of cytokinins in plants is controlled by flavoprotein cytokinin dehydrogenase (CKX, EC 1.5.99.12). Cleavage of the cytokinin substrate proceeds via a ternary complex involving covalently bound FAD cofactor and a quinonic electron acceptor [1,2]. Maize kernels 3 weeks after pollination and 2-week-old maize seedlings were examined. CKX activity was monitored using a spectrophotometric assay of the product aldehyde with 4-aminophenol[3]. Histochemical localization of CKX activity was done with PMS/NBT redox-dye system. Activity of laccase was stained with 3,3'-diaminobenzidine. Immunochemical detection of CKX protein was done using specific antibody against the enzyme from maize [4]. CKX from maize showed the capability of using oxidation products of guaiacol, catechol, caffeic acid, acetosyringone, some flavonoids and several other compounds as electron acceptors. Further experiments revealed that the electron acceptors may be generated *in vivo* from plant phenolics by other enzymatic systems, e.g. peroxidase or laccase/catechol oxidase. Histochemical localizations of CKX by activity staining and immunochemistry using optical and confocal microscopy showed that CKX is most abundant in aleurone layer of maize kernels and in phloem cells of the seedling shoots. Co-staining of laccase/catechol oxidase activities in those tissues suggests possible cooperation of the enzymes in cytokinin degradation. Presence of unknown low molecular electron acceptors of CKX was detected in phloem exudates collected from maize seedlings.

References

1. Bilyeu K. D., Laskey J. G. and Morris R. O.: *Plant Growth Regul.* **39**, 195 (2003)
2. Frébortová J., Fraaije M. W., Galuszka P., Šebela M., Peč P., Hrbáč J., Bilyeu K. D., English J. T. and Frébort I.: *Biochem. J.* **380**, 121 (2004)
3. Frébort I., Šebela M., Galuszka P., Werner T., Schmülling T. and Peč P.: *Anal. Biochem.* **306**, 1 (2002)
4. Morris R. O., Bilyeu K. D., Laskey J. G. and Cheikh N. N.: *Biochem. Biophys. Res. Commun.* **255**, 328 (1999)

Acknowledgement

This work was supported by the grant 522/03/0979 from the Grant Agency, Czech Republic.

1P17

PREPARATION OF A SPECIFIC ANTI-AMINOALDEHYDE DEHYDROGENASE ANTIBODY AND ITS APPLICATION FOR MICROSCOPIC LOCALISATION OF THE ENZYME FROM PEA

František Brauner[a,*], Lenka Luhová[a], Marek Šebela[a], Radek Novotný[b], Ludmila Zajoncová[a], Riccardo Angelini[c] and Pavel Peč[a]

[a]*Department of Biochemistry, Faculty of Science, Palacký University, Šlechtitelů 11, CZ-783 71 Olomouc, Czech Republic*
[b]*Department of Microscopic Methodology, Faculty of Medicine, Palacký University, I. P. Pavlova 35, CZ-775 20 Olomouc, Czech Republic*
[c]*Department of Biology, University „Roma Tre", Viale Guglielmo Marconi 446, I-00146 Rome, Italy*

** E-mail: brauner@gytool.cz*

The studied enzyme - aminoaldehyde dehydrogenase (AMADH, EC 1.2.1.19) from pea (*Pisum sativum*) - belongs to the group of NAD^+-dependent dehydrogenases[1]. It catalyzes the oxidation of aminoaldehydes to the corresponding amino acids. It is involved in catabolic pathways of polyamines, which have been studied due to their regulatory properties in living organisms. Polyamines are oxidatively degraded by amine oxidases. There are two types of these enzymes: Cu-amine oxidases specific for diamines (DAO, EC 1.4.3.6.) and FAD-polyamine oxidases (PAO, EC 1.5.3.-.)[2,3]. Spermidine and spermine oxidation by PAO brings about the formation of 4-aminobutyraldehyde (ABAL) and N-(3-aminopropyl)-4-aminobutyraldehyde (APBAL), respectively, with the additional formation of 1,3-diaminopropane and H_2O_2. The diamines 1,3-diaminopropane and putrescine are converted to 3-aminopropionaldehyde (APAL) and ABAL, respectively. DAO participate in putrescine oxidation, however, the enzymatic system metabolising 1,3-diaminopropane in plants have not been thoroughly studied up to date. APAL and ABAL are further metabolised by AMADH to β-alanine and γ-butyric acid - GABA[1]. Plant AMADH has been found in many representatives of Fabaceae and Poaceae. Properties of the pea enzyme has recently been characterised and its role in polyamine metabolism discussed[4].

AMADH localisation was studied in pea seedlings. An antiserum raised against purified pea AMADH protein was used to study the tissue distribution of this enzyme using immunocytochemical methods. The specificity of the polyclonal anti-AMADH rabbit antibody was verified by both immunoblotting and Ouchterlony immunodiffusion method. As a result, the antiserum could be used for immunocytochemical detections without any prior purification. Immunocytochemical localisation of AMADH under light microscope was carried out by an immunogold technique. The analysis confirmed previously published results obtained by a histochemical method, where the enzyme was visualised in tissues with the help of a nitroblue tetrazolium-based staining system[5]. Pea AMADH was localised in the roots, hypocotyls and epicotyls of etiolated pea seedlings. The enzyme was particularly seen in cells belonging to the pericycle, vascular cambium

and endodermis. A weak signal of gold particles was also found in the epidermis and rhizodermis.

At the cellular level, the distribution of AMADH was studied by transmission electron microscopy. Gold particles were observed in the intracellular space, particularly in the cytoplasm and vacuoles. No signal was observed in the nucleus, endoplasmic reticulum, Golgi apparatus, mitochondria, peroxisome, chloroplast and the other plastids. AMADH protein was also localised in the cell wall. Our results indicate that AMADH may participate in the reactions of GABA production in the cytoplasm. In the cell wall, the enzyme probably functions in polyamine degaradation pathway initiated by the reaction of amine oxidase.

References

1. Awal H. M. A., Yoshida Y., Doe M. and Hirasawa E.: *Phytochemistry* **40**, 393 (1995)
2. Tiburcio A. F., Altabella T., Borrell A. and Masgrau C: *Physiol. Plant*. **100**, 664 (1997)
3. Šebela M., Radová A., Angelini R., Tavladoraki P., Frébort I. and Peč P.: *Plant Sci.* **160**, 197 (2001)
4. Šebela M., Brauner F., Radová A., Jacobsen S., Havliš J., Galuszka P. and Peč P.: *Biochim. Biophys. Acta* **1480**, 329 (2000)
5. Šebela M., Luhová L., Brauner F., Galuszka P., Radová A. and Peč P.: *Plant Physiol. Biochem.* **39**, 831 (2001)

Acknowledgement

This work was supported by the grant MSM 153100010 from the Ministry of Education, Youth and Sports of the Czech Republic and by the project 14BI6 within Czech and Italian bilateral scientific co-operation (granted by the Czech Ministry of Education, Youth and Sports and Italian Ministry of Foreign Affairs).

1P18

AMINOALDEHYDE DEHYDROGENASE ACTIVITY IN DEFENCE RESPONSES TO MECHANICAL INJURY OF PEA SEEDLINGS

Lenka Luhová*, František Brauner, Marek Petřivalský, Marek Šebela and Pavel Peč

Department of Biochemistry, Faculty of Science, Palacký University, Šlechtitelů 11, 78371 Olomouc, Czech Republic

** E-mail: luhova@prfholnt.upol.cz*

Polyamine catabolism in plants is mediated by amine oxidases. There are two types of these enzymes: copper-containing (Cu-AOs, EC 1.4.3.6) and FAD-containing (polyamine oxidases, PAOs, EC 1.5.3.-). Besides hydrogen peroxide, aminoaldehydes are formed in the reactions of Cu-AOs and PAOs[1]. These aminoaldehydes are further metabolised by NAD^+-dependent aminoaldehyde dehydrogenases (AMADHs, EC 1.2.1.19) to the corresponding amino acids[2]. Pea seedling AMADH has recently been purified to homogeneity and characterised in detail[3]. Plant Cu-AOs and PAOs are associated with the cell wall. A coupled function has been suggested for these enzymes and peroxidase (POD) localised in the apoplast. Both Cu-AOs and PAOs could represent sources of H_2O_2 for cross-linking reactions mediated by the peroxidase and thus may play an important physiologic role. A close relationship between the ligno-suberisation process and response to pathogen invasion catalysed by POD and AOs, which occur during wound healing of injured plants, has been reported[4]. AMADH activity has been localised in cells belonging to the pericycle, endodermis and vascular cambium in different organs of pea seedlings[5].

The influence of mechanical stress on the activity of the mentioned enzymes in plants was investigated. First we studied the distribution of Cu-AO, POD and AMADH activities in intact pea seedlings. A gradual decrease in Cu-AO and POD activities was observed from the first to the fourth internode. Interestingly, AMADH activity was similar in all the internodes. A significant increase in Cu-AO and POD activities appeared after a mechanical injury of the fourth internode. In the same plants, AMADH activity increased until the fourth day after the healing processes had started and then it decreased. In control plants, AMADH activity could not be detected in the epidermis by histochemical methods. However, a violet colour that appeared at the place of the mechanical injury signalised an increased activity of this enzyme in wounded plants. This clearly showed that this enzyme also participates in plant response against stress. We further focused on the production of polyamines and γ-aminobutyric acid (GABA) in the wounded plants. A major part of GABA production in plant is covered by the reaction of glutamate decarboxylase. However, there is another minor pathway, which originates from putrescine oxidation to 4-aminobutyraldehyde[6]. Polyamine concentration reached a maximum on the second and third day after the injury. During healing of the wounded pea plants, a large accumulation of GABA was observed, namely in the first three days after the mechanical injury. In conclusion, the results obtained with

polyamines and GABA correlated well with the observed time distribution of activity changes of Cu-AO, POD and AMADH.

References

1. Frébort I. and Adachi O.: *J. Ferment Bioeng.* **80**, 625 (1995)
2. Šebela M., Frébort I., Petřivalský M. and Peč P.: In *Studies in Natural Products Chemistry, Vol. 26 - Bioactive Natural Products, Part G* (Atta-ur-Rahman, ed.), Elsevier, Amsterdam 2002, p.1259
3. Šebela M., Brauner F., Radová A., Jacobsen S., Havliš J., Galuszka P. and Peč P.: *Biochim. Biophys. Acta* **1480**, 329 (2000)
4. Scalet M., Federico R. and Angelini R.: *J. Plant Physiol*. **137**, 571 (1991)
5. Šebela M., Luhová L., Brauner F., Galuszka P., Radová A. and Peč P.: *Plant Physiol. Biochem.* **39**, 831 (2001)
6. Shelp B. J., Bown A. W. and McLean M. D.: *Trends Plant Sci.* **4**, 446 (1999)

Acknowledgement

This work was supported by the grant MSM 153100010 from the Ministry of Education, Youth and Sports of the Czech Republic.

1P19

OCCURRENCE AND LOCALISATION OF AMINOALDEHYDE DEHYDROGENASE ACTIVITY IN POACEAE AND FABACEAE PLANTS

Lenka Luhová*, Jana Procházková, František Brauner, Marek Šebela and Pavel Peč

Department of Biochemistry, Faculty of Science, Palacký University, Šlechtitelů 11, 783 71 Olomouc, Czech Republic

** E-mail: luhova@prfholnt.upol.cz*

Polyamines are regulatory substances involved in key developmental processes[1,2]. The catabolic regulation of polyamines is mediated by quinoprotein Cu-amine oxidases (Cu-AOs; EC1.4.3.6) and flavoprotein FAD-polyamine oxidases (PAOs; EC 1.5.3.-)[2,3]. In plants, these enzymes produce aminoaldehydes, which are further metabolised by NAD^+-dependent aminoaldehyde dehydrogenases (AMADHs, EC 1.2.1.19 or 1.2.1.54). AMADHs have been found in legumes and grasses[4], the enzymes from pea and oats were purified to homogeneity and characterised[5,6]. Pea AMADH has been localised in tissues of etiolated seedlings using histochemistry[7].

Here we report a screening study directed to the occurrence of AMADH in various plant species belonging to legumes (Fabaceae) and grasses (Poaceae). Fenugreek was chosen as a legume, the cereals barley, maize, oats, rye, triticale and wheat were

representatives of grasses. 3-Aminopropionaldehyde (APAL) served as a substrate for AMADH assay. Activity level of the enzyme was significantly higher in fenugreek then in the rest of the listed plants. As regards to activity distribution, significant differences were demonstrated. In the studied grasses, highest AMADH activity was measured in the roots. An inverse trend was found for fenugreek, where the activity appeared particularly in the above-ground parts of seedlings. AMADH activity changes during early development stages of the studied plants (2-14 days after germination of seeds) were similar in both roots and above-ground parts. The highest activity was detected in 2-day-old seedlings, and then a slow decrease followed until the day 5. A new flat activity maximum was observed between the days 6 and 9.

Using a nitroblue tetrazolium-based staining system, AMADH activity in all the studied cereals was histochemically detected in the central cylinder of the root, coleoptile and primary leaf. With the exception of wheat, AMADH activity was also localised in the root endodermis. Wheat, barley and oat additionally provided a positive staining in the leaf epidermis. In triticale, interestingly, the activity was further visualised in the rhisodermis. The histochemical localisation of AMADH in the legume fenugreek corresponded well to previously published results obtained with pea seedlings, where the enzyme was visualised in cells belonging to the pericycle, vascular cambium and endodermis[7]. However, a weak colour signal was additionally found in the epidermis and rhizodermis.

An anti-pea AMADH rabbit antiserum was raised by immunisation and examined for possible application in immunohistochemical localisations of AMADH proteins in plants. The antiserum was subjected to both immunoblotting and Ouchterlony immunodiffusion tests to evaluate its specificity and cross-reactivity. Positive precipitation results were achieved with extracts of all the studied plants indicating the presence of AMADH proteins. The anti-pea AMADH polyclonal antibody was specific and the antiserum could be used without any further purification. There appeared identity lines in the Ouchterlony tests performed with legumes (pea and fenugreek) only and also within the studied cereals. Instead, in comparison between legumes and cereals the results showed non-identical antigenic epitopes in the corresponding AMADHs. The prepared anti-pea AMADH antibody was finally utilised as a primary antibody in immunohistochemical localisation experiments using light microscope. A commercial goat anti-rabbit IgG conjugate with colloidal gold particles (10 nm) was used as a secondary antibody. The immunogold patterns observed resembled the mentioned results of histochemistry.

References

1. Federico R. and Angelini R.: In *Biochemistry and Physiology of Polyamines in Plants* (Slocum R. D. and Flores H. E., eds.), CRC Press, Boca Raton 1991, p. 41
2. Tiburcio A. F., Altabella T., Borrell A. and Masgrau C: *Physiol. Plant*. **100**, 664 (1997)
3. Šebela M., Radová A., Angelini R., Tavladoraki P., Frébort I. and Peč P.: *Plant Sci.* **160**, 197 (2001)
4. Flores H. E. and Filner P.: *Plant Growth Regul.* **3**, 277 (1985)
5. Šebela M., Brauner F., Radová A., JacobsenS., Havliš J., Galuszka P. and Peč P.: *Biochim. Biophys. Acta* **1480**, 329 (2000)

6. Livingstone J. R., Yoshida I., Tarui Y., Hirooka K., Yamamoto Y., Tsutui N. and Hirasawa E.: *J. Plant Res.* **115**, 393 (2002)
7. Šebela M., Luhová L., Brauner F., Galuszka P., Radová A. and Peč P.: *Plant Physiol. Biochem.* **39**, 831 (2001)

Acknowledgement

This work was supported by the grant MSM 153100010 from the Ministry of Education, Youth and Sports of the Czech Republic.

1P20

BIOCHEMICAL AND IMMUNOCHEMICAL PROPERTIES OF CD36 PROTEIN AND PRELIMINARY STUDY OF RNA INTERFERENCE

Jarmila Zídková[a,*], Kateřina Kontrová[a], Václav Zídek[b], Karel Mikulík[c], Petra Palečková[a], Jiří Sajdok[a], Jana Pěknicová[d], Zdeněk Knejzlík[a] and Michal Pravenec[d]

[a]*Department of Biochemistry, Institute of Chemical Technology Prague, Technická 5, 16628 Praha 6, Czech Republic*
[b]*Institute of Physiology,* [c]*Institute of Microbiology and* [d]*Institute of Molecular Genetics, Academy of Sciences, Czech Republic*

** E-mail: Jarmila.Zidkova@vscht.cz*

The integral membrane protein CD36 is a hydrophobic protein on mammary epithelial cells, platelets, capillary endothelial cells and other cell types. Protein CD36 - platelet glycoprotein IV is recently best known also as a receptor for thrombospondin-1 and collagen and as such may have an important role in platelet activation and thrombus formation. CD36 also functions as a signal transduction molecule. Spontaneously Hypertensive Rat (SHR) displays many of the features of human metabolic disease syndroms, thus SHR can be used as a model of mutation in CD36 and study of its CD36 protein.

CD36 from heart muscle has been isolated from both SHR and the WKY (Wistar Kyoto) as a control by six step isolation with subsequent HPLC ion exchange chromatography.

CD36 has been isolated from purified platelet membranes of SHR, WKY as a control and also transgenic rat platelets.

Analysis of purified platelet CD36 by two-dimensional electrophoresis has revealed formation of multimeric CD36. There have been differences between preparations from SHR and control WKY platelet CD36. Immunoblotting also identified CD36 on whole platelet and on the surface of U937. Platelest of different strain also differ in number of receptors per cell. Results with transgenic line of rat SHR 10 and SHR 19 were studied.

Indirect fluorescence technique has been used for detecting CD36 proteins on the surface of cells. There have been significant differences in the expression of CD36 protein on the surface of blood platelets of SHR and WK rat strains and substantial higher number of CD36 positive platelets in control strain of rats.

Preparation of raw CD36 from rat platelet was subsequently purified on protein G-agarose using specific interaction with monoclonal antibodies anti human CD36. Posttranslation modifications phosphorylation and mainly glycosylation of CD36 from rat platelet has been studied. Monoclonal anti-human thrombospondin receptor by Biodesign Int. US has been used for monitoring of rat CD36. Rabbit polyclonal antibodies raised against purified rat muscle CD36 gave a strong band on immunoblot and on immunoprecipitation with solubilized human and rat platelet membranes.

RNA interference (iRNA) is a highly conserved gene silencing mechanism that uses double-stranded RNA (dsRNA) as a signal to degradation of homologous mRNA in sequence to the dsRNA. The method is useful for reverse genetic analysis of mammalian gene function in any aspect of cell biology such as gene expression, metabolic pathways or cell cycle. The techniques make possible analysis of gene function, provided that transfectable cell lines are used and that single cell-based assays (e.g immunofluorescence) are used for phenotypic screening. The high sequence-specificity of siRNA and relative simplicity of administering dsRNA to organism whose genomes have been sequenced is promising candidate for biomedical application.

One way of detecting CD36 gene knockdown is to use a specific antibody by immunofluorescence techniques (e.g. rhodamine, fluorescein isothiocyanate) or by Western blotting of cell extract.

Real time reverse transcription polymerase chain reaction (RT PCR) is widely used to quantify mRNA. Being a very powerful and sensitive method it will be used to quantify mRNA expression level during knockdown of CD36 mRNA.

In our preliminary results we have found significant differences between transfected and control nontransfected 3T3 fibroblast cell culture. 72 hours of cell cultivation seems to be optimal for transfection observation. Dose dependent transfection, optimal transfection media and work with cell culture has been already thoroughly tested.

Acknowledgement

This work was partially supported by the grant 301/03/0751 from the Grant Agency, Czech Republic.

1P21

UTILIZATION OF LECTIN AFFINITY MICROREACTORS FOR SPECIFIC ISOLATION OF SIGNATURE GLYCOPEPTIDES

Šárka Ouzká[a,*], Markéta Bártová[b], Raluca Stefanescu[c], Michael Przybylski[c] and Zuzana Bílková[b]

[a]*Department of Analytical Chemistry and* [b]*Department of Biological and Biochemical Sciences, University of Pardubice, nám. Čs. legií 565, 53210 Pardubice, Czech Republic*
[c]*Department of Chemistry, University of Konstanz, Germany*

** E-mail: sarka.ouzka@upce.cz*

The principle of lectin affinity chromatography is suitable for study and isolation of various glycoforms of signature peptides of highly heterogeneous biomolecules. This type of chromatography combines simplicity with high resolution for peptide mapping technique.

Lectins are defined as a sugar-binding multivalent proteins or glycoproteins of non-immune origin which are devoid of enzymatic activity towards sugars to which they bind and do not require free glycosidic hydroxyl groups on these sugars for their binding. They agglutine cells and/or precipitate glycoconjugates[1,2]. Lectins when immobilized and employed as the affinity ligands for the purification of glycoconjugates offer an advantage since only mild conditions need be applied to elute the protein of interest. The metal ions Ca^{2+} and Mn^{2+} are essential constituents of the sugar binding sites and are also essential for the tertiary structure of lectins.

Selected biotinylated lectins (Vector Laboratories, USA): Concanavalin A with affinity to α-D-mannose and α-D-glucose residues, *Griffonia simplicifolia* I with affinity to N-acetyl-α-D-galactosamine and α-D-galactose residues, *Griffonia simplicifolia* II with affinity to α- or β-N-acetylglucosamine residues have been immobilized using high affinity interaction streptavidin - biotin on magnetic microparticles functionalized by carboxyl group (IMCh, CAS) using EDAC and S-NHS technique.

As a model glycoprotein for study of post-translation modification – conserved N-glycosylation - we have selected highly heterogeneous molecule of IgG. IgG conserved oligosaccharide moiety on asparagine 297 is the complex biantennary type and exhibits heterogeneity with respect to the terminal sugars attached. The minimal structure observed for normal human IgG is a heptasaccharide having terminal N-acetylglucosamine residues, the possible addition of galactose and subsequently sialic acid contributes to the generation of multiple glycoforms of IgG.

Newly developed enzyme microreactors (IMERs) with immobilized neuraminidase (sialidase) and β-D-galactosidase have been used for modification of oligosaccharide chains of IgG. Neuraminidase (sialidase) from *Clostridium perfringens* is exoglycosidase, which removes α-linked N-acetylneuramine acid from glycoside chain. β-D-galactosidase from bovine liver catalyzes the hydrolysis of lactose and many β-D-galactopyranosides.

Lectin affinity chromatography using immobilized lectins affinity reactors (IMARs) have been used to select various glycoforms of signature peptides from TPCK-tryptic

digest of IgG. After lectin affinity selection of these various glycoforms elution fraction have been identified by MALDI-TOF-MS.

References

1. Kocourek J. and Hořejší V.: *Nature* **290**, 188, (1981)
2. Satish P. R. and Surolia A.: *J. Biochem. Biophys. Methods* **49**, 625, (2001)

Acknowledgement

This work was supported by the grants from the Grant Agency of the Czech Republic 203/02/0023 and Ministry of Education, Czech Republic MSM 253100002.

1P22

YodA, A NOVEL *ESCHERICHIA COLI* CADMIUM-BINDING PROTEIN

Tatjana Stojnev*, Jana Harichová and Peter Ferianc

Department of General Microbiology, Institute of Molecular Biology, Center of Excellence of the Slovak Academy of Sciences, Dúbravská cesta 21, 84551 Bratislava, Slovakia

** E-mail: Tatjana.Stojnev@savba.sk*

Bacteria generally need to express only part of their genome to become structural and functional units of an environment. Continual changes in environmental conditions can then be accommodated by complex but efficient mechanisms that alter the pattern of gene expression. While the exhaustion of a building block may require the induction of a particular operon, severe stress conditions causing growth arrest are more complex, requiring global control systems for regulation above the operon level. Cadmium, an extremely toxic heavy metal with no known biological function, causes growth arrest of *Escherichia coli* cells accompanied by elevated expression of the entire set of genes, which together make up the cadmium stress stimulon[1]. Some of cadmium-induced proteins (CDPs) belong to global regulatory networks, which include the SOS, oxidative stress, heat-shock and stringent response networks[1]. However, only a limited number of the proteins in these regulons are induced during cadmium exposure and the synthesis of these CDPs constitutes a minor fraction of the overall cellular response. In addition, these general stress responses are only transiently activated during cadmium induced growth inhibition. When proliferation resumes, these regulons are down regulated and expression reaches a new steady-state level[1]. Other CDPs, however, are specific to cadmium stress. These CDPs retain an elevated production level even in accommodated cells. One of these proteins has been identified as the product of ORF o216[1], later renamed yodA. The yodA ORF encodes 216 amino acid residues of the YodA protein

and the increased synthesis of YodA during cadmium stress was found probably to be a result of transcriptional activation from one single promoter upstream the structural yodA gene[2]. In addition, the YodA protein was crystallized in several crystal forms by *in vitro* experiments[3], and its crystal structure has been determined[4]. The protein is composed of two domains: a main lipocalin/calycin-like domain and a helical domain. According to proposed three-dimensional model for this receptor, YodA may be a metal-binding protein[4].

In the present work we have reported regulation of yodA gene, localization of YodA in cell and, to be ensuring that YodA is able to bind metal *in vivo* conditions as well, we have constructed a mutant of *E. coli* with an insertional inactivation of the yodA gene.

The rate of YodA synthesis began to increase 15 min after cadmium addition. The increased synthesis of YodA during cadmium stress was found probably to be a result of yodA expression. Analysis of a transcriptional gene fusion, PyodA-lacZ, demonstrated that basal expression of yodA is low during exponential growth and expression is increased greater than 50-fold by addition of cadmium to growing cells. However, challenging cells with additional metals such as zinc, copper, cobalt and nickel did not increase the level of yodA expression. Hydrogen peroxide also increased yodA expression but after a time lag compared with the cadmium response, whereas the superoxide-generating agent paraquat failed to do so. Cadmium-induced transcription of yodA was shown to be dependent on soxS, fur, and relA/spoT of the stringent response but not on oxyR. Although primarily a cytoplasmic protein in nonstressed cells, YodA was shown to be exported to the periplasm upon exposure to cadmium.

YodA mutants were isolated after insertional mutagenesis of yodA clone pAL11 (kindly supported by T. Nyström laboratory) by use of the kanamycin resistance gene block from Pharmacia Biochemicals. The mutation was crossed into the chromosome of strain K4633 (recD) and subsequently introduced into strain MC4100 by standard P1 transduction procedures. Kanamycin-resistant recombinants were then analyzed by Western blot to confirm their inability to synthesize protein YodA. One of these mutants, JH6503, devoid of YodA expression, was further tested for its growth ability in presence of cadmium and for ability to bind cadmium. γ-Labeled ^{109}Cd experiments confirmed ability of wild type YodA to bind cadmium whereas the mutant failed to do so. In addition, yodA mutant cells have lagged in growth ability in presence of the metal, related to the degree of a growth level of wild type cells. Hydrogen peroxide also lagged growth rate of the mutant cells, but not significantly. However, challenging cells with additional metals such as nickel did not significantly affect on growth rate of the mutant. This growth phenotype was found to be the result of the loss of its ability to bind cadmium.

References

1. Ferianc P., Farewell A. and Nyström T: *Microbiology* **144**, 1045 (1998)
2. Puškárová A., Ferianc P., Kormanec J., Homerová D., Farewell A. and Nyström T: *Microbiology* **148**, 3801 (2002)
3. David G., Blondeau K., Renouard M. and Lewit-Bentley A: *Acta Cryst.* **D58**, 1243 (2002)
4. David G., Blondeau K., Schiltz M., Penel S. and Lewit-Bentley A: *J. Biol. Chem.* **278**, 43728 (2003)

1P23

STRUCTURE-FUNCTION CHARACTERIZATION OF CHIMERICAL GLUCOAMYLASES FROM YEAST *SACCHAROMYCOPSIS FIBULIGERA*

Juraj Gašperík* and Eva Hostinová

Institute of Molecular Biology, Slovak Academy of Sciences, Dúbravská cesta 21, 84551 Bratislava, Slovakia

** E-mail: juraj.gasperik@savba.sk*

Exo-acting starch degrading enzymes called glucoamylases can be divided into two groups: enzymes capable to degrade exclusively soluble starch and enzymes capable to degrade also insoluble, raw starch.

It has been known that an external domain, so called starch binding domain (SBD) is responsible for raw starch degradation. This domain is connected to the main glucoamylase chain through a short linker[1].

Three wild type strains of the dimorphous yeast *S. fibuligera* produce glucoamylases with a high degree of homology with different catalytic activity, ability to degrade raw starch, ability to renature after a thermal denaturation. These three strains: HUT 7212, KZ and IFO 0111 produce glucoamylases Glu, Gla and Glm, respectively.

Glu glucoamylase is our model enzyme with RTG determined 3D structure[2]. The enzyme is capable to bind to raw starch but not capable to digest it. Among three tested glucoamylases has a highest catalytic activity. Its ability to renature after thermal denaturation is relatively good.

Gla glucoamylase differs from Glu enzyme only in 6 aa residues. Its catalytic activity is about 50% lower in comparison to Glu enzyme. Serine 457 instead of glycin is responsible for the reduced specific activity[3]. Affinity of Gla towards raw starch is similar as at Glu enzyme. Gla enzyme is capable of an excellent renaturation after heat treatment at 100°C[4].

Glm is the only known yeast glucoamylase capable of raw starch digestion. Its catalytic activity is comparable with Glu enzyme. The enzyme represents a unique structure among a broad spectrum of amylolytic enzymes: although capable of raw starch degradation it does not contain a separate starch binding domain. Starch binding site of the enzyme is integrated within a catalytic domain[5]. The enzyme exposed to 70°C loses ability to renature. The 3D model of Glm was calculated on the base of its similarity to the Glu enzyme.

The goal of the work was to prepare a series of chimerical glucoamylases of *S. fibuligera* from the wild type genes encoding the above mentioned enzymes with the aim to obtain glucoamylases with improved technological properties. The chimerical genes were constructed by exchanges of various lengths of N-, and C-termini of Glu, Gla and Glm, in the identical restriction sites. After expression in *Saccharomyces cerevisiae*, the physico-chemical properties of the purified recombinant enzymes were compared.

References

1. Sumitami J., Tottori T., Kawaguchi T. and Arai M.: *Biochem. J.* **350**, 477 (2000)

2. Ševčík J., Solovicová A., Hostinová E., Gašperík J., Wilson K. S. and Dauter Z.: *Acta Cryst.* **D54**, 854 (1998)
3. Solovicová A., Christensen T., Hostinová E., Gašperík J., Ševčík J. and Svensson B.: *Eur. J. Biochem.* **264**, 756 (1999)
4. Gašperík J. and Hostinová E.: *Current Microbiol.* **27**, 11 (1993)
5. Hostinová E., Solovicová A., Dvorský R. and Gašperík J.: *Arch. Biochem. Biophys.* **411**,189 (2003)

1P24

LOCALIZATION OF STARCH-BINDING SITE IN *SACCHAROMYCOPSIS FIBULIGERA* GLUCOAMYLASE

Eva Hostinová*, Jozef Ševčík, Adriana Solovicová and Juraj Gašperík

Institute of Molecular Biology, Slovak Academy of Sciences, Dúbravská cesta 21, 84551 Bratislava, Slovakia

** E-mail: eva.hostinova@savba.sk*

Various strains of food-born yeast *Saccharomycopsis fibuligera* produce extracellular glucoamylases with a high degree of homology in primary and tertiary structures that differ in physico-chemical properties[1,2].

Glucoamylase Glu produced by *S. fibuligera* HUT7212 strain is one of the best-described glucoamylases. 3D structure of the enzyme has been determined in a complex with TRIS molecule bound in the active site[3]. Here we present the 3D structure of Glu in complex with the specific amylase inhibitor acarbose, having a structure similar to starch. It was found out that Glu binds two molecules of acarbose. One acarbose molecule, as expected, was bound in the active site of the enzyme and the other one interacted with four aa residues (R15, H447, D450, T462) localized in the region distinct from the catalytic site (unpublished results).

During characterization of glucoamylase Glu we have found out that this enzyme, although unable of raw starch digestion, has a strong affinity towards this substrate.

Glucoamylase Glm produced by *S. fibuligera* IFO 0111 strain is the only yeast amylolytic enzyme capable of raw starch digestion. Glm, highly homologous to Glu, has a unique structure because it does not contain a separate starch-binding domain (SBD) typical for all known raw starch degrading amylases. Our preliminary results show that the raw starch-binding site (SBS) is an integral part of the catalytic domain of Glm[4].

From comparison of Glu and Glm 3D structures we deduce that one acarbose molecule might be bound in the region of starch binding site. To prove this hypothesis, the double mutant of Glu, in aa residues interacting with acarbose (H447A, D450A) outside of the active site, was prepared. The recombinant protein, expressed in *Saccharomyces cerevisiae* was isolated, characterized and compared with the wild type

enzyme. The mutant retained, on soluble starch, the full catalytic activity of the wild type enzyme but lost the ability to adsorb on raw starch. It is evident that the mutation influenced the binding of enzyme on insoluble substrate and that we localized the region of the putative starch binding site.

References

1. Hostinová E.: *Gen. Physiol. Biophys.* **17**, 19 (1998)
2. Gašperík J. and Hostinová E.: Current Microbiol. 27, 11 (1993)
3. Ševčík J., Solovicová A., Hostinová E., Gašperík J., Wilson K. S. and Dauter Z.: *Acta Cryst.* **D54**, 854 (1998)
4. Hostinová E., Solovicová A., Dvorský R. and Gašperík J.: *Arch. Biochem. Biophys.* **411**, 189 (2003)

1P25

PURIFICATION AND CHARACTERIZATION OF IRON(III) REDUCTASES FROM *PARACOCCUS DENITRIFICANS*

Radek Tesařík[a,*], Jiří Mazoch[b], Jaroslav Turánek[a], Igor Kučera[b] and Vojtěch Sedláček[b]

[a]*Veterinary Research Institute, Hudcova 70, 621 32, Brno, Czech Republic*
[b]*Department of Biochemistry, Faculty of Science, Masaryk University, Kotlářská 2, 61137, Brno, Czech Republic*

** E-mail: tesarik@vri.cz*

Ferric iron reductases, which catalyze the reduction of various complexed forms of Fe(III), are widely spread within bacterial kingdom[1]. Conversion of Fe(III) to more soluble Fe(II) generally increases the bioavailability of iron. Some of these enzymes are associated with cytoplasmic membrane, some are present in soluble form in cytoplasm or periplasm[2], and some are excreted into extracellular medium[3]. In our work, two soluble enzymes (FerA and FerB) catalyzing the reduction of a number of iron(III) complexes by NADH, were purified to homogeneity from the aerobically grown iron-limited culture of *Paracoccus denitrificans* using a combination of anion-exchange chromatography, chromatofocusing and gel permeation chromatography and characterised with respect to kinetics of enzyme reaction. Fe(III)-nitrilotriacetate was chosen as the best artificial substrate for activity measurements. FerA (pI 6.9) is a monomer with a molecular mass of 19 kDa, whereas FerB (pI 5.5) exhibited a molecular mass of about 55 kDa and consists of probably two identical subunits. Both NADH and NADPH serve as electron donors for FerB whereas FerA uses NADH exclusively. FerA can be classified as an NADH:flavin oxidoreductase with a sequential reaction

mechanism. It requires the addition of FMN or riboflavin for activity on Fe(III) substrates. Observations on reducibility of Fe(III) chelated by vicinal dihydroxy ligands support the view that FerA takes part in releasing iron from the catechol type siderophores synthesized by *P. denitrificans.* Contrary to FerA, the purified FerB contains a noncovalently bound redox-active FAD coenzyme, does not reduce free FMN at an appreciable rate, and gives a ping-pong type kinetic pattern with NADH and Fe(III)-nitrilotriacetate as substrates. N-terminal sequences of FerA, FerB were determined and database searches revealed that the sequence of FerB is homologous to chromate reductase of *Pseudomonas putida*. Indeed, FerB has chromate reductase activity. Besides this, it also readily reduces quinones like ubiquinone-0 (Q_0) or unsubstituted *p*-benzoquinone.

References

1. Schröder I., Johnson E. and de Vries S.: *FEMS Microbiol. Rev.* **27**, 427 (2003)
2. Fontecave M., Coves J. and Pierre J. L.: *BioMetals* **7**, 3 (1994)
3. Vartivarian S. E. and Cowart R. E.: *Arch. Biochem. Biophys.* **364**, 75 (1999)

Acknowledgement

This work was supported by grant 203/01/1589 from the Grant Agency and VZ-0002716201 from the Ministry of Agriculture, Czech Republic.

1P26

CHARACTERIZATION AND CRYSTALLISATION OF *STREPTOMYCES COELICOLOR* A3(2) MALATE DEHYDROGENASE

Darina Mikulášová*, Ivana Janská, Petra Štefanková and Marta Kollárová

Department of Biochemistry, Faculty of Sciences, Comenius University, Mlynská dolina CH-1, 842 15 Bratislava, Slovakia

** E-mail: mikulaso@fns.uniba.sk*

Streptomycetes are unique among bacteria in their mycelial, sporulating life cycle, which involves complex regulation of gene expression in space and time. These characteristics of members of the genus *Streptomyces* have generated considerable interest in their genetics and physiology.

Malate dehydrogenase (MDH) is an integral enzyme in several metabolic processes. The reaction catalyzed is the reversible oxidation of L-malate to oxaloacetate using either $NAD(H)^+$ or $NADP(H)^+$ as a coenzyme.

Malate dehydrogenases from different sources have been characterized extensively, including crystal structure analyses of malate dehydrogenases from mesophilic, thermopholic, halophilic and psychrophilic organisms.

We isolated the *Streptomyces coelicolor* A3(2) malate dehydrogenase from overexpression strain *E. coli* IB831 and the His-tag from the isolated malate dehydrogenase was cut by biotinylated thrombine. Isolated *Streptomyces coelicolor* A3(2) malate dehydrogenase without His-tag we used for estimation of pH optima, molecular mass determination of subunit, coenzyme specificity and for crystallisation screening.

Acknowledgement

This study was founded by Slovak Grant Agency VEGA 1/0035/03.

1P27

THIOREDOXIN-THIOREDOXIN REDUCTASE SYSTEM OF *STREPTOMYCES COELICOLOR*: OVEREXPRESSION, ISOLATION AND CHARACTERIZATION

Petra Štefanková[a,*], Jana Maderová[b], Marta Kollárová[a], Imrich Barák[a], Diana Tomchick[b] and Zbyszek Otwinowski[b]

[a]*Department of Biochemistry, Faculty of Sciences, Comenius University, Mlynská dolina CH-1, 842 15 Bratislava, Slovakia*
[b]*Department of Biochemistry, Southwestern Medical Center, University of Texas, Dalas, TX, USA*

** E-mail: pstefankova@fns.uniba.sk*

Thioredoxin, thioredoxin reductase and coenzyme NADPH together form thioredoxin system, which is ubiquitous from Archae to man. Thioredoxins are ubiquitous small-molecular-mass heat-stable proteins containing a reducible disulfide bridge with the amino acid sequence Cys-X-X-Cys. Thioredoxin reductases, flavoprotein homodimeric enzymes, catalyse the NADPH-dependent reduction of thioredoxin disulfide[1]. Reduced thioredoxin acts as a major ubiquitous disulfide reductase responsible for maintaining proteins in their reduced state. Glutathion can act in preservation of thiol-disulfide status in the cells similar to thioredoxin. Interestingly, glutathione is not produced by actinomycetales, so Streptomyces are suitable model for study of thioredoxin system.

Streptomyces are Gram-positive spore-forming soil bacteria with specific life cycle. Streptomyces strain *S. coelicolor* is geneticaly the best known representative of the

genus. The 8.667.507 base pair linear chromosome of this organism contains the largest number of genes so far discovered in a bacterium[1].

In presented work we over-expressed thioredoxin and thioredoxin reductase from *S. coelicolor* with His.Taq fusion on N-terminal end of proteins in *E. coli*. Proteins were purified by metal chelate affinity chromatography. Homogenous proteins were used for investigation of protein stability, their role in the redox control in the cell and study of protein-protein interaction. Thioredoxin was used for the crystallization trials and crystallization conditions for thioredoxin from *S. coelicolor* were determined. We have obtained the crystals suitable for X-ray diffraction analyses. The preliminary X-ray study has showed that protein crystallized in P222 space group with cell parameters 33.2, 43.5, 143.9, 90.0, 90.0, 90.0.

References

1. Gilbert H. F.: *Adv. Enzymol. Relat. Areas Mol. Biol.* **63**, 69 (1990)

Acknowledgement

This work was supported by the research grant from the Slovak Grant Agency VEGA 1/0035/03.

1P28

TUMOR-ASSOCIATED CARBONIC ANHYDRASE IX CONTRIBUTES TO ACIDIFICATION OF EXTRACELLULAR pH IN RESPONSE TO HYPOXIA

Alžbeta Hulíková, Eliška Švastová, Miriam Zaťovičová, Adriana Gibadulinová, Silvia Pastoreková and Jaromír Pastorek

Centre of Molecular Medicine, Institute of Virology, Slovak Academy of Sciences, Dúbravská cesta 9, Bratislava 845 05, Slovak Republic

** E-mail: viruhula@savba.sk*

Acidic extracellular pH (pHe) is a characteristic feature of tumor microenvironment that facilitates tumor progression via multiple effects including increased invasion and metastasis. It has been believed to result from aerobic and anaerobic glycolysis that generates excess of lactic acid, which is then poorly removed by inadequate tumor vasculature. This is especially important in hypoxic tumor cells that are largely dependent on glycolysis for energy production. However, lactic acid is not the only source of interstitial acidity, because tumors grown from glycolysis-deficient cells are also acidic. Recent physiological studies strongly support involvement of CO_2 metabolism driven by carbonic anhydrases (CA), which catalyze the reversible hydration of carbon dioxide to bicarbonate and proton. Mammalian CAs exist in 15 isoforms that

differ by domain composition, subcellular localization, enzyme activity and function. Transmembrane carbonic anhydrase IX (CA IX) is the only isoenzyme, which is tightly associated with human tumors and absent from the corresponding normal tissues. CA IX is a highly active enzyme and its level is strongly inducible by tumor hypoxia. Based on the extracellular localization of the enzyme active site, CA IX has been proposed to regulate pH across the plasma membrane. However, despite strong indirect arguments in favor of this concept, there was no real evidence for active participation of CA IX in the pH control. In order to bring such direct evidence, we focused on the capacity of CA IX to acidify extracellular medium in cell culture, using as a model MDCK epithelial cells transfected with the cDNA encoding the human CA IX (MDCK-CA IX) and control mock-transfected MDCK cells (MDCK-neo). MDCK-neo cells did not contain any endogenous CA IX protein, whereas MDCK-CA IX cells ectopically expressed CA IX from the constitutive promoter and the level of CA IX protein was therefore independent of oxygen supply as documented by Western blotting analysis. Incubation of the cells in normoxia versus hypoxia (2% O_2) for 48 h resulted in marked differences in their pHe. MDCK-CA IX and MDCK-neo cells showed approximately the same pHe under normoxia. In both cell types, the values of pHe were more acidic in hypoxia than in normoxia due to increased production of lactate. However, pHe of hypoxic MDCK-CA IX cells was further decreased and became significantly lower (~ 0.4) than that of hypoxic MDCK-neo cells. Because there was no significant difference in lactate production from MDCK-CA IX compared to MDCK-neo cells under hypoxia, this finding clearly indicated direct contribution of CA IX. Moreover, the levels of CA IX protein were similar in normoxia and hypoxia, so this effect could be assigned to hypoxic stimulation of CA IX catalytic activity. Expression of the CA IX-DCA protein lacking the central CA domain with the active enzyme site did not lead to hypoxia-related acidification of pHe, whereas CA IX-DPG variant with deletion of the N-terminal region behaved similarly as the wild-type CA IX protein. This result confirmed the involvement of the catalytic activity of CA IX in the extracellular acidosis. In conclusion, our study brought the first evidence for the role of CA IX in the pH control. We showed that hypoxia activates the catalytic performance of CA IX leading to acidification of the extracellular pH, what may have important implications for tumor growth and progression.

1P29

GENETIC CHARACTERIZATION OF INDUCIBLE NAD$^+$-DEPENDENT ALCOHOL DEHYDROGENASE (ADH) FROM *CORYNEBACTERIUM GLUTAMICUM* R

Anna Kotrbova-Kozak[a], Pavel Kotrba[a], Kateřina Kontrová[a], Masaiuki Inui[b], Hydeki Yukawa[a] and Jiři Sajdok[a,*]

[a]*Department of Biochemistry and Microbiology, Institute of Chemical Technology, Technická 5, 16628 Praha, Czech Republic*
[b]*Research Institute of Innovative Technology for the Earth, Kyoto, Japan*

** E-mail: Sajdokj@vscht.cz*

Gram-positive high-GC soil bacterium *Corynebacterium glutamicum* has a long history of commercial use as producer of various amino acids, notably lysine and glutamic acid. *C. glutamicum* grows on a variety of carbohydrates and organic compounds as carbon and energy sources.

Nowadays, however, ethanol became energy source of interest for its potential as a fuel additive, matching features of petroleum, and as a starting material for manufacture of large number of chemicals, such as acetaldehyde, butanol and ethylene. This recent call brought about number of new ethanol-producing microorganisms. Among them, engineered *C. glutamicum* expressing recombinant NAD$^+$/Zn-dependent alcohol dehydrogenase (ADH) from *Zymomonas mobilis* was reported which can efficiently produce ethanol.

Wild-type bacterium can not produce ethanol by using its own ADH that serves rather catabolic role. The work presented here describes isolation and genetic characterization of adhA gene encoding ADH from *C. glutamicum* R (CgADH). The deduced 36.9-kDa CgADH of 345 amino acids shares more than 45% identity with NAD$^+$-cofactored Zn-dependent ADHs from *Pseudomonas aeuginosa*, *Sinorhizobium melioti* and *Bacillus stearothermophilus*. Chromosomal inactivation of the adhA gene and its complementation with extrachromosomal adhA in disruptant mutant demonstrated that adhA plays an essential role in utilization of ethanol and *n*-propanol. Although the same NAD$^+$-dependent ADH activity was induced in wild-type cells, but not in adhA-null cells, also by methanol, 2-propanol and butanol, these alcohols did not support growth of *C. glutamicum* R. The RNA hybridization analysis revealed that adhA transcript is present in cells grown on ethanol and *n*-propanol, but not on glucose, thereby suggesting that this ADH is an inducible enzyme. Glucose repressed ADH activity in growing cells and its negative regulation was dominant over presence of inducer ethanol. The ADH activity was detected only after all glucose was consumed. Two DNA features located in the adhA promoter region were proposed possible regulatory elements, responsible for induction and/or repression of adhA transcription. Set of plasmids was constructed with various deletions of putative regulatory DNA elements. The biochemical properties of recombinant CgADH are present.

1P30

IDENTIFICATION OF CEREAL GRAIN PROTEINS BY PEPTIDE MASS FINGERPRINTING AND A FRAGMENTATION ANALYSIS OF IN-GEL DIGESTED NONSPECIFIC PEPTIDES BY MALDI-TOF MS

Josef Chmelík[a,*], Pavel Řehulka[a] and Günter Allmaier[b]

[a]*Institute of Analytical Chemistry of the Academy of Sciences, Veveří 97, 61142 Brno, Czech Republic*
[b]*Institute of Chemical Technologies and Analysis, Vienna University of Technology, Vienna, Austria*

** E-mail: chmelik@iach.cz*

Investigation of the proteome of various species is a rather difficult task. In contrast to the static genome, the proteome is dynamic and reflexes the real state of life. The modern solution of this problem was enabled by the recent developments in molecular biology, bioinformatics and mass spectrometry. It brought the protein sequence databases and the sensitive mass spectrometry instruments suitable for investigation of proteins. The combination of suitable separation technique together with exact mass spectrometry measurements and the use of bioinformatics give new possibilities to study the proteome of various organisms. The proteomic approach to the characterisation of the metabolic and storage protein content in cereal grains may bring fundamental information on the relationships between protein composition and food quality (e.g. dough properties, malting, problems with celiac disease).

A MALDI-TOF mass spectrum of an in-gel digest often contains not only expected peptide signals, but also peaks of different origin (autolysis peptides, post translationally modified peptides, peptides from nonspecific cleavages, contaminants in the sample etc.). Peptides originating from unexpected enzyme activity or as a product of the chemical treatment during protein cleavage and/or peptide extraction are termed as "nonspecific peptides".

This work is focused on identification of low molecular mass proteins from aqueous extract of barley grain and high molecular mass proteins from ethanol extract of wheat grain by using peptide mass fingerprinting and post-source decay analysis of nonspecific peptides both for protein identification and for increasing of the protein sequence coverage by MALDI-TOF MS. Seamless post source decay analyses of tryptic and nonspecific peptides were used for the successful protein identification of low molecular mass proteins isolated from barley grains. The mixture of these proteins was located in a single band on the electrophoretic gel and it was not possible to identify the proteins just by peptide mass fingerprinting with considering only specific cleavage sites. Post source decay analysis of nonspecific peptides enabled both protein identification and increase of protein sequence coverage. Several prolamins were determined in wheat grains by fragmentation analysis by MALDI-QIT MS.

Acknowledgement

This work was supported by the grant 203/02/0023 from the Grant Agency of the Czech Republic.

ACTA UNIVERSITATIS PALACKIANAE OLOMUCENSIS
FACULTAS RERUM NATURALIUM 2004 CHEMICA 43S

PROCEEDINGS OF THE XIX. MEETING OF CZECH AND SLOVAK SOCIETIES FOR BIOCHEMISTRY AND MOLECULAR BIOLOGY

Olomouc, August 31 – September 3, 2004

SESSION 2: BIOCHEMISTRY OF CARBOHYDRATES AND LIPIDS

2L01

MASS SPECTROMETRY: A UNIVERSAL TOOL FOR PROBING OF PROTEIN GLYCOSYLATION

Petr Man[a,*], Petr Novák[a], Jan Sklenář[b], Karel Bezouška[b] and Vladimír Havlíček[a]

[a]Laboratory of Molecular Structure Characterization, and [b]Laboratory of Protein Architecture, Institute of Microbiology, Academy of Sciences, Vídeňská 1083, 14220 Praha 4, Czech Republic

** E-mail: halada@biomed.cas.cz*

Advances in proteomics strongly encourage the scientific community to treat protein not only as a set of amino acids but to pay attention to all possible protein modifications and conformations. Apart from the protein phosphorylation, the glycosylation fulfils one of the key roles in protein recognition, interaction and stability.
The talk will cover general approaches for detailed mapping of protein glycosylation, including the most recent techniques. Besides that, several examples how mass spectrometry was used in studies dealing with glycosylation will be shown.

2L02

THE IMPORTANCE OF HAVING GLYCOSYLATED CLEAVABLE PROPEPTIDE: THE STORY OF β-N-ACETYLHEXOSAMINIDASE

Petr Pompach[a,*], Petr Man[a], Vladimír Havlíček[b], Ondřej Plíhal[a], Jan Sklenář[a], Vladimír Křen[c] and Karel Bezouška[a]

[a]Laboratory of Protein Architecture, [b]Laboratory of Molecular Structure Characterization and [c]Laboratory of Biotransformations, Institute of Microbiology, Academy of Sciences, Vídeňská 1083, 14220 Praha 4, Czech Republic

** E-mail: pompach@biomed.cas.cz*

Fungal β-N-acetylhexosaminidases (chitobiases, EC 3.2.1.30) are secreted enzymes that hydrolyze chitobiose into monosaccharides. The enzymes are physiologically important for the formation of septa in molds, germ tubes in yeast, and fruit bodies in fungi[1]. Moreover, they have found many applications in biotechnologies due to their unique abilities to synthesize new and unusual oligosaccharide structures[2].

We have cloned and sequenced β-N-acetylhexosaminidase from the collection strain of *Aspergillus oryzae* CCF1066[3]. This enzyme is composed of four polypeptide segments. The N-terminal signal peptide is cleaved intracellularly. The zincin-like and

catalytical domains define the enzyme as a member of family 20 glycohydrolases. Homology modeling revealed significant similarity with the two crystallized bacterial β-N-acetylhexosaminidases.

Fungal β-N-acetylhexosaminidases contain a unique N-terminal propeptide that is processed intracellularly before the secretion of the enzyme. Detailed pulse-chase and inhibition studies revealed that the propeptide is processed very early during the biosynthesis, just after the addition of N-glycans. The propeptide must be processed in order to asssist in enzyme refolding, activation, and dimerization. Monomeric enzyme subunits devoid of the propeptide are inactive, cannot dimerize, and may not be secreted from the cell (unpublished results). Dimers containing a single propeptide are secreted at only half the rate of those containing both propeptides, and have lower specific activity.

The unique propeptide properties are undoubtedly dictated by its unusual structural features. Therefore we turned our attention toward the characterization of its primary structure. The propeptide has a prolin rich C-terminal part with several potential O-glycosylation sites. The glycosylation pattern is extremely difficult to solve as there are at least two or three glycans attached to serin or threonin. Moreover, the glycosylation appeared rather resistant to chemical cleavage and it also protected the C-terminal part of the propeptide from proteolysis. Partial primary structure including glycosylation was solved by tandem mass spectrometry, namely collision induced dissociation (CID). Final characterization using electron capture dissociation (ECD) combined with high resolution and high mass accuracy experiments on an FT-ICR mass spectrometer is under progress.

References

1. Hearn V. M., Escott G. M., Glyn E., Evans V. and Adams D. J.: *Microbios* **93**, 85 (1998)
2. Weignerová L., Vavrušková P., Pišvejcová A., Thiem J. and Křen V.: *Carbohydr. Res.* **338**, 1003 (2003)
3. Hušáková L., Herkommerová-Rajnochová E., Semeňuk T., Kuzma M., Rauvolfová J., Přikrylová V., Ettrich R., Plíhal O., Bezouška K. and Křen V.: *Adv. Synth. Catal.* **345**, 735 (2003)

Acknowledgement

Supported by the Ministry of Education (MSM 113100001), by the Institutional Research Concept AVOZ5020903, and by the Grant Agency, Czech Republic (203/04/1045).

2L03

PA-IIL LIKE LECTINS: A COMMON FEATURE OF HIGH ADAPTABILITY OF SOME OPPORTUNISTIC BACTERIA

Michaela Wimmerová[a,b,*], Edward P. Mitchell[c], Martina Budová[a], Charles Sabin[d], Nikola Kostlánová[a], Stephanie Perret[d], Gianluca Cioci[d], Nechama Gilboa-Garber[e] and Anne Imberty[d]

[a]*National Centre for Biomolecular Research and* [b]*Department of Biochemistry, Masaryk University, Kotlářská 2, 61137 Brno, Czech Republic*
[c]*Experiments Division, ESRF, BP 220, 38043 Grenoble, France*
[d]*CERMAV-CNRS, 601 rue de la Chimie, BP 53, 38041 Grenoble, France*
[e]*Faculty of Life Sciences, Bar-Ilan University, Ramat Gan 52900, Israel*

** E-mail: michaw@chemi.muni.cz*

Enormous potential of sugar structures gives them a crucial importance in recognition and signalling events. Carbohydrate-mediated recognition plays an important role in the ability of parasitic organisms to adhere to the surface of the host cell in the first step of their invasion and infectivity. For example, *Pseudomonas aeruginosa* galactose- and fucose-binding lectins (PA-IL and PA-IIL) contribute to the virulence of this pathogenic bacterium, which is a major cause of morbidity and mortality in cystic fibrosis patients[1,2]. Moreover, the PA-IIL lectin displays an affinity for fucose in micromolar range, unusually high for monosaccharide binding. This characteristics is correlated to the remarkable presence of two calcium ions in the binding site of the protein[3].

Database searching in newly sequenced bacterial genomes revealed the presence of PA-IIL like proteins within a limited number of other opportunistic pathogens. All of them are soil inhabitants, are phylogenetically related and in past were usually considered as *Pseudomonas* spp. At the present time, PA-IIL like gene have been identified in the genomes of phytopathogen *Ralstonia solanacearum* and of human opportunistic pathogens *Chromobacterium violaceum* and *Burholderia cenocepacia*. The latter was found to cause life threatening pulmonary infections in cystic fibrosis patients at a mortality rate of 80%[4].

PA-IIL like proteins from *R. solanacearum* (RS-IIL) and *C. violaceum* (CV-IIL) have been fully characterized. RS-IIL has been purified from bacteria by affinity chromatography[5] whereas CV-IIL has been obtained in the recombinant form. The three lectins have been compared for their specificity (enzyme amplification method), their affinity for monosaccharides (isothermal titration microcalorimetry experiments) and their crystal structures. Comparison of the structures of the PA-IIL/fucose and RS-IIL/mannose complexes allow us to rationalize the basis of the unusual high specificity of both proteins for monosaccharides and the importance of three amino acid motif for fine tuning of the lectin specificity.

References

1. Gilboa-Garber N.: *Methods Enzymol.* **83**, 378 (1982)

2. Imberty A., Wimmerová M., Mitchell E. P., Gilboa-Garber N.: *Microb. Infect.* **6**, 222 (2004)
3. Mitchell E., Houles C., Sudakevitz D., Wimmerová M., Gautier C., Pérez S., Wu A. M., Gilboa-Garber N. and Imberty A.: *Nature Struct. Biol.* **9**, 918 (2002)
4. Mahenthiralingam E., Baldwin A. and Vandamme P. J.: *Med. Microbiol.* **51**, 533 (2002)
5. Sudakevitz D., Kostlánová N., Blatman-Jan G., Mitchell E., Lerrer B., Wimmerová M., Katcoff D. J., Imberty A. and Gilboa-Garber N.: *Mol. Microbiol.* **52**, 691 (2004)

2P01

ANALYSIS OF STARCH DEGRADATION PRODUCTS AND OTHER CARBOHYDRATES FROM SPRUCE NEEDLES USING VARIOUS ANALYTICAL TECHNIQUES

Jana Cabálková[a,*] and Josef Chmelík[b]

[a]*Brno University of Technology, Faculty of Chemistry, Purkyňova 118, 612 00 Brno, Czech Republic*
[b]*Josef Chmelík, Institute of Analytical Chemistry, Academy of Sciences of the Czech Republic, Veveří 97, 61142 Brno*

** E-mail: cabalkova@fch.vutbr.cz*

An understanding of the carbohydrate biochemistry in biological processes has been reported in many papers with considerable interest to study in detail their composition and structures in various species. Recently a great amount of research deals with photosynthesis and growth of broad-leaved and coniferous species simulated by long-term exposure to elevated CO_2 concentration.

The aim of this study was to investigate the possibility of producing fractions of starch with granule size distribution, using various separation techniques: size exclusion chromatography (SEC) equipped with multi-angle light scattering (MALS) and refractive index (RI) detectors, asymmetrical flow field flow fractionation (AsFlFFF) with MALS and RI detectors, by high performance liquid chromatography (HPLC) with RI detector, and matrix assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS). The study is focused on determination small (up to tetrasaccharides) and oligosaccharides from Norway spruce (*Picea abies* [L.] Karst) needles grown under different CO_2 concentrations. Three different needle samples grown in shady site were studied. They were one-year old needles grown in control, ambient and elevated CO_2 concentration, respectively.

Starch degradation products were analysed by AsFlFFF-MALS-RI and SEC-MALS-RI for determination of molar mass distribution and size distribution. Separation of low molecular weight fractions of carbohydrates using HPLC-RI led to the

identification of xylose, glucose, fructose, derivatives of pinitol, maltose, myo-inositol, sucrose and raffinose. MALDI-TOF MS spectra confirmed the presence from mono- to tetrasaccharides and also higher oligosaccharides. It was found that elevated CO_2 concentration in the atmosphere had significant effect on the carbohydrate composition.

Acknowledgement

This work was supported by the grants 526/03/1182 from the Grant Agency of the Czech Republic and FRVŠ no. G4/1685/2004.

2P02

CV-IIL: NEW LECTIN FROM *CHROMOBACTERIUM VIOLACEUM*

Martina Budová[a,*], Stephanie Perret[b], Edward P. Mitchell[c], Gianluca Cioci[b], Anne Imberty[b] and Michaela Wimmerová[a,d]

[a]*National Centre for Biomolecular Research and* [d]*Department of Biochemistry, Masaryk University, Kotlářská 2, 61137 Brno, Czech Republic*
[b]*CERMAV-CNRS, 601 rue de la Chimie, BP 53, 38041 Grenoble, France*
[c]*Experiments Division, ESRF, BP 220, 38043 Grenoble, France*

** E-mail: budova@chemi.muni.cz*

Bacterium *Chromobacterium violaceum*, a Gram-negative saprophyte from soil and water, is usually considered non-pathogenic to human. However, infections in animals, including human, can be quite varied, ranging from mild diarrhoea to septicaemia leading to a rapid death. This bacterium has been found to be highly abundant in the water and borders of the Negro river, a major component of the Brazilian Amazon. It produces the violacein pigment, which exhibits an antimicrobial activity particularly against soil amoebae and trypanosomes. Because of its pharmaceutical interest, *C. violaceum* genome has been fully sequenced by the Brazilian National Genome Project Consortium[1]. The genome contains extensive but incomplete arrays of ORFs coding for proteins associated with mammalian pathogenicity, probably involved in the occasional but often fatal cases of human *C. violaceum* infection.

Homology search in the *C. violaceum* genome revealed that gene cv1741 displays homology with gene lecB from human pathogen *Pseudomonas aeruginosa*. Product of the gene lecB is the fucose-binding lectin PA-IIL that can play a crucial role in adhesion and specific recognition of a host by the pathogen and contributes to its virulence[2]. Similar properties of chromobacterial protein CV-IIL, the gene product of cv1741, could be expected.

The recombinant CV-IIL protein has been prepared for structural and biochemical characterisation. The cv1741 gene was cloned into pET25b vector, and the protein expressed in *E. coli* TUNER (DE3) cells has been purified by affinity chromatography

on mannose-agarose. Purification yielded 25 mg of pure protein CV-IIL per litre of cultivation media and MS analysis confirmed purity and molecular mass of the obtained product. Competitive binding assays using ELLA methodology showed that CV-IIL displays high affinity towards L-fucose and D-mannose. Crystals of CV-IIL/fucose and CV-IIL/mannose complexes have been grown using PEG precipitants. Diffraction data have been measured at ESRF and structures have been solved at 1.1 Å resolution. Analysis of the binding sites allows to rationalize the high affinity of the lectin for monosaccharides.

References

1. Ribeiro De Vasconcelos A.T. and 109 others [Brazilian National Genome Project Consortium]: *Proc. Natl. Acad. Sci. U.S.A.* **100**, 11660 (2003)
2. Imberty A., Wimmerová M., Mitchell E. P. and Gilboa-Garber N.: *Microb. Infect.* **6**, 222 (2004)

ACTA UNIVERSITATIS PALACKIANAE OLOMUCENSIS
FACULTAS RERUM NATURALIUM 2004 CHEMICA 43S

PROCEEDINGS OF THE XIX. MEETING OF CZECH AND SLOVAK SOCIETIES FOR BIOCHEMISTRY AND MOLECULAR BIOLOGY

Olomouc, August 31 – September 3, 2004

SESSION 3: MOLECULAR PROCESSES IN CELL, REGULATION AND SIGNALIZATION

3L01

FUNCTIONAL INTERACTIONS BETWEEN NUCLEAR RECEPTORS: CONSEQUENCES ON XENOBIOTIC DETOXIFICATION PATHWAYS

Jean-Marc Pascussi[a], Zdeněk Dvořák[b], Martin Modrianský[b], Jitka Ulrichová[b,]*, Jean-Claude Ourlin[a], Sabine Gerbal-Chaloin[a], Lionel Drocourt[a] and Patrick Maurel[a]

[a]*Physiopathologie Hepatique, INSERM U632, Route de Mende 19, 34293 Montpellier, France*
[b]*Institute of Medical Chemistry and Biochemistry, Faculty of Medicine, Palacký University, Hněvotínská 3, 77515 Olomouc, Czech Republic*

** E-mail: ulricho@tunw.upol.cz*

During species evolution, an arsenal of gene superfamilies has been set-up in order to cope with the constant risks presented by xenobiotics. This arsenal comprizes two main groups of genes. First, those encoding xenobiotic metabolizing and transporting systems (XMTS), including notably the superfamilies of cytochrome P450s (CYP1-CYP3), conjugation enzymes and xenobiotic transporter proteins. Second, those encoding specific receptors capable of controlling the coordinated expression of XMTS in response to xenobiotics. Because these receptors are able to interact with and be activated by intracellular xenobiotics, they are often designated xenosensors. These include notably the aryl hydrocarbon receptor (AhR), a member of the PAS family of transcription factors, and the pregnane X receptor (PXR) and the constitutive androstane receptor (CAR), members of the nuclear receptor superfamily. Recent investigations on nuclear receptors and other transcription factors involved in the regulation of genes encoding XMTS reveal that the xenobiotic-dependent signaling pathways are embedded in, and establish functional interactions with, a tangle of regulatory networks involving the glucocorticoid, the vitamin D receptor and other nuclear receptors controlling cholesterol/bile salt homeostasis such as small heterodimerization partner (SHP), lanosterol X receptor (LXR) and farnesyl X receptor (FXR). An existence of functional cascade XMEs/PXR,CAR/GR was proposed and confirmed[1]. Notably, we investigated the role of microtubules (MT) on GR and thus on xenobiotics metabolism. We found that MT disruption in human hepatocytes leads to CYP-PXR/CAR-hGR cascade impairment with consequent CYPs 2B6, 2C8/9, 3A4, receptors PXR and CAR, and tyrosine aminotransferase mRNAs down-regulation. In the other hand, we observed that CAR, PXR and VDR share target genes, such as CYP3A4, CYP2B6 and CYP2C9 but also CYP24. Such functional interferences provide new views, first for understanding how physiopathological stimuli affect xenobiotic metabolism and second how xenobiotic might excert adverse effects by interfering with endocrine regulation pathways. The aim of this lecture is to focus on these interferences and cross-talks between xenosensors and transcription factors and/or nuclear receptors, and their physiological and toxicological consequences.

References

1. Dvořák Z., Modrianský M., Pichard-Garcia L., Balaguer P., Vilarem M. J., Ulrichová J., Maurel P. and Pascussi J. M.: *Mol. Pharmacol.* **64**, 160 (2003)

Acknowledgement

This presentation was supported by mobility grant Barrande 2004-021-1.

3L02

REGULATED EXPRESSION AND A POTENTIAL ROLE FOR FIBROBLAST GROWTH FACTOR RECEPTORS AND BASIC FIBROBLAST GROWTH FACTOR IN HUMAN EMBRYONIC STEM CELLS

Petr Dvořák[a, c, d, *], Dana Dvořáková[b], Stanislava Kosková[b], Martina Vodinská[a], Daniel Krekáč[a, c], and Aleš Hampl[a, c, d]

[a]*Department of Molecular Embryology, Institute of Experimental Medicine of Academy of Sciences of the Czech Republic, 14220 Prague, Czech Republic*
[b]*Department of Internal Medicine – Hematooncology, University Hospital Brno, Czech Republic*
[c]*Department of Molecular Embryology, Mendel University of Agriculture and Forestry Brno, 61300 Brno, Czech Republic*
[d]*Center for Cell Therapy and Tissue Repair, Charles University, Prague, Czech Republic*

** E-mail: dvorakp@mendelu.cz*

Human embryonic stem (HES) cells are pluripotent stem cells derived from blastocyst stage embryo, with capacity to form all adult cell types. Several groups have now shown that HES cells differentiate *in vitro* to specialized cells of great clinical interest. Thus, it seems likely that HES cells will rapidly become central to biomedical research. However, growth factor signaling pathways that modulate cell fate decision in human embryonic stem (HES) cells are largely unknown.

We analyzed the expression pattern of fibroblast growth factor receptors (FGFR1, 2, 4, and 4) and molecular isoforms of basic fibroblast growth factor (bFGF) in undifferentiated and differentiated HES cells. We show that undifferentiated HES cells that are cultured in low density express all four FGFRs in the following pattern: FGFR1 is highly expressed and dominant; FGFR3 is also highly expressed, however at lower levels; FGFR4 shows even lower expression; and FGFR2 is only weakly expressed. This expression pattern is changed when undifferentiated HES cells are cultured in high density or have initiated differentiation. Several fold upregulation of FGFR1 and FGFR4, and downregulation of FGFR3 characterize such changed expression pattern.

Notably, undifferentiated HES cells synthesize both low (exportable) and high (nuclearly localized) molecular mass isoforms of bFGF and this expression pattern remains stable under conditions that allow differentiation. We next examined HES cell response to exogenous bFGF. Although extracellular signal-regulated kinases (ERK1/2) could be activated, bFGF does not significantly affect proliferation or differentiation of HES cells. Interestingly, increased concentration of exogenous bFGF led to reduced spreading of HES cells on feeder layer of mouse embryonal fibroblasts.

Since soluble bFGF might has the potential to modulate cell attachment and spreading by the formation of a trimolecular complex between bFGF, FGFR1, and heparan sulfate expressed on fibroblasts, we suggest that such interaction has functional significance also in HES cells.

3L03

CYCLING-UNRELATED FUNCTIONS OF CELL CYCLE REGULATORS

Aleš Hampl[a, b, c, *] and Petr Dvořák[a, b, c]

[a]*Department of Molecular Embryology, Institute of Experimental Medicine of Academy of Sciences of the Czech Republic, 14220 Prague, Czech Republic*
[b]*Department of Molecular Embryology, Mendel University of Agriculture and Forestry Brno, 61300 Brno, Czech Republic*
[c]*Center for Cell Therapy and Tissue Repair, Charles University, 15018 Prague, Czech republic*

** E-mail: hampl@mendelu.cz*

Proper establishment of differentiated cell phenotype during embryonal, fetal, and postnatal period of life is always the result of a concerted integration of a multitude of proliferation and differentiation cues. In the complex processing of differentiation-inducing signals, very important role is played by the components of cell cycle regulating machinery, including cyclin dependent kinases (CDKs), their cyclins, and inhibitors of cyclin dependent kinases (CKIs). As the primary function of CDKs and their partners is to regulate the progression of cell cycle, withdrawal from cell cycle is their most obvious differentiation-associated function. Still, there is an increasing body of evidence that cell cycle regulators may play a role in certain aspects of differentiation, other then differentiation-associated cell cycle exit. In the past few years, we employed several cell and tissue types to address such cycling-independent role(s) of the selected cell cycle regulators. We offered the model of how p27 CKI and cyclin D3 might participate in establishment and maintenance of luteinized phenotype[1]. We demonstrated that the amounts of p27 and D-type cyclins themselves can not determine the presence or absence of the pluripotent phenotype of embryonal carcinoma (EC) cells, and that coregulation of their quantities may serve some specific differentiation-associated function[2]. The existence of such coregulation of D-type cyclins and p27 has been shown also in various tissues by experiments employing normal and p27-deficient mice[3]. In mouse embryonic stem (ES) cells, p27 has been shown to protect the differentiating cells from dying by apoptosis[4]. Finally, unexpected expression and modification of p27, D-type cyclins, CDK4, and CDK6 proteins was found in developing mouse oocytes[5].

References

1. Hampl A., Pacherník J. and Dvořák P.: *Biol. Reprod.* **62**, 1393 (2000)
2. Preclíková H., Bryja V., Pacherník J., Krejčí P., Dvořák P. and Hampl A.: *Cell Growth & Differ.* **13**, 421 (2002)
3. Bryja V., Pacherník J., Faldíková L., Krejčí P., Pogue R., Nevriva I., Dvořák P. and Hampl A.: *Biochim. Biophys. Acta* **1691**, 105 (2004).
4. Bryja V., Pachernik J., Soucek K., Horvath V., Dvorak P. and Hampl A.: *Cell. Mol. Life Sci.* **61**, 1384 (2004).

Acknowledgement

This research was supported in part by the Grant Agency of the Czech Republic (GA 204/01/0905), by the Academy of Sciences of the Czech Republic (AV 0Z5039906), and by the Ministry of Education, Youth, and Sports (MSM 432100001, LN 00A065).

3L04

A ROLE OF THE p21(WAF1/Cip1) PROTEIN IN THE DIFFERENTIATION OF F9 MOUSE EMBRYONAL CARCINOMA CELLS INTO PARIETAL ENDODERM

Blanka Drdová and Jiří Vachtenheim*

Laboratory of Molecular Biology, University Hospital Bulovka, Budínova 2, 18000 Praha 8, Czech Republic

** E-mail: jivach@upn.anet.cz*

Combined treatment with retinoic acid and dibutyryl-cAMP induces differentiation of F9 embryonal carcinoma cells into cells with a phenotype resembling parietal endoderm. We show that the level of cyclin-dependent kinase inhibitor p21(WAF1/Cip1) (p21) is dramatically elevated during this differentiation via both the reduction of the proteasome-dependent degradation and accumulation of mRNA. The induction of differentiation markers could not be achieved by ectopic p21 alone and still required treatment with differentiation agents. A clone of F9 cells in which expression of p21 was reduced by a stable transfection of the p21-antisense construct transcribed lower level of mRNA for thrombomodulin, a parietal endoderm-specific marker. Conversely, a clone overexpressing p21, upon differentiation, revealed upregulated levels of thrombomodulin mRNA, without any effect on the cell cycle. Furthermore, p21 activated the thrombomodulin promoter in transient reporter assays and the promoter activity in differentiated cells was reduced by cotransfecting p21-specific siRNA. The p21 mutant defective in binding to cyclin E activated the thrombomodulin promoter as efficiently as the wild type protein. As the differentiation of F9 cells into parietal endoderm-like cells requires the cAMP-PKA-CREB-p300 signaling axis, the results together suggest that the cyclin-dependent kinase inhibitor p21 may specifically promote the cAMP-dependent differentiation pathway in these cells, independently of its function in cell cycle regulation.

Acknowledgement

Supported by an institutional research project MZ00000064211 from the Ministry of Health, Czech Republic.

3L05

CELLULAR AND MOUSE MODELS OF DEREGULATED SIGNALING PATHWAYS IN LEUKEMIA

Sylvia Takáčová[a,*], Monika Priwitzerová[a], Marie Jarošová[b] and Vladimír Divoký[a,b]

[a]*Laboratory of Genetic Engineering, Department of Biology, Faculty of Medicine, Hněvotínská 3, 775 15 Olomouc, Czech Republic*
[b]*Department of Hemato-oncology, University Hospital Olomouc, I. P. Pavlova 6, 775 20 Olomouc, Czech Republic*

** E-mail: stakacova@hotmail.com*

Hematopoiesis is a process in which a stem cell differentiates into multiple cell types. Multiple growth factors, receptors and transcription factors affecting this process are primary regulators of survival, proliferation and differentiation of hematopoietic progenitors. Many of the genes encoding these signaling factors are mutated in leukemia. We focus our research on two key signaling molecules implicated in leukemogenesis: tyrosine kinase Bcr/Abl and transcription factor MLL (Mixed Lineage Leukemia).

A specific inhibitor STI571 (imatinib mesylate, IM) inhibits Bcr/Abl oncogenic tyrosine kinase activity[1]; however, resistance to this drug represents an important clinical problem. We study cellular and molecular mechanisms of resistance of Bcr/Abl-positive chronic myeloid leukemia to IM. In the resistant KBM5$^{STI571-R}$ cells (created at M.D. Anderson Cancer Center, Houston, TX), the activity of the Bcr/Abl kinase (level of autophosphorylation) was incompletely inhibited by IM. We described occurrence of a single point mutation within a kinase ATP-binding pocket leading to a substitution of a threonine-to-isoleucine at position 315 of Abl (T315I) in a proportion of copies of the BCR/ABL gene in IM-resistant cells[2]. This mutation abolishes IM binding to Abl-ATP-binding pocket, while ATP binding is not affected[3].

MLL protein is a transcription factor with histone methyltransferase activity that can be activated into an oncoprotein by diverse mutations in human acute leukemias[4]. The high diversity of MLL mutations and fusion proteins, as a consequence of chromosomal translocations with a variety of partner genes suggests multiple molecular mechanisms for leukemogenic conversion of MLL and cellular transformation. We use double-replacement gene targeting in mouse embryonic stem cells to replace a mouse MLL gene with its inducible oncogenic form - a MLL-ENL fusion gene, which is one of the most common fusions in MLL - associated leukemias[5]. This animal model should be useful for studies of the early events in leukemogenic transformation.

References

1. Druker B. J., Tamura S., Buchdunger E., Ohno S., Segal G. M., Fanning S., Zimmermann J. and Lydon N. B.: *Nat. Med.* **2**, 561 (1996)
2. Ricci C., Scappini B., Divoký V., Gatto S., Onida F., Verstovsek S., Kantarjian H. M. and Beran M.: *Cancer Res.* **62**, 5995 (2002)

3. Gorre M. E., Mohammed M., Ellwood K., Hsu N., Paquette R., Rao P. N. and Sawyers C. L.: *Science* **293**, 876 (2001)
4. Milne T. A., Briggs S. D., Brock H. W., Martin M. E., Gibbs D., Allis C. D. and Hess J. L.: Mol. *Cell.* **10**, 1107 (2002)
5. Zeisig B. B., Milne T., Garcia-Cuellar M. P., Schreiner S., Martin M. E., Fuchs U., Borkhardt A., Chanda S. K., Walker J., Soden R., Hess J. L. and Slany R. K.: *Mol. Cell. Biol.* **24**, 617 (2004)

Acknowledgement

This research is supported by grants 301/01/0489 and 301/04/1239 from the Grant Agency, Czech Republic, and NC/6652 and NR/7849 from the Internal Grant Agency of the Ministry of Health, Czech Republic.

3L06

p73 PROTEIN, A HOMOLOGUE OF p53 TUMOUR SUPPRESSOR GENE

Pavla Češková* and Bořivoj Vojtěšek

Department of Experimental Oncology, Masaryk Memorial Cancer Institute, Žlutý kopec 7, 65653 Brno, Czech Republic

** E-mail: ceskova@mou.cz*

The p53 is the well known tumour suppressor protein which is involved in control of the cell cycle, apoptosis and senescence. The realisation that p53 in fact belongs to a family of related genes came in 1997 when Kaghad et al.[1] discovered cDNA encoding the p73 protein. The TP73 gene maps to the chromosome region 1p36.2-3 known for a long time as a part of the chromosome where some of tumour suppressor genes are localised.

Although both genes are showing many similarities, substantial differences can be found between them. In contrast to the p53-deficient mice, which exhibit increased susceptibility to spontaneous tumorigenesis, mice functionally deficient for all p73 isoforms exhibit profound defects, which indicated the role of p73 protein in neurogenesis, sensory pathways and homeostatic control[2]. The p73 protein is not a classic Knudson-type tumour suppressor gene like p53 and its role in tumorigenesis is still unclear. The p73 protein is an important component of the cellular response to DNA damage, and may play a critical role in tumour suppression and chemosensitivity.

Different splicing of TP73 gene is responsible for formation of many p73 C- and N-terminal isoforms. The C-terminal region of the p73 protein affects the binding ability of the protein to the DNA and seems to be important for many specific protein-protein interactions, which take part in different regulation processes. Detailed analysis of the p73 protein in tumour tissues has revealed that it is necessary to distinguish between the

N-terminal non-deleted TA-p73 isoforms and the N-terminally truncated, transactivation-deficient DN-p73 isoforms, which has been shown to possess oncogenic activity.

Each of the p73 isoforms has different potential for protein–protein and DNA–protein interactions. Together with p63 isoforms and through their oligomerisation domains they may also interact with each other and with the p53 protein, forming a complex network finely tuning the actual physiological cell state and its response to different stress stimuli.

Our studies are focused on the comparison of p73 alfa, beta, gama and delta isoforms (i) in potential for *in vitro* DNA binding ability, (ii) on the comparison of their *ex vivo* transactivation abilities and (iii) on the comparison of different p73 isoforms in the ability to induce apoptosis.

References

1. Kaghad M., Bonnet H., Yang A., Creancier L., Biscan J. C., Valent A., Minty A., Chalon P., Lelias J. M., Dumont X., Ferrara P., McKeon F. and Caput D.: *Cell* **90**, 809 (1997)
2. Yang A., Walker N., Bronson R., Kaghad M., Oosterwegel M., Bonnin J., Vagner C., Bonnet H., Dikkes P., Sharpe A., McKeon F. and Caput D.: *Nature* **404**, 99 (2000)

Acknowledgement

This work was supported by the grant NS 301/04/P023 from the Grant Agency of the Czech Republic and grant IAA4004110 from the IGA AV CR.

3L07

RECENT RESEARCH ADVANCEMENT IN BACTERIAL CELL DIVISION

Imrich Barák*

Institute of Molecular Biology, Centre of Excellence for Molecular Medicine, Slovak Academy of Sciences, Dúbravská cesta 21, 84551 Bratislava, Slovakia

** E-mail: imrich.barak@savba.sk*

The basic process of cell division is conceptually similar in eukaryotic and prokaryotic cells. This process is characterized by creating the division septum between the duplicated chromosomes. There are several advantages of studying cell division in prokaryotes. In general, it is still more simple process in bacteria than in eukaryotic cells and there are several outstanding model as *Escherichia coli* and *Bacillus subtilis*. The process of cell division is intensively studied on molecular level for decades but there are still many unanswered basic questions. Probably most is known about the

mechanism of cell division of rod shaped bacteria, mainly Gram-negative *Escherichia coli* and Gram-positive *B. subtilis*. There are known a lot of players in this process but mechanism of where, how and when cells form the division septum with high fidelity is often postulated in fully different models. Probably the most controversial question in cell division of rod shaped bacteria is where to divide, in other words, how the position of the division site is determined. At least two distinct mechanisms are involved in accurate placement of the division machinery: the Min system and nucleoid occlusion.

Generally, the cell must coordinate the cell division with the faithful segregation of the newly duplicated chromosomes to each daughter cell. This is followed in vegetatively growing *B. subtilis* cell by complex mechanism. Strikingly, at the beginning of sporulation, instead of splitting two chromosomes, they form an elongated structure known as axial filament. The chromosomes in this predivisional sporangium are oriented with their replication origin regions to opposite poles of the cell. One protein candidate involved in recruiting the chromosomes to this sites is the DivIVA protein[1], which forms an oligomer like structures[2,3]. The movement of two chromosomes is under the control of the phosphorelay system[4]. There are three known proteins involved in chromosome segregation during sporulation – Spo0J, RacA and Soj DNA-binding proteins. Soj has an ability to undergo co-operative relocation from nucleoid to nucleoid[5] or pole to pole[6] and this movement requires Spo0J protein, which binds to condensation regions at the chromosome near the oriC. RacA was shown that also binds at the chromosome and is a part of the mechanism that attaches the two chromosomes to the poles, likely by contacting DivIVA protein already localized at the cell pole[7].

Cell division as a fundamental cellular process still holds many secrets which are waiting to be unraveled. The major challenges now lie in understanding of assembly and disassembly of the protein complexes at the site of division. To understand the molecular mechanisms of these processes would require state of art experimental methods to solve the structure not only particular proteins but rather the protein complexes and their proper interpretation to explain such phenomena as asymmetry of protein localization, protein oscillation, protein spiral formation and other.

References

1. Thomaides H. B., Freeman M., El Karoui M. and Errington J.: *Genes Dev.* **15**, 1662 (2001)
2. Muchová K., Kutejová E., Scott D. J., Brannigan J. A., Lewis R. J., Wilkinson A. J. and Barák I.: *Microbiology* **148**: 807 (2002)
3. Stahlberg H., Kutejová E., Muchová K., Gregorini M., Lustig A., Müller S. A., Olivieri V., Engel A., Wilkinson A. J. and Barák I.: *Mol. Microbiol*., in press (2004)
4. Graumann P. L. and Losick R.: *J. Bacteriol.* **183**, 4052 (2001)
5. Marston A. L. and Errington J.: *Mol. Cell* **4**, 673 (1999)
6. Quisel J. D., Lin D. C. H. and Grossman A. D.: *Mol. Cell* **4**, 665 (1999)
7. Ben-Yehuda S., Rudner D. Z. and Losick R.: *Science* **299**, 532 (2003)

Acknowledgement

Work in author‘s laboratory was supported by grant 2/1004/22 from the Slovak Academy of Sciences and The Wellcome Trust Collaborative Research Initiative Grant 066732/Z/01/Z.

3L08

PHARMACOLOGICAL INHIBITORS OF PROTEIN KINASES

Vladimír Kryštof*

Laboratory of Growth Regulators, Faculty of Science, Palacký University & Institute of Experimental Botany, Academy of Science, Šlechtitelů 11, 783 71 Olomouc, Czech Republic

** E-mail: krystof@aix.upol.cz*

Protein kinase superfamily, constituting about 1.7% of all human genes, represents central information network in the cell[1]. Besides intra and intercellular signal transduction, kinases also control metabolism, transcription, cell cycle progression, apoptosis, differentiation, maintain cell shape and movement, ensure functioning of the nervous and immune systems. All of these diverse processes are provided by the transfer of γ-phosphate from ATP to the side-chain hydroxyls of serine/threonine or tyrosine in a substrate protein, a reaction referred to as phosphorylation. Small phosphate group then stimulates conformational changes of the protein, leading to de/activation of its functions, e.g. enzymatic, receptor or binding activity[1,2].

Mutations and deregulations of protein kinases play essential roles in many human diseases, and that makes them potential targets for pharmacological intervention. A number of inhibition mechanisms was already designed, but a great majority of described kinase inhibitors are ATP competitors. The development of an active compound requires several steps, including identification of a leading structure, either by *in vitro* assay or by virtual screening *in silico*, subsequent structure-activity relationships studies, culminating in the design and synthesis of an optimized pharmacological candidate and verification of its therapeutic properties. Examples of successful development of protein kinase inhibitors will be demonstrated on drugs that are already being evaluated clinically, including cyclin-dependent kinase inhibitor roscovitine and BCR/Abl inhibitor STI571.

References

1. Manning G., Whyte B. D., Martinez R., Hunter T. and Sudarsanam S.: *Science* **298**, 1912 (2002)
2. Bridges A. J.: *Chem. Rev.* **101**, 2541 (2001)

Acknowledgement

The author was supported by the grants from GACR (204/03/D231) and GA ASCR (KJB4038301).

3L09

MOLECULAR MECHANISM OF PLANT DEFENSE INDUCED BY ELICITINS, PROTEINACEOUS ELICITORS OF *PHYTOPHTHORA* SP.

Vladimir Mikeš[a,*], Jan Lochman[a], Tomáš Kašparovský[a], Jiří Damborský[b], Michel Ponchet[c] and Jean-Pierre Blein[d]

[a]*Department of Biochemistry and* [b]*National Centre for Biomolecular Research, Masaryk University, Kotlářská 2, 61137 Brno, Czech Republic*
[c]*UMR IPMSV, Sophia Antipolis, France*
[d]*UMR 692, INRA, Dijon, France*

** E-mail: mikes@chemi.muni.cz*

Elicitins are secreted specifically by the fungi *Oomycete* genera *Pythium* and *Phytophthora*[1,2]. Biological function of elicitins is currently unknown. The response is induced by the interaction of elicitins with a putative receptor located on the cytoplasmic membrane. The transfer of signal through the receptor triggers phosphorylation-dephosphorylation cascades in the tobacco resulting in alkalization of the extracellular medium, efflux of potassium and chloride ions, influx of calcium, production of the active species from oxygen[3,4], changes in composition of the cell wall and the induction of acquired systemic resistance. Binding of sterols to the cavity of elicitins seems to be essential for consecutive association of the elicitin with a receptor and induction of a biological response in a plant[5]. We prepared a series of mutants of cryptogein, one of the most potent elicitors of this group, with altered capacity of binding sterols. We compared the physicochemical parameters of sterol-cryptogein binding, their ability to induce the synthesis of active oxygen species, their necrotic activity on tobacco suspension cells and the ability to induce the expression of pathogen related (PR) proteins in plants. The results showed that some of the early events are proportional to the affinity of cryptogein to bind sterols whereas the others (necrotic effect and the induction of PR protein synthesis) seemed to be dependent on the overall cryptogein structure.

References

1. Ricci P., Bonnet P., Huet J. C., Sallantin M., Beauvais-Cante F., Bruneteau M., Billard V., Michel G. and Pernollet J. C.: *Eur. J. Biochem*. **183**, 555 (1989)
2. Huet J.C., Lecaer J. P., Nespoulous C. and Pernollet J. C.: *Mol. Plant-Microb. Interact*. **8**, 302 (1995)
3. Blein J. P., Milat M. L. and Ricci P.: *Plant Physiol*. **95**, 486 (1991)
4. Rusterucci C., Stallaert V., Milat M. L., Pugin A., Ricci P. and Blein J. P.: *Plant Physiol.* **111**, 885 (1996)
5. Mikeš V., Milat M. L., Ponchet M., Ricci P. and Blein J. P.: *FEBS Lett*. **416**, 190 (1997)

Acknowledgement

This work was supported by the grants Barrande 2002-042-2 (Czech Republic, France) and 522/02/0925 (Grant Agency of the Czech Republic).

3L10

IDENTIFICATION AND CHARACTERIZATION OF rpoE REGULON IN *SALMONELLA TYPHIMURIUM*

Henrieta Mitická[a,*], Bronislava Rezuchová[a], Gary Rowley[b], Mark Roberts[b] and Jan Kormanec[a]

[a]*Institute of Molecular Biology, Centre of Excellence for Molecular Medicine, Slovak Academy of Sciences, Dúbravská cesta 21, 84551 Bratislava, Slovakia*
[b]*Department of Veterinary Pathology, Glasgow University Veterinary School, Glasgow G61 1OH, U.K.*

** E-mail: miticka@hotmail.com*

Salmonella species are intracellular bacterial pathogens, which cause a spectrum of diseases in human and animals ranging from mild enteritidis to systematic infections. The extracytoplasmatic stress response sigma factor, RpoE, encoded by rpoE gene, has been shown to be critical to *Salmonella typhimurium* virulence and for resistance to oxidative stress, antimicrobial peptides, survival in stationary phase and the starvation stress response[1]. We analyzed expression of rpoE in bacteria grown in different condition by S1-nuclease mapping using RNA and *in vivo* reporter gene fusions. Three promoters, rpoEp1, rpoEp2 and rpoEp3, were located upstream of the rpoE gene in *S. typhimurium*. The promoters were differentially expressed during growth and under several stress conditions. Expression from the rpoEp3 promoter was absent in the rpoE mutant, demonstrating its dependence upon RpoE in *S. typhimurium*[2]. Using the RpoE-dependent rpoEp3 promoter, we have optimized *E. coli* two-plasmid system for identification of RpoE-dependent genes. The method is based on the assumption that the *E. coli* RNA-polymerase core enzyme interacts with a particular heterologous sigma factor expressed from the first plasmid. The resulting holoenzyme would recognize a promoter present in a library of chromosomal fragments cloned in the second compatible plasmid, upstream of the lacZa reporter gene. After screening of 240,000 colonies of the *S. typhimurium* SL1344 library, 36 positive clones containing *S. typhimurium* RpoE-dependent promoters were identified and the promoters located by S1-nuclease mapping. All the promoters contained sequences similar to the consensus sequence of RpoE-dependent promoters in *E. coli*. Analyzing their sequences with database we identified genes governed by the RpoE-dependent promoters. They are important as transcriptional factors, regulatory genes, genes of primary metabolism functions, periplasmic folding factors, lipopolysaccharide biogenesis and genes of unknown functions. Expression of several genes has been verified in *S. typhimurium.* The RpoE-dependent promoters were induced at the transition into stationary phase and under cold shock.

References

1. Humphreys S., Stevenson A., Bacon A., Weinhard A. A. and Roberts M.: *Inf. and Immun*. **67**, 1560 (1999)
2. Mitická H., Rowley G., Rezuchová B., Homerová D., Humphreys S., Farn J., Roberts M. and Kormanec J.: *FEMS Microbiol. Lett.* **226**, 307 (2003)

Acknowledgement

This work was supported by the grant 2/3010/23 from the Slovak Academy of Sciences and the Wellcome Trust grant 065027/Z/01/Z.

3L11

USING OF AFFINITY CHROMATOGRAPHY FOR ISOLATION OF PLANT STEROL-BINDING PROTEINS

Marek Kamlar[a,b], Tomáš Macek[b,*] and Ladislav Kohout[c]

[a]*Department of Biochemistry and Microbiology, Institute of Chemical Technology, Technická 3, 16628 Praha 6, Czech Republic*
[b]*Department. of Natural Products and* [c]*Department of Steroid Chemistry, Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Flemingovo nám. 2, 16610 Praha 6, Czech Republic*

** E-mail: tom.macek@uochb.cas.cz*

Plant sterols are large group of natural products with wide spectrum of action. We sort them to brassinosteroids, ecdysteroids, steroid saponins, cardenolids etc. Nowadays we tested especially brassinosteroids (BRs), steroid phytohormones affecting differentiation of plants, formation of biomass, photomorphogenesis, but also senescence or stress tolerance[1].

The molecular mechanism of their action is still unclear. It is supposed that BRs act in plants after binding to a sterol-binding protein (SBP), which complexes with receptor in the membrane, but also binding of BR to the membrane receptor alone is not excluded[2,3].

We attempted to isolate and identify such proteins by bioaffinity chromatography. For our experiments we used various types of synthetic analogues of natural brassinosteroids (e.g. with carboxyl group introduced into the side chain, or on ring B of the steroid skeleton)[4] immobilised on polymeric carriers. These bioaffinity matrices were tested for affinity to brassinosteroid-binding proteins or receptors[5].

Here we report on the identification of one of these proteins isolated from tobacco callus during affinity chromatography on polymeric matrices and subsequent electrophoresis. One of the most abundant proteins recovered from the column was sequenced from N-terminal and exhibited a 100 % homology with osmotin-like protein precursor (OLPA)[6], an already known pathogenesis-related (PR) protein from tobacco, which has connection with plant adaptation to abiotic and biotic stress caused by drying, wounding or other factors, pathogens like *Candida albicans*, *Trichoderma reesei* etc [7].

This is especially interesting in connection with the fact, that brassinosteroids can influence the stress response of higher plants, including stress caused by pathogens[8].

Recently the same experimental approach has been applied also to work with ecdysteroids, another group of plant steroids.

References

1. Szekeres M., Nemeth K., Koncz-Kalman Zs., Mathur J., Kauschmann A., Altmann T., Redei G. P., Nagy F., Schell J. and Koncz Cs.: *Cell* **85**, 171 (1996)
2. Müssig C. and Altmann T.: *Trends Endocrinol. Metab.* **12**, 398 (2001)
3. Li J.: *Curr. Opin. Plant Biol.* **6**, 494 (2003)
4. Kohout L., Chodounská H., Macek T. and Strnad M.: *Collect. Czech Chem. Comm.* **65**, 1 (2000)
5. Macek T., Kamlar M., Ježek R., Pišvejcová A., Koncz Cs. and Kohout L.: *Chem. Listy* **97**, S285 (2003)
6. Kamlar M., Macek T., Koncz Cs. and Kohout L: *Chem. Listy* **97**, S274 (2003)
7. Abad L., D'Urzo M. P., Liu D., Narasimhan M. L., Reuveni M., Zhu J.K., Niu X., Singh N. K., Hasegawa P. M. and Bressan R. A.: *Plant Sci.* **118**, 11 (1996)
8. Kohout L.: *Collect. Czech Chem. Comm*. **59**, 457 (1994)

Acknowledgement

This work was sponsored by the grant A4055204 from the Grant Agency of the Academy of Sciences of the Czech Republic, research project Z4 055 905 and by grant 658G4 from the Fund of Advancement of Universities.

3L12

TRANSCRIPTION REGULATION OF POTENTIAL VIRULENCE FACTORS IN *COXIELLA BURNETII*

Jana Melničáková[a,*], Dale Howe[b], Robert A. Heinzen[b] and Imrich Barák[c]

[a]*Institute of Virology, Slovak Academy of Sciences, Dúbravská cesta 9, 84245 Bratislava, Slovakia*
[b]*Rocky Mountain Laboratories, NIAID/NIH, Hamilton, MO, USA*
[c]*Institute of Molecular Biology, Slovak Academy of Sciences, Dúbravská cesta 21, 84551 Bratislava, Slovakia*

** E-mail: virujaci@mail.com*

Coxiella burnetii is the only obligate intracellular parasite that is able to survive normally bactericidal phagolysosomal environment of the host cell. It undergoes biphasic live cycle with two distinct morphological forms, extremely resistant

extracellular cell variant able to transmit by aerosol and intracellular variant capable to abolish bactericidal influences of the host cell. Previous research of sigma factor RpoS indicates its important role in transition between cell variants and involvement in pathogenesis of *C. burnetii*[1].

In our study, based on the high level of homology between both *Escherichia coli* and *C. burnetii* RpoS, we showed complementation of *E. coli* RpoS deficiency with *C. burnetii* RpoS. We applied two-compatible vector system in *E. coli* to identify *C. burnetii* genes specifically regulated by RpoS. Using this two-vector system we identified 116 RpoS-dependent lacZα fusions from *C. burnetii* library. DNA sequence analysis revealed RpoS-recognized promoters and alignment of these promoter regions showed the high level of similarity in their putative –10 promoter regions[2]. The specific binding of *C. burnetii* RpoS onto selected promoters was checked by β-galactosidase assays of lacZα fusions of these promoters. Gel shift assay provided evidence of binding RpoS onto sodC promoter. To further investigate transcription profiles of *C. burnetii* genes after infection of the host cells, real time PCR assays were performed. These analyses showed first time the possibility to study transcription profiles of *C. burnetii* genes as early as 2 hours post-infection.

Development of the gene disruption methods in *C. burnetii* requires improvement of DNA transformation procedure. There are at least three bottlenecks in this methodology. Firstly, the use of proper antiobiotic selection markers is crucial. Thus, we checked ampicillin and chloramphenicol as selection markers for their suitability for transformation procedure. Using foci forming unit assay, lower bacteriostatic concentrations of ampicillin (25 mg/ml) and chloramphenicol (10 mg/ml) were assessed. *C. burnetii* cells were checked by fluorescence and confocal microscopy for evaluation of influence of these antibiotics on *C. burnetii* cell cycle in the host cells. Secondly, the clonal isolation of transformed *C. burnetii* cells is important for subsequent genotypic and phenotypic characterization. We have used a newly developed technique which allows sterilely excise parasitophorous vacuoles harboring *C. burnetii* into eppendorf tube and re-infect them into freshly prepared monolayer of the host cell line. Finaly, it was important to find suitable plasmids to obtain integration into chromosome. For this purpose, we designed different vectors. We prepared shuttle vector with cat, gfp and internal fragment of *C. burnetii* gene to achieve integration into chromosome as a consequence of autonomous plasmid replication in bacterial cytoplasm after its successful introduction into *C. burnetii*. We also constructed the second, a low copy number vector, carrying the same selection markers. This vector should allow integration into chromosome without plasmid replication. Finally, we used previously published ColE1- plasmid pGFP-CB, which was supposed to integrate into chromosome and replicate in cytoplasm, respectively[3]. After construction of all these vectors and introduction of above mentioned methods, we are well prepared for successful genetic transformation of *C. burnetii* and to achieve homologous recombination.

References

1. Seshadri R. and Samuel J. E.: *Infect. Immun*. **69**, 4874 (2001)
2. Melničáková J., Lukáčová M., Howe D., Heinzen R. A. and Barák I.: *Ann. NY Acad. Sci.* **990**, 591 (2003)

3. Lukáčová M, Valková D., Quevedo Diaz M., Perečko D. and Barák I.: *FEMS Microbiol. Lett.* **175**, 255 (1999)

Acknowledgement

The work in the author's laboratory was supported by grants 2/7057/20 and 2/1004/21 from the Slovak Academy of Sciences (M.L. and I.B) and by NIH grant RR15553 (R.A.H.).

3P01

ROLE OF PHOSPHATIDYLCHOLINE (PC) TURNOVER PRODUCTS IN TRANSCRIPTIONAL REGULATION OF PHOSPHOLIPID BIOSYNTHETIC GENES

Roman Holič* and Peter Griač

Institute of Animal Biochemistry and Genetics, Slovak Academy of Sciences, Moyzesova 61, 90028 Ivanka pri Dunaji, Slovakia

** E-mail: roman.holic@savba.sk*

The majority of yeast phospholipid structural genes contain in their promoters inositol-sensitive upstream activating sequence (UASINO) and are regulated in response to the availability of soluble precursors inositol and choline. The coordinately regulated genes are derepressed in the absence of inositol and repressed in the presence of inositol. The INO1 gene (encoding inositol 1-phosphate synthase) is the most regulated gene of the entire regulon and frequently serves as a reporter gene for the whole set of phospholipid biosynthetic genes. Transcription of the INO1 gene responds also to the changes of phospholipid metabolism. It was shown, that under some conditions, repression of the INO1 gene in the presence of inositol and choline depends on the functional Sec14p. Sec14p is an essential protein in *Saccharomyces cerevisiae*. Sec14p was originally identified as a phosphatidylinositol transfer protein (PITP) which catalyzes *in vitro* transport of phosphatidylinositol (PI) and PC between artificial and biological membranes. Sec14p is involved in protein secretion and regulation of lipid synthesis and turnover *in vivo*. Five sequence homologues to Sec14p (Sfh1-5 – sec fourteen homologues) were found in *S. cerevisiae*. Two of them, Sfh2p and Sfh4p, when overexpressed, complemented very well the otherwise essential Sec14p requirement in growth and secretion. Sfh2p and Sfh4p also regulated phospholipase D1 mediated PC turnover, however, in opposite way than Sec14p.

In this study we were interested, which one of Sec14p homologues is able to correct the deregulation of INO1 caused by missing Sec14p. We also tested response of the INO1 gene expression to metabolic signal generated via phospholipase D1. Our results

show that none of the Sec14 homologues was able to substitute for Sec14p in its regulatory aspects toward phospholipid biosynthesis. We observed that the INO1 gene expression is deregulated in yeast cells with high PC turnover. Removal of phospholipase D1 activity (Pld1p) corrected the INO1 regulation in yeast strains with otherwise high PC turnover and resulting deregulation of the INO1 gene. Two products, phosphatidic acid (PA) and choline, are generated from every molecule of PC when PC turnover occurs via a PLD-mediated route. Overall cellular levels of PA were measured using steady-state 32P labelling. Results show that higher steady-state cellular levels of PA correspond with the deregulated levels of INO1 transcription in the presence of inositol. Overall, our data support the model that relative levels of PA act as a metabolic signal for INO1 derepression[1]. However, the mechanism, how changes in PA levels are transmitted to the transcriptional machinery remains to be elucidated.

References

1. Henry S. and Patton-Vogt J.: *Prog. Nucleic Acid Res. Mol. Biol*. **61**, 133 (1998)

Acknowledgement

Study was supported by VEGA 2/4130/4 and APVT (Slovak Republic) 51-016502.

3P02

YEAST *SACCHAROMYCES CEREVISIAE* AS A MODEL FOR STUDYING MOLECULAR MECHANISMS OF HUMAN NEURODEGENERATIVE DISORDER NIEMANN PICK SYNDROME TYPE C

Lucia Hronská[a,]*, Alena Pichová[b], Roger Schneiter[c] and Ivan Hapala[a]

[a]*Institute of Animal Biochemistry and Genetics, Slovak Academy of Sciences, Moyzesova 61, 90028 Ivanka pri Dunaji, Slovakia*
[b]*Institute of Genetics, Salzburg University, Austria*
[c]*Institute of Biochemistry, Graz University of Technology, Austria*

** E-mail: lucia.hronska@savba.sk*

Several years ago Carstea et al.[1] identified gene responsible for the majority of cases of fatal neurodegenerative disorder Niemann Pick type C syndrome in humans. Intense studies are now in progress to understand the cellular and molecular function of this human Npc1 protein and its orthologues in other organisms suggested to be involved in transport and homeostasis of cholesterol in higher eukaryotes. Perturbation of Npc1 function causes accumulation of cholesterol and other compounds in the endosomal/lysosomal compartment in mammalian cells. A gene with 60 % homology to

human NPC1 gene has been identified in the yeast *S. cerevisiae*[2]. No phenotype has been described for the deletion of this yeast NCR1 gene so far.

One of research objectives in our laboratory is the study of possible mechanisms of uptake and intracellular transport of sterols in *S. cerevisiae*. We established an assay for monitoring sterol uptake and intracellular fate of external sterols in non-growing yeast cells deprived of sterol in anaerobiosis-like conditions. This enabled us to describe sterol homeostasis/transport-related changes in *S. cerevisiae* strain lacking the NCR1 gene. We have also shown analogy in the dynamic intracellular distribution of the yeast Ncr1-GFP (non-functional) and GFP-Ncr1 (potentially functional) fusion proteins with the human Npc1 mutant and wild-type proteins suggesting functional homology of the two homologues. Our results show that yeast *S. cerevisiae* is an excellent model for study of the functions of Npc1/Ncr1 proteins and of the molecular mechanisms behind the cholesterol storage disease – Niemann Pick type C syndrome as well as for the general mechanisms involved in intracellular transport and homeostasis of sterols in eukaryotic cells.

References

1. Carstea E. D., Morris J. A., Coleman K. G., Loftus S. K., Zhang D., Cummings C., Gu J., Rosenfeld M. A., Pavan W. J., Krizman D. B., Nagle J., Polymeropoulos M. H., Sturley S. L., Ioannou Y. A., Higgins M. E., Comly M., Cooney A., Brown A., Kaneski C. R., Blanchette-Mackie E. J., Dwyer N. K., Neufeld E. B., Chang T. Y., Liscum L., Tagle D. A., et al.: *Science* **277**, 228 (1997)
2. Loftus S. K., Morris J. A., Carstea E. D., Gu J. Z., Cummings C., Brown A., Ellison J., Ohno K., Rosenfeld M. A., Tagle D. A., Pentchev P. G. and Pavan W. J.: *Science* **277**, 232 (1997)

Acknowledgement

This work was supported by grants APVT-51-016502 (I.H.), Aktion Austria-Slovakia 36s2 (I.H. and R.S.) and CEEPUS Mobility Grant (L.H.).

3P03

INTERACTION OF ENVELOPE GLYCOPROTEINS WITH MATRIX PROTEIN OF MASON-PFIZER MONKEY VIRUS

Lenka Sedláčková*, Jan Lipov and Tomáš Ruml

Department of Biochemistry and Mikrobiology, Institute of Chemical Technology, Technická 3, 16628 Praha 6, Czech Republic

** E-mail: lenka.sedlackova@vscht.cz*

Retroviruses are enveloped viruses, which are released from infected cells by budding through cellular membrane. During this process envelope glycoproteins (Env) are incorporated into the virus particle. Env complex consits of the surface (SU) and the transmembrane (TM) glycoproteins, which are noncovalently associated. The SU glycoprotein is responsible for cellular tropism of the virus, whereas the TM glycoprotein mediates virus-cell membrane fusion during viral entry.

Incorporation of glycoproteins to budding virions is generally believed to be mediated by a specific interaction between cytoplasmic domain of TM and polyprotein precursor Gag, which is precursor of viral structural proteins. Some biochemical evidence for a stable interaction between $Pr55^{Gag}$ and cytoplasmic domain of gp41 in immature HIV-1 particles has been reported and some results suggest that particularly matrix protein (MA), which is localized under lipid envelope of the mature virion, is required for incorporation of glycoproteins[1-3].

Mason-Pfizer Monkey Virus (M-PMV) is characterized by the assembly of intracytoplasmic immature particles, which are afterwards transported to the plasma membrane and are released by budding. M-PMV is in this way different from HIV-1, which assembles directly at the plasma membrane at the same time with budding. We are interested in the interaction between cytoplasmic domain of TM (gp22) and MA, optionally with $Pr78^{Gag}$. To determine whether interaction between gp22 and $Pr78^{Gag}$ is stable also in M-PMV immature virions, we use sedimentation of virions through detergent layer.

We have prepared vectors for expression MA, Env and TM in tissue cultures for the study of their properties *in vivo*. We have expressed the proteins and they were confirmed to be localized in a membrane fraction by sedimentation flotation centrifugation. Preliminary results indicate that MA is associated with the plasma membrane in regions called „lipid rafts“ and that Env and TM are incorporated into these regions. The possibility that targeting of the MA to these regions is induced by specific interaction with glycoproteins is under current investigation. The experiments with *Vaccinia* virus overexpression system should show whether MA forms enveloped particles containing co-expressed glycoproteins[4].

References

1. Wyma D. J., Kotov A. and Aiken C.: *J. Virol.* **74**, 9381 (2000)
2. Cosson P.: *EMBO J.* **15**, 5783 (1996)

3. Freed E. O. and Martin M. A.: *J.Virol.* **70**, 341 (1996)
4. Gonzalez S. A., Affranchino J. L., Gelderblom H. R. and Burny A.: *Virology* **194**, 548 (1993)

3P04

THE ROLE OF MAP KINASE ACTIVATION AND p53 PHOSPHORYLATION IN APOPTOSIS INDUCED BY POLYCYCLIC AROMATIC HYDROCARBONS

Zdeněk Andrysík[a,*], Kateřina Chramostová[a], Lenka Šindlerová[a], Alois Kozubík[a], Alena Gábelová[b], Miroslav Machala[c] and Jan Vondráček[a,c]

[a]*Laboratory of Cytokinetics, Institute of Biophysics, Academy of Sciences of the Czech Republic, Královopolská 135, 612 65 Brno, Czech Republic*
[b]*Department of Mutagenesis and Carcinogenesis, Cancer Research Institute of Slovak Academy of Sciences, Vlárská 7, 833 91 Bratislava, Slovak Republic*
[c]*Veterinary Research Institute, Hudcova 70, 621 32 Brno, Czech Republic*

** E-mail: andrysik@ibp.cz*

The liver plays a primary role in both metabolic clearance and activation of chemical carcinogens, such as polycyclic aromatic hydrocarbons (PAHs). These compounds exert many toxic effects including genotoxicity, tumor promotion and apoptosis induction. The p53 tumor suppressor plays a major role in processes associated with cell death, cell cycle arrest and DNA repair. Phosphorylation of p53 at serine 15 in response on treatment with various genotoxic agents promotes both cellular accumulation and functional activation of p53[1]. In the regulation of cell survival, there are essential signals transduced by three groups of mitogen-activated protein kinases (MAPKs) represented by extracellular signal regulated kinases (ERKs), Jun N-terminal kinases (JNKs) and p38 kinases. The regulation of apoptosis by MAPKs is a complex and often controversial process. The relevance of the original hypothesis that ERKs are important for cell survival whereas JNKs and p38s respond to stress and participate in apoptosis induction is not absolute, since ERKs might also control[1-3].

In our study we used a set of genotoxic PAHs - benzo[a]pyrene (BaP), dibenzo[a,l]pyrene (DBalP), 7*H*-dibenzo[*c,g*]carbazole (DBC) and its two derivatives, *N*-methyldibenzo[*c,g*]carbazole (MeDBC) and *5,9*-dimethyldibenzo[*c,g*]carbazole (diMeDBC), in order to study induction of apoptosis, p53 phosphorylation at Ser 15 and activation of MAPKs in rat hepatic epithelial WB-F344 cells[4]. These cells represent a cellular model of liver epithelial „stem-like" cells that could be a potential target for liver carcinogens[5]. We found that BaP, DBalP, DBC and diMeDBC induced apoptosis, which was preceded by S-phase cell cycle arrest, p53 phosphorylation and ERK1/2 activation. These results suggested that induction of apoptosis was associated with the

genotoxic effects of PAHs. Contrary to that, MeDBC did not induce either apoptosis or p53 phosphorylation and ERK1/2 activation at concentrations up to 10 μM.

We next used BaP and DBalP as model compounds to study the effects of PAHs in more detail. Inhibition of MEK1/2 kinases by U0126 inhibitor blocked ERK1/2 activity and prevented apoptosis induced by PAHs. Pifithrin-alpha, which is as an inhibitor of cell death associated with genotoxic damage, prevented ERK1/2 activation after either BaP or DBalP treatment. Inhibition of JNK pathway by SP 600125 as well as inhibition of p38 kinase by SB 203580 both diminished p53 phosphorylation and decreased the fraction of apoptotic cells in WB-F344 population. These data suggest interactions between p53 and MAPK pathways in apoptosis induction by genotoxic PAHs in rat liver epithelial cells are complex and that all three types of MAPKs might be involved in cell death control.

References

1. Kwon Y. W., Ueda S., Ueno M., Yodoi J. and Masutani H.: *J. Biol. Chem.* **277**, 1837 (2002)
2. Wada T. and Penninger J. M.: *Oncogene* **23**, 2838 (2004)
3. Chen S., Nguyen N., Tamura K., Karin M. and Tukey R. H.: *J. Biol. Chem.* **278**, 19526 (2003)
4. Chramostová K., Vondráček J., Šindlerová L., Vojtěšek B., Kozubík A. and Machala M.: *Toxicol. Appl. Pharmacol.* **196**, 136 (2004)
5. Teak M. S., Smith J. D., Nelson K. G. and Grisham J. W.: *Exp. Cell Res.* **154**, 38 (1984)

Acknowledgement

This work was supported by grants 525/01/D076 from the Grant Agency of the Czech Republic and KJB6004407 from the Grant Agency of the Academy of Sciences of the Czech Republic.

3P05

MASON-PFIZER MONKEY VIRUS MATRIX PROTEIN – THE STRUCTURAL STUDY

Jan Lipov[a,*], Jiří Vlach[b], Václav Veverka[b], Michaela Rumlová[c], Richard Hrabal[b] and Tomáš Ruml[a]

[a]*Department of Biochemistry and Microbiology and* [b]*NMR laboratory, Institute of Chemical Technology, Technická 5, 16628 Praha 6*
[c]*Department of Biochemistry, Institute of Organic Chemistry and Biochemistry, Czech Academy of Sciences, Praha, Czech Republic*

** E-mail: jan.lipov@vscht.cz*

The Mason-Pfizer Monkey Virus (M-PMV) is a prototype of D-type retroviruses. Morphogenetic pathway of retroviral capsid formation represents the main difference between C- and D- type retroviruses. Unlike the immature capsid of D-type retroviruses is assembled from polyprotein precursors Gag in the specific place in cytoplasm and then transported to the plasma membrane of host cell, the C-type capsid is assembled at the plasma membrane. However, one of the crucial steps of the life cycle of both types of retroviruses is the targeting of polyprotein precursors Gag to the site of assembly. It is known that the matrix protein (MA), which forms the N-terminal part of polyprotein Gag, plays essential role during this process. MA protein plays also a role in other steps of retroviral life cycle – it probably serves as a transport signal of preintegration complex during its targeting to the host nucleus.

It was shown that single point mutation in position 55 of the matrix protein of M-PMV changes its morphogenesis from D- to C- type.

The aim of this work is to obtain the structural data of mutant M-PMV matrix protein R55F. We could take assumption about structural domains of the MA and their importance for particular functions of this protein from comparison of obtained data with the structure of wild type MA .

The affinity chromatography of histidine tagged fusion proteins was used for purification of bacterially expressed MA protein. It is very important for structural measurements to obtain non-modified protein, therefore specific M-PMV protease was used to liberate the histidine tag. Then the gel chromatography was performed followed by concentration of the pure matrix protein up to 1 mM.

The NMR experiments showed dramatic changes in the orientation of MA protein helices I and II compared to the published structure of the wild type MA, which is consistent with our hypothesis on the possible large functional changes of the MA of M-PMV caused by this point mutation.

3P06

HUMAN MITOCHONDRIAL Lon PROTEASE – STRUCTURE AND FUNCTION

Gabriela Ondrovičová[a], Tong Liu[b], Katarína Slezáková[a], Natalia Pharkhomenko[c], Oleksandr Gakh[c], Dušan Perečko[a], Jiří Janata[c], Carolyn K. Suzuki[b] and Eva Kutejová[a,*]

[a]*Department of Enzymology, Institute of Molecular Biology, Slovak Academy of Sciences, Dúbravská cesta 21, 84551 Bratislava, Slovakia*
[b]*Department of Biochemistry and Molecular Biology, UMDNJ- New Jersey Medical School, USA*
[c]*Institute of Microbiology, Academy of Sciences of the Czech Republic, Praha, Czech Republic*

** E-mail: evakutej@hotmail.com*

The ATP-dependent Lon (La) protease is a multi-functional enzyme conserved from archea to mammalian mitochondria[1-3]. Mitochondrial Lon is a homo-oligomeric complex, in which each monomer carries separate site for the binding and hydrolysis of both ATP and protein substrate[4,5]. The binding the substrate polypeptide to Lon stimulates its ATPase activity[6]. ATP hydrolysis is required for the processive unfolding of a substrate that permits peptide bond cleavage. Lon belongs to a unique group of proteases that also binds DNA. *In vivo* studies show that Lon has role in DNA maintenance and expression. In *Saccharomyces cerevisiae*, strains lacking mitochondrial Lon suffer large deletion in mtDNA and do not process and express mitochondrial RNA transcripts that contained introns [7-9].

We purified the recombinant mitochondrial human Lon (hLon) protease and using the crosslinking by glutaraldehyde we demonstrated, that the hLon complex composes of six or seven identical subunits similar to the yeast mitochondrial homologue[4]. To understand better the roles governing substrate selection, we have analyzed the degradation of the matrix processing peptidase (MPP) by the hLon. Both subunits of MPP are recognized and degraded by the hLon and stimulate its ATPase activity. Using the mass spectrometry we identified 42 yeast and 59 rat MPPα peptides produced by Lon, which covered 64% and 73% of each respective protein.

We also demonstrate, that hLon binds to GT-rich DNA sequences found throughout the guanine-rich heavy strand of human mtDNA as well as to GU-rich RNA. ATP inhibits hLon binding to DNA and RNA, whereas protein substrate increases the affinity of the proteases for nucleic acid[10]. We propose that DNA and RNA binding by the human mitochondrial Lon protease is regulated by conformational changes within the holoenzyme that are induced differentially by nucleotide and protein substrate.

References

1. Goldberg A. L., Moerschell R. P., Cung C. H. and Maurizi M. R.: *Methods Enzymol.* **244**, 350 (1994)
2. Gottesman S., Wickner S. and Maurizi M. R.: *Genes Dev.* **11**, 181 (1997)

3. Suzuki C. K., Rep M., van Dijl J. M., Suda K., Griwell L. A. and Schatz G.: *Trends Biochem. Sci*. **22**, 118 (1997)
4. Stahlberg H., Kutejová E., Suda K., Wolpensinger B., Lustig A., Schatz G., Engel A. and Suzuki C. K.: *Proc. Natl. Acad. Sci. U.S.A.* **96**, 6787 (1999)
5. van Dijl J. M., Kutejová E., Suda K. Perečko D., Schatz G. and Suzuki C. K.: *Proc. Natl. Acad. Sci. U.S.A*. **95**, 10584 (1998)
6. Menon A. S. and Goldberg A. L.: *J. Biol. Chem*. **262**, 14929 (1987)
7. Suzuki C. K., Suda K., Wang N. and Schatz G.: *Science* **264**, 273 (1994)
8. van Dyck L., Pearce D. and Sherman F.: *J. Biol. Chem*. **269**, 238 (1994)
9. van Dyck L., Neupert W. and Langer T.: *Genes Dev.* **12**, 1515 (1998)
10. Liu T., Lu B., Lee I., Ondrovičová G., Kutejová E. and Suzuki C. K.: *J. Biol. Chem.* **279**, 13902 (2004)

Acknowledgement

We are grateful to Helena Kolesárová for the technical assistance. This work was supported by the National Institute of Health Grant GM61095, by Basil O'Coonor Scholars Award-March of Dimes Grant FY99-797, by grant from the Foundation of University of Medicine and Dentistry of New Jersey, by Slovak Grant Agency Grant VEGA 2/2086/22, by Twinning Program-National Science Foundation/National Academy of Sciences and by the grant 204/98/0416 from the Grant Agency, Czech Republic.

3P07

CHARACTERIZATION OF THE CLONES DERIVED FROM THE HT 29 COLORECTAL CANCER CELL LINE

Eva Šloncová*, Dana Kučerová, Martina Vojtěchová, Jolana Turečková, Zdena Tuháčková and Vlasta Sovová

Institute of Molecular Genetics, Academy of Sciences, Flemingovo nám. 2, 16637 Praha 6, Czech Republic

** E-mail: slon@img.cas.cz*

During our analyses of the HT29 colorectal carcinoma cell line we have obtained heterogenic differentiation response of these cells to butyrate. Therefore, we established several clones of HT29 cells differing in some morphological and molecular markers.

Analyses of more than ten clones showed that besides the presence of different cell types, also other factors are involved in heterogenic response of HT 29 cells to butyrate. Yet untreated clones differ in the expression of several molecular markers, e.g. MUC 1 and p53 expression, subcellular localization of β-catenin and of its phosphorylated form.

Great differences were observed in sensitivity of individual clones to sodium butyrate. Extremely different responses to butyrate measured by the activity of alkaline phosphatase were found in G 9 and H 8 clones that were analysed in detail; e.g. the effects of phosphoinositol 3-kinase (PI3K) overexpression on butyrate-induced differentiation were studied.

3P08

INTERACTIONS BETWEEN PLANT PHOSPHOLIPASE D AND CYTOSKELETON

Jan Linek[a,*], Zuzana Novotná[a], Martin Potocký[a], Jan Martinec[b] and Olga Valentová[a]

[a]*Department of Biochemistry and Microbiology, Institute of Chemical Technology, Technická 5, 16000 Praha 6, Czech Republic*
[b]*Institute of Experimental Botany, Academy of Sciences, Praha, Czech Republic*
** E-mail: linekj@email.cz*

Phospholipase D (PLD), which was identified as an ubiquitous microbial, plant and animal enzyme, plays an important role in numerous cellular processes. In plants, PLD has been proposed to participate in cellular events such as abscisic acid signalling, response to various biotic and abiotic stress cues, including water stress, wounding and pathogen attack. Recently Gardiner et al.[1] reported that the microtubule-associated protein in *Arabidopsis* is one of plasma membrane-localized PLD.

Human PLD2 was found to interact directly with actin and the exact domain involved in this interaction was determined[2]. After sequence analysis of plant phospholipases the closer homolog coresponding to this domain was found in *Nicotiana tabacum* PLDβ. To elucidate the possible interaction, bacterially expressed glutathione S-transferase fusion proteins of NtPLDβ and NtPLDα fragments were prepared and immobilized on Sepharose for further experiments. Pull-down assays with animal actin and plant extracts were performed to investigate the actin-PLD binding. Additionaly was examined the role of phosphorylation on this interaction, because our previous experiments[3] revealed that exactly the same fragment comprises residues which are phosphorylated *in vitro* and thus could modulate the possible binding of actin. Phosphatase inhibitors and protein kinase were used for supporting our hypothesis. To explore the influence of PLD activity by actin, tobacco plasma membrane was treated with different actin concentration and times used. To draw the complex and detailed scheme of cytoskeleton-related PLD function, further experimentation is required.

References

1. Gardiner J. C., Harper J. D. I., Weerakoon N. D., Collings D. A., Ritchie S., Gilroy S., Cyr R. J. and Marc J.: *Plant Cell* **13**, 2143 (2001)

2. Lee S., Park J. B., Kim J. H., Kim Y., Kim J. H., Shin K.-J., Lee J. S., Ha S. H., Suh P.-G. and Ryu S. H.: *J. Biol. Chem.* **276**, 28252 (2001).
3. Novotná Z., Linek J., Hynek R., Martinec J., Potocký M. and Valentová O.: *FEBS Lett*. **554**, 50 (2003).

3P09

ANALYSES OF POSTTRANSLATIONAL MODIFICATIONS OF p53 PROTEIN IN CELL LINES TREATED USING DIFFERENT DRUGS USED IN TUMOUR TREATMENT

Šárka Helánová*, Petr Müller, Roman Hrstka, Pavla Češková and Bořivoj Vojtěšek

Department of Experimental Oncology, Masaryk Memorial Cancer Institute, Žlutý kopec 7, 65653 Brno, Czech Republic

** E-mail: shelanova@mou.cz*

Posttranscriptional modification of p53 protein by phosphorylation has been proposed to be an important mechanism of p53 stabilisation and functional regulation. Phosphorylation of p53 at serine 392 in particular activates specific DNA binding functions by stabilising p53 tetramer formation.

Phosphoprotein p53 binds sequence-specifically to DNA and is responsible for activation and/or inactivation of p53 target genes. It is well known that different functions of p53 protein are regulated by posttranscriptional modifications such as phosphorylation or acetylation. Phosphorylation of serine 15 is an important mechanism, by which interaction between p53 and MDM2 is disrupted, leading to (i) wild-type p53 stabilisation, (ii) disruption of p53 transport from the nucleus and (iii) stimulation of transcriptional activity associated with increased interaction of p53 with coactivator p300. Second critical modification of p53 protein is phosphorylation of serine 392 in C-terminal domain. Oncogenic studies support the evidence that point mutations in gene encoding p53 protein are responsible for creation of conformationally different p53 protein. In the DNA binding domain of p53, there are two functional classes of mutations: one affects the residues that directly contact DNA, while the other plays a critical role in stabilising the structural integrity of this domain. These mentioned phosphorylations might affect conformational changes of wild-type and mutated p53 as well as changes in its DNA binding activity. Real meaning of p53 phosphorylation on serine 15 and 392 and relation to tumorigenesis is still unknown. The role of different types of cellular stress on p53 posttranslational modifications is also unclear.

Aim of our study was to find out the relation between p53 posttranscriptional modifications and p53 status in tumour-derived cell lines treated using different anti-tumour drugs. Our results show that using different anti-tumour drugs we obtained variance in p53 phosphorylation at serine 15 and serine 392. We found that these

phosphorylations affect neither DNA-binding activity nor stability of mutant p53 protein compare to wild-type p53 protein.

Acknowledgement

The work was supported by the grant 7131-3/2002 from the Internal Grant Agency, Ministry of Health, Czech Republic.

3P10

THE INFLUENCE OF 285 AMINO ACID SUBSTITUTION IN p53 PROTEIN ON ITS DNA BINDING AND TRANSACTIVATION ABILITY

Kristýna Brožková*, Pavla Češková and Bořivoj Vojtěšek

Department of Experimental Oncology, Masaryk Memorial Cancer Institute, Žlutý kopec 7, 65653 Brno, Czech Republic

** E-mail: brozkova@mou.cz*

The TP53 is well known tumour suppressor gene which encodes a nuclear transcription factor involved in the regulation of the cell cycle. Loss of normal p53 function can lead to uncontrolled cell proliferation and neoplastic transformation. Mutation in tumour suppresor p53 appears in approximately 50% of human tumours and 95 % of these tumorigenic mutations lie in the core DNA-binding domain[1].

The mutation in codon 285 belongs to so-called hot spot mutations which appear more frequently in tumours and significantly alter the DNA binding site of p53. The replacement of Glu 285 with Lys or Val is associated with bladder tumours (or other tumours of unspecified urinary organs) and breast cancer. Glu 285 is part of alpha helix forming loop-sheet-helix motif which stabilise the surrounding structure of DNA binding domain. According to the new results[2] Glu 285 mutation is temperature sensitive hot-spot mutation. The transcriptional activity of this mutated protein is dependent on temperature as decrease of temperature to 32°C reactivate the protein. The mutant p53 protein at 32°C behaves like wild-type p53 protein.

To study the impact of temperature and amino acid substitution in 285 position on ability of p53 protein to bind DNA and transactivate the target genes we prepared a set of plasmids coding p53 proteins with Glu 285 replacement (Lys, Val, Ala). We purified bacterially expressed p53 proteins (wild-type and mutant p53 protein 285 Lys, 285 Val, 285 Ala) using heparin chromatography and gel filtration. Using EMSA (electromobility shift assay) we studied temperature impact on DNA binding ability of these purified proteins *in vitro*. For *in vivo* studies we used a system of transient transfection of wt and mutated p53 proteins into p53-null cell line H1299-RGC (cell line carrying β-galactosidase reporter gene under RGC promotor).

Our results from *ex vivo* experiments show, that replacement of Glu 285 with Lys or Val results to temperature sensitive behaviour in comparison to replacement Glu 285 with Ala.

References

1. Bullock N. A. and Fersht A. R.: Nat. Rev. Cancer **1**, 68 (2001)
2. Shiraishi K., Kato S., Han S. Y., Liu W., Otsuka K., Sakayori M., Ishida T., Takeda M., Kanamaru R., Ohuchi N. and Ishioka C.: *J. Biol. Chem.* **279**, 348 (2004)

Acknowledegement

This work was supported by the grant NS 7131-3 from the Grant Agency of the Ministry of Health of the Czech Republic and by the grant NS 301/04/P023 from the Grant Agency of the Czech Republic.

3P11

INVESTIGATION OF UVA CYTOTOXICITY ON HUMAN KERATINOCYTES

Alena Svobodová*, Jitka Psotová and Daniela Walterová

Institute of Medical Chemistry and Biochemistry, Faculty of Medicine, Palacký University, Hněvotínská 3, 77515 Olomouc, Czech Republic

** E-mail: alf.svoboda@seznam.cz*

UV radiation forms a part of the electromagnetic spectrum with wavelengths between 200 nm and 400 nm. It is divided into three categories UVA (320–400 nm), UVB (280-320 nm), and UVC (200-280 nm)[1-3]. UVA is not absorbed by the ozone layer and more than 90% reaches us. It penetrates deep into the epidermis and dermis of the skin. Intense or extensive exposure to UVA can damage underlying structures in the corium and cause premature photoaging of the skin. UVA-induced responses in cells happen mainly because of oxidative processes initiated by endogenous photosensitization. After UVA exposure reactive oxygen species (ROS) such as singlet oxygen, H_2O_2, and hydroxyl free radicals are generated. ROS can cause damage to cellular proteins, lipids, nucleic acids, and saccharides. UVA injury tends to cause necrosis of endothelial cells, thus damaging the dermal blood vessels, impair the immune system, and lead to cancer[3-6].

In our study we investigated effects of various UVA doses (10, 20, 30 and 40 J/cm^2) on human keratinocyte cell line HaCaT, extensively used as an *in vitro* model of epidermal skin to study the effect of UV. HaCaT (10^5 cells/cm^2) were grown in Dulbecco's modified Eagles's medium supplemented with 7% fetal calf serum for 48 h, then washed and irradiated in PBS. After UVA exposure PBS was replaced with serum-

free medium and cells were incubated for 4 h. The cytotoxic effect of UVA was evaluated using following parameters: neutral red retention, bromodeoxyuridine incorporation into cellular DNA, intracellular ATP and GSH levels. The Comet assay was employed to detect DNA single strand breakage.

Our results showed that UVA exposure provokes decrease in cell viability, cell proliferation and intracellular ATP and GSH levels. UVA also causes DNA damage (single strand breakage). All studied cytotoxicity parameters were found dose-dependent. These pilot results will serve for further studies of photoprotective effects of herbal compounds.

References

1. Afaq F., Adhami V. M., Ahmad N. and Mukhtar H: *Front. Biosci*. **7**, 784 (2002)
2. Clydesdale G. J., Dandie G. W. and Muller H. K: *Immunol. Cell Biol.* **79**, 547 (2001)
3. Duthie M. S., Kimber I. and Norval M: *Br. J. Dermatol.* **140**, 995 (1999)
4. Afaq F. and Mukhtar H: *J. Photochem. Photobiol. B* **63**, 61 (2001)
5. Afaq F. and Mukhtar H.: *Skin Pharmacol. Appl. Skin Physiol.* **15**, 297 (2002)
6. Trautinger F.: *Clin. Exp. Dermatol*. **26**, 573 (2001)

Acknowledgement

This work was supported by the grant 303/02/1097 from the Grant Agency, 22/G3/2004 from the University Development Fund and MSM 151100003 from the Ministry of Education, Youth and Sports, Czech Republic.

3P12

EFFECT OF SANGUINARINE AND CHELERYTHRINE ON PROTEIN KINASE C ACTIVITY IN NEUTROPHIL-LIKE CELLS

Jiří Vrba*, Jitka Ulrichová and Martin Modrianský

Institute of Medical Chemistry and Biochemistry, Faculty of Medicine, Palacký University, Hněvotínská 3, 77515 Olomouc, Czech Republic

** E-mail: vrbambv@seznam.cz*

Sanguinarine (SA) and chelerythrine (CHE) are members of quaternary benzo[c]phenanthridine alkaloids of isoquinoline type and possess antimicrobial and anti-inflammatory properties. We previously showed that both SA and CHE inhibit phorbol myristate acetate (PMA)-induced oxidative burst in DMSO-differentiated HL-60 cells with IC50 of 1.8 μM and 2.9 μM, respectively. Because PMA triggers assembly of NADPH oxidase, resulting in extracellular superoxide generation, via direct activation

of protein kinase C (PKC) and CHE is a known PKC inhibitor, we examined whether SA or CHE inhibition of oxidative burst is connected with PKC inhibition. Western blot analyses of the whole cell lysates obtained from differentiated HL-60 cells treated with alkaloids showed that the concentrations of SA and CHE necessary for efficient PKC activity inhibition are about ten-fold higher than those inhibiting superoxide generation. Furthermore, SA and CHE affect PKC activity in differentiated HL-60 cells differently from staurosporine, a potent PKC inhibitor. SA and CHE preferentially decrease phosphorylation of approx. 40 kDa protein while staurosporine, which inhibits both PMA-induced oxidative burst and PKC activity with comparable effectiveness, decreases phosphorylation of approx. 35 kDa protein first. Dihydrosanguinarine and dihydrochelerythrine, which lack positive charge in their structures, and berberine, another quaternary alkaloid from isoquinoline group, affected neither PMA-induced oxidative burst nor PKC activity. Comparison of effects of SA and CHE with structurally similar, but ineffective compounds accentuates the importance of unique $C=N^+$ region with bonded methyl group in the molecules of SA and CHE. As low concentrations of SA or CHE do not affect the phosphorylating activity of PKC, which activates NADPH oxidase assembly via phosphorylation of its components, we conclude that SA and CHE inhibit oxidative burst in DMSO-differentiated HL-60 cells primarily by direct inhibition of NADPH oxidase.

Acknowledgement

This research was supported by grant MSM 151100003 from the Ministry of Education, Youth and Sports, Czech Republic.

3P13

FATE OF RIBOSOMAL GENE UNITS ASSOCIATED WITH NUCLEOLAR UPSTREAM BINDING FACTOR UBF IN LYMPHOBLASTS TREATED WITH ANTIPROLIFERATIVE AGENTS

Ivan Kalousek* and Petra Otevřelová

Department of Cell Biology, Institute of Hematology and Blood Transfusion, U nemocnice 1, 128 20 Praha 2, Czech Republic

** E-mail: kal@uhkt.cz*

Phytohemagglutinin (PHA) induced transformation of quiescent T lymphocytes into proliferating cells is accompanied by extensive unwinding of multicopy rDNA gene resulting in fibrilar centers dispersion[1]. The transformation is supported by the strong promotion of transcription of rRNA and translation and phosphorylation of nucleolar transcription factor UBF[2,3]. To investigate the mechanism of ribosomal gene unwinding we examined cultures of peripheral blood T lymphocytes induced to the entry to the G1 phase by PHA in the presence of various growth inhibitors, using the method of anti-

UBF immunofluorescence microscopy. Briefly: Lymphocytes treated with PHA and inhibitor were harvested and cytocentrifuged on glass slides at 2000 rpm for 4 min, fixed and permeabilized with acetone / metanol 1:1 for 10 min at -20 °C. Then the cells were incubated with rabbit polyclonal anti-UBF antiserum diluted 1:100 in PBS for 45 min at 37 °C. After washing in PBS, the cells were incubated with a fluorescein-conjugated GARIgG diluted 1:50 in PBS for 30 min at 37 °C. After repeated washing in PBS the cells were mounted in Vectashield medium. Samples were analyzed using Olympus IMT-2 apparatus supplemented with equipment for digital scanning. In cells arrested in G1 phase by the RNA polymerase I activity independent manner the structural mobilization of UBF positive ribosomal genes was independent of the presence of antiproliferative agent in the culture media. In cultures containing a histone deacetylase inhibitor *n*-butyric acid, an inhibitor of nucleotide incorporation and DNA synthesis azido-deoxythymidine AZT, G1/S transition blocking agents deferoxamine mesylate and thymidine, a specific DNA polymerases inhibitor aphidicolin or a selective inhibitor of cycline dependent kinases olomoucine the process started immediately after addition of PHA (and inhibitor) and culminated after 40 hrs of incubation. However, in the presence of olomoucine a small fraction of apoptotic cells and bodies containing fragments of UBF positive rDNA were detected. Many other antiproliferative inhibitors inactivated intra-nucleolar transformations in G1 phase. In the presence of camptothecine which stabilizes covalent intermediates of DNA by inhibition of topoisomerase I, bleomycin sulfate which causes DNA strand scissions cleaving N-glycosidic and phosphodiester bonds and in the presence of hydroxyurea a potent inhibitor of ribonucleotide reductase, enzyme involved in repair of endamaged DNA, unwinding of multicopy ribosomal gene was more frequently interrupted in the middle of the process and replaced by an apoptotic fragmentation of the nucleus accompanied by exclusion of UBF-rDNA positive bodies. In the presence of rRNA transcription suppressor actinomycin D which inhibits RNA polymerases by complexing with DNA via deoxyguanosine residues, in the presence of cisplatin which inhibits polymerases generating intrastrand d(GpG) and d(ApG) crosslinks and in the presence of adenylyl-imidodiphosphate AMP-PNP which acts as competitive inhibitor of ATP-dependent enzymes the unwinding was strictly repressed and nuclear fragmentation and UBF exclusion proceeded.

Our data suggest, that the dispersion of UBF positive fibrilar centers as a demonstration of unwindig of multicopy ribosomal gene is associated with an undisturbed interaction of RNA polymerase I and associated factors with rDNA sequences. We speculate, that the process is induced by intermolecular forces raising as the consequence of the formation of initiation complex and/or the production of rRNA transcript.

References

1. Ochs R. L. and Smetana K.: *Exp. Cell Res.* **184**, 552 (1989)
2. Čabart P. and Kalousek I.: *Cell Mol. Biol. (Noisy-le-grand)* **44**, 343 (1998)
3. Kalousek I. and Křížková P.: *Cell Mol. Biol. (Noisy-le-grand)* **46**, 1163 (2000)

Acknowledgement

This work was supported by Grants IGA NR/7998-3 and CEZMZ 0023736001 from the Ministry of Health of the Czech Republic.

3P14

ROLE OF PHOSPHOLIPASES IN ALUMINUM TOXICITY IN PLANTS

Přemysl Pejchar[a,*], Zuzana Novotná[b], Olga Valentová[b] and Jan Martinec[a]

[a]*Institute of Experimental Botany, Academy of Sciences, Rozvojová 135, 16502 Praha 6, Czech Republic*
[b]*Department of Biochemistry and Microbiology, Institute of Chemical Technology, Technická 5, 16000 Praha 6, Czech Republic*

** E-mail: pejchar@ueb.cas.cz*

Aluminum (Al) is highly cytotoxic metal to plants, but the molecular base of Al toxicity is still far from being understood. The most important physiological consequence of Al toxicity is a cessation of root growth and changes in root morphology suggesting a role of the root cytoskeleton as a target of Al toxicity[1]. A major focus of plant cytoskeleton research is the identification of signals and signal transduction cascades that regulate cytoskeleton dynamics[2]. The important role of phospholipid degrading enzymes, phospholipase C (PLC) and phospholipase D (PLD) in regulation of cytoskeletal dynamics in animal organisms is now evident[3]. The aim of work is to study molecular mechanism of aluminum toxicity with special respect to the role of plant phospholipid-cytoskeleton signalling. Here we present the first part of the work - role of phospholipases in aluminum toxicity.

Fluorescently labelled substrates (BODIPY-phosphatidylcholine and BODIPY-phosphatidic acid) were incorporated into the tobacco cell line BY-2. Labelled cells were then treated with $AlCl_3$. Products of phospholipases action were analysed in different time intervals in methanol/chloroform cell extracts by HP-TLC and/or HPLC.

Results show a changes of the amount of lysophosphatidylcholine (product of PLA2 action), diacylglycerol (product of PLC) as well as phosphatidic acid (product of PLD) already after 30 min of aluminum (0.1 mM $AlCl_3$) treatment. Al^{3+} affect level of phospholipase products in dose-dependent manner. Quantitative changes in unknown products of other members of phospholipid signalling pathway were observed as well and their identification is currently under investigation.

References

1. Jones D. L. and Kochian L. V.: *FEBS Lett.* **400**, 51 (1997)
2. Schwarzerová K., Zelenková S., Nick P. and Opatrný Z.: *Plant Cell Physiol.* **43**, 207 (2002)
3. Liscovitch M., Czarny M., Fiucci G., Lavie Y. and Tang X.: *Biochim. Biophys. Acta* **1439**, 245 (1999)

Acknowledgement

This work was supported by the grant MSM 223300006 from the Ministry of Education, Youth and Sports, Czech Republic.

3P15

DIAPHORASE CAN METABOLIZE SOME VASORELAXANTS TO NO AND ELIMINATE NO SCAVENGING EFFECT OF 2-PHENYL-4,4,5,5,-TETRAMETHYLIMIDAZOLINE-1-OXYL-3-OXIDE (PTIO)

Petr Bartík*, Karel Chalupský, Simona Eklová and Gustav Entlicher

Department of Biochemistry, Faculty of Science, Charles University, Hlavova 8, 12840 Praha 2, Czech Republic

** E-mail: bartikpetr@seznam.cz*

Diaphorase was studied as a possible oxidoreductase participating in NO production from some vasorelaxants. In the presence of NADH or NADPH, diaphorase can convert selected NO donors, glycerol trinitrate (GTN) and formaldoxime (FAL) to nitrites and nitrates with NO as an intermediate. This activity of diaphorase was inhibited by diphenyleneiodonium (DPI) (inhibitor of some NADPH-dependent flavoprotein oxidoreductases) while it remained not inhibited by NG-nitro-L-arginine methyl ester (inhibitor of NO-synthase) and 7-ethoxyresorufin (inhibitor of cytochrome P-450 1A1 and cytochrome P-450 NADPH-dependent reductase). Existence of NO as an intermediate of the reaction was supported by results of electron paramagnetic resonance spectroscopy. In addition to ability to affect the above mentioned NO donors diaphorase was able to reduce 2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) and thus eliminate its NO scavenging effect. Also this activity of diaphorase was inhibitable by DPI. Reaction of diaphorase with GTN and PTIO were not affected by superoxide dismutase (SOD) or catalase. Reaction of FAL with diaphorase was lowered with SOD by 38% indicating the partial participation of superoxide anion probably generated by reaction of diaphorase with NADH or NADPH. Catalase had no effect.

3P16

CELL SYSTEM FOR GENE THERAPY USING EGFP-NLS FUSION PROTEIN EXPRESSION: TESTING OF OLIGONUCLEOTIDE NUCLEAR UPTAKE

Šárka Mikolajská[a,*], Hana Radilová[a] and Martin Bunček[b]

[a]*Department of Biochemical Sciences, Faculty of Pharmacy, Charles University, Heyrovského 1203, 50005 Hradec Králové, Czech Republic*
[b]*Generi Biotech, Machkova 587, 50011 Hradec Králové, Czech Republic*

** E-mail: mikolajska@faf.cuni.cz*

Gene therapy based on the antigene strategy uses triplex forming oligonucleotides (TFO) that can bind to the target sequence of dsDNA in nucleus[1,2]. Short TFO are complementarily bound in a sequence-specific manner to the specific region of the gene (most often control, but also transcribed region) in order to generate triple-helical structure. Formation of this triple-helical complex leads to the depression of the gene expression.

We prepared system for testing of fluorescence labeled TFO entering into the cells and into the nucleus. We have cloned an retroviral express vector encoding the gene for EGFP-NLS fusion protein. Enhanced green fluorescent protein (EGFP) is widely used as a reporter gene for its fluorescence due to the active chromofore that is formed by the autocatalytic cyclization of three amino acids[3]. Nuclear localization signal (NLS) drives energy-dependent nuclear transport through the nuclear pore complex. NLS sequence ensures in our case localization of EGFP in the nucleus due to its recognition signals and affinity to the nuclear import. EGFP mediates green fluorescent nuclei.

Retroviral system was used to prepare stable cell line with EGFP expression in the nucleus. PT67 packaging cell line was tranfected by pLNCX2 EGFP-NLS expression vector. We investigated EGFP entry from cytoplasm to nucleus several hours after transfection. After selection in 300 μg/ml G418 for about two weeks we collected medium containing viral particles and infected target Hep2 cell line. After selection in 400 μg/ml G418 we gained stable infected Hep2 cell line with EGFP in 100% nuclei.

We tested the cytoplasmic and nuclear uptake of different modifications of triplex forming oligonucleotides such as phosphorothioate, cholesterol and 2´O-Me-RNA on the system described above. Modified TFOs were fluorescent labeled with 5´Cy5. We microscopically evaluated the TFO delivery into the cell compartments in the terms of fluorescence changes.

References

1. Malvy C., Harel-Bellan A. and Pritchard L. L.: *Triple helix forming oligonucleotides*, Kluwer Academic Publishers, The Netherlands 1999
2. Gorlich D.: *Curr. Opin. Cell Biol*. **9**, 412 (1997)
3. Phillips G. J.: *FEMS Microbiol. Lett.* **204**, 9 (2001)

Acknowledgement

The work was supported by the Ministry of Education Youth and Sports (LN00B125), University Development Fund (160/51/439) and Generi Biotech s.r.o.

3P17

DIFFERENCES IN THE EXPRESSION OF TRANSCRIPTS ENCODING THE REGULATORY AND EFFECTOR COMPONENTS OF THE APOPTOSOME PATHWAY IN NON-SMALL CELL LUNG CARCINOMA AND THE LUNG

Evžen Křepela[a], Jan Procházka[a,*], Pavel Fiala[b] and Pavel Selinger[c]

[a]Department of Molecular and Cellular Biology, [b]Department of Chest Surgery and [c]Department of Pathology, Clinic of Pneumology and Chest Surgery, University Hospital Na Bulovce, Budínova 2, 18081 Praha 8, Czech Republic

** E-mail: prochazj@fnb.cz*

Deregulated proliferation of tumor cells and their capability to evade apoptosis are the major driving forces of growth and progression of malignant tumors[1,2]. Recent studies provide evidence that the intrinsic apoptosis apparatus, which plays a significant role in generating and amplifying cell death signals, is frequently suppressed in various tumor types including non-small cell lung carcinomas[3,4]. In order to find out whether the expression of apoptosome pathway-related transcripts is altered in non-small cell lung carcinoma (NSCLC) we investigated the levels of Apaf-1, procaspase-9, procaspase-3, procaspase-7, procaspase-6 and Smac mRNAs in NSCLC and lung tissue samples from surgically treated patients. The indicated target transcripts and beta-actin mRNA (an internal reference transcript) were quantitated by means of real time RT-PCR assays with using gene specific oligonucleotide primers and fluorogenic TaqMan probes and an input of 200 ng of total RNA which was isolated from the tumors and lungs by the Trizol method. The levels of the beta-actin mRNA-normalized expression of target mRNAs in NSCLCs and lungs were calculated by the 2^-deltaCT method[4]. It was found that the levels of the beta-actin mRNA-normalized expression of the apoptosome pathway-related transcripts in NSCLCs and lungs were different for five of the six analyzed transcripts. The expression of Apaf-1 mRNA in NSCLCs was significantly lower as compared with the lungs. On the contrary, the expression of procaspase-9, procaspase-3, procaspase-6 and Smac mRNAs was significantly higher in NSCLCs than in the lungs while the expression of procaspase-7 mRNA in the tumors and lungs was not significantly different. With respect to NSCLC staging and in comparison with the lungs, the expression of Apaf-1 mRNA was significantly decreased in both stage I tumors and stage II+III tumors. On the other hand, the expression of procaspase-3 and Smac mRNAs was significantly elevated in both stage I tumors and stage II+III tumors whereas the expression of procaspase-9 and procaspase-6 mRNAs was significantly increased only in stage I tumors. Concerning the most prevalent histopathological types of NSCLC, i.e. SQCLC and LAC, the expression of procaspase-3, procaspase-7 and Smac mRNAs in these NSCLC types was not significantly different whereas the expression of Apaf-1, procaspase-9 and procaspase-6 mRNAs was significantly higher in LACs as compared with SQCLCs. The results of the present study indicate that transcripts encoding the main regulatory and effector components of the apoptosome pathway are expressed in NSCLCs and that among them only Apaf-1 mRNA is expressed at a reduced quantity as compared with the lungs. The latter finding would

suggest that downregulation of Apaf-1 mRNA expression in NSCLCs might contribute to suppression of the apoptosome pathway activation in these tumors. However, even though the expression of Apaf-1 mRNA in NSCLCs is reduced the tumors contain a significantly elevated Apaf-1 protein level as compared to the lungs and the levels of Apaf-1 protein in LACs and SQCLCs are comparably high[4]. These findings point to the possibility that the tumors may have an enhanced capability to set the Apaf-1 mRNA molecules into the translationally competent state. In this respect, there is evidence that the translation initiation of Apaf-1 mRNA requires sequential binding of the protein upstream of N-ras and polypyrimidine tract binding protein to an IRES element which is located in the 5' untranslated region of Apaf-1 mRNA[5,6]. It remains to be determined whether the increased Apaf-1 protein level in the presence of reduced Apaf-1 mRNA expression in NSCLCs results from overexpression of the Apaf-1 mRNA IRES trans-acting factors in the tumors.

References

1. Hanahan D. and Weinberg R. A.: *Cell* **100**, 57 (2000)
2. Green D. R. and Evan G. I.: *Cancer Cell* **1**, 19 (2002)
3. Ferraro E., Corvaro M. and Cecconi F.: *J. Cell. Mol. Med.* **7**, 21 (2003)
4. Křepela E., Procházka J., Liu X., Fiala P. and Kinkor Z.: *Biol. Chem.* **385**, 153 (2004)
5. Mitchell S. A., Brown E. C., Coldwell M. J., Jackson R. J. and Willis A. E.: *Mol. Cell Biol*. **21**, 336 (2001)
6. Mitchell S. A., Spriggs K. A., Coldwell M. J., Jackson R. J. and Willis A. E.: *Mol. Cell* **11**, 757 (2003)

Acknowledgement

This work was supported by the research project MZ00000064211 from the Ministry of Health, Czech Republic.

3P18

INCREASED EXPRESSION OF TRANSCRIPTS ENCODING SURVIVIN AND PROTEINASE INHIBITOR-9 IN NON-SMALL CELL LUNG CARCINOMAS

Evžen Křepela[a,*], Jan Procházka[a], Pavel Fiala[b], Petr Zatloukal[b] and Pavel Selinger[c]

[a]Department of Molecular and Cellular Biology, [b]Department of Chest Surgery and [c]Department of Pathology, Clinic of Pneumology and Chest Surgery, University Hospital Na Bulovce, Budínova 2, 18081 Praha 8, Czech Republic

** E-mail: krepelae@fnb.cz*

Failure to activate caspase zymogens may account for resistance of tumor cells to apoptosis. There is evidence that the major intrinsic caspase zymogens activation pathway, which is triggered via the homodimerization-mediated activation of procaspase-9 in apoptosomes, can be disabled in tumor cells through overexpression of apoptosis inhibitors such as caspase-9S, XIAP and survivin[1-3]. Moreover, the proteolytic activation of caspase zymogens by granzyme B upon its perforin-assisted entry from cytotoxic lymphocytes into the cytoplasm of tumor cells can be suppressed through overexpression of the granzyme B-inactivating cytosolic proteinase inhibitor-9 in target tumor cells[4]. To find out whether transcripts encoding the above mentioned apoptosis inhibitors are upregulated in non-small cell lung carcinoma (NSCLC) we investigated their expression levels in NSCLC and lung tissue samples from surgically treated patients. The expression of caspase-9S, XIAP, survivin and proteinase inhibitor-9 mRNAs (target transcripts) and beta-actin mRNA (an internal reference transcript) was quantitated by means of real time RT-PCR assays with using gene specific oligonucleotide primers and fluorogenic TaqMan probes and an input of 200 ng of total RNA which was isolated from the tumors and lungs by the Trizol method. The levels of the beta-actin mRNA-normalized expression of target mRNAs in NSCLCs and lungs were calculated by the 2^-deltaCT method[5]. It was found that the expression of caspase-9S mRNA and XIAP mRNA in NSCLC tissues and lungs was not significantly different. In addition, subsequent analysis showed that the caspase-9S mRNA/procaspase-9 mRNA expression ratio in the majority of examined tumors was lower than 0.2. The expression of both proteinase inhibitor-9 mRNA and survivin mRNA was significantly higher in NSCLC tissues than in lungs. The increase of proteinase inhibitor-9 mRNA expression in the tumors by more than 1.5-fold was observed in 45% of all examined tumor-lung matched pairs. By contrast, the expression of survivin mRNA was elevated in all examined NSCLC tissues and its increase ranged from 1.6-fold to 755-fold. There was no statistically significant difference in the expression of caspase-9S mRNA, XIAP mRNA or proteinase inhibitor-9 mRNA among the major histopathological types of NSCLC including squamous cell lung carcinoma (SQCLC), lung adenocarcinoma (LAC) and large cell lung carcinoma (LCLC). On the other hand, the expression of survivin mRNA was significantly higher in LCLC than in SQCLC and LAC and it tended to be higher in SQCLC than in LAC. The results of the present study provide evidence that survivin mRNA is overexpressed in the majority of NSCLCs while the expression of proteinase inhibitor-9 mRNA is heightened in a subset of NSCLCs. These

findings suggest that upregulated expression of both survivin and proteinase inhibitor-9 mRNAs may be instrumental for the maintenance of apoptosis resistance in NSCLCs. The comparably high expression of XIAP mRNA in the majority of studied NSCLCs and lungs suggests that the possible involvement of XIAP in apoptosis resistance of NSCLCs may stem from the IRES-dependent enhancement of XIAP mRNA translation in the tumors (c.f. 6). The low expression ratio of caspase-9S/procaspase-9 mRNAs in most examined tumors indicates that the expression of caspase-9S mRNA splice variant, encoding a catalytically incompetent protein, should not significantly contribute to apoptosis resistance of NSCLCs.

References

1. Chalfant C. E., Rathman K., Pinkerman R. L., Wood R. E., Obeid L. M., Ogretmen B. and Hannun Y. A.: *J. Biol. Chem*. **277**, 12587 (2002)
2. Yang L., Mashima T., Sato S., Mochizuki M., Sakamoto H., Yamori T., Oh-Hara T. and Tsuruo T.: *Cancer Res*. **63**, 831 (2003)
3. Marusawa H., Matsuzawa S., Welsh K., Zou H., Armstrong R., Tamm I. and Reed J. C.: *EMBO J.* **22**, 2729 (2003)
4. Medema J. P., de Jong J., Peltenburg L. T., Verdegaal E. M., Gorter A., Bres S. A., Franken K. L., Hahne M., Albar J. P., Melief C. J. and Offringa R.: *Proc. Natl. Acad. Sci. U.S.A.* **98**, 11515 (2001)
5. Křepela E., Procházka J., Liu X., Fiala P. and Kinkor Z.: *Biol. Chem.* **385**, 153 (2004)
6. Holčík M., Gordon B. W. and Korneluk R. G.: *Mol. Cell Biol.* **23**, 288 (2003)

Acknowledgement

This work was supported by the grant NR/7860-5 from the Ministry of Health, Czech Republic.

3P19

IDENTIFICATION AND CHARACTERIZATION OF GENES DEPENDENT UPON STRESS RESPONSE SIGMA FACTOR SigB IN *STAPHYLOCOCCUS AUREUS*

Jan Kormanec[a], Markus Bischoff[b], Alexis Dumoulin[b] and Dagmar Homerová[a,*]

[a]*Institute of Molecular Biology, Centre of Excellence for Molecular Medicine, Slovak Academy of Sciences, Dúbravská cesta 21, 84551 Bratislava, Slovakia*
[b]*Institute of Medical Microbiology, University of Zurich, CH-8028 Zurich, Switzerland*

** E-mail: dagmar.homerova@savba.sk*

We have recently established a method for identification of promoters recognized by a particular sigma factor of RNA polymerase. The method was based on the assumption that *Escherichia coli* RNA polymerase core enzyme would interact with a heterologous sigma factor expression of which is induced from one plasmid, and the resulting holoenzyme may recognize a promoter present in a library of chromosomal fragments cloned in the second compatible plasmid upstream of the lacZa reporter gene[1,2]. We optimized this method for identification of *Staphylococcus aureus* promoters that are recognized by the general stress response sigma factor SigB[3]. The method was optimized using the previously characterized SigB-dependent asp23p promoter and *S. aureus* sigB gene under control of arabinose-induced PBAD promoter. The asp23p promoter was active in the two-plasmid system only after arabinose-induced expression of *S. aureus* SigB. To confirm the transcriptional start of the promoter in *E. coli* two-plasmid system, S1-nuclease mapping was performed using the RNA from the *E. coli* two-plasmid system containing *S. aureus* asp23p and induced *S. aureus* SigB. The transcriptional analysis revealed an identical transcription start point as in *S. aureus*. Therefore, we have used this two-plasmid system for identification of other *S. aureus* sigB-dependent promoters. We have identified 18 *S. aureus* SigB-dependent promoters, 12 of which reported here for the first time to be SigB-dependent. The promoters were confirmed and transcriptional start points of the promoters were determined by S1-nuclease mapping. Sequences of identified promoters exhibited high similarity and allowed to establish a *S. aureus* SigB consensus promoter (GttTaa - N12-15 - gGGTAt) that was highly homologues to that of SigB of *B. subtilis*. The promoters governed expression of genes encoding proteins proposed to be involved in various cellular functions, including the stress response genes and virulence-associated clfA gene for fibrinogen-binding clumping factor.

References

1. Novaková R., Ševčíková B. and Kormanec J.: *Gene* **208**, 43 (1998)
2. Rezuchová B. and Kormanec J.: *J. Microbiol. Methods* **45**, 103 (2001)
3. Homerová D., Bischoff M., Dumolin A. and Kormanec J.: *FEMS Microbiol. Lett.* **232**, 173 (2004)

Acknowledgement

This work was supported by the grant 2/3010/23 from the Slovak Academy of Sciences and grant 560030 from Forschungskredit der Universität Zürich.

3P20

REGULATION OF TWO ALTERNATIVE SIGMA FACTORS, SigB AND SigH, IN CONNECTION WITH STRESS RESPONSE AND DIFFERENTIATION IN *STREPTOMYCES COELICOLOR* A3(2)

Vladislava Mazuraková*, Beatrica Sevčíková and Jan Kormanec

Institute of Molecular Biology, Centre of Excellence for Molecular Medicine, Slovak Academy of Sciences, Dúbravská cesta 21, 84551 Bratislava, Slovakia

** E-mail: vladalubirada@yahoo.com*

Gram-positive soil bacteria *Streptomyces* undergo a complex cycle of morphological differentiation resembling that of filamentous fungi. In their natural habitat, they are exposed to various stresses, including desiccation, cold shock and heat shock. In order to overcome unfavourable conditions, bacteria activate alternative sigma factors, which govern expression of genes having a various roles during these stress conditions. In *Bacillus subtilis*, the general stress response is governed by an alternative sigma factor SigB. We previously identified the sigH gene, encoding a stress-response sigma factor SigH in *S. coelicolor* A3(2) that was homologous to *B. subtilis* SigB. The gene is part of an operon ushY, ushX, sigH, and its expression is directed by four promoters expressed differentially during development and in response to several stress conditions[1]. Disruption of sigH in *S. coelicolor* affected response to salt-stress conditions and morphological differentiation, septation of aerial hyphae into spores[2]. The operon is autoregulated by means of salt-stress induced sigHp2 promoter that is directly recognized by RNA polymerase containing SigH. UshX, encoded by an upstream gene, has been shown to be specific anti-sigma factor for SigH. Interestingly, SigH has been shown to be proteolytically activated during growth and differentiation[3,4]. The results indicated that SigH has a dual role in *S. coelicolor* in osmotic stress response and in morphological differentiation, at the specific stage, aerial mycelium septation. Thus, in *Streptomyces*, the process of differentiation has been shown to be connected with the response to salt-stress by means of SigH. However, *S. coelicolor* contains nine close homologues of SigH, and other homologue, SigB, has been shown to have a similar function. *S. coelicolor* sigB mutant was much more sensitive to hyperosmolarity and, in contrast to sigH, it was impaired in aerial mycelium formation. Similarly, also sigB is part of an operon rsbB, rsbA, sigB, with rsbA encoding likely anti-sigma factor, and its expression is directed by two promoters, with one promoter dependent upon SigB and preferentially induced by salt-stress[5]. By Western blot analysis, we have shown that, in contrast to SigH, SigB is not proteolytically processed, and is dramatically induced after salt-stress conditions. This induction is mediated at transcriptional level by means of SigB-dependent sigBp1 promoter. Taken together, similar to SigH, also SigB has a dual role in stress response and morphological differentiation, though in different developmental stage. To clarify in more detail the *in vivo* function of both sigma factor, a stable null mutant was prepared in *S. coelicolor* A3(2) by replacement of a whole sigB operon and sigH operon with a selectable marker. Interestingly, deletion of both operons resulted in strong, precocious sporulation and had no obvious effect on growth of *S.*

coelicolor in the presence of high osmotic conditions. The mutants produced higher levels of actinorhodin. These results are in contrast with the previous disruption of sigB and sigH genes where the mutants were sensitive to hyperosmolarity and affected different stages of *S. coelicolor* development. The results indicated a complicated regulation of the both operons in relation to stress response and differentiation in *S. coelicolor* A3(2).

References

1. Kormanec J., Ševčíková B., Halgasová N., Knirschová R. and Rezuchová B.: *FEMS Microbiol. Lett.* **189**, 31 (2000)
2. Ševčíková B., Benada O., Kofronová O. and Kormanec J.: *Arch. Microbiol.* **177**, 98 (2001)
3. Ševčíková B. and Kormanec J.: *FEMS Microbiol. Lett.* **209**, 229 (2002)
4. Viollier P. H., Weihofen A., Folcher M. and Thompson C. J.: *J. Mol. Biol.* **325**, 637 (2003)
5. Cho Y-H., Lee E-J., Ahn B-E. and Roe J-H.: *Mol. Microbiol*. **42**, 205 (2001)

Acknowledgement

This work was supported by the grant 2/3010/23 from the Slovak Academy of Sciences.

3P21

MOLECULAR ANALYSIS OF CATALASE-1 FROM *COMAMONAS TERRIGENA* N3H REVEALS ITS PHYSIOLOGICAL ROLE IN THE ANTIOXIDANT DEFENCE

Marcel Zámocký, Viera Boháčová, Jana Godočíková, Juraj Gašperík and Bystrík Polek*

Department of Cell Biology, Institute of Molecular Biology, Slovak Academy of Sciences

** E-mail: Bystrik.Polek@savba.sk*

Catalases are essential components of the cellular equipment to cope with the oxidative stress. Bacterial isolates *Comamonas terrigena* N3H selected from soil contaminated with crude oil and *Comamonas testosteroni* isolated from sludge of waste treatment plant, exhibited much higher level in total catalase activity than the same species from the stock culture collections[1]. A gradual increase in total catalase activity during bacterial growth was observed in all strains. We have focused on the most abundant heme-containing catalase-1 from the bacterium *Comamonas terrigena* N3H. This oxidative stress-induced enzyme was isolated from exponential phase cells grown in the presence of peroxyacetic acid and purified to homogeneity. Main enzymatic characteristics of this metalloenzyme are: the specific catalytic activity of 55,900 U/mg,

allosteric behaviour in peroxidic reaction, a broad pH optimum and a rather atypical electronic spectrum. The molecular weight of the subunit of this homodimeric protein was determined as 55,417 Da in the mass spectrometry analysis. The Qq-TOF mass analysis method allowed us to sequence short tryptic fragments of this catalase. Five such fragments with a total length of 57 amino acids together with several enzymatic properties allowed the classification of this hydroperoxidase as belonging to clade III of monofunctional catalases[2]. The highest sequence similarity is with the catalase from *Vibrio fischeri*. These known sequence motifs were exploited for the construction of degenerated primers applied for isolation of the complete gene from the genome of this bacterium. The presented results imply the significance of this inducible enzyme in the prevention of toxic effects of oxidative stress for bacterial cells.

References

1. Boháčová V., Godočíková J., Zámocký M. and Polek B.: *Biologia* **57**, 813 (2002)
2. Zámocký M., Godočíková J., Gašperík J., Koller B. and Polek B.: *Protein Expression Purif.*, in press (2004)

Acknowledgement

This work was supported by VEGA grant 2/2056/22.

3P22

CONSTRUCTION OF *CORYNEBACTERIUM GLUTAMICUM* STRAINS OVERPRODUCING VALINE

Veronika Elišáková*, Jiří Holátko and Miroslav Pátek

Institute of Microbiology, Academy of Sciences, Vídeňská 1083, 14220 Praha 4, Czech Republic

** E-mail: elisak@biomed.cas.cz*

Gram-positive bacterium *Corynebacterium glutamicum* is widely used in industrial production of amino acids, mainly of lysine and threonine. In this work, production of valine, one of the essential amino acids, was increased by modulating the metabolic flux through its own and related biosynthesis pathways. In *C. glutamicum*, biosynthesis pathway of valine is partly shared with pathways of isoleucine, leucine and pantothenate. The process includes complex regulation at different levels, such as multivalent regulation of gene expression by transcriptional attenuation, multivalent inhibition of enzymes, homologous reactions catalysed by a single enzyme and also distinct affinity of key enzyme to alternative substrates. Features involved in this regulatory network were analysed in detail. The regulation of transcription and strength of particular

promoters were studied and the activity of key enzyme was determined, as well. Successive interventions at different selective points of pathway led gradually to the increased production of the desired amino acid. The first step of modulation was the abolishment of feedback inhibition of the key enzyme, acetohydroxy acid synthase. The binding site for feedback inhibitors (valine, leucine and isoleucine) was modified by site-directed mutagenesis and the production of valine by resulting feedback-resistant mutant was thus increased. The competing pathways were interrupted at branching points by deletions of the corresponding genes (ilvA, leuA and panB for the biosynthesis of isoleucine, leucine and pantothenate, respectively). Although these deletions provided more intermediates for valine production, they caused auxotrophy of the resulting strains, as well. The growth medium had to be therefore supplemented with leucine, isoleucine and pantothenate, which might prove uneconomic in industrial usage. To overcome this negative effect, the transcription of respective genes was decreased by mutagenesis of their promoters. The acquired strains with leaky phenotype and self-limited in growth yielded higher concentration of valine. In parallel with direct modifications within the *C. glutamicum* chromosome, the ilvBNC operon coding for the first two enzymes of valine and isoleucine pathway was cloned in multi-copy plasmid vector. Various combinations of mentioned alterations were constructed and their cumulative effect on valine production was analysed. Further goals of this project are increasing the expression of the genes ilvD and ilvE that determine the last two reactions of valine biosynthesis.

3P23

PLASMID VECTORS FOR GENE MANIPULATIONS IN *RHODOCOCCUS ERYTHROPOLIS*

Martin Veselý, Monika Knoppová* and Miroslav Pátek

Institute of Microbiology, Academy of Sciences, Vídeňská 1083, 14220 Praha 4, Czech Republic

** E-mail: knoppova@biomed.cas.cz*

Various strains of *Rhodococcus* degrade a number of aromatic compounds like phenols, chlorobenzoates and polychlorinated biphenyls (PCB). Knowledge of their genetics and development of tools and methods for gene manipulations are prerequisites for purposeful improving the efficiency of degrading abilities of these Gram-positive bacteria. We have chosen the phenol-degrading strain of *Rhodococcus erythropolis* for the development of host-vector system. We constructed plasmid vectors based on the cryptic plasmids pSR1 (3.1 kb) and pGA1 (4.8 kb) from the related *Corynebacterium glutamicum* and optimized the electrotransformation procedure to obtain in total $7x10^4$ transformants using 1 mg plasmid DNA. In contrast to indigenous plasmids of

Rhodococcus strains, mechanism of replication, copy number control and stability determinants were studied in these small *C. glutamicum* plasmids. On the basis of the plasmid pSR1, the stable cloning shuttle vector pSRK21 (*Escherichia coli – C. glutamicum - R. erythropolis*) with wide cloning choice (6 unique cloning sites) and providing a positive selection of recombinant molecules based on the a-complementation of β-galactosidase was constructed. Based on the plasmid pSRK21, the vector pSRKgfpuv carrying a reliable fluorescent marker (green fluorescent protein) was constructed. Another marker gene successfully tested for the function in *R. erythropolis* was the cat gene coding for chloramphenicol acetyltransferase. Further vectors for special purposes were derived from these basic vectors. A promoter-probe vector pPRE11 containing the rsgfp reporter gene coding for red-shifted green florescent protein is based on the pSR1 replicon. Its function was tested by cloning several promoters and evaluating their activity in *R. erythropolis* and in *C. glutamicum* as well. The vector carrying the sacB gene as a counter-selectable marker allowing the unmarked manipulations (deletions, integrations, mutations and replacements) within the *R. erythropolis* chromosome was used, as well. The vectors were utilized for cloning of the genes coding for enzymes of catechol degradation pathway, of 16S rRNA gene and for analysis of *R. erythropolis* promoters.

3P24

PHAGE-HOST INTERACTIONS BETWEEN CORYNEPHAGE BFK 20 AND DIVERSE CORYNEBACTERIAL STRAINS

Nora Halgašová*, Tomáš Majtán, Jana Ugorčáková, Jozef Timko and Gabriela Bukovská

Institute of Molecular Biology, Slovak Academy of Sciences, Dúbravská cesta 21, 84551 Bratislava, Slovakia

** E-mail: Nora.Halgasova@savba.sk*

Reply to bacteriophages infection reflects diversity of theirs bacterial hosts. Many native barriers against phages infections were detected in bacterial strains. The most extensively studied are those of lactococci[1,2], where four different resistance mechanisms were identified: inhibition of bacteriophage adsorption, blocking of phage DNA injection, DNA restriction/modification systems and mechanisms of abortive infection.

In case of corynephage BFK 20 infection, only one from the six investigated corynebacterial strains, *Brevibacterium flavum* CCM 251, is liable to lysis. Five other examined strains, *Brevibacterium flavum* ATCC 21127, *Brevibacterium flavum* ATCC 21128, *Brevibacterium flavum* ATCC 21474, *Brevibacterium lactofermentum* BLOB and *Corynebacterium glutamicum* RM3 prevent the lysis using different defence

mechanisms. Inhibition of the phage adsorption seems to be the exclusive defence system in *C. glutamicum* RM3. The adsorption barrier functions as strong resistance mechanism also in *B. lactofermentum* BLOB, but seems not to be the solely defence system in this strain. Using Southern hybridization we detected presence of phage DNA in infected cells of *B. lactofermentum*. In all tested *B. flavum* strains we observed significant amount of phage DNA indicating an intensive BFK20 DNA replication. We intended to compare replication rate of BFK 20 DNA in tested strains. Replication of phage DNA in these strains and also in *B. lactofermentum* was monitored at different time points after BFK 20 infection. We propose presence of some abortive infection mechanism in non-lysed *B. flavum* ATCC strains and also in *B. lactofermentum.* Our results indicated action of different resistance mechanisms in examined corynebacterial strains against corynephage BFK 20 infection.

References

1. Forde A. and Fitzgerald G. F.: *A. van Leeuwenhoek* **76**, 89 (1999)
2. Coffey A. and Ross R. P.: *A. van Leeuwenhoek* **82**, 303 (2002)

Acknowledgement

This work was supported by the VEGA Grant 2/2058/22.

3P25

PROTEINS INVOLVED IN ACTIVATION OF sF IN THE PROCESS OF SPORULATION IN *BACILLUS SUBTILIS*

Zuzana Chromíková* and Imrich Barák

Institute of Molecular Biology, Slovak Academy of Sciences, Dúbravská cesta 21, 84551 Bratislava, Slovakia

* *E-mail:* zuzana.chromikova@savba.sk

Sporulation is the one of the crucial responses of *B. subtilis* cells to hostile environment conditions. In this process the cell divides asymmetrically and thus gives rise to unequal sized progeny, the bigger mother cell and the smaller prespore. Since both daughter cells differ not only in size but also in expression of genes, they have also a different fate. The mother cell, that supplies and nourishes the prespore lyses in the end of the process, while prespore develops into the heat resistant spore. Therefore the process of sporulation is often taken as the simplest example of primary cellular differentiation.

Differential gene expression is in both cells governed by compartment-specific s-subunits of RNA-polymerase, so called s-factors. Differentiation of gene expression begins with activation of the first compartment-specific s factor, sF, which is activated in

the prespore. sF is synthesized in predivisional cell as SpoIIAC together with other two members of the spoIIA operon, SpoIIAB and SpoIIAA. Immediatelly after its synthesis it is bound by anti-s factor SpoIIAB, and thus it is held in an inactive state[1]. However, in the presence of dephosphorylated SpoIIAA molecules SpoIIAB binds to SpoIIAA and sF is released[2]. Since SpoIIAB is a protein-kinase, SpoIIAA gets phosphorylated and SpoIIAB is free again to block another sF[3]. In addition, there is a SpoIIE phosphatase that dephosphorylates SpoIIAA-P, and enables sF activation[4-8] and cell survival. SpoIIE is a multidomain bifunctional protein with several transmembrane helices. This protein plays the crucial role in mediating sF activation and also it is essential for completion of sporulation septum. In addition, it is known to interact with important cell division protein, FtsZ[9,10]. It is proposed that SpoIIE protein is involved in the checkpoint that couples formation of sporulation septum to activation of the first cell-type specific s-factor. Although the molecular mechanism of sF activation is partially known, there are still several questions to ask, for example why is sF activated only after the sporulation septum is finalized and why it is active specifically in the prespore. The process of transient genetic asymmetry also contributes to compartment-specific activation of sF[11,12], but precise mechanism of this process is still unknown.

In order to test interactions between proteins involved in sF activation, we prepared strains expressing the key proteins of this process: SpoIIAA, SpoIIAB, SpoIIE, as well as strains expressing mutated proteins: SpoIIAAS58A , SpoIIAAS58D, SpoIIE71. All of mutated protein strains were subjected to genetic analysis. We were also successful in purifying of all proteins mentioned above together with FtsZ. We intend to test interactions between various combinations of these proteins using surface-plasmon resonance methodology. We believe that biochemical study of interactions between proteins that take part in the process of sF activation, as well as study of interactions between corresponding mutant proteins, will reveal the specific mechanism which leads to compartmentalization of gene expression during *B. subtilis* cell differentiation.

References

1. Duncan L. and Losick R.: *Proc. Natl. Acad. Sci. U.S.A*. **90**, 2325 (1993)
2. Diederich B., Wilkinson J. F., Magnin T., Najafi S. M. A., Errington J. and Yudkin M. D.: *Genes & Dev*. **8**, 2653 (1994)
3. Min K. T., Hilditch C. M., Diederich B., Errington J. and Yudkin M. D.: *Cell* **74**, 735 (1993)
4. Duncan L., Alper S., Arigoni F., Losick R. and Stragier P.: *Science* **270**, 641 (1995)
5. Barák I., Behari J., Olmedo G., Guzman P., Brown D. P., Cashio E., Walker D., Westpheling J. and Youngman P.: *Mol. Microbiol.* **19**, 1047 (1996)
6. Barák I. and Youngman P.: *J. Bacteriol.* **178**, 4984 (1996)
7. Arigoni F., Guérout-Fleury A. M. , Barák I. and Stragier P.: *Mol. Microbiol.* **31**, 1407 (1996)
8. Feucht A., Magnin T., Yudkin M. D. and Errington J.: *Genes & Dev*. **10**, 794 (1996)
9. Lucet I., Feucht A., Yudkin M. D. and Errington J.: *EMBO J.* **19**, 1467 (2000)
10. Prepiak P., Chromiková Z. and Barák I.: *Folia Microbiol.* **46**, 292 (2001)
11. Frandsen N., Barák I., Karmazyn-Campelli C. and Stragier P.: *Genes & Dev.* **13**, 394 (1999)
12. Dworkin J. and Losick R.: *Cell* **107**, 339 (2001)

3P26

PROGRAMMED BACTERIAL CELL DEATH – SpoIISAB SYSTEM

Patrik Florek[a,*], Katarína Muchová[a], Richard J. Lewis[b] and Imrich Barák[a]

Institute of Molecular Biology, Slovak Academy of Sciences, Dúbravská cesta 21, 84551 Bratislava, Slovakia
School of Cell and Molecular Biosciences, The Medical School, University of Newcastle, Newcastle NE2 4HH, UK

** E-mail: patrik.florek@savba.sk*

The spoIIS locus of *Bacillus subtilis* encodes two proteins, SpoIISA and SpoIISB. SpoIISA is a 248-residue protein containing three predicted transmembrane domains with the last two-thirds of protein being located in the cytoplasm. SpoIISB is a hydrophilic 56-residue protein, absence of which leads to strong sporulation defect, but which has no effect during exponential growth[1].

In this study we investigated toxic properties of heterogically expressed SpoIISA protein from *B. subtilis* in *Escherichia coli* cells. The spoIISA gene was inserted next to the tightly regulated arabinose promoter in pBAD24 plasmid[2]. Expression of SpoIISA was induced by adding arabinose in the middle of exponential phase, which led to instant growth arrest of *Escherichia coli* cells. In contrast to this observation, co-expression of SpoIISA and SpoIISB from the same arabinose promoter does not impair cells growth at all.

In addition to this experiments, we also tested killer effect of putative SpoIISA protein from *Bacillus cereus*[3]. This 245-residue protein shares only 16.9% identity with its putative homologue from *B. subtilis*, however, both proteins have almost identical predicted topology in cell membrane. The gene coding for SpoIISA-like protein from *B. cereus* was cloned in the pBAD24 vector downstream of the arabinose promoter and its expression in *E. coli* cell was induced in the middle exponential phase by adding of xylose. As in case of SpoIISA from *B. subtilis*, we observed cells growth arrest immediately after addition of arabinose.

Altogether, these facts led us to conclusion that predicted SpoIISA protein from *B. cereus* and SpoIISA from *B. subtilis* are toxic for *E. coli* cells and could have similar mode of action and perhaps share common cellular targets for both *E. coli* and *Bacilli*. To further study the mechanism of SpoIISA function and protein-protein interaction of SpoIISA - SpoIISB, we analyzed biochemical properties of these proteins *in vitro*.

References

1. Adler E., Barák I. and Stragier P.: *J. Bacteriol.* **183**, 3574 (2001)
2. Guzman L.-G., Belin D., Carson M. J. and Beckwith J.: *J. Bacteriol*. **177**, 4121 (1995)
3. Ivanova N., Sorokin A., Anderson I., Galleron N., Candelon B., Kapatral V., Bhattacharyya A., Reznik G., Mikhailova N., Lapidus A., Chu L., Mazur M., Goltsman E., Larsen N., D'Souza M., Walunas T., Grechkin Y., Pusch G.,

Haselkorn R., Fonstein M., Ehrlich S. D., Overbeek R. and Kyrpides N.: *Nature* **423**, 87 (2003)

Acknowledgement

Work in I.B. laboratory is supported by grant 2/1004/22 from the Slovak Academy of Sciences and The Wellcome Trust Collaborative Research Initiative Grant (066732/Z/01/Z).

3P27

MICROARRAY DATA NORMALIZATION: DEVELOPMENT OF NEW POSITIVE CONTROL BASED ON RED FLUORESCENT PROTEIN

Tomáš Majtán*, Gabriela Bukovská and Jozef Timko

Institute of Molecular Biology, Slovak Academy of Sciences, Dúbravská cesta 21, 84551 Bratislava, Slovakia

** E-mail: Tomas.Majtan@savba.sk*

DNA microarray technology is a powerful high-throughput technique that was recently developed in order to analyze expression of thousands of genes simultaneously in a short time. The applications of microarrays extend beyond the boundaries of basic biology into diagnostics, environmental monitoring, pharmacology, toxicology and biotechnology.

There are basically two types of microarrays that have been developed so far: complementary DNA (cDNA) microarray and oligonucleotide microarray. These DNA chips technologies are currently distinguished by the sizes of arrayed DNA fragments, the methods of arraying, the chemistries and linkers for attaching DNA to the chip and the hybridization and detection methods. Each microarray experiment consists of array fabrication, target preparation, hybridization and data analysis. During most biological experiments as well as microarrays there are several systematic variables that can affect the measurement of mRNA levels, making it difficult to compose direct comparison. Sources of the variations include the inherent errors from sample handling, slide-to-slide variation, difference in labeling or hybridization efficiency and variations during image analysis. Normalization is a process of minimizing these variations thus removing non-biological influences on biological data. Microarray data need to be normalized so that we can compare results from different labeling dyes, different genes on the same array and results from different arrays. One important issue included in normalization is utilization of various negative and positive controls which are printed onto a microarray along with test genes. Negative controls are helpful to set up background, signal-to-noise ratio or efficiency of printing pin washing. Positive controls from genes whose regulation is known or stable (e.g. housekeeping genes) are useful to check on biological

experiment and data analysis. Positive "spiked" controls give us the most useful signals, which may serve to check the quality of microarray printing, efficiency of reverse transcription and fluorescent labeling procedure, specificity and sensitivity of hybridization, dynamic range of fluorescence signal and also as "landing lights" for analytical software.

For this purpose we have prepared the system, which can be used to generate an universal exogenous positive "spiked" control. It is based on expression vector pET26b with insertion of red fluorescent protein DsRed coding sequence located downstream to T7 promoter. Desired mRNA is prepared by *in vitro* transcription reaction catalyzed with phage T7 RNA polymerase. DNA probe attached onto the microarray slide can be prepared by PCR amplification using commercially available T7 promoter and T7 terminator primer pair. Except of this there are available several commercial systems (e.g. Spotreport-10 Array Validation System, Stratagene). The unique of our system relies on fact that DsRed gene is inserted in frame with pET26b initiation codon, which may be important point for *in vitro* translation. Thus generated protein may be used as a positive control in other microarray-related experiments: protein arrays.

Acknowledgement

This work was supported by the VEGA Grant 2/2058/22.

3P28

BFK20 PHAGE: STUDY OF ESSENTIAL GENE PROTEINS EXPRESSION

Gabriela Bukovská*, Jana Ugorčáková, Nora Halgašová, Jarmila Holbová, Stanislava Gunišová and Jozef Timko

Institute of Molecular Biology, Slovak Academy of Sciences, Dúbravská cesta 21, 84551 Bratislava, Slovakia

** E-mail: Gabriela.Bukovska@savba.sk*

Non-pathogenic members of the genera *Corynebacterium* and *Brevibacterium* are of industrial interest and have been traditionally used as industrial producers in many biotechnological processes. Often negative phenomenon is the presence of bacteriophage lytic infection in bacterial culture during fermentation. Up to now there are not known any universal methods to solve this problem effectively. Detail knowledge about bacteriophage and relation between phage and host cell on molecular level could help to solve this problem by finding new strategies for bacterial infection defense.

In our laboratory we isolated and characterized the corynephage BFK20, lytic phage of *Brevibacterium flavum* CCM 251, industrial production strain. BFK20 seems to be a very good object for molecular biology studies in corynebacterial species. We determined entire nucleotide sequence of phage BFK20 genome. Its genome consists of

a double stranded DNA molecule 42,975 bp long. By using bioinformatics analysis and amino acid homology search we established assumed organization of its genome and identified the function of 16 ORFs, from total of 55 ORFs on BFK20 genome (EMBL, Acc.No: AJ278322).

We focused our study on identification and protein expression of ORFs, which represent replication and lytic cassettes. We considered ORF45 (putative replication origin), ORF47 (putative helicase), ORF49 (putative RepA like protein) and ORF50 (putative DNA polymerase) as genes involved in DNA replication mechanism of phage BFK20.

Most phages accomplish host lysis using at least two phage encoded enzymes: endolysin and holin. The lytic cassette genes of BFK20 involve ORF30 as hypothetical lytic gene-endolysin consists of 570 bp encoding 190 amino acids. On the basis of structural characteristic for putative holin proteins (transmembrane domains, highly charged C-terminal area) we identified two ORFs 32 and 33 as those with characteristics of putative holins.

By using PCR method we have amplified regions of ORFs from replication and lytic cassettes and have cloned these fragments separately into *E. coli* vector. We have over-expressed potential proteins in pET28a expression system and purified them with advantage of preparative affinity chromatography on HiTrap affinity column.

As the *in vivo* complementation data available suggest that any holin will work with any endolysin, even when holin and endolysin pairs are chosen from phages of Gram-positive and Gram-negative hosts we cloned and identified holin gene on the base of its function by *in vivo* complementation of mutated S holin from lambda phage with putative holin gene on pTH32 and pTH33 plasmids.

In future we will characterized all purified proteins on structural, biochemical and functional levels.

Acknowledgement

This work was supported by the VEGA Grant 2/2058/22.

3P29

HYPOXIA REDUCES eNOS EXPRESSION IN HYPOXIC RAT PLACENTAS

Jana Bíbová[a,*], Karel Chalupský[a], Sylvie Hojná[a], A. Hrdličková[c] and Václav Hampl[a]

[a]*Department of Physiology, 2nd Medical School, Charles University, Plzeňská 221, 15000 Praha 5, Czech Republic*
[b]*Institute of Physiology, Academy of Science, Praha, Czech Republic*
[c]*Department of Biochemistry, Faculty of Science, Charles University, Praha, Czech Republic*

** E-mail: jana.bibova@lfmotol.cuni.cz*

Hypoxia in fetoplacental vessels is believed to be one of the most important factors in the pathogenesis of fetal and neonatal morbidity, such as intrauterine growth retardation.

Chronic hypoxia regulates the expression of endothelial NO synthase in different species and tissues. Typically the expression of endothelial NO synthase (eNOS) rises under chronic conditions. The expression in rat placenta has never been determined in hypoxia. The aim of our study was to analyze the effect of chronic hypoxia on the expression of endothelial NO synthase (eNOS) in rat placenta.

Female rats were exposed to hypoxia (10% O_2) for the last 10 days of pregnancy. Control group of rats remained in normoxia. Experiments were conducted in accordance with the Guide for the Care and Use od Laboratory Animals as adopted and promulgated by U.S. National Institutes of Health (agreement number B 67 900). One day before the calculated day of delivery placentas were removed under thiopental anesthesia (60 mg/kg). Placentas were rinsed in saline solution and immediately homogenized. eNOS expression was determined by western blot with immunodetection using rabbit anti eNOS (St. Cruise). The results were quantified by densitometry.

We found significantly lower expression of eNOS in hypoxic placentas compared with normoxic controls. We suppose, that lower expression of eNOS could contribute to the lower blood flow through the hypoxic placenta and deterioration of fetal blood oxygenation and nutrition.

Acknowledgement

Supported by the Grant Agency of Charles University (55/2001/230015).

3P30

GENE THERAPY: THE USE OF THE RETROVIRAL GENE TRANSFER TECHNOLOGY FOR THE DEVELOPMENT OF TFO BINDING AFFINITY TESTING SYSTEM

Hana Radilová[a,*], Šárka Mikolajská[a] and Martin Bunček[b]

[a]*Department of Biochemical Sciences, Fakulty of Pharmacy, Charles University, Heyrovského 1203, 50005 Hradec Králové, Czech Republic*
[b]*Laboratory of Gene Therapy, Generi Biotech, Machkova 587, 50011 Hradec Králové, Czech Republic*

** E-mail: radilova@faf.cuni.cz*

Gene therapy, at present a very fast evolving part of molecular biology, includes a number of methods. One of the most promising methods is the method of changing the expression of individual genes. Triplex forming oligonucleotides (TFOs) can be used in order to reduce or completely prevent the expression of a given gene. TFOs are designed in a way that they can bind to a specific region of a gene, such as to the regulatory sequence or to the coding sequence thus forming triplex structures of DNA.

We synthesize modified TFOs that we test in human cell cultures. For this purpose we developed a system that enables testing of the binding affinity of individual TFOs to the target DNA sequences. We designed retroviral testing vectors containing triplex target sites (TTS) for TFOs (i.e., polypurine sequences in the coding regions of the pol and nef genes, regions conserved among HIV-1 isolates). TTS were cloned into the regulatory sequence or in frame into the coding sequence of the reporter gene encoding luciferase.

We transfected packaging cell line PT67 by these retroviral vectors and selected stable transfected colonies in selective growth medium with blasticidin. We infected the human cell line Hep2 by infectious virus produced by this stable packaging cell line.

We use the stable transfected cell line Hep2 for TFO binding affinity testing. This testing system is based on measuring the activity of luciferase enzyme that catalyze oxidation of the substrate D-luciferin into oxyluciferin in the presence of ATP. Light emitted during this reaction can be measured by luminometer. According to the level of the luciferase gene expression we find out whether the TFO is bound to the target sequence or not.

Acknowledgement

The work was supported by the grants 954/2004 and LNB0125 from the Ministry of Education, Youth and Sports, and by Generi Biotech, s.r.o.

3P31

INHIBITION OF GJIC BY NEW HYDROPHOBIC PLATINUM(IV) COMPLEX LA-12 THROUGH ACTIVATION OF MEK-1/2

Lubomír Procházka[a,*], Jaroslav Turánek[a], Radek Tesařík[a], Zdeněk Andrysík[b], Alois Kozubík[b], Petr Sova[c], Aleš Kroutil[c], František Žák[c], Adolf Mistr[c] and Miroslav Machala[a]

[a]*Veterinary Research Institute, Hudcova 70, 621 32, Brno, Czech Republic*
[b]*Institute of Biophysics, Academy of Sciences of the Czech Republic, Královopolská 135, 612 65 Brno, Czech Republic*
[c]*PLIVA-Lachema, Karásek 1, 621 33 Brno, Czech Republic*

** E-mail: prochazka@vri.cz*

The aim of this study was to characterize the influence of a new hydrophobic platinum(IV) complex on GJIC. The new platinum(IV) complex is more toxic than cisplatin (CDDP) against the panel of cancer cell lines[1,2]. The loss of intercellular communication (GJIC) is caused by closing of gap junction channels. This is related to phosphorylation of connexins cx43, which form the gap junction channel. We have observed that a new platinum(IV) complex LA-12 caused the rapid inhibition of GJIC in WBF344 cell line. The extent of inhibition GJIC was measured by Lucifer Yellow Dye Transfer Assay which is based on measuring of fluorescence dye distribution among confluent cells. Using the western blott techniques and inhibitors of signal transduction pathways showed linkage between phosphorylation of gap junction channels (closing the channels) and LA-12 pretreatment. Phosphorylation of cx43 is related to activation of MEK-1/2 proteinkinases. Inhibitor of MEK-1/2 (U0126) prevents the gap junction closing but does not influence the progress of apoptosis which is the result of DNA damage by platinum complexes. The hypothesis that the signal for phosphorylation of cx43 is transferred from MEK-1/2 to Erk-1/2 was tested using western blott and antibodies for activated Erk. We found that Erk kinases are not activated by MEK-1/2 as subsequence of LA-12 pre-treatment. Inhibition of GJIC appears sooner than characteristic morphological features of apoptosis. We have also determined that limit concentration which causes the inhibition of GJIC is similar to IC_{50} (LA-12) for WBF344 cell line. In comparison to LA-12 complex, CDDP does not inhibit GJIC and is less cytotoxic. In summary, platinum(IV) complex LA-12 inhibits GJIC through activation MEK-1/2. This complex is more toxic than CDDP, and induction of apoptosis is faster in comparison to CDDP.

References

1. Žák F., Turánek J., Kroutil A., Sova P., Mistr A., Poulová A., Mikolín P., Žák Z., Kašná A., Zaluská D., Neča J., Šindlerová L. and Kozubík A.: *J. Med. Chem.* **47**, 761 (2004)
2. Turánek J., Kašná A. and Záluská D.: *Anti-Cancer Drugs*, in press (2004)

Acknowledgement

This work was supported by the grants from the Ministry of Agriculture of the Czech Republic 0002716201 and the Ministry of Industry and Trade PZ-Z2/29.

3P32

MULTILEVEL REGULATION OF A HYPOXIA-INDUCIBLE CARBONIC ANHYDRASE IX IN HYPOXIC AND REOXYGENATED TUMOR CELLS *IN VITRO*

Monika Rafajová[a,*], Miriam Zaťovičová[a], Richard Kettmann[b], Jaromír Pastorek[a] and Silvia Pastoreková[a]

[a]*Center of Molecular Medicine, Institute of Virology, Slovak Academy of Sciences, Dúbravská cesta 9, 84505 Bratislava, Slovakia*
[b]*Department of Applied Biochemistry and Biology, Faculty of Agronomy, Gembloux Agricultural University, Belgium*

** E-mail: virumoni@savba.sk*

Hypoxia is a key factor of tumor microenvironment that contributes to tumor progression and induces a number of cellular responses including metabolic shift to anaerobic glycolysis, cell cycle arrest, apoptosis, neoangiogenesis, and development of an aggressive phenotype. The primary response to the lack of oxygen at the molecular level is the stabilization of a subunit of HIF-1 transcriptional complex that activates the genes whose products are involved in adaptation to the hypoxic stress, such as vascular endothelial growth factor (VEGF) and glucose transporters (GLUT-1, GLUT-3). Carbonic anhydrase IX (CA IX) is strongly induced by hypoxia via HIF-1. Moreover, it is almost exclusively associated with various tumors, while being absent from the corresponding normal tissues. Therefore, CA IX was proposed as a promising intrinsic marker of tumor hypoxia with diagnostic and prognostic value. However, lack of the full overlap between the intratumoral distribution of CA IX and either HIF-1a or VEGF indicates that regulation of CA IX may differ from the regulation of other hypoxic markers. To explain this phenomenon, we performed *in vitro* study of hypoxia-mediated induction and reoxygenation-affected stability of CA IX mRNA and protein in comparison to HIF-1a, VEGF, GLUT-1 and GLUT-3. Expression of mRNA was analyzed in HeLa cervical carcinoma cells cultivated in a hypoxic incubator with 1% O_2 atmosphere by semi-quantitative RT PCR using hypoxia-independent b-2 microglobulin mRNA as an internal standard. We found that the transcriptional activation in hypoxia and persistence of transcripts in subsequent reoxygenation slightly differ between CA IX and other HIF-regulated genes with respect to the time-course and degree of induction. In addition, transcription as well as CA IX protein levels are further increased by

reduced glucose or bicarbonate concentration, which are the adverse factors of hypoxia. Posttranslational stability of CA IX was assessed by monitoring the quantity of biotinylated protein extracted in different time intervals from the cells metabolically labeled immediately after shift to reoxygenation. The analysis has shown that the stability of CA IX with the half life corresponding to about 38 hours is considerably higher than the stability of other hypoxic markers and is independent of the duration of the foregoing hypoxia. Thus, we assume that the immunohistochemical detection of CA IX in tumor tissues can reveal not only the areas that actually suffer from hypoxia, but also those tumor regions that were switched to reoxygenation. Further experiments revealed that CA IX can be post-translationally regulated also via shedding of its ectodomain to extracellular space, which can be reduced by the metalloproteinase inhibitor batimastat. Altogether, our results show that CA IX expression is regulated at several levels of the biosynthetic pathway and that understanding of this complex regulation may have potential implications both for basic molecular characterization of CA IX and for interpretation of the clinical data related to intratumoral distribution of CA IX.

3P33

TRANSCRIPTIONAL REGULATION OF Prnd GENE CODING FOR DOPPEL (PRION LIKE PROTEIN)

Jana Nagyová, Jaromír Pastorek and Juraj Kopáček*

Centre of Molecular Medicine, Institute of Virology, Slovak Academy of Sciences, Dúbravská cesta 9, 84505 Bratislava, Slovakia

** E-mail: virukopa@savba.sk*

Doppel (Dpl) is a recently discovered protein with homology to prion protein (PrPc). The Dpl protein resembles an N-terminally truncated PrPc protein lacking the octamer repeats. Dpl coding gene (Prnd) is located 16 kb downstream of murine PrPc gene and is thought to result from the ancestral duplication of this gene. Despite the fact that both proteins share only about 25% sequence homology on amino acids level, their structural features are well preserved. Most abundant place of PrPc expression is the adult brain. Dpl has more restricted expression pattern with higher levels in the testis and heart. However, Dpl expression in the brain is neonatally regulated and restricted to the brain endothelial cells.

Based on the fact that Dpl expression is tissue and developmentally regulated we decided to characterize Dpl promoter region and elucidate its activity in different cell types. For that purpose several constructs of Dpl promoter region were prepared and introduced into luciferase vector. After transfection of different cell lines promoter

activities were measured. It was found that region from –62 bp to +27 bp (with respect to the start of transcription), which contains putative TATA box and SP1 binding sites displays very low transcriptional activity in contrast to the region from – 63 bp to –188 bp which has shown the highest transcriptional activity especially in testicular cell line (GC-1spg). In this region, using the method of footprint and gelshift, we identified sequences for several transcription factors out of which NF-Y is seemed to be the most important for the expression of Dpl gene.

3P34

ANTIPROLIFERATIVE ACTIVITY OF OLOMOUCINE II, A NOVEL CYCLIN-DEPENDENT KINASE INHIBITOR

Vladimír Kryštof[a,*], Martin Orság[a], Michal Otyepka[b], Bořivoj Vojtěšek[c] and Miroslav Strnad[a]

[a]*Laboratory of Growth Regulators, Faculty of Science, Palacký University & Institute of Experimental Botany, Academy of Sciences, Šlechtitelů 11, 783 71 Olomouc, Czech Republic*
[b]*Department of Physical Chemistry, Faculty of Science, Palacký University, tř. Svobody 26, 771 46 Olomouc, Czech Republic*
[c]*Department of Experimental Oncology, Masaryk Memorial Cancer Institute, Žlutý kopec 7, 656 53 Brno, Czech Republic*

** E-mail: krystof@aix.upol.cz*

Altered phosphorylation of cellular proteins stimulated an extensive search for protein kinase inhibitors as drugs. Cyclin-dependent kinases (CDKs), enzymes involved in diverse cellular processes, including cell division cycle, transcription, differentiation and apoptosis, are suitable targets of such inhibitors with potential application in cancer therapy[1]. One of the first reported specific CDK inhibitors, olomoucine, had became a leading compound of the development based on structure-activity relatioships, that led to the synthesis of roscovitine[2] and olomoucine II[3], respectively. Both derivatives show an enhanced CDK inhibitory activity, increased selectivity and antiproliferative activity. Moreover, roscovitine has been also shown to induce nuclear accumulation of tumour suppressor p53 and enhance p53-dependent transcription in human cancer cells[4]. The novel derivative olomoucine II inhibits CDKs more efficiently and causes cell cycle blocks at G1/S and G2/M transitions stronger than roscovitine. Also the transcriptional activity of p53 in cells treated with olomoucine II was substantialy higher than in cells treated with roscovitine. The results demonstrate our previous findings that the antiproliferative activity of purine CDK inhibitors is caused via inhibition of cyclin-

dependent kinases and induction of p53 and highlight the potential application of CDK inhibitors in therapy of cell cycle connected diseases.

References

1. Meijer L. and Raymond E.: *Acc. Chem. Res.* **36**, 417 (2003)
2. Havlíček L., Hanuš J., Veselý J., Leclerc S., Meijer L., Shaw G. and Strnad M.: *J Med. Chem.* **40**, 408 (1997)
3. Kryštof V., Lenobel R., Havlíček L., Kuzma M. and Strnad M.: *Bioorg. Med. Chem. Lett.* **12**, 3283 (2002)
4. Kotala V., Uldrijan S., Horký M., Trbušek M., Strnad M. and Vojtěšek B.: *Cell. Mol. Life Sci.* **58**, 1333 (2001)

Acknowledgement

The work was supported by the GACR grant 204/03/D231.

3P35

DIFFERENTIAL SENSITIVITY OF TWO CYTOKININ RECEPTORS OF *ARABIDOPSIS THALIANA*, CRE1/AHK4 AND AHK3, TOWARDS DIFFERENT CYTOKININ METABOLITES

Lukáš Spíchal[a,*], Natalia Yu. Rakova[b], Michael Riefler[c], Takeshi Mizuno[d], Miroslav Strnad[a], Georgy A. Romanov[b] and Thomas Schmülling[c]

[a]*Laboratory of Growth Regulators, Palacký University & Institute of Experimental Botany Academy of Sciences, Šlechtitelů 11, 78371 Olomouc, Czech Republic*
[b]*Institute of Plant Physiology, Russian Academy of Sciences, Moscow, Russia*
[c]*Institute of Biology/Applied Genetics, Free University of Berlin, Berlin, Germany*
[d]*Laboratory of Molecular Microbiology, School of Agriculture, Nagoya University, Chikusa-ku, Nagoya, Japan*

** E-mail: spichy@post.cz*

Strains of *Escherichia coli* that express two different cytokinin receptors of *Arabidopsis thaliana*, CRE1/AHK4 and AHK3, were used to study the relative sensitivity of these receptors to different cytokinin metabolites[1-4]. Both receptors were most sensitive to the bases *trans*-zeatin and isopentenyl adenine but differed significantly in the recognition of other cytokinin metabolites. In particular, CRE1/AHK4 recognized only *trans*-zeatin while AHK3 recognized also *cis*-zeatin and dihydrozeatin, although with a lower sensitivity. Similarly, AHK3 was activated by cytokinin ribosides and ribotides, which did not activate CRE1/AHK4. Comparisons using the ARR5::GUS fusion gene as a cytokinin reporter in *Arabidopsis*[5] showed

similar relative degrees of responses in planta, with the exception that aromatic cytokinins showed much higher activities than in the bacterial assay. These results indicate that the diverse cytokinin metabolites might have specific functions in the numerous cytokinin-regulated processes, which may depend in turn on different receptors and their associated signalling pathways. The importance of a precise control of local concentrations of defined cytokinin metabolites to regulate the respective downstream event is corroborated.

References

1. Spíchal L., Rakova N. Y., Riefler M., Mizuno T., Strnad M., Romanov G. A. and Schmülling T.: *Plant Cell Physiol*., in press (2004)
2. Inoue T., Higushi M., Hashimoto Y., Seki M., Kobayashi M., Kato T., Tabata S., Shinozaki K. and Kakimoto T.: *Nature* **409**, 1060 (2001)
3. Suzuki T., Miwa K., Ishikawa K., Yamada H., Aiba H. and Mizuno T.: *Plant Cell Physiol.* **42**, 107 (2001)
4. Yamada H., Suzuki T., Terada K., Takei K., Ishikawa K., Miwa K. and Mizuno T.: *Plant Cell Physiol.* **42**, 1017 (2001)
5. Romanov G. A., Kieber J. and Schmülling T.: *FEBS Lett.* **515**, 39 (2002)

3P36

THE EFFECT OF CARVEDILOL ON THE SUPEROXIDE GENERATION IN AND MYELOPEROXIDASE RELEASE FROM ACTIVATED HUMAN NEUTROPHILS *IN VITRO*

Jana Pečivová[a,*], Tatiana Macíčková[a], Antonin Lojek[b], Rado Nosál[a], Milan Číž[b] and Viera Fabryová[c]

[a]*Institute of Experimental Pharmacology, Slovak Academy of Sciences, Dúbravská cesta 9, 84104 Bratislava, Slovak Republic*
[b]*Institute of Biophysics, Academy of Sciences of the Czech Republic, Královopolská 135, 612 65 Brno, Czech Republic*
[c]*Department of Hematology and Transfusiology, University Hospital, Bratislava, Slovak Republic*

** E-mail: exfajape@savba.sk*

Azurophilic granules of neutrophils (N) contain in huge amount the heme-containing enzyme myeloperoxidase, which is gradually released either intracellularly into the formed phagosomes or intra and extracellularly upon stimulation of cells. Together with the membraneous NADPH oxidase, myeloperoxidase is involved in the

formation of reactive oxygen species and oxidation of biological material. Myeloperoxidase also modulates a variety of aspects of the inflammatory response.

Because virtually every disease state involves some degree of oxidative stress, we studied the effect of carvedilol (pharmacologically unique agent that possesses multiple pharmacological actions) (0.1-100 μmol/l) on respiratory burst and myeloperoxidase release from isolated human neutrophils stimulated by particle phagocytosis (OZ) or by activation with soluble stimulus (chemotactic tripeptide FMLP). Superoxide formation was measured in isolated human N as superoxide dismutase inhibitable reduction of cytochrome *c*, absorbance was measured at 550 nm in a spectrophotometer Hewlet Packard 8452 A. The activity of myeloperoxidase was assayed by determining the oxidation of *o*-dianisidine in the presence of hydrogen peroxide in a spectrophotometer Hewlet Packard 8452 A at 463 nm.

Carvedilol dose dependently decreased OZ stimulated superoxide generation. Wortmannin, a specific inhibitor of 1-phosphatidylinositol 3-kinase, inhibited significantly fMLP stimulated superoxide generation only. Carvedilol (100 μmol/l) with wortmannin[100 nmol/l] decreased superoxide generation after both stimuli (OZ, FMLP). Carvedilol decreased OZ and FMLP stimulated MPO release dose dependently (1, 10, 100 μmol/l), while in the lowest (0.1 μmol/l) concentration it was without effect.

Acknowledgement

This study was supported by the grant APVT–51-029602 and Project of Scientific and Technical Cooperation (Slovak Republic/Czech Republic) No.131.

3P37

RESPONSE REGULATOR Spo0A – THE SWITCH BETWEEN VEGETATIVE GROWTH AND CELL DIFFERENTIATION

Katarína Muchová* and Imrich Barák

Institute of Molecular Biology, Centre of Excellence for Molecular Medicine, Slovak Academy of Sciences, Dúbravská cesta 21, 84551 Bratislava, Slovakia

** E-mail: katarina.muchova@savba.sk*

Sporulation in *Bacillus subtilis*, an adaptive response to unfavorable environmental conditions, is controlled by an expanded two-component signal transduction system termed phosphorelay[1]. This system consisting of several sensor kinases and two phosphorelay proteins, Spo0B and Spo0F, ensures phosphorylation of the master response regulator of sporulation initiation, Spo0A[2]. If a threshold concentration of Spo0A~P is attained, sporulation commences. The importance of Spo0A as an activator or repressor of transcription of many stationary-phase and sporulation-specific genes is

highlighted by the finding that expression of more than 500 genes is dependent on Spo0A[3].

Transcription factor Spo0A has two domains, an N-terminal phosphoacceptor domain connected to a C-terminal DNA binding domain by a flexible hinge. The phosphoacceptor domain mediates dimerization of Spo0A on phosphorylation[4]. Phosphorylation-induced dimerization increases the affinity of Spo0A for tandemly repeated 0A-boxes within the promoter regions of target genes. Unphosphorylated Spo0A cannot bind to 0A boxes *in vitro*[5] and thus cannot regulate transcription. It seems that the function of receiver domain is to inhibit the effector domain and this inhibition is overcome by phosphorylation.

The crystal structures of the receiver domain (N-Spo0A) in both phosphorylated and unphosphorylated form[6,7] as well as effector domain (C-Spo0A) have been solved recently[8]. Comparison of the structure of N-Spo0A~P with the structures of unphosphorylated response regulators suggested that the major structural change accompanying phosphorylation is the reorientation of the side chain of Thr84 which moves towards the phosphoryl group of phosphorylated Asp56 to make a charge-dipol contact to an oxygen of the phosphoryl group. This is accompanied by a structural rearrangement of the side chain of Phe105 from outward to an inward orientation with respect to the protein core. This "aromatic switch" seems to be a concerved aspect of signalling in majority of response regulators. Mutational analysis was used to evaluate the contribution of Phe105 and neighboring residue Tyr104 to signalling in Spo0A[9]. This analysis together with biochemical data suggested that dimerization and signal transduction between the two domains of Spo0A are mediated principally by the alfa4-beta5-alfa5 signalling surface in the receiver domain. Despite many biochemical, mutational and structural data concerning the function of Spo0A, the molecular mechanism of transduction of the phosphorylation signal from N-Spo0A to C-Spo0A remains still an unanswered question.

References

1. Hoch J. A.: *Annu. Rev. Microbiol.* **47**, 441 (1993)
2. Burbulys D., Trach K. A. and Hoch J. A.: *Cell* **64**, 545 (1991)
3. Molle V., Fujita M., Jensen S. T., Eichenberger P., Gonzales-Pastor J. E., Liu J. S. and Losick R.: *Mol. Microbiol*. **50**, 1683 (2003)
4. Lewis R. J., Scott D. J., Brannigan J. A., Ladds J. C., Cervin M. A., Spiegelman G. B., Hoggett J. G., Barak I. and Wilkinson A. J.: *J. Mol. Biol*. **316**, 235 (2002)
5. Ladds J. C., Muchová K., Blaškovič D., Lewis R. J., Brannigan J. A., Wilkinson A. J. and Barák I.: *FEMS Microbial Lett*. **223**, 153 (2003)
6. Lewis R. J., Brannigan J. A., Muchová K., Barák I. and Wilkinson A. J.: *J. Mol. Biol.* **294**, 9 (1999)
7. Lewis R. J., Muchová K., Brannigan J. A., Barák I., Leonard G. and Wilkinson A. J.: *J. Mol. Biol.* **297**, 757 (2000)
8. Lewis R. J., Krzywda S., Brannigan J. A., Turkenburg J. P., Muchová K., Dodson E. J., Barák I. and Wilkinson A. J.: *Mol. Microbiol.* **38**, 198 (2000)
9. Muchová K., Lewis R. J., Perečko D., Brannigan J. A., Ladds J. C., Leech A., Wilkinson A. J. and Barák I.: *Mol. Microbiol.*, in press (2004)

Acknowledgement

This work was supported by grant 2/1004/22 from the Slovak Academy of Sciences and Wellcome Trust Project and Collaborative Research Initiative Grants (056247/Z/98/Z and 066732/Z/01/Z, respectively).

ACTA UNIVERSITATIS PALACKIANAE OLOMUCENSIS
FACULTAS RERUM NATURALIUM 2004 CHEMICA 43S

PROCEEDINGS OF THE XIX. MEETING OF CZECH AND SLOVAK SOCIETIES FOR BIOCHEMISTRY AND MOLECULAR BIOLOGY

Olomouc, August 31 – September 3, 2004

SESSION 4: BIOENERGETICS AND BIOMEMBRANES, PHOTOSYNTHESIS

4L01

THE CHEMILUMINESCENCE OF CHLOROPHYLL *a* IRRADIATED BY LASER

Iva Šnyrychová[a] and Martina Bancířová[b,*]

[a]*Department of Experimental Physics and* [b]*Department of Physical Chemistry, Fakulty of Science, Palacký University, tř. Svobody 26, 771 46 Olomouc, Czech Republic*

** E-mail: bancir@aix.upol.cz*

Reactive oxygen species (ROS) are presently thought to play important role in an increasing number of physiological and pathological processes in living organisms.

The photodynamic effect[1] involves the combination of light, an organic dye – photosensitizer - and molecular oxygen. Upon irradiation by laser, the photosensitizer is excited to the first excited singlet state, which can react in two ways. Type I mechanism involves hydrogen-atom abstraction or electron-transfer reactions to yield free radicals and radicals ions. Type II results from an energy transfer and generates singlet oxygen. It has been observed, that some types of photosensitizers are specifically taken up tumor tissue. The photodynamic generation of reactive oxygen species is the base of the cancer treatment known as photodynamic therapy (PDT).

Various chemiluminescent compounds (e.g. 5-amino-2,3-dihydro-1,4-phthalazine-dione - luminol) have been studied in order to find suitable and specific probes for detection of ROS. Recently Cypridina luciferin analogues CLA (2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-a]pyrazin-3-one) and MCLA (2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-a]pyrazin-3-one) are thought to emit light only when reacting with superoxide anion and singlet oxygen[2,3].

One of the sources of singlet oxygen is chloroplast, where chlorophyll acts as a photosensitizer. It was shown, that minimal photon emission of singlet oxygen (1268 nm) can be observed with illuminated isolated photosystem II reaction centers without secondary electron acceptors. These acceptors under physiological conditions stabilize charge separation of a special pair of chlorophyll molecules in the reaction center. When the electron transport chain is damaged or when its capacity is insufficient according to the high rate of excited chlorophyll formation, chloroplast is endangered by singlet oxygen. The absorbed energy that cannot be used for charge separation can be transferred by oxygen via excited triplet state of chlorophyll[4]. Naturally occurring carotenoids such as β-carotenoids are highly important for photosynthetic organisms because they act as protectors against photooxidative damage[1].

Luminol, CLA and MCLA chemiluminescence were studied in three ROS generating chemical systems *in vitro* (HRP - H_2O_2 system, $CuSO_4$ - H_2O_2 system, and NaClO - H_2O_2 system). Measure-ments were carried out in phosphate buffer (pH 7.4) at 25°C and 37°C using luminometer Fluoroscan Ascent FL. Superoxide dismutase and sodium azide were used as specific scavenger of superoxide anion radical and quencher of singlet oxygen, respectively. Their effect was compared with that of Trolox (water soluble analogue of vitamin E), which should scavenge all reactive species present in the

reaction mixture, and tryptophan. As the photodynamic systems we used e.g. phthalocyanines and chlorophyll *a*.

The declared specifity of Cypridina luciferin analogues chemiluminescence based on superoxide and singlet oxygen and the suitability of this prove is discussed. Both MCLA and CLA are able to visualize the photodynamic effect of irradiated extract of chlorophyll *a*. β-Carotene suppressed the chemiluminescence of MCLA in the presence of chlorophyll *a* in both cases with and without laser irradiation. It can act as a quencher of singlet oxygen and excited triplet chlorophyll, but it can also react with ROS (especially singlet oxygen) forming xanthophylls[1,5]. All these effects combine together and result in strong antioxidant effect of β-carotene and carotenoids in general. It is not clear whether the chemiluminescence of CLA and MCLA is initiated by the reaction with singlet oxygen or superoxide or other reactive oxygen species formed by the photodynamic effect.

References

1. Bensasson R. V., Land E. J. and Truscott T. G.: *Excited states and free radicals in biology and medicine*, Oxford University Press (1993)
2. Nakano M.: *Methods Enzymol.* **186**, 585 (1990)
3. Nakano M.: *Cell. Mol. Neurobiol.* **18**, 565 (1998)
4. Elstner E. F. and Osswald W.: *Proc. Royal Soc. Edinburg* **102B**, 131 (1994)
5. Montenegro M. A., Nazareno M. A., Durantini E. N. and Borsarelli D. C.: *Photochem. Photobiol.* **75**, 353 (2002)

Acknowledgement

This research was supported by the grant MSM 153100008 from the Ministry of Education, Youth and Sports, Czech Republic.

4L02

BIOTIC STRESS CAUSED BY POTYVIRAL INFECTION – EFFECTS ON ANAPLEROTIC METABOLIC PATHWAYS IN TOBACCO

Karel Müller[a,*], Helena Ryšlavá[a], Helena Synková[b], Renata Pechová[c], Veronika Doubnerová[a] and Noemi Čeřovská[b]

[a]*Department of Biochemistry and* [c]*Department of Plant Physiology, Faculty of Science, Charles University, Hlavova 2030, 12840 Praha 2, Czech Republic*
[b]*Institute of Experimental Botany, Academy of Science, Praha, Czech Republic*

** E-mail: caia@volny.cz*

Biotic stress caused by Potato virus YNTN (PVY) was studied in *Nicotina tabacum* L. cv. Petit Havana SR1. Our attention was focused on activities of Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase, EC 4.1.1.39), PEPC (phosphoenolpyruvate carboxylase, EC. 4.1.1.31), and NADP-ME (NADP-malic enzyme, EC 1.1.1.40).

While the activity of Rubisco in PVY infected tobacco plants decreased, the activity of PEPC increased twice. NADP-ME activity in PVY infected tobacco was five times higher than in control non-infected plants. This enzyme was found to be the most sensitive indicator of the progress of viral infection. NADP-ME catalyses the conversion of malate to pyruvate and CO_2 and NADP is reduced to NADPH. That was the reason to carry out more detailed study of this enzyme. No new isozymes were detected in infected plants by non-denaturating PAGE stained for enzyme activity. To study kinetic parameters of NADP-ME, a thorough isolation was done. NADP-ME was purified by DEAE-cellulose and gel chromatography. We determined specific activity, K_m for malate, NADP, pH optimum and effect of several metabolites. Histochemical localisation was done with NBT-PMS activity staining on hand cut fresh section of the leaf tissue.

Acknowledgement

The research was supported by the project 206/03/0310 from the Grant Agency of the Czech Republic.

4L03

Ca^{2+} CONTROL OF METHANOGENESIS IN METHANOARCHAEON *METHANOTHERMOBACTER THERMAUTOTROPHICUS*

Peter Šmigáň*, Monika Vidová, Stanislav Šurín and Alan Majerník

Institute of Animal Biochemistry and Genetics, Slovak Academy of Science, Moyzesova 61, 90028 Ivanka pri Dunaji, Slovakia

** E-mail: Peter.Smigan@savba.sk*

It is well established that the intracellular Ca^{2+} concentration regulates the different cell functions in mammalian cells[1]. In procaryotes, Ca^{2+} ions are also involved in various processes[2]. Intracellular free Ca^{2+} concentrations are generally maintained at submicromolar levels (0.1-1.0 μM). The low Ca^{2+} level in the cell is maintained either by primary ATP- dependent calcium pumps or by secondary transport systems.

The driving force for Ca^{2+} ions efflux in different bacteria is a proton electrochemical gradient or a sodium electrochemical gradient. Only few data on Ca^{2+} cycling are available in Archaea including methanoarchaea.

Evidence have been obtained in *Halobacterium halobium* and *Methanothermobacter thermautotrophicus* belonging to the Archaea that they also posses the systems for transport of Ca^{2+} ions[3-5]. We have suggested the model in which Ca^{2+} influx pathway is represented by a membrane potential-driven uniport, whereas Ca^{2+} efflux is mediated by two transport systems Na^+/Ca^{2+} and H^+/Ca^{2+} antiporters respectively whose participation in the total efflux is dependent on the energy of corresponding electrochemical gradients of driving force[5]. This model corresponds well with the specific features of bioenergetic system in methanoarchaea. They create two electrochemical gradients (protons and sodium ions), and Na^+/H^+ antiport has in this complex bioenergetic machinery a key function[6,7]. Recently we have found out that Na^+/H^+ antiport activity in the cell of *M. thermautotrophicus* is under the control of calcium ions[7].

In this work the role of Ca^{2+} ions in control of methanogenesis has been investigated. Methane formation by non-growing cells of *M. thermoautotrophicus* was strongly dependent on the Ca^{2+} concentration. Methanogenesis of these cells loaded with Ca^{2+} by means of calcium ionophore A23 187 exerted stimulation by 40%. The methanogenesis was strongly inhibited in the presence of Co^{2+} and Ni^{2+}, that are inorganic antagonists of Ca^{2+} transport in these cells. The methanogenesis was strongly inhibited by Co^{2+} and Ni^{2+} respectively, even in the presence of Na^+ ions. On the other hand Na^+/H^+ exchange was not inhibited by these ions. The growth of these methanoarchaea was inhibited in the presence of calcium chelator EGTA, calcium ionophore A 23 187 and inhibitor of Ca^{2+} influx into mitochondria ruthenium red. Membrane potential was inhibited in the presence of EGTA only by 11%. ATP synthesis driven by an electrogenic potassium efflux was not inhibited in the presence of EGTA. ATP synthesis of these cells loaded with Ca^{2+} by means of calcium ionophore A23 187 did not exert stimulation. The results obtained show that calcium ions are a prerequisite for optimal methane formation in these cells.

Taken together, the results obtained demonstrate that methanogenesis is under the simultaneous control of Ca^{2+} and Na^{+} ions. Our data are in agreement with the model in which Na^{+}/Ca^{2+} exchange mediated by Na^{+}/Ca^{2+} exchanger might be involved in the regulation of methanogenesis.

References

1. Carafoli E.: *Proc. Natl. Acad. Sci. USA* **99**, 1115 (2002)
2. Norris V., Grant S., Freestone P., Canvin J., Sheikh F. N and Toth J.: *J. Bacteriol.* **178**, 3677 (2000)
3. Belliveau J. W. and Lanyi J. K.: *Arch. Biochem. Biophys.* **186**, 98 (1978)
4. Varečka Ľ., Šmigáň P. and Greksák M.: *FEMS Microbiol. Lett.* **107**, 241 (1993)
5. Varečka Ľ., Šmigáň P. and Greksák M.: *FEBS Lett.* **399**, 171 (1996)
6. Deppenmeier U., Miller V. and Gottschalk G.: *Arch. Microbiol.* **165**, 149 (1996)
7. Majerník A., Šmigáň P. and Greksák M.: *Biochem. Mol. Biol. Int.* **43**, 123 (1997)

Acknowledgement

This investigation was supported in part from the Science and Technology Assistance Agency (Slovak Republic) APVT-51-016502 and by a Research Grant VEGA 2/3047/23 from the Slovak Academy of Sciences.

4L04

PURIFICATION AND CHARACTERIZATION OF A HUMAN ELECTRON TRANSFER FLAVOPROTEIN-UBIQUINONE OXIDOREDUCTASE

Martin Šimkovič[a,*] and Frank Frerman[b]

[a]*Department of Biochemistry and Microbiology, Slovak University of Technology, Radlinského 9, 812 37 Bratislava, Slovakia*
[b]*Department of Pediatrics, University of Colorado Health Sciences Center, 4200 East Ninth Avenue, Denver, CO 80262, USA*

** E-mail: martin.simkovic@stuba.sk*

Electron transfer flavoprotein-ubiquinone oxidoreductase (ETF-QO) is a mitochondrial enzyme that catalyzes electron transfer from electron transfer flavoprotein (ETF) to coenzyme Q (CoQ) in the mitochondrial respiratory chain. It is the membrane protein (~ 64 kDa) containing one equivalent of FAD and a $[4Fe\text{-}4S]^{2+/1+}$ cluster, and is one of the simplest quinone oxidoreductases in the respiratory chain[1,2]. The protein serves as the input site to the main respiratory chain for reducing equivalents derived from the nine acyl-CoA dehydrogenases and two *N*-methyl-dehydrogenases[1,2]. Despite

its central role in the oxidation of fatty acids and some amino acids, relatively little is known about ETF-QO.

Based on the redox potentials of the FAD, iron-sulfur cluster and ubiquinone determined by EPR-spectroelectrochemical analysis of the porcine ETF-QO[3], it had been assumed that electrons from ETF enter ETF-QO through the flavin (E_0' = +28 mV, semiquinone/oxidized couple) and are transferred to the iron-sulfur cluster (E_0' = +47 mV), which is the reductant of ubiquinone ($E_0' \approx$ +100 mV). However, the distance between the iron-sulfur cluster and the 1,4-benzoquinone head group of ubiquinone is 19 Å, making it unlikely that the iron-sulfur cluster is the immediate reductant of ubiquinone[4]. In contrast, the isoalloxazine-benzoquinone distance is 8.5 Å which would be favorable for electron transfer[5].

Ramsay et al.[6] and Watmough et al.[7] determined steady state kinetic constants of porcine ETF-QO with the water-soluble ubiquinone analogue, CoQ_1 and with porcine ETF as varied substrates. Beckmann and Frerman also demonstrated that porcine ETF-QO catalyzes the equilibration of ETF redox states and determined kinetic constants for the disproportionation and comproportionation reactions[2,8]. Ramsay *et al.* suggested that the disproportionation of ETF semiquinone (eq 1) catalyzed by ETF-QO may be physiologically significant since it is faster than the overall rate of electron transfer from a primary dehydrogenase to ubiquinone[6].

$$2ETF_{1e^-} \rightleftarrows ETF_{ox} + ETF_{2e^-} \qquad \text{(eq 1)}$$

The discovery of glutaric acidemia type II, an often fatal metabolic disorder of fatty acids and amino acids caused by a deficiency in either ETF or ETF-QO, resulted in a greater interest in the human ETF-QO[9]. The first time a cDNA encoding the human protein was cloned by Goodman *et al.*[10]. Although the protein was successfully expressed in *Saccharomyces cerevisiae*, the expression gave only low yields of the human ETF-QO. In this study we describe an expression system for human ETF-QO in insect cells using a baculovirus vector[11]. We report catalytic and spectral properties of the recombinant protein and show that the recombinant protein provides an appropriate system for investigating structure/function relationships in ETF-QO. Moreover, we have focused on the characterization of ubiquinone site, using alternate substrates and quinone analogs and inhibitors that could serve as useful structural and mechanistic probes[12].

Reference

1. Ruzicka F. J. and Beinert H.: *J. Biol. Chem.* **252**, 8440 (1977)
2. Beckmann J. D. and Frerman F. E.: *Biochemistry* **24**, 3913 (1985)
3. Paulsen K. E., Orville A. M., Frerman F. E., Lipscomb J. D. and Stankovich M. T.: *Biochemistry* **31**, 11755 (1992)
4. Kim J.-J. P., Zhang J. and Frerman F. E.: In *Flavins and Flavoproteins 2002* (Chapman S., Perham R. and Scrutton N., eds.), Rudolf Weber, Berlin 2002, p. 77
5. Page C. C., Moser C. C., Chen X. and Dutton P. L.: *Nature* **402**, 47 (1999)
6. Ramsay R. R., Steenkamp D. J. and Husain M.: *Biochem. J.* **241**, 883 (1987)
7. Watmough N. J., Loehr J. P., Drake S. K. and Frerman F. E.: Biochemistry **30**, 1317 (1992)
8. Beckmann J. D. and Frerman F. E.: *Biochemistry* **24**, 3922 (1985)

9. Frerman F. E. and Goodman S. I.: In *The Metabolic & Molecular Bases of Inherited Disease, 8th ed.* (Scriver C. R., Beaudet A. L., Sly W. S. and Valle D., eds.), McGraw-Hill, New York 2001, p. 2357
10. Goodman S. I., Axtell K. M., Bindoff L. A., Beard S. E., Gill R. E. and Frerman F. E.: *Eur. J. Biochem.* **219**, 277 (1994)
11. Šimkovič M., DeGala G. D., Eaton S. S. and Frerman F. E.: *Biochem. J.* **364**, 659 (2002)
12. Šimkovič M. and Frerman F. E.: *Biochem J.* **378**, 633 (2004)

Acknowledgement

This research was supported by a grant HD 08315 from the National Institutes of Health.

4L05

MODULATION OF (BASAL) CA^{2+} FLUXES ACROSS THE PLASMA MEMBRANE BY PROTONMOTIVE FORCE

Ľudovít Varečka[a,*], Roman Hudec[a], Boris Lakatoš[a], Martin Šimkovič[a], Karin Kaiserová[a] and Jozef Orlický[b]

[a]*Department of Biochemistry and Microbiology, Faculty of Chemical and Food Technology, Slovak University of Technology, Radlinského 9, 81237 Bratislava, Slovak Republic*
[b]*Institute of Molecular Physiology and Genetics, Slovak Academy of Sciences, Vlárska 5, 834 04 Bratislava, Slovak Republic*

** E-mail: ludovit.varecka@stuba.sk*

The notion that the transport of ions across the cell plasma membranes consists of "leak" and "pump" is generally accepted. In the studies of Ca^{2+} homeostasis of mammalian cells, the „leak" is represented by several sorts of Ca^{2+}-selective ion channels while the „pump" are Ca^{2+}-ATPase(s) (PMCA) and Na^{+}/Ca^{2+} exchangers localised in the plasma membrane and ER (Golgi)-located Ca^{2+}-ATPases (SERCA)[1]. In microbial and plant cells, the character of channels is not yet understood but the Ca^{2+}-ATPases were found in several microorganisms[2-5].

Human red blood cell (RBC) Ca^{2+}-ATPase was found to catalyse the ATP-driven electroneutral (or at least partially charge-compensated) Ca^{2+}/H^{+} exchange[6]. Due to the structural homology of PMCA of eucaryotic organisms[2-5] it could be inferred that this is a general feature of PMCA. As the cyclic basal Ca^{2+} influx reaches values in the order of 10^{-5} mol/l cells.h, concomitant changes in H^{+} fluxes should acidify the cytoplasm in a short time.

Effects of uncoupler on the basal Ca^{2+} fluxes in human RBC[7], human white blood cells (WBC), in filamentous fungus *Trichoderma viride* (Trv)[8] and in yeast *Saccharomyces cerevisiae* (Sac) were studied by means of $^{45}Ca^{2+}$ and a repeated washing procedure with EDTA-containing solution. The results showed that the rate of the $^{45}Ca^{2+}$ influx posesses characteristics of the carrier-mediated transport (saturation with Ca^{2+}, pH- and temperature-dependence) in all cells. In human RBC, the basal $^{45}Ca^{2+}$ transport is fully compensated with the activity of the Ca^{2+}-ATPase, so that there is no net $^{45}Ca^{2+}$ uptake during the experiment but other organisms could store a part of Ca^{2+} taken up by cells. Uncoupler inhibited the $^{45}Ca^{2+}$ influx under conditions which excluded the involvement of anion channel activity. In human WBC, the effect of uncoupler on the $^{45}Ca^{2+}$ was strongly dependent on the monovalent ion composition being slightly inhibitory at low K^+ concentration but stimulatory at high K^+ concentrations. In *T. viride* both Ca^{2+} influx and Ca^{2+} efflux are stimulated by uncoupler, whereas in *S. cerevisiae*, uncoupler stimulated only the Ca^{2+} efflux and the Ca^{2+} influx was rather inhibited or unaffected by this compound. Thus, the effect of uncoupler on the basal Ca^{2+} fluxes reveals the role of protonmotive force in modulation of this important cellular characteristics, although underlying mechanisms may be different in different cell types.

References

1. Carafoli E., Santella L., Branca D. and Brini M.: *Crit. Rev. Biochem. Mol. Biol.* **36**, 107 (2001)
2. Benito B., Garciadeblas B. and Rodriguez-Navarro A.: *Mol. Microbiol.* **35**, 1079 (2000)
3. Sze H., Liang F., Hwang I., Curran A. C. and Harper J. F.: *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **51**, 433 (2000)
4. Axelsen K. B. and Palmgren M. G.: *Plant Physiol*. **126**, 696 (2001)
5. Carafoli E. and Brini M.: *Curr. Opin. Chem. Biol*. **4**, 152 (2000)
6. Niggli V., Sigel E. and Carafoli E.: *J. Biol. Chem*. **257**, 2350 (1982)
7. Hudec R., Lakatoš B., Kaiserová K., Orlický J. and Varečka Ľ.: *Biochim. Biophys. Acta* **1661**, 204 (2004)
8. Šimkovič M., Kryštofová S. and Varečka Ľ.: *Can. J. Microbiol*. **46**, 312 (2000)

Acknowledgement

This work was supported by grants VEGA 1/0109/23, VEGA 2/3188/23, and APVT 51-013802.

4P01

MECHANISMS OF ACTIVITY OF POLYENE ANTIMYCOTICS IN YEAST: CONTRIBUTION OF CELL WALL AND PLASMA MEMBRANE CHARACTERISTICS TO SELECTIVE RESISTANCE TO NYSTATIN AND AMPHOTERICIN B

Katarína Strhanová*, Vlasta Klobučníková and Ivan Hapala

Institute of Animal Biochemistry and Genetics, Slovak Academy of Science, Moyzesova 61, 90028 Ivanka pri Dunaji, Slovakia

** E-mail: Katarina.Strhanova@savba.sk*

Mycoses have developed to one of serious health care problems due to widespread prevalence and increasing severity of symptoms, especially in immunocompromised individuals. Polyene macrolides represent an important class of clinically applied antimycotics. Their name reflects the presence of alternating conjugated double bonds that constitute a part of their macrolide ring structure. The polyene antibiotics are all products of *Streptomyces* species.

Two dominant representatives of this group are nystatin used in combating for superficial fungal infections and amphotericin B applied against systemic fungal infections. Their positive features include relatively broad spectrum of antifungal activity including dimorphic pathogens such as *Coccidioides immitis*, *Histoplasma capsulatum*, *Blastomyces dermatitidis* and *Paracoccidioides brasiliensis* and primary fungicidal mode of action. However, they bring also severe side effects (ranging from phlebitis at the infusion site and chills to renal toxicity) that are linked to mechanisms of their activity. The antifungal activity of these antimycotics is based on formation of aqueous pores in sterol-containing membranes that cause leak of essential ions and metabolites and death of fungal cells.

Nystatin and amphotericin B are very similar in structure, however, they show different pattern of specificity to target pathogens and different extent of side effects in human organism. Detailed information about the molecular mechanisms of their activity might thus help to tailor the antifungal therapy of individual mycoses more efficiently.

Although the yeast *Saccharomyces cerevisiae* has a low pathogenic potential in humans, it is often used to study various aspects of fungal pathogenicity. Resistance to polyene antimycotics in this yeast is reported to be based primarily on qualitative and quantitative changes in sterol content. In an attempt to elucidate other factors modulating the sensitivity to this antimycotic group we designed a specific selection scheme for isolation of mutants showing resistance to nystatin and amphotericin B independently on ergosterol content. We succeeded in isolation of two mutant clones of *Saccharomyces cerevisiae* that show selective resistance to either nystatin or amphotericin B. The genetic characterization of these mutants showed that they are bearing independent mutations with different mode of inheritance. The detailed phenotypic characterization indicates that the modes of resistance in mutant clones are significantly influenced by the properties of cell wall. Some presented data indicate that the mechanisms of activity of

nystatin and amphotericin B may show significant differences in spite of their structural similarity.

Acknowledgement

The work was supported by grants APVT-51-016502 and VEGA 2-4130-24.

4P02

ISOLATION AND CHARACTERIZATION OF AN AMILORIDE–RESISTANT MUTANT OF *METHANOTHERMOBACTER THERMAUTOTROPHICUS*

Stanislav Šurín*, Monika Vidová, Alan Majerník, Lubomíra Čuboňová and Peter Šmigáň

Institute of Animal Biochemistry and Genetics, Slovak Academy of Science, Moyzesova 61, 90028 Ivanka pri Dunaji, Slovakia

** E-mail: Stanislav.Surin@savba.sk*

Methanogens as a member of the Archaea, possessing a specialized kind of metabolism belong to a group of microorganisms containing H^+ and Na^+-cycles[1]. Methanogenesis in these microorganisms is coupled to the generation of a primary H^+-electrochemical gradient and Na^+- electrochemical gradients. In some methanogens, both ion gradients are directly used for ATP synthesis.

The existence of Na^+/H^+ antiporter as an universal device for linking the H^+ cycle with Na^+ cycle was confirmed in some methanogens and this antiporter might be the basic regulatory element in the complex bioenergetic strategy of these cells[2].

To understand the function and interrelationship of H^+, Na^+ energetic subsystems and Na^+/H^+ antiporter, a genetic elimination of the components of the bioenergetic machinery was successfully used recently[3]. Isolation and characterization of a mutant with lesion in Na^+/H^+ antiporter could help us to solve the problem of a function of Na^+/H^+ antiporter in this complex bioenergetic system .

The intention of this work is to present the first case of an amiloride resistant mutant of methanoarchea with lesion in Na^+/H^+ antiporter.

The classical inhibitor of Na^+/H^+ antiporter amiloride in different concentrations completely inhibited growth and methanogenesis. To select spontaneous amiloride resistant mutant the cells were plated on solid medium with different amiloride concentrations in an anaerobic chamber. The resistant colony to 2 mM amiloride was picked and purified. The growth of this mutant in both liquid and solid media with 2mM amiloride exerted good growth in comparison with the wild type cells which growth was inhibited under these conditions completely. Mutant cells exhibited higher production of methane than wild-type cells. Na^+/H^+ antiporter activity was considerably diminished in the mutant cells. The ATP synthesis driven either by potassium diffussion potential in

the presence of sodium ions or by methanogenesis was considerably higher in the mutant cells. The results presented here strongly support the idea that in this mutant Na^+-dependent bioenergetic system(s) is modified.

References

1. Deppenmeyer U., Muller V. and Gottschalk G.: *Arch. Microbiol.* **165**, 149 (1996)
2. Majerník A., Šmigáň P. and Greksák M.: *Biochem. Mol. Biol. Internat.* **43**, 123 (1997)
3. Šmigáň P., Polák P., Majerník A. and Greksák M.: *FEBS Lett.* **420**, 93 (1997)

Acknowledgement

This work was supported in part from the Science and Technology Assistance Agency (Slovak Republic) APVT –51-016502 and by Research grant VEGA 2/3047/23 from the Slovak Academy of Sciences.

4P03

TOPOGRAPHICAL HETEROGENEITY OF ACTIVITIES OF ANTIOXIDANT ENZYMES ASCORBATE PEROXIDASE AND GLUTATHION REDUCTASE AND LIPID PEROXIDATION IN WHEAT PLANTS SENESCING UNDER DIFFERENT LIGHT LEVELS

Kateřina Slouková*, Miroslava Hunková, Martina Špundová and Jan Nauš

Laboratory of Biophysics, Department of Experimental Physics, Faculty of Science, Palacky University, tř, Svodody 26, 771 46 Olomouc, Czech Republic

** E-mail: katka.sloukova@email.cz*

Senescence and accompanying oxidative processes are heterogeneous within the plant[1] and are affected by the level of cultivating light. The antioxidant enzymes ascorbate peroxidase (APX) and glutathion reductase (GR) are the main enzymes of the ascorbate-glutathione cycle that is a part of the water-water cycle in chloroplasts[2] but also appears in other organelles[3]. The water-water cycle scavenges active oxygen species (superoxide and hydrogen peroxide) photogenerated by thylakoid membranes. Under high light condition the water-water cycle also dissipates the excess energy. However, the changes of APX activity during senescence seem to be species specific. Lascano et al.[4] found a decrease for wheat whereas Kanazawa et al.[5] found an increase for cucumber. Moreover, the results depend on the reference quantity[6] and light conditions during senescence[5].

In our research the plants of spring wheat (*Triticum aestivum* L. cv. Saxana) were cultivated in pots at autumn season in a greenhouse at three light levels (unshaded and two shaded variants, 70 % and 40 % of incident light). In the growth phase of third to

fifth leaf (37 to 49 days old plants) the activities of the antioxidant enzymes APX and GR were followed within two weeks at three leaf levels (from the first to the third leaf counting in the order of emergency). The oxidative processes in plant tissue were documented by measurements of lipid peroxidation being mostly of non-enzymatic character in this case[7].

Both the effect of heterogeneous senescence and plant shading were demonstrated. The senescence was indicated by a decrease of chlorophyll and total protein content on the leaf area basis. The GR and APX activities decreased when expressed on the fresh weight, dry weight and leaf area basis, whereas they were constant or slightly increasing when related to the total protein or chlorophyll (a+b) content. The results show the importance of the reference quantity and lead to different physiological interpretations of the obtained dependencies. Whereas a decrease of activities expressed on the leaf area basis indicates a controlled degradation of enzymes during senescence, the stability of the measured activities on the protein basis reflect a protection of the remaining protein moiety.

The peroxidation of lipids expressed on the fresh weight basis increased both with the age of leaf and upon lowering the light intensity. The course of senescence and oxidative processes were different in different leaf levels. A more pronounced senescence found in older leaves was expressed by a higher loss of pigments, by a higher decrease in antioxidant enzyme activities on the leaf area (and similarly also fresh weight and dry weight) basis, and by an increase of lipid peroxidation. The plant shading has enhanced the topographic gradients of different quantities[1] and accelerated the senescence effects predominantly in the first leaf. The activities expressed on the leaf area basis decreased more for GR than for APX. However, as the activities of antioxidant enzymes did not decrease on the protein or chlorophyll basis, we suggest to explain this finding by a tendency to protect the remaining functional part of protein systems (e.g. photosynthesis) against oxidation whereas the lipidic systems are less protected from oxidation.

References

1. Matoušková M., Nauš J. and Flašarová M..: *Photosynthetica* **37**, 281 (1999)
2. Asada K.: *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **355**, 1419 (2000)
3. Jiménez A., Hernandez J. A., Del Rio L. A. and Sevilla F.: *Plant Physiol.* **114**, 275 (1997)
4. Lascano H. R., Antonicelli G. E., Luna C. M., Melchiorre M. N., Gómez L. D., Racca R. W., Trippi V. S. and Casano L. M.: *Aust. J. Plant Physiol.* **28**, 1095 (2001)
5. Kanazawa S., Sano S., Koshiba T. and Ushimaru T.: *Physiol. Plantarum* **109**, 211 (2000)
6. Logan B., Demmig-Adams B., Adams W. and Grace S.: *J. Exp. Bot.* **49**, 1869 (1998)
7. Berger S., Weichert H., Porzel A., Wasternack C., Kuhn H. and Feussner I.: *Biochim. Biophys. Acta* **1533**, 266 (2001)

Acknowledgement

The research was supported by the grants MSM 153100010 from Ministry of Education, Youth and Sports, and 522/01/P098 from the Grant Agency, Czech Republic.

4P04

THE IMPACT OF CADMIUM ON THE PHOTOSYNTHESIS AND THE ACTIVITY OF ENZYME GLUTAMATE KINASE IN BARLEY

Dana Hradecká[a], Jiří Dudjak[b] and Ludmila Staszková[b,*]

[a]*Department of Plant Crop Production and* [b]*Department of Chemistry, Czech Agricultural University, Kamýcká 957, 16521 Praha 6, Czech Republic*

** E-mail: staszkova@af.czu.cz*

Heavy metals as pollutants limiting plant productivity, and their impact on growth processes are now intensively studied. Stresses, caused by high concentration of heavy metals, decrease photosynthesis as well as the activity of various enzymes. Plant protects itself against the stress by the specific reactions, consisting of the synthesis of special metabolites e.g. proline, or plant hormones. The object of our research was to evaluate energy distribution during the photosynthesis of barley stressed by cadmium and to describe the role of natural plant hormone 24-epibrassinolide (24-epi) during the stress. The process was monitored by the measurement of the activity of enzyme glutamate kinase. The enzyme catalyses the first step of proline biosynthesis. Proline is degraded through the glutamic acid to δ-aminolevuloinic acid, which is the main building unit of the porphyrine skeleton of chlorophyll. This fact illustrates the relation of proline to photosynthesis.

Convenient method for the evaluation of energy balance of photosynthesis, based on the relation between chlorophyll fluorescence and its photosynthetic activity which might be changed by stresses, is well known as the method of Fast Fluorescent Induction (FFI)[1].

Plantlets of barley cv. Kompakt have been grown in nutritive solution (control), and treated with the supplement of Cd^{2+} ions (10^{-6} M) for 4 weeks. During the experiment FFI has been measured for three times (45% light of 6 diods saturated by 12 V batery), by fluorometer PEA Hansatech Instr. The activity of the enzyme glutamate kinase was determined by a modification of the hydroxamate method [2,3]. Subsequently, 24-epi, made at the Institute of Organic Chemistry and Biochemistry CAS (1 ml of 10^{-6} M), has been applied. To evaluate the effect of 24-epi, FFI was measured 1, 2, 3, 4, 5, and 6 hours after the addition of the brassinosteroid. Plant growth analysis has been performed on 10 plants of each variant to describe the impact of Cd^{2+} ions and 24-epi on growth.

Cd^{2+} ions shortened stems by 45%, and roots by 65%. Dry matter decreased in stems but only a little in roots. Assimilative area diminished by 75%. Chlorophyll content fell by 85%. The treatment with 24-epi caused rapid increase of chlorophyll to a level measured in non-treated control plants. Cd^{2+} ions negatively influenced the energy balance of the photosynthesis. After six hours, 24-epi caused significant expansion of Tfm(ms), whereas Fv/Fm, Fo, Fv a Fm increased only slightly. The activity of enzyme glutamate kinase grew up to 138.1% in comparison with the control plants. Proline concentration was enhanced, which supported Cd^{2+} stress resistance.

References

1. Kautský A. and Hirsch J.: *Naturwissenschaften* **19**, 96 (1931)
2. Vašáková L. and Štefl M.: *Collect. Czech. Chem. Commun.* **47**, 349 (1982)
3. Yoshinaga F., Tsuchida T. and Okumura S.: *Agric. Biol. Chem.* **39**, 6 (1975)

Acknowledgement

Supported by grants 525/02/03/01 from the Grant Agency and MSM412100002 from the Ministry of Education, Youth and Sports, Czech Republic.

4P05

CHARACTERIZATION OF CHLOROPHYLL-CONTAINING PROTEIN COMPLEXES SEPARATED BY NATIVE DERIPHAT-POLYACRYAMIDE GEL ELECTROPHORESIS

Lenka Lípová*, Petr Ilík and Pavel Krchňák

Department of Experimental Physics, Fakulty of Science, Palacký University, tř. Svobody 26, 771 46 Olomouc, Czech Republic

** E-mail: lipovalenka@seznam.cz*

At present the native chlorophyll-containing protein complexes (PPCs) from thylakoid membranes of chloroplasts are fractionalized by non-ionic glycosidic surfactant decylmaltoside, which preserves pigment-protein interactions in PPCs. The native PPCs are then partly charged by addition of the surfactant Deriphat 160 (N-lauryl-beta-iminodipropionate) and separated by mild polyacrylamide gel electrophoresis (Deriphat-PAGE)[1]. Many green zones with PPCs accompanied by few free pigments (FP, complexed with surfactant in micelles) in resulting electrophoreograms indicate a sufficient and mild separation of PPCs. In this work we showed which gel density is most suitable for separation of PPCs and characterized the separated PPCs by their molecular weights and chlorophyll fluorescence emission and excitation spectra at 77 K.

The best separation of PPCs – 7 green zones with PPCs and a relatively faint yellow-green FP zone - was performed with 7 % polyacrylamide gel (percentage of acrylamide). The gel with all zones was scanned by the home-made 2-D monochromatic gel densitometer[2,3] which allows scanning of the electrophoreogram at the wavelength of the red absorption maximum of chlorophyll *a* in PPCs (670 nm). Resulting 2-D densitograms are of high contrast in comparison with the densitograms scanned at white light[3,4].

We compared our electrophoreograms with those reported by Lee and Thornber[5] who used same method and plant material. Apart from the standard zones - PSI (photosystem I), CCI (core complex of PSI), CCII (core complex of photosystem II),

LHCo (LHC oligomer) and LHCm (LHC monomers) - we observed two additional zones in our electrophoreograms. Comparing densities and migration distances of our green zones with those of the standard zones reported by Lee and Thorber[5] we concluded that the additional zones appeared between PSI and CCI and between LHCo and LHCm. On the basis of molecular weights of PPCs in these new zones (determined by native PAGE) and their characterization by chlorophyll fluorescence emission and excitation spectra we attributed them to the disintegrated PSI and separated internal antennae of PSII (CP47 and CP43), respectively. We also characterized all other separated PPCs by their molecular weights (using native PAGE) and chlorophyll fluorescence spectra at 77 K that has not been performed yet.

References

1. Peter G. F. and Thornber J. P.: In *Methods in Plant Biochemistry, Vol. 5* (Rogers L. J., ed.), Academic Press, London 1991, p. 195
2. Ilík P., Krchňák P., Tomek P. and Nauš J.: *J. Biochem. Biophys. Meth.* **51**, 273 (2002)
3. Krchňák P., Ilík P. and Nauš J.: *Jemná mechanika a optika* **1**, 15 (2002)
4. Lee A. I. and Thornber J. P.: *Plant Physiol.* **107**, 565 (1995)

Acknowledgement

This work was supported by the grant MSM 153100010 from the Ministry of Education, Youth and Sports, Czech Republic.

4P06

ACTIVATION OF MITOCHONDRIAL GLYCEROL-3-PHOSPHATE DEHYDROGENASE BY DIFFERENT ANALOGUES OF COENZYME Q

Hana Rauchová[a,b,*], Ivan Mikšík[b], Romana Fato[c], Christian Bergamini[c] and Giorgio Lenaz[c]

[a]*Center for Experimental Cardiovascular Research and* [b]*Institute of Physiology, Academy of Sciences, Vídeňská 1083, 14220 Praha 4, Czech Republic*
[c]*Department of Biochemistry, University of Bologna*

** E-mail: rauchova@biomed.cas.cz*

Nuclear-encoded, mitochondrial flavoprotein-dependent glycerol-3-phosphate dehydrogenase (mGPDH; EC 1.1.99.5) embedded in the inner mitochondrial membrane catalyzes the transfer of hydrogen from glycerol-3-phosphate to coenzyme Q pool in the respiratory chain[1,2]. As a component of glycerol-3-phosphate shuttle, it plays (together with its cytosolic counterpart) a role in the transfer of reducing equivalents from cytosol

into mitochondria. High activity of mGPDH (and also of the above shuttle) was found in insect flight muscle where this enzyme enables the utilization of cytosolic reducing equivalents as supplementary respiratory substrate during flying. An active glycerol-3-phosphate shuttle (with enough high mGPDH activity) was also described in mammalian brown adipose tissue where the function of this cycle is connected with the regulation of norepinephrine-induced thermogenesis. The amount of mGPDH can be markedly induced by thyroid hormones so that it can be used as a suitable marker for the evaluation of thyroid status both in acute or chronic experiments[3,4]. Recent studies also show the enhancement of mGPDH activity by dehydroepiandrosterone and its different metabolites[5,6]. In our previous studies it was found that the activity of mGPDH *in situ* in the mitochondrial membrane is strongly stimulated by a coenzyme Q (CoQ) homologues with short isoprenoids chain: CoQ_3 and CoQ_1[2,7,8].

The aim of our present study was to test other different analogues of CoQ. We used idebenone (6-(10-hydroxydecyl)-2,3-dimethoxy-5-methyl-1,4-benzoquinone) and its two derivatives: methoxyidebenone and acetylidebenone and three other compounds in which aliphatic chain of CoQ was substituted by thiazoimidazol type groups.

The experiments were performed on isolated mitochondria (frozen-thawed) from brown adipose tissue of cold-adapted male hamster (*Mesocricetus auratus*). The activity of mitochondrial glycerol-3-phosphate dehydrogenase was measured spectrophotometrically at room temperature as glycerol-3-phosphate cytochrome *c* oxidoreductase (following the reduction of cytochrome *c* at 550 nm) or as dehydrogenase (following the reduction of the artificial hydrophilic electron acceptor 2,6-dichlorophenol-indophenol at 600 nm). Reaction was started by addition of 25 mM glycerol-3-phosphate. Idebenone and its two derivatives were diluted in absolute ethanol, the other compounds in DMSO. The protein was determined using the Folin phenol reagent using bovine serum albumin as a standard. The data were expressed as the means + S.E.M.

We found that the activity of mGPDH is highly stimulated by idebenone and its two derivatives (10 μM idebenone increases activity by 73 %). The stimulation of enzyme activity is not markedly affected by the modification of the end of aliphatic chain (hydroxyl group, methoxyl group or acetyl group). The substitution of the long aliphatic chain of CoQ by thiazoimidazol type groups also stimulates activity but the effect on mGPDH activity is lower than that of idebenone(s). On the other hand, it is known that idebenone has an inhibitory effects on the mitochondrial Complex I activity[9]. Italian coworkers have also found that two idebenone derivatives have a similar effect on NADH-CoQ1 activity.

The present results can support our previous data[8], suggesting differences between mGPDH and other dehydrogenases of respiratory chain in the transfer of reducing equivalents to the CoQ pool.

References

1. Gong D. W., Bi S., Weintraub B. D. and Reitman M: *DNA Cell Biol.* **17**, 301 (1998)
2. Rauchová H., Battino M., Fato R., Lenaz G. and Drahota Z: *J. Bioenerg. Biomembr.* **24**, 235 (1992)
3. Lotková H., Rauchová H. and Drahota Z: *Physiol. Res.* **50**, 333 (2001)
4. Rauchová H., Zachařová G. and Soukup T: *Horm. Metab. Res.* **36**, in press (2004)
5. Su C.-Y. and Lardy H. A: *J. Biochem.* **110**, 207 (1991)

6. Reich I. L., Reich H. J., Kneet N. and Lardy H: *Steroids* **67**, 221 (2002)
7. Rauchová H., Fato R., Drahota Z. and Lenaz G: *Chem. Papers* **52**, 396 (1998)
8. Rauchová H., Drahota Z., Rauch P., Fato R. and Lenaz G: *Acta Biochim. Pol.* **50**, 405 (2003)
9. Esposti M. D., Ngo A., Ghelli A., Benelli B., Carelli V., McLennan H. and Linnane A. W: *Arch. Biochem. Biophys.* **330**, 395 (1996)

Acknowledgement

This study was supported in part by grant 305/04/0500 from the Grant Agency of the Czech Republic and by a joint project 43/BI4 in the framework of Czech-Italian scientific and technological cooperation.

ACTA UNIVERSITATIS PALACKIANAE OLOMUCENSIS
FACULTAS RERUM NATURALIUM 2004 CHEMICA 43S

PROCEEDINGS OF THE XIX. MEETING OF CZECH AND SLOVAK SOCIETIES FOR BIOCHEMISTRY AND MOLECULAR BIOLOGY

Olomouc, August 31 – September 3, 2004

SESSION 5: BIOTECHNOLOGY AND FOOD BIOCHEMISTRY

5L01

A COLD-ACTIVE β-GALACTOSIDASE FROM THE ANTARCTIC BACTERIUM *ARTHROBACTER* SP. C2-2

Petra Lipovová[a,] *, Hynek Strnad[a], Vojtěch Spiwok[a], Blanka Králová[a] and Nicholas J. Russell[b]

[a]*Department of Biochemistry and Microbiology, Institute of Chemical Technology, Technická 3, 16628 Praha 6, Czech Republic*
[b]*Department of Agricultural Sciences, University of London Imperial College at Wye,UK*

** E-mail: karasovp@vscht.cz*

Psychrotrophic microorganisms are important in global ecology as a large proportion of our planet is cold. To retain rates of metabolic processes at low temperatures, psychrotrophs must contain enzymes with high specific activity in the cold. Such enzymes offer a great potential in biotechnology and in food processing.

β-Galactosidase is the enzyme widely used for preparation of lactose-free milk and biosynthesis of galactooligosaccharides in dairy technologies. The aim of this project is to find β-galactosidase with high activity in the cold.

The Antarctic bacterium *Arthrobacter* sp. C2-2 contains at least two cold-active isoenzymes of β-galactosidase. The C2-2-1 isoenzyme was cloned, purified and characterized. It is a homotetramer, each subunit being composed of 1023 amino acids. It shows greater activity towards lactose as a substrate compared with artificial chromogenic substrates. The C2-2-1 isoenzyme is particularly cold-active, retaining 30-68 % of activity at 10 ^{o}C compared with maximum values. The temperature optimum of the purified enzyme differed for lactose (40 ^{o}C) and for the chromogenic substrate *o*-nitrophenyl-β-D-galactopyranoside (20 ^{o}C). The enzyme is losing all activity within 15 min at 50 ^{o}C. The isoelectric point of the enzyme was 5.9. Dithiotreitol and Mg^{2+} ions are strong activators, whereas Cu^{2+}, Al^{3+} and Tris are strong inhibitors of the activity. The enzyme exhibited transglycosylation ability. The highest concentration of trisaccharides (34 mM) was formed after 10 hours at 15 ^{o}C. Therefore, the C2-2-1 β-galactosidase isoenzyme of *Arthrobacter* sp. C2-2 could be used as a biotechnological tool in the production of lactose-reduced dairy products at refrigeration temperatures.

Acknowledgement

This work was performed as part of the project 204/02/0843 supported by the Grant Agency and research grant MSM 223300006 from the Ministry of Education, Youth and Sports, Czech Republic. Sequencing part of this project was supported by the grant LN00A079 from the Center for Integrated Genimics.

5L02

OUR FAVORITE DRINKS AND THEIR ANTIOXIDANT CAPACITY

Martina Bancířová*, Veronika Jarešová, Tereza Novotná, Jitka Řepíková, Alena Veinlichová and Tomáš Zelený

Department of Physical Chemistry, Faculty of Science, Palacký University, tř. Svobody 26, 771 46 Olomouc, Czech Republic

** E-mail: bancir@aix.upol.cz*

Atmospheric pollutants are an important source of oxidative stress to terrestrial plants and to animals. Reduced visibility, a yellow brown haze, and the presence of irritating substances in the atmosphere characterize photochemical smog. The chemical reactions occurring in photochemical smog are very complex.

Oxidative stress may give a rise to many diseases dependent on damage of tissues, e.g. cardiovascular diseases, rheumatoid arthritis and some cancers. Amongst the oxidant species particular attention has focused upon free radicals, defined as any chemical species capable of independent existence that contains an unpaired electron. Free radicals are usually unstable and highly reactive, being capable of pooling electrons out of surrounding molecules. The levels of such harmful compounds in living organisms can be controlled enzymatically or by involvement of molecules known as „scavengers". They include vitamins (e.g. vitamin C and E), proteins and enzymes (glutathione peroxidase, catalase, superoxide dismutase). Fruit and vegetables contain several classes of compounds that when ingested can potentially contribute to antioxidant protection. The previous studies demonstrated that topical application of some antioxidant in healthy volunteers significantly increased the antioxidative potential of skin biosurface, thus highlighting the effectiveness of a natural antioxidant biotechnology in the antiaging management of skin[1]. Observations on cancer causation are some 150 years old, but actual detailed research on elements bearing on cancer started at the beginning of the twentieth century. Studies in humans documented certain lifestyle-related factors to lead to cancer. In addition, reactive oxygen species (ROS) are involved in both the early steps in cancer and in developmental aspects. Thus, foods containing antioxidants such as vegetables, fruits, soy products, cocoa and tea that counteract ROS are protective in cancer causation and development[2].

The lifetime of free radicals is extremely short, and if they are not immediately neutralized by a physiological acceptor, they can damage biological systems to such an extent that a chain reaction is set off, which may even cause a variety of pathological conditions. All aerobic organisms have developed more or less complex systems to neutralize free radicals before their potentially harmful effect is activated, most of which derive directly or indirectly from O_2. Nutritional elements are also extremely important.

The protective effect against oxidative stress of the dietary intake of low molecular weight antioxidant compounds has been widely recognized[3-8]. A positive correlation between moderate consumption of red wine and beneficial effects on various pathological conditions is widely accepted. Foods that have potential or definite antioxidant capacities are mainly vegetables and fruits, as well as beverages like red

wine, tea and beer. Wine is well appreciated because of its organoleptic qualities and is also a source of antioxidant substances such as flavonoids and resveratrol. A positive correlation between moderate consumption of red wine and beneficial effects on various pathological conditions is widely accepted.

They report several methods for the determination of the total antioxidant capacity (TAC), all of them based on a system to produce free radicals, coupled to the measurement of the sample's capacity to inhibit this production. The antioxidant effect can be measured by chemiluminescence, fluorescence or CO production inhibition. A widely used method is TAC determination by measurement of the length of inhibition time of luminol radicals dependent chemiluminescence, assuming that it is directly proportional to the total antioxidant potential. This potential is quantified by comparison with a reference substance (Trolox) calibration curve.

We have tested wines, beers, fruit and vegetable juices, and teas. They were fresh, stored in the fridge, and frozen. We show the brief survey of changes of their antioxidant capacity in dependence on type and time of the storage.

References

1. Calabrese V., Scapagnini G., Randazzo S. D., Randazzo G., Catalano C., Geraci G. and Morganti P.: *Drug Exp. Clin. Res.* **25**, 281 (1999)
2. Weisburger J. H.: *Mut. Res.-Fund. Mol. Mech. Mut.* **480** (Sp. Iss. SI SEP 1), 23 (2001)
3. Packer L., Hiramatsu M. and Yoshikawa T.: *Antioxidant food supplements in human health.* Academic Press, London 1999
4. Huxley P. R. and Haw N.: *Eur. J. Clin. Nutr.* **57**, 904 (2003)
5. Safari M. R. and Sheikh N.: *Prostag. Leukotr. Ess.* **69**, 73 (2003)
6. Conklin K.: *Nutr. Cancer* **37**, 1 (2000)
7. Wollin S. D., Jones P. J.: *J. Nutr.* **131**, 1401 (2001)
8. Zhuang H., Kim Z. S. and Koehler R. C.: *Ann. N. Y. Acad. Sci.* **993**, 276 (2003)

Acknowledgement

This research was supported by the grant MSM 153100008 from the Ministry of Education, Youth and Sports.

5L03

BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF EIGHT CADMIUM-RESISTANT BACTERIAL ISOLATES FROM A CADMIUM-CONTAMINATED SEWAGE SLUDGE

Katarína Chovanová[a], Vladimír Kmeť[b], Jana Harichová[a] and Peter Ferianc[a,*]

[a]*Department of General Microbiology, Institute of Molecular Biology, Center of Excellence of the Slovak Academy of Science, Dúbravská cesta 21, 84551 Bratislava, Slovakia*
[b]*Institute of Animal Physiology, Slovak Academy of Science, Košice, Slovakia*

* *E-mail: Peter.Ferianc@savba.sk*

Among bacteria participating in polluted environment communities predominate bacteria of those genera which are known to be involved in biodegradation of organic pollutants. Often they belong to the genus *Pseudomonas*, *Comamonas* or *Acinetobacter*. However, in environments contaminated not only with organic pollutants but also with heavy metals, species diversity and metabolic activities of the microorganisms is reduced, and the metal-tolerant bacterial populations are developed[1] with species of *Pseudomonas* and/or acidophylic bacteria predominating[2,3]. As a response to heavy metal challenge, multiple-metal ion-resistant bacteria evolved which contain a variety of plasmid-encoded metal resistance determinants, e. g. *Staphylococcus aureus*[4] and *Alcaligenes eutrophus* (*Ralstonia metallidurans*) strain CH34 which is known as biosorbent of heavy metals[5,6]. The ability of metal bioaccumulation by another Gram-negative bacterial species, such as *Escherichia coli*, *Pseudomonas putida*, *Pseudomonas syringae*, *Pseudomonas aeruginosa*, was established on production of intracellular cadmium-binding proteins. In particular, cadmium, as a toxic heavy metal, constitutes a major problem in industrialized nations, because the presence of this metal in environment mostly endangers public health. The identification of more bacterial strains that could uptake metals with high efficiency and specificity has attracted increasing attention both from a medical and from a biotechnological point of view.

The aim of this work was to identify and characterize in physiological and molecular terms some cadmium-resistant bacterial strains from a culturable microbial community occupying a cadmium contaminated sewage sludge. In addition, the potential exploitation of cadmium-resistant bacterial strains in bioremediation processes aimed at heavy metal removal from contaminated environments is discussed in this study.

In total, sixty-eight bacterial isolates were obtained from sewage sludge, which contained approximately 7.5 μg cadmium per g of dry weight. All isolates growing on mineral medium supplemented with cadmium ions were distinguished on the basis of color, size and morphology. The results revealed that fifty-four of the isolates were identified as Gram-negative bacteria with short rods predominating. All fifty-four Gram-negative isolates were tested for their growth characteristics in liquid mineral medium[7] without $CdCl_2$ treatment (control sample) or supplemented with cadmium, and according to growth rate and length of lag-phase, only eight isolates were chosen for further characterization. These eight isolates were biochemically profiled using either API 20 E,

API 20 NE systems or ENTERO tests, and by key conventional and confirmation tests. Biochemical tests assigned the eight isolates to six bacterial species, *Alcaligenes xylosoxidans*, *Comamonas testosteroni*, *Klebsiella planticola*, *Pseudomonas putida*, *Pseudomonas fluorescens*, and *Serratia liquefaciens*. However, the ARDRA analysis of each of the eight isolates enabled five different ARDRA patterns to be recognized. *P. putida* and *P. fluorescens*, identified by biochemical tests as two different species, ARDRA analysis clustered these two strains to the same cluster indicating only one species. In addition, similarly to ARDRA patterns, whole cell protein pattern analysis of each of the eight isolates enabled rather five as six different protein patterns to be recognized. These results suggesting a relatively high interspecific variability in the culturable cadmium-resistant microbial community. Analysis of plasmid content revealed that only two *K. planticola* strains harboured plasmids suggesting a certain degree of genetic variability between these two isolates. Differentiation among strains of the same ARDRA group was shown by analysis of whole cell protein patterns. Furthermore, cadmium-resistant bacterial isolates were able to remove cadmium from solution and the efficiency of cadmium removal correlated with the amount of additionaly synthesized proteins in the cell fractions.

Perhaps bacterial strains described here could be used in bioremediation processes aimed at heavy metal removal from contaminated environments.

References

1. Knotek-Smith H. M., Deobald L. A., Ederer M. and Crawford D. L: *Biometals* **16**, 251 (2003)
2. Babich H. and Stotzky G: *Environ. Res.* **36**, 111 (1985)
3. Dopson M., Baker-Austin C., Koppineedi P. R. and Bond P. L: *Microbiology* **149**, 1959 (2003)
4. Novick R. P. and Roth C: *J. Bacteriol.* **95**, 1335 (1968)
5. Diels L. and Mergeay M: *Appl. Environ. Microbiol.* **56**, 1485 (1990)
6. Nies D. H: *Plasmid* **27**, 17 (1992)
7. Higham D. P., Sadler P. J. and Scawen M. D: *Science* **225**, 1043 (1984)

5P01

EFFECTS OF ARSENIC ON ELEMENTAL SULFUR OXIDATION IN *ACIDITHIOBACILLUS FERROOXIDANS*

Blanka Pokorná*, Martin Mandl and Pavla Gavlasová

Department of Biochemistry, Faculty of Science, Masaryk University, Kotlářská 2, 61137 Brno, Czech Republic

** E-mail: mandl@chemi.muni.cz*

Arsenic ions are released in the environment during bacterial arsenopyrite oxidation in sulfide wastes or biohydrometallurgy. Elemental sulfur can be formed as an intermediate during arsenopyrite oxidation. Its further biooxidation to sulfuric acid is inhibited by arsenic ions. Therefore this study investigated the inhibitory effects of arsenic on the oxidation of elemental sulfur by *Acidithiobacillus ferrooxidans*. The arsenic effects on bacterial iron oxidation have been previously described[1].

The kinetic analysis of the inhibition of sulfur biooxidation by both As(III) and As(V) in bacterial suspension demonstrated a non-competitive type of inhibition. The inhibition constants (K_i) were 1.3 and 46.8 mM for As(III) and As(V), respectively. Inhibitory effects of arsenic on sulfur oxidation were much higher than on iron oxidation based on the above K_i values and those for iron oxidation[1]. Considering the observation of the defined kinetics of enzyme inhibition, a specific enzyme inhibition can be assumed at a level of one component of the electron transport. This qualitative characteristic was the same for both sulfur and iron oxidation. The Michaelis constant for sulfur changed, probably due to changes in physiological state of bacteria.

We observed an As(V)-reductase activity which could increase the inhibitory effect of As(V). As(V) was reduced to As(III) in the growing culture after a lag phase in the reduction rate. The activity was directly connected with the living cells and could have a negative effect due to production of the more toxic As(III). However, the low rate of As(V) reduction indicated the low concentration of sulfur metabolites or the low rate constant of reduction reaction. To explain the As(V)-reductase activity, different chemical As(V) reductions were investigated using possible sulfur metabolites. We assume that the As(V)-reductase activity is probably due to the effect of thiosulfate or sulfite which can be produced in the sulfur-oxidizing cultures[2]. In addition to these metabolites, there is another possibility of As(V) reduction by a metabolic pigment iodinin[3].

References

1. Mandl M., Hrbáč D. and Dočekalová H.: *Biotechnol. Lett.* **18**, 333 (1996)
2. Česková P., Mandl M., Helánová S. and Kašparovská J.: *Biotechnol. Bioeng.* **78**, 24 (2002)
3. Česková P., Žák Z., Johnson D. B., Janiczek O. and Mandl M.: *Folia Microbiol.* **47**, 78 (2002)

Acknowledgement

Supported by grant No. 525/04/1309 from the Grant Agency of the Czech Republic.

5P02

FERROFLUID MODIFIED *SACCHAROMYCES CEREVISIAE* CELLS

Mirka Šafaříková[a,*], Luciano P. Silva[b], Ana P. S. Lemos[b], Zulmira G. M. Lacava[b], Ricardo B. Azevedo[b], Paulo C. de Morais[c] and Ivo Šafařík[a]

[a]*Laboratory of Biochemistry and Microbiology, Institute of Landscape Ecology, Na Sádkách 7, 370 0 České Budějovice, Czech Republic*
[b]*Instituto de Ciencias Biológicas and* [c]*Instituto de Física, Universidade de Brasília, Brasil*

** E-mail: mirkasaf@uek.cas.cz*

Magnetic adsorbents can be used for the separation of various types of compounds both from solutions and suspensions. *Saccharomyces cerevisiae* cells interact with some types of water based magnetic fluids (MF), leading to the formation of magnetically labeled cells which could be easily manipulated in an external magnetic field[1,2]. This material is a promising adsorbent for various xenobiotics present in water.

Six different procedures have been used to study the processes of magnetic modification of *Saccharomyces cerevisiae* cells (1 - cultured and incubated with MF; 2 - cultured, incubated with MF and heated; 3 – no cultivation and incubated with MF; 4 – no cultivation, incubated with MF and heated; 5 - cultured, heated and incubated with MF; 6 – no cultivation, heated and incubated with MF). After the treatment cells were analyzed with scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Our results indicated, as observed by a smooth surface in the SEM, that only group 1 and group 2 cells (i.e., actively growing cells) are able to uptake MF. The results were confirmed by TEM analysis that showed particles between the cellular wall and plasma membrane, i.e. in the periplasmic space, and even some particles within the cytoplasm of those cells, despite the presence of some aggregated particles on their surface. A higher number of particles were observed inside group 2 cells when compared to group 1. In all the other groups, SEM and TEM analysis showed that most MF were attached to the surface of the cells, and just a few inside them.

Cell modified with perchloric acid stabilized MF (according to the procedure No. 2) were used for the adsorption of various triphenylmethane and heteropolyaromatic dyes. Maximum adsorption capacities were relatively high (20 – 430 mg dye/g adsorbent) although they significantly differed according to the dyes structures.

References

1. Šafařík I., Ptáčková, L. and Šafaříková M.: *Eur. Cells Mater.* **3** (Suppl. 2), 52 (2002)
2. Azevedo R. B., Silva L. P., Lemos A. P. C., Báo S. N., Lacava Z. G. M., Šafařík I., Šafaříková M. and Morais P. C.: *IEEE Trans. Magn.* **39**, 2660 (2003)

5P03

BACTERIAL PYRITE OXIDATION UNDER DIFFERENT METABOLIC STATES OF BACTERIA

Šárka Helánová[a,*], Martin Mandl[a], Oldřich Janiczek[a], Kevin B. Hallberg[b], Pavel Bouchal[a] and Alena Španová[c]

[a]*Department of Biochemistry and Department of Microbiology, Faculty of Science, Masaryk University, Kotlářská 2, 61137 Brno, Czech Republic*
[b]*School of Biological Sciences, University of Wales Bangor, UK*

* E-mail: shelanova@mou.cz

Pyrite biooxidation is an important process in sulfide wastes and biohydrometallurgy resulting in environmental acidification. Induction of iron-oxidizing system in bacteria grown on sulfur seems to be an essential factor for pyrite oxidation. Electrochemical, proteomic and kinetic differences during bacterial pyrite oxidation by cultures of *Acidithiobacillus ferrooxidans* grown on sulfur or iron were investigated. Based on electrochemical parameters of pyrite electrode, which was described for arsenopyrite oxidation studies[1], iron-oxidizing bacteria were able to oxidize pyrite since the beginning. Sulfur-oxidizing *A. ferrooxidans* provided rather reductive conditions at the beginning and was not able to oxidize pyrite immediately. Kinetic observations with pyrite concentrate demonstrated a lag phase in the ability of pyrite oxidation. Induction of iron-oxidizing system during the lag phase is assumed.

Systematic evaluation of *A. ferrooxidans* two-dimensional proteome maps was performed in order to investigate the differences in protein composition of bacteria oxidizing iron or sulfur. Number of up- or down-regulated proteins was detected. Successful identification of some protein spots affected by the growth changes was performed by peptide mass fingerprinting via MALDI-TOF MS.

Sequencing of amplified 16S RNA gene, 1.4 kb pairs, confirmed the homogeneity of each investigated population. These data indicate that the substrate-dependent alterations of proteomic properties are the result of differences in oxidation metabolism of both populations.

References

1. Zeman J., Mandl M. and Mrnuštíková P.: *Biotechnol. Tech.* **9**, 111 (1995).

Acknowledgement

Supported by grant 525/04/1309 from the Grant Agency of the Czech Republic.

5P04

BLACK OR GREEN TEA?

Martina Bancířová[a,*] and Jarmila Medková[b]

[a]*Department of Physical Chemistry and* [b]*Department of Botany, Faculty of Science, Palacký University, tř. Svobody 26, 771 46 Olomouc, Czech Republic*

** E-mail: bancir@aix.upol.cz*

The lifetime of free radicals is extremely short, and if a physiological acceptor does not immediately neutralize them, they can damage biological systems. All aerobic organisms have developed more or less complex systems to neutralize free radicals before their potentially harmful effect is activated. Nutritional elements are also extremely important. Foods that have potential or definite antioxidant capacities are mainly vegetables and fruits, as well as beverages like red wine, tea and beer.

Tea is a source of epigallocatechin gallate in green tea, and theaflavin and the associated thearubigins, in black tea. Cancer in the colon, breast, prostate and pancreas may be caused by a new class of carcinogens - the heterocyclic amines, formed during the broiling or frying of creatinine-containing foods, including fish and meats. Their formation and action can be inhibited by antioxidants such as those in soy and tea. Black tea is a powerful chemopreventor of reactive oxygen species and was found to be more efficient than green tea[1].

A sensitive and simple chemiluminescent (CL) method for measuring antioxidant activity was developed. The determination of TEAC (Trolox equivalent antioxidant capacity) is based on the inhibition of CL intensity of luminol by an antioxidant. The system is based on the oxidation reaction of luminol catalysed by the peroxidase. Luminol occurs in an alkaline environment by means of oxidizing agents like hydrogen peroxide and in the presence of peroxidase as catalyst. A generally accepted scheme envisages the oxidation of luminol by formation of a complex between oxidant and peroxidase to produce a luminol radical. These radicals then react in further reactions giving rise to an endoperoxide which is subsequently decomposed into the dianion 3-aminophthalate, in an electronically excited state which, as previously reported, emits light returning to its basic state. Consequently, in the luminol-peroxidase system, light is emitted after oxidation of the luminol catalysed by the enzyme using hydrogen peroxidase in a highly specific fashion as a proton acceptor. To develop an inhibition assay, emission needs to be constant for the time required to measure such effect, so as to attribute the changes in light emission solely to the sample. If the production of radical intermediates is constant, emission will be inhibited for a period directly correlated to the amount of antioxidants present. The time obtained is fitted to the relative calibration curve and will yield the antioxidant capacity of the sample. The value obtained (in seconds) was a function of the antioxidant capacity of the sample examined. This antioxidant capacity was expressed, fitting the times obtained on the relative calibration curve, in concentration of Trolox (mM).

Antimicrobial activity was tested as a minimum inhibitory concentration (MIC) by broth microdilution method on Gram-positive (*Enterococcus faecalis*, *Staphylococcus*

aureus) and Gram-negative (*Pseudomonas aeruginosa*, *Escherichia coli*) bacterial strains. The minimum bactericidal concentration (MBC) is determined by plating out standard aliquots of the supernatant from each well that shows no visible bacterial growth. The MBC is then defined as the lowest concentration of each antibiotic that kills 99.9% of the bacteria in the original inoculum. In many cases, the MIC and MBC of antibiotics are equivalent - that is, as wells that shows no growth do so because all the bacteria in the well have been killed.

The microdilution method is the most commonly used method to test the sensitivity of bacteria. Microtiter wells containing serial twofold dilutions of samples are incolulated with a standard inoculum of the bacterium in question, the plates are incubated overnight, and the wells are then examined for the presence of bacterial growth. The lowest concentration of each sample dilution series that prevents bacterial growth is considered to be the minimum inhibitory concentration of the sample. We compared MIC of the different types of black and green tea (34 samples; they were obtained from the common tea-rooms). Preparation of tea solution: leaves were dissolved in hot water (under 100 °C) for 3 min (2 g/100 ml). The extracted solutions were filtered and allowed to cool at room temperature. The tea solutions were made fresh.

The results are preliminary only. The relationship between antioxidant capacity and antimicrobial activity was confirmed but cannot be generalize yet. We cannot verify generally accepted theory that the green tea is more powerful.

References

1. Sarkar A. and Bhaduri A.: *Biochem. Biophys. Res. Com*. **284**, 173 (2001)

Acknowledgement

This research was supported by the grant MSM 153100008 from the Ministry of Education, Youth and Sports.

5P05

DETERMINATION OF DEOXYNIVALENOL (DON), ZEARALENONE (ZEA) AND T-2 TOXIN CONTENT IN MODEL SET OF WHEAT VARIETIES – COMPARISON OF TWO ANALYTICAL METHODS (HPLC AND ELISA)

Eva Matějová*, Světlana Sýkorová and Marie Berová

Department of Breeding Methods, Institute of Plant Crop Production, Drnovská 507, 16106 Praha 6, Czech Republic

** E-mail: matejova@vurv.cz*

Trichothecenes are produced by several genera of fungi; especially by the genus *Fusarium*, which is known to attack various cereals. They are found to inhibit protein synthesis, DNA and RNA synthesis, and have immunosuppressive and haemorrhaging effects. Acute and sub acute toxicity is characterized by feed refusal and weight loss, and increased susceptibility to infectious diseases[1].

A model set of 9 wheat samples was sown in three replicates into plots 3 m^2. The first variant of the trial (I - Infection) was inoculated with spore suspension of *F. culmorum* (isolate B). The fungicide Horizon 250 EW was applied (by the methodology) to the second variant of the trial (IF – Infection + fungicide) before the inoculation. The rest of variants were controls (K – spraying with water) and (KF- K + fungicide).

DON, ZEA and T-2 toxin content was determined by using two methods. The first one was enzyme quantitative immunoassay technique (ELISA) and commercial kits Ridascreen FAST DON, Ridascreen FAST ZEA and Ridascreen FAST T-2 Toxin manufactured by R-Biopharm, Germany. As the second method was used analytical method employing extraction acetonitrile:water (84:16, v:v) and clean-up procedure using column MycoSep 225. The determination of DON and ZEA was performed by liquid chromatography with UV or FL detection. The results of both methods correlated for DON content significantly ($r = 0.935$).

The concentrations of DON and ZEA of the variant I were several time higher than the concentrations of the variant IF. The content of tested mycotoxins in the control samples were found below LOD.

Last year our laboratory participated in laboratory proficiency testing FAPAS for determination of DON in wheat flour. Our result carried out by ELISA was satisfactory. The standard deviation in z-score was 0.5. In laboratories which participated in this testing outweighed quantification of DON by HPLC, then GC, ELISA and the least used method was TLC/HPLTC. The highest number of results lying in z-score outside $|z| \pm 2$ (unsatisfactory) had TLC/HPTLC and the lowest number had ELISA.

References

1. Langseth W. and Rundberget T.: *J. Chromatogr. A* **815**, 103 (1998)

5P06

IDENTIFICATION OF BARLEY VARIETIES USING COMBINATIONS OF VARIOUS ELECTROPHORETIC METHODS FOR THE ANALYSIS OF THE STORAGE AND ENZYME PROTEINS

Jana Bradová*, Světlana Sýkorová and Helena Šrámová

Department of Breeding Methods, Institute of Plant Crop Production, Drnovská 507, 16106 Praha 6, Czech Republic

** E-mail: bradova@vurv.cz*

Organisms are characterized by variants differing in the contents of storage proteins and enzymes. The phenomenon of protein polymorphism enables the utilization of proteins as genetic markers. Protein genetic markers of barley, i.e. hordein proteins of barley grain as well as certain enzymes of germinating seed, can be used not only for marking of some commercial characteristics but especially for the identification of the barley varieties.

We used electrophoretic methods for the analysis of hordein coming from standard seed samples of spring and winter barley varieties registered in the Czech Republic in 2003 and received from the Division of Plant Variety Testing of the Central Institute for Supervising and Testing in Agriculture (CISTA).

Four procedures for the electrophoretic separation were used and compared, namely a) starch gel electrophoresis (SGE) according to the Czech State Standard (ČSN 46 1085-1)[1]; b) polyacrylamide gel electrophoresis recommended by the International Seed Testing Association (PAGE ISTA) and cited in ČSN 46 1085-2[2]; c) SDS-PAGE for hordeins recommended by the Union for the Protection of New Varieties of Plants (UPOV)[3]; and d) PAGE for the determination of alleles of esterase loci Est 1, Est 2, Est 4 and Est 5 using Tris-glycine buffer, pH 8,3[4].

The signal hordein genes are used for the evaluation of the genetic structure of barley varieties. The varieties can be evaluated as pure lines – homogeneous (one-line) in the hordein composition, or as hordein polymorphic populations (multi-line).

The number of hordein lines and their relative frequency in a variety population, as determined by the electrophoretic analysis of hordeins, are important for the identification of the variety. This results from the fact that some varieties do not differ from others by the pattern of the hordein lines but by the proportion of the hordein lines in the respective variety.

Grains of 44 spring varieties and of 26 winter varieties of common barley were used for the electrophoretic analysis of hordeins while their seven-day-old leaves were used for the analysis of esterase. The patterns obtained were compared to detect the intra- and intervarietal polymorphism. Out of the total number of 44 varieties of spring habit, 24 % varieties were found to be polymorphic by hordein composition and 9 % by the esterase composition. All varieties of the winter barley assortment were homogeneous in view of both hordein and esterase; they can be characterized, consequently, as pure lines.

As the hordein polymorphism is lower than that of wheat gliadins, it is more difficult to determine the genuineness of the barley varieties of which many have

identical hordein patterns. Therefore, the varieties identical in hordein, as determined by SGE, were further evaluated by PAGE ISTA, SDS -PAGE, and by means of the esterase markers. To evaluate the possibility of differentiation between identical varieties on the basis of the methods given above, the varieties have been included in the identity classes of the hordein and esterase patterns[5].

The differentiation between the hordein-identical varieties Camera, Monaco, and Tiffany can be used as an example. The variety of the winter malting barley Tiffany has the hordein electrophoretic pattern (HRD A3 - HRD B3 - HRD F2) identical with that of the fodder-type varieties Camera and Monaco. However, Tiffany differs from Camera and Monaco in the alleles of the hordein loci as found by SDS PAGE (Tifanny: HRD D1 - HRD C10 - HRD B6; Camera, Monaco: HRD D1 - HRD C8 - HRD B6). Furthermore, it is possible to distinguish the varieties Camera and Monaco by means of the esterase loci (Camera: Est1 Ca – Est2 Fr – Est4 At – Est5 Pi; Monaco: Est1 Ca – Est2 Dr – Est4 Su – Est5 Pi).

References

1. ČSN 46 1085-1: *Common wheat and barley – Determination the variety trueness and purity - Part 1: Electrophoresis of proteins in starch gel (SGE).* ČNI Praha, 1998
2. ČSN 46 1085-2: *Common wheat and barley – Determination the variety trueness and purity - Part 2: Electrophoresis of proteins in polyacrylamide gel (PAGE).* ČNI Praha 1998
3. UPOV: *Guidelines for the conduct of tests for distinctness, uniformity and stability. Barley. (Aditional usefull explanation).* TG/19/10 94-11-04
4. Sýkorová S. and Šašek A.: *Genetika a šlechtění* **32**, 123 (1996)
5. Bradová J., Sýkorová S., Šašek A. and Černý J.: *Rostlinná výroba* **47**, 167 (2001)

Acknowledgement

This project was supported by the Ministry of Agriculture of Czech Republic (QF3050).

5P07

IDENTIFICATION OF REGISTERED POTATO CULTIVARS (*SOLANUM TUBEROSUM* L.) BY ELECTROPHORESIS OF TUBER PROTEINS

Světlana Sýkorová*, Jana Bradová and Marie Berová

Department of Breeding Methods, Institute of Plant Crop Production, Drnovská 507, 16106 Praha 6, Czech Republic

* *E-mail:* sykorova@vurv.cz

Plant cultivars belong among the most important factors in the process of the agricultural development. Potatoes represent a very fundamental and irreplaceable crop and food for which the importance of cultivar as a quality carrier is implicit. Potatoes enter the food chain as soon as they are put into circulation through the sale to consumers; they are subject to the Food Law of the Czech Republic (No. 110/1997). In connection with these facts, the cultivar verification and identification as well as the possibility of the cultivar genuineness control have become an obvious necessity. The methods of the cultivar identification based on the morphological characteristics are not too reliable on account of their dependence on the environmental conditions. On the other hand, the stable characteristics of tubers (e.g. soluble tuber proteins and enzymes) are considered as the most appropriate for the cultivar identification.

Potato tubers constitute a relatively homogeneous tissue out of which the soluble tuber proteins can be easily extracted. The soluble proteins obtained from freshly harvested mature tubers consist predominantly of albumins (60 %) and globulins (20 %). A glycoprotein of M.w. 45 kD – patatin - constitutes up to 20% of the soluble proteins; the main protein of the albumin fraction, tuberin, is a dimer of M.w. 66 kD possessing esterase activity[1]. Electrophoresis on starch (SGE) or polyacrylamide gel (PAGE) in alkaline or acid buffer is regarded as an appropriate and effective method for the separation of the tuber proteins. The advantage of this approach resides in the fact that the electrophoretic patterns of the tuber proteins do not depend on the environmental conditions (year, locality, fertilization, term of storage in the cold stock)[1]. Stegemann and Loeschcke[2] worked up a catalogue of the European potato cultivars based on the variability of the protein and esterase electrophoretic patterns. Also UPOV (International Union for the Protection of New Varieties of Plants) recommended the utilization of the tuber protein and enzyme polymorphism for the control of distinctness, uniformity, and stability (DUS-tests)[3,4].

In the Research Institute of Crop Production in Prague, two methods of PAGE - UPOV were used for the characterization and identification of the registered potato cultivars. Extracts of sap were first prepared from tubers (freshly harvested proved to be optimal) which were deep-frozen at -20 °C and then allowed to thaw at room temperature; the sap could be subsequently easily squeezed out into centrifuge tubes. The first of the recommended methods for the tuber protein and esterase electrophoresis is based on the system of only one polyacrylamide gel and Tris-borate buffer (pH 7.9)[3], while the second one, used mainly for patatin electrophoresis, utilizes the system of stacking and resolving gels and Tris-glycine buffer (pH 8.9)[4]. The

electrophoretic patterns obtained were compared with cultivar etalons, the values of REM (relative electrophoretic mobility) of the individual bands were counted and the matrix of the identity index was constructed.

Potato cultivars were found by both methods used to be distinguishable and identifiable. Our results prove that a reliable identification of potato cultivars is feasible by means of tuber protein electrophoresis. The electrophoretic patterns of the tuber proteins and enzymes are considerably specific for individual cultivars and genetically conditioned, independent on the year and locality; they remain unchanged in dormant tubers for several months of storage in the cold. The methods of the cultivar identification and verification given above can be successfully utilized both in the plant breeding and for the control of the cultivar genuineness, e.g. on the potato market.

References

1. Desborough S. L.: *Potato (Solanum Tuberosum L.). Isozymes in Plant genetics and breeding, Part B* (Tanksley S. D. and Orton T. J., eds.), Elsevier, Amsterdam 1983, p. 167
2. Stegemann H. and Loeschcke V.: *Potato Res.* **20**, 101 (1977)
3. *Bundessortenamt – Anlage zur Richtlinie zur Prüfung der Unterscheidbarkeit, Homogenität und Beständigkeit von Kortoffel* (April 1996)
4. UPOV TWA 31/6 *Draft test guedelines for potato,* document TG/23/6 (Proj.1) *(Rio de Janeiro 23.-27.9.2002)*

Acknowledgement

This project was supported by the grants QF3050 and 1B44011 from the Ministry of Agriculture, Czech Republic.

5P08

THE IMPACT OF BRASSINOSTEROIDS ON THE QUALITY OF FOOD WHEAT

Dana Hradecká[a,*], Alena Hejtmánková[b], Ludmila Staszková[b] and Milan, Kroutil[b]

[a]*Department of Crop Production and* [b]*Department of Chemistry, Czech University of Agriculture, 16521 Praha 6, Kamýcká 957, Czech Republic*

** E-mail: hradecka@af.czu.cz*

Wheat grain quality is determined by genotype, weather conditions and farming methods. Stress conditions can force a plant to induce biochemical adaptive responses which could also be controlled by exogenous application of phytohormons e.g.

brassinosteroids. Brassinosteroids are defined as polyhydroxy steroids based on 5α-cholestane skeleton. They are active during organogenesis, stimulate germinability and root formation, retard ageing, etc[1-4]. They can also support plant resistance to certain pathogens. Long-term stress conditions, especially in post-floral period, destroy the baking quality of grains. Temperature and the duration of maturation period influence certain enzyme activities as well as the content of Ca^{2+}, Mg^{2+}, and K^{+}. The amount of mineral ions subsequently influences baking parameters such as wet gluten content, gluten extensibility, activity of α-amylase and proteolytic enzymes etc. The impact of brassinosteroids on these parameters has been studied for three years (2001-2003) at CAU experimental fields in Prague.

During heading (49DC), the wheat experimental plots (10 m^2, variety Sandra) were treated by 1 litre of 1.10^{-6} M solution of the following tested compounds: 4154BR, 48911A-Olefin, 4861 salt LEV, A-BOC-Gly, BOC-Gly-CHOL-4872 and 4831CYKlO. The effect was compared with the activity of naturally occurring steroid phytohormone, 24-epibrassinolide (24-epi), used as a standard.

Ca^{2+}, K^{+}, Mg^{2+}, Cu^{2+} and Zn^{2+} contents were determined by Varian SpectrAA 400. Wet gluten content, extensibility and falling number were specified by standard methods (No 461011-9, No ISO 712 and No ISO 3093 respectively).

The amount of the Ca^{2+} and Zn^{2+} in grains increased after they were treated by majority of the tested brassinosteroids. The differences in weather conditions between the particular years also influenced the grain quality parameters. The accumulation of Mg^{2+} and Zn^{2+} ions correlated positively with the temperature. Negative correlation between the rainfall and Ca^{2+}, K^{+}, and Cu^{2+} contents was also found; high temperatures decreased the amount of Cu^{2+} ions. Falling number, wet gluten content and gluten extensibility are determined by weather conditions during post-floral period and by the duration of this period. Brassinosteroids could improve these quality parameters and could be suggested for good farming practices to ensure the grain quality.

References

1. Kefeli I. I., Prusakovova L. D. and Čížová N. A.: *Prirodnyje i sintetičeskije regujlatory ontogeneza rastěnij*, Moskva 1990, p. 165
2. Chripac V.A., Zhabinski V. N and Malevannaja N. N.: *Plant Growth Regulat. Soc. Amer.* **24**, 101 (1997)
3. Procházka S., Macháčková I., Krekule J. and Šebánek J.: *Fyziologie rostlin,* Academia, Praha 1995, p. 484
4. Sakurai A., Yokota T. and Clouse S. D.: *Brassinosteroids,* p. 253, Springer, Tokyo (1999)

Acknowledgement

Supported by the grant MSM 412100002 from the Ministry of Education, Youth and Sports, Czech Republic.

5P09

DIPSTICKS IN SCREENING FOR *LISTERIA* CONTAMINATION IN FOOD SAMPLES

Martina Blažková[a,]*, Ludmila Karamonová[a], Pavel Rauch[a] and Gary Wyatt[b]

[a]*Department of Biochemistry and Microbiology, Institute of Chemical Technology, Technická 5, 16628 Praha, Czech Republic*
[b]*Institute of Food Research, Norwich, UK*

** E-mail: martina.blazkova@vscht.cz*

Listeria are gram-positive, non-spore-forming, rod-shaped bacteria. Of the six known species of the *Listeria* genus, *Listeria monocytogenes* deserves particular mention as a human and animal pathogen, while *L. ivanovii* is pathogenic only in animals and *L. innocua*, *L. seeligeri*, *L. grayi*, and *L. welshimeri* are mostly considered harmless environmental bacteria. Severe disease listeriosis caused by pathogenic bacteria from genus *Listeria* is one of the most deadly bacterial infections currently known. These bacteria are well equipped to survive food processing technologies. For example, they tolerate high concentrations of salt and relatively low pHs, and worst of all, they are able to multiply at refrigeration temperatures. This makes *Listeria* microorganisms a serious threat to food safety and ranks them among the microorganisms that most concern the food industry. The foods most frequently implicated are raw milk, soft cheeses (particularly those made from unpasteurized milk), ice cream, raw vegetables, fermented raw-meat sausages, raw and cooked poultry and raw and smoked fish.

The main goal of our project was to develop rapid and specific method for detection of *Listeria* contamination in food. Two polyclonal antisera had previously been developed. With these antisera ELISAs in microtitrate plates had been set up, one specific for *Listeria* spp., and the other specific for *L. monocytogenes*. In the present study optimized method was transformed into dipstick format of ELISA. We tested different kinds of artificially contaminated food samples (milk, ice-cream, salad-dressing…). Achieved results were compared with the developed ELISA in microtitrate plates.

Acknowledgement

This work was supported by the Grant Agency of the Czech Republic (525/03/0350).

5P10

CLONING AND EXPRESSION OF crt GENES FROM *ERWINIA CAROTOVORA*

Jana Pokorná*, Jana Hrdličková, Michaela Drábková, Jitka Kubešová and Ivana Márová

Institute of Food Chemistry and Biotechnology, Faculty of Chemistry, Brno Institute of Technology, Purkyňova 118, 612 00 Brno, Czech Republic

** E-mail: janca.p@post.cz*

The bacteria *Erwinia carotovora* is gram-negative epiphytic non-photosynthetic bacterium with aerobic metabolism, which produces carotenoids (phytoene, lycopene, β-carotene, β-cryptoxanthin, zeaxanthin, zeaxanthin glucosides) and pectinolytic, cellulolytic and proteolytic enzymes. Carotenoids are industrially significant pigments with antioxidant activity, which act as protective agents against reactive oxygen species and UV irradiation. Carotenoid production in *E. carotovora* is encoded by crt gene cluster containing 6 genes localized on dsDNA.

In this work possibility of cloning and expression of crt gene cluster isolated from *Erwinia carotovora* in recipient strain *E. coli* DH5α, *Erwinia carotovora* and *Saccharomyces cerevisiae* was tested. Isolation of crt genes was proved using XbaI, EcoRI and HindIII restriction endonucleases. Plasmid vector pHSG298 and shuttle vector pAUR135 with insert on size about 1200 bp was used for transformation of chemically competent cells. Transformants were selected based on genotype as well as fenotype changes: a) resistance of transformants to kanamycin encoded by pHSG298 or to aureobasidin A encoded by pAUR135, b) formation of orange-coloured *E. coli* colonies, c) isolation and analysis of size of recombinant plasmid, d) HPLC analysis of carotenoids produced by recombinant cells. In individual transformants lutein, lycopene, β-carotene and phytoene were demonstrated. Amount of carotenoids isolated from *E. coli* cells transformed by pHSG298/crt was higher than those from *E. carotovora* cells.

5P11

COMPARISON OF DIFFERENT METHODS FOR ISOLATION AND PURIFICATION OF GENOMIC DNA FROM THERMOPHILIC BACTERIA *BACILLUS ACIDOCALDARIUS* AND *THERMUS* SP.

Lucie Piterková*, Karel Melzoch and Martina Skarkeová

Department of Fermentation Chemistry and Bioengineering, Institute of Chemical Technology, Technická 5, 16628 Praha, Czech Republic

** E-mail: Lucie.Piterkova@vscht.cz*

DNA of the thermophilic microorganisms[1], which are very interesting from the general biological point of view because they are able to live and reproduce themselves in the range of the temperature which is lethal for other microorganisms, was isolated every time from the same amount of the liquid culture of *Bacillus acidocaldarius* (CCM 2855) and *Thermus* sp. (CCM 4199)[2] cultivated at 65°C for 24 h on the shaking machines in the LB medium.

There were used 6 different methods for isolation and purification of genomic DNA[3]. In three cases it was used enzymatic extraction of DNA by using different concentration of enzymes, time and temperature of incubation followed by different procedures of precipitation and purification of DNA. In two cases it was dealt with chemical extraction by a combination of phenol and chloroform, which were used in different amounts, number of steps but also in the mixture and finally it was used the commerce kit HIGHT PURE PCR Templetate preparation Kit (Roche, USA) for isolation of nucleic acids from bacteria or yeast.

Obtained DNA was visualized by horizontal electrophoresis on 1% agarose gel containing ethidium bromide (1x TBE buffer, 75 V, constant current, 150 min) and quantified by spectrophotometric measurements using wavelengths 260 and 280 nm. Gained values enabled us to count the yield of DNA and the purity quotient of isolated DNA.

The best results were obtained by using commerce kit ROCHE when the concentration of 600 μg/ml of DNA with relative small impurities of proteins was achieved. Other methods gave also sufficient amount of yield but the big difference was noted in the purity value of isolated DNA (by other methods) which fluctuated in the ratio A_{260}/A_{280} between 0.4 – 2.8 in comparison with 3.9 by using kit.

References

1. Kristjansson J. K.: *Thermophilic bacteria*, CRC Press, Boca Raton 1992
2. Sharp R. and Williams R: *Thermus Species*, Plenum Press, New York 1995
3. Kršek M. and Wellington E. M. H.: *J. Microbiol. Meth*. **39**, 1 (1999)

5P12

SIMULTANEOUS DETERMINATION OF AMINES AND POLYAMINES BY A DUAL-WORKING ELECTRODE

Ludmila Zajoncová*, Marek Petřivalský and Pavel Peč

Department of Biochemistry, Faculty of Science, Palacký University, Šlechtitelů 11, 783 71 Olomouc, Czech Republic

** E-mail: zajoncov@prfholnt.upol.cz*

Polyamines are widely present in bacteria, plants and animal tissues and play an important role in growth processes. Biogenic amines are formed upon degradation of proteins and amino acids by decarboxylase-positive microorganisms and thereby present a good index of food decomposition. The determination of amines and polyamines in the flow system was enabled by the application of a dual peroxide electrode equipped with enzyme membranes. The membranes were obtained by immobilisation of pea seedling amine oxidase or oat polyamine oxidase on a cellophane, co-crosslinked by gelatin-glutaraldehyde together with bovine serum albumine[1]. We have assembled a biosensor using amine oxidase (AO) to measure the total amine content and polyamine oxidase (PAO) for specific determination of spermidine and spermine. The direct electrochemical oxidation of the produced hydrogen peroxide occurs at high positive potentials, which exposes the biosensor to interference from other easily oxidisable substances like ascorbate, urate or paracetamol. A cellulose acetate membrane covering the Pt-Yr electrode was used to eliminate almost completely interfering effects[2].

The new biosensor has the limit of detection 10 $nmol.l^{-1}$ putrescine and 20 $nmol.l^{-1}$ spermidine (determined with respect to a signal-to-noise ratio 3:1). A linear range of current response were 0.05 – 10 $mmol.l^{-1}$ putrescine and 0.1 – 5 $mmol.l^{-1}$ spermidine, with a response time of 10 s (for putrescine) and 20 s (for spermidine). The determination of biogenic amines in food samples by the use of biosensor was compared to established HPLC method using benzoyl derivatives of polyamines. The biosensor was stable at least three months and retained 80 % of its original activity. The lifetime of dry enzyme membranes stored at 4°C in a refrigerator was approximately one year.

References

1. Zajoncová L., Jílek M., Beranová V. and Peč P.: *Biosens. Bioelectron*., in press (2004)
2. Carsol M. A. and Mascini M.: *Talanta* **50**, 141 (1999)

ACTA UNIVERSITATIS PALACKIANAE OLOMUCENSIS
FACULTAS RERUM NATURALIUM 2004 CHEMICA 43S

PROCEEDINGS OF THE XIX. MEETING OF CZECH AND SLOVAK SOCIETIES FOR BIOCHEMISTRY AND MOLECULAR BIOLOGY

Olomouc, August 31 – September 3, 2004

SESSION 6: CLINICAL BIOCHEMISTRY, PATHOBIOCHEMISTRY AND IMMUNOCHEMISTRY

6L01

OLIGO-CHIP BASED ASSAY FOR DETECTION OF TICK-BORNE BACTERIA

Dušan Blaškovič* and Imrich Barák

Institute of Molecular Biology, Slovak Academy of Sciences, Dúbravská cesta 21, 84551 Bratislava, Slovakia

** E-mail: dusan.blaskovic@savba.sk*

Number of pathogenic tick-borne bacteria cause diseases in humans and animals. Wide range of conventional methods as PCR, immunodetection, cultivation, reverse line blot or microscopy is available for identification of bacterial pathogens. Although these methods are used for many years, they do not allow simultaneous detection of wide spectrums of bacteria. DNA chips represent robust technology for fast, sensitive, relatively cheap and simultaneous detection of microorganisms in biological samples.

We have developed oligonucleotide-chip for detection of selected tick-borne bacteria as *Borreliae*, *Rickettsiae*, *Anaplasmae* and *Ehrlichiae*. The chip contains oligonucleotide probes targeting variable fragments of 16S rRNA. Oligonucleotide probes were designed by use of PRIMROSE software[1] or manually, their specificity was checked by BLAST software[2], and cross-reactive probes were excluded. Oligo-chip was manufactured by immobilization of specific probes on a chemically modified surface of glass slide. Fluorescently labeled fragments of 16S rRNA were amplified by PCR, using kingdom specific universal primers designed for convenient amplification of target 16S rDNA from any bacteria. Using universal primers, we have been able to obtain PCR product from 1 pg of bacterial DNA and genomic DNA isolated from tick infected by selected bacteria. Hybridization signals obtained after hybridization of oligo-chip with PCR amplified, fluorescently labeled 16S rDNA fragment allowed us to discriminate between tick-borne bacteria specified above. Use of universal primers for target DNA amplification in combination with probes designed from variable regions of 16S rRNA genes and possibility to include other probes to our oligo-chip is making our assay universally applicable for identification of any bacteria with known sequence of 16S rRNA gene. Apply of our method together with existing methods can increase accuracy of methods currently used in clinical laboratories by overcoming some of their limitations.

References

1. Ashelford K. E., Weightman A. J. and Fry J. C.: *Nucleic Acids Res.* **30**, 3481 (2002)
2. Altschul S. F., Madden T. L., Schaffer A. A., Zhang J., Zhang Z., Miller W. and Lipman D. J: *Nucleic Acids Res.* **25**, 3389 (1997)

6L02

THE APPLICATION OF HUMAN MICROSATELLITE GENOTYPING IN THE ANALYSIS OF DEGRADED DNA: MINIPLEX KITS

Jiří Drábek[a,*], Denise Chung[b], Kerry Opel[b] and Bruce McCord[b]

[a]*Department of Biochemistry, Faculty of Science, Palacký University, Šlechtitelů 11, 78371 Olomouc, Czech Republic*
[b]*Department of Chemistry and Biochemistry, Ohio University, Athens, USA*
** E-mail: drabek@prfholnt.upol.cz*

Microsatellite typing is an indespensible tool for human identification in natural and human catastrophes. However, highly fragmented DNA in degraded forensic samples presents a challenge for current commercial STR typing kits. The longest amplicons in the PowerPlex 16 from Promega and in the AmpFLSTR Identifiler from Applied Biosystems exceed 350 bp while the average lengths of a degraded DNA length may be well under these sizes. A series of redesigned primer kits with shorter amplicons have been recently developed to address this issue[1]. These primer sets, known as Miniplexes, contain 3 or 6 multiplexed STR loci with the primer binding sites located as close as possible to the polymorphic region. Five different miniplexes cover the entire set of 13 Combined DNA Index System STRs. The initial results demonstrated that all Miniplex primer sets performed satisfactorily on a fragmented DNA template with the exception of Penta E locus. In this study we discuss results obtained in experiments performed to partially satisfy developmental validation requirements of Scientific Working Group on DNA Analysis Methods for Miniplex 2 (D5S818, D8S1179, and D16S539), Miniplex 4 (vWA, D18S51, and D13S317), and BigMini (TH01, TPOX, CSF1PO, D7S820, FGA, and D21S11). Namely, we examined the effect of template size on the efficiency of amplification, concordance with established commercial systems, and detection sensitivity in ABI310 capillary electrophoresis.

Degraded DNA was prepared by digesting a series of samples with DNase I as well as obtained from collaborative arrangements with the Anthropology Departments of Ohio University and the University of Tennessee Forensic Anthropology Center. A concordance study was undertaken to examine 500+ samples in order to address potential primer binding site mutations. Discrepant samples were reamplified by Miniplexes, PowerPlex 16 and Identifiler to check for clerical errors and then sequenced. The sensitivity was assessed as the minimum DNA template amount correctly typable using peak height ratios of 60% as an objective criterion.

The loss of the peak intensity as the amplicon sizes became larger was clearly evident for the PowerPlex 16 system; on the other hand, the Miniplex primer sets were capable of producing complete profiles for all tested samples even at template fragment lengths below 222 base pairs. For DNA extracts from environmentally exposed bone samples, the Miniplex sets yielded higher allele peaks than a commercial kits and in one case provided a full STR profile where only partial sets had been obtained previously[2].

Full concordance was observed in 99.77% (6369 out of 6384) STR allele calls compared[3]. In discrepant samples, four types of deletions (TGTC, TATC, AGAG, and CCATCCAT) and one type of mutation (TG to CA) were discovered.

Miniplexes successfully amplified DNA at concentrations well below recommended values for commercial systems. Typical results showed sensitivities below 100 pg of template. However, we found that amplification efficiency was affected when more than 3 loci were included in the multiplex reaction.

References

1. Butler J. M., Shen Y. and McCord B. R.: *J. Forensic Sci.* **48**, 1054 (2003)
2. Chung D., Drábek J., Opel K. L., Butler J. M. and McCord B. R.: *J. Forensic Sci*., in press (2004)
3. Drábek J., Chung D., Butler J. M. and McCord B. R.: *J. Forensic Sci.* **49**, 1 (2004)

6L03

THE EFFECT OF CARVEDILOL ON THE OXIDATIVE BURST OF RAT LEUKOCYTES ACTIVATED WITH DIFFERENT TYPES OF STIMULI

Antonín Lojek[a,*], Radomír Nosál'[b], Katarína Drábiková[b], Jana Pečivová[b] and Milan Číž[a]

[a]*Laboratory of Pathophysiology of Free Radicals, Institute of Biophysics, Academy of Sciences of the Czech Republic, Královopolská 135, 61265 Brno*
[b]*Institute of Experimental Pharmacology, Slovak Academy of Sciences*

** E-mail: alojek@ibp.cz*

Carvedilol is a unique cardiovascular drug with multiple therapeutic potential. While membrane adrenoreceptor blockage as well as the effects of carvedilol on endothelial and smooth muscle cells are relatively well described, the antioxidative properties of carvedilol and the influence of carvedilol on blood leukocytes are not completely documented. The major aim of the study was to provide new information on the effects of carvedilol on the oxidative burst of leukocytes.

Leukocyte rich plasma was prepared using dextran sedimentation of heparinized rat whole blood. Then the leukocyte rich plasma was washed and isolated leukocytes were resuspended in HBSS. Leukocyte-derived free radical production activated with different kinds of stimuli (opsonized zymosan particles - OZP, bacterial polypeptide FMLP, calcium ionophore - CaI, phorbol myristate acetate - PMA) was measured in the absence or in the presence of carvedilol using luminol-enhanced chemiluminescence (CL). CL activity was detected in microtitre plate luminometer Immunotech LM-O1T (Czech Republic) and analysed for its kinetics within 1 h after the activation.

Carvedilol in a concentration of 100 μmol/l completely inhibited CL activity originated from cells activated by any of the activators tested. FMLP- and CaI-activated

CL was significantly inhibited also with 10 and 1 μmol/l carvedilol while OZP-activated CL was inhibited only sligtly with 10 μmol/l. PMA-activated CL was not decreased by carvedilol in a concentration of 10 μmol/l. Concentrations of carvedilol lower than 1 μmol/l had no effect on the CL of leukocytes.

The antioxidative properties of carvedilol were confirmed in following experiments. The antioxidative potential to individual reactive oxygen metabolites decreased in the following order: hydroxyl radicals > hydrogen peroxide > superoxide radicals > peroxyl radicals. These antioxidative properties of carvedilol can explain the decreased cell-derived CL only partially. The question what is the reason of different effect of carvedilol on cells activated by different types of stimuli remains to be elucidated.

Acknowledgement

This study was supported by grants 305/04/0896 from the Grant Agency and Kontakt 131 from the Ministry of Education, Youth and Sports, Czech Republic.

6L04

LEUKEMIC CELLS TREATED WITH PHOTODYNAMIC THERAPY (ALA-PDT) EXHIBIT DIFFERENT CELL RESPONSE IN THE EXPRESSION AND/OR PHOSPHORYLATION OF SOME HEAT SHOCK PROTEINS

Michaela Pluskalová[a,*], Dana Grebeňová[a], Petr Halada[b] and Zbyněk Hrkal[a]

[a]*Department of Cell Biology, Institute of Hematology and Blood Transfusion, U Nemocnice 1, 12820 Praha 2, Czech Republic*
[b]*Laboratory of Molecular Structure Characterization, Institute of Microbiology, Academy of Sciences, Vídeňská 1083, 14220 Praha 4, Czech Republic*

** E-mail: pluskalm@uhkt.cz*

New developing approach to the cancer treatment is a photodynamic therapy (PDT). PDT is based on the application of a photosensitive compound. Subsequent irradiation with visible light leads to energy-transfer processes and induces activation of oxygen molecules present in tissues. Highly reactive singlet oxygen molecules are formed. These cause oxidation of cellular compounds (proteins, lipids) leading to the cell death.

In this work a variation of PDT was used, based on the administration of 5-aminolevulinic acid (ALA), the distant precursor of heme in biosynthetic pathway. Protoporphyrin IX, the compound with excellent photosensitizing properties, is accumulated in cancer cells due to defective heme biosynthesis in most cancer cell types.

Promyelocytic leukemia cell line HL60 and chronic myelogenous leukemia cell line K562 were employed to reveal the specific cancer cell response to ALA-PDT. Apoptosis was induced in HL60 cells due to ALA-PDT treatment. Some specific signs of the

induction of apoptotis were also detected in K562 cells; however the process was interrupted before the execution phase of apoptosis.

The effects of ALA-PDT on the proteome of HL60 and K562 cells were examined by two-dimensional electrophoresis (2D-PAGE). The 2D-peptide or phosphopeptide maps of ALA-induced cells (treatment with 1 mM ALA for 4 h) as well as cells subjected to ALA-PDT (irradiation of ALA-induced cells with blue light dose of 18 J/cm^2) were obtained and compared by image analysis. Protein spots that differed in the position, density or fluorescence intensity were subjected to mass spectromery analysis (MALDI-TOF) and identified by bioinformatics means. Among these the molecular chaperons were also identified. Subsequent study was focused on the responsibility of molecular chaperons in the regulation of different cell response to ALA-PDT. Both pro- and anti-apoptotic responses of several heat shock proteins were determined.

Molecular chaperon Hsp60 is a mitochondrial heat shock protein, which is important for the folding of nascent proteins, for a protein function and a proteolytic degradation of incorrectly folded proteins. Hsp60 was affected in response to ALA-PDT in HL60 cells as uncovered by proteomic analysis. Protein level of Hsp60 increased by over 40 % in HL60 cells, while it remained unchanged in K562 cells. In this case the increased expression of Hsp60 was in accordance with the execution of apoptosis in leukemic cells.

Cytoplasmic protein heat shock 90 (Hsp90) is an essential molecular chaperone that is found even in unstressed eukaryotic cells in the abundance level (1 – 2 %). Hsp90 associates with client proteins and its role appears to be particulary in activation or maturation of client proteins rather than their folding de novo. Several important proteins that are key components of signal tranduction pathways belong to Hsp90 client proteins. However Hsp90 association is also required for the stability and function of multiple mutated and over-expressed signaling proteins that promote growth and/or survival of cancer cells. Hsp90 client proteins include mutated p53, Bcr-Abl, Raf-1, Akt or ErbB2.

Oncogene Bcr-Abl tyrosine kinase was determined as the primary molecular cause of the chronic myelogenous leukemia disease. Association of Bcr-Abl with Hsp90 was found to be necessary for its stability. Phosphorylation of Hsp90 causes dissociation from its client protein, which is than more sensitive to proteasome degradation targeting.

The number of Hsp90 spots on protein and phosphoprotein maps increased in response to ALA-PDT whereas the protein level of Hsp90 remained unchanged. ALA-PDT induced phosphorylation of Hsp90, which led to its dissociation from the client proteins and the protein level of oncogene Bcr-Abl decreased in K562 cells to 30 % in three hours after treatment. The apoptotis- inducing signal was determined due to ALA-PDT in K562 cells. Cytochrome *c* was released to cytosol and might induce the execution phase of apoptosis as in HL60 cells. However, any signs of execution process were not detected in K562 cells in comparison with HL60 cells.

Protein expression level of another molecular chaperone - Hsp27 was found much higher in K562 cells in HL60 cells. Hsp27 inhibits cytochrome *c*-dependent activation of procaspase-9. High protein level of Hsp27 disables the execution of apoptosis in K562 cells. Any alternative apoptotic pathway was not induced.

Acknowledgement

The work was supported by grant NL 7681-3 from the Internal Grant Agency, Ministry of Health, Czech Republic.

6L05

INFLUENCE OF *CHLORELLA PYRENOIDOSA* INTAKE ON LIPID METABOLISM IN SUBJECTS WITH HYPERLIPIDAEMIA

Ivana Márová[a,]*, Simona Macuchová[a], Rostislav Kotrla[b] and Petr Ptáček[a]

[a]*Institute of Food Chemistry and Biotechnology, Faculty of Chemistry, Brno University of Technology, Purkyňova 118, 61200 Brno, Czech Republic*
[b]*Department of Clinical Biochemistry, Municipal Hospital, Kyjov, Czech Republic*

** E-mail: marova@fch.vutbr.cz*

The aim of this work was to study influence of *Chlorella pyrenoidosa* intake on lipid metabolism in hyperlipidemic subjects. A total of 30 subjects enrolled in a 7-month study were divided into 2 groups: A - 11 controls, B - 19 subjects with hyperlipidaemia. During the 1st period of experiment (3 months) took all groups normal diet. During consecutive 90 days (2nd period) both groups A and B were treated by *Chlorella pyrenoidosa* supplement (2 g daily). During the 3rd period (1 month) rate of antioxidants utilization in tested organisms was followed. Some metabolic parameters, serum carotenoids, tocopherols, flavonoids and total antioxidant status (TAS) were measured in each subject regularly in 60-day intervals. Additionally, antioxidant (TAS kit, Randox) and antimutagenic (test strain *S. cerevisiae* D7) effects of *Chlorella pyrenoidosa* were analysed.

Levels of antioxidants and TAS in the control group exhibited typical seasonal decrease in the 2nd period. After 90-day of *Chlorella* treatment statistically significant changes in some parameters of lipid metabolism were observed in group B (total cholesterol 6.08±1.26 vs 5.88±1.03 mM,; LDL-cholesterol 3.89±0.68 vs 3.66±0.81 mM,; TAG 2.08±0.78 vs 1.72±0.84 mM; AI 3.58±1.14 vs 3.34±0.84), while in group A no significant changes were observed (except 1.2 x decrease of TAG level). Both groups A and B exhibited significant increase in levels of some antioxidants: 1.7 x retinol, 1.6 x lutein, 2.3 x lycopene, 1.4 x β-carotene and 1.7 x rutin. *Chlorella* exhibited high antimutagenic effect due probably by presence of chlorophyll and other antioxidants.

Chlorella pyrenoidosa intake can positively influence lipid metabolism, but supplementation will be proved for long time.

6L06

PIEZOELECTRIC IMMUNOSENSORS

Petr Skládal*

Department of Biochemistry, Faculty of Science, Masaryk University, Kotlářská 2, 611 3 Brno, Czech Republic

** E-mail: skladal@chemi.muni.cz*

The piezoelectric transducer – quartz crystal microbalance - represents an affordable tool for direct and real-time monitoring of immunoaffinity interactions. The measuring set-up suitable for convenient measurement in a flow-through arrangement was developed and applied for bioaffinity studies as well as for immunoassays. For clinical applications, the piezoelectric immunosensor for rapid determination of albuminuria was developed using competitive assay with antibody in solution. Another studies involved interactions of the tumour suppressor protein p53 with antibodies corresponding to several regulatory regions of p53. The covalently immobilised protein p53 retained its activity and it was able to interact with antibodies as well as with several protein kinases resulting in modulation of the physiological activity. The interactions of osteoprotegerin (OPG) with several antibodies were studied. Monoclonal anti-OPG antibodies (5H3, 4E6H9 and HU-OPG1.3) were immobilized on the sensing surface modified with covalently attached monolayer of protein A. Binding of both OPG standard and recombinant OPG-Fc chimeric protein was followed in real time. All antibodies were able to bind OPG and OPG-Fc, though in the case of MAb 4E6H9 the immunocomplexes dissociated quickly in the absence of OPG. In all cases, the dissociation of MAb-OPG complexes was faster compared to MAb-OPGFc complexes. For the developed OPG immunosensor, the lowest detectable concentration of OPG was 60 ng/ml. The assays of OPG in serum samples were performed using both piezoelectric immunosensors and compared with assays carried out using the standard ELISA procedure. The developed immunosensors seem promising for rapid determination of different markers in real clinical samples. Additional examples of piezoelectric immunosensors will include determination of pesticides in water, immunosensing of polychlorinated biphenyls in organic solvents and rapid detection of bacteria.

6P01

GENOTYPING OF THE CATECHOL-O-METHYLTRANSFERASE GENE BY REAL-TIME PCR

Martin Beránek[a,*], Jiří Horáček[b] and Vladimír Palička[a]

[a]*Institute of Clinical Biochemistry and Diagnostics, Charles University Hospital, Sokolská 581, 500 05 Hradec Králové, Czech Republic*
[b]*Prague Psychiatric Centre, 3rd Faculty of Medicine of Charles University, Prague, Czech Republic*

** E-mail: beranek@lfhk.cuni.cz*

Biotransformation of catechols in the human body depends on catalytic activity of catechol-O-methyltransferase (COMT; EC 2.1.1.6). This enzyme exists as membrane-bound (MB) and soluble (S) isoforms differing by 50 amino acid residues. The COMT gene maps to chromosome 22q11.2. Biallelic genetic polymorphism at codon 108/158 (S-/MB-COMT) influences thermostability and consequently enzyme activity of COMT. Slower biodegradation of catecholamine neurotransmitters in the organism could contribute to the etiology of mental disorders (velo-cardio-facial syndrome, rapid cycling bipolar disorder, obsessive-compulsive disorder, type 1 alcoholism, schizophrenia) and breast cancer. The aim of this study was to develop a rapid real-time PCR method for COMT genotyping via melting point analysis with hybridization probes. Primers and fluorescent hybridization probes were designed using Roche LightCycler Probe Design Software. Amplification was carried out in a final volume of 10 µl in disposable glass capillaries (Roche Diagnostics, Mannheim, Germany). Each reaction contained 1 µl of LightCycler FastStart DNA Master Hybridization Probes (Roche), 2 mmol/l $MgCl_2$, 5 pmol of primers, 1 pmol each of hybridization probes, and 50–100 ng of genomic DNA. The thermocycling conditions were: 95 °C for 10 min (initial denaturation and activation of Taq polymerase) and 45 cycles of 95 °C for 2 s, 55 °C for 5 s, and 72 °C for 5 s. After amplification, melting analysis was performed by denaturation at 95 °C for 30 s, annealing at 40 °C for 90 s, and slow heating to 75 °C with a ramp rate of 0.1 °C/s.

The genotyping was performed on DNA samples of 118 volunteers with their informed consent. 58 subjects (49%) were heterozygous Met/Val, 25 subjects (21%) were homozygous Met/Met, and 35 subjects (30%) were homozygous Val/Val. The reliability of the results was confirmed by a PCR/RFLP method. Both techniques gave identical results. The estimation of Val-108/158 allele frequency was about 54%, and Met-108/158 allele about 46%. Real-time PCR is a rapid and accurate approach to detection of a single-nucleotide polymorphism at COMT codon 108/158.

6P02

THE EFFECT OF SEROTONIN ON PHAGOCYTE-DERIVED REACTIVE OXYGEN SPECIES PRODUCTION

Milan Číž[a,*], Daniela Komrsková[a], Viera Jančinová[b], Katarína Drábiková[b], Radomír Nosál[b] and Antonín Lojek[a]

[a]*Laboratory of Pathophysiology of Free Radicals, Institute of Biophysics, Academy of Sciences of the Czech Republic, Královopolská 135, 61265 Brno*
[b]*Institute of Experimental Pharmacology, Slovak Academy of Sciences*

** E-mail: milanciz@ibp.cz*

Specific uptake of serotonin by macrophages may regulate various constituents of the immune system and immune functions. Serotonin, the major secretory product of activated platelets, was also widely reported to modulate the function of PMNLs. This modulation is complex and data available are rather controversial. Serotonin could act as a true scavenger of reactive oxygen species generated during the respiratory burst of stimulated phagocytes, caused aggregation and degranulation of PMNLs, inhibited the migration of mononuclear leucocytes. The aim of the present study was to clarify the mechanisms of serotonin action on neutrophils and monocytes/macrophages. The effect of serotonin in a concentration range of 10^{-2} - 10^{-7} M on various parameters of oxidative burst of phagocytes was studied using various luminol-enhanced chemiluminescence (CL) methods .

The oxidative burst of opsonized zymosan-activated neutrophils in human whole blood was assessed using Luminometer 1251 (BioOrbit, Finland). CL values were expressed as maxima of the curves obtained during 60 min measurements. Serotonin inhibited the CL response of the cells in a dose dependent manner: by 95% (concentration 10^{-3} M), 88% (10^{-4} M), and 57% (10^{-5} M). Lower concentrations of serotonin were ineffective.

Since luminol-enhanced CL of phagocytes is widely considered to be dependent on a reaction of the myeloperoxidase (MPO) system, the effect of serotonin on the activity of MPO was studied in further experiments. Lysed HL-60 cells were used as a source of MPO and the activity of the enzyme was evaluated using bromide-dependent CL reaction. In this case, serotonin again exerted a dose dependent inhibition of the MPO activity: 99% inhibition (10^{-3} M serotonin), 95% inhibition (10^{-4} M serotonin), 27% inhibition (10^{-5} M serotonin) and 17% inhibition (10^{-6} M serotonin). The lowest concentration of serotonin (10^{-7} M) did not inhibit the activity of MPO at all.

The CL was used to follow up the total peroxyl-radical antioxidative parameter (TRAP) of serotonin. Peroxyl radicals were generated at a constant rate by thermal decomposition of ABAP. These peroxyl radicals were scavenged when antioxidants were present and the TRAP value (expressed as mmol of peroxyl radicals scavenged by 1 l of the sample) was proportional to the period when the CL signal was diminished. Only the two highest concentrations of serotonin showed a peroxyl radical-scavenging effect: 4959 mmol/l and 410 mmol/l at concentrations of 10^{-3} M and 10^{-4} M, respectively.

Antioxidant properties of serotonin were further studied in various chemical systems producing individual ROS: hypoxanthine/xanthine oxidase system (produced superoxide anion), hydrogen peroxide/ferrous sulphate system (produced hydroxyl radical) and hydrogen peroxide itself. ROS production was measured in a microtitre plate luminometer LM-01T (Immunotech, Czech Republic). Serotonin exerted the strongest antioxidant potential against hydroxyl radical and the weakest antioxidant potential against hydrogen peroxide. The percentage of inhibition was as follows for individual serotonin concentrations: 10-3 M – 100%, 98%, and 96% (CL driven by superoxide anion, hydroxyl radical and hydrogen peroxide, respectively); 10-4 M - 97%, 82%, and 63%; 10^{-5} M - 69%, 34%, 0%; 10^{-6} M - 22%, 0%, and 0%. Serotonin at the concentration of 10^{-7} M did not inhibit CL in any of the chemical systems mentioned above.

It can be concluded that serotonin could affect the oxidative burst of phagocytes. Both decreasing the generation of ROS and direct scavenging of ROS were responsible for its inhibitory effects.

Acknowledgement

The study was supported by the grant 524/04/0897 from the Grant Agency of the Czech Republic.

6P03

DIFFERENTIAL EXPRESSION OF PROGELATINASE B/PROMMP-9 IN PARANEOPLASTIC PLEURAL EFFUSIONS

Jaromír Kotyza[a,*], Miloš Pešek[b], Bernd Werle[c] and David Havel[b]

[a]*Institute of Biochemistry, Faculty of Medicine, Charles University, Karlovarská 48, 30166 Plzeň, Czech Republic*
[b]*Clinis of Tuberculosis and Respiratory Diseases, University Hospital, Edvarda Beneše 13, 305 99 Plzeň, Czech Republic*
[c]*Medizinische Klinik Heidelberg, Ruperto-Carola-University Heidelberg, Germany*

** E-mail: jaromir.kotyza@lfp.cuni.cz*

Previous analyses of pleural exudates revealed high levels of gelatinases, a subclass of matrix metalloproteinases (MMPs), associated with invasive processes during cancer progression and inflammatory reactions. Whereas the expression of major gelatinase A (MMP-2) seems to be constitutive, the expression of gelatinase B (MMP-9) is apparently inducible under pathological conditions. However, reports on the degree of expression of MMP-9 among clinically relevant effusion types have so far been contradictory. To elucidate expression of MMP-9 in pleural effusions of paraneoplastic origin, we analyzed pleural fluids of lung cancer patients (n=130) by substrate zymography and enzyme-linked immunosorbent assay, and compared proMMP-9 levels with

parainfectious effusions (n=33), and transudates (n=12). We found significant differences among the three groups, with the highest concentrations in parainfectious exudates, and the lowest in transudates. However, data analysis of paraneoplastic exudates revealed many patients, expressing considerably higher amounts of proMMP-9 then the major paraneoplastic subgroup around a relatively low median value. A subsequent sorting based on histological origin of malignancies showed significantly higher values in exudates associated with metastatic tumors (n=26), when compared to primary tumor effusions. No significant differences were found between metastase associated effusions and parainfectious exudates. Values of proMMP-9 correlated significantly with levels of C-reactive protein, a systemic marker of inflammation. These observations are discussed with respect to a possible contribution of inflammation onto the neoplastic process.

6P04

PREPARATION AND IMMUNOGENICITY OF YEAST MANNAN CONJUGATE

Slavo Bystrický*, Richard Ďurana, Ema Paulovičová and Eva Machová

Institute of Chemistry, Slovak Academy of Sciences, Dúbravska cesta 9, 85238 Bratislava, Slovakia

chembyst@savba.sk

Mannans, yeast surface polysaccharide antigens (*Candida* spp.) were partially oxidized and conjugated to protein carrier. Prepared conjugates were immunogenic in mice and re-injection elicited significant increase of specific serum antibody levels. The results obtained by ELISA method indicate that the yeast mannan conjugates prepared by the simple scheme could be considered as candidate for preventive treatment in the some potentially risk population (cellular and humoral immune deficiences).

Acknowledgement

The work was supported by grant VEGA No. 2/4144/24 and APVT project 51-015902.

6P05

MONOCLONAL ANTIBODY SPECIFIC FOR BOVINE MHC CLASS I ANTIGENS

Jana Antalíková, Michal Simon, Lubica Horovská* and Jana Valentovičová

Institute of Animal Biochemistry and Genetics, Slovak Academy of Sciences, Moyzesova, 90028 Ivanka pri Dunaji, Slovakia

** E-mail: Lubica.Horovska@savba.sk*

The MHC Class I molecules present endogenously synthetized peptides to CD8+ cytotoxic T cells[1] and these molecules (MHC I) are also recognized by inhibitory receptors of natural killer cells[2]. The MHC I class molecules of man and other studied species are expressed on most nucleated cells[1]. They consist of heterodimers of highly polymorphic α chains (M_r 44 kDa) non-covalently associated with the invariant β2-microglobulin subunit (M_r 12 kDa)[3]. Knowledge about MHC class I structure and their biological function has been advanced by the extensive development of monoclonal antibody (mAb) reagents. In cattle, most of the MHC class molecule (denoted BoLA) analyses were performed by cross-reactive mAb W6/32[4,5] commonly used for human MHC study. Only a few mouse mAb generated against bovine cells were reported[6].

This paper describes the antibody IVA-26 formed against bovine cells detecting MHC class I molecule of several species and showing analogical features with mAb MEM-147[7] that recognizes MHC class I on human cells. The indirect immunofluorescent analysis revealed that mAb IVA-26 gave positive reaction with whole population of bovine, ovine, pig and human lymphocytes. The IVA-26 was also bound to some population of bovine, pig and human granulocytes and weak fluorescence was found on thrombocytes of the same species. IVA-26 immunoprecipitated proteins with molecular weight of 45 kDa (α chain), 14 kDa (β2m) and 58 kDa (α + β2m) from lyzates of detergent-solubilized, surface-biotinylated bovine lymphocytes. IVA-26 also reacted with β2m molecules of human lymphocyte lysates.

References

1. York I. A. and Rock K. L.: *Annu. Rev. Immunol.* **14**, 369 (1996)
2. Lanier L. L.: *Annu. Rev. Immunol.* **16**, 359 (1998)
3. Ploegh H. L., Orr H. T. and Strominger J. L.: *Cell* **24**, 287 (1981)
4. Parham P., Barnstable C. J. and Bodmer W. F.: *J. Immunol.* **123**, 342 (1979)
5. Kahn-Perles B., Boyer C., Arnold B., Sanderson A. R., Ferrier P. and Lemonnier F. A.: *J. Immunol.* **138**, 2190 (1987)
6. Bensaid A., Kaushal A., MacHugh N. D., Shapiro S. Z. and Teale J.: *Anim. Genet.* **20**, 241 (1989)
7. Tran T. M., Ivanyi P., Hilgert I., Brdička T., Pla M., Breur B., Flieger M., Ivasková E. and Hořejší V.: *Immunogenetics* **53**, 440 (2001)

6P06

ASSOCIATION OF CD9 AND CD41/61 (αIIBβ3) MOLECULES IN BOVINE PLATELET MEMBRANES

Jana Antalíková*, Jana Tomášková, Michal Simon and Ľubica Horovská

Institute of Animal Biochemistry and Genetics, Slovak Academy of Sciences, Moyzesova, 90028 Ivanka pri Dunaji, Slovakia

** E-mail: Jana.Antalikova@savba.sk*

CD9 is a widely expressed transmembrane protein of the tetraspanin superfamily (also known as TM4SF) with a broad tissue distribution. The tetraspanins are involved in many cellular functions as adhesion, migration, co-stimulation, signal transduction, and differentiation[1]. The wide diversity of functions attributed to tetraspanins is probably related to the association of these molecules with a wide variety of partner proteins in tetraspanin web. Not only tetraspanins are associated with each other, but they also associate with many Ig superfamily proteins, proteoglycans, complement regulatory proteins, integrins, growth factors and signalling enzymes[2]. CD9 forms complexes with CD81 or CD63 molecules which associate with αvβ1 or α6β1 integrins[1]. In platelets CD9 is associated with the integrin αIIbβ3 (CD41/61)[3]. In order to establish the complex of CD9 with another proteins in bovine platelet membranes we solubilized the platelets with non-ionic detergent Brij 35. We found that the integrin CD41/61 and the tetraspanin CD9 can associate in a protein complex that is present in resting untreated bovine platelets. Both antibodies anti CD41/61 (IVA 38) and anti CD9 (IVA50) immunoprecipitated similar pattern of protein bands including the co-precipitation of CD41/61 and CD9. Western blot and reimmunoprecipitation confirmed the presence of β3 subunit in mAb IVA50 immunoprecipitates. In addition CD9 was detected in IVA 38 immunoprecipitates by Western blotting and reimmunoprecipitation. The depletion experiment indicated that CD9 is completely associated with CD41/61 in bovine platelet membranes.

References

1. Maecker H. T., Todd S. C. and Levy S.: *FASEB J.* **11**, 428 (1997)
2. Boucherix C. and Rubinstein E.: *Cell. Mol. Life Sci.* **58**, 1189 (2001)
3. Indig F. E., Diaz-Gonzales F. and Ginsberg M. H.: *Biochem J.* **327**, 291 (1997)

6P07

METHODS FOR DETECTON OF K-RAS MUTATIONS - EARLY DIAGNOSE OF CANCER

Jana Ugorčáková[a,*], Tibor Hlavatý[b], Gabriela Bukovská[a], Nora Halgašová[a] and Jozef Timko[a]

[a]*Institute of Molecular Biochemistry, Slovak Academy of Sciences, Dúbravská cesta 21, 84551 Bratislava, Slovakia*
[b]*1st Clinic of Internal Medicine, Faculty of Medicine, Comenius University, Bratislava, Slovakia*

** E-mail: Jana.Ugorcakova@savba.sk*

K-ras (Kirsten) onkogen belongs to the supra-family of ras onkogens. K-RAS protein is located on the cytoplasmic side of cell membrane and functions as a signal transducer that promotes cell growth and cell division. In the active state, K-RAS binds and cleaves GTP. A defective K-RAS releases GTPase activity, becomes continuously activated and promotes unregulated, proliferative cell growth[1]. Mutation in K-ras so lead toward uncontrolled growth and cell division. Since K-ras is a signal transducer, only one copy of K-ras is sufficient to be inappropriately activated for unregulated cell growth to occur. High frequency of K-ras point mutations used to be detected in several malignances like pancreatic cancer (at 75-100% patients), colorectal cancer (40-50%), lung carcinoma (15-40%). There is mainly 3 exactly defined mutations of first exon 12 codon of these gene.

Various methods have been described for detecting mutant K-ras alleles in the presence of excess healthy alleles. All methods are established on utilization of PCR amplification. Detection of mutations on the cloned PCR products by the allel-specific hybridization was one of the first laboratory techniques in this area, but for technical severity showed as unsuitable for routine exploitation. One of utilizable methods is "mutant-enriched" PCR[2,3]. Disadvantage of this method is low reproducibility and a lot of false-positive results. Detection of rare mutant alleles can be achieved using enriched PCR, which requires multiple rounds of PCR, or using restriction endonuclease-mediated selective (REMS)-PCR, which achieves the same result in single round of PCR[4,5].

Fischer at al.[6] developed method to achieve an enrichment of mutant allels by the removing of wild-type alleles by differential hybridization to complementary oligonucleotides. The nonbound fraction is reamplified by PCR and submited to a second round of absorption. The technique, termed subtractive interactive PCR (siPCR) allows the detection of K-ras mutations in pancreatic juice with high sensitivity and reliability. Rapid method for detection of K-ras mutations in bile by peptide nucleic acid-mediated PCR was developed by Chen et al.[7]. Authors used RT PCR with a cysteine-specific (TGT) sensor probe, what can rapidly detect K-ras gene mutations in bile and diagnose malignant biliary obstruction with high specificity. Various authors have described methods like PCR-SSCP, "dot blot" hybridization and

immunohistochemical analysis[1,8]. For complexity and cost-noneffective severity of these methods, their use for current screening is not suitable.

Recent years research brings diagnostics of K-ras onkogen by exploitation "biochip array" methods[9]. High effort of researches is to develope methods using DNA and protein microarray[10,11].

Current methods for detection of K-ras gene mutations are time-consuming. We aimed to develop a one-step PCR technique to detect K-ras mutations in diferent samples of tissue. We work for development of clinical and laboratory method that can detect presence of even small amounts of mutated K-ras gene in biological sample at reliable sensitivity and specificity and prove that this method is useful for diagnosis of pancreatic cancer.

References

1. Doolittle B. R., Emanuel J., Tuttle C. and Costa J.: *Exp. Mol. Pathol.* **70**, 289 (2001)
2. Smith-Ravin J., England J., Talbot I. C. and Bodmer W.: *Gut* **36**, 81 (1995)
3. Nollau P., Moser C., Weinland G. and Wagener C.: *Int. J. Cancer* **66**, 332 (1996)
4. Ward R., Hawkins N., O'Connor T., Impey H., Roberts N., Fuery C. and Todd A.: *Am. J. Pathol.* **153**, 373 (1998)
5. Fuery C. J., Impey H. L., Roberts N. J., Applegate T. L., Ward R. L., Hawkins N. J., Sheehan C. A., O'Grady R. and Todd A. V.: *Clin. Chem.* **46**, 620 (2000)
6. Fischer C., Buthe J., Nollau P., Hollerbach S., Schulmann K., Schmiegel W., Wagener C. and Tschentscher P.: *Lab. Invest.* **81**, 827 (2001)
7. Chen C. Y., Shiesh S. C.and Wu S. J.: *Clin. Chem.* **50**, 481 (2004)
8. Song M. M., Nio Y., Dong M., Tamura K., Furuse K., Tian Y. L., He S. G. and Shen K.: *J. Surg. Oncol. Nov.* **75**, 176 (2000)
9. Prix L., Uciechowski P., Bockmann B., Giesing M. and Schuetz A. J.: *Clin. Chem.* **48**, 428 (2002)
10. Li M., Lin Y. M, Hasegawa S., Shimokawa T., Murata K., Kameyama M., Ishikawa O., Katagiri T., Tsunoda T., Nakamura Y. and Furukawa Y.: *Int. J. Oncol.* **24**, 305 (2004)
11. Ohnami S., Aoki K., Yoshida K., Ohnami S., Hatanaka K., Suzuki K., Sasaki H and Yoshida T.: *Biochem. Biophys. Res. Com.* **309**, 798 (2003)

Acknowledgement

This work was supported by the VEGA Grant 1/0256/03.

6P08

PREPARATION AND BIOLOGICAL APPLICATION OF LIPOSOMAL MURAMYL GLYCOPEPTIDES

Andrea Kašná[a,b,*], Veronika Kvardová[a,b], Jaroslav Turánek[b], Ivan Rychlík[b], Josef Krejčí[b] and Miroslav Ledvina[c]

[a] *Masaryk University, Kotlářská 2, 611 37 Brno, Czech Republic*
[b] *Veterinary Research Institute, Hudcova 70, 621 32 Brno, Czech Republic*
[c] *Institute of Organic Chemistry and Biochemistry, Prague, Czech Republic*

** E-mail: kasna@vri.cz*

We have designed and synthesized new types of analogues with changes in both the sugar and the peptide parts of the molecule that now induce a high level of immunostimulation and adjuvant activity but with adverse side effects suppressed. There are several important groups of such compounds including muramylglycopeptides, analogues of muramyldipeptide (MDP), glucosaminyl-muramyldipeptide (GMDP), and desmuramylpeptides. Lipophilic residues have also been introduced to improve adjuvant incorporation into liposomes, which themselves represent suitable and versatile carriers for lipophilic drugs or antigens and are useful potential vehicles for the construction of vaccines. We designed and constructed a stirred thermostated cell which was connected to a liquid delivery system for the rapid production of multilamellar liposomes by the proliposome-liposome method. This method is based on the conversion of the initial proliposome preparation into a liposome dispersion by dilution under strictly controlled conditions. Liposomal preparations of immunomodulators were characterized by electron microscopy and dynamic light scattering instrument. Biological activity of liposomal preparations was tested on mice infection model. Liposomal immunomodulators N-L18-norAbu-GMDP and B30-norAbu-MDP were very effective at modulating innate immunity against salmonelosis in mice (within the range of 3 - 50 μg per mouse) by intranasal/peroral application. Both liposomal preparations stimulated innate immunity and radioprotective effects in sublethally irradiated mice.

Acknowledgement

This work was supported by the grants from the Ministry of Agriculture of the Czech Republic (grant No. VZ 0002716201, NAZV Contract No: QF 3115), and the Academy of Sciences (IGA AV ČR No: Z4055905).

ACTA UNIVERSITATIS PALACKIANAE OLOMUCENSIS
FACULTAS RERUM NATURALIUM 2004 CHEMICA 43S

PROCEEDINGS OF THE XIX. MEETING OF CZECH AND SLOVAK SOCIETIES FOR BIOCHEMISTRY AND MOLECULAR BIOLOGY

Olomouc, August 31 – September 3, 2004

SESSION 7: BIOCHEMISTRY OF NATURAL COMPOUNDS AND XENOBIOCHEMISTRY

7L01

LESSONS FROM KNOWN P450 STRUCTURES

Pavel Anzenbacher[a,]*, Eva Anzenbacherová[b] and Michal Otyepka[c]

[a]*Instititute of Pharmacology and* [b]*Institute of Medical Chemistry and Biochemistry, Faculty of Medicine, Palacký University, Hněvotínská 3, 77515 Olomouc, Czech Republic*
[c]*Department of Physical Chemistry, Faculty of Science, Palacký University, tř. Svobody 8, 77146 Olomouc, Czech Republic*

** E-mail: anzen@tunw.upol.cz*

There is a considerable progress last five years in knowledge of the P450 (CYP) structures which allows to draw out at least some conclusions important for function of the respective enzymes. First, the structure of mammalian (rabbit) liver microsomal CYP2C5 was published in 2000 which allowed for detailed comparisons of all important human liver microsomal P450s. The next milestone was the structure of CYP2C9 presented in full in 2003 showing the first human liver microsomal P450 in the absence as well as in the presence of substrate. Third success was the resolution of the CYP3A4 structure presented this year.

The structures show that although the overall „P450 fold“ is preserved, there are considerable differences in the ways the substrates approach the active site and in the properties of the active site itself. Relative flexibility and openness of the CYP3A4 active site was expected by the modeling as well as by the spectroscopic approaches. The CYP2C9 structure opens a possibility to study in more detail the modes by which the mutations in the protein structure are reflected in altered enzyme activities. CYP2E1 structure has been predicted from data known for CYP2C9; here, interesting conclusions based on interspecies (human, pig, minipig, rat) differences are possible based on variability of amino acid sequence in discrete sites in the primary structure.

Acknowledgement

Support through the grant 203/02/1152 from the Grant Agency, Czech Republic, is gratefully acknowledged.

7L02

INDUCTION OF DRUG METABOLIZING ENZYMES – MECHANISM AND CONSEQUENCES

Lenka Skálová* and Barbora Szotáková

Department of Biochemical Sciences, Faculty of Pharmacy, Charles University, Heyrovského 1203, 50005 Hradec Králové, Czech Republic

** E-mail: skaloval@faf.cuni.cz*

The significantly increased level of drug metabolizing enzymes due to presence of xenobiotic in organism is commonly termed induction and this phenomenon has been studied for decades. Previously, induction seemed to be unique property of special isoforms of cytochrome P450. Nowadays, induction is considered as common feature of the most of biotransformation enzymes and drug transporters. It represents one of the adaptive response of organism on chemical stress[1,2].

In pharmacotherapy induction of drug metabolizing enzymes may cause the undesired drug-drug interactions and adverse drug reactions. Lack of knowledge of the inductive capability of drug can result in therapeutic failure or toxicity due to increased formation of inactive or active drug metabolites[3]. In case of antibiotic or antiparasitic treatment the decrease of plasma levels of active compound due to induction of biotransformation enzymes may contribute to development of bacteria or parasites resistance.

The mechanisms of induction are primarily based on activation of special intracellular receptors which act as ligand-dependent transcription factors. They regulate the expression of a battery of genes depending on species and tissues. The most important receptors involved in induction of drug metabolizing enzymes are aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), pregnane X receptor (PXR), glucocorticoid receptor (GR) and peroxisome proliferator activated receptor (PPAR). Their ligand-mediated activation may lead not only to induction of phase I and phase II biotransformation enzymes and ATP-dependent transporters but also to alteration of endogenous metabolism and cell cycle. Thus the consequences of xenobiotic interaction with these receptors are believed to be more complex and serious then was considered previously[4].

References

1. Mankowski D. C. and Ekins S.: *Current Drug Metabolism* **4**, 381 (2003)
2. Conney A. H.: *Annu. Rev. Pharmacol. Toxicol.* **43**, 1 (2003)
3. Hanschin C., Podvinec M. and Meyer U. A.: *Drug News Perspect.* **16**, 423 (2003)
4. Denison M. S. and Nagy S. R.: *Annu. Rev. Pharmcol. Toxicol.* **43**, 309 (2003)

Acknowledgement

This project was supported by the Grant Agency of the Czech Republic (524/03/1361) and the Ministry of Education, Youth and Sport, Czech Republic (MSM 111600002).

7L03

USE OF PHOTOLABILE COMPOUNDS TO STUDY A MEMBRANE TOPOLOGY OF CYTOCHROMES P450

Martin Karabec[a,*], Petr Hodek[a] and Miroslav Šulc[b]

[a]*Department of Biochemistry, Faculty of Science, Charles University, Hlavova 2030/8, 12840 Praha 2, Czech Republic*
[b]*Institute of Microbiology, Academy of Sciences of the Czech Republic*

** E-mail: karabec@natur.cuni.cz*

The cytochromes P450 are located in membrane of endoplasmic reticulum. The membrane topology of the mammalian P450 cytochromes has been studied intensively by computational approaches, proteolysis, chemical modification, genetic engineering, and immunochemistry. Recent results show that cytochrome P450 core is anchored in membrane by one or two transmembrane helixes, located at the NH_2- terminal end. Most likely, an additional parts of the enzyme macromolecule might be inserted into membrane.

We are going to approach this topic by using of photoaffinity labeling, which is a chemical modification technique employed in study protein structure and interactions. Highly reactive species, which is able to modify amino acid residues of a protein, is generated by UV-light irradiation at certain place and exact time.

For phenobarbital-inducible cytochrome P450 2B4 diamantane like probe 3-azidiamantane [spiro-(diazirine-3,3'-diamantane)] was designed. The probe is deuterized to make it easier for identification by mass spectroscopy.

Specific deuterization of diamantane molecule is carried out by reduction of diamantane thioacetal in presence of Raney nickel saturated with deuterium.

To examine topology of cytochrome P450 membrane embedding we use bilayer of phosphatidylcholine as an artificial membrane. The hydrophobicity of this membrane assured high solubility of our hydrophobic label in the enzyme environment. Upon photolysis (366 nm) deuterized 3-azidiamantane gives highly reactive carbene intermediate, which modifies amino acid residues, contained in transmembrane peptides or in other parts of the enzyme that might be submerged into the bilayer.

Modified enzyme is cleaved by proteases and peptides are separated on HPLC RP-C18. Separated peptides are analyzed on MALDI TOF and MS-DECA for the presence of the deuterized probe. Labeled peptides should show a peak doublet (shift m/z=2), because our photolabile probe was used as an eqiumolar mixture (1:1) of deuterized and non-deuterized 3-azidiamantane during our experiment.

7L04

ESTROGEN RECEPTOR-MEDIATED ACTIVITY OF BLOOD EXTRACTS OF HUMAN MALES DIFFERENTLY EXPOSED TO PERSISTENT CHLORINATED AROMATICS

Martina Plíšková[a,*], Jiří Neča[a], Jan Vondráček[a], Anton Kočan[b], Jan Petrík[b], Tomáš Trnovec[b] and Miroslav Machala[a]

[a]*Veterinary Research Institute, Hudcova 70, 62132 Brno, Czech Republic*
[b]*Institute of Preventive and Clinical Medicine, Limbová 14, 833 01 Bratislava, Slovak Republic*

** E-mail: mpliskova@centrum.cz*

In a large epidemiological study, over 2000 human blood samples were collected from two differently polluted areas in the Eastern Slovakia in order to investigate whether the frequences of specific disease conditions, such as diabetes and cancer, modulations of steroid hormone levels, neurobehavioral perturbations, and other adverse effects could be potentially associated with a long-term exposure to polychlorinated biphenyls (PCBs) and other persistent organochlorine polutants. In this study, 150 male serum samples were extracted by hexane/diethyl ether and the total extracts were tested for the overall estrogen receptor (ER)-mediated activity, using human breast carcinoma T47D.Luc cells, stably transfected with a luciferase reporter gene under the control of estrogen responsive elements (ER-CALUX assay[1]). The ER activation was determined both in the total extract-treated samples and in cells treated with fraction of persistent organic pollutants (POPs), obtained by H_2SO_4/silica fractionation. Antiestrogenicity of the fraction of POPs (i.e. decrease in 17β-estradiol response) was determined as well. The estrogenic and antiestrogenic activities were compared with the data on serum concentrations of 17β-estradiol, *p,p'*-DDE, prevalent PCB congeners and with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalents (TEQs) determined in serum samples by the DR-CALUX assay.

The total extracts of human male serum samples, containing both non-persistent compounds (hormones and xenoestrogens) and POPs, elicited an estrogenic response. The overall estrogenic responses were marginally repressed in the serum samples collected from the polluted region and their levels corresponded with serum concentrations of estradiol. Contrary to that, no correlations were found with a sum of PCBs, TEQ values on dioxin-like activity or concentrations of *p,p'*-DDE.

Both estrogenic and antiestrogenic activites of the fractions of POPs present in the blood were identified in several of samples. However, the POPs fractions from the less-polluted background area elicited ER-mediated activity with higher incidence, than the samples from the region contaminated with PCBs and other POPs. In contrast, the antiestrogenic activity was found more frequently in the samples from the PCB-contaminated region. Principle component analysis (PCA) revealed potential association between antiestrogenicity and sum of PCBs or TEQ values.

For this reason, relative *in vitro* potencies of individual 18 prevalent and indicator PCB congeners to either induce estrogenic activity or to decrease the 17β-estradiol

response were evaluated in the ER-CALUX assay. Low-molecular-weight indicator PCBs 28 and 52, present in the serum only at low concentrations, induced relatively strongly the ER-dependent reporter construct activity; a partial estrogenicity was found also for PCBs 74, 66, 99 and 105. On the other hand, the prevalent PCB congeners Nos. 138, 153, 180, 187, 199, and 203 significantly inhibited 17β-estradiol-induced luciferase activity. A significant, albeit only partial, antiestrogenic activities were induced by additional prevalently occuring congeners 156, 170, 194 and by PCB 101. Reconstituted mixture of prevalent PCBs elicited a strong dose-dependent antiestrogenicity. Mono-*ortho*-chlorinated PCB 118 and coplanar PCB 126 showed no type of activity in the assay. The results of *in vitro* evaluation of activity of individual PCB congeners suggest that the prevalent higher-molecular-weight congeners might contribute to the antiestrogenic activity observed in the samples from the polluted area. However, the antiestrogenicity of individual PCBs was apparently not associated with coplanarity of PCB molecule and aryl hydrocarbon receptor activation.

In summary, persistent organic pollutants, especially PCBs, might contribute to decrease in overall estrogenic activity in human male serum and they may cause a weak antiestrogenic effect. Prevalent PCB congeners showed antiestrogenic activity *in vitro* and it seems reasonable to conclude that these compounds were responsible for the weak antiestrogenic effects observed in serum samples. Nevertheless, a major part of total estrogenic activity determined in serum samples was elicited by endogenous hormone 17β-estradiol. These results might suggest that modulations of levels of estradiol (e.g. by perturbations in its biosynthesis or catabolism) could present more significant endocrine-disrupting effects of POPs than the direct modulations of ER-mediated activity.

References

1. Legler J., van den Brink C. E., Brouwer A., Murk A. J., van der Saag P. T., Vethaak A. D. and van der Burg B.: *Toxicol. Sci.* **48**, 55 (1999)

Acknowledgement

This work was supported by the 5th FP EU grant No. QLK4-CT-2000-00488.

7L05

DEREGULATION OF CELL CYCLE CONTROL IN RAT LIVER EPITHELIAL CELLS BY ARYL HYDROCARBON RECEPTOR LIGANDS

Jan Vondráček[a,*], Kateřina Chramostová[a], Vítězslav Bryja[b], Lenka Šindlerová[a], Zdeněk Andrysík[a], Pavel Krčmář[c], Aleš Hampl[d], Alois Kozubík[a] and Miroslav Machala[c]

[a]*Institute of Biophysics, Academy of Sciences, Královopolská 135, 61265 Brno, Czech Republic*
[b]*Institute of Experimental Medicine, Academy of Sciences, Praha, Czech Republic*
[c]*Veterinary Research Institute, Brno, Czech Republic*
[d]*Laboratory of Molecular Embryology, Mendel University, Brno*

** E-mail: hivrisek@ibp.cz*

The maintenance of a balance between cell gain and cell loss is essential for proper liver function. It is now generally accepted that progenitor cells exist in liver, are activated in various liver diseases and can form a potential target cell population for both tumor initiating and tumor promoting chemicals in mammalian liver[1]. This presentation summarizes the results of our recent studies of effects of various types of aryl hydrocarbon receptor (AhR) ligands, including both persistent and non-persistent environmental contaminants such as polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs), hydroxylated PCBs, and model flavonoids on the release of rat liver epithelial stem-like WB-F344 cells from contact inhibition, which is supposed to be an significant mode of action of tumor promoters.

The exact role of AhR in regulating cell proliferation and apoptosis remains unclear, since ligand-dependent activation of AhR has been shown to induce cell cycle arrest, proliferation, differentiation or apoptosis, depending on the cellular model used[2]. AhR can directly interact with retinoblastoma protein in hepatic cells, forming protein complexes that can efficiently block cell cycle progression by inducing G1 arrest, or to induce the expression of inhibitors of cyclin-dependent kinases, such as p27Kip1[2]. On the other hand, it has been suggested that AhR could play a stimulatory role in cell proliferation, either directly or by mediating a release from contact inhibition[3,4]. It has been reported that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) can release rat liver epithelial cells from contact inhibition by upregulating cyclin A expression and cyclin A/cdk2 activity[4].

We found that the PAHs that are both AhR ligands and only weak mutagens[5], several dioxin-like PCBs or their derivatives are able to release WB-F344 cells from contact inhibition. Furthermore, we investigated the effects of model flavonoid compounds, beta-naphthoflavone (AhR agonist), alpha-naphthoflavone (both agonist and antagonist of AhR) and 3'-methoxy-4'-nitroflavone (a weak agonist or antagonist of AhR) on contact inhibition and cytochrome P450 1A1 (CYP1A1) mRNA expression. All three compounds released WB-F344 cells from contact inhibition, again suggesting that various types of AhR ligands might produce a similar effect.

All active compounds induced both an increase of percentage of cells in S-phase of cell cycle and cell proliferation. The release from contact inhibition corresponded with

induction of CYP1A1 mRNA levels, suggesting that the AhR activation might play a role in the observed effects on cell cycle an cell proliferation. Using two model compounds, PCB 126 (a strong AhR ligand) and PCB 153 (di-ortho PCB congener which does not activate AhR), we studied their effects on proteins controlling G0/G1-S-phase transition and early S-phase progression. PCB 126 and not PCB 153 was found to strongly upregulate cyclin A expression. PCB 126 also induced a significant increase of cyclin D2 protein levels. Contrary to that, the levels of p27Kip1, a major cdk2 inhibitor known to be involved in contact inhibition of cell growth and S-phase entry, remained unaffected by either PCB 153 or PCB 126. Cdk2 activity is strongly inhibited in confluent WB-F344 cell population. Both total cdk2 and cyclin A/cdk2 complex activities were significantly increased by PCB 126 treatment. The expression of cyclin E, another cdk2 partner, was not affected by PCB 126. Despite the observed upregulation of cyclin D2, no increase of cdk4 activity was observed.

Taken together, our data support the hypothesis suggesting that various AhR ligands can release rat liver epithelial cells from contact inhibition by increasing cyclin A protein levels, which in turn leads to upregulation of cyclin A/cdk2 activity and initiation of DNA replication. This mechanism could play a role in tumor promoting effects of AhR-activating compounds in hepatocarcinogenesis.

References

1. Roskams T. A., Libbrecht L. and Desmet V. J.: *Semin. Liver Dis*. **23**, 385 (2003)
2. Puga A., Xia Y. and Elferink C.: *Chem. Biol. Interact.* **141**, 117 (2002)
3. Ma Q. and Whitlock J. P. Jr.: *J. Biol. Chem.* **272**, 8878 (1997)
4. Dietrich C., Faust D., Budt S., Moskwa M., Kunz A., Bock K. W. and Oesch F.: *Toxicol. Appl. Pharmacol.* **183**, 117 (2002)
5. Chramostová K., Vondráček J., Šindlerová L., Vojtěšek B., Kozubík A. and Machala M.: *Toxicol. Appl. Pharmacol.* **196**, 136 (2004)

Acknowledgement

This work was supported by grants 525/03/1527 from the Grant Agency of the Czech Republic and KJB6004407 from the Grant Agency of the Academy of Sciences, Czech Republic.

7L06

PGP MEDIATED MULTIDRUG RESISTANCE OF MOUSE LEUKEMIC CELL LINE L1210/VCR

Albert Breier[a,]*, Miroslav Barančik[b], Ivana Kupsáková[b], Jozef Orlický[a], Zdena Sulová[a], Vierka Boháčová[a], Peter Dočolomanský[a] and Roderik Fiala[a]

[a]*Institute of Molecular Physiology and Genetics, Slovak Academy of Sciences, Vlárska 5, 83334 Bratislava, Slovakia*
[b]*Institute for Heart Research, Slovak Academy of Sciences, Dúbravská cesta 9, 84005 Bratislava, Slovakia*

** E-mail: Albert.Breier@savba.sk*

Mouse leukemic cell line L1210/VCR used in our studies was prepared by adaptation of parental sensitive L1210 cell line to vincristine[1]. In L1210/VCR cells was observed also increased cross-resistance to other cytostatics such as vinblastine, doxorubicin, mitomycin C, and actinomycin D[2]. Important factors influencing the realization of MDR in L1210/VCR cells are flexibility of structure, lipophilicity and molecular weight of used cytostatics[2]. Multidrug resistant cell line L1210/VCR is characterized by over expression of PGP[3] but not by over-expression of other ATP dependent drug-efflux pump called "multidrug resistance protein" – MRP. Activities of glutathione S-transferase in L1210/VCR cells were not differing with activity of this enzyme in parental L1210 cells[4]. Extrusions of calcein AM or Fluo 3 (fluorescent PGP and MRP substrates) from L1210/VCR cells may be inhibited by PGP antagonizing agents (verapamil and cyclosporine) but not by inhibitors of MRP – probenecid. Inhibitors p38 and ERK mitogen activated protein kinase cascades were found to influence the MDR of our cells[5,6]. Derivatives of pentoxifylline represents potent inhibitors of PGP mediated MDR of L1210/VCR cells[7].

References

1. Poleková L., Barančík M., Mrázová T., Pirker R., Wallner J., Sulová Z. and Breier A.: *Neoplasma* **39**, 73 (1994)
2. Breier A., Drobná Z., Dočolomanský P. and Barančík M.: *Neoplasma* **47**, 100 (2000)
3. Fiala R., Sulová Z., El-Saggan A. H., Uhrík B., Liptaj T., Dovinová I., Hanušovská E., Drobná Z., Barančík M. and Breier A.: *Biochim. Biophys. Acta* **1639**, 213 (2003)
4. Boháčová V., Kvačkajová J., Barančík M., Drobná Z. and Breier A.: *Physiol. Res*. **49**, 447 (2000)
5. Barančík M., Boháčová V., Kvačkajová J., Hudecová S., Križanová O. and Breier A.: *Eur. J. Pharm. Sci.* **14**, 29 (2001)
6. Kišucká J., Barančík M., Boháčová V. and Breier A.: *Gen. Physiol. Biophys.* **20**, 339 (2001)
7. Kupsáková I., Rybár A., Dočolomanský P., Drobná Z., Stein U., Walther W., Barančík M. and Breier A.: *Eur. J. Pharm. Sci.* **21**, 283 (2004)

Acknowledgement

This work was supported in part by VEGA grant No 2/3122 and Slovak Science and Technology Assistance Agency grant no. APVT-51-013802.

7L07

THE ROLE OF CYTOSOLIC REDUCTASES IN ANTHRACYCLINE TOXICITY

Helena Kaiserová[a,*], Eva Kvasničková[a], Ladislava Schröterová[b] and Vladimír Geršl[b]

Department of Biochemical Sciences, Faculty of Pharmacy, and [b]Department of Pharmacology, Faculty of Medicine, Charles University, Heyrovského 1203, 50005 Hradec Králové, Czech Republic

* *E-mail:* kaiserova@faf.cuni.cz

Anthracycline antibiotics are among the most effective chemotherapeutics of cancer. However, their clinical use is limited by cardiotoxic side effects which are most seriously manifested by a development of cardiomyopathy upon chronic administration of cumulative doses of anthracyclines. Pathophysiology of anthracycline-induced myocardial damage is obviously complex. In addition to generally accepted theory of oxidative damage by reactive oxygen species formed by metabolization of anthracyclines via cytochomes P450[1] the formation of C13-hydroxymetabolites (C13-OL) has been considered as a potential culprit of chronic anthracycline toxicity[2]. Even though these metabolites affect mostly cardiac cells, their major amount is produced by liver[3]. Three cytosolic reductases have been described to form C13-OL: aldehyde reductase (ALR1, 1.1.1.2), carbonyl reductase (CR, 1.1.1.184) and dihydrodiol dehydrogenase (AKR1C2, 1.3.1.20)[4]. The aim of this work was to evaluate and compare the activities of these enzymes in a model daunorubicin-induced cardiomyopathy in rabbit *in vivo* and in isolated intact rabbit hepatocytes incubated with daunorubicin *in vitro*. Enzyme activities were determined spectrophotometrically at pH 6.0 and 8.5 which are the optimal pH of CR, AKR1C2 and ALR1 respectively. The second aim of the study was to evaluate the contribution of CR and AKR1C2 to reductase activity at pH 6.0. For *in vivo* study we used the well established model of experimental cardiomyopathy in rabbit[5]. There were three different groups of animals in the experiment. In experimental group (n=9) the animals were treated by daunorubicin 50 mg/m^2 once a week and the animals´ heart functions were monitored by invasive and noninvasive methods during the whole experiment. The animals of control group (n=10) were under shame operations. In both groups the animals were repeatedly anaesthetized with ketamin and once with pentobarbital (before sacrificing them). Intact group animals did not receive neither any medications nor other manipulations. This group served us

for evaluation of potential influence of anaesthesia and/or heart functions measurements on enzyme acitivities. Comparing experimental and control groups we assumed that repeated administration of daunorubicin to rabbits led to statistically significant ($p<0.05$) increase of reductase activity at pH 6.0 (CR+AKR1C2) to 140% of control. On the other hand the reductase activity at pH 8.5 remained unaffected. Comparing control and intact groups we found out that neither anaesthesia nor heart functions examination had effect on reductase activity. In the experiment *in vitro* we observed enzyme activities in isolated intact rabbit hepatocytes after their incubation with 2 μM and 10 μM daunorubicin or with cultivation media only (control). The time of incubation was 24 hours. We concluded that the presence of daunorubicin in cultivation media in both concentrations of the drug provoked significant ($p<0.05$) increase of reductase activities at pH 6.0 (CR+AKR1C2). In 2 μM concentration of daunorubicin the increase of activity was about 150% of control, while in 10 μM concentration of daunorubicin the increase was slighter – only about 120% of control. The activity at pH 8.5 remained on control levels. Which of those two enzymes was really responsible for the increase of activity after daunorubicin remained unknown. We performed one pilot inhibition study on subcellular fractions of liver homogenate of intact animals using specific inhibitors – quercitrin for CR and flufenamic acid (NSAID) for AKR1C2. By evaluation of inhibition curves we found out that AKR1C2 participated on the total acitivity at pH 6.0 of 15-25% so in this condition the major enzyme seemed to be CR. Nevertheless, at the time we were not competent to claim that it was CR which was responsible for an increase of reductase activity after daunorubicin treatment. Further studies are necessary. However, we found the „induction“ effect of daunorubicin potentially serious and worth of further investigations.

References

1. Keizer H.G., Pinedo H. M., Schuurhuis G. J. and Joenje H.: *Pharmac. Ther.* **47**, 219 (1990)
2. Cusack B. J., Mushlin P. S., Voulelis L. D., Li X., Boucek R. J. Jr. and Olson R. D.: *Toxicol. Appl. Pharm*. **118**, 177 (1993)
3. Pröpper D. and Maser E.: *Pharmacol. Toxicol.* **80**, 240 (1997)
4. Ax W., Soldan M., Koch L. and Maser E.: *Biochem. Pharmacol.* **59**, 293 (2000)
5. Klimtová I., Šimůnek T., Mazurová Y., Hrdina R., Geršl V. and Adamcová M.: *Hum. Exp. Toxicol*. **21**, 649 (2002)

Acknowledgement

This work was supported by grant 305/03/1511 from the Grant Agency, Czech Republic, and Research Centre LNB0125.

7L08

IDENTIFICATION OF GENOTOXICITY OF *o*-ANISIDINE AND ITS CARCINOGENIC POTENTIAL FOR HUMANS: EVIDENCE FOR *o* - ANISIDINE-DERIVED DNA ADDUCT FORMATION IN RATS AND *IN VITRO* AFTER ITS METABOLIC ACTIVATION BY HUMAN CYTOCHROMES P450

Marie Stiborová[a,]*, Markéta Mikšanová[a], Miroslav Šulc[a], Heinz H. Schmeiser[b] and Eva Frei[b]

[a]*Department of Biochemistry, Faculty of Science, Charles University, Albertov 2030, 128 40 Praha 2, Czech Republic*
[b]*Department of Molecular Toxicology, German Cancer Research Center, In Neuenheimer Feld 280, 69120 Heidelberg, Germany*

** E-mail: stiborov@natur.cuni.cz*

2-Methoxyaniline (*o*-anisidine) is an important pollutant and a potent carcinogen for rodents. The mechanism of its carcinogenicity was investigated in this study. Here, we used two direct independent methods, namely ^{32}P postlabelling and ^{14}C-labeled *o*-anisidine to show that *o*-anisidine binds covalently to DNA *in vitro* after its activation by human hepatic microsomes. We also investigated the capacity of *o*-anisidine to form DNA adducts *in vivo*. Male Wistar rats, used as animal models, were i.p. treated with *o*-anisidine, and DNA samples from various organs were analyzed by ^{32}P-postlabeling. Two *o*-anisidine-specific DNA adducts, identical to those found *in vitro*, were detected in urinary bladder, and to a lesser extent, in liver, kidney and spleen. The highest level of DNA adducts was found in the target organ of the *o*-anisidine carcinogenicity, the urinary bladder (4.1 adducts per 107 nucleotides). These results, demonstrating *o*-anisidine covalent binding to DNA after metabolic activation *in vitro* and *in vivo*, indicate a genotoxic mechanism of *o*-anisidine carcinogenicity. To provide information on the nature of the adducts formed *in vivo*, they were co-chromatographed in two independent systems with standardized deoxyguanosine adducts produced by reaction of an *o*-anisidine metabolite, N-(2-methoxyphenyl)hydroxylamine, with deoxyguanosine 3'-monophosphate *in vitro*. Based on this analysis, two DNA adducts found in rats were identified to be deoxyguanosine adducts derived from this *o*-anisidine metabolite. Formation of *o*-anisidine-DNA adducts during the *o*-anisidine oxidation by human hepatic microsomes, identical with those formed in rat *in vivo* indicates a potential of human hepatic microsomal enzymes to activate this carcinogen in human. Indeed, the proximate carcinogenic metabolite of *o*-anisidine, N-(2-methoxyphenyl)hydroxylamine, is produced by oxidation catalyzed with human hepatic microsomes. Understanding which human enzymes are involved in such metabolic activation is useful in evaluating individual susceptibility to this environmental carcinogen. We compared the ability of nine human hepatic microsome samples to catalyze *o*-anisidine oxidation. The role of specific cytochrome P450 (CYP) enzymes in the human hepatic microsomal metabolism of *o*-anisidine was investigated by correlating the CYP-dependent catalytic activities in each microsomal sample with the levels of individual metabolites formed by the same microsomes and by examining the effects of agents that can inhibit specific CYP

enzymes in *o*-anisidine metabolism. Based on these studies, we attribute most of *o*-anisidine oxidation in human microsomes to CYP2E1. Using microsomes from *Baculovirus* transfected insect cells expressing recombinant human CYP enzymes and purified CYP enzymes, the participation of this enzyme in *o*-anisidine oxidation was confirmed. The results of our study, the first report on the potential of the human microsomal CYP enzymes to contribute to the activation of *o*-anisidine, strongly suggest a carcinogenic potency of this rodent carcinogen for humans.

Acknowledgement

Supported by Grant Agency of the Czech Republic (grant 203/03/0283) and the Ministry of Education of the Czech Republic (grant MSM 113100001).

7L09

MODE OF CYTOTOXIC ACTION OF ORACIN METABOLITE – 11-DIHYDROORACIN (DHO)

Milan Miko[a,]* and Radomír Souček[b]

Department of Biochemistry and Microbiology, Slovak Technical University, Radlinského 9, 81237 Bratislava
[b]REaD VUFB, Slovakia

** E-mail: milan.miko@stuba.sk*

Oracin, 6-[2-(2-hydroxyethyl)aminoethyl]-5,11-dioxo-5,6-dihydro-11H-indenol-[1,2-*c*] isoquinoline, is a potential cytostatic drug for oral use and presently in phase II of clinical trials. Major advantages of this novel chemotherapeutic are the posibility of oral administration, its negative results in the Ames test on mutagenity, and the lack of cardiotoxicity. Metabolic studies on oracin have revealed that the principal metabolite in all laboratory animals is 11-dihydrooracin (DHO), which is produced by carbonyl reduction of the parent compound[1]. The aim of the present study was to study the cytotoxicity and mode of action of DHO. The degree of inhibition of ^{14}C-adenine and ^{14}C-valine incorporation into TCA-insoluble fraction of Ehrlich ascites carcinoma (EAC) cells was used to measure the cytotoxic activity (screening). The inhibitory effect was characterized by IC50 values (molar concentration of compound reguired for 50% reduction of the incorporation rate). The IC50 for adenine was 475.0 (DHO) and 66.0 mmol/l (oracin). Similar results were obtained with IC50 valine more than 600 (DHO) and 196 mmol/l (oracin). As is evident cytotoxic activity of DHO is much lower than that of the parent compound. In a first approach to determine the mode of action of the cytotoxicaly active DHO, the kinetics of DNA, RNA and protein synthesis inhibition were examined using isotope incorporation. This method has been described in detail[2].

Only when the time course is known it is possible to state at what time and concentration the inhibitory effect appears. Therefore we followed inhibitory effects of DHO upon biosynthesis of macromolecules, indicated by incorporation of ^{14}C-adenine and ^{14}C-valine into TCA-insoluble material of Ehrlich ascites cells. DHO inhibited incorporation of both precursors into appropriate macromolecules of EAC cells, the extent of inhibition being dependent on both time and concentration of DHO in the incubation medium. Incorporation of ^{14}C-adenine is more inhibited than incorporation of ^{14}C-valine as was shown already in screening.

As ^{14}C-adenine is incorporated into both DNA and RNA, we determined which of these nucleic acids was more sensitive. Biosynthesis of RNA, indicated by incorporation of ^{14}C-uridine was more inhibited than biosynthesis of DNA indicated by incorporation of ^{14}C-thymidine. Since the biosynthesis of macromolecules is a process requiring energy, we studied effect of DHO on energy-producing processes, i.e. on aerobic glycolysis and endogenous respiration of EAC cells. DHO stimulated aerobic glucose concentration and lactic acid formation in Ehrlich cells at all tested concentrations in little measure only. Inhibition of endogenous respiration of EAC cells was observed only at higher concentrations of DHO (more than 180 mmol/l). Exogenous respiration of EAC cells with succinate as substrate was decreased by concentrations of DHB higher than 700 mmol/l. As on of probable mode of action we studied membrane effect of DHO after 120 min incubation. DHO released intracellular proteins from Ehrlich cells and increased the activity of „marker“ enzyme lactate dehydrogenase in incubation medium in the highest concentrations only. It is necessary to take in mind also DHO intercalation properties for interpretation of its inhibition effects. Further, only recently topoisomerase II has been identified as the site of oracin action[3]. The effect of the drug on activity of topoisomerase II (EC 5.99.1.3) which was isolated from nuclei of EAC cells was examined by the relaxation and decantation assays. Oracin has been shown to be an inhibitor of topoisomerase II, and may represent a novel class of inhibitor for this enzyme.

References

1. Wsol V., Szotaková B., Skálová L. and Maser E.: *Chem. Biol. Interactions* **459**, 143 (2003)
2. Miko M., Poturnajová M. and Souček R.: *Neoplasma* **49**, 167 (2002)
3. Miko M., Turná J., Stuchlík S. and Souček R.: *12th Mediteranean Congress of Chemotherapy*, Marrakesh (Morocco), November 11-14, 2000, (Himmich H, ed.), Monduzi Editore, Bologna 2000, p. 331

Acknowledgement

This study was supported by the Grant Agency VEGA No 1/1173/04

7L10

GRAPE PRODUCTS AS A SOURCE OF NATURAL ANTIOXIDANTS

Alexey Kondrashov*

Institute of Medical Chemistry, 1st Faculty of Medicine, Charles University, Kateřinská 32, 12108 Praha, Czech Republic

** E-mail: Alexey.kondrasov@lf1.cuni.cz*

Grape products contain polyphenols, including flavonoids, which can inhibit low density lipoproteins (LDL) oxidation, reduce platelet aggregation and have been associated with lower rates of cardivascular desease[1-4]. These polyphenols are:

1. Flavonols (quercetin and quercetin glycosides, myricetin and myricetin glycosides)
2. Flavanols (catechin and epicatechin)
3. Phenolics (caftaric acid, caffeic acid, coutaric acid, coumaric acid, gallic acid)
4. Stylbenes (*trans*-, *cis*-resveratrol, astringin, astringinin)
5. Monomeric anthocyanins (delphinidin glucoside, cyanidin glucoside, penidin glucoside, petunidin glucoside, malvidin glucoside)
6. Polymeric phenols (procyanidin dimmers, polymeric anthocyanins, tannins)[5].

There are 7-10 times more total flavonoids in red wine compared to white wine[6,7]. Thus red wine polyphenolic compounds attracted considerable interest in many research works. The underlying pathogenesis of cardiovascular desease is atherosclerotic vascular desease. The etiology of atherosclerosis is very complicated and includes the interaction among four cell systems, such as endothelial cells, platelets, vascular smooth muscle cells and monocytes/macrophages[8-9]. Recent researches show that grape products, such as red wine, dealcoholized red wine and purple grape juice possess three properties unrelated to alcohol content that may reduce the rate of development of atherosclerotic narrowing of arteries. These properties are antiplatelet, antioxidant and improvement in endothelial cell function[6,8].

Human studies suggest that protective antioxidant effect of red wine is related to phenolic compounds including procyanidins by their direct action on peroxyl radicals, formed by oxidative attack on lipid membranes and lipoproteins, a potential mechanism for their protection of LDL *in vivo*[2,10,11]. Red wine polyphenols can make LDL-cholesterol less susceptible to *ex vivo* oxidation[12].

Red wine also improves endotelial function by enhancing platelet and endothelial production of nitric oxide (NO), then platelets and monocytes are less likely to stick on the wall and release their growth factors and initiate vascular smooth muscle cells (VSMC) proliferation and migration[8,13]. This migration of VSMC produces intimal thickening and causes narrowing of the arterial lumen[8,9,14].

This suggests that the polyphenols in red wine and other grape products inhibit the initiation of atherosclerosis by different mechanisms.

References

1. Frankel E. N., Kanner J., German G. B., Parks E. and Kinsella J. E.: *Lancet* **341**, 454 (1993)
2. Fauconneau B., Waffo-Teguo P., Huguet F., Decendit A. and Merillon J. M.: *Life Sci.* **61**, 2103 (1997)
3. Freedman J. E., Parker C. 3rd., Li L., Perlman J. A., Frei B., Ivanov V., Deak L. R., Iafrati M. D. and Folts J. D.: *Circulation* **103**, 2792 (2001)
4. Cook N. C. and Samman S.: *J. Nutr. Biochem.* **7**, 66 (1996)
5. Howard A., Chopra M., Thurnham D., Strain J., Fuhrman B. and Aviram M.: *Med. Hypotheses* **59**, 101 (2002)
6. Serafini M., Maiani G. and Ferro-Luzzi A.: *J. Nutr*. **128**, 1003 (1998)
7. Waterhouse A. L. and Teissedre P.: In *Wine* (Watkins T. R., ed.), Oxford University Press, Cary, NC, 1997, p. 12
8. Folts J. D.: *Adv. Exp. Med. Biol.* **505**, 95 (2002)
9. Rabbani L. E. and Loscalzo J.: *Atherosclerosis* **105**, 1 (1994)
10. Sies H.: *Eur. J. Biochem.* **215**, 213 (1993)
11. Esterbauer H., Gebicki J., Puhl H. and Jurgens G.: *Free Radic. Biol. Med.* **13**, 341 (1992)
12. Vinson J. A., Jang J., Dabbagh Y. A., Serry M. M. and Cai S.: *J. Agric. Food Chem.* **43**, 2798 (1995)
13. Fitzpatrick D. F., Hirschfield S. L. and Coffey R. G.: *Am. J. Physiol.* **265**, 774 (1993)
14. Navab M., Berliner J. A., Watson A. D., Hama S. Y., Territo M. C., Lusis A. J., Shih D. M., Van Lenten B. J., Frank J. S., Demer L. L., Edwards P. A. and Fogelman A. M.: *Arterioscler. Thromb. Vasc. Biol*. **16**, 831 (1996)

7L11

FLAVONOID STRUCTURAL REQUIREMENTS FOR MODULATION OF CYP19 ACTIVITY

Petr Hodek[a,]*, Zuzana Opltová[a], Stanislav Smrček[b] and Marie Stiborová[a]

[a]*Department of Biochemistry and* [b]*Department of Organic Chemistry, Faculty of Science, Charles University, Hlavova 2030/8, 128 40 Prague 2, Czech Republic*

* *E-mail:* hodek@natur.cuni.cz

Flavonoids represent a group of phytochemicals exhibiting a wide range of biological activities. Owing to the structural similarity with the estrogen skeleton flavonoids show an estrogenic or anti-estrogenic activity. They also inhibit enzyme activity of cytochrome P450 CYP19, aromatase, catalyzing aromatization of the A ring of male sex steroids resulting in estrogens. Since flavonoids in human diet may reduce the risk of hormone-dependent breast and prostate cancers, their structure–function relationship is extensively studied.

The most effective aromatase flavonoid inhibitor is synthetic 7,8-benzoflavone (ANF). As the presence of C4 oxo-group of the flavonoid skeleton (likely approaching the heme iron) seems to be crucial for the CYP19 inhibition, the substitution of oxygen atom for sulphur in this position was undertaken to increase the inhibition capability. Resulting thio-analogue of ANF (SANF), however, stimulated CYP19-dependent formation of estrone from androstendione (160% of control) and dibenzylfluoresceine metabolism (200% of control). To examine in details this unique flavonoid behavior, series of flavonoids (5,6-benzoflavone, flavone, 7-hydroxyflavone, 5,7-dihydroxyflavone) and their thio-analogues were examined for the modulation of the enzymatic activity of a steroidogenic CYP19. From all flavonoids (including their thio-analogues) tested in this study only thio-analogue of 5,6-benzoflavone exerted the stimulatory effect similar to SANF, the other compounds were only inhibitors of CYP19. For example, IC50 determined for flavone was 5 times lowered by sulphur substitution as originally expected for flavonoid thio-analogues. Flavonoid structural requirements for CYP19 activity stimulation and inhibition are discussed in the context of CYP19 homology model.

Acknowledgement

The work was supported by the grant MSM-113100001 from the Ministry of Education, Youth and Sports, Czech Republic.

7L12

EFFECT OF SELECTED ALKALOIDS WITH ANTI-INFLAMMATORY ACTIVITY ON CYTOCHROME P450 1A EXPRESSION AND ACTIVITY

Radim Vrzal*, Zdeněk Dvořák, Adéla Zdařilová and Jitka Ulrichová

Institute of Medical Chemistry and Biochemistry, Faculty of Medicine, Palacký University, Hněvotínská 3, 77515 Olomouc, Czech Republic

** E-mail: radim.vrzal@email.cz*

Cytochrome P450 (CYP) is a superfamily of enzymes, some of them participating in the phase I of biotransformation of xenobiotics. Two members of this family, CYP1A1 and CYP1A2, are involved in the metabolism of dioxins, nitrosamines and polycyclic aromatic hydrocarbons (PAHs). These compounds are transformed into reactive metabolites, which in turn modify the nucleotides and form adducts with the DNA. In addition, expression of CYP1A enzymes is highly inducible by dioxins and PAHs. The whole process induction-increased reactive metabolites production – DNA damage leads to carcinogenesis.

It is well known that some inflammatory cytokines like tumor necrosis factor alpha (TNFα) or interleukine 6 (IL-6), suppress the expression of P450 enzymes, including 1A1/2 isoforms. Since pro-inflammatory factors are involved in CYP1A regulation, the question "How can anti-inflammatory agents affect CYP1A expression?" arises.

In this study the effect of four structurally different alkaloids with anti-inflammatory activity i.e. colchicine (COL), sanguinarine (SA), chelerythrine (CHE), berberine (BE) on cytochrome P450 CYP1A1/2 activity is presented. Specific CYP1A enzyme activity was assessed as 7-ethoxyresorufine-O-deethylase activity (EROD) in human hepatoma cell line - HepG2.

Cells were treated for 48 h with tested compounds and in parallel with DMSO and/or TCDD (2 nM final) for negative and/or positive control, respectively. Colchicine (COL) in 1 μM concentration decreased CYP1A activity (15% inhibition), while other compounds (SA, CHE, BE) did not display any significant effect on CYP1A basal activity, assessed as EROD activity. In TCDD treated cells, the EROD activity raised cca 20 fold as compared with DMSO. Dioxin dependent CYP1A expression was strongly impaired in COL treated cell, in concentration dependent manner (98% inhibition for COL 1 μM). A bit milder inhibition was reached in BE treated cells (57% inhibition for BE 1 μM). SA and CHE did not affect CYP1A inducible expression, however, in 1 μM concentration significantly decreased EROD activity (30% and 19%, resp.). In case of SA, the decrease of EROD activity could be due to enzyme activity inhibition, when IC50 value of 16 μM was described[1].

In further experiments, the enzyme kinetic studies in human liver microsomes together with CYP1A protein content determination by western blot will be performed using tested compounds, to elucidate the cause for CYP1A inducible activity by tested compounds.

References

1. Vrba J., Kosina P., Ulrichová J. and Modrianský M.: *Toxicol. Lett*., in press (2004)

Acknowledgement

The authors gratefully acknowledge financial support from the Ministry of Education of the Czech Republic grant MSM 151100003 and Grant Agency of the Czech Republic grant 303/04/P074.

7P01

IMMUNOCHEMICAL SCREENING OF ISOFLAVONES IN THE RUTACEAE FAMILY

Michaela Vítková[a,*], Radka Koblovská[b], Zuzana Macková[b], Bořivoj Klejdus[c], Ladislav Kokoška[d] and Oldřich Lapčík[b]

[a]*Department of Biochemistry and Microbiology and* [b]*Department of Chemistry of Natural Compounds, Institute of Chemical Technology, Technická 3, 16628 Praha 6, Czech Republic*
[b]*Department of Chemistry and Biochemistry, Mendel University of Agriculture and Forestry, Brno, Czech Republic*
[c]*Institute of Tropical and Subtropical Crops, University of Agriculture in Prague, Czech Republic*

** E-mail: vitkovam@vscht.cz*

Isoflavones (3-phenyl chromones) are phytoalexins abundant in the Fabaceae, however their biosynthesis was described in over 20 other families, e.g. Rosaceae, Iridaceae, Asteraceae, Poaceae. Molecular biology data indicate occurrence of genes for isoflavonoid metabolic pathway even in higher number of families[1], although it is difficult to obtain the evidence of their biochemical activity. Recently, we have found isoflavonoids in the Rutaceae family[1]. In our recent research, we combine immunochemical approaches and HPLC-MS.

Five immunoassays for early products of the isoflavonoid pathway (i.e. daidzein, formononetin, genistein and biochanin A and their glycosides) were adapted for phytochemical studies. Tested plant material (leaves from apical branches) was frozen immediately after collecting and kept in –20 °C until lyophilized. A limited number of samples were divided to two aliquots of which one was dried in laboratory temperature in shadow. Dry samples were pulverized and extracted using methanol/water 7:3 (v/v) as the extraction solvent.

Immunoanalysis of crude extracts possesses semi-quantitative data, enabling to exclude negative samples and to optimize the amount of material for further analysis.

Pre-selected samples were fractionated by HPLC, analyzed again and the retention times of immunoreactive fractions were compared to those of authentic standards. Immunochemically positive samples were evaluated by HPLC-MS-SIM in order to confirm the identity of individual compounds (unpublished data).

Isoflavonoids were detected in all plants under study, belonging to the Rutaceae family (60 species from 7 genera). The samples dried in the laboratory temperature displayed by 50-150% higher content of isoflavonoid immunoreactivity than the freeze-dried samples. Three Ruta and six Citrus species were selected for detailed analysis. All Ruta and Citrus plants contained numerous isoflavonoids, aglycones as well as glycosides in a mg/kg level. The 4´-methoxyisoflavones (i.e. biochanin and formononetin) were more abundant than 4´-hydroxyisoflavones (i.e. genistein and daidzein). The Ruta species were relatively rich in sissotrin (i.e. 7-glucoside of biochanin A) while all Citrus species contained by several orders of magnitude lower levels of this substance.

References

1. Jung W., Yu O., Lau S.-M. C., O'Keefe D. P., Odell J., Fader G. and McGonigle B.: *Nat. Biotechnol.* **18**, 208 (2000)

Acknowledgement

This study was supported by Grant 525/03/0352 from the Grant Agency of the Czech Republic.

7P02

EVALUATION OF THE CYTOTOXIC AND MUTAGENIC POTENTIAL OF BERBERINE AND THE ABILITY TO INDUCE THE APOPTOSIS

Silvia Letašiová[a,*], Marica Theiszová[a], Luboš Čipák[b] and Soňa Jantová[a]

[a]*Department of Biochemistry and Microbiology, Slovak Technical University, Radlinského 9, 81137 Bratislava, Slovakia*
[b]*Department of Tumor Therapy, Institute of Experimental Oncology, Slovak Academy of Sciences, Bratislava, Slovakia*

** E-mail: silvia.letasiova@stuba.sk*

Berberine, an isoquinoline alkaloid, is an active component of *Berberis aquifolium* (Oregon grape), *Berberis aristata* (tree tumeric), *Berberis vulgaris* (barberry), *Coptis chinensis* (coptis or goldenthread) and *Hydrastis canadensis* (goldenseal). It occurs in the rhizomes, outer bark of stems and roots of the plants. Berberine has a wide range of pharmacological and biochemical effects[1]. It has demonstrated significant antimicrobial

activity against a variety of microorganisms including bacteria, fungi, protozoans, viruses, chlamydia, and helminths. It can also be used as an antidiarrhes, antihypertension, antiarrhytmias and antiinflamantory agent. Berberine is demonstrated to possess antitumor activity[2].

In our previous experiments we have investigated the effect of berberine on proliferation, cell cycle profile and apoptosis in human tumour HeLa and murine leukemia L1210 cell lines. We have shown that berberine induces apoptosis in L1210 cells[3]. In this study, the growth inhibition assay was used for another human cancer and mouse non-cancer cell lines. Furthermore, cell cycle effects and apoptosis/necrosis in human promonocytic U937 and in mouse fibroblast 3T3 cell lines treated with berberine were monitored.

Cytotoxicity was measured by direct counting of cells in Burker chamber. Berberine acted cytotoxically on both cell lines. The mouse fibroblast 3T3 cells were much more sensitive to berberine treatment than U937 cells. The highest tested concentrations had an acute cytotoxic effect manifested by degeneration of cell population. The value of IC100 was below 100 $\mu g\ ml^{-1}$ for U937 cells and 50 $\mu g\ ml^{-1}$ for 3T3 cells. For cancer cell line after the long-term influence the value of IC50 was found to be less than 4 $\mu g\ ml^{-1}$. It corresponds with the criterion of the National Cancer Institute, that if the IC50 value is less than 4 $\mu g\ ml^{-1}$, the compound can be classified as a potential anticancer drug. The cell cycle profile was measured by flow cytometry. DNA was stained by propidium iodide. No effect of berberine on cell cycle profile of U937 and 3T3 cells was detected, however, berberine induced apoptosis of U937 cells. The apoptotic DNA fragmentation – apoptosis was measured by electrophoresis in agarose gel complemented with ethidium bromide. Analysis showed berberine induced apoptosis of cancer U937 cell line but did not induce apoptosis of non-cancer 3T3 cell line. On the other hand, at the highest concentrations cell lysis/necrosis of berberine-treated 3T3 cells was observed. The mutagenic potentiator effect of berberine was determined by the hypoxanthin – guanine – phosphoribosyl transferase (HGPRT) assay. The results obtained suggest that berberine is not mutagenic compound.

References

1. Kuo C. L., Chi C. W. and Liu T. Y.: *Cancer Lett*. **203**, 127 (2004)
2. Tang W., Hemm I. and Bertram B.: *Planta Med.* **69**, 97 (2002)
3. Jantová S., Čipák L., Čerňaková M. and Košťálová D.: *J. Pharm. Pharmacol.* **55**, 1143 (2003)

Acknowledgement

This study was supported by the Grant Agency VEGA (1/1173/04).

7P03

DETERMINATION OF MIDAZOLAM FROM PERFUSE MEDIUM AS A PROBE SUBSTRATE OF CYTOCHROME P450 3A1/2 ACTIVITY IN RATS

Jan Juřica[a,*], Miroslav Dostálek[b], Josef Tomandl[a] and Eva Hadašová[b]

[a]*Institute of Biochemistry and* [b]*Institute of Pharmacology, Faculty of Medicine, Masaryk Univerzity, Komenského nám. 2, 66243 Brno, Czech Republic*

** E-mail: jurica@med.muni.cz*

Midazolam, CAS [59467-70-8] is a short-acting benzodiazepine, commonly used in intravenous anesthesia induction, short-term sedation and oral hypnotic medication[1].

Hydroxylation of midazolam is used as a valuable tool for measurement of metabolic activity of the 3A1/2 isoform of cytochrome P-450 in rats (CYP3A1/2)[2].

There is an orthologue CYP3A4/5 isoform in humans, which shows 78% similarity to CYP3A1/2 in amino acid sequence. Due to this similarity it is possible to predict the metabolic activity of human CYP3A4/5 by the measurement of rat CYP3A1/2 activity[3].

CYP3A4/5 is the most abundant CYP enzyme expressed in human liver and small intestine[4] and plays a pivot role in the metabolism of almost half of commonly used drugs including nifedipine, felodipine, cyclosporine, erythromycine, midazolam, triazolam, alprazolam, statins, verapamil, dilthiazem, testosteron, etc.[5].

We describe a new, simple, rapid, sensitive and reproducible HPLC method for determination of midazolam and its three metabolites, 1'- and 4'-hydroxymidazolam and 1',4'-dihydroxymidazolam from perfuse rat liver medium. After simple one step extraction, the samples were analyzed on C18 column (150 x 4.6 mm, 5µm) using UV detection. The mobile phase consisted of 10 mM acetate buffer, pH 4.7 and acetonitrile, the flow rate was 1 ml/min. The retention times of all compounds including diazepam as an internal standard were up to 11 minutes.

This method is applicable for modeling and description of possible pharmacological interactions on rat CYP3A1/2 or human CYP3A4/5, respectively.

References

1. Reves J. G., Fragen R. J., Vinik H. R. and Greenblatt D. J: *Anesthesiology* **62**, 310 (1985)
2. Kobayashi K., Urashima K. and Shimada N., Chiba K.: *Biochem. Pharmacol*. **63**, 889 (2002)
3. Souček P. and Gut I.: *Xenobiotica* **22**, 83 (1992)
4. Guengerich F. P: *Annu. Rev. Pharmacol. Toxicol.* **39**, 1 (1999)
5. Anzenbacher P. and Anzenbacherová E: *Cell. Mol. Life Sci.* **58**, 737 (2001)

Acknowledgement

This work was supported by the projects from the Ministry of Education, Youth and Sports, Czech Republic FRVŠ G3/447 and MSM 141100001. The authors gratefully acknowledge Hoffmann-LaRoche, Basel, Switzerland for the supply of pure midazolam.

7P04

APPLICABILITY OF THE BACTERIAL SHORT-TERM TESTS TO DETECT GENOTOXIC ACTIVITY OF BISQUATERNARY AMMONIUM SALTS

Tomáš Majtán[a,*] and Viktor Majtán[b]

[a]*Institute of Molecular Biology, Slovak Academy of Sciences, Dúbravská cesta 21, 84551 Bratislava, Slovakia*
[b]*Institute of Preventive and Clinical Medicine, Slovak Medical University*
** E-mail: Tomas.Majtan@savba.sk*

The bisquaternary ammonium salts (BQAS) belong to the group of cationic surface active compounds. Due to their surface activity they were succesfully used for the elimination of plasmids, mainly of resistance plasmid[1]. These compounds represent a pharmaceutically interesting substances with surfactant, solubilizing and antimicrobial properties and they represent promissing components of disinfectants. Bacterial short-term tests are widely used to detect potential carcinogens and mutagens, at least the large fraction of these agents which are genotoxic, i.e. which act directly or indirectly on the genome. Efforts have been made to develop test systems relying on a single strain. Phage induction assay[2] is based on the ability of chemicals to induce prophage of a lysogenic strain, i.e. to induce a vegetative evolution of temperate phage. SOS chromotest[3] is a colorimetric assay which measures the expression of genes induced by genotoxic agents in *Escherichia coli* PQ37, by means of a fusion with the structural gene for β-galactosidase. Applicability of both tests is to detect DNA damaging agents which induce SOS response. We report here about the genotoxic activity of 5 newly-synthesized BQAS derived from L-tartaric acid in both prophage induction test and SOS chromotest. In the prophage induction assay BQAS were used at their subinhibitory concentrations. The evaluation of results showed that the strongest induction of a prophage of the lysogenic *Salmonella typhimurium* strain was caused by the compounds with decyl and dodecyl at 1/2 of the MIC. In the SOS chromotest, which was done in absence of metabolizing mixture S9, we found no genotoxic activity of BQAS tested independently of long of alkyl chain. The main advantage of these tests is that they require only one tester strain and response is obtained within a few hours.

References

1. Sýkora P., Čeplíková V., Foltýnová Z., Horniak L. and Ebringer L.: *Folia Microbiol.* **36**, 240 (1991)
2. Moreau P., Bailone A. and Devoret R.: Proc. Natl. Acad. Sci. U.S.A. **73**, 3700 (1976)
3. Quillardet P., Huisman O., Dari R. and Hofnung M.: Proc. Natl. Acad. Sci. U.S.A. **79**, 5971 (1982)

7P05

MODE OF ACTION OF CYTOTOXIC ACTIVE HALOGENOSALICYLATECOPPER(II) COMPLEXES

Silvia Letašiová*, Milan Miko, Milan Melník and Dušan Valigura

Department of Biochemistry and Microbiology, Slovak Technical University, Radlinského 9, 81137 Bratislava, Slovakia

** E-mail: silvia.letasiova@stuba.sk*

Screening of cytotoxic activity of 29 novel derivatives of halogenosalicylate copper(II) complexes (HSCC) using Ehrlich ascites carcinoma (EAC) cells has been carried out *in vitro*. Derivatives of salicylic acid (4-Cl, 5-Cl, 3,5-Cl_2, 5-Br, 3,5-Br_2, 5-I and 3,5-I_2) were somewhat more active than salicylic acid. Copper(II) complexes of the respective acids are even more active than only single acids.

On the basis of primary screening one of the most active compound, namely dihydrate of bis(3,5-diiodosalicylato)copper(II) complex (compound 7.2) was chosen for further detail biochemical studies. To define further the mechanism of action of selected drug, the kinetics of DNA, RNA and protein synthesis were examined using isotope incorporation. This method has been described in detail[1]. Biosynthesis of nucleic acids in EAC cells was followed using ^{14}C-adenine and ^{14}C-valine into acid-insoluble fractions of the cells. Compound 7.2 inhibited incorporation of both precursors into appropriate macromolecules of Ehrlich cells, the extent of inhibition being dependent on both time and concentration of the drug in the incubation medium. At the highest concentration tested nearly complete inhibition of incorporation of both precursors occured in cancer cells. The results appear to indicate that the inhibition takes place immediately on addition of the drug in the cancer cell suspension, i. e. without the appearance of a lag phase. Biosynthesis of proteins, indicated by incorporation of ^{14}C-valine, is more sensitive than the biosynthesis of nucleic acids.

As ^{14}C-adenine is incorporated into both DNA and RNA, we determined which of these nucleic acids was more sensitive. We observed similar effects as in the case of ^{14}C-adenine and ^{14}C-valine. The complete inhibition of thymidine and uridine incorporation was reached at the highest concentration of the drug (75 and 150 μmol/l). The lowest concetration of the drug inhibited incorporation of both precursors in proportion to the tested concentrations. The incorporation of all precursors was followed in the incubation medium containing glucose as a sole energy source.

Since as macromolecule biosynthesis is an energy-requiring process, we followed the effects of the drug (7.2) on energy-producing processes, i. e. on aerobic glucose consumption, lactic acid formation, content of total (T-SH) and non-protein (NP-SH) thiol groups, endogenous as well as exogenous respiration in Ehrlich ascites carcinoma cells. Ehrlich cells consumed glucose from the medium linearly with the time, even if its concentration fell by more than 50 % of its original value. A likewise proportional increase in lactate concentration in the medium was seen in control cells. Drug in the concentration range from 18.75 to 150 μmol/l inhibited aerobic glycolysis of EAC cells proportionally to the tested concentrations. Inhibition of glycolysis is the consequence of

decreased level of both thiol groups in Ehrlich cells. At the same time all 29 compounds stimulated endogenous respiration of cancer cells. On the other hand, drug 7.2 inhibited exogenous respiration with succinate as substrate in the concentration range from 6 to 35 μmol/l.

References

1. Miko M. and Devinsky F.: *Inter. J. Biochem. Cell Biol*. **30**, 1253 (1998)

Acknowledgement

This study was supported by the Grant Agency VEGA (1/1173/04).

7P06

DNA ADDUCTS FORMED BY ANTICANCER DRUG ELLIPTICINE *IN VIVO*

Dagmar Aimová[a,*], Tereza Somolová[a], Eva Frei[b] and Marie Stiborová[a]

[a]*Department of Biochemistry, Faculty of Science, Charles University, Albertov 2030, 128 40 Praha 2, Czech Republic*
[b]*Department of Molecular Toxicology, German Cancer Research Center, In Neuenheimer Feld 280, 69120 Heidelberg, Germany*

** E-mail: aimova@seznam.cz*

Ellipticine is a potent antineoplastic agent, whose mode of action is considered to be based mainly on DNA intercalation and/or inhibition of topoisomerase II. We found that ellipticine also forms covalent DNA adducts *in vitro* and that the formation of DNA adducts is dependent on the activation of ellipticine by cytochrome P450 (CYP)[1]. This implicates the potential importance of several CYPs in producing more active ellipticine metabolite(s). Here, we investigated the capacity of ellipticine to form DNA adducts *in vivo*. Male Wistar rats were treated with ellipticine, and DNA from various organs was analyzed by ^{32}P-postlabeling. Ellipticine-specific DNA adduct patterns, similar to those found *in vitro*, were detected in most test organs. The highest level of DNA adducts was found in liver, followed by spleen, lung, kidney, heart and brain. One major and one minor ellipticine-DNA adducts were found in DNA of all these organs of rats exposed to ellipticine. Besides these, two or three additional adducts were detected in DNA of liver, kidney, lung and heart. The predominant adduct formed in rat tissues *in vivo* was identical to the deoxyguanosine adduct generated in DNA by ellipticine *in vitro*. Correlation studies showed that the formation of this major DNA adduct *in vivo* is mediated by CYP3A1- and CYP1A-dependent reactions. The additional aim of the present work was to study whether ellipticine could influence the expression of the major CYPs participating in its metabolism. An expression of CYP1A1/2 proteins in

liver of rats of both sexes is strongly induced by treatment of animals with ellipticine. The expression levels of CYP1A1/2 in treated rats are one order of magnitude higher than those in control animals. The CYP1A1/2 induction is strongly dependent on concentration of ellipticine applied to experimental animals and on the time of their exposition. The induction of other isoforms of cytochromes P450 (CYP2B, 2E1, 3A) was negligible. The results presented here are the first report showing the formation of CYP-mediated covalent DNA adducts by ellipticine *in vivo*, and confirm the formation of covalent DNA adducts as a new mode of ellipticine action. In addition, they indicate that a long-term treatment of humans with ellipticine might stimulate its pharmacological efficiency against cancer diseases.

References

1. Stiborová M., Bieler C. A., Wiessler M. and Frei E.: *Biochem. Pharmacol.* **62**, 1675 (2001)

Acknowledgement

Supported by Grant Agency of the Czech Republic (203/01/0996) and the Ministry of Education, Youth and Sports, Czech Republic (grant MSM 1131 00001).

7P07

IDENTIFICATION OF HUMAN ENZYMES GENERATING DNA ADDUCTS FROM THE ENVIRONMENTAL POLLUTANT 3-NITROBENZANTHRONE (3-NBA) AND EVALUATION OF ITS POTENCY TO INDUCE THESE ENZYMES

Helena Rýdlová[a,*], Marie Stiborová[a], Heinz H. Schmeiser[b], Eva Frei[c], Volker M. Arlt[d] and David Phillips[d]

[a]*Department of Biochemistry, Faculty of Science, Charles University, Albertov 6, 12843 Praha 2, Czech Republic*
[b]*Division of Molecular Toxicology, German Cancer Research Center, Heidelberg, Germany*
[c]*Division of Molecular Biology, German Cancer Research Center, Heidelberg, Germany*
[d]*Section of Molecular Carcinogenesis, Institute of Cancer Research, Sutton, United Kingdom*

** E-mail: helili@centrum.cz*

Determining the capability of humans to metabolize the potent mutagen 3-NBA and understanding which human enzymes are involved in its activation are important in the assessment of individual susceptibility to this environmental contaminant found in diesel exhaust and ambient air pollution[1]. We compared the ability of human hepatic

microsomal and cytosolic samples to catalyze DNA adduct formation by 3-NBA. Using the ^{32}P-postlabelling method we found that both hepatic subcellular fractions were competent to mediate formation of DNA adducts qualitatively similar to those found *in vivo* in rats. To define the role of human microsomal and cytosolic reductases in the activation of 3-NBA, we investigated the modulation of 3-NBA-DNA adduct formation by cofactors or selective inhibitors of the reductases present in both subcellular fractions. Most of the activation of 3-NBA in human hepatic microsomes is mediated by NADPH:CYP reductase, but participation of CYP1A1/2, 2B6 and 2D6 cannot be excluded. In cytosols, NAD(P)H:quinone oxidoreductase (NQO1) is the major enzyme involved in 3-NBA activation. With human recombinant enzymes the role of these enzymes in the formation of 3-NBA-DNA adducts was confirmed. The role of conjugation enzymes (sulfotransferases, SULTs, and N-acetyltransferases, NATs) was also examined. We found the major and minor role of hepatic SULTs and NATs in activation of 3-NBA, respectively. Using cytosols containing individual human recombinant SULTs, participation of SULT1A1 and, to a lesser extent, SULT1A2 in 3-NBA activation was corroborated.

Another target of the study was to evaluate the effect of 3-NBA on the expression of major enzymes activating this pollutant. Utilizing Western blotting and consecutive immunoquantification employing chicken polyclonal antibodies raised against various CYPs, the expression of CYP1A1/2 was found to be strongly induced in rats treated with the compound. Furthermore, EROD (7-ethoxyresorufin O-deethylation) activity, corresponding to both CYP1A1 and 1A2, was significantly increased in the pre-treated rats. The activity of NQO1 was also enhanced in these rats. The results demonstrate the potential of human NADPH:CYP reductase, CYP1A1/2, 2B6 and 2D6 as well as NQO1 and SULT1A1/2 to contribute to the metabolic activation of 3-NBA and imply the potency of this chemical to generate precarcinogenic lesions in humans.

References

1. Enya T., Suzuki H., Watanabe T., Hirayama T. and Hisamatsu Y.: *Environ. Sci. Technol.* **31**, 2772 (1997)

Acknowledgement

Supported by the grant MSM 113100001 from the Ministry of Education, Youth and Sports, Czech Republic.

7P08

EFFECTS OF NITROBENZOATECOPPER(II) COMPLEXES ON ENERGY-REQUIRING AND ENERGY-PRODUCING PROCESSES IN EHRLICH CELLS

Milan Miko[a,*], Jarmila Lakomá[a], Milan Melnik[b] and Dušan Valigura[b]

[a]*Department of Biochemistry and Microbiology and* [b]*Department of Inorganic Chemistry, Slovak Technical University, Radlinského 9, 81137 Bratislava, Slovakia*

** E-mail: milan.miko@stuba.sk*

The primary aim of our group is the identification and evaluation of potencial anticancer agents. A new class of nitrobenzoatecopper(II) complexes has been synthesized and studied on energy-requiring and energy-producing processes in Ehrlich ascites carcinoma (EAC) cells *in vitro*.

The first step in our program is primary biochemical screening of cytotoxic activity *in vitro*. On the basis of primary screening, one of the most active compound, namely monohydrate of diethanol-tetrakis(4-nitrobenzoate)copper(II) complex (compound number 4.4.) was chosen for further detailed studies. As an energy-requiring process, we followed biosynthesis of macromolecules (DNA, RNA and proteins) in time and concentration dependence.

Effect of the compound on the kinetics of DNA, RNA and protein synthesis inhibition were examined using isotope incorporation. At the highest concentration (100 μmol/l), biosynthesis of proteins indicated by the incorporation of ^{14}C-valine into TCA insoluble fraction of EAC cells was fully inhibited immediately after addition of the drug to the suspension of the cells. Biosynthesis of nucleic acids indicated by incorporation of ^{14}C-adenine was also inhibited, however the degree of inhibition was about 90 %. The lower concentration of the drug decreased the incorporation of ^{14}C-valine by higher degree than incorporation of ^{14}C-adenine.

As ^{14}C-adenine is incorporated into both DNA and RNA, we determined which of these nucleic acids were more sensitive. The highest concentrations of the drug (100 and 50 μmol/l) inhibited biosynthesis of DNA (incorporation of ^{14}C-thymidine) and RNA (incorporation of ^{14}C-uridine) approximately at the same degree (80-90 %). The lowest concentration (12.5 μmol/l) of the compound no. 4.4. also inhibited incorporation of both precursors, however incorporation of ^{14}C-thymidine was more sensitive. The incorporation of all precursors was followed in the medium containing glucose as sole energy source.

Since biosynthesis of macromolecules is an energy-requiring process, therefore we followed effect of compound no 4.4. on energy-yielding processes in EAC cells. We studied effect of the drug on aerobic glycolysis of EAC cells after two hour incubation. Glycolysis was studied by measuring of glucose consumption and lactate formation. All tested concentrations inhibited aerobic glycolysis in concentration and time dependence. In order to outline the possible mechanism of inhibition of aerobic glycolysis, levels of total (T-SH) and non-protein (NP-SH) thiol groups were investigated. The concentration of both thiol groups was decreased in concentration dependence. The decrease of T-SH groups was higher than NP-SH groups.

At the same time, we studied effect of the selected drug on endogenous as well as exogenous respiration of EAC cells with succinate as substrate. All tested concentrations (45 – 230 μmol/l) stimulated endogenous respiration. The maximum of stimulation of endogenous respiration was induced by concentrations 90 to 140 μmol/l. The drug is able released the endogenous respiration previously inhibited by oligomycine. Therefore, the mode of action of the drug is similar to the uncoupler of oxidative phosphorylation.

The observed inhibition of macromolecular biosynthesis can be the consequence of the direct drug interference with energy-producing processes in tumour cells, or interaction of the drug with the biological membranes of the cells.

Acknowledgement

This study was supported by the Grant Agency VEGA (1/1173/04).

7P09

BINDING OF PEROXIDASE-MEDIATED RADICALS OF 1-PHENYAZO-2-NAPHTHOL (SUDAN I) AND ELLIPTICINE TO GLYCOPROTEINS

Václav Martínek[a,*], Karel Bezouška[a], Miroslav Šulc[a], Eva Frei[b] and Marie Stiborová[a]

[a]*Department of Biochemistry, Faculty of Science, Charles University, Hlavova 2030/8, 12840 Praha 2, Czech Republic*
[b]*Division of Molecular Toxicology, German Cancer Research Center, Heidelberg, Germany*

** E-mail: vacmar@natur.cuni.cz*

Glycosylation is one of the major naturally occurring modifications of the covalent structure of proteins. Although much is known about the structure and biosynthesis of oligosaccharides in glycoproteins, the central question of how glycosylation contributes to the glycoprotein structure and function is not entirely clear. Many specific effects have been observed, such as regulation of intracellular traffic and localization of proteins, modulation of their immunological properties, participation in cell-cell interactions, stabilization of protein conformation, protection of proteins from proteolysis, enhancement in their solubility, and many others[1]. In the present work, we show another role of carbohydrate moiety of glycoproteins; it might also contribute to protect proteins from covalent modification of their molecules by radicals.

To study this subject, several model proteins with different degree of glycosylation were examined for their modification by radicals generated from two xenobiotics, 1-phenylazo-2-hydroxynaphthalene (Sudan I) and ellipticine. Both these chemicals form radicals during their oxidation by peroxidases. Sudan I is a liver and urinary bladder carcinogen in mammals. This carcinogen is metabolically activated by cytochromes P450 and peroxidases to reactive species binding covalently to nucleic acids and

proteins[2,3]. Ellipticine is an alkaloid exhibiting significant antineoplastic and anti-HIV activities. This anticancer agent is oxidized by peroxidases via a radical mechanism and this reaction might participate in enhancing its pharmacological efficiencies[4]. The levels of binding of radicals generated from both xenobiotics by peroxidases are dependent on degree of protein glycosylation, being highest for non-glycosylated proteins: human serum albumin, bovine serum albumin and lysozyme, and followed by asilofetuin > calf fetuin > chicken ovomucoid > Tamm-Horsfall glycoprotein and a1-acid glycoprotein. The results showed in the study demonstrate for the first time a novel role of carbohydrate parts of glycoprotein molecules, namely, their protective effects against modification by xenobiotic-derived radicals.

References

1. Wang C., Eufemi M., Turano C. and Giartosio A: *Biochemistry* **35**, 7299 (1996)
2. Stiborová M., Frei E., Schmeiser H. H., Wiessler M. and Hradec J: *Carcinogenesis* **11**, 1843 (1990)
3. Stiborová M., Martínek V., Rýdlová H., Hodek P., Schmeiser H.H. and Frei E: *Cancer Res*. **62**, 5678 (2002)
4. Frei E., Borek-Dohalska L., Wiessler M. and Stiborová M.: *Proc. Am. Assoc. Cancer Res*. **42**, 252 (2001)

Acknowledgement

Supported by the Ministry of Education, Youth and Sports, Czech Republic (MSM 113100001).

7P10

CHLOROCATECHOL CATABOLIC ENZYMES FROM *ACHROMOBACTER XYLOSOXIDANS* A8

Zdeněk Chodora[a,*], Věra Jenčová[a], Hynek Strnad[a], Miluše Hroudová[a] and Václav Pačes[b]

[a]*Department of Biochemistry and Microbiology, Institute of Chemical Technology, Technická 5, 16628 Praha 6, Czech Republic*
[b]*Institute of Molecular Genetics, Academy of Sciences of the Czech Republic*

** E-mail: chodoraz@vscht.cz*

Pollution with chloroaromatic compounds is a serious problem. Several bacterial strains that degrade chloroaromatics under aerobic or anaerobic conditions have been isolated from contaminated sites. It was shown that often the modified *ortho*-cleavage pathway (MOCP) is involved in the degradation process under aerobic conditions. In

MOCP are degraded chlorocatechols that are generated from various chlorinated aromatics throughout convergent pathways.

Chlorocatechols are mainly catabolized by MOCP that involves four enzymes, namely chlorocatechol-1,2-dioxygenase, chloromuconate cycloisomerase, dienelactone hydrolase and maleylacetate reductase and by that chlorocatechols are demineralized to the β-ketoadipate, that is intermediate for the TCA cycle. The chlorocatechol degradation genes are worldwide distributed and the frequent location of these genes on conjugative plasmids indicates that their transfer can explain the widespread distribution. Moreover, some chlorocatechol catabolic genes are the part of IS elements or in their proximity are genes encoding transposases. This also suggests the possibility of a horizontal transfer.

This work is focused on characterisation of genes of modified *ortho*-cleavage pathway from the *Achromobacter xylosoxidans* A8 (mocpR-ABCD). The *A. xylosoxidans* strain A8 was isolated in the Czech Republic from PCB contaminated soil. It is able to use 2-chlorobenzoate (2-CB) and 2,5-dichlorobenzoate (2,5-DCB) as sole sources of carbon and energy. The genome of this strain contains two large conjugative plasmids pA81 and pA82. A cluster of genes homologous to genes of a modified *ortho*-cleavage pathway was identified during the sequence analysis of pA81. Hybridization analysis confirmed that mocp genes are located only on pA81, which is thereby essential for the degradation of CBs by this strain.

Individual genes were cloned and expressed in *Escherichia coli* BL21(DE3). Proteins of chlorocatechol-1,2-dioxygenase (Mocp A), chloromuconate cycloisomerase (Mocp B), dienelactone hydrolase (Mocp C) and maleylacetate reductase (Mocp D) were purified to a homogenicity suitable for enzyme assays. Basic characteristics were accomplished: molecule weighs, subunit composition and isoelectric point were described. Optimum conditions (temperature, pH and ionic strength) of reaction were defined and kinetic parameters were confirmed by reactions with suitable substrates.

7P11

PLASMID PA81 FROM CHLOROBENZOATE DEGRADING STRAIN *ACHROMOBACTER XYLOSOXIDANS* A8

Věra Jenčová[a,*], Zdeněk Chodora[a], Hynek Strnad[a] and Václav Pačes[b]

[a]*Department of Biochemistry and Microbiology, Institute of Chemical Technology, Technická 5, 16628 Praha 6, Czech Republic*
[b]*Institute of Molecular Genetics, Academy of Sciences of the Czech Republic*

* *E-mail:* jencova@tc.cas.cz

The *Achromobacter xylosoxidans* strain A8 was isolated in the Czech Republic from PCB contaminated soil. It is able to use 2-clorobenzoate (2-CB) and 2,5-dichlorobenzoate (2,5-DCB) as sole sources of carbon and energy. We analyzed the *A. xylosoxidans* A8 with the aim to characterize genes involved in the degradation of CBs.

The genome of this strain contains two large conjugative plasmids pA81 (~ 100 kbp) and pA82 (~ 250 kbp). The genes encoding enzymes involved in the degradation pathway were found to be situated directly on the plasmid pA81. On the basis of the pA81 sequence analysis we identified clusters responsible for the plasmid replication, conjugative transfer, stable inheritance, degradation of xenobiotics and transposition. Comparison of the plasmid „backbone" constituted by regions of replication control and stability and two Tra1/Tra2 regions responsible for conjugative transfer shows the highest similarity to the backbone of IncPß plasmids. Members of this plasmid group can replicate in broad-host spectra of organisms and usually carry multi-antibiotic resistance determinants and degradative cassettes. Concerning degradation genes of pA81, we identified *ortho*-halobenzoate dioxygenase responsible for the initial aromatic ring hydroxylation, cluster of modified *ortho*-cleavage pathway responsible for following chlorocatechol degradation and cluster encoding salicilate hydroxylase allowing salicylate degradation by this strain. These catabolic genes are either the part of IS elements or in their proximity are located genes encoding transposases. Sequence comparison of these determinants shows very high identity to clusters identified in organisms from the very different part of the world.

The similarity of pA81 identified clusters to those identified in organisms from widely varying points on the globe underscores the importance of horizontal gene transfer in the global evolution of microbial communities. In this regard, pA81 and other related mobile elements may play central roles

7P12

PEROXIDASE-MEDIATED ELLIPTICINE-DNA ADDUCT FORMATION EXPLAINS THE SELECTIVE EFFICIENCY OF THIS ANTICANCER DRUG AGAINST BREAST CANCER AND LEUKEMIA

Jitka Poljaková[a,*], Jan Šejbal[a], Miroslav Šulc[a], Eva Frei[b] and Marie Stiborová[a]

[a]*Department of Biochemistry, Faculty of Science, Charles University, Albertov 2030, 128 40 Prague 2, Czech Republic*
[b]*Department of Molecular Toxicology, German Cancer Reserach Center, Im Neuenheimer Feld 280, 69 120 Heidelberg, Germany*

** E-mail: Jitka.Poljakova@seznam.cz*

Ellipticine and its more soluble derivatives exhibit promising results in the treatment of breast cancer and acute myeloblastic leukemia. The CYP-dependent activation leading to DNA adduct formation, we found to be generated *in vitro*, is a novel mechanism for the ellipticine pharmacological action[1]. Target tumor cells express, beside several CYPs also peroxidases (myeloperoxidase, MPO or lactoperoxidase, LP) in higher levels than cells of peritumoral tissues. Since the participation of peroxidases in metabolism of ellipticine has not been identified yet, the aim of the present work is to extend our knowledge on this issue. Ellipticine is oxidized by LP and MPO via a radical mechanism. Using mass spectrometry (MALDI-TOF, EI) and NMR we identify that ellipticine is primarily oxidized to a one-electron reaction product (radical) producing a dimer as the major metabolite. Peroxidase attacks a nitrogen atom of a pyrrole ring of the ellipticine skeleton to form a radical, which is subsequently bound to carbon C9 in the second molecule of ellipticine. Its formation is inhibited by radical trapping agents (glutathione, NADH) and by DNA. Another ellipticine metabolite formed by peroxidases is N(2)-oxide of ellipticine. The same metabolite is formed also by CYP-mediated reactions. During oxidation of ellipticine by peroxidases, two ellipticine-DNA adducts, which were generated by CYP-mediated reaction[1], are also formed. Identities of adducts formed by CYPs and peroxidases were confirmed by co-chromatography on HPLC. Deoxyguanosine was determined as a target deoxynucleoside for ellipticine covalent binding in DNA. The involvement of myeloperoxidase and lactoperoxidase in an increase of ellipticine pharmacological efficiency in the target tumor tissue is discussed.

References

1. Stiborová M., Bieler C.A., Wiessler M. and Frei E.: *Biochem. Pharmacol.* **62**, 1675 (2001)

Acknowledgement

Supported by Grant Agency of the Czech Republic (grant 203/01/0996) and the Ministry of Education of the Czech Republic (grant MSM 1131 00001).

7P13

CHROMATOGRAPHIC METHOD FOR DETERMINATION OF CLOPIDOGREL AND ITS MAIN METABOLITES PRODUCED BY HEPATIC MICROSOMES

Michal Šavlík*, Lenka Skálová and Vladimír Wsól

Department of Biochemical Sciences, Faculty of Pharmacy, Charles University, Heyrovského 1203, CZ-50005 Hradec Králové, Czech Republic

** E-mail: savlik@faf.cuni.cz*

Atherosclerosis is very common disease affecting vascular cell wall and causing disturbances in vessel functions. During progression of atherosclerosis, lipids and monocytes accumulate in the intima of arteries and form atheromatous plaque. Subsequent decreased blood flow caused by constriction of vessels and other factors (e.g. fatty streak formation, plaque rupture, inflammation process) potentially damage the endothelial cell layer. Under physiological conditions platelets circulate in close contact with endothelial cells lining of the vessel wall without adhering to it. Alteration of endothelium caused by inflammation or plaque rupture and exposure of subendothelial structures activate platelets and triggers their aggregation and thrombus formation, which is usually associated with ischemic events. Administration of inhibitors of platelet function is one of the possible ways to reduce the risk of blood vessel occlusion induced by intravascular coagulation.

Clopidogrel is antiplatelet drug, which is indicated for the prevention of vascular thrombotic events in patients at risk. It is a thienopyridine derivative that comprises a class of ADP receptor antagonists. Clopidogrel is inactive prodrug. Very reactive and unstable active metabolite is produced in the liver and subsequently binds by covalent bond to ADP receptor[1]. Irreversible inactivation of ADP receptor on the surface of platelets discontinues cascade of events leading to figrinogen cleavage and fibrin strands formation.

Clopidogrel is pure S enantiomer. The R enantiomer devoids of antithrombotic activity and can provoke side events not observed with clopidogrel. Clopidogrel is rapidly and extensively metabolised by hepatic cytochromes to form both active and inactive metabolites. Clopidogrel is changed to the active metabolite via intermediates, all of which keep one chiral centre. During last step when the active clopidogrel metabolite is created, a new chiral centre is formed. Active metabolite is one of eight stereoisomers with identical primary chemical structure[2].

The goal of our work was to develop chromatographic method that can be used for separation of main clopidogrel metabolites produced by incubation of parent substance with hepatic microsomal fraction.

The metabolites were isolated from the incubation mixture with the use of the liquid-liquid extraction to lipophilic organic solvent. Extraction was performed to ethylacetate at neutral pH. Separation was performed on Discovery C18 column (150×4 mm i.d., 5 μm particle size), the mobile phase consisted of a mixture of methanol and 20 mM butylamine phosphate buffer of pH 6.6 in ratio 60:40 pumped at flow rate 0.8

ml/min. The UV detector was operated at 230 nm. The retention times for clopidogrel and its main metabolite SR26334 were 26.1 and 5.7 min, respectively.

References

1. Pereillo J. M.: *Drug Metab. Dispos*. **30**, 1288 (2002)
2. Weber A. A.: *Br. J. Clin. Pharmacol*. **52**, 333 (2001)

Acknowledgement

This project was supported by FRVŠ grant 973/G6/2004.

7P14

PIG HEPATOCYTES IN SUSPENSION AND CULTURE FOR MODELING OF DRUG METABOLISM

Eva Anzenbacherová[a,*], Jana Baranová[a], Martin Modrianský[b], Zdeněk Dvořák[b], Jitka Ulrichová[b] and Pavel Anzenbacher[b]

[a]*Institute of Pharmacology and* [b]*Institute of Medical Chemistry and Biochemistry, Faculty of Medicine, Palacký University, Hněvotínská 3, 77515 Olomouc, Czech Republic*

* *E-mail: anzeneva@tunw.upol.cz*

Fresh suspension of pig hepatocytes as well as pig hepatocytes in culture was used for modeling of metabolism of selected CYP forms. Substrate of CYP3A, nifedipine, of CYP2E1, chlorzoxazone, of CYP2A, coumarin, and of CYP2D, propafenone were selected. Metabolites were in all cases found in higher concentrations in the medium than in the cells apparently due to their higher polarity than polarity of the parent compounds. In suspension of freshly prepared hepatocytes, the activities of all four CYP forms are functional documenting their applicability to metabolic studies. The results with the cultured cells show that the activity of CYP2E1 and CYP2A forms declines to zero to 30% of the values found in the suspension. This is not the case of CYP3A and CYP2D activities. Cultured pig hepatocytes may be probably suitable for studies of processes mediated by CYP3A and CYP2D forms. Freshly prepared suspensions of pig hepatocytes apparently have the advantage of reliable expression of the most important liver microsomal CYP activities. Another advantage of the use of pig hepatocytes is their practically unlimited availability.

Acknowledgement

The work at this project was supported by the Ministry of Education, Youth and Sports, Czech Republic (MSM 151100003).

7P15

PRESENCE AND ACTIVITY OF CYTOCHROME P450 ENZYMES IN HEART AND PANCREAS OF PIG AND RAT ORIGIN

Jana Baranová[a,*], Eva Anzenbacherová[a], Jitka Psotová[a] and Pavel Anzenbacher[a]

[a]*Institute of Pharmacology and* [b]*Institute of Medical Chemistry and Biochemistry, Faculty of Medicine, Palacký University, Hněvotínská 3, 77515 Olomouc, Czech Republic*

* *E-mail:* jana.baranova@post.sk

Presence and activity of cytochromes P450 was investigated in pig heart microsomes, in newborn rat cardiomyocytes and in the pig pancreas. In all tissues, the CYP2E1 form was found by immunoblotting with chemiluminiscence detection and its presence was confirmed by determination of the specific activity (chlorzoxazone 6-hydroxylation or *p*-nitrophenol hydroxylation). Presence and activity of another cytochrome P450, the CYP2C9 form, was found only in the fresh cardiomyocytes by both the Western blotting as well as by the determination of the CYP2C9 specific activity (diclofenac 4′-hydroxylation). The fact that cytochromes P450 are fully functional in the heart may have serious consequences e.g. for the metabolism of cardioactive drugs as well as for biosynthesis of endogenous regulators in this tissue.

Acknowledgement

The work at this project was supported by COST B15.50 project from the Ministry of Education, Youth and Sports, Czech Republic.

7P16

NOVEL AND CLASSICAL TAXANES: METABOLISM AND TRANSPORT

Radka Václavíková[a,*], Marie Ehrlichová[b], Stanislav Horský[a], Petr Šimek[c], Pavel Souček[a], Iwao Ojima[d] and Ivan Gut[a]

[a]*Department of Biotransformations, National Institute of Public Health, Šrobárova 48, 10042 Praha 10, Czech Republic*
[b]*Institute of Molecular Genetics, Academy of Sciences, Czech Republic*
[c]*Entomologic Institute, Academy of Sciences, Czech Republic*
[d]*Department of Chemistry, Institute of Chemical Biology & Drug Discovery, State University of New York, USA*

** E-mail: rvaclavikova@szu.cz*

Classical taxanes paclitaxel (Taxol) and docetaxel (Taxotere) are among the most important anticancer drugs used in breast, ovarian and lung cancer therapy. Although both paclitaxel and docetaxel possess potent antitumor activity, treatment with these drugs often results in a number of undesirable side effects and suffers from multidrug resistance (MDR). That is why the second generation of taxanes with better pharmacological properties and anticancer activity are synthesized[1].

We have investigated so far unreported metabolism of three novel taxanes described SB-T-1214, SB-T-1216 and SB-T-1103. The drugs exhibit excellent cytotoxicity against several human cancer cell lines (breast, ovarian, non-small lung and colon) and extremely high potency against the drug resistant breast cancer cell lines[2]. *In vitro* systems of human, rat, minipig and pig liver microsomes or human cDNA-expressed P450 enzymes (CYP1A2, 1B1, 2A6, 2C9, 2E1 and 3A4) were incubated with either of the novel taxanes to study metabolic pathways. HPLC and MS/MS analysis were used to analyze and identify their metabolites. We have found nine metabolites of SB-T-1214, eight metabolites of SB-T-1216 and eleven metabolites of SB-T-1103 in liver microsomes from different species. Some of those metabolites were formed in all species. Six, seven and five main metabolites of SB-T-1214, SB-T-1216 and SB-T-1103, respectively, were identified by MS/MS.

In view of the fact that treatment with classical taxanes leads to development of MDR, we also compared transport of the novel taxanes with classical taxanes in adriamycin-sensitive (MDA-MB-435) and resistant (NCI/ADR-RES) human breast cancer cells. Our results suggest that both examined cell lines absorbed similar amounts of the novel taxanes. In contrast, accumulation of paclitaxel by NCI/ADR-RES cells was 10-fold lower than in MDA-MB-435 cells. Likewise, the efflux of paclitaxel from NCI/ADR-RES cells was 2.5-fold higher than in the sensitive MDA-MB-435 cells.

This study describes metabolic profile and interspecies variability in the metabolism of three novel taxanes and also their transport in human breast cancer cells in comparison with classical taxanes. All three novel taxanes exert substantially higher activity toward resistant tumor cells than classical taxanes. Thus, they seem to be potential drugs for more succesful cancer therapy.

References

1. Miller M. L. and Ojima I.: *The Chemical Record* **1**, 195 (2001)
2. Ojima I., Slater J. C., Michaud E., Kuduk S. D., Bounaud P. Y., Vrignaud P., Bissery M. C., Veith J. M., Pera P. and Bernacki R. J.: *J. Med. Chem*. **39**, 3889 (1996)

Acknowledgement

This study was supported by Grant Agency of the Ministry of Health of the Czech Republic (NL/7567-3) and Grant Agency of the Czech Republic (305/04/0403).

7P17

MULTIDRUG RESISTANT L1210/VCR CELLS EXPRESSING P-GLYCOPROTEIN ARE MORE SENSITIVE TO HIGH EXTRACELLULAR CALCIUM AS DRUG SENSITIVE L1210 CELLS

Zdena Sulová*, Jozef Orlický, Roderik Fiala and Albert Breier

Laboratory of Electron Microscopy, Institute of Molecular Physiology and Genetics, Slovak Academy of Sciences, Vlárska 5, 83334 Bratislava, Slovakia

** E-mail: Zdena.Sulova@savba.sk*

L1210/VCR cell line was obtained by adaptation of the L1210 mouse leukaemic cells to vincristine and showed an over-expression of P-glycoprotein (P-gp) accompanied with multidrug resistance (MDR). Substances influencing calcium homeostasis could effectively reverse MDR of these cells. Therefore in the present study we have compared the parental L1210 and MDR L1210/VCR cells in respect to their sensitivity to high extracellular calcium concentration. MDR cells were observed to be more sensitive to high extracellular calcium as parental cells. This effect could not be antagonised by calcium blockers verapamil and flunarizine. More pronounced calcium uptake was observed for MDR cells. High extracellular calcium did not influence the P-gp mediated extrusion of Calcein AM as P-gp substrate. These results indicate that calcium enters and consequently damages the MDR cells to a higher extent than parental cells.

7P18

THE USE OF MINIPIG LIVER MICROSOMES AS A MODEL SYSTEM IN THE STUDY OF OXIDATIVE STRESS INDUCED BY XENOBIOTICS

Eliška Kondrová[a,*], Pavel Stopka[b] and Pavel Souček[a]

[a]*Centre of Industrial Hygiene and Occupational Health, National Institute of Public Health, Šrobárova 48, 10042 Praha 10, Czech Republic*
[b]*Department of Bioinorganic Chemistry, Institute of Inorganic Chemistry, Academy of Sciences, Czech Republic*

** E-mail: ekondrova@szu.cz*

Our previous results show that rat is not suitable as a model species to study CYP destruction and oxidative stress resulting from CYP interaction with xenobiotics. Minipigs on the other hand contain main biotransformation enzymes in amounts and activities comparable to humans and are considered to be suitable as model animals for the study of some human biotransformation pathways. The aim of our present study was to evaluate the properties of minipig liver microsomes as a model system for *in vitro* testing of interactions between biotransformational enzymes and xenobiotics, especially with respect to oxidative stress.

We incubated minipig liver microsomes in the presence of different concentrations of NADPH and substrates (catechol, benzoquinone, hydroquinone) and determined CYP content spectrophotometrically. We assayed lipid peroxidation (spectrophotometrically) and the formation of hydroxyl radicals (both spectrophotometrically and by ESR).

The extent of CYP destruction by NADPH-mediated futile cycle was similar as has been reported for human microsomes, the observed CYP destruction caused by metabolites of benzene on the other hand differed from values found for majority of human samples. The detected values of lipid peroxidation were very low and it was impossible to observe the effect of NADPH and substrates. The formation of hydroxyl radicals by NADPH was concentration-dependent and did not correlate with CYP destruction.

Our results further support the use of minipigs rather than rats as a model species for the study of interactions between human biotransformational enzymes and xenobiotics. However, no ideal species exists for this purpose. One of the reasons for the difficulties in obtaining a model system is high interindividual variability of human liver samples.

Acknowledgement

The study was supported by grants of the Grant Agency of the Czech Republic 203/02/1152 and 203/01/0944.

7P19

THE EFFECT OF COMMERCIALLY MANUFACTURED DISINFECTANTS ON THE METABOLISM OF *STENOTROPHOMONAS MALTOPHILIA*

Viktor Majtán* and Ľubica Majtánová

Institute of Preventive and Clinical Medicine, Slovak Medical University, Limbová 12, 83303 Bratislava 37, Slovakia

** E-mail: majtan@upkm.sk*

The *in vitro* antibacterial effect of 14 commercially manufactured disinfectants on a nosocomial pathogen *Stenotrophomonas maltophilia* was studied. The substances tested represented 5 quaternary ammonium substances (QAS) and 9 QAS combinated with other ingredients. The antimicrobial efficacy was characterized by influencing the growth of bacterial cells expressed by MIC and ED50 values as well as by the inhibition of the incorporation rate of [^{14}C] adenine and [^{14}C] leucine. The disinfectants were divided into four groups according to their efficacy. The first group comprised substances with strong inhibitory effect (MIC 0.045-0.09 μg/ml) where belonged Benzalkonium chloride and Triquart. The second group represented substances with good antibacterial efficacy (MIC 0.19-0.78 μg/ml), the third group comprised substances with MIC values up to 1.56-25 μg/ml. Cetrimide with very low activity (MIC 50-100 μg/ml) belonged to fourth group. The effect of substances studied on the biosynthetic processes expressed by R values (IC50 Ade : IC50 Leu) showed that those values were < 1 only after treatment with Hexaquart plus, Diesen forte, Sokrena, Forten, ID 212 and Cetrimide. Much lower IC50 Ade, IC50 Leu values of the disinfectants studied suggested interference with nucleic acid synthesis and protein synthesis which was expressed by inhibition of both precursors. The most effective in inhibition of endogenous respiration were Cetrimide and Microbac forte, which its inhibited nearly to 50% at 0.78 μg/ml concentration against control. The respiration was totaly inhibited by Almyrol, Diesen forte, Microbac forte at 6.25 μg/ml concentration and by substances FD312, Triquart, Hexaquart plus, Hexaqurt S, ID212, TPH 5225 at 12.5 μg/ml concentration. The tested substances suppressed the growth of *Stenotrophomonas maltophilia* probably through interference with energy-yielding and energy-requiring processes of the cells.

7P20

CYTOTOXICITY AND APOPTOSIS INDUCED BY 5-MORPHOLIN-4-YL-3-(5-NITROTHIEN-2-YL)[1,2,4]TRIAZOLO[4,3-C]QUINAZOLINE IN LEUKEMIC CELL LINE L1210

Silvia Letašiová*, Renáta Ovádeková and Soňa Jantová

Department of Biochemistry and Microbiology, Slovak Technical University, Radlinského 9, 81237 Bratislava

** E-mail: silvia.letasiova@stuba.sk*

Many derivatives of quinazoline (benzopyrimidine; 1,3-benzodiazine) are used in the pharmaceutical industry, in medicine and in agriculture due to their antimicrobial, antiinflammatory, diuretic, anticonvulsant, antiallergic, antihypertensive, and antiparkinsonian effects. As documented in the literature, many quinazolines act as anticancer active agents[1-3] and they also act as antimetabolites from the group of folic acid analogues. They are antifolate thymidylate synthase inhibitors and some of these are now in clinical development[4].

In view of the above mentioned wide-ranging effect of quinazolines, a series of substituted triazolo[1,5-c]quinazoline derivatives was prepared[5]. The primary screening of the fifteen above mentioned quinazoline derivatives on human tumor HeLa cells showed that 5-morpholin-4-yl-3-(5-nitrothien-2-yl)[1,2,4]triazolo[4,3-*c*] quinazoline (MNTQ) was the most cytotoxic active compound.

In this study, the growth inhibition assay was used for human cancer HeLa cells and mouse cancer cell line L1210. Furthermore, apoptosis/necrosis in L1210 cells treated with MNTQ were monitored. Cytotoxicity was measured by direct counting of cells in Burker chamber. MNTQ acted cytotoxically on both cell lines. We found the highest concentrations of MNTQ (100, 75, 50, 10, 5, 1 $\mu g.ml^{-1}$) induced acute cytotoxic effect, however other tested concentrations (1 $\mu g.ml^{-1}$) manifested a concentration/dependent cytotoxic effect. The mouse leukemic cells L1210 were much more sensitive to MNTQ treatment than HeLa cells. The value of IC100 was below 10 $\mu g\ ml^{-1}$ for HeLa cells and 1 $\mu g\ ml^{-1}$ for L1210 cells. For both cancer cell lines after the long-term influence the value of IC50 was found to be less than 4 $\mu g.ml^{-1}$. It corresponds with the criterion of the National Cancer Institute, that if the IC50 value is less than 4 $\mu g.ml^{-1}$, the compound can be classified as a potential anticancer drug. The apoptotic DNA fragmentation was measured by electrophoresis in agarose gel complemented with ethidium bromide. DNA fragmentation was detected in L1210 cells treated with 5, 1, 0.5, 0.1, 0.01, 0.001 and 0.0001 $\mu g.ml^{-1}$ of MNTQ after 24, 48 and 72 h of treatment. Analysis showed that MNTQ induced apoptosis of cancer cell line L1210 after 48 and 72 h of treatment, but didn‘t induce apoptosis after 24 h of influence. MNTQ demonstrated a concentration/dependent increase of apoptotic DNA fragmentation. On the other hand, at the concentrations 100, 75, 50 and 10 $\mu g.ml^{-1}$ cell lysis/necrosis of MNTQ-treated HeLa and L1210 cells was observed.

References

1. Hirata A., Ogawa S., Kometani T., Kuwano T., Naito S., Kuwano M. and Ono M.: *Cancer Res.* **62**, 2554 (2002)
2. Tamura T.: Nippon Geka Gakkai Zasshi **103**, 233 (2002)
3. Jantová S., Čipák Ľ., Slameňová D., Horváth V. and Rauko P.: *Toxicol. in Vitro* **17**, 457 (2003)
4. Albanell J., Rojo F., Averbuch S., Feyereislova A., Mascaro J. M., Herbst R., LoRusso P., Rischin D., Sauleda S., Gee J., Nicholson R. I. and Baselga J.: *J. Clin. Oncol.* **20**, 110 (2002)
5. Špirková K. and Stankovský Š.: Collect. Czech. Chem. Commun. **56**, 1117 (1991)

Acknowledgement

This study was supported by the Grant Agency VEGA (1/1173/04).

7P21

BIOTRANSFORMATION ENZYME ACTIVITIES IN MOUFLON IN HEALTH AND DISEASE

Barbora Szotáková*, Lenka Štorkánová, Jiří Lamka and Lenka Skálová

Department of Biochemical Sciences, Faculty of Pharmacy, Charles University, Heyrovského 1203, 50005 Hradec Králové, Czech Republic

** E-mail: szotako@faf.cuni.cz*

Mouflon (*Ovis musimon*) is a wild form of domestic sheep (*Ovis ammon f. aries*). Formerly they were classified as a cloven-hoofed game only, but later (in 1990s) they became an important farm animal, too. However, they have always been considered as a species very sensitive to parasitic diseases. Infections with *Fasciola hepatica* and *Dicrocoelium dendriticum* can be acquired on some pastures in Europe and in Czech Republic by cattle, sheep or mouflon as intermediate hosts of these parasites may find suitable habitats in these regions[1,2]. Therefore, breeding of mouflon in free grounds, game enclosures as well as in farms necessitates regular administration of anthelmintics[3]. With regard to the broad spectrum of helminths, which infect mouflons, wide-spectrum anthelmintics are appropriate to use. Benzimidazole derivative albendazole (ABZ, methyl-5-propylthio-1H-benzimidazol-2-yl-carbamate) represents available and commonly administered drug for mouflon helminthoses treatment.

Dicroceliosis was characterized by bile ductular proliferation and enlargement of the bile duct surface area caused by hyperplastic cholangitis in septal bile ducts. The aim of the study was to investigate the changes in biotransformation enzyme activities caused

by dicroceliosis in mouflon and to find out if dicroceliosis affect metabolism of anthelmintic ABZ.

Adult male healthy mouflons (5 animals) were from game enclosure Janovice and adult male dicroceliosis infected mouflons (5 animals) were from game enclosure Vlkov. Activities of several biotransformation enzymes toward their specific substrates were measured in hepatic microsomal and cytosolic fractions. Significant increase in activity of flavine monooxygenases (FMO) and significant decrease in activity of glutathione-S-transferase was observed in ill mouflons comparing to healthy animals. No other biotransformation enzymes tested were affected by dicroceliosis.

In vitro metabolism of ABZ in microsomes from healthy and ill mouflons was also investigated. Concentrations of two main ABZ metabolites – pharmacologically active ABZ sulfoxide enantiomers and pharmacologically inactive ABZ sulfon were analysed using HPLC. Significant increase of velocity of formation of (+)-ABZ sulphoxide enantiomer (which is catalysed by FMO[4]) was observed in hepatic microsomes from ill animals.

References

1. Eckert J. and Hertzberg H.: *Vet. Parasitol.* **54**, 103 (1994)
2. Lavin S., Marco I., Vilafranca M., Feliu C. and Vinas L.: *Vet. Rec*. **143**, 396 (1998)
3. Lanusse C. E. and Prichard R. K.: *Drug Metab. Rev.* **25**, 235 (1993)
4. Cristofol C., Virkel G., Alvarez L., Arboix M. and Lanusse C. E.: *Biopharm. Drug Dispos.* **21**, 303 (2000)

Acknowledgement

This project was supported by Grant Agency of the Czech Republic, grant No 524/03/1361 and by the Czech Ministry of Education, Youth and Sport, project MSM 111600002.

7P22

THE PHASE I BIOTRANSFORMATION OF ANTI-OBESITY DRUG SIBUTRAMINE IS STEREOSELECTIVE IN RAT HEPATOCYTES

Marek Link*, Romana Novotná, Barbora Szotáková, Bohumila Suchanová, Radim Král, Vladimír Wsól and Lenka Skálová

Department of Biochemical Sciences, Faculty of Pharmacy, Charles University, Heyrovského 1203, 50005 Hradec Králové, Czech Republic

** E-mail: link@faf.cuni.cz*

Nowadays the obesity represents serious problem especially in European and American populations. Sibutramine hydrochloride monohydrate represents one of the few established and well-proven agents available for obesity treatment[1]. Sibutramine (N-{1-[1-(4-chlorophenyl)cyclobutyl]-3-methylbutyl}-N,N-dimethyl amine) is a terciary amine with one chiral centre. It is sold as racemic mixture under the trademark Meridia or Reductil. The weight loss of patient produced by sibutramine is thought to be due to combination of serotonine- and norepinephrine-mediated mechanisms that increase both satiety and energy expenditure[1].

In organism sibutramine is rapidly demethylated to form active metabolites M1 and M2. These metabolites undergo further biotransformation to form inactive metabolites and conjugates. Sibutramine and both M1 and M2 metabolites are chiral compounds. Enantioselective pharmacodynamic profile of these enantiomers was reported. The (R)-enantiomers acted as more potent monoamine reuptake inhibitors than the (S)-enantiomers[2].

Presented study was designed to evaluate the stereoselectivity in phase I of sibutramine biotransformation in rat. Primary cultures of rat hepatocytes were used as *in vitro* model systems. Rac-sibutramine or individual R-sibutramine and S-sibutramine enantiomers served as substrates. Aliquots of culture medium were collected during 24-hour incubation. Time-dependent and concentration-dependent consumption of substrates and formation of metabolites in hepatocytes were measured. Novel analytical method for the determination of sibutramine and its phase I metabolites in culture medium using isocratic reversed phase high performance liquid chromatography with UV detection was developed.

When R-sibutramine was used as a substrate, M1 and M2 represented the only metabolites in culture medium. In addition to M1 and M2, other metabolite denoted as M3 was found as a biotransformation product of S- and rac-sibutramine. Using LC-MS metabolite M3 was identified as hydroxy-derivative of M2. When S-sibutramine was used as a substrate the concentration of active metabolites M1 and M2 after 8-hour incubation was significantly lower comparing to R- and rac-sibutramine. With respect to these results, faster deactivation of S-enantiomer in organism may be expected. As S-sibutramine also displays lower biological activity, the use of R-enantiomer instead of racemic mixture would be reasonable in obesity treatment.

References

1. Stock M. J.: *Int. J. Obesity* **21** (Suppl 1), S25 (1997)
2. Glick S. D., Haskew R. E. and Maisonneuve M.: *Eur. J. Pharmacol.* **397**, 93 (2000)

Acknowledgement

This project was supported by Czech Ministry of Education, Youth and Sport, Research project LN00B125 and FRVŠ grant 969/G6/2004.

7P23

FLUBENDAZOLE AND MEBENDAZOLE DO NOT CHANGE CYP1A ACTIVITIES IN CULTURES OF PHEASANT HEPATOCYTES

Michal Šavlík*, Pavlína Polášková, Barbora Szotáková, Jiří Lamka and Lenka Skálová

Department of Biochemical Sciences, Faculty of Pharmacy, Charles University, Heyrovského 1203, 50005 Hradec Králové, Czech Republic

** E-mail: savlik@faf.cuni.cz*

Mebendazole and flubendazole are members of benzimidazole anthelmintics family that are often used in veterinary medicine for treatment and prevention of parasital diseases.

It is well known fact that administration of some substances may lead to changes of biotransformation enzymes activities, which can result in unexpected alterations of drug biotransformation velocity[1]. Changes in plasmatic concentrations of drugs have more or less serious consequences and can decrease efficiency of therapy or cause its failure. In case of antiparasital treatment drug resistance of helminthes can be facilitated.

Many of benzimidazole anthelmintics were proven to be modulators of cytochrome P450 activities in laboratory animals as well as in farm animals[2,3]. Nevertheless, very little studies were performed on a pheasant. Inter-species diversities of biotransformation enzymes encourage scientists to perform studies on target animals. Additionally, since pheasants are food-producing animals, influence of changed metabolism velocity on occurrence of drug residues in animal products has to be considered.

Primary cultures of pheasant hepatocytes have been used as a model system in order to investigate possible influence of flubendazole and mebendazole on P450 1A activities. After 48 hours of incubation of cells with tested substances (0.1 – 5 mM), enzyme activities were measured. Ethoxyresorufin O-deethylation (EROD) and methoxyresorufin O-deethylation (MROD) served as a marker of CYP1A activities. CYP1A protein content in microsomal fraction of treated cells was determined by Western blotting.

We observed moderate changes in P450 activities depending on concentration of tested drugs and on the way in which the hepatocytes were treated. Our results did not show significant effect of flubendazole or mebendazole on CYP1A activities in *in vitro* study on pheasant hepatocytes. No changes of CYP1A protein content were detected. Observed slight induction at low concentrations of flubendazole is not pharmacologically important. Decreased viability of the cells probably contributed to enzyme activity inhibition found at higher concentrations of both drugs. Plasmatic concentrations in real organism do not reach levels necessary for observed inhibition effect therefore above mentioned decrease of enzyme activities probably do not have relevant clinical consequences.

In conclusion, the treatment of pheasant by both anthelmintics tested seems to be safe in a view of CYP1A induction or inhibition.

References

1. Correia M. A.: In *Basic & Clinical Pharmacology.* 6th edn. (Katzung B., ed.) Appleton & Lange, USA 1995
2. Baliharová V., Velík J., Lamka J., Balarinová R. and Skálová L.: *Res. Vet. Sci.* **75**, 231 (2003)
3. Baliharová V., Skálová L., Maas R. F., De Vrieze G., Bull S. and Fink-Gremmels J.: *J. Pharm. Pharmacol.* **55**, 773 (2003)

Acknowledgement

This project was supported by grant 524/03/1361 from the Grant Agency, Czech Republic.

7P24

ARACHIDONIC ACID RELEASE AND FORMATION OF REACTIVE OXYGEN SPECIES INDUCED BY METHYLATED ANTHRACENE DERIVATIVES IN RAT LIVER EPITHELIAL CELLS

Lenka Šindlerová[a,*], Lenka Umannová[b], Jan Vondráček[a], Kateřina Chramostová[a], Jiří Neča[c], Kateřina Pěnčíková[c], Milan Číž[d], Antonín Lojek[d], Alois Kozubík[a], Brad L. Upham[e] and Miroslav Machala[e]

[a]*Department of Cytokinetics and* [d]*Laboratory of Pathophysiology of Free Radicals, Biophysical Institute, Academy of Sciences, Královopolská 135, 612 65 Brno, Czech Republic*
[b]*RECETOX, Faculty of Science, Masaryk University, Kamenice 126/3, 625 00 Brno, Czech Republic*
[c]*Department of Chemistry and Toxicology, Research Institute of Veterinary Medicine, Hudcova 70, 621 32 Brno, Czech Republic*
[e]*Michigan State University, Eats Lansing, Michigan 48824, USA*

** E-mail: sindler@ibp.cz*

The methylated derivatives of polycyclic aromatic hydrocarbons are important environmental pollutants that are also found at high levels in cigarette smoke. Methylated anthracenes are present in cigarette smoke in concentration 60-fold higher than benzo(a)pyrene[1]. It has been hypothesized that they could play a role in tumor promoting effects of cigarette smoke condesates, such as inhibition of gap junctional intercellular communication (GJIC), an epigenetic event involved in the removal of an initiated cell from growth suppression. The position of methyl group has been shown to significantly affect the biological effects of methylated anthracenes on GJIC inhibition in rat liver epithelial cells, as the compounds with substitution forming bay-like region have been shown to inhibit GJIC and activate mitogen-activated protein kinases[2]. Moreover, it has been recently shown that 1-methylanthracene (1-MeA) can induce a release of ^{3}H-arachidonate and apoptosis in endothelial cells by a mechanism that involves activation of phospholipase A2[3]. Arachidonic acid (AA)-induced cell death is often associated with oxidative stress, although a causal relationship has not yet been made.

In the present study, we investigated effects of methylated anthracenes on the release of AA, formation of reactive oxygen species (ROS), GJIC inhibition, induction of aryl hydrocarbon receptor (AhR)-mediated activity and cytokinetics in rat liver epithelial WB-F344 cells, a cellular model used for studies on tumor promoting effects of xenobiotics. Moreover, we have studied effects of arachidonic acid itself on cell proliferation and cell death in WB-F344 cells. We found that 1-MeA and 9-MeA, both carrying methyl substitution forming a bay-like region, inhibited GJIC in concentration-dependent manner and stimulated a rapid and transient ROS formation, detected by lucigenin-dependent chemiluminescence. Contrary to that, 2-MeA failed to inhibit GJIC and induce ROS production. Also, 1-MeA and not 2-MeA induced a release of AA from WB-F344 cells assessed by HPLC. Neither compound was cytotoxic at the range up to

100 μM concentration and they did not affect cell proliferation in confluent WB-F344 cells. All three compounds induced only a weak AhR-mediated activity. Unlike in endothelial cells, arachidonic acid had no direct apoptotic effect on WB-F344 cells and it did not inhibit their proliferation at concentrations up to 20 μM.

Taken together, our data seem to suggest that bay-like methylated anthracenes can inhibit GJIC and induce AA release, but also stimulate ROS formation. These rapid effects were not associated with modulation of cell proliferation and cell death, however they might be associated with GJIC inhibition, an important tumor-promoting process, and/or perturbation of intracellular lipid signaling. Future studies should investigate the exact mechanisms of ROS formation and AA release control, as both ROS and AA can play a significant role in modulation of cell signaling and cytokinetics.

References

1. Severson R. F., Snook M. E., Higman H. C., Chortyk O. T. and Akin F. J.: In *Carcinogenesis*, Freudenthal R. I. and Jones P.W. , eds.), Raven Press, New York 1976, p. 269
2. Rummel A. M., Trosko J. E., Wilson M. R. and Upham B. L.: *Toxicol. Sci.* **49**, 232 (1999)
3. Tithof P. K., Elgayyar M., Cho Y., Guan W., Fisher A. B. and Peters-Golden M.: *FASEB J.* **16**, 1463 (2002)

Acknowledgement

This work was supported by grants 525/03/1527 from the Grant Agency and MZE 0002716201 from the Ministry of Agriculture, Czech Republic.

7P25

THE DEVELOPMENT OF IMMUNOCHEMICAL METHOD FOR N-METHYLCARBAMATE PESTICIDES

Barbora Mičková[a,*], Pavel Rauch[a], Angel Montoya[b], Fabiana Fini[c] and Stefano Girotti[c]

[a]*Department of Biochemistry and Microbiology, Institute of Chemical Technology, Technická 3, 16628 Praha 6, Czech Republic*
[b]*Centro de Investigación e Innovación en Bioingenieria, Polytechnical University Valencia, Spain*
[c]*Istituto di Scienze Chimiche, Universita` di Bologna, Italy*

** E-mail: mickovab@vscht.cz*

N-methylcarbamate pesticides were introduced worldwide as substituents of persistent organochlorine compounds, due to their broad spectrum of activity and their

low bioaccumulation potentials. Though pesticides are indispensable chemicals in modern civilization, the exposure to their residues poses a potential hazard for humans, especially for children.

Traditional methods of analysis (e.g. HPLC/MS), although sensitive, are expensive and time-consuming and require specialized instrumentation. Because pesticide residues occur in water, soil and food crop, there is a growing demand for increased analytical capability and capacity of the laboratories involved in pollution monitoring. In this respect, immunochemical methods are gaining importance as analytical techniques in the agrochemical field, since they provide the analytical chemists with a rapid, sensitive and cost-effective alternative to chromatographic methods.

The aim of the present work was the development of new rapid and simple immunochemical method for the determination of carbofuran, carbaryl and methiocarb pesticides in environmental samples. For experiments were chosen enzyme-linked immunosorbent assays (ELISA) and lateral flow immunoassays (LFIA). During the experiments the indirect ELISA formats were optimized, using monoclonal and polyclonal antibodies and the corresponding hapten-ovalbumin conjugates, with chemiluminescent and colorimetric detection. The advantages of the chemiluminescent assay over the colorimetric one are the detection of pesticides in a wider range of concentrations and lower consumption of immunoreagents.

7P26

METABOLISM OF FLOBUFEN IN MAN – A PRELIMINARY STUDY

Yogeeta Babú*, Radim Král and Vladimír Wsól

Department of Biochemical Sciences, Faculty of Pharmacy, Charles University, Heyrovského 1203, 500 05 Hradec Králové, Czech Republic

** E-mail: babu@faf.cuni.cz*

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most widely used drugs. Although being a heterogeneous group of compounds, all of them have in common, to a certain extent, the therapeutic properties as well as the side effects. Their mechanism of action consists predominantly of the inhibition of both forms of cyclooxygenase (COX) - COX-1, constitutive and COX-2, induced during inflammation – and, therefore, they inhibit the synthesis of prostaglandins and tromboxanes[1].

Flobufen (F), 4-(2´,4´-difluorobiphenyl-4-yl)-2-methyl-4-oxobutanoic acid, is an aryloxoalkanoic acid derivative which mainly inhibits 5-lipoxygenase, even though inhibition of COX and antagonist effects on LTB4 receptors may also be observed[2]. Due to this fact, F is considered to be an anti-rheumatic drug. It displays intermediate to strong efficacy and lengthened effect, which enables a single daily administration. F exhibits excellent gastric tolerance when compared with other NSAIDs.

Metabolism of F has been studied in different species. The main phase I metabolite arising is 4-dihydroflobufen, 4-(2′,4′-difluorobiphenyl-4-yl)-2-methyl-4-hydroxy-butanoic acid (DHF)[3,4]. Studies performed in rats revealed yet another metabolite arising in hepatocytes[4] and urine and faeces[4,5], 2-(2′,4′-difluorobiphenyl-4-yl)-acetic acid (M 17203). In rat, DHF is converted into M-17203 by a process similar to the β-oxidation of fatty acids[6]. F and DHF are both chiral molecules, with one and two stereogenic centres, respectively. Studies concerning chiral point of view were already undertaken[4,6-9].

Concerning phase II biotransformation of F, recently concluded studies in rats showed that the main metabolite formed *in vivo* in this species was a conjugate of M-17203 with the amino acid taurine. The same result was obtained in incubations with primary culture of hepatocytes. Other *in vitro* experiments, namely microsomal, cytosolic and mitochondrial incubations gave rise to other metabolites such as F-glucuronide, M-17203-glucuronide and a conjugate of DHF with glutathione.

This study focused the metabolism of F in Man. Preliminary *in vitro* experiments established DHF as the major phase I metabolite. M-17203 is not formed, however a new unknown metabolite has arisen. Moreover, results from primary culture of hepatocytes revealed a phase II metabolite. The structure of this metabolite was determined by online LC-MS (electrospray ionization, ion trap detector) and it was identified as DHF-glucuronide. Further assays are being performed in order to determine the structure of the unknown metabolite as well as to verify whether other conjugates may be formed.

References

1. Vane J. R. and Botting R. M.: *Am. J. Med.* **104**, 2S (1998)
2. Kuchař M., Vosatka V., Poppová M., Kněžová E., Panajotovová V., Tomková H. and Taimr J.: *Collect. Czech Chem. Commun.* **60**, 1026 (1995)
3. Kvasničková E., Szotáková B., Wsól V., Trejtnar F., Skálová L., Hais I. M., Kuchař M. and Poppová M.: *Exp. Toxic. Pathol.* **51**, 88 (1999)
4. Wsól V., Král R., Szotáková B., Trejtnar F. and Flieger F.: *Chirality* **13**, 754 (2001)
5. Lapka R., Brejcha S., Smolík S. and Franc Z.: *Česk. Farm.* **39**, 443 (1990)
6. Král R., Skálová L., Szotaková B., Velík J., Schroterová L., Babú Y. N. and Wsól V.: *BMC Pharm.* **3**, 5 (2003)
7. Trejtnar F., Wsól V., Szotákova B., Skálová L., Pavek P. and Kuchař M.: *Chirality* **11**, 781 (1999)
8. Skálová L., Král R., Szotáková B., Babú Y. N., Pichard-Garcia L. and Wsól V.: *Chirality* **15**, 433 (2003)
9. Král R., Skálová L., Szotáková B., Babú Y. N. and Wsól V.: *Chirality* **16**, 1 (2004)

Acknowledgement

This work is supported by the Ministry of Education, Youth and Sports of Czech Republic, Research Centre LN00B125.

7P27

STUDY OF ANTIOXIDANT AND ANTIMUTAGENIC EFFECT OF PLANT FOODS

Petr Ptáček[a,*], Ivana Márová[a], Andrea Mikulcová[a] and Miloslav Pekař[b]

[a]*Department of Food Chemistry and Biotechnology and* [b]*Department of Applied Chemistry, Faculty of Chemistry, Brno University of Technology, Purkyňova 118, 612 00 Brno, Czech Republic*

** E-mail: petr.33@centrum.cz*

Important factors in the induction of mutagenesis and carcinogenesis are oxygen free radicals. The diet contains a wide variety of compound appear to act as antioxidants and antimutagens. Thus, the possibility to alter cell response to mutagen by dietary factors has opened a new frontier for cancer control. Short-time mutagenicity assays have been used effectively to screen for mutagens and potential carcinogens in the human environment. The same procedures are increasingly being used to identify antimutagens and potential anticarcinogens. In this work antimutgenic effects of some of plant extracts with high content of flavonoids and carotenoids and their active components were analyzed using *Saccharomyces cerevisiae* D7 strain. This test is commonly used to evaluate genotoxicity of various substances. The frequency of spontaneous convertants at the tryptophan locus trp5-12/trp5-27 and revertants at the isoleucine locus ilv1-92/ilv1-92 was tested using extracts from several fruit, vegetables, food additives, "green foods" - *Chlorella*, green barley and some standard antioxidants – carotenoids, tocopherols, ascorbate and flavonoids. In each assay positive and negative controls were included. For positive controls 4-nitroquinoline-1-oxide was used as standard mutagen. Content of antioxidants in natural extracts was analysed by high-performance liquid chromatography. Simultaneously, antioxidant activity of all tested extracts and standards was determined using Randox "Total antioxidant status" kit. Most of plant foods exhibited positive (20 - 40% of inhibition) or high positive (more than 40%) antimutagenic activity. High positive antimutagenicity exhibited extracts of spinach (65%), broccoli (58%) and *Chlorella* (54%). Positive antimutagenicity was observed using extracts from paprika, carrot and most of standard antioxidants. Some kinds of vegetables as beet-root, cabbage, cauliflower and green barley exhibited mild mutagenic effect in *S. cerevisiae* D7 test. No correlation between total antioxidant status of plant extracts and their antimutagenic activity was found, although foods with high antioxidant potential obviously act as positive antimutagens. According to our results, high content of antioxidants could not in several cases lead to benefical biological effect of complex foods. The chemical properties of antioxidants *in vivo* may be modified by interactions with other molecules in the food and may be significantly different from those of standard solutions.

7P28

STUDY OF ANTIMUTAGENIC PROPERTIES OF SOME SORTS OF TEA USING *EUGLENA GRACILIS* TEST SYSTEM

Jitka Kubešová[a,]*, Andrea Mikulcová[a], Ivana Márová[a], Simona Macuchová[a] and Ladislav Omelka[b]

[a]*Department of Food Chemistry and Biotechnology and* [b]*Department of Applied Chemistry, Faculty of Chemistry, Brno University of Technology, Purkyňova 118, 612 00 Brno, Czech Republic*

** E-mail: kubesova@fch.vutbr.cz*

Oxygen free radicals and lipid peroxidation processes are important factors in the induction of mutagenesis and carcinogenesis, and are considered to be major contributors to DNA damage. The powerful reactivity of active oxygen species can cause functional damage to man, triggering mutagenesis, carcinogenesis, circulatory disturbances and aging. In this work biological effects of some sorts of tea were analysed using test with *Euglena gracilis* and the test with yeast strain *Saccharomyces cerevisiae* D7 cells as relative test. The tea extracts were allowed to be positive antimutagens based on their ability to inhibit the mutagenic effects of standard mutagens (aminofluorene and nitropyrene in the Ames test, 4-nitroquinoline-1-oxide in *S. cerevisiae* D7 test and ofloxacin in *E. gracilis* test). Five sorts of green tea, one sort of white tea, two sorts of rooibos tea and two sorts of fermented black tea were extracted by hot water. Content of tea catechins in tested extracts was determined by RP-HPLC. Total antioxidant capacity of tea extracts was analyzed using the Randox kit as well as by TRAP method. Content of tea catechins in tested samples ranged in 500 – 2000 mg/100 g of dry tea and 2000 – 3000 mg/100 g of (-)catechin and (-)catechin gallate, respectively. All tested extracts exhibited high antioxidant capacity and high positive antimutagenicity. The highest antimutagenic effect exhibited most of green tea extracts, mild antimutagenic effect was observed using white tea and rooibos. No antimutagenic effect exhibited totally fermented tea. In most samples significant dependence of antimutagenic effect on extract concentration was observed. Catechin standards did not show so high antimutagenic effects as green tea extracts, so, catechins probably are not major component responsible for complex antigenotoxic effects of tea extracts. Many plant foods and beverages exhibited some antimutagenic and antioxidant activity. Green tea belong to the most active as well as popular dietary factors. Consumption of these substances in diet could be one of the most practical method for prevention of degenerative diseases (cancer, atherosclerosis, diabetes etc.) avaliable to the general public.

7P29

NONGENOTOXIC AND GENOTOXIC EFFECTS OF METHYLCHRYSENES IN RAT LIVER CELLULAR MODELS

Kateřina Pěnčíková[a,*], Jan Vondráček[b], Kateřina Chramostová[c], Zdeněk Andrysík[b], Lenka Šindlerová[b], Alois Kozubík[b] and Miroslav Machala[a]

[a]*Department of Chemistry and Toxicology, Veterinary Research Institute, Hudcova 70, 62132 Brno, Czech Republic*
[b]*Laboratory of Cytokinetics, Institute of Biophysics, Academy of Sciences, Brno, Czech Republic*

** E-mail: pencikova@vri.cz*

Methylchrysenes belong to a large class of polycyclic aromatic hydrocarbons (PAHs) that exert their mutagenic and/or carcinogenic activities after metabolic activation[1]. Similarly to other PAHs, the not yet investigated nongenotoxic effects of methylchrysenes might contribute significantly to their carcinogenicity, tumor promoting activity, as well as to various endocrine disrupting processes.

The aim of this study was to assess aryl hydrocarbon receptor (AhR)-inducing activity of methylchrysenes, which may lead both to induction of expression of CYP1A1, a major bioactivating enzyme, and to an increased proliferation of confluent cells, a possible tumor-promoting event[2]. The AhR activation was determined in the DR-CALUX bioassay using rat hepatoma H4IIEGud.Luc1.1 cells, stably transfected with a luciferase reporter gene. Cell numbers and modulation of cell cycle were determined by cell counting and flow cytometry, respectively. The genotoxic events, possibly associated with induction of apoptosis, were investigated by detection of Ser15 phosphorylation of p53 tumor suppressor protein and evaluation of fragmentation of nuclei in rat liver epithelial WB-F344 cell line[2]. Moreover, the inhibition of gap junctional intercellular communication, which is considered to be a complementary event associated with tumor promotion, was determined in WB-F344 cells following an acute exposure to PAHs under study[4].

All methylchrysene derivatives elicited the AhR-mediated activity at nanomolar concentrations; their relative potencies were comparable with the most potent PAHs. In accordance with this finding, a percentage of cells entering S phase and total number of cells increased at similar concentration range. On the other hand, methylchrysenes did not induce p53 phosphorylation or an excessive amount of apoptosis in rat liver epithelial cells. Both 5-methyl- and 6-methylchrysene inhibited significantly GJIC, while other test compounds were inactive. In summary, monomethylated chrysenes can induce *in vitro* effects associated with tumor promotion in model liver cells, while they do not act as strong genotoxins comparable to benzo[a]pyrene or dibenzo[a,l]pyrene. Therefore, the nongenotoxic modes of action could play a significant role in carcinogenicity of methylated chrysenes.

References

1. Cheung Y. L., Gray T. J. and Ioannides C.: *Toxicology* **81**, 69 (1993)

2. Chramostová K., Vondráček J., Šindlerová L, Vojtěšek B., Kozubík A. and Machala M.: *Toxicol. Appl. Pharmacol.* **196**, 136 (2004)
3. Machala M., Vondráček J., Bláha L., Cigánek M. and Neča J.: *Mutation Res*. **497**, 49 (2001)
4. Bláha L., Kapplová P., Vondráček J., Upham B. and Machala M.: *Toxicol. Sci*. **65**, 43 (2002)

Acknowledgement

This work was supported by the grant MZE 0002716201 from the Ministry of Agriculture, Czech Republic.

7P30

FLUO-3, AM CAN BE USED AS AN AGENT REFLECTING FUNCTION OF P-GLYCOPROTEIN IN L1210/VCR CELLS

Roderik Fiala*, Jozef Orlický, Zdena Sulová, Alexandra Zahradníková Jr. and Albert Breier

Institute of Molecular Physiology and Genetics, Slovak Academy of Sciences, Vlárska 5, 83334 Bratislava, Slovak Republic

** E-mail: usrdrdmj@savba.sk*

Multidrug resistance (MDR) phenotype of L1210/Vcr cell line, acquired by selection for vincristine, is predominantly mediated by P-glycoprotein (Pgp). Eneroth et al.[1] have shown that calcein AM (Cal), a fluorescent substrate for Pgp, is suitable for measuring of transport activity of Pgp. Expression of Pgp in the cells prevents them to be filled with the fluorescent marker. To detect the activity of Pgp, verapamil (Ver) or cyclosporin A (CsA) has to be used as known Pgp inhibitors. Multidrug resistance protein (MRP), another drug efflux pump, may be inhibited by probenecid (Pro), i.e. the inhibitor of wide variety of anion transporters. Ver, but not Pro, is able to induce the loading of L1210/Vcr cells by Cal that is measurable by FACS. Another dye, Fluo-3, AM (Fluo), has a similar behaviour like Cal. Using confocal microscopy we have proved that L1210/Vcr cells, in contrast to parental sensitive cells, are not filled with Fluo. Marking cells with the dye can be achieved using inhibitors of Pgp like Ver or CsA but not by Pro. These results indicate that Fluo is usable for detection of Pgp function in various MDR tissue cells.

Refrences

1. Eneroth A., Astrom E., Hoogstraate J., Schrenk D., Conrad S., Kauffmann H. M. and Gjellan K.: *Eur. J. Pharm. Sci*. **12**, 205 (2001)

Acknowledgement

Suported by VEGA 2/3122, 2/4155 and APVT-51- 013802.

ACTA UNIVERSITATIS PALACKIANAE OLOMUCENSIS
FACULTAS RERUM NATURALIUM 2004 CHEMICA 43S

PROCEEDINGS OF THE XIX. MEETING OF CZECH AND SLOVAK SOCIETIES FOR BIOCHEMISTRY AND MOLECULAR BIOLOGY

Olomouc, August 31 – September 3, 2004

SESSION 8: BIOINFORMATICS, STRUCTURAL BIOLOGY AND PROTEIN ENGINEERING

8L01

INTERESTING EVOLUTIONARY RELATIONSHIPS IN THE α-AMYLASE FAMILY: THE IDEAS FOR PROTEIN ENGINEERING AND DESIGN

Štefan Janeček*

Institute of Molecular Biology, Slovak Academy of Sciences, Dúbravská cesta 21, 84551 Bratislava, Slovak Republic

** E-mail: Stefan.Janecek@savba.sk*

α-Amylase enzyme family was established more than 10 years ago. In general, the members of the family should: (i) hydrolyse or form by transglycosylation the α-glucosidic bonds; (ii) have four to seven conserved sequence regions in their amino acid sequences; and (iii) contain three catalytic residues Asp, Glu and Asp corresponding to Asp206, Glu230 and Asp297 of Taka-amylase A. The scope of the α-amylase family is enormous: more than one thousand known amino acid sequences cover about 30 various enzyme specificities from hydrolases, transferases and isomerases that, in the sequence-based classification system of glycoside hydrolases (GH), form the clan GH-H containing the families GH-13, GH-70 and GH-77. The real diversity of the α-amylase family can also be documented by the extreme sequence divergence, since there are only 3 invariantly conserved amino acid residues within the entire family: just the above-mentioned catalytic triad. The attractiveness of the family is strengthened by the existence of the other amylolytic family, the family GH-57, containing the amylase, amylopullulanase, 4-α-glucanotransferase and α-galactosidase specificities, the sequences of which are different from those of the members of the clan GH-H.

Bioinformatics analysis of the α-amylase family members has revealed several evolutionary relationships that could serve as ideas for future protein engineering and design, such as: (i) the existence of subfamilies based on the subtle sequence differences; (ii) evolutionary relatedness of taxonomically distantly related producers (archaeons and plants); (iii) eventuality of the horizontal gene transfer (e.g., in the genome of *Microbulbifer degradans*); (iv) similarity of non-amylolytic proteins to the α-amylase family enzymes (e.g., the proteins involved in cystinuria in mammals); and many others.

8L02

BIOINFORMATICS ANALYSIS OF THE FAMILY GH-57 GLYCOSIDE HYDROLASES FOCUSED ON THE AMYLOPULLULANASE FROM *THERMOCOCCUS HYDROTHERMALIS*

Richard Zona* and Štefan Janeček

Institute of Molecular Biology, Slovak Academy of Sciences, Dúbravská cesta 21, 84551 Bratislava, Slovak Republic

** E-mail: Richard.Zona@savba.sk*

Amylolytic enzymes forms a large group of enzymes acting on starch and related oligo- and polysaccharides. The majority of these enzymes have been grouped into the α-amylase family that, in the sequence-based classification of glycoside hydrolases, constitutes the clan GH-H covering the three glycoside hydrolase families (GHs) 13, 70, and 77. All members of GH-H clan are multidomain proteins with catalytic (β/α)8-barrel fold (TIM-barrel) employing a common catalytic machinery and retaining mechanism of glycosidic bond cleavage. The GH-13 is the main family containing almost 30 various enzyme specificities, such as cyclodextrin glucanotransferase, oligo-1,6-glucosidase, neopullulanase, amylosucrase, etc., in addition to the α-amylase. Recently the closely related members of GH-13 were grouped into subfamilies. The GH-70 consists of glucan synthesising glucosyltransferases with circularly permuted form of catalytic (β/α)8-barrel. The GH-77 covers 4-α-glucanotransferases that lack the domain C positioned C-terminally to catalytic (β/α)8-barrel in the GH-13. The characteristic feature joining the entire clan GH-H is the existence of 4 up to 7 conserved sequence regions common for all members.

More than 16 years ago a sequence of a heat stable α-amylase from a thermophilic bacterium *Dictyoglomus thermophilum* was published. Despite the fact that the sequence has coded for an α-amylase, it did not exhibit any detectable similarities with known sequences of GH-13. Later a similar sequence of the α-amylase from the hyperthermophilic archaeon *Pyrococcus furiosus* was determined. These two sequences became the basis for a new amylolytic family GH-57. The main reason for establishing GH-57 was the fact that the two α-amylases lack the conserved sequence regions characteristic for the GH-13 α-amylases.

Very recently the structure of 4-α-glucanotransferase from *Thermococcus litoralis* as a (β/α)7-barrel fold, i.e. an incomplete TIM barrel, with the two catalytic residues, Glu123 and Asp214, has became available. However, the detailed alignment of the GH-57 complete sequences has not been done yet. Until now only either partial or selected sequences were compared.

This study brings the first detailed comparison and alignment of all available and complete amino acid sequences of GH-57 members. It is focused also on the evolutionary characterization of the N- and C-terminal parts of *Thermococcus hydrothermalis* amylopullulanase (Thchy.apu) subfamily.

To collect the sequences, the CAZy server (http://afmb.cnrs-mrs.fr/CAZY/; November 2003) and Pfam database (http://www.sanger.ac.uk/Software/Pfam/; July

2003; entry PF03065) were used. The sequences were retrieved from GenBank and SwissProt. Owing to the sequence diversity problem, alignment of the GH-57 family was done manually. Partial and pair-wise alignments were performed using the program CLUSTALW. The method used for building the evolutionary trees was the neighbour-joining method. The trees were drawn with the TreeView program. In order to detect raw data about similarities between sequences from the GH-57 family, the BLAST tools were used.

In our work we prepared the set of 59 amino acid sequences of the GH-57 (α-amylases, 4-α-glucanotransferases, α-galactosidases, amylopullulanases, hypotetical proteins) with extreme sequence diversity, illustrated especially by the sequence lengths, which vary from 346 to 1641 amino acid residues. Based on the sequence alignment and detailed study of primary structures we built the evolutionary trees and all GH-57 members were classified into the subfamilies. We also identified 5 conserved sequence regions (15 HQP, 246 GNVEVT, 288 WAAESA, 392 TLDGENPVEN, 539 AEASDWFWW; Thchy.apu numbering), which are valid for the whole GH-57 family. We also suggested the amino acid residues potentially important for the entire GH-57: His15, Glu249, Glu291, Asp394, Glu396, Asp543 (Thchy.apu numbering), which will be experimentally verified by site-directed mutagenesis. Two of the residues, Glu291 and Asp394, have already been experimentally confirmed as catalytic nucleophile and the proton donor, respectively.

Based on sequence similarities and known three-dimensional structure of *Thermococcus litoralis* 4-α-glucanotransferase we suggested β-strands creating the catalytic TIM-barrel and succeeding β-sandwich domain in Thchy.apu (cca 900 residues from the N-terminal end). In an effort to evolutionary characterise the subfamily around the Thchy.apu we also found that the N-terminal parts exhibit closer evolutionary relationships in comparison with the C-terminal parts.

8L03

RALSTONIA SOLANACEARUM LECTINS: STRUCTURE-FUNCTIONAL INSIGHT INTO THEIR SPECIFICITY

Nikola Kostlánová[a,*], Edward Mitchell[b], Stefanie Offermann[c], Dvora Sudakevitz[d], Catherine Gautier[c], Jean Gagnon[d], Nechama Gilboa-Garber[e], Anne Imberty[c] and Michaela Wimmerová[a,f]

[a]*National Centre for Biomolecular Research and* [f]*Department of Biochemistry, Masaryk University, Kotlářská 2, 61137 Brno, Czech Republic*
[b]*Experiments Division, ESRF, BP 220, 38043 Grenoble, France*
[c]*CERMAV-CNRS, 601 rue de la Chimie, BP 53, 38041 Grenoble, France*
[d]*Laboratoire d'Immunologie, Hospital Sud, Grenoble, France*
[e]*Faculty of Life Sciences, Bar-Ilan University, Ramat Gan 52900, Israel*

** E-mail: nikol@chemi.muni.cz*

The adhesion of parasitic organisms at different developmental stages to the surface of the host cell is the first step for invasion and infectivity. Based on the enormous potential of sugar structures to code biological information and on presence of lectins (carbohydrate-binding proteins) as receptor for such structures both in host tissue and parasites, lectin – carbohydrate interactions should play a crucial role in many recognition events.

Ralstonia solanacearum is a plant pathogenic bacterium commonly found in the soils of tropical and subtropical countries where it devastates cultures of many crop plants. Certain strains are adapted to milder environmental conditions and have recently been isolated in northern European countries. This organism, responsible for bacterial wilts, can infect over 200 plants species belonging to over 28 botanical families. Major agricultural hosts include tobacco, tomato, potato, eggplant, pepper and banana. Since there are no known treatments or resistant species, identifying targets for the bacterium in host plants could help develop strategy against it.

The contribution describes three *R. solanacearum* lectins that have been discovered in bacterium extract - RSL (9.9 kDa), RS-IIL (11.6 kDa) and RS20L (20 kDa) using mannose affinity chromatography. RSL exhibits high specificity to fucose and mannose and partial sequence homology to mushroom *Aleuria aurantia* lectin AAL[1]. RS-IIL lectin has high sequence similarity with the fucose binding lectin PA-IIL from the phylogenetically related animal pathogen *Pseudomonas solanacearum*[2]. Their 3D structures are also similar but they differ in sugar specifity[3]. RS20L binds mannose and has no sequence similarity to any known lectin amino acid sequence.

References

1. Wimmerová M., Mitchell E., Sanchez J. F., Gautier C. and Imberty A.: *J. Biol. Chem*. **278**, 27059 (2003)
2. Imberty A., Wimmerová M., Mitchell E. P. and Gilboa-Garber N.: *Microb. Infect.* **6**, 222 (2004)
3. Sudakevitz D., Kostlánová N., Blatman-Jan G., Mitchell E., Lerrer B., Wimmerová M., Katcoff D. J., Imberty A. and Gilboa-Garber N.: *Mol. Microbiol*. **52**, 691 (2004)

8L04

CYTOKININ OXIDASE/DEHYDROGENASE GENE FAMILY: SEARCH FOR NOVEL CEREAL GENES

Petr Galuszka[a,*], Jitka Frébortová[b], Tomáš Werner[c], Mamoru Yamada[d], Thomas Schmülling[c] and Ivo Frébort[a]

[a]*Division of Molecular Biology, Department of Biochemistry, Palacký University, Šlechtitelů 11, 783 71 Olomouc, Czech Republic*
[b]*Laboratory of Growth Regulators, Palacký University/Institute of Experimental Botany of the Academy of Sciences, Šlechtitelů 11, 783 71 Olomouc, Czech Republic*
[c]*Institute of Biology-Applied Genetics, Free University of Berlin, Albrecht-Thaer-Weg 6, D-14195 Berlin, Germany*
[d]*Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, 1677-1 Yoshida, 753-8515 Yamaguchi, Japan*

** E-mail: galuszka@prfholnt.upol.cz*

Level of growth regulators - cytokinins is mainly controlled by catabolic enzyme cytokinin oxidase/dehydrogenase (CKX, EC 1.5.12.99) in plants[1]. CKX proteins are encoded by a small gene family fully described in rice and *Arabidopsis*[2]. Several CKX genes were cloned and their function was studied by overexpression in *Arabidopsis* and tobacco plants. Most of them cause cytokinin-deficiency and various phenotypic alterations[3]. Genetic manipulation of CKX activity holds the promise in improving agricultural traits of crop plants in near future.

The cloning of two novel genes that encode cytokinin oxidase/dehydrogenase in barley was accomplished. Transformation of both genes into *Arabidopsis* and tobacco shows that at least one of the genes codes for a functional enzyme, as its expression caused a cytokinin-deficient phenotype in the heterologous host plants. Additional cloning of two gene fragments and an *in silico* search in the public EST clone databases revealed the presence of at least thirteen more members of the cytokinin oxidase/dehydrogenase gene family in barley and wheat. The expression of three selected barley genes was analyzed by RT-PCR and found to be tissue-specific with peak expression during kernel development. One barley CKX was characterized in detail after heterologous expression in *Arabidopsis*. Interestingly, this enzyme shows pH optimum at 4.5 and a preference for cytokinin ribosides as substrates, which may indicate its vacuolar targeting. Biochemical properties of cytokinin degradation enzymes extracted from different barley tissue will be also presented.

1. Schmülling T., Werner T., Riefler M., Krupková E. and Bartrina y Manns I.: *J. Plant Res*. **116**, 241 (2003)
2. Bilyeu K. D., Cole J. L., Laskey J. G., Riekhof W. R., Esparza T. J., Kramer M. D. and Morris R. O.: *Plant Physiol*. **125**, 378 (2001)
3. Werner T., Motyka V., Laucou V., Smets R., van Onckelen H. and Schmülling T.: *Plant Cell* **15**, 1 (2003)

8L05

SITE-DIRECTED MUTAGENESIS OF THE CYTOKININ DEHYDROGENASE GENE AtCKX2 FROM *ARABIDOPSIS THALIANA*

Hana Popelková[a,*], Jitka Frébortová[b], Petr Galuszka[a] and Ivo Frébort[a]

[a]*Division of Molecular Biology, Department of Biochemistry, Palacký University, Šlechtitelů 11, CZ-78371 Olomouc, Czech Republic*
[b]*Laboratory of Growth Regulators, Palacký University/Institute of Experimental Botany of the Academy of Sciences, Šlechtitelů 11, CZ-78371 Olomouc, Czech Republic*

** E-mail: hanapo@prfholnt.upol.cz*

Cell division, shoot development, growth of lateral buds, photosynthesis, chloroplast development, and delay of leaf senescence[1] are processes in plants that are regulated by hormones cytokinins. A level of these hormones is regulated by their natural degradation catalyzed by the enzyme cytokinin dehydrogenase (CKX; EC 1.5.99.12). N^6-chain cleavage of cytokinins[2] occurs by a dehydrogenation mechanism in the active site of the CKX enzyme. Biochemical experiments show that the enzyme mediates this reaction by means of the covalently bound FAD cofactor that forms ternary complex with cytokinin substrate and electron acceptor[3,4]. In a majority of plants, cytokinin dehydrogenase is present in several gene forms. Multiple sequence alignment of all known CKXs reveals that they share high sequence homology in the FAD-binding and the C-terminal domains. FAD binding domain contains highly conserved N-terminal motif GHS where His is the FAD-binding residue. C-terminus of CKXs contains the motifs PHPW, HFG and DP. Cytokinin dehydrogenases have been found to have common sequence characteristic with some other covalent flavoproteins such as berberine bridge enzyme, 6-hydroxy-D-nicotine oxidase, D-arabino-1,4-lactone oxidase, L-gulono-γ-lactone oxidase, and hexose oxidase. These flavoproteins bind covalently FAD cofactor through His residue in the same N-terminal GHS motif as that found in CKXs. Multiple sequence alignment of CKXs with these flavoproteins[5] has revealed that, besides GHS motif, all mentioned proteins share 100 % homology in DP pair of residues. This indicates that GHS and DP motifs are sequence characteristics of a certain subgroup of covalent flavoproteins[6]. On the other hand, the PHPW and HFG motifs seem to be specific for CKX. Homological model of CKX, generated using flavoproteins vanillyl-alcohol oxidase, and *p*-cresol methylhydroxylase[5], has shown that protein C-terminus plugs the FAD-containing cavity to keep FAD molecule in a hydrophobic environment. To explore an importance of the CKX C-terminus in a more detail, site-directed mutagenesis was employed on the one gene form of the *Arabidopsis thaliana* CKX (AtCKX2). The cDNA of AtCKX2 has been previously cloned into pYES2 plasmid[7] and subsequently subcloned into the pDR197 yeast expression vector. In this work, two mutants of AtCKX2 were produced; the truncated mutant (D489@) having a shorter amino acid sequence by 13 residues and thus lacking the DPKKLLSPGQDIF domain with the highly homological DP motif, and the point mutant (DP489,490AA) having the DP motif replaced by alanines in the amino acid sequence otherwise same as that of the wild type enzyme. The cDNA of mutants was synthesized using specific

oligonucleotides, and cloned into the pDR197 expression vector. The wild type and mutant AtCKX2-pDR197 constructs were used to transform the yeast *Saccharomyces cerevisiae*. The CKX proteins overexpressed in yeast were secreted into the growth medium. The medium was afterwards concentrated by ultrafiltration to obtain a higher concentration of the CKX protein. Expression yield of the wild type and mutant enzymes were biochemically tested using SDS-PAGE and 4-aminophenol activity assay[4]. Gel bands corresponding to CKX were identified using a hybride mass analyser quadrupole-time-of-flight (Q-TOF Micro, Micromass-Waters, UK). Data will be discussed in relation to knowledge available on the CKX protein as well as on other covalent flavoproteins.

Reference

1. Skoog F. and Amstrong C. O.: *Annu. Rev. Plant Physiol.* **21**, 359 (1970)
2. Letham D. S. and Palni L. M. S.: *Annu. Rev. Plant Physiol.* **34**, 163 (1983)
3. Galuszka P., Frébort I., Šebela M., Sauer P., Jacobsen S. and Peč P.: *Eur. J. Biochem.* **268**, 450 (2001)
4. Frébortová J., Fraaije M. W., Galuszka P., Šebela M., Peč P., Hrbáč J., Novák O., Bilyeu K. D., English J. T. and Frébort I.: *Biochem. J.* **380**, 121 (2004)
5. Popelková H., Galuszka P., Frébortová J., Bilyeu K. D. and Frébort I.: In *Recent Research Developments in Proteins, Vol. 2*, (Pandalai S. G., ed.), Transworld Research Network, Kerala, India 2004, p. 63
6. Fraaije M., Van Berkel W. J. H., Benen J. A. E., Visser J. and Mattevi A.: *Trends Biochem. Sci.* **23**, 206 (1998)
7. Werner T., Motyka V., Strnad M. and Schmülling T.: *Proc. Natl. Acad. Sci. U.S.A.* **98**, 10487 (2001)

Acknowledgement

The presented work has been supported by the grant 1K03007 from the Ministry of Education, Youth and Sports, Czech Republic.

8L06

ATP-BINDING SITE OF Na^+/K^+-ATPASE

Martin Kubala*

Department of Protein Structures, Institute of Physiology, Czech Academy of Sciences, Vídeňská 1083, Praha 4, Czech Republic

** E-mail: mkubala@centrum.cz*

Cells evolved several types of membrane transporters, but mechanism of their function is not completely understood yet. The aim of our study was the transporter of sodium and potassium ions, Na^+/K^+-ATPase, often called sodium pump. Na^+/K^+-ATPase is a member of P-type ATPases family that contains enzymes with similar structural and functional features. A significant progress to understanding of the structure of these enzymes brought recent studies of crystal structure of Ca^{2+}-ATPase in both conformations of this enzyme[1,2].

Under ideal physiological conditions, the concentration of sodium ions is about 30 times lower in the cytoplasm than in the extracellular milieu, the proportion for potassium ions is reverse. However, the integrity of cytoplasmic membrane, which is a natural barrier impermeable for ions, can be affected by several physiological processes, typically e.g. by nervous impulse. Then the concentrations of ions can shift toward equilibrium. The gradient in concentrations must be restored and this process requires energy. The energy for the transport is provided by the hydrolysis of ATP. Na^+/K^+-ATPase has high affinity for sodium ions (K_D = 0.6 mM) and low affinity for potassium ions (10 mM) on the cytoplasmic side. On the extracellular side the affinities are reverse, the dissociation constant for sodium ions is 600 mM and for potassium ions only 0.2 mM[3]. It has been found that hydrolysis of one molecule of ATP results in an outlet of 3 Na^+ ions and an uptake of only 2 K^+ ions.

The single ATP binding site was identified on the major cytoplasmic loop connecting transmembrane helices 4 and 5 (H4-H5-loop) of the catalytic subunit, which can be expressed and isolated from bacteria retaining its native three-dimensional structure. We tested influence of point mutations on the ATP-binding affinity using the fluorescence analog 2'(or 3')-trinitrophenyl adenosine-5'-triphosphate (TNP-ATP) and a part of the H4-H5-loop (Leu354-Ile604) of Na^+/K^+-ATPase[4,5]. We found that besides the previously reported amino acid residues Lys480, Lys501, Gly502 and Arg544, further four amino acid residues, Asp443, Glu446, Phe475 and Gln482, contribute to the enzyme-ATP interaction[6-8]. This set of amino acids forming the ATP-binding pocket of Na^+/K^+-ATPase is complete, as deduced from our computer model[9]. This model is based on the crystal structure of Ca^{2+}-ATPase from sarco(endo)plasmic reticulum (SERCA), which is a close relative of Na^+/K^+-ATPase in the P-type ATPases superfamily. On the other hand, only some minor effects were caused by mutations of Ile417, Asn422, Ser445, Ser477 and Glu505. Moreover, we found that the hydrogen bond between Arg423 and Glu472 is essential for the proper connection of the opposite halves of the ATP-binding pocket[8].

References

1. Toyoshima C., Nakasako M., Nomura H. and Ogawa H.: *Nature* **405**, 647 (2000)
2. Toyoshima C. and Nomura H.: *Nature* **418**, 605 (2002)
3. Jorgensen P.L. and Pedersen P. A.: *Biochim. Biophys. Acta* **1505**, 57 (2001)
4. Kubala M., Plášek J. and Amler E.: *Eur. Biophys. J.* **32**, 363 (2003)
5. Kubala M., Plášek J. and Amler E.: *Physiol. Res.* **53**, 109 (2004)
6. Kubala M., Hofbauerová K., Ettrich R., Kopecký V., Krumscheid R., Plášek J., Teisinger J., Schoner W. and Amler E.: *Biochem. Biophys. Res. Commun.* **297**, 154 (2002)
7. Kubala M., Teisinger J., Ettrich R., Hofbauerová K., Kopecký V. Jr., Baumruk V., Krumscheid R., Plášek J., Schoner W. and Amler E.: *Biochemistry* **42**, 6446 (2003)
8. Lánský Z., Kubala M., Ettrich R., Kuty M., Plášek J., Teisinger J., Schoner W. and Amler E.: *Biochemistry*, in press (2004)
9. Ettrich R., Melicherčík M., Teisinger J., Ettrichová O., Krumscheid R., Hofbauerová K., Kvasnička P., Schoner W. and Amler E.: *J. Mol. Model*. **7**, 184 (2001)

8L07

A MOLECULAR DYNAMICS STUDY OF THE CYCLIN-DEPENDENT KINASE-2 (CDK2) WITH SUBSTRATE PEPTIDE (HHASPRK) INHIBITION BY PHOSPHORYLATION

Iveta Bártová[a,*], Michal Otyepka[b], Zdeněk Kříž[a] and Jaroslav Koča[a]

[a]*National Centre for Biomolecular Research, Masaryk University, Kotlářská 2, 611 37 Brno, Czech Republic*
[b]*Department of Physical Chemistry, Faculty of Science, Palacký University, tř. Svobody 26, 771 46 Olomouc, Czech Republic*

** E-mail: iveta@chemi.muni.cz*

The cyclin-dependent kinase-2, CDK2, controls the eukaryotic cell cycle at the G1 – S boundary. CDK2 catalyzes the phosphoryl transfer of the adenosine-5´-triphosphate (ATP) α-phosphate to serine or threonine hydroxyl in the protein substrate. The CDK2 activity is regulated by complex mechanism including binding to positive regulatory subunit (Cyclin A or Cyclin E) and phosphorylation at positive regulatory site in the activation segment (T-loop)[1]. The CDK2 activity is inhibited in several ways, for example, by (de)phosphorylation, interaction with various artificial and natural protein inhibitors[2,3], etc. The CDK2 can be also negatively regulated by phosphorylation at Y15 and, to a lesser extent, at T14 residue in the inhibition segment (G-loop)[4]. Mechanism of the CDK2 inhibition by phosphorylation is known from the kinetics experiments but the structural aspects of inhibition remains unclear. The first attempt to explain the

mechanism of inhibition by phosphorylation came from molecular dynamics simulations on the fully active CDK2 but without any peptide substrate[5].

This work broadens the previous study describing behavior of the fully active CDK2 (pT160-CDK2/Cyclin A/ATP complex) with bound the substrate peptide (HHASPRK) and CDK2 inhibited by phosphorylation at T14, Y15, and T14/Y15 residues in the G-loop using molecular dynamics simulations with the Cornell et al. force field as implemented in the AMBER 6.0 software package. The inhibited complexes of CDK2 were prepared from X-ray structure of the pT160-CDK2/Cyclin A/HHASPRK/ATP complex (1QMZ PDB ID code) by "in silico" phosphorylation of the T14 and/or Y15 residues. Enzyme dynamics was studied during 15 ns long trajectory for the fully active CDK2 and 10 ns long trajectories for all inhibited CDK2. Differences in conformational behavior of key residues for substrate binding and phosphoryl transfer of fully active vs. inhibited CDK2 will be presented and compared to the previous work[5].

References

1. Morgan D. O.: *Annu. Rev. Cell Dev. Biol.* **13**, 261 (1997)
2. Otyepka M., Kříž Z. and Koča J.: *J. Biol. Struct. Dyn.* **20**, 141 (2002)
3. Davies T. D., Pratt D. J., Endicott J. A., Johnson L. N. and Noble M. E. M.: *Pharmacol. Ther.* **93**, 125 (2002)
4. De Bondt H. L., Rosenblatt J. and Jančařík J.: *Nature* **363**, 595 (1993)
5. Bártová I., Otyepka M., Kříž Z. and Koča J.: *Protein Sci.* **13**, in press (2004)

8L08

ENZYMATIC AND NONENZYMATIC DEHALOGENATION OF 1,2-DICHLOROETHANE AND 1,2-DIBROMOETHANE

Michal Otyepka[a,*], Michal Boháč[b], Zbyněk Prokop[b], Eva Otyepková[a] and Jiří Damborský[b]

[a]*Department of Physical Chemistry, Faculty of Science, Palacký University, tř. Svobody 26, 771 46 Olomouc, Czech Republic*
[b]*National Centre for Biomolecular Research, Masaryk University, Kotlářská 2, 611 37 Brno, Czech Republic*

** E-mail: otyepka@prfnw.upol.cz*

1,2-Dichloroethane (DCE) and 1,2-dibromoethane (DBE) are toxic and mutagenic halogenated compounds. As many other synthetic halogenated aliphatic compounds, both are rather resistant to biodegradation and persist in the environment[1]. Nevertheless, several bacterial cultures that are able to use DCE as the only carbon and halogen source have been isolated. The most efficient catalysis has been observed with the haloalkane

dehalogenase DhlA from *Xanthobacter autotrophicus* GJ10 and substantially low activity with haloalkane dehalogenase LinB from *Sphingomonas paucimobilis* UT26[2]. A crystallographic analysis of LinB-DCE complex showed non-productive binding of DCE to the enzyme active site, while molecular docking suggested that DCE molecule can possibly bind to the active site but is prevented by chloride ion and/or water molecules[3].

The different reactivity of DCE and DBE was examined by quantum mechanical calculations as well as by molecular dynamics simulations of enzyme-substrate complexes The calculations showed that the difference in DCE and DBE reactivity cannot be simply explained by the difference in the activation barriers of respective reactions, but the active site flexibility, solvation effects and solvent dynamics must also be taken into account.

References

1. Mabey W. and Mill T: *J. Phys. Chem. Ref. Data* **7**, 383 (1978)
2. Prokop Z., Monincová M., Chaloupková R., Klvaňa M., Nagata Y., Janssen D. B. and Damborský J: *J. Biol. Chem.* **278**, 45094 (2003)
3. Oakley A. J., Prokop Z., Boháč M., Kmuníček J., Jedlička T., Monincová M., Kuta-Smatanova I., Nagata Y., Damborský J. and Wilce M. C. J: *Biochemistry* **41**, 4847 (2002)

8L09

COLD-ACTIVE ENZYMES STUDIED BY COMPARATIVE MOLECULAR DYNAMICS SIMULATION

Vojtěch Spiwok[a,]*, Petra Lipovová[a], Tereza Skálová[b], Eva Buchtelová[b], Jindřich Hašek[b] and Blanka Králová[a]

[a]*Department of Biochemistry and Microbiology, Institute of Chemical Technology, Technická 3, 16628 Praha 6, Czech Republic*
[b]*Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic*

* *E-mail: spiwokv@vscht.cz*

Enzymes from cold adapted species (cold-active enzymes) remain active at low temperatures. Therefore, they are promising catalysts for modern biotechnologies. Rationale of this adaptation is complex and is not completely understood. The idea of relationship between flexibility of an enzyme and its catalytic activity at low temperature is often proposed. We simulated molecular dynamics of five pairs of enzymes – one cold-active enzyme and one counterpart from mesophilic or thermophilic species in each pair. Enzymes involved in this study were α-amylases, citrate synthases, malate dehydrogenases, alkaline proteases and xylanases. For each enzyme we obtained 1 ns trajectory in NPT ensemble at constant temperature of 300 K with explicit solvent.

Comparison of flexibility profiles (expressed as root-mean-square fluctuations) showed that highly flexible parts of protein are located into loop regions. Citrate synthase, malate dehydrogenase and alkaline protease from cold adapted sources were more flexible compared to their meso- or thermophilic counterparts. Site with elevated flexibility were located into loops that are possibly involved in opening and closing of active site pocket. In a dynamics of cold adapted xylanase we observed higher flexibility in active site region. Protein - water interactions were also studied. We observed that these interactions are enhanced in enzymes from thermophilic species. Results indicate, that protein dynamics plays an important role in catalytic processes where structural rearrangements take place.

Acknowledgement

Presented work was supported by Czech Science Foundation (GACR 204/02/0843) and by Foundation for Development of Universities (FRVS G4/1564/2003).

8L10

FLEXIBILITY OF PROTEINS AS OBSERVED IN STRUCTURES DETERMINED BY DIFFRACTION METHODS

Jozef Ševčík*

Institute of Molecular Biology, Slovak Academy of Sciences, Dúbravská cesta 21, 84551 Bratislava, Slovak Republic

** E-mail: jozef.sevcik@savba.sk*

Structures determined by X-ray diffraction methods are generaly considered as rigid folds. In spite of that one can observe substantial flexibility in some regions of the protein molecules which are comparable to that suggested by NMR.

In past two decades we have solved, among others, 23 structures of three ribonucleases from *Streptomyces aureofaciens*, RNase Sa, Sa2 and Sa3, including their mutants and complexes with inhibitors. All structures were solved at high resolution ranging from 1.8 – 0.85 Å at room as well as at cryogenic temperatures. Coordinate accuracy of these structures varies from 0.05 Å in the atomic resolution structures to around 0.12 Å in structures at lower resolutions.

S. aureofaciens ribonucleases are guanylate endoribonucleases which highly specifically hydrolyse the phosphodiester bonds of RNA at the 3'-side of guanosine nucleotides. These enzymes belong to the prokaryotic subgroup of microbial ribonucleases. In spite of relatively high identity of primary sequence, and apparently identical specificity and function, several physico-chemical properties (isoelectric point, activity, thermal stability) differ substantially between the three proteins. In addition,

RNase Sa3 possesses cytotoxic activity against human erythroleukemia cells, not observed for the other two enzymes.

Structures of the three ribonucleases were used in the study of the mechanism of catalysis, conformational stability of proteins, protein-protein and protein-nucleic acid recognition. Besides that structures of ribonucleases identified a number of flexible segments in their molecules. Several examples can be quoted, as the mobility of the loop around a tyrosine residue which forms the bottom of the active site, two main-chain conformations observed in several parts of the structure, the flexibility of the surface loops due to crystal contacts, and the flexibility of the segments showing open and closed conformations of the active site.

The structures of RNase Sa, Sa2 and Sa3 are closely similar. Superposition of 41 molecules taken from 23 structures (34 molecules of RNase Sa, five of RNase Sa2 and two of Sa3) at first sight might be viewed as having some similarity to an ensemble of NMR structures. Each of the structures in this superposition is derived directly from experimental X-ray data, thus there is an intrinsic error in the coordinates and one would expect some variation between the 41 molecules. The variation evident in superposition has four major components: the intrinsic experimental error, which should have a normal distribution with r.m.s. differences around 0.1 Å, differences caused by the various crystallization conditions and the packing in the various crystal forms, differences caused by the formation of complexes with inhibitors and differences due to the amino acid substitutions between the three enzymes.

Based on a relatively large number of structures, it can be concluded that the structures of *Streptomycete* ribonucleases possess substantial flexibility which might be related to their function. It is believed that these observations are not related to ribonucleases only but under favourable circumstances could be observed for most of proteins.

8L11

STRUCTURES OF THE COMPLEXES OF RIBONUCLEASE Sa2 WITH MONONUCLEOTIDES

Vladena Hlinková*, Ľubica Urbánková and Jozef Ševčík

Institute of Molecular Biology, Slovak Academy of Sciences, Dúbravská cesta 21, 84551 Bratislava, Slovak Republic

** E-mail: umbivlad@savba.sk*

Ribonucleases Sa2 and Sa are extracellular guanylspecific ribonucleases isolated from *Streptomyces aureofaciens*, strains BMK and R8/26, respectively. Both enzymes

specifically hydrolyse the phosphodiester bond of RNA at the 3´-side of guanosine nucleotides[1,2]. The identity of the primary structures of RNase Sa2 and Sa is 65% and tertiary structures of these proteins are almost identical. The amino acids Glu56 and His86 (RNase Sa2 numbering), which are involved in catalytic reaction, are conserved in both enzymes. In spite of that, the kinetic parameters of the two enzymes significantly differ. For better understanding the structure-function relationship of RNase Sa2, we have solved four structures of the complexes of the enzyme with mononucleotides guanosine-3'-monofosfate (3´-GMP) and guanosine-2'-monofosfate (2´-GMP), which act as inhibitors of the enzyme (complexes 1, 2 and 3) and exo-guanosine-2´,3´-cyclophosphorothioate (2´,3´-GCPT), representing the analogue of the reaction intermediate (complex 4). Crystals of the complexes were prepared by diffusion of the ligands into the drops containing crystals of RNase Sa2[3]. The structures were solved with the program MOLREP[4] and refined with the program REFMAC5[5] against data around 2.0 Å to final crystallographic R-factors in the range of 20-23%.

In the asymmetric unit of the complex 1 there are four molecules of the RNase Sa2/3' GMP complexes. The base of 3'-GMP forms three hydrogen bonds with the amide groups of Glu40, Asn41, Arg42 and two hydrogen bonds with carboxyl group of Glu43. The base is stabilised also by hydrophobic interactions with the aromatic rings of Phe39 and Tyr87, which form the bottom of the active site. The phosphate group of 3´-GMP forms several hydrogen bonds with Arg67, Arg71, His86 and Tyr87.

The asymmetric unit of the complexes 2, 3 and 4 contains three protein molecules (A, B, C). In all complexes, the mononucleotides (3´-GMP, 2´-GMP and 2´,3´-GCPT) are bound to the active site of molecule B because it is the only one which is not blocked by the neighbor molecules in the crystal. In molecule A there is a sulfate anion bound to the phosphate-binding site. The active site of molecule C is filled only with water molecules. By comparison of the active sites of all three molecules the conformational changes of several amino acids were observed due to different occupation (mononucleotide, sulfate anion, water molecules) of the active sites. Observed conformational changes of important amino acids in the active site show their flexibility which can be related to the substrate binding and cleaving.

As the active site amino acids are conserved in ribonucleases Sa2 and Sa and the structures of the active sites are nearly identical, mononucleotides are bound in a similar way[6,7]. Comparison of the active sites shows that the main difference is in substitution of Arg34 and Arg45 in RNase Sa2 by Gln32 and Val43 in Sa. None of these residues is directly involved in catalytic reaction. In spite of that it is expected, that these residues play an important role in the function of the enzyme as it was shown by site directed mutagenesis in ribonuclease Bi (*Bacillus intermedius*). Mutation of the arginine to valine increased k_{cat} seven fold[8]. To explain differences in enzymatic activity between RNase Sa2 and Sa, besides observed differences in the structures, which partially clarified the problem, experiments with site-directed mutagenesis are also planned.

Reference

1. Bačová M., Zelinková E. and Zelinka J.: *Biochim. Biophys. Acta* **235**, 335 (1971)
2. Nazarov V.: *Ph.D. thesis*. Institute of Molecular Biology, Slovak Academy of Sciences (1991)
3. Hlinková V., Urbániková L. and Ševčík J.: *Biologia* **57**, 823 (2002)

4. Vagin A. and Teplyakov A. J.: *Appl. Cryst.* **30**, 1022 (1997)
5. Murshudov G., Vagin A. and Dodson E. J.: *Acta Cryst.* **A53**, 240 (2001)
6. Ševčík J., Dodson E. J. and Dodson G. G.: *Acta Cryst*. **B47**, 240 (1991)
7. Ševčík J., Zegers I., Wyns L., Duter Z. and Wilson K.: *Eur. J. Biochem.* **216**, 301 (1993)
8. Okorokov A. L., Panov K. I., Kolbanovskaya E. Yu., Karpeisky M. Ya., Polyakov, K. M., Wilkinson, A. J. and Dodson G. G.: *FEBS Lett.* **384**, 143 (1996)

8L12

EFFECT OF MUTATIONS ON PROTEIN CRYSTALLIZATION

Ľubica Urbániková[a,*], Lubomír Janda, Vladena Hlinková and Jozef Ševčík

[a]*Institute of Molecular Biology, Slovak Academy of Sciences, Dúbravská cesta 21, 84551 Bratislava, Slovak Republic*
[b]*Institute of Biochemistry and Molecular Cell Biology, Vienna BioCenter, Vienna, Austria*

** E-mail: lubica.urbanikova@savba.sk*

Preparation of highly ordered protein crystals is essential for using X-ray crystallography technique, which is still the most powerfull tool for determining the three-dimensional structure. At high technical level of data collection at synchrotron sources and software developments for data processing and structure determination and refinement, protein crystallization still remains more art than science and has become the major limiting step. Structural genomics projects show that only 10-30 % of proteins of crystallographic purity yield high quality crystals necessary for data collection and structure determination. Conditions at which protein might produce highly ordered crystals cannot be computed, they must be identified empirically. Crystallization is influenced by extremely large number of parameters from which the most important is protein itself. It is well known that protein modifications may have a dramatic effect on crystallization behavior.

We have crystallized a number of proteins and determined their tertiary structures. As far as the effect of protein modification on crystal formation is concerned, the most relevant results were obtained in crystallization of ribonucleases Sa and Sa3 produced by *Streptomyces aureofaciens* and actin-binding domain of cytoskeletal protein plectin from Mus musculus.

We have observed that some point mutations of RNase Sa surface amino acids introduced changes in the number and arrangement of molecules in the asymmetric unit and the ability of the protein to form crystal. Several mutants of RNase Sa crystallized more easily in comparison with the wild-type, some failed to crystallized or crystals were unstable or of poor quality not suitable for data collection.

All RNase Sa mutants were crystallized under similar conditions. Most mutations of surface residues (Ser›Ala, Thr›Ala, Tyr›Phe) had no effect on crystal packing or quality. Mutation Gln›Lys caused changes in the crystal packing. Introduction of five lysines significantly decreased stability and quality of obtained crystals.

RNase Sa3 crystallized under different conditions in two crystal forms with completely different crystal packing[1]. In spite of that, tryptophan residue formed intermolecular contacts in both cases. RNase Sa does not contain any tryptophan residue. Introduction of tryptophan into RNase Sa at equivalent position as in RNase Sa3 changed the crystal packing and tryptophan was again involved in formation of crystal contacts.

For crystallization of actin–binding domain of plectin two isoforms differing in the presence/absence of an additional exon consisting of five amino acids (His-Trp-Arg-Ala-Glu) were used. The exon-containing isoform yielded two crystal forms under different conditions[2,3]. In spite of different crystal symmetry and arrangement of molecules in the two crystal forms, tryptophan residue from the exon always formed contacts with the neighbor molecules. The exon-free isoform failed to crystallize.

Results of our experiments concerning importance of some amino-acids for formation of intermolecular contacts in crystal was supported also by systematic analysis of structures in PDB. We believe that our findings could be useful for prediction of mutations which might improve crystallizability of proteins and optimize crystal packing.

References

1. Ševčík J., Urbániková Ľ., Leland P. A. and Raines R. T.: *J. Biol. Chem.* **277**, 47325 (2002)
2. Urbániková Ľ., Janda Ľ., Popov A. S., Wiche G. and Ševčík J.: *Acta Cryst.* **D58**, 1368 (2002)
3. Ševčík J., Urbániková Ľ., Košťan J., Janda L. and Wiche G.: *Eur. J. Biochem.* **271**, 1873 (2004)

8P01

ASSEMBLY OF M-PMV RETROVIRAL CAPSIDS *IN VITRO*

Šárka Haubová[a,*], Pavel Ulbrich[a], Michaela Rumlová[b] and Tomáš Ruml[a]

[a]*Department of Biochemistry and Microbiology, Institute of Chemical Technology, Prague, Technická 5, 166 28 Praha 6, Czech Republic*
[a]*Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic*

** E-mail: haubovas@vscht.cz*

Retroviruses are subject of intensive studies not only in context with AIDS pandemia and oncogenic effect on several organisms including human. The understanding of retroviral capsid assembly mechanisms and nucleic acids incorporation could significantly contribute to further use of these systems for therapeutic purposes. Our research is focused on study of forming of Mason-Pfizer monkey virus (M-PMV) immature capsids *in vitro*. M-PMV as the prototype of D-type retrovirus is an excellent model for assembly studies. These retroviruses are characterized by the assembly of intracytoplasmic capsids, which make their way to the plasma membrane and are released by budding. Gag polyproteins of all retroviruses contain domains corresponding to the structural proteins which are important for driving the assembly process, namely matrix (MA), capsid (CA) and nucleocapsid (NC) proteins.

We have found specific conditions under which we can prepare properly assembled capsids *in vitro* from structural polyprotein Gag and his deletion mutants. Our interest has been focused on minimized Gag domain, namely fused capsid and nucleocapsid proteins, which contain domains responsible for interactions during capsid assembly process. Due to the fact that N-terminal proline of CA has significant effect on the shape of formed capsids we studied both CANC and CANC lacking this N-terminal proline. We determined the influence of different conditions on capsid assembly process; namely concentration of zinc ions, concentration of salts, pH, temperature and presence of oligonucleotides or RNA. Formed capsids were analysed using sucrose gradient ultracentrifugation and by electron microscopy. We confirmed that presence of oligonucleotides or RNA facilitated particle assembly.

The system described here might contribute to understanding of capsid assembly mechanism and to a development of inhibitors of capsid assembly. Moreover the *in vitro* incorporation of specific genes represents a promising system for construction of gene therapy vectors.

8P02

LIMITATION OF HETEROLOGOUS EXPRESSION OF HONEYBEE ROYAL JELLY APALBUMIN-1 IN *ESCHERICHIA COLI* BY RARELY CODONS USAGE

Juraj Majtán*, Katarína Bílková and Jozef Šimúth

Laboratory of Genetic Engineering, Institute of Chemistry, Slovak Academy of Sciences, Dúbravská cesta 9, 84538 Bratislava, Slovakia

** E-mail: chemmajt@savba.sk*

Royal jelly (RJ) is a natural food rich in numerous nutritive substances, high in protein content (12-15 %). The proteins have an irreplaceable role in nutrition and protection of the honeybee brood against pathogens. They occur in largest amounts in RJ which contains as much as 40 % proteins per dry matter. Sequential analysis showed that almost 90 % of RJ proteins form one protein family with 70 % identity with high content of Arg, Leu a Ile[1]. Previously, we have expressed apalbumin-1 and apalbumin-2 (previously named as MRJP1 or MRJP2 respectively) using pQE32 system in *E. coli* M15 [pREP4], however without achieving a sufficient expression level [2,3].

Although *E. coli* has a remarkable capacity to produce large quantities of protein, there are limits when the codon usage in the mRNA of the recombinant gene differs from the *E. coli* host cells. A subset of codons, namely AGG/AGA (arginine), AUA (isoleucine), CUA (leucine), CGA (arginine), and CCC (proline), are the least used codons in *E. coli* that correspond to rare tRNAs. In order to obtain large amounts of the (His)6-tagged fusion recombinant apalbumin-1, we subcloned cDNA encoding apalbumin-1 without signal peptide sequence into pET28 system. We investigated level of expression of recombinant aplabumin-1 in conventional *E. coli* BL-21(DE3) and modified *E. coli* BL-21-Codon plus(DE3)-RIL with extra copies of *E. coli* argU, ileY and leuW tRNA genes. The cells were induced by addition of IPTG into culture medium.

Exprimed protein was produced in insoluble form as inclusion bodies in both types of *E. coli* cells. The inclusion bodies were solubilized effectively with denaturing buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl, pH 8.0). Level of expression of recombinant apalbumin-1 in modified *E. coli* BL-21-Codon plus(DE3)-RIL was significantly higher compared to the expression level achieved by conventional *E. coli* BL-21(DE3) cells and the expression level of apalbumin-1 in pQE32 system in *E. coli* M15 [pREP4]. We isolated and purified recombinant protein by nickel affinity chromatography[4]. Pure recombinant apalbumin-1 was obtained by electroelution from the polyacrylamide gel in electroelution buffer (200 mM glycine, 25 mM Tris-HCl, pH 8.3).

Apalbumin-1 is the most abundant glycoprotein of royal jelly, which creates free assembling of regular filamentous structure[5]. We have found that apalbumin-1 is a regular component of honeybee products and thus is a suitable marker tool for proving adulteration of honey by means of immunochemical detection. Its presence in all tested samples of honeys and honeybee pollen was confirmed by Western-blot analysis using

polyclonal antibodies raised against recombinant apalbumin-1. It has been found that major RJ-proteins, apalbumin-1 and apalbumin-2 stimulate mouse macrophages to release TNF-α, which demonstrates that physiologically active proteins of honey could be used for its biological valuation[6]. The recombinant apalbumin-1 is used to like marker tool for proving adulteration of honey by imunochemical detection[7] and for study if the stimulating effect of apalbumin-1 on production of TNF-α is derived from their specific amino acids sequentional domains or from sugar moieties.

References

1. Schmitzová J., Klaudíny J., Albert Š., Schröder W., Schreckengost W., Hanes J., Judová J. and Šimúth J.: *Cell. Mol. Life Sci.* **54**, 1020 (1998)
2. Bíliková K., Klaudíny J. and Šimúth J.: *Biologia* **54**, 733 (1999)
3. Júdová J., Klaudíny J. and Šimúth J.: *Biologia* **53**, 777 (1998)
4. Hochuli E., Bannwarth W., Dobeli H., Gentz R. and Stűber D.: *Bio/Technology* **6**, 1321 (1988)
5. Šimúth J.: *Apidologie* **32**, 69 (2001)
6. Šimúth J., Bíliková K., Kováčová E., Kuzmová Z. and Schröder W.: *J. Agric. Food Chem.*, in press (2004)
7. Šimúth J., Bíliková K., Kováčová E. and Majtán J.: *Prihláška vynálezu:* PV-PP 0302-2003

Acknowledgement

This work has been supported by VEGA grant Agency of Ministry of Education of Slovak Republic and Slovak Academy of Sciences Nr. 2/4059/04.

8P03

BIOINFORMATICS ANALYSIS OF STARCH-BINDING DOMAIN

Richard Zona[a], Martin Machovič[b,*] and Štefan Janeček[a]

[a]*Institute of Molecular Biology, Centre of Excellence for Molecular Medicine, Slovak Academy of Sciences, Bratislava, Slovakia*
[b]*Department of Biotechnologies, Faculty of Natural Sciences, University of SS. Cyril and Methodius, nam. J. Herdu 2, 91701 Trnava, Slovakia*

** E-mail: machro@orangemail.sk*

Amylolytic enzymes are multidomain proteins. The three best-known amylases, the α-amylase, β-amylase and glucoamylase, differ structurally and functionally from each other. In the sequence-based classification of glycoside hydrolases (GH) they form their own independent enzyme families without mutual sequence similarities: GH-13, GH-14 and GH-15, respectively.

The family GH-13 covers about 30 various enzyme specificities. It forms, together with the families GH-70 and GH-77, the clan GH-H. Sequentially not similar α-amylases and related amylolytic enzymes were grouped into the family GH-57, some amylolytic enzymes being also found in the family GH-31. The amylolytic enzymes belonging to the clan GH-H (GH-13, GH-70 and GH-77) can be discriminated from those belonging to families GH-14, GH-15, GH-31 and GH-57 by their amino acid sequences and three-dimensional structures. The families moreover employ different reaction mechanisms and catalytic machineries. The members of the families GH-13 (α-amylases) and GH-14 (β-amylases) adopt the structure of a parallel (β/α)8-barrel catalytic domain, while the catalytic domain of the GH-15 members (glucoamylases) is the helical (α/α)6-barrel. Concerning the reaction mechanism, α-amylases and related enzymes employ the retaining mechanism whereas the β-amylases and glucoamylases use the inverting mechanism.

Approximately 10% of amylolytic enzymes are able to bind and degrade the raw starch. Usually a distinct domain or a module is responsible for this property. Based on their sequences the starch-binding domains (SBD) have been classified into the families of carbohydrate-binding modules (CBM). At present, there are five SBD families: CBM-20, CBM-21, CBM-25, CBM-26 and CBM-34.

This work has been focused on the families CBM-20 and CBM-21 of SBDs. The CBM-20 motif belongs to the most deeply studied SBD families. It is positioned almost exclusively at the C-terminal end of amylolytic enzymes from the families GH-13, GH-14 and GH-15. Its three-dimensional structure has already been determined. On the other hand, there is a lack of information on structure-function relationships of CBM-21 motif. It has been known as the N-terminally positioned SBD. Nowadays many non-amylolytic proteins (especially from sequenced genomes) have been recognised to possess the segments in their amino acid sequences that exhibit the unambiguous similarities with the real SBD of CBM-20 and CBM-21, e.g. laforin, genethonin and protein phosphatases. These facts have evoked the interest aimed at the rigorous bioinformatics analysis of the two CBM families.

The eventual relatedness between the C-terminally positioned (CBM-20) and the N-terminally positioned (CBM-21) SBDs was indicated 15 years ago. We therefore, in the first step, analysed the sequences of both families separately taking into account the above-mentioned lack of information concerning the family CBM-21. Then the CBM-20 sequence-structural features were tried to being identified in the sequences of CBM-21 in an effort to reveal the amino acid residues corresponding each other in both families. For this reason the HCA method, a sensitive method for finding the correspondences at high sequence divergence, was used. Finally a sequence alignment was prepared that served for calculation of the common CBM-20-CBM-21 evolutionary tree. Based on the results of this work it has been proposed that these two SBD families could constitute a CBM clan.

ACTA UNIVERSITATIS PALACKIANAE OLOMUCENSIS
FACULTAS RERUM NATURALIUM 2004 CHEMICA 43S

PROCEEDINGS OF THE XIX. MEETING OF CZECH AND SLOVAK SOCIETIES FOR BIOCHEMISTRY AND MOLECULAR BIOLOGY

Olomouc, August 31 – September 3, 2004

AUTHOR INDEX

ACTA UNIVERSITATIS PALACKIANAE OLOMUCENSIS
FACULTAS RERUM NATURALIUM

CHEMICA 43S
SUPPLEMENT

Published by the Palacký University Olomouc

Editor in-Chief: doc. RNDr. Ivo Frébort, CSc., Ph.D.;
doc. RNDr. Daniela Walterová, CSc.; prof. RNDr. Pavel Peč, CSc.
Professional Editor: prof. RNDr. Juraj Ševčík, Dr.

Printed by Palacký University Press
First Edition

ISBN 80-244-0882-1
ISSN 0232-0061