



# **Yarrowia lipolytica, a yeast expression system adapted to the genetic engineering of complex proteins: directed mutagenesis of a fungal laccase**

Catherine Madzak, Claude Jolival, M. C. Mimmi, Agathe Brault, Eliane Caminade, Christian Mougin, Jean-Marie Beckerich

## **► To cite this version:**

Catherine Madzak, Claude Jolival, M. C. Mimmi, Agathe Brault, Eliane Caminade, et al.. Yarrowia lipolytica, a yeast expression system adapted to the genetic engineering of complex proteins: directed mutagenesis of a fungal laccase. IUBMB 50th Anniversary Symposium, Feb 2005, Budapest, Hungary. 1 p. hal-01600556

**HAL Id: hal-01600556**

**<https://hal.science/hal-01600556>**

Submitted on 4 Jun 2020

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

# L1-Protein Diagnostics, Protein Determination

## L1-001

### Reference measurement procedures and external quality assessment schemes

H. Reinauer<sup>1</sup> and P. Kaiser<sup>1</sup>

<sup>1</sup>INSTAND e.V., Düsseldorf, Germany, <sup>2</sup>Reference Laboratories, INSTAND e.V., Düsseldorf, Germany.

E-mail: instand@instand-ev.de

External quality assessment schemes are evaluated by method-dependent consensus values or by method independent target values, determined with reference measurement procedures. The reference measurement procedures yield values having a high trueness and precision with a defined uncertainty of measurement. The great advantage of the reference measurement procedure is that the comparability of results in the different medical laboratories is promoted and improved intending in the same reference interval for an analyte in all laboratories. At the same time the manufacturers are motivated to calibrate their analytical systems in such a way, that the best comparability of values in the external quality assessment schemes is given. The European regulation in that field is the "In-vitro-diagnostic medical device directive". This directive gives an additional role to the external quality assessment schemes that is the vigilance of the market. The vigilance procedure of the diagnostic market intends to register and inform the manufacturers and the competent authorities of any incidence in the diagnostic system. To perform this vigilance procedure in an appropriate way a European standard has been developed (EN 14136). The interaction between EQAS-organizers the manufacturers and the routine laboratories will improve the quality of measurements in the medical laboratories in favour of the patients. One typical reference method measurement procedure will be presented to determine the concentration of digoxin and digitoxin in EQA-samples. The reference measurement procedures have already proved their significance within epidemiological studies.

## L1-002

### International standardization of protein measurement

C. C. Heuck

Institut für Laboratoriumsdiagnostik Universitätskliniken, Düsseldorf, Germany. E-mail: heuck@med.uni-duesseldorf.de

International standardization has wide ranging implications ensuring the quality of biologicals, including proteins, for the diagnosis, prevention and treatment of disease. The highest authority for the international standardization of biologicals is the World Health Assembly (WHA). The secretariat of the Assembly is the World Health Organization (WHO). The WHO Expert Committee of Biological Standardization prepares recommendations on international standards and reference materials that are proposed to the WHA for adoption. Two concepts guided the setting of international standards. The first concept emerged from the practical experience that medical therapy is not effective unless biological products of a defined quality are used. Since internationally accepted reference methods for characterization of biological products did not exist, candidate materials were assessed by an international collaborative study and the standard was defined by consensus. The standard received an arbitrary international unit (IU) that had no relation to the physicochemical properties of the material. The concept proved to be effective for the standardization of biologicals used for prevention (e.g. vaccines) and therapy (e.g. heparin, insulin). As a major

disadvantage, subsequent preparations of an international standard again require a collaborative evaluation, where other techniques may be used for assessment, and the candidate material must be matched with the 1. international standard. With the development of analysis in biochemistry it appeared that the established international standards did not meet diagnostic requirements, either because the materials were not suitable for analytical measurement, or because results that were obtained using various analytical methods were not comparable, although the methods were calibrated with the international standards. Therefore, another concept developed concomitantly with the progress in the molecular characterization of biologicals. As prerequisite highly purified candidate materials are evaluated by analytical methods of highest metrological order and the standard has an assigned international scientific unit (e.g. Mol). The concept is more accurate, however it is also much more complex. Advantageously, a new international reference preparation does not need to be matched with another material provided that the materials have the same physicochemical criteria. The two existing concepts may lead to conflicting situations. International reference preparations of the same biological prepared according to one or the other concept that were established either for a therapeutic or a diagnostic purpose may not be comparable. At present, the conflicting situation was avoided by a diplomatic agreement, where the WHO Committee for Standardization of Biologicals limited its recommendations to materials that are used for preventive care and therapy, whereas international professional societies in collaboration with national and supranational authorities established reference materials for diagnostic purposes. The *In-Vitro* Device Directive of the European Commission complicated international standardization. According to the directive traceability is mandatory, but manufacturers are not obliged to match their diagnostic test systems with international standards. It is obvious that this solution undermines the international efforts in diagnostic standardization.

## L1-003

### IFCC reference method for HbA1c in human blood: a new concept for protein standardization by HPLC-MS

U. Kobold

Analytics, Rare Reagents Diagnostics R&D, Roche Diagnostics GmbH, Penzberg, Germany. E-mail: uwe.kobold@roche.com

HbA1c ( $\beta$ -N-terminal glycosylated hemoglobin) is the most important marker for monitoring the long-term therapy of diabetic patients. A new principle for quantification of proteins in biological fluids based on electrospray ionization mass spectrometry has been developed. The approach was used to set up a highly specific and accurate reference method for the determination of HbA1c in human blood. The method is based on the measurement of the specific N-terminal residues of the hemoglobin  $\beta$ -chains. Enzymatic cleavage of the whole hemoglobin molecule with endoproteinase Glu-C has been optimized to obtain the N-terminal hexapeptides from both HbA1c and HbA0. These peptides are separated by reversed phase HPLC in the first dimension and the ratios are quantitated in the second dimension by electrospray-ionization mass spectrometry. With this new and highly specific method, it has been possible to overcome the insufficient resolution of present protein separation system for HbA1c. The approach has been validated by the IFCC working group on HbA1c standardization and has been approved by the IFCC, it will be the major part of the upcoming reference system

for the international standardization of HbA1c/glycohemoglobin. This approach clearly shows, that proteins can be detected and quantified highly specifically and accurately based on their unique structural features by electrospray mass spectrometry.

## L1-004

### The HUPO Brain Proteome Project

H. E. Meyer

Medical Proteom-Center, Ruhr-University Bochum, Bochum, Germany. E-mail: [helmut.e.meyer](mailto:helmut.e.meyer)

The brain is the most complex tissue of higher organisms, differing from other organs due to its many different cell types, its structure at the cellular and tissue level. At the same time, the brain is of paramount interest to medical research and pharmaceutical industry because of the social impact of the more common neurological diseases such as Alzheimer, Parkinson, Multiple Sclerosis, Prion Diseases and Stroke. The prevalence of some of these diseases is increasingly high, e.g. every fifth person over 80 years in industrial countries is suffering from Alzheimer. The HUPO Brain Proteome Project (HBPP, [www.hbpp.org](http://www.hbpp.org)) was established under the patronage of the Human Proteome Organization (HUPO) in order to help coordinating worldwide neuroproteomic efforts, as well as to set up standards. It aims at the defining and deciphering of normal brain proteome including polymorphisms, modifications, histological localization as well as identification of brain derived proteins in bodily fluids. With the help of differential protein expression profiling by techniques and methods available within the participating groups, disease-related proteins involved in neurodegenerative diseases and aging will be identified with a focus on Alzheimer's disease (AD), Parkinson's disease (PD) and aging. The validation and functional characterization of these proteins will hopefully lead to early onset biomarkers for diagnosis and therapeutic strategies. Numerous meetings and sessions already took place, e.g. the HUPO BPP workshops at Castle Mickeln, Germany (September 2003) and in Paris, France (April 2004). In addition, two pilot studies were initiated analysing mouse brain samples from three different stages (E16, P7, 8 weeks) as well as human brain tissues from autopsy and biopsy under standardized starting points in differential proteomics, peptidomics and transcriptomics approaches. The results will be collected in a Data Collection Center that has been elaborated together with the HUPO Proteomics Standardization Initiative (HUPO PSI), re-processed and published in 2005. The master plan phase will be started with a mouse model workshop in spring 2005.

## L1-005

### Rapid screening of Bothrops snake venoms for peptides using high performance liquid chromatography coupled to tandem nano-electrospray mass spectrometry

G. H. M. F. Souza<sup>1,2</sup>, D. R. Ifa<sup>2</sup>, E. S. Ferro<sup>3</sup>, M. N. Eberlin<sup>2</sup> and S. Hyslop<sup>1</sup>

<sup>1</sup>Biochemistry and Pharmacology Laboratory, Department of Pharmacology, Faculty of Medical Sciences, State University of Campinas (UNICAMP), Campinas, Sao Paulo, Brazil, <sup>2</sup>Thomson Mass Spectrometry Laboratory, Institute of Chemistry, State University of Campinas (UNICAMP), Campinas, Sao Paulo, Brazil, <sup>3</sup>Cellular Communication Laboratory, Department of Cell Biology and Development, Cell Biology Program, Institute of Biomedical Sciences, University of São Paulo (USP), Sao Paulo, Sao Paulo, Brazil. E-mail: [desouz@fcm.unicamp.br](mailto:desouz@fcm.unicamp.br)

Snake venoms contain a variety of peptides, including atrial natriuretic-like peptides, bradykinin-potentiating peptides (BPPs),

sarafotoxins and waglerins. We used nano-LC/MS/MS to examine the peptides present in several Bothrops snake venoms. Venom samples (20 mg) were dissolved in ultrapure water and centrifuged to remove undissolved material. The venom solution was then filtered through Amicon ultrafilters with a molecular mass cutoff 5 kDa. Samples of the filtrate were then analyzed by mass spectrometry using CapLC tandem Q-TOF mass spectrometer. The peptides were sequenced by collision-induced dissociation (CID) with argon and by *de novo* analysis. Sixty-nine peptides were identified, approximately one-third of which were BPPs or putative BPPs (11 of these BPPs have already been reported in the literature). One BPP (QGGWPRPGPEIPP) was found in all of the venoms and may represent a specific marker for this genus since this peptide was not found in venom of the South American rattlesnake, *Crotalus durissus terrificus*. An unrooted tree obtained by sequence alignment and phylogeny analysis showed that the peptides formed four broad groups, one of which contained the BPPs. The remaining peptides formed heterogenous groups. Searches of protein databases yielded very few matches for these peptides, indicating that they did not result primarily from the degradation of venom proteins. These peptides may be the products of specific, as yet unknown genes, or may be derived from the degradation of unidentified cellular proteins by intracellular proteasomes, with the products of degradation subsequently being released into the venom gland lumen. Peptides they may contribute to the cardiovascular effects of the venoms (e.g. hypotension).

**Acknowledgment:** Financial support: CAPES and FAPESP.

## L1-006

### Proteome analysis of normal and transformed thyroid cells

R. Schiavone, L. Zilli, V. Zonno, C. Storelli and S. Vilella  
Laboratory of Physiology, Department of Biological and Environmental Sciences and Technologies, University of Lecce, Lecce, Italy. E-mail: [sebastiano.vilella@unile.it](mailto:sebastiano.vilella@unile.it)

In the present study we used the two-dimensional polyacrylamide gel electrophoresis (2-DE) and matrix associated laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) to compare the protein expression in two cell lines (PC-Cl3 and PC-E1A + raf). PC-Cl3 is a immortalized rat thyroid cell line which *in vitro* retain biochemical characteristics of differentiated thyroid cell. PC-E1A + raf originates by stable transfection with E1A and v-raf oncogenes of the PC-Cl3 cells, and displays a malignant phenotype. The protein profiles differed between the two cell lines as revealed by visual inspection and by image analysis software. We identified  $414 \pm 10$  ( $n = 10$ ) spots in PC-Cl3, among these 11 significantly decreased, eight were absent and six increased in 2-D gel obtained with PC-E1A + raf. Five of these spots were analyzed with MALDI-TOF, but only three showed a significant match in the databases used in the bioinformatics analysis (Profound, Mascot, ad MS-fit). In particular, one of the spot showed homology with a GAPDH (glyceraldehydes-3-phosphate dehydrogenase, one with H(+) transporting ATP-synthase and one with PEBP (phosphatidylethanolamine-binding protein). The present work shows that the transfection with E1A and v-raf oncogenes of the PC-Cl3 cells causes the change of protein expression of PC-Cl3. Two out of three of these identified spots (ATP-synthase and GAPDH) seems to be involved in the cell apoptosis.

## L1-007P

### Thyroid assessment in mothers of primary congenital hypothyroid newborns

G. Asadi Karam, A. Rashidi, M. Rezaeian, A. Gafarzadeh, M. Mahmoodi and M. Khaksari  
*Biochemistry, Rafsanjan Medical University, Rafsanjan, Iran.*  
*E-mail: asadi\_ka@yahoo.com*

**Objective:** To determine etiologic significance of maternal thyroid disease on incidence of primary congenital hypothyroidism in newborns.

**Methods:** Thyroid function was assessed in 96 mothers of hypothyroid newborns and 96 mothers of non-hypothyroid newborns as control. Maternal blood samples were taken at the time of follow-up serum sampling of the infants for measurements of TSH, T4, T3, FTI and Anti-peroxidase (Anti-TPO). Results were compared with similar data from the control population.

**Results:** The mothers of congenitally hypothyroid infants had higher TSH, T3 and Anti-TPO concentrations compared with control population ( $P = 0.018$ ,  $0.001$  and  $0.031$ , respectively). We could not find significant difference of T4 and FTI between two groups.

**Conclusion:** These results indicate that most cases of primary congenital hypothyroidism were attributable to maternal thyroid disease. Because of the high prevalence of thyroid disease in Iran, we recommend thyroid assessment of pregnant women.

## L1-008P

### Characterization of metals bound to biomolecules (metallomics) in pine nuts (*Pinus pinea*) by size-exclusion-reversed-phase LC-ICP-MS

J. L. G. Ariza and A. A. Borrego  
*Environmental and Bioanalytical Chemistry, University of Huelva, Huelva, Spain. E-mail: ariza@uhu.es*

Pine nuts are complex plant foods with lipids 50–70%, proteins 10–20%, and carbohydrates 10–20% [1]. The composition of plant foods is important for varied reasons such as nutritional value, toxicity, pollution, geographic origin of plants, and so on [2]. On the other hand, the determination of the presence of metals in living organisms is necessary to estimate the intake of essential elements and to evaluate the potential health risks caused by the presence of high element levels. Speciation studies have been traditionally used for the determination of the oxidation state of the elements and their alkylation grade since their toxicity is strongly dependent of these parameters. Thus, Cr(VI) is much more toxic than Cr(III), trialkyltin is more toxic than di- or monoalkyltin, and methylmercury is about one thousand more toxic than  $Hg^{2+}$ . On the other hand, inorganic species of arsenic are more harmful than mono- or dimethylarsenic, and bigger molecules such as arsenobetaine is virtually innocuous [3]. Other metals play important roles in living organisms, and are involved on oxygen transport, act as enzyme cofactors or can interact in antioxidative processes, etc. The aim of this work is to identify some of these metals (Cu, Mn, Cd, Ni, Zn, Se and others) and the biomolecules associated to them. This study has been focused on the determination of the molecular size distribution patterns characterization of the above-mentioned elements. The analytical methodology was based on size-exclusion liquid chromatography (SEC) on-line coupled to in serie DAD, inductively coupled plasma mass spectrometry (ICP-MS) system to get the metal species molecular weight profile. After that, the metal bound to high molecular weight fraction was resolved by using reversed phase liquid chromatography (RP-HPLC).

## References

1. Fraser GE. *Asia Pac J Clin Nutr* 2000; **9**: S28.
2. Wuilloud RG, Kannamkumarath SS, Caruso JA. *Anal Bioanal Chem* 2004; **379**: 495–503.
3. Gómez-Ariza JL, García-Barrera T, Lorenzo F, Bernal V, Villegas M.J, Olivera V. *Anal Chim Acta* 2004; **524**.

## L1-009P

### Clinical relevance of the serum dipeptidyl peptidase IV DPP IV/CD26 activity in adult patients with Crohn's disease and ulcerative colitis

J. Varljen<sup>1</sup>, L. Baticic<sup>1</sup>, D. Detel<sup>1</sup>, N. Varljen<sup>1</sup> and B. M. Sincic<sup>2</sup>  
<sup>1</sup>Department of Chemistry and Biochemistry, Faculty of Medicine, University of Rijeka, Rijeka, Croatia, <sup>2</sup>Department of Gastroenterology, Clinical Hospital Centre Rijeka, University of Rijeka, Rijeka, Croatia. E-mail: larabaticic@hotmail.com

The dipeptidyl peptidase IV (DPP IV/CD26), a serine-type protease, is a membrane anchored enzyme widely expressed in many cell types. DPP IV is also present in a soluble form in the serum. The aim of this study was to evaluate the clinical relevance of the changes in serum DPP IV activity in adult patients with inflammatory bowel diseases (Crohn's disease, CD and Ulcerative colitis, UC). The study was performed on 59 patients, 35 with CD (age  $42.60 \pm 15.89$ ; 17 males, 18 females), and 24 with UC (age  $46.62 \pm 16.97$ ; 13 males, 11 females). The diagnosis of CD or UC were established on the basis of clinical history, laboratory, endoscopic and histological data. The control group included 68 healthy donors (age  $41.01 \pm 11.85$ ; 33 males, 35 females). The CD activity was evaluated using the Crohn's Disease Activity Index (CDAI) while the UC activity was evaluated by the Truelove-Witts (TW) classification. Both serum DPP IV activities in patients with active CD ( $35.31 \pm 1.55$  U/l) and UC ( $33.93 \pm 2.13$  U/l) were statistically significantly decreased compared with healthy controls ( $48.37 \pm 1.10$  U/l) ( $P < 0.001$ ). The levels of DPP IV activity in CD and UC patients were inversely correlated with the CDAI and TW score, respectively. As it has been shown that there is no statistically significant difference in the serum DPP IV activity between patients with CD and UC, it can be concluded that the DPP IV could not be a good marker for the differential diagnosis.

## L1-010P

### Increased serum complement C3 and C4 levels in autism: a correlation with severity and language disability

A. Chauhan<sup>1</sup>, V. Chauhan<sup>1</sup> and I. Cohen<sup>2</sup>

<sup>1</sup>Laboratory of Cellular Neurochemistry, Department of Neurochemistry, NYS Institute for Basic Research, Staten Island, New York United States of America, <sup>2</sup>Laboratory of Behav. Assess. & Res., Department of Psychology, NYS Institute for Basic Research, Staten Island, New York United States of America. E-mail: abhachauhan@aol.com

Autism is severe neuro-developmental disorder in children with poorly understood etiology and pathology. Recently, we have reported increased oxidative stress in autism [1]. In this study, complement C3 and C4 levels were determined nephelometrically in serum samples from children with autism, and their developmentally normal non-autistic siblings. Diagnosis was based on information provided by parent interviews using the Autism Diagnostic Interview-Revised (ADI-R) criteria, and by direct observation of child using Autism Diagnostic Observation-Generic (ADOS-G) criteria. Pervasive Developmental Disorder Behavior Inventory (PDDBI) Scale, an independent measure of severity of autism, was

obtained on all subjects using information provided by parents. The levels of both C3 and C4 complement proteins were observed to be significantly higher in autistic subjects as compared to their normal siblings. A strong correlation was observed between increased C3/C4 levels and (a) severity of autism as indicated by PDDBI scale, and (b) language disability. C3 and C4 are the most abundant complement proteins in blood that facilitate immunological and inflammatory responses, and their increased levels are generally linked to acute inflammatory reactions and tissue inflammation. Our results suggest that inflammatory reactions may play a role in the pathogenesis of autism.

#### Reference

1. Chauhan *et al. Life Sci* 2004; 75: 2539–2549.

#### L1-011P

##### **Self-assembly of a globular protein into native-like and enzymatically active aggregates that subsequently reorganize to form amyloid structures**

G. Plakoutsi<sup>1</sup>, F. Bemporad<sup>1</sup>, N. Taddei<sup>1</sup>, C. M. Dobson<sup>2</sup> and F. Chiti<sup>1</sup>

<sup>1</sup>Dipartimento di Scienze Biochimiche, University of Florence, Firenze, Italy, <sup>2</sup>Department of Chemistry, University of Cambridge, Cambridge, UK. E-mail: fchiti@scibio.unifi.it

The conversion of the acylphosphatase from *S. solfataricus* (Sso AcP) into amyloid aggregates was studied under conditions in which this globular protein is initially in its native state and hence in conditions that are relevant to the physiological environments of living organisms. Native Sso AcP was found to self-assemble very rapidly, on the time scale of a few seconds, into aggregate structures in which the individual molecules retain enzymatic activity, have a secondary structure content only marginally different from that of the native state, as deduced from circular dichroism and Fourier-transform infrared spectroscopies, and are not yet able to bind to amyloid diagnostic dyes such as thioflavin T and Congo red. These initial aggregated species convert subsequently into a second type of aggregates that have lost completely the enzymatic activity, have an extensive  $\beta$ -sheet structure, bind to thioflavin T and Congo red and have a morphology that bears close resemblance to the toxic amyloid protofibrils that have been imaged for a number of other protein systems. A kinetic analysis indicates that the structural conversion of the enzymatically active and ThT-negative aggregates into the ThT-binding protofibrils occurs with a rate that is two-orders of magnitude faster than unfolding under the same conditions. Moreover, such conversion is direct and does not require the disassembly of the initial aggregates before the ThT-binding protofibrils can form. The observation of an aggregation process that leads to the formation of enzymatically active aggregates and facilitates further maturation of the aggregates into amyloid structures is unprecedented and may have physiological implications.

#### L1-012P

##### **The determination of hydroxyproline in wound fluid collagen hydrolysate by derivatization and HPLC**

N. Dashti<sup>1</sup>, M. Ansari<sup>1</sup>, M. Shabani<sup>2</sup> and S. Vardasbi<sup>1</sup>

<sup>1</sup>Department of Allied Health Services, Tehran University of Medical Sciences, Tehran, Iran, <sup>2</sup>Department of Biochemistry, Iran University of Medical Sciences, Tehran, Iran.

E-mail: dashti@sina.tums.ac.ir

Nitric oxide(NO) is a small radical, formed from the amino acid L-arginine by three distinct isoforms of nitric oxide synthase. NO plays an important role in wound healing. Because of the limited

utility of authentic NO gas and short half- life of NO *in vivo*, NONOates have been widely used as NO donor drugs. In this study DETA NONOate was applied on full thickness thermal wound and its effect on wound fluid collagen synthesis was investigated. Twelve Sprague–Dawley rats were transferred to separate metabolic cages and the dorsal surface of each rat was given full thickness thermal wound. During wound healing (21 days) the test wounds ( $n = 6$ ) were treated with 100  $\mu$ mol DETA NONOate in phosphate buffer on the day of wounding and every 3 days. Wound fluid was collected using circular sponges. The rate of collagen synthesis was determined by quantitative estimation of hydroxyproline. PTC was used for derivatization of amino acids from wound fluid collagen hydrolysate followed by HPLC analysis. The rate of wound healing was determined by video image analysis. The results of this assay indicate nitric oxide can increase the rate of collagen synthesis in the test group ( $1.22 \pm 0.13$  mg vs.  $0.118 \pm 0.017$  mg for controls,  $P < 0.005$ ). There is also a significant difference ( $P < 0.001$ ) in wound closure profiles between the test and control groups. These experiments indicate nitric oxide can increase the rate of wound healing by increasing the rate of collagen synthesis at the wound site.

#### L1-013P

##### **Timothy grass pollen extracts : biochemical and immunological characterization**

R. Dibiani, M. Azarkan and D. Baeyens-Volant

Protein Chemistry, of Brussels, Brussels, Belgium.

E-mail: rdibiani@ulb.ac.be

Grass pollen allergy ranks among the most frequently observed seasonal respiratory allergies world-wide, affecting up to 25% of the general population. Detection of grass pollen sensitization in epidemiological and clinical studies partially relies on *in vivo* testing by means of skin prick testing (SPT) using allergenic extracts from one or more grass species. This seemingly simple test is subject to multiple variables that can affect the diagnosis result. These variables range from the quality of the allergen preparation to the technique used. Recently, attention has been focused on the quality of allergen extracts; nevertheless, extreme variability in the composition and content in major and minor allergens of products used for SPT and immunotherapy is still frequently reported. The variability seems to be related to the variations in the equipment, reagents and processes that are used to produce the extracts, as well as to the origin of the raw material (pollen). The aim of this study was to characterize extracts of timothy grass (*Phleum pratense*) pollen purchased from different companies. The extractions were realized according to a well defined procedure in terms of extraction ratio, temperature and time. The extracts were investigated regarding their protein concentration and composition, allergens content and immunological activity. Comparative electrophoretic, chromatographic and immunological analyses were carried out. Such studies constitute an important step towards improving simplified diagnosis and specific immunotherapy of grass pollen allergy. It will allow the selection of key allergens and contribute to our understanding of allergic reactions.

#### L1-014P

##### **Interaction of the flavonol quercetin with human target proteins**

M. Böhl, S. V. Tokalov, B. Kind, A. Franz, Y. Henker and

H. O. Gutzeit

Institute of Zoology, Technische Universität Dresden, Dresden, Germany. E-mail: herwig.gutzeit@mailbox.tu-dresden.de

The flavonoid quercetin is common in food and numerous interesting biological effects have been reported. We have exploited

the property of quercetin to change the spectral properties upon binding to specific target proteins. The protein autofluorescence induced by excitation at 285 nm is quenched by the quercetin ligand and in the visible spectrum fluorescence may be induced (Ex485nm, Em545nm). These effects were studied in detail using bovine serum albumin and insulin as model proteins. The induced fluorescence can be exploited to localize target proteins in living cells by fluorescence microscopy. A high concentration of target proteins was found to be present in nuclei. By making use of the induced spectral changes we identified some major target proteins of quercetin in nuclear extracts of human leukaemia cells (HL-60). We have fractionated nuclear proteins by column chromatography, probed the fractions with quercetin, analysed the spectra in a fluorescence spectrophotometer, and finally separated promising protein fractions on SDS-PAGE. Single bands were cut out and the proteins identified by MALDI-MS. In this way we identified, amongst others, actin as a quercetin binding protein. The interaction was confirmed using purified actin. This protein has recently been shown to play an essential role in transcription. This experimental approach opens up new ways in interpreting and predicting biological and pharmacological effects of drugs of interest.

### L1-015P

#### Immunoproteomic approach identifies novel proteins of *Aspergillus fumigatus* with specific IgE immunoreactivity

P. Gautam<sup>1</sup>, T. M. Gupta<sup>1</sup>, W. N. Gade<sup>2</sup>, R. Sirdeshmukh<sup>3</sup> and P. U. Sarma<sup>4</sup>

<sup>1</sup>Laboratory of CSIR, IGIB, Department of Molecular Biochemistry and Diagnostics Division, Mall Road, Delhi, India, <sup>2</sup>Laboratory of Proteomics, Department of Biotechnology, University of Pune, Pune, Maharashtra, India, <sup>3</sup>Laboratory of CSIR, Department of Proteomics, CCMB, Hyderabad, Andhra Pradesh, India, <sup>4</sup>Laboratory of IARI, Department of Plant Pathology, Pusa Road, Delhi, India. E-mail: poonamgautam@rediffmail.com

Allergic bronchopulmonary aspergillosis (ABPA), a severe respiratory disease in humans, is caused by allergenic/antigenic proteins of *Aspergillus fumigatus* inducing type I and type III hypersensitivity reactions. Since a number of secretory proteins are reported to be allergenic/antigenic/virulent factors which are in direct contact to the host tissue mediating important host-pathogen interactions, an immunoproteomic studies have been undertaken to map secretory allergenic proteins by modern proteomic approach to identify novel allergens with specific IgE immunoreactivity. Comparative analysis of 2-DE gels (coomassie-stained) and specific IgE immunoblots of *A. fumigatus* culture filtrate proteins at different time intervals was performed. We observed a total of 159 proteins in culture filtrate of *A. fumigatus* out of which 75 proteins showed specific IgE immunoreactivity. Third week culture filtrate had maximum number of proteins and specific IgE immunoreactive proteins. MALDI-TOF analysis of 33 spots showing specific IgE immunoreactivity led to the identification of 25 proteins, 19 of which are new to *A. fumigatus* proteome database and six are known allergens of *A. fumigatus*. Out of 19 proteins function is known for eight proteins in other fungi: NADPH dependent alcohol dehydrogenase, mitochondrial matrix acyl carrier protein, DST, SEC5, two putative GSTs, 60S ribosomal subunit, cytochrome P450, acid proteinase precursor and eleven were hypothetical proteins. For eight proteins conclusive match could not be obtained. Availability of allergenic proteome and 19 novel allergens/antigens would facilitate a sensitive and specific diagnosis, immunotherapy and further understanding of the biology of the fungus.

### L1-016P

#### Binding of IgM antibodies to bovine serum albumin as a biomarker for type 1 diabetes mellitus

M. Haroun

Department of Bioscience and Technology, IGSR, University of Alexandria, Alexandria, Egypt. E-mail: mharounag@yahoo.com

The aim of this study was to investigate the humoral immune response to bovine serum albumin (BSA) and their relation to the pathophysiology of type 1 diabetes mellitus. Serum immunoglobulin M (IgM) concentration was measured by enzyme-linked immunosorbent assay (ELISA) in 25 adult patients with newly diagnosed type 1 diabetes mellitus, and 25 matched normal adult individuals. Binding of BSA to diabetic serum immunoglobulins led to an over-estimation in the levels of IgM in human diabetic sera. The increase detected by ELISA and turbidimetric assay varied between 10% and 109%. If anti-BSA antibodies were present in serum, they might interfere with the ELISA assay, thus a suitable method was employed to minimize such interference. Initial results before purification from the interfering anti-BSA antibodies suggested that diabetic patients had incremented levels of IgM in their sera. It was found that normal individuals had a mean IgM level of 1.67 mg/ml and diabetic individuals had a mean IgM level of 2.30 mg/ml ( $P < 0.0003$ ). However, the mean level of IgM in diabetic sera after purification from anti-BSA antibodies was 1.69 mg/ml. Therefore, there was no significant difference in IgM level in patients with type 1 diabetes mellitus purified from anti-BSA antibodies, as compared to normal individuals ( $P < 0.84$ ). In conclusion, a high level of heterophile antibodies reactive with BSA commonly associates with type 1 diabetes and may well play a role in the complex immunopathogenetic interactions. However, the demonstration of the binding of IgM antibodies to BSA in patients with newly diagnosed type 1 diabetes initiated a controversial debate on the utility of BSA antibodies as a disease marker.

### L1-017P

#### Biomarker discovery for the early diagnosis of cervical cancer

P. Horvatovich<sup>1</sup>, N. Govorukhina<sup>1</sup>, T. H. Reijmers<sup>2</sup>, S. Nyangoma<sup>2</sup>, R. C. Jansen<sup>2</sup> and R. Bischoff<sup>1</sup>

<sup>1</sup>Department of Analytical Biochemistry, Centre for Pharmacy, University of Groningen, Groningen, The Netherlands, <sup>2</sup>Groningen Bioinformatics Centre, University of Groningen, Haren, The Netherlands. E-mail: p.l.horvatovich@rug.nl

**Aim:** Development of an analytical method for the comparative analysis of serum samples in the benefit of biomarker discovery for cervical cancer diagnosis.

**Introduction:** Cervical carcinoma is the second most frequent carcinoma in women worldwide, while in the developing countries cervical carcinoma is the most frequent carcinoma in women. In an approach to perform comparative analyses of samples from a serum bank from patients in a longitudinal and cross-sectional manner, we have developed a methodology for the comparative analysis of depleted, trypsin digested serum samples by LC-MS followed by data pre-processing and multivariate statistics.

**Results:** Optimization of the analytical method from sample preparation (clotting time, various depletion methods of the most abundant proteins) to the final LC-MS analysis were performed to lower the within sample variability. Further improvement of the reproducibility of the overall procedure was achieved by the use of horse heart Cytochrom C as internal

standard added to the sample prior to sample preparation. The obtained LC-MS data were pre-processed prior to statistical analysis by alignment of retention time and the normalization of intensity of the chromatograms by using specific internal standard peaks prior to selecting the most "information rich"  $m/z$  traces. The selected chromatographic traces containing a significantly decreased level of spikes and noise, were subjected to unsupervised multivariate statistics (principal component analysis). In an effort to validate the methodology, we spiked various amounts of horse heart Cytochrome C into the original serum and found that samples containing the internal standard were discriminated from the non-spiked samples down to a level of 1 pmol in 20  $\mu$ l serum.

**Perspectives:** In order to further improve the sensitivity of the overall method, we performed comparative studies using an on-line nanoLC-MALDI spotter set-up combined with MALDI-TOF/(TOF)-MS\*. Using the described methodology, we are presently performing a comparative, longitudinal study with samples from early and late stage cervical cancer patients prior to and after treatment. Furthermore, we are comparing samples from patients with a positive or negative prognosis in order to define new discriminatory biomarkers or biomarker patterns.

\*Collaboration with Dr. Theo Luider (Erasmus Medical Centre, Rotterdam).

## L1-018P

### Assessment of urinary and serum cystatin C in determination of renal function in children with renal scar

H. Islekel<sup>1</sup>, U. Yis<sup>2</sup>, Z. Altun<sup>1</sup>, A. Soyulu<sup>2</sup>, M. Turkmen<sup>2</sup> and S. Kavukcu<sup>2</sup>

<sup>1</sup>Department of Biochemistry, Dokuz Eylul University School of Medicine, Izmir, Turkey, <sup>2</sup>Department of Pediatrics, Dokuz Eylul University School of Medicine, Izmir, Turkey.

E-mail: huray.islekel@deu.edu.tr

Urinary tract infection (UTI) is a frequently encountered problem in childhood, leading to scar development in renal tissue with sometimes impaired renal functions. The existing routine laboratory renal function tests might not reflect slight changes or have some other limitations due to age, body mass, etc. dependence in the follow-up of children with pyelonephritis. The aim of this study was to evaluate changes in plasma and urine concentrations of cystatin C, a novel glomerular filtration rate marker; creatinine, sodium, phosphorus, as well as urine N-acetyl-beta-D-glucosaminidase (NAG) activity, a sensitive and specific tubular damage marker and microalbumin level in children with renal scar. The study group comprised children with pyelonephritis ( $n = 18$ ) with renal scar and ( $n = 10$ ) without scar both groups diagnosed with dimercaptosuccinic acid (DMSA) renal scans. Cystatin C in urine and serum was determined using ELISA. There were no significant differences between renal scar positive and negative patients regarding age, gender, body weight and length, serum cystatin C, serum creatinine, creatinine clearance, tubular phosphate reabsorption, fractional sodium excretion, microalbuminuria and urinary cystatin C and NAG levels. However, there was a significant correlation between 1/serum cystatin C and both endogen creatinine clearance ( $r = 0.385$ ,  $P = 0.047$ ) and glomerular filtration rate (GFR) calculated with Schwartz formula ( $r = 0.396$ ,  $P = 0.041$ ) while the same correlation could not be found with 1/serum creatinine. This data suggest that children with renal scar diagnosed with DMSA does not show any change in serum, urine cystatin C and other renal function tests. However, cystatin C-based GFR estimate is better than creatinine in those patients.

## L1-019P

### Developmental expression of neogenin protein in human brain

N. Jovanov-Milosevic, Z. Krsnik and I. Kostovic

Croatian Institute for Brain Research, Medical faculty, University of Zagreb, Zagreb, Croatia. E-mail: njovanov@hiim.hr

Neogenin (NGN) protein was first identified in a chicken as developmentally highly regulated protein in neuronal tissue, suggesting a role in the generation of the fully functional nervous system. To address possible role of NGN in a critical period for human brain development we used an affinity-purified antibody raised against NGN of human origin in Western blot, immunoperoxidase histochemistry and immunofluorescent multilabeling analyzing NGN cell-origin and its spatio-temporal expression, on postmortem human fetal brains, staged from 10th week of gestation (w.g.) to newborn. The most prominent feature was perinuclear and extracellular expression in subventricular subcallosal zone below anterior extent of corpus callosum (cc) in cells with low neuron-specific nuclei protein (NeuN) expression or activated caspase-3 and on the surfaces of growing fibers in ventral cc at midgestation. Furthermore, strong cellular NGN expression was displayed where fibers of fornix diverge from cc, as well as in the fornix fibers on the insertion of plexus chorioideus. At about 30 w.g. the most prominent was expression confined to bushy astroglial like cell-population in the developing putamen. From 35 w.g. to newborn we could only observe a very low level of NGN expression in cells in subventricular zone laterally to and in cc.

In conclusion, NGN is expressed during important gestational developmental window showing topographically very specific localization confined to a small number of differentiating cells or cells undergoing apoptosis and to some midline growing fibers. In developing human brain NGN expression decreases and disappears during latest fetal stage.

## L1-020P

### The investigation of serum N-acetyl- $\beta$ -D-glucosaminidase and its isoenzymes as markers of the progression of diabetic complications in IDDM

V. B. Jovanovic<sup>1</sup>, J. M. Acimovic<sup>1</sup>, V. S. Dimitrijevic-Sreckovic<sup>2</sup> and L. M. Mandic<sup>1</sup>

<sup>1</sup>Faculty of Chemistry, University of Belgrade, Belgrade, Serbia and Montenegro, <sup>2</sup>Faculty of Medicine, University of Belgrade, Belgrade, Serbia and Montenegro.

E-mail: vjovanovic@chem.bg.ac.yu

Significantly increased of serum N-acetyl- $\beta$ -D-glucosaminidase (NAG, EC 3.2.1.30) activity in diabetic patients, especially in diabetics with secondary complications was found. However, the results obtained for total NAG and its relationship with development of the secondary diabetic complications are often contradictory and unexplained. Consequently, we have attempted to establish whether total NAG and/or NAG isoenzymes can provide additional diagnostic information regarding diabetic status and the complications of diabetes. The serum NAG isoenzymes in control ( $n = 18$ ) and in four groups of IDDM patients (1st – without complications,  $n = 20$ ; 2nd – with retinopathy,  $n = 6$ ; 3rd – with retinopathy and neuropathy,  $n = 11$ ; 4th – with retinopathy, neuropathy and nephropathy,  $n = 12$ ) were separated by ion-exchange chromatography on DEAE cellulose. In all diabetic groups there were a statistically significantly increase ( $P < 0.001$ ;  $P < 0.01$ ) of total NAG activity compared to the control. Analysis of isoenzyme profiles in all diabetic groups showed significantly decreased ( $P < 0.001$ ) contribution of the B form to total NAG activity ( $15.1 \pm 4.5\%$ ;  $16.3 \pm 3.4\%$ ;  $18.3 \pm 6.0\%$ ;

22.5 ± 6.7%, resp.) and significantly increased ( $P < 0.001$ ) of the A form (84.6 ± 5.1%; 83.7 ± 3.4%; 81.8 ± 6.0%, 77.5 ± 6.7%, resp.) compared to the control group ( $B = 32.0 \pm 2.7\%$ ;  $A = 68.0 \pm 2.7\%$ ). The statistically significant differences in the percent fractions of the B form between the first and the fourth group of diabetics ( $P < 0.001$ ), and also between the second and the fourth group ( $P < 0.05$ ) were found. Significant differences in total NAG activity between all diabetic groups were found. It was concluded that the serum total NAG activity is better marker for the differentiation and progression of diabetic complications than the B isoenzyme.

### L1-021P

#### Enhanced protein identification of 2D-gel spots utilizing a novel multiplexed PSD technique

M. Kennedy<sup>1</sup>, M. Snel<sup>2</sup>, E. Claude<sup>2</sup>, D. Kenny<sup>2</sup>, T. McKenna<sup>2</sup> and J. Langridge<sup>2</sup>

<sup>1</sup>MS Technologies Centre, Waters Corporation, Almere, The Netherlands, <sup>2</sup>MS Technologies Centre, Waters Corporation, Manchester, UK. E-mail: matt\_kennedy@waters.com

Peptide mass fingerprinting (PMF) is an established technique for identifying proteins. In PMF experiments, proteins are digested using a site selective protease, e.g. trypsin and the masses of the peptides produced are measured, typically using MALDI TOF mass spectrometry. Proteins are then identified by comparing these masses with those expected from an *in silico* digestion of proteins in protein sequence databanks. This approach can be very successful, but crucially relies on several factors:

- Proteins under investigation must be present in the databank being interrogated.
- Proteins must be isolated effectively from other proteins, as protein mixtures lead to ambiguous databank search results.
- Proteolytic digestion must produce at least four peptides falling in the  $m/z$  range of 600–4000 and yielding mass spectral peaks of similar intensity.

Due to these limitations, between 10% and 60% of proteins studied are not identified using PMF. The specificity of protein identification experiments can be enhanced, by obtaining partial sequence information from these peptides by MS/MS or PSD experiments. Here we present an evaluation of a novel post source decay (PSD) experiment as an enhancement to PMF. The PSD experiment described differs from conventional PSD, as data are obtained simultaneously from a number of peptide ions. Parallel acquisition is made possible by assigning PSD fragments to associated precursor ions by determining the kinetic energy of PSD fragments. This novel approach simplifies the PSD experiment, reduces sample consumption and increases the number of peptides analysed. A comparison of parallel PSD and PMF was made using a 2d gel separated cytosolic fraction of *E. coli*. Improvement of the rate of protein identification is demonstrated.

### L1-022P

#### The presence of 11beta-hydroxysteroid dehydrogenase type I in human granulocytes

B. Legeza<sup>1</sup>, G. Banhegyi<sup>1</sup>, J. Mandl<sup>1</sup>, A. Benedetti<sup>2</sup> and T. Kardon<sup>1</sup>

<sup>1</sup>Department of Medical Chemistry, Molecular Biology and Pathobiochemistry, Semmelweis University, Budapest, Hungary,

<sup>2</sup>Department of Pathophysiology, Experimental Medicine and Public Health, University of Siena, Siena, Italy.

E-mail: kardon@puskin.sote.hu

Glucocorticoids are known to induce apoptosis in human lymphocytes, while they delay it in polymorphonuclear cells. The

mechanisms underlying these phenomena are unclear. Our work-group has previously shown that S3483, a chlorogenic acid derivative inhibitor of the glucose-6-phosphate transporter, induces a fast-onset apoptosis in human granulocytes. This result evidenced that the inactive glucose-6-phosphate transporter is at least partly responsible for decreased granulocyte numbers in glycogen storage disease 1b, where the transporter is genetically defective. We supposed that the absence of an active transporter causes alterations in the redox state of the intraluminal space of the ER, which in turn triggers ER-derived apoptosis. In our experiments we now investigated whether cortisol inhibits S3483-induced apoptosis. After one hour, cortisol was effective in inhibition of apoptosis caused by S3483 in human granulocytes. In hepatocytes the ER-localized 11beta-hydroxysteroid dehydrogenase type I (11beta-HDH) is responsible for metabolism of cortisol. This enzyme is an oxidoreductase and uses NADP(H) as cofactor. Availability of the oxidized/reduced forms of the cofactor is a determinant of the direction of the catalysed reaction. We detected 11beta-HDH with immunoblot in granulocyte ER. We presume that the presence of this enzyme in granulocyte ER could maintain an appropriate level of NADPH by cortisol dehydrogenation even in the absence of glucose-6-phosphate, thus preventing the cells from induced apoptosis.

### L1-023P

#### Finding markers of gastric cancer from patients' sera using proteome pattern recognition

J. U. Kweon<sup>1,2</sup>, Y. S. Shin<sup>1,2</sup>, K. N. Kang<sup>2,3</sup>, K. H. Lee<sup>1,2</sup>, S. Y. Ohn<sup>3</sup>, C. W. Kim<sup>1,2</sup> and M. Y. Han<sup>2,4</sup>

<sup>1</sup>Pathology and Cancer Research Institute, Seoul National University College of Medicine, Seoul, South Korea, <sup>2</sup>BioInfra, Inc., Seoul, South Korea, <sup>3</sup>Department of Computer Engineering, Hankuk Aviation University, Seoul, South Korea, <sup>4</sup>Green Cross Institute of Medical Genetic, Seoul, South Korea. E-mail: ourscent@hanmail.net

Gastric cancers are one of the major causes of cancer death in Asian countries. Recently, proteomic approaches have been used in an attempt to identify new diagnostic markers. Characterizing the proteome will be complicated because it exists in many different states and thus enters the issue of differential protein expression. We have focused on the development of strategies for identifying sera markers. Serum proteins are useful diagnostic tools and alteration of the expression of some serum proteins is an early sign of an altered physiology and may be indicative of disease. Advances in proteomics technology, particularly in mass spectrometry, are now providing an excellent opportunity to develop high throughput, accurate testing tools that can aid in disease diagnosis and prognosis.

The Protein-Chip system uses surface enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF-MS) to perform rapidly the separation, detection and analysis of proteins directly from unprocessed biological samples. Using a case-control study design, 288 serum samples from patients with gastric cancer (n=144), and normal controls (n=144) were analyzed on strong anion-exchange surfaces.

By comparing with normal and stomach cancer serum samples, we found that at least five proteins were significantly changed. The result shows that SELDI profiling of serum could be used to accurately distinguish patients with gastric cancer from normal controls. These protein markers can also be used as targets for further study in drug design and prognostic markers of stomach cancer therapy. In the future, we are going to validate that the proteins can be used as diagnostic markers.



**L1-024P****Alpha-amylase production in aqueous two-phase systems by *Bacillus subtilis***

Z. Konsoula and M. Liakopoulou-Kyriakides

Laboratory of Organic Chemistry, Department of Chemical Engineering, Aristotle University, Thessaloniki, Greece.

E-mail: markyr@eng.auth.gr

The production of  $\alpha$ -amylase by *Bacillus subtilis* was studied in polyethylene glycol (PEG 10 000)/dextran (505 000) aqueous two-phase systems at various concentrations. An increase in the PEG concentration from 7 to 25% (w/w) in the aqueous two-phase system resulted in an increase in the phase volume ratio with a concomitant decrease in the partition coefficient ( $K$ ) and recovery of  $\alpha$ -amylase in the top phase. However, varying dextran concentrations from 2.5 to 10% (w/w) decreased both the phase volume ratio and the partition coefficient of  $\alpha$ -amylase. At dextran concentrations lower than 2.5% (w/w) the phases could not separate. The purification ratio was found to increase with increasing PEG concentration up to 9% (w/w), while it was slightly decreased at higher concentrations. The PEG 9% (w/w) and dextran 2.5% (w/w) system was found to be optimum for cultivation of *Bacillus subtilis*, where more than 95% of the produced  $\alpha$ -amylase was selectively partitioned to the upper phase giving a purification factor of 2.3. In this system the  $\alpha$ -amylase activity in the top phase, which reached 93 U/ml after 48 h of cultivation, was 1.2 times higher in comparison to the homogeneous medium. It was observed that *Bacillus subtilis* secreted 84% of the total  $\alpha$ -amylase produced within 24 h, while the respective time exceeded 33 h in the homogeneous medium. The bacterial cells were microscopically observed to partition totally to the bottom phase in the system used. The dextran-rich bottom's phase affinity for cells was exploited in the development of a semicontinuous cell recycle system, in which the bottom phase was reused and the cell-free top phase was withdrawn. In the first two uses  $\alpha$ -amylase activity in the top phase exceeded 90 U/ml, while in the third use it was reduced to 84 U/ml. Concluding the use of the PEG 9% (w/w)/dextran 2.5% (w/w) aqueous two-phase system enabled the recirculation of the bacterial cells and the reduction of the required time of cultivation, thus contributing significantly to the reduction of the cost of the fermentation.

**L1-025P****Protein markers for identification of cotton resistance to phytopathogens and environmental stress**E. V. Kamilova<sup>1</sup>, S. Molinari<sup>2</sup> and S. Y. Yunuskhonov<sup>1</sup><sup>1</sup>Biochemistry Genetics, Institute of Genetics and Plant Experimental Biology, Academy of Science, Tashkent, Uzbekistan,<sup>2</sup>Biochemistry, Istituto per la Protezione delle Piante, CNR, Bari, Italy. E-mail: khelen@tki.uz

Agriculture is the leading economic activity in Uzbekistan (448 900 km<sup>2</sup>, 24.5 million inhabitants), providing the basis for food self-sufficiency and a source of export products. A major problem of biochemical researches in agriculture is creation methods of estimation and forecasting qualities of the future crop yield, to choice from large number of selection materials the forms, valuable to desirable tags. Such problem can be decided by detection of molecular markers of plants, spanned to major important and valuable tags. *Fusarium oxysporum* f. sp. *vasinfectum* (Alkinson) Snyder and Hansen, was obtained from Centraalbureau voor Schimmelcultures, Utrecht, NL (CBS 174.30), as Freeze-dried material. Reactivated spores were inoculated on corn meal agar (CMA) with antibiotics (ampicillin 50 mg/l, strep-

tomycin 50 mg/l) and incubated at 25 °C for culturing. For detection assays, fifteen varieties of cotton (*Gossypium hirsutum* or *G. barnadense*) were used. After incubation for about 15 days, conidia were harvested in sterile distilled water. We made artificial inoculation of cotton plants in age of 2–4 true leaf on the steam of sufficiently lignified plants by injection 0.5 ml suspension of pathogen's spores. Concentration of spores suspension was  $1 \times 10^6 = 1 \times 183\,000$  spores/ml, checked by hemacytometer. Symptoms were appeared in 3 weeks after inoculation, plants appeared wilting, yellowing and defoliated plants, that are typical of disease symptoms. The vascular tissue of affected plants exhibited a brown/chocolate discoloration through the entire main stem. Cotton plants of 17 varieties and lines of *Gossypium hirsutum* L. (C-6524, C-4534, AN-512, Novbahor, AN-UZBEKISTAN-4, Gulbahor, AN-Bayaut-2, Shodlik-9, N-4727, Ilgor, L-183, Karcic, L-Bagdad, etc.) and *Gossypium barbadense* L. (Navo, Igod, etc.) were grown in greenhouse in controlled environmental conditions. In cotton seeds and leaf total protein assay was detected by Lowry method with bovine serum albumin like standard. Peroxidase activity was determined with syringaldazine as a substrate in a 1-ml reaction mixture. Superoxide dismutase (SOD) by Ravindranath + Fridovich was studied in cotton seeds with electrophoresis Native Page 12.5% PAD, using Pharmacia LKB, PhastSystem equipment, isofocusing in 3–9 pH gradient. We may detect increasing of peroxidase activity like response to fusarium infection. A correlation has been reported between the peroxidase contents of healthy seedlings, seeds and their resistance to fusarium wilt, that may be molecular markers. Superoxide dismutase (SOD) shows polymorphism as between *G. hirsutum* L. and *G. barbadense* L., as between different varieties of *G. hirsutum* L. We detected positive correlation ( $k = 0.78$ ) between total protein content in seeds and resistance to drought of cotton plants.

**L1-026P****Activity-based profiling of membrane-bound metalloproteinases**T. Klein<sup>1</sup>, M. Leeuwenburgh<sup>2</sup>, H. Overkleeft<sup>2</sup>, H. F. Kauffman<sup>3</sup> and R. Bischoff<sup>1</sup><sup>1</sup>Analytical Biochemistry, Department of Pharmacy, University of Groningen, Groningen, The Netherlands, <sup>2</sup>Gorlaeus Laboratory, Leiden Institute of Chemistry, University of Leiden, Leiden, The Netherlands, <sup>3</sup>Allergology and Lung Disease, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands. E-mail: t.klein@rug.nl

**Aim:** Development of a profiling method for active ADAMs (A Disintegrin And Metalloprotease) in biological samples using synthetic hydroxamate-based inhibitors.

**Background:** Metalloprotease activity plays an important role in various pathophysiologic processes. Profiling of actual activity of ADAMs in biological samples may provide valuable insights into their role in disease.

**Methods:** Newly synthesized metalloprotease inhibitors were tested for efficacy in enzymatic activity assays (ADAM17, MMP12) and a cellular model based on TNF $\alpha$  shedding in the human bronchial epithelia cell line 16HBE. Successful inhibitors were immobilized on Sepharose beads and used for batch extraction of spiked recombinant ADAM17 from buffer and cell lysate and native ADAM17 from a lysate of the human lung carcinoma cell line A549. Extracted ADAM17 was detected by Western blotting.

**Results:** The new hydroxamate-based inhibitors had low nanomolar IC<sub>50</sub> values for both MMP12 (4.80–41.8 nM) and ADAM17 (10.7–58.2 nM). Extraction of recombinant ADAM17 was successful and activity-dependant from both buffer and cell

lysate. Furthermore native ADAM17 was extracted from the A549 lysate in an activity-dependant manner.

**Future prospects:** Positive identification of extracted active metalloproteases by mass spectrometric techniques (MALDI-TOF, LC-nanoESI-MS). Incorporation of the inhibitor beads into a fully integrated online affinity SPE-tryptic digestion-LC-nanoESI-MS system for activity dependant profiling of metalloproteases in biological samples.

### L1-027P

#### The degree of ConA binding and of level of plasma fibronectin in tumor diseases of thyroid gland

N. V. Lutay

*Laboratory of Biochemistry, Department of Biochemistry and General Chemistry, Dnepropetrovsk State Medical Academy, Dnepropetrovsk, Ukraine. E-mail: starter79@mail.ru*

Fibronectins (FN) are a class of high-molecular weight glycoproteins abundantly present in plasma and extracellular matrix of a human organism. Multiple functions of these proteins have been described including their role in the malignancy and the cellular adhesion as well as in the opsonization and the hemostasis, etc. Our research has been carried out in order to investigate changes in the expression and the structural alteration of FN in norm as well as in thyroid tumor diseases. We have measured plasma FN level and its degree of ConA binding. Plasma taken from nine healthy persons and 14 patients including seven patients with thyroid benign tumor and seven patients with thyroid papillary carcinoma was studied. It was established that in the normal group the FN concentration was at an average  $369 \pm 24$  µg/ml, whereas the average plasma FN level of both groups of patients was lower than that one of the normal group in the preoperative period ( $302 \pm 31$  µg/ml for patients with benign thyroid tumor and  $278 \pm 71$  µg/ml for patients with cancer thyroid). In a week after surgical operation and hormonal treatment the level of plasma FN of these groups increased approximately to normal. The degree of binding ConA by FN has been measured by method of the rocket affinity immunoelectrophoresis. The degree of interaction between FN and ConA has been evaluated as a ratio of the precipitate area that was formed by FN with antiserum in absence of ConA to the precipitate area which was formed in the same conditions in the presence of ConA. Our results showed that the degree of binding plasma FN with ConA of patients with benign thyroid tumor and of patients with cancer thyroid was 1.66 and 1.91 times lower than that healthy persons respectively ( $P < 0.05$ ) in the preoperative period. A week after a surgical operation there were no significant changes in the degree of binding ConA by FN in comparison with its initial value.

### L1-028P

#### Dynamic expression of hepatic leptin in CCl<sub>4</sub> and fat food induced liver fibrosis in rats

C. Liu, B. Sun and H. Xun

*Institute of Liver Disease, Shanghai University of Traditional Chinese Medicine, Shanghai, PR China.*

*E-mail: chenghai\_liu@yahoo.com.cn*

**Background and aims:** Leptin is a 16-kDa peptide product of ob gene, with the biological functions such as controlling energy balance and food intake and fatty metabolism etc. Recent studies showed that leptin could augment inflammatory and profibrogenic response in animal liver, stimulated collagen expression in cultured hepatic stellate cells, and played an important role in the hepatic fibrogenesis. However it is still unclear that the liver itself express leptin, and what pattern of expression changes during

hepatic fibrosis formation. It is known that liver fibrosis in nonalcoholic steatohepatitis (NASH) mainly relate to the hepatic lipid peroxidation, but the fibrotic and fatty degenerative degrees are not agreed, suggesting that there is other mechanism underlying fibrogenesis in steatohepatitis. In the study, we aim to investigate hepatic leptin expression, its change pattern during fibrogenesis and its role in fibrogenesis of steatohepatitis.

**Methods:** The liver fibrotic model was induced by hypodermal injection of CCl<sub>4</sub> and intake of food with high fat and lower protein for 6w, and model rats were sacrificed at 2d, 1w, 2w, 3w, 4w, 5w and 6w respectively, normal rats at 2d, 3w and 6w. The hepatic fatty degeneration was checked with HE and oil red O stain, collagen accumulations stained with Sirius red. Serum liver function (including AST, ALT, Alb, TBil) were assayed with kit, hepatic SOD MDA GSH, Hydroxyproline (Hyp) and triglycerol (TG) content were measured with biochemical method, serum leptin was assayed with ELISA, hepatic leptin protein expression was immuno-histochemically stained and analyzed with Western blot. The leptin cDNA was generated with primers according to its sequence at GenBank, and used to probe the leptin mRNA expression in liver. And correlations between leptin protein and hepatic Hyp, TG and lipid peroxidation, etc. were analyzed respectively.

**Result:** The rat liver does express both leptin mRNA and protein, its protein mainly stained at hepatic sinusoids, portal area and inflammatory regions, and its mRNA and protein expression increased as the liver fibrosis developed. The hepatic leptin protein had significant positive relation with hepatic Hyp,  $\alpha$ -SAM and MDA levels respectively, serum leptin changed as rats weight varied with a positive correlation, but had no relation to hepatic leptin and Hyp levels.

**Conclusion:** The liver expressed leptin gene and protein, which increased as the hepatic fibrosis developed, and hepatic leptin expression contributed to the liver fibrogenesis in CCl<sub>4</sub> induced rats with characteristic of steatohepatitis besides the hepatic lipid peroxidation mechanisms.

### L1-029P

#### C/EBP delta expression during rat hepatocytes differentiation and inflammation

M. Mihailovic, D. Bogojevic, S. Ivanovic-Matic, M. Vidakovic, A. Uskokovic, N. Grdovic, J. Arambasic and S. Dinic

*Department of Molecular Biology, Institute for Biological Research, Belgrade, Serbia and Montenegro.*

*E-mail: mista@ibiss.bg.ac.yu*

CCAAT/enhancer binding protein (C/EBP) delta, member of C/EBP liver-enriched transcription factors, is associated with cell proliferation and the acute-phase response (APR). The APR is a defense reaction of an organism in response to a variety of different insults in which liver plays a very important role. Increased expression of C/EBP delta was observed during early adipocyte differentiation. Since the role C/EBP delta in hepatocyte differentiation is unknown, we wanted to determine whether C/EBP delta is expressed throughout rat liver development under normal and acute-phase conditions. In view of the importance of the dynamic spatial and solubility partitioning of gene regulators in the nucleus for their appropriate functioning, we examined the partitioning of C/EBP delta between two nuclear protein fractions, soluble – the nuclear extract, and insoluble – the nuclear matrix. Western analysis of the soluble cytosol fraction revealed the presence of very low concentrations of C/EBP delta suggesting that the C/EBP delta protein pool was mostly localized in the nucleus. Using Western analysis C/EBP delta was established in the nuclear extract and nuclear matrix throughout rat liver development and in the adult. During the APR, C/EBP delta increased in the nuclear extract but remained unchanged in the

nuclear matrix of fetal and postnatal rats, whereas it increased in both the nuclear extract and nuclear matrix of the adult. According to our results we can assume that C/EBP delta is involved in hepatocyte differentiation and that its activity is regulated by different mechanisms during development and in the adult.

### L1-030P

#### Antifilaggrin antibodies in serum and synovial fluid samples of patients with rheumatoid arthritis

A. Magyar<sup>1</sup>, M. Brózik<sup>2</sup>, L. Kis<sup>1</sup>, K. Tamás<sup>1</sup>, U. Böhm<sup>2</sup>, K. Merétey<sup>2</sup> and F. Hudecz<sup>1,3</sup>

<sup>1</sup>Research Group of Peptide Chemistry, Hungarian Academy of Sciences, Eötvös Loránd University, Budapest, Hungary, <sup>2</sup>National Institute of Rheumatology, Budapest, Hungary, <sup>3</sup>Department of Organic Chemistry, Eötvös Loránd University, Budapest, Hungary. E-mail: magyar@szerves.chem.elte.hu

Autoantibodies directed against citrulline-containing proteins have high specificity of rheumatoid arthritis (RA). Citrullinated proteins are formed by posttranslational modification, namely by deimination of arginine residues in protein sequences by peptidylarginine deiminase enzymes. The presence of deiminated proteins has been demonstrated in the inflamed synovium of humans and also in animal models of RA. It has been shown that anti-filaggrin antibodies (AFA) can be detected at a very early stage of the disease and their level correlates with the severity and the progression of RA. Filaggrin, containing citrulline instead of arginine has been recently identified as major antigenic protein recognized by AFA. The aim of this study was to compare the recognition patterns of antibodies in paired serum and synovial fluid samples of RA patients towards citrullinated peptide sequences and to investigate whether or not they comprise the same antibody population. Peptides corresponding to the most antigenic six filaggrin regions [1] and the N- or C-terminal shortened version of the peptide 306SHQESTRGRSRGRSGRSGS324 were synthesized. In all peptides the arginines were replaced by citrullines systematically. We used surface-attached peptides multipin approach for the screening procedure. The selected peptides and the non-citrullinated control peptides – with Cys on the N- or C-terminal – were prepared also on Rink-amid resin by solid-phase peptide synthesis, using Fmoc strategy. The peptides, purified by FPLC and analysed by MS, were covalently coupled to sulphydryl functionalized microplates. Reactivity of paired sera and synovial fluid samples were analysed in ELISA.

**Acknowledgment:** This work was supported by OTKA TO37876, GVOP-3.1.1.-2004-05-0183/3.0.

#### Reference

- Schellekens GA *et al.* *J Clin Invest* 1998; 101: 273–281.

### L1-031P

#### Orthopoxvirus tumor necrosis factor binding proteins and gamma-interferon binding proteins: expression, purification and some characteristics

T. S. Nepomnyashchih<sup>1</sup>, L. R. Lebedev<sup>2</sup>, G. V. Kochneva<sup>1</sup>, A. A. Grajdantceva<sup>1</sup>, I. A. Ryazankin<sup>1</sup>, S. N. Shchelkunov<sup>1</sup> and I. P. Gileva<sup>1</sup>

<sup>1</sup>Laboratory of Molecular Biology of Genomes, Department State Research Center of Virology and Biotechnology Vector, Novosibirsk, Russian Federation, <sup>2</sup>Department Center of Engineering Immunology, Novosibirsk, Russian Federation. E-mail: antonec@ngs.ru

G2R, J2R and I4R genes for TNF-binding proteins (CrmBs) of variola virus (VARV), monkeypox virus (MPXV) and cowpox

virus (CPXV) respectively and B9R genes for IFN-gamma-binding proteins (INFBPs) VARV and MPXV, were isolated from viral genomes and expressed in the insect Sf21 cells using baculovirus expression system. Secreted recombinant CrmBs and INFBPs were isolated from culture medium of infected Sf21 cells using affinity chromatography and analyzed through SDS-PAAG. Under nonreducing condition affino-purified VARV-CrmB and INFBPs (but not MPXV- or CPXV-CrmBs) were detectable as 90 and 60 kDa disulfide-linked complexes that dissociate to 47 and 30 kDa forms respectively under reducing condition. Biological properties of recombinant CrmBs were studied *in vitro* using TNF cytolytic activity inhibition and *in vivo* using LPS-induced septic shock model. *In vitro* VARV-, MPXV- and CPXV-CrmBs inhibited cytolytic activity of human, murine and rabbit TNFs, some mutant forms of human TNF and human LT in species-specific manner. *In vitro* VARV-CrmB inhibited cytolytic activity of TNF hundred times more effectively than human recombinant receptor II. Only VARV-CrmB, but not MPXV- or CPXV-CrmBs decreased LPS-induced mortality of BALB/c mouse *in vivo*. Biological properties of recombinant INFBPs were studied *in vitro* using inhibition of antiviral activity of human gamma-IFN on L68 cells.

Understanding the mechanisms of actions of such proteins may provide insight into the development of novel immune-modulating therapies.

### L1-032P

#### Alkaline phosphatase retained in HepG2 hepatocarcinoma cells vs. alkaline phosphatase released to culture medium: different aberrant glycosylation

A. Nowrouzi and R. Yazdanparast

Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran. E-mail: nowrouzi@ut.ac.ir

Liver alkaline phosphatase (AP) accounts for as much as 90% of the AP present in human serum; however, its determination both qualitatively and quantitatively is difficult due to close molecular weights of different AP isoenzymes also present in serum. To escape the confounding interference from other AP forms released by other tissues of the body when studies are aimed at only one form, it is possible to conduct studies on cell cultures. This study used the HepG2 hepatocarcinoma cell line to characterize the kinds of AP produced in this particular form of liver cancer. The AP released to the cell culture medium was compared to the AP retained in the cell membranes with respect to: PAGE electrophoretic mobility, elution through Sephadex G-200, Con A lectin affinity elution profile, sensitivity to heat denaturation, and inhibition by amino acid inhibitors as L-Phe, L-Leu, L-Homoarginine and levamisole. The results showed that both APs were distinctly different from normal liver AP and from each other both electrophoretically and with respect to ConA profiles; however they closely resembled the healthy liver AP under heat and inhibitors. It was concluded that the HepG2 APs were similar to normal liver AP (tissue non-specific AP) with regard to their primary structure but differed from it and from each other with respect to their carbohydrate moieties. The observation that cell membrane AP did not produce the activity bands similar to the intense band of the culture medium even after phospholipase C treatment reminds the question about the role of carbohydrate modification in the release of the enzyme from the cell surface to the medium especially in cancer.

**L1-033P****Isolation and characterization of two nucleases from sap of *Chelidonium maius* L.**

R. Nawrot<sup>1</sup>, K. Lesniewicz<sup>2</sup>, L. Plesner<sup>1</sup> and A. Gozdicka-Jozefiak<sup>1</sup>

<sup>1</sup>Department of Molecular Virology, Adam Mickiewicz University, Poznań, Poland, <sup>2</sup>Department of Plant Molecular Biology, Adam Mickiewicz University, Poznań, Poland.  
E-mail: rnawrot@amu.edu.pl

*Chelidonium maius* L., which belongs to the family *Papaveraceae*, is a rich source of various biologically active substances and serves as a valuable material for pharmaceutical industry. The extracts from *Ch. maius* whole plants and milky juice have been applied for a long time in folk medicine to remove condylomas and warts, treat liver and fight fever. It was also found that they have antitumor, antimicrobial, antifungal and fungistatic properties.

*Ch. maius* curing properties were determined by alkaloids present in plants in the form of salts of organic acids [1]. Two glycoproteins from *Ch. maius* sap which exhibit lectin CM and DNase activity were also isolated in our laboratory and partially characterized [2]. Results of this study suggest that some substances showing biological activity other than alkaloids are present in the extract from *Ch. maius*. Lately using gelshift method for separation of nuclease from this plant's sap, we have isolated two nucleases of 17 and 32 kDa. Studying the effect of pH and ions ( $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Zn}^{2+}$ ) we identified that both proteins require  $\text{Ca}^{2+}$  ions and pH 8.0 for their activity. Loss of nuclease activity was observed in pH 5.5 and in 1 mM EDTA. The nuclease of 17 kDa was observed in milky juice of plant during the early summer, and nuclease 32 kDa during the whole vegetation period. Crude extract of proteins stimulates the proliferation of human lymphocytes and has hemagglutination activity towards group B human erythrocytes. Both proteins are probably involved in development and differentiation of plant and defence against different pathogens.

**References**

1. Hegnauer R. Chemotaxonomie der Pflanzen, Birkhäuser Verlag, Basel Boston Berlin, 1990.
2. Fik et al. *Acta Biochim Pol* 2000; 47: 413–420.

**L1-034P****Clinical evaluation of leukocyte Arylsulphatase A, serum interleukin-6 and urinary heparan sulphate estimations in breast cancer patients and their relationship to the tamoxifen treatment**

P. Oner<sup>1</sup>, Y. O. Iyidogan<sup>1</sup>, H. Kocak<sup>1</sup>, A. Lama<sup>1</sup>, S. Bekpinar<sup>1</sup>, F. Gurdol<sup>1</sup>, N. Unur<sup>1</sup> and Z. K. Ozbek<sup>1</sup>

<sup>1</sup>Biochemistry, Istanbul, Istanbul, Turkey, <sup>2</sup>Radiation Oncology, SSK, Istanbul, Turkey, <sup>3</sup>Biochemistry, SSK, Istanbul, Turkey.  
E-mail: permuron@istanbul.edu.tr

To evaluate the clinical significance of the possible changes in the activity of leukocyte Arylsulphatase A (AS-A), in the levels of serum interleukin-6 (IL-6), total urinary glycosaminoglycans (GAGs) and urinary heparan sulphate (HS) in response to tamoxifen (TAM) therapy in mastectomized breast cancer patients. A total of 34 patients aged between 30 and 82 years were administered TAM at a dose of 20 mg twice daily, for 3 and 6 months. Blood and urine samples were obtained at the start of the study, 3 and 6 months after TAM therapy. There were no significant differences between baseline leukocyte AS-A activity and that of measured after 3 months of TAM treatment. However, enzyme activity measured at the end of 6-month TAM treatment was significantly higher than the values both obtained at the beginning

and third month after therapy ( $P = 0.022$ ). TAM treatment did not have any effect on serum IL-6, urinary HS and GAG levels. Unchanged levels of serum IL-6, urinary HS and GAG both at the beginning and after 3 and 6 months of TAM therapy may show either an insufficient TAM treatment period for these parameters to reflect disease regression or the lack of sensitivity of these variables to therapy. Elevated leukocyte AS-A activity following 6 months of TAM therapy may result from altered integrity of lysosomal membrane due to the malignant degenerative processes which could not be suppressed by a short-term drug treatment.

**L1-035P*****Borrelia garinii* hypothetical protein from erp gene family**

R. Ranka and V. Baumanis

Biomedical Research and Study Centre, University of Latvia, Riga, Latvia. E-mail: renate\_r@biomed.lu.lv

Three members of the genus *Borrelia* (*Borrelia burgdorferi sensu stricto*, *Borrelia garinii* and *Borrelia afzelii*) cause Lyme disease (*Lyme borreliosis*). In the United States, *B. burgdorferi* ss is the only causative agent for this disease, whereas in Europe *B. afzelii* and *B. garinii* are major contributors to the reported case numbers. The nuclear genomes of all *B. burgdorferi* species consist of one linear chromosome and varying amounts of several linear and circular plasmids. All isolates of the spirochete *Borrelia burgdorferi* contain multiple, different plasmids of the cp32 family, each of which contains a locus encoding Erp surface proteins. Many of these proteins are known to bind host complement regulatory factor H, enabling the bacteria to avoid killing by the alternative complement pathway during vertebrate infection. Interestingly, *B. burgdorferi sensu stricto* and *B. afzelii*, but not *B. garinii*, bind the complement inhibitor factor H to protect themselves against complement-mediated opsonophagocytosis and killing.

The complement sensitivity of *B. garinii* may explain its preference to cause infections in the CNS, however, this item remains to be investigated. In addition, outer membrane surface proteins from Erp family were shown to be immunogenic, so that they are potential targets in the serodiagnosis of Lyme disease. Our recent studies showed that all three clinically relevant genotypes are distributed in tick population in Latvia. However, unlike *Borrelia burgdorferi sensu stricto*, there is very little information about erp genes and proteins of *B. afzelii* and *B. garinii*. *B. garinii* strain IP90 was used in our investigation. PCR amplification with different sets of primers and following sequencing indicated the presence of potential gene in *Borrelia garinii* genome similar to those of erp gene family. DNA analysis and possible cloning and expression of this potential protein will give additional information about the pathogenesis of *B. garinii* infection and may be useful in serodiagnosis of Lyme disease.

**Acknowledgment:** This work has been supported by European Social Fund.

**L1-036P****Activity of peroxidase and superoxide dismutase proteins under presence of some anticancer preparations**

A. E. Zakaryan and N. A. Sargsyan

Laboratory of Medical Biophysics, Department of Biophysics, Yerevan State University, Yerevan, Armenia.  
E-mail: naira\_sarkisyan@yahoo.com

Process of lipid peroxidation plays an important role in the normal functionality of organisms. The regulation of this

process is made by enzymatic and non-enzymatic systems of antioxidant defense. During different processes such balance might be disturbed. And different pharmacological preparations can regulate this balance and restore it. This work considers the level of activities of integral proteins (peroxidase and superoxide dismutase enzymes) in the biological object and possible changes of their activities in the presence of some anticancer preparations. For experiments there was used the spectrophotometric methods of investigation of the activity of antioxidant enzymes. As biological target there was used homogenate of brain of cow. During investigations it was found that experimental anticancer preparations diminished the activity of both enzymes in the biological material. Also it was found that anticancer preparations differently affected the activities of those enzymes and had the different level of activities for each enzyme. For example, colchicine suppressed the activity of peroxidase by 44% and superoxide dismutase by 57%, cyclophosphamide decreased the activity of peroxidase by 14%, and thiophosphamide diminished the activity of superoxide dismutase by 27%. So it has been shown that the investigated anticancer preparations had a strong antioxidant activity and can be used in the antioxidant therapy. In conclusion, it can be said that one of the mechanisms of action of anticancer preparations is the decrease of the activity of enzymes, which might be of much importance for understanding the functions and usage of different enzymes in the medicine.

#### L1-037P

##### Identification of staphylococcal exoproteins by two-dimensional gel electrophoresis and mass spectrometry

G. Schlosser<sup>1,2</sup>, M. Cuccurullo<sup>1</sup>, G. Cacace<sup>1</sup>, A. Sorrentino<sup>1</sup>, A. Malorni<sup>1</sup> and G. Pocsfalvi<sup>1</sup>

<sup>1</sup>Proteomic and Biomolecular Mass Spectrometry Center, Institute of Food Science and Technology, Avellino, Italy, <sup>2</sup>Research Group of Peptide Chemistry, Hungarian Academy of Sciences, Budapest, Hungary. E-mail: schlosser@chemres.hu

*Staphylococcus aureus* is one of the major human infectious bacteria causing community- and hospital-acquired infections. These range from fairly benign cutaneous infections to potentially fatal diseases, e.g. toxic shock syndrome. *Staphylococcus aureus* is highly efficient at overcoming antibiotic treatment. Nearly all strains secrete a group of enzymes (hemolysins, nucleases, proteases, lipases) and various staphylococcal enterotoxins. Whole genome sequencing of two *Staphylococcus aureus* strains has been published in 2001, however, it is still little known about how the expression of the various exoproteins of different strains depends on bacterial growth conditions. The aim of this work was the description of the exoprotein patterns of the different strains studied. Exoprotein fractions of four *Staphylococcus aureus* strains were obtained by precipitation from the culture broth. Proteins were separated by two-dimensional gel electrophoresis. 2D gels showed characteristic expression patterns, in which intensive protein spots were obtained in the 20–30 kDa region at basic (8–9) pH and in the 40–90 kDa region at acidic (4–6) pH. The separated protein spots, mainly derived from cytotoxins, were digested by trypsin, and the peptides were analyzed by MALDI-TOF mass spectrometry and/or sequenced by nanoESI-MS/MS or MALDI-MS/MS. Based on the MALDI peptide fingerprints and sequences, the proteins were identified by database search. Approximately 70 different proteins were identified, including the most important enterotoxins (TSST-1, enterotoxin A, B, C, K, Q).

#### L1-038P

##### Immobilized metal affinity on expanded bed chromatography for preparative isolation and purification of the Chagas' disease marker recombinant proteins CRA-His6 and FRA-His6

J. G. Silva Jr<sup>1</sup>, H. J. Nascimento<sup>2</sup> and E. D. da Silva<sup>2</sup>

<sup>1</sup>Laboratory of Protein Chemistry, Department of Biochemistry, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ Brazil, <sup>2</sup>BioManguinhos, Fundação Oswaldo Cruz. E-mail: godinho@bio.fiocruz.br

Many *Trypanosoma cruzi* antigens have been chosen and designed for diagnostic purposes; two of them the antigens CRA (Cytoplasmic repetitive antigen) and FRA (Flagellar repetitive antigen) have been used successfully in the *T. cruzi* infection diagnosis. These two antigens were initially produced as recombinant proteins linked to  $\beta$ -galactosidase or glutathione-S-transferase and isolated employing affinity chromatography. Recently, the CRA and FRA were tagged with His<sub>6</sub> and isolated by immobilized metal affinity chromatography (IMAC) using a new media named IMAC-Streamline (Ge Health & Care). This particle supports high upward and downward flow rates and can be used without further sample processing and feedstock dilution, and so cutting steps of the downstream process, which means less time and cost. The recombinant antigens over-expressed in *Escherichia coli* were extracted through sonication and applied in a previously expanded streamline 50 column, initial dimensions 50 × 180 mm (expansion rate of 1.66), whose matrices was linked to Ni. The crude feed passed upward through the expanded bed at a flow rate of 30 ml/min. After CRA or FRA capture the loosely bound material was washed out with 25 mM Imidazole buffer. The settled bed was positioned to its initial position (180 mm) and the target molecules were eluted downward with a step gradient of 250 mM Imidazole. The fractions observed at 280 nm were collected, desalted by ultrafiltration on a 10 kDa cut-off membrane and finally purified by anion exchange chromatography in a Resource Q (HR 16/10 column). The homogeneity was analyzed by HPLC-RP C4 and SDS-PAGE 12%.

#### L1-039P

##### Biophysical aspects of RNA-protein folding features in A/B type hnRNP proteins and their implications in human systemic autoimmune pathology

E. Süleymanoğlu

Laboratory of Physical Chemistry, Department of Pharmaceutical Chemistry, Gazi mahalleli, Ankara, Turkey. E-mail: erhan@berlin.com

Institut Curie, UMR-216 du CNRS, Genetique et Chimie des Genomes Eucaryotes; bat. 110, Centre Universitaire Paris, Sud, 91405-Orsay Cedex, France In human cells, the heterogeneous nuclear ribonucleoproteins (hnRNPs) are represented by a group of polypeptides, with various molecular properties, comprising the most abundant constituents of the cell nucleus. The A/B type heterogeneous nuclear ribonucleoproteins (hnRNPs) form a subgroup of closely related proteins, characterized by a modular structure: their N-terminal part consists of two adjacent RNA-binding domains (RBDs), whereas their auxiliary domain is involved in protein-protein interactions. These evolutionarily conserved RNA-protein assemblies have been considered promising for the prognostics and monitoring of systemic autoimmune diseases and anti-hnRNP autoantibodies are regularly reported in patients suffering from different rheumatic disorders. The strong correlation between particular autoimmune target and the type of disorder is well established. However, little is known about the factors that

determine the antigenicity of hnRNPs. In addition to the mode and degree of exposure, structural characteristics appear to play an important role for the capacity of a ribonucleoprotein to trigger the immune response towards autoimmune reactions. Our efforts focus on certain biophysical issues with particular emphasis on their functional implications and relevance to development of disease-specific epitope formation. Our recent models on these less-well-characterized functions of hnRNP A/B proteins will be presented. The data are based on thermodynamic stability measurements and sequence-specific RNA-binding preferences of hnRNP-A0, A1/A1B, A2/B1, D0, as well as the recently cloned and characterized new member of this family of proteins, the human hnRNP A3. Employing circular dichroism and fluorescence spectroscopy, coupled with UV cross-linking assay to follow the RNA-binding, we report a link between overall RNA-folding patterns within hnRNP A/B particles and how this could exert a number of physiological and pathological effects.

#### L1-040P

##### **A micro-total analysis system with chemiluminescence detection and its application to detection of cancer marker**

**K. Tsukagoshi**, N. Jinno and R. Nakajima  
Department of Chemical Engineering and Materials Science,  
Doshisha University, Kyotanabe, Kyoto, Japan.  
E-mail: ktsukago@mail.doshisha.ac.jp

A novel concept involving a micro-total analysis system incorporating an immunoassay and chemiluminescence detection was developed. The analysis system performed three processes on a microchip: an immune reaction for high selectivity, electrophoresis for formation and transportation of the sample plug, and chemiluminescence detection for high sensitivity. The three processes were compactly integrated onto the microchip. The chemiluminescence reaction of isoluminol isothiocyanate (ILITC) (as a chemiluminescence reagent for labeling) – microperoxidase (as a catalyst) – hydrogen peroxide (as an oxidant) was adopted. The microchip contained two micro-channels that crossed at an intersection, while the ends of the micro-channels accessed four reservoirs. As the first process, the immune reaction was performed using an antibody-immobilized glass bead. The glass bead was placed in one of the reservoirs along with antigen (analyte) and a known amount of ILITC-labeled antigen to set up a competitive immune reaction. For electrophoresis, as the second process, the reactant after the immune reaction was fed electrophoretically into the intersection resulting in a sample plug. The sample plug was then moved into another reservoir containing hydrogen peroxide solution. At this point, chemiluminescence detection was performed as the third process: the labeled antigen mixed with the hydrogen peroxide and the catalyst included in the migration buffer to produce chemiluminescence. Chemiluminescence was detected by a photomultiplier tube located under the reservoir. The micro-total analysis system described here was capable of determining, with high selectivity and sensitivity, human serum albumin or immunosuppressive acidic protein as a cancer marker in human serum.

#### L1-041P

##### **Purification of soybean lipoxygenase**

**S. Tukul**, D. Yildirim, R. Bilgin and G. Yucebilgin  
Department of Chemistry, University of Cukurova, Adana, Turkey.  
E-mail: rbilgin@cu.edu.tr

Lipoxygenases (EC 1.13.11.12) catalyse the dioxygenation of fatty acids which contain one or more 1(Z),5(Z)-pentadiene systems, yielding chiral (E,Z) conjugated hydroperoxy fatty acids. Lipoxygenases cause the whitening of bread by oxidizing the carotenoids, increase the loaf volume and play a role in the formation

of volatile flavour compounds. Risinoleic acid and derivatives which are formed by the action of lipoxygenases are used in soap, dye, varnish, resin and plastic products. In this study lipoxygenase has been purified from two types of soybean seeds encoded as A 3935 and Sa 88 by Field Crops Department of C.U. Agricultural Faculty. For this purpose soybean seeds homogenized, ultracentrifuged, fractioned with ammonium sulphate precipitation and applied on hydrophobic interaction chromatographic columns. After hydrophobic interaction chromatographic separation lipoxygenase was purified 16.5-fold in A 3935 soybean samples and was purified 100.5-fold in Sa 88 soybean samples. Specific activity of enzyme in A 3935 and Sa 88 was found as 53.83 U/mg prot. and 516.36 U/mg prot., respectively.

#### L1-042P

##### **A diagnostic test for active onchocerciasis based on stage specific polypeptides predominantly expressed in adult *Onchocerca* spp.**

F. Cho-Ngwa<sup>1</sup>, J. A. Metuge<sup>1</sup>, K. O. Gronvik<sup>2</sup> and V. P. Titanji<sup>1</sup>  
<sup>1</sup>Biotechnology Unit, University of Buea, Buea, South West Province Cameroon, <sup>2</sup>Department of Vaccine Research, National Veterinary Institute, Uppsala, Sweden. E-mail: vpkttitanji@yahoo.co.uk

Onchocerciasis or river blindness remains an important health problem affecting 17 million persons in Africa, the Yemen and Central America. But there are currently no permanent cures for the infection, since ivermectin the widely used drug kills the juveniles but not the adults of *Onchocerca volvulus*, the causative agent of the disease. Adult *Onchocerca*-specific tests are required to aid the development of new macrofilaricides in onchocerciasis. Herein, we describe the development of such a test based on the use of a cocktail of three monoclonal antibodies (mabs), termed UB1, UB6 and UB7 in a sandwich ELISA protocol. The mabs did not cross-react with extracts of *Ascaris suum*, *Loa loa* and *O. ochengi* microfilariae. They were all IgG1 molecules. Each mab recognized about 15 polypeptides spanning the range 20–220 kDa on Western blots in both *O. volvulus* and *O. ochengi* crude extracts. The purified targets were recognized specifically by bovine and human onchocerciasis antisera. Immunohistochemical staining confirmed their adult worm specificity and showed their binding to the hypodermis. The developed test could detect target antigens at 100 pg/ml and had a specificity of 94.1% for both the bovine and human infections and sensitivities that were, respectively, 90% and 48.8% from the human and bovine infections. We conclude that the test is potentially useful in macrofilaricide evaluation and perhaps in follow-up of ivermectin-treated populations. The sequencing and bioinformatic analysis of the dominant mab targets for chemotherapeutic goals are envisaged.

**Acknowledgment:** Supported by a grant from the International Programme In the Chemical Sciences (IPICS), Uppsala University, Sweden (CAM 01 Project).

#### L1-043P

##### **Improving prostate cancer diagnosis using PSA glycosylation**

**G. Tabarés**<sup>1</sup>, S. Barrabés<sup>1</sup>, L. Pagès<sup>1</sup>, J. Comet<sup>3</sup>, M. Ramírez<sup>2</sup>, R. N. Aleixandre<sup>2</sup>, W. Hösel<sup>4</sup>, R. Peracaula<sup>1</sup> and R. de Llorens<sup>1</sup>  
<sup>1</sup>Biochemistry, Biology, University of Girona, Girona, Spain,  
<sup>2</sup>ICS Girona Laboratory, Hospital Dr. J. Trueta, Girona, Spain,  
<sup>3</sup>Urology, Hospital Dr. J. Trueta, Girona, Spain, <sup>4</sup>Protein Chemistry, Roche Diagnostics, Penzberg, Germany.  
E-mail: gloria.tabares@udg.es

Prostate carcinoma (PCa) is the most common cancer in men from developed countries and it is the second highest cause of

cancer mortality in men. Prostate specific antigen (PSA) is currently used as a marker for the detection and monitoring of PCa. However, PSA serum levels below 15 ng/mL cannot well differentiate between PCa and other benign pathologies as benign prostatic hyperplasia (BPH). Different approaches have been used to improve the specificity of PSA as a tumour marker, but none of them has reached a high improvement in PCa and BPH diagnostics so far. PSA is a glycoprotein secreted mainly by the prostate. By sequencing, the glycan structures of PSA from seminal plasma (SP) and the media of the prostate cancer cell line LNCaP were different: PSA glycans present in LNCaP media were neutral and fucosylated, and PSA glycans from SP carry sialic acid. The objective was to study the glycosylation of PSA from serum of PCa and BPH patients, and to compare it with PSA from SP and LNCaP conditioned media. Glycan characterization of PSA from different biological fluids has been done by establishing different immunological assays combined with lectin detection, and by analyzing the degree of sialylation with a sialyltransferase assay. Moreover isoforms of PSA were characterized by two-dimensional electrophoresis. It is possible to differentiate between PSA glycans from PCa patient serums and seminal plasma by using the lectin as well as the sialyltransferase assays, because PSA from serum of PCa patients showed less sialic acid content than PSA from seminal plasma. By two-dimensional electrophoresis, PSA from serum of PCa patients showed sialylated and neutral forms. They were also observed in PSA from seminal plasma, but in different proportions. These results warrant further studies to find out whether anomalous glycosylation of PSA could be useful for cancer diagnosis.

#### L1-044P

##### Possible change of activity of glycolytic enzymes in RBC of tumor-bearing animals

J. I. Voronkova<sup>1</sup>, E. D. Zhabitskaya<sup>1</sup>, N. I. Shtemenko<sup>1</sup> and A. V. Shtemenko<sup>2</sup>

<sup>1</sup>Laboratory of Biochemistry, Department of Biophysics and Biochemistry, Dnepropetrovsk National University, Dnepropetrovsk, Ukraine, <sup>2</sup>Laboratory of Synthesis of Metal-Organic Substances, Department of Inorganic Chemistry, Ukrainian State University of Chemical Technology, Dnepropetrovsk, Ukraine. E-mail: ashtem@a-teleport.com

Glucose content (Glu, Mmol/l) was measured in plasma and red blood cells (RBC) hemolysates in blood of healthy and Guerin's carcinoma-bearing Wistar rats under influence of cis-platine (CP) and cluster rhenium compounds with organic ligands (CROL) introduction. Cytological control and Hb level measurements were made. In healthy animal Glu content in plasma is normally 4.5–7.2 and in RBC 30–36. It is explained by the fact that RBC are unique cells that have no nuclei and mitochondria, that's why the only source of energy is glycolysis and the cell has to support rather high concentration of glucose to survive. Introduction of CP (RBC – hemolytic) to healthy animals caused not very significant increase of Glu in plasma and RBC (approximately on 3–4%). Introduction of CROL (RBC – stabilizer) to healthy animals caused not very significant increase of Glu in plasma but very strong decrease in RBC (10–15). Development of the tumor caused the changing of the picture: decrease of Glu in RBC (to 25–27) but not in plasma that is followed by strong hemolytic anemia. Introduction of CP to tumor-bearing animals caused practically no changes in plasma Glu content but extremely high concentration of Glu in RBC (up to 60). Introduction of CROL to tumor-bearing animals led to approximately normal values of Glu as in plasma as in RBC. According to the obtained data we guess that development of tumor is connected with changing of activity of glycolytic enzymes action in RBC with the growth of their sensitivity to CP.

#### L1-045P

##### Identification of potential biomarkers in the macrovascular pathogenesis of diabetes mellitus

K. K. Yue<sup>1</sup>, W. C. Cho<sup>1</sup>, T.-T. Yip<sup>2</sup>, C. H. Cheng<sup>3</sup> and A. W. Leung<sup>1</sup>

<sup>1</sup>School of Chinese Medicine, Hong Kong Baptist University, Kowloon, Hong Kong, <sup>2</sup>Ciphergen Biosystems Inc., Fremont, CA, USA, <sup>3</sup>Department of Biochemistry, Chinese University of Hong Kong, New Territories, Hong Kong. E-mail: kkmue@hkbu.edu.hk

Diabetes mellitus (DM), with its rapid growth rate, is becoming an alarming threat to health of mankind, especially in developing countries such as China, India, etc. The purpose of this study was to discover potential biomarkers to serve as indicators of the pathogenesis of DM.

**Methods:** Tissue lysate were prepared from descending aorta of 102 diabetic and 85 control male Sprague-Dawley (S.D.) rats obtained at the 4th and 8th week after injection of streptozotocin (STZ), when redox changes and oxidative stress were found respectively. Sera were also obtained from these rats. The protein profiles were then studied employing surface-enhanced laser desorption-ionization time-of-flight mass spectrometry (SELDI-TOF MS) technology with detection sensitivity in the attomole (10–18) range, using immobilized metal affinity capture (IMAC3/IMAC30) and weak cation exchange (WCX2) arrays.

**Results:** Four potential biomarkers (at  $m/z$  10 003, 10 040, 11 110 and 11 275 Da) were identified in the descending aorta at the 8th week. In addition, a range of three peaks (at  $m/z$  5125, 5143 and 5162 Da) and two single biomarkers (at  $m/z$  6148 and 13 930 Da) were discovered to have high classification values (accuracy rate all 100%) in the sera to differentiate the diabetic group and the control group at both time intervals.

**Conclusions:** Comparing diabetic and non-diabetic control rats, potential biomarkers with significant changes of peak intensities and high classification values were found. These potential biomarkers will provide valuable insight on the pathogenesis of DM and macrovascular complications.

#### L1-046P

##### Blood proteins under metal-organic substances treatment

E. D. Zabitskaya<sup>1</sup>, N. I. Shtemenko<sup>1</sup>, O. A. Sorochnan<sup>1</sup>, M. V. Gorelaya<sup>1</sup> and A. V. Shtemenko<sup>2</sup>

<sup>1</sup>Laboratory of Biochemistry, Department of Biophysics and Biochemistry, Dnepropetrovsk National University, Dnepropetrovsk, Ukraine, <sup>2</sup>Laboratory of Synthesis of Metal-organic Substances, Department of Inorganic Chemistry, Ukrainian State University of Chemical Technology, Dnepropetrovsk, Ukraine. E-mail: ashtem@a-teleport.com

Cluster rhenium compounds with organic ligands (CROL) were investigated in experiments *in vitro* and *in vivo* in comparison with other antitumor agents. It was found that some CROL had weak antitumor, cell-stabilizing, antiradical and antioxidant activity, low toxicity, changed morphology of cells, etc. CROL with GABA and isobutyric acid as ligands did not cause the essential changes in content of sulfur-containing and other amino acids in plasma and red blood cells (RBC) of healthy and Guerin-carcinoma-bearing rats. Cis-platine (CP) with hemolytic effect and CROL-adamantane with RBC-stabilizing effect essentially changed the level of some amino acids thus showing activation of proteolysis in the cells and in plasma. Mechanism of such activation is strongly dependent from the nature of ligands in CROL. Influence of CROL on the interaction between IgG-antibody (Ab), IgM-Ab, IgA-Ab was studied and their impact on the

formation of the complexes Ag–Ab was shown. Changes of antigenic properties *in vitro* may be connected with conformational shifts in proteins, which did not bring perturbation in complementarity of Ag–Ab reactive sites. In models *in vivo* CROL

showed as albumin interacting agents and did not change the immune status of animals in comparison with other antitumor metal-organic substances.

## L2 – Microarrays for Proteome Analysis

### L2-001

#### Current state-of-the-art in protein microarrays

P. Van Hummelen

*MicroArray Facility, Flanders Institute of Biotechnology (VIB), Leuven, Belgium. E-mail: paul.vanhummelen@vib.be*

The completion of the human genome project truly set off a new race in technological developments for biological and clinical research that entered us into the so-called Omics-Era. This race is currently lead with great length by the Transcriptome but may soon be surpassed by an accelerating Proteome. Indeed, array based technologies for DNA or RNA research has been used successfully for a variety of applications but the use of protein microarrays is still limited. This has to do with obvious bigger challenges in the protein field, like the ~100 fold higher complexity, its dynamic nature, the critical structure-activity relationship, identification problems and the lack of amplification methods. Technical hurdles that have to be solved include: high-throughput methods for collecting the array-elements (e.g. antibodies, peptides, proteins etc.), immobilizing strategies that maintain the active conformation, labeling methods, sensitive detection, interpretation of the results, etc. However, there is no lack of new innovative ways to address all these issues. Recent array binding strategies include three dimensional deposition (MIST), and development of special linker/spacers and coatings. New detection strategies were reported in the field of BioMEMS with nanoscale electronic sensors, mass spectrometry like SELDI-MS, detection at atomic level using Kelvin Nanoprobes, or others like surface potential detection and planar waveguide. It is of obvious importance to develop an efficient and sensitive protein array platform because it will dramatically accelerate the pace of protein function research leading to understanding of diseases, discovery of drug targets and diagnostic biomarkers.

### L2-002

#### Making it small: microarrays for a multiplexed protein analysis

S. Kramer<sup>1</sup>, O. Poetz<sup>2</sup>, T. Knorpp<sup>1</sup>, J. M. Schwenk<sup>2</sup>, T. O. Joos<sup>2</sup> and M. F. Templin<sup>1</sup>

<sup>1</sup>Department of Assay Development, NMI at the University of Tübingen, Reutlingen, Germany, <sup>2</sup>Department of Biochemistry, NMI at the University of Tübingen, Reutlingen, Germany. E-mail: templin@nmi.de

Array-based assay systems allow the determination of hundreds of molecular parameters in a single experiment. DNA-chips proved to be powerful tools for generating genome-wide transcriptional profiles and their use has led to a wealth of new insights. Within the last years, microarray technology has been adapted to analyze proteins and different analytical systems are under development that are likely to evolve into key technologies for the characterization of complex samples. The use of miniaturized and multiplexed assay systems allows the measurement of up to several dozens of different analytes from limiting amount of sample material. This gives the opportunity to monitor the status of different proteins of interest in one experiment. We have established different protein microarray based systems that are

capable of detecting marker proteins directly from tumour biopsy material and from human serum. The examples given include (i) the multiplex detection and quantification of cytokines during sepsis (ii) the analysis of the phosphorylation state of key regulator molecules (iii) the analysis of tumours using different protein microarray-based analysis systems.

### L2-003

#### The use of protein arrays in proteomic applications

M. A. Coleman, K. A. Miller, P. T. Beernink, D. M. Yoshikawa and J. S. Albala

*BioSciences, Lawrence Livermore National Laboratory, Livermore, CA, USA. E-mail: coleman16@llnl.gov*

The use of protein arrays and their importance in proteomic applications continues to be at the forefront of scientific discovery and innovative technology development. To date, array-based approaches have proven to be a powerful tool for protein expression profiling, novel biomarker discovery, and for the examination of protein, DNA, and small molecule interactions. Our laboratory has developed several approaches for characterizing DNA repair-related and chromatin-remodeling processes using both protein profiling and protein-protein interactions in a microarray format. This was accomplished using an assembly line that takes advantage of multiple molecular techniques. First, methods were developed for producing recombinant proteins using both *in vitro* (IVT) and *in vivo* expression techniques. Second, methods for arraying and protein attachment were devised. Third, direct and indirect detection strategies requiring the use of fluorescently labeled molecules were employed. This approach was used to demonstrate that the RAD51B DNA repair protein interacts with histones and not nucleosome complexes. Novel nucleosome-specific interactions were also demonstrated with the SWI/SNF protein, SMARCA1. These array-based chromatin interaction assays were further validated using traditional molecular techniques.

### L2-004

#### Generation and recent applications of protein and antibody arrays

D. J. Cahill

*Centre for Human Proteomics, Royal College of Surgeons, Dublin, Ireland. E-mail: chp@rcsi.ie*

We are involved in developing and applying high density arraying technologies and automation to generate high content, high-density protein arrays and antibody arrays. One of the main focuses is to provide the protein content to put on the arrays. We have previously generated a human brain cDNA expression library [1], containing over 10 000 different human proteins. To optimize the generation of protein arrays, we have previously tested different expression system [2, 3] and different surfaces (glass, plastic, coated surfaces, membranes) to optimize the protein chip microscope slide format [4]. Also, antibody arrays have been tested on these chip surfaces, with the aim to develop chip-based



assays and for possible applications in diagnostics [5]. The microarray technology was extended to allow ELISA to be performed in this high-density format using conventional hardware [6]. We have demonstrated cell-free protein expression in nanowell plates in chip format and have monitored the enzymatic activity of those *in vitro* expressed proteins [7]. We have previously demonstrated the use of proteins in proteomics [8] and in large-scale selection systems [9]. Our second focus is to apply this technology to study interactions, such as peptide-protein interactions, and to elucidate the specificity and cross-reactivity of binders, such as monoclonal antibodies, and to profile the antibody repertoire in body fluids, such as human serum. We have previously demonstrated the proof of principle of screening these high density, high content protein arrays with monoclonal antibodies and sera from patients with autoimmune diseases [10]. We have recently used this approach to study Dilated Cardiomyopathy (DCM). Currently, experiments are underway for profiling the serum antibody repertoire in cancer, such as colon cancer and in the autoimmune diseases, Alopecia areata and Multiple Sclerosis. We have recently screened the protein arrays with labeled peptides and have found interacting peptides, and verified the interactions [11]. We have recently generated a mouse Th-1 protein expression library, and used it to profile sera from mouse autoimmune disease model. In this presentation, recent results of the applications of protein arrays in peptide screening, profiling the binding of antibodies and the disease-associated antibody repertoire in human serum will be presented.

**Acknowledgements:** D.J.C. gratefully acknowledges funding from Science Foundation Ireland (SFI), Dublin 2, Ireland (Award 02/CE1/B141).

#### References

1. Büsow K, Cahill D, Nietfeld, W *et al.*, *Nucleic Acids Res* 1998; **26**(21): 5007–5008.
2. Lueking A, Holz C, Gotthold C *et al.*, *Prot Express Purifi.* 2000; **20**: 372–378.
3. Holz C, Lueking A, Bovekamp L *et al.*, *Genome Res* 2001; **11**: 1730–1735.
4. Angenendt P, Glöckler J, Sobek J *et al.*, *Chromatography A* 2003; **1009**: 97–104.
5. Angenendt P, Glöckler J, Murphy D *et al.*, *Anal Biochem* 2002; **309**: 253–260.
6. Angenendt P, Glöckler J, Konthur Z *et al.*, *Anal Chem* 2003; **75**: 4368–4372.
7. Angenendt P, Nyarsik L, Szaflarski W *et al.*, *Anal Chem* 2004; **76**: 1844–184.
8. Schmidt F, Lueking A, Nordhoff E *et al.*, *Electrophoresis* 2002; **23**: 621–625.
9. Arap W, Kolonin MG, Trepel M *et al.*, *Nature Medicine* 2002; **8**(2): 121–127.
10. Lueking A, Possling A, Huber O *et al.*, *Mol Cell Proteom* 2003; **2**: 1342–1349.
11. Larkin D, Murphy D, Reilly DF *et al.*, *J Biol Chem* 2004; **279**(26): 27286–93.

#### L2-005

##### Chemical microarrays for screening the proteome for drug and target identification

L. G. Puskás<sup>1</sup>, G. Dormán<sup>2</sup>, L. Űrge<sup>2</sup>, F. Darvas<sup>2</sup>, L. Z. Fehér<sup>1</sup> and L. Hackler<sup>1</sup>

<sup>1</sup>Laboratory of Functional Genomics, HAS, Biological Research Center, Szeged, Hungary, <sup>2</sup>Comgenex Inc., Budapest, Hungary. E-mail: pusi@brc.hu

Special surfaces have been developed to make them capable for immobilizing small chemical compounds and to create small molecule microarrays. These types of microarrays can be used for chemical genomics approaches, especially for analyzing the

interactions between proteins and ligand binding proteins. In our system we used a special modified surface having a dendrimer-like structure and a ligand-microarray platform to analyze and compare complete proteomes of different cells. This technique has the advantage that protein target as well as their ligands could be identified in the same experiment and there is no need for the definition of the target and for purification of target proteins. We used our ligand microarrays to search for specific ligands that give differential signals after incubation with complete protein extracts of primary melanocytes and different melanoma cell lines. Positive ligands were further tested *in vitro* to reveal their effects on the growth characteristics of melanoma cells and to determine their phenotypic effects on other cell lines.

#### L2-006

##### High-throughput antibody based protein profiling using automated image analysis on cells and cell lines

S. Strömberg<sup>1</sup>, A.-C. Andersson<sup>1</sup>, K. Wester<sup>1</sup>, J. Kononen<sup>2</sup>, C. Kampf<sup>1</sup> and F. Ponten<sup>1</sup>

<sup>1</sup>Rudbeck laboratory, Department of Genetics and Pathology, Uppsala University, Uppsala, Sweden, <sup>2</sup>Beecher Instruments, Sun Prairie, Wisconsin, USA. E-mail: sara.stromberg@genpat.uu.se

Antibody-based proteomics provides a tool for exploring protein expression on a genome-wide scale. The ultimate goal is to map distribution and subcellular localization of all human proteins in all organs, tissues and cells. The Tissue MicroArray (TMA) technology provides the possibility to explore protein expression in a multitude of normal and diseased tissues in a high throughput manner. However, methods for including cells from *in vitro* cultured cell lines and cell aspirate material appear sparse. Here we describe the use of cells in a tissue microarray setting for analysis of protein expression using immunohistochemistry, creating an artificial tissue composed of cells in a gel matrix. Sections from cell TMA were immunohistochemically stained with a panel of in-house generated monospecific antibodies. Staining parameters such as intensity, fraction and subcellular localization were semi-quantitatively evaluated by manual scoring of cells in a microscope. An application for automated image analysis was developed and confirmed a high quality of the cell preparation method. Results from manual scoring were compared to the automated system. The outcome revealed that automated intensity scores correlated well with manual scoring, suggesting that this automatic approach for evaluation of immunohistochemical staining of cells, may be preferable as compared to manual, subjective scoring. Subcellular localization could also be detected with the automated image analysis system, receiving individual intensity values for specific subcellular localizations. We conclude that this modified TMA technique including cell samples, together with a customized automated image analysis system provide a valuable tool for high throughput analysis of protein expression in cells.

#### L2-007P

##### The effect of Poly (ADP) Ribose Polymerase inhibitors on Taxol induced cell death in cultured cells

Z. Bognar, G. Varbiro, B. Radnai, A. Tapodi, K. Hanto, P. Jakus, F. Gallyas Jr and B. Sumegi

Institute of Biochemistry, University of Pecs, Pécs, Hungary. E-mail: zita.bognar@aok.pte.hu

Taxol, a widely used tumor agent stabilizes tubulin dimmers, and it also exerted direct mitochondrial effect. PARP is also thought to be involved in the signaling and regulation of apoptotic

process. In this study we analyzed effect of PARP inhibition on Taxol induced toxicity in various models. The effect of Taxol was analyzed in cells in the presence of PJ-34, in PARP knockout cells, in cells where PARP was suppressed by siRNA and in cells over-expressing the N-terminal DNA-binding domain of PARP. The effects of PARP inhibition on the Akt and MAP kinase pathway were analyzed by western blot. The gene expression patterns in the various models were compared by DNA microarray. The cell toxicity induced by taxol was significantly inhibited by 10  $\mu$ M of PJ-34. When PARP activity was inhibited in cell expressing in large quantity the N-terminal DNA-binding domain of PARP, thus competing to binding sites with cellular PARP, the taxol induced toxicity was also decrease. Similar results were observed in PARP knockout cells, and in cells where PARP expression was suppressed by siRNA. The inhibition of PARP is known to influence well described survival pathways, however in our case no alteration in the AKT-phospho-AKT or MAP-kinase pathway were found. When the gene expression pattern of taxol treatment in the presence or absence of PJ-34 was compared numerous gene were found to be either over expressed or suppressed in the presence of the PARP inhibitor. With inhibition of PARP activity taxol induced cytotoxicity was significantly decreased. This lead us to the conclusion, that the presence and function of PARP is necessary for the potent effect of Taxol. To reveal the exact mechanism by which PARP influences the taxol cytotoxicity, we used to DNA-microarray analysis to reveal the alteration in gene expression pattern in the presence of PARP inhibitors, and identified genes with altered expression. Further studies are now ongoing to reveal the exact mechanism in details.

## L2-008P

### NF $\kappa$ B dependent induction of COX-2 expression by amiodarone in liver cells.

K. Hanto<sup>1,2</sup>, B. Radnai<sup>2</sup>, Z. Bognar<sup>2</sup>, V. Nemeth<sup>2</sup>, G. Varbio<sup>2</sup>, A. Tapodi<sup>2</sup>, F. Gallyas Jr<sup>2</sup>, B. Sumegi<sup>2</sup> and K. Toth<sup>1</sup>

<sup>1</sup>First Department of Medicine, University of Pecs, Medical School, Pécs, Hungary, <sup>2</sup>Department of Biochemistry and Medical Chemistry, University of Pecs, Medical School, Pécs, Hungary.  
E-mail: hantokata@yahoo.com

Amiodarone is a frequently used antiarrhythmic drug in the clinical practice, but its application is often limited by its serious side effects. The most severe is pulmonary fibrosis, however the fibrosis of the liver has also been described. Therefore we have investigated the possible mechanisms responsible for the pathological alterations in the liver caused by amiodarone. WRL-68 human liver cells were treated with different concentrations of amiodarone or its metabolite, desethyl-amiodarone. In an other experiment, the same agents were used for various times. We have detected the activation level of Cox-2 and iNOS by Western blotting using monoclonal antibodies. Alternatively nuclear fractions from the same experiments were isolated for the detection of the transcription factor NF  $\kappa$ B by a transcription factor assay kit. DNA microarray was used to determine the changes in the inflammatory gene expression levels. Our results show that both amiodarone and its metabolite induced COX-2 and iNOS expression in a concentration dependent manner. Amiodarone at the concentration of as low as 5  $\mu$ M increased the transcription of COX-2 and iNOS as detected by the elevated level of NF $\kappa$ B in the nuclear fraction of the cells with a sandwich ELISA technique. Amiodarone treatment significantly induced the overexpression of the genes related to the inflammatory and apoptotic pathways. Our results indicate that the mechanisms responsible for the side effects of amiodarone can be explained by the onset of inflammatory process caused by COX-2 and iNOS through an NF  $\kappa$ B dependent pathway.

## L2-009P

### Biomarker discovery strategies: comparison of 2D gel based, bottom-up and top-down proteomics approaches with diabetic serum samples

A. Paulus, S. Freeby, A. Posch, T. Wehr and K. Academia  
Protein Separation Division, Bio-Rad Laboratories, Hercules, CA, USA. E-mail: aran\_paulus@bio-rad.com

Biomarker discovery and validation is an important part of most proteomics programs in academia and biotech industry. The goal is to identify and verify up or down regulated proteins that are part of a disease-specific pathway and thus allow early diagnosis. Typically, biological material of a disease sample and a control sample are subjected to fractionation, separation and quantitative analysis to determine possible markers. Currently three major approaches to separate and identify protein biomarkers are pursued: 2D gel separation followed by image analysis, spot cutting, digestion and MS identification, bottom-up proteomics where a more or less complex protein mixture is first digested into an even more complex tryptic peptide mix before separated with two-dimensional HPLC with on-line MS identification and lastly top-down proteomics where the intact protein mixture is separated via HPLC methods prior to high-resolution MS identification with FT-ICR-MS. We have used serum from diabetic patients and normal serum as control in a biomarker study. After removal of albumin and IgG and fractionation by isoelectric point, we compared the three approaches in terms of times of analysis, times of data evaluation and interpretation, cost of equipment and number of identified differentially expressed proteins.

## L2-010P

### Generation of mono-specific antibodies using Protein Epitope Signature Tags (PrEST): A comparative study based on immunohistochemistry on tissue microarrays

L. Paavilainen<sup>1</sup>, S. Strömberg<sup>1</sup>, A.-C. Andersson<sup>1</sup>, C. Kampf<sup>1</sup>, H. Wernérus<sup>2</sup>, P. Nilsson<sup>2</sup>, M. Uhlén<sup>2</sup>, F. Pontén<sup>1</sup> and K. Wester<sup>1</sup>

<sup>1</sup>Rudbeck Laboratory, Department of Genetics and Pathology, Uppsala University, Uppsala, Sweden, <sup>2</sup>Department of Biotechnology, Royal Institute of Technology (KTH), Stockholm, Sweden.  
E-mail: Linda.Paavilainen@genpat.uu.se

Human Proteome Resource (HPR) is an antibody based proteomics project aiming to map the human proteome utilizing HUGO information. In this study, a strategy for generating mono-specific antibodies using unique Protein Epitope Signature Tags (PrESTs) as immunogens is presented. A PrEST represents a unique region comprising 100–150 amino acid residues of a protein. The amino acid sequence for each PrEST was designed using in-house developed bioinformatic software and human genome sequence information (Ensembl database, <http://ensembl.org/>). Recombinant proteins (PrESTs) were generated using RT-PCR and cloning. The PrESTs were used for immunization and polyclonal antisera were affinity purified using the PrEST as ligand. The purified antibodies, here called mono-specific antibodies, were generated towards a set of well-characterized proteins where commercial antibodies are readily available. All antibodies were compared using immunohistochemistry on tissue microarray (TMA) designs. The different designs represent a wide spectrum of normal and cancer tissues. The outcome of the immunohistochemistry was evaluated and the different antibody stainings were compared regarding distribution. The result

revealed that mono-specific antibodies showed identical or similar staining patterns when compared to corresponding commercial analogues. As a conclusion, the highly concordant antibody

staining patterns confirm our strategy for high-affinity antibodies and thus can further be used for generation of a protein atlas for the human genome.

## L3 – Genomics in Protein Biotechnology/Protein Biotechnology

### L3-001

#### Recombinant antibodies for analytics

B. Hock, C. Kraft, J. Wiebe and K. Kramer

Chair of Cell Biology, Center of Life and Food Sciences,  
Technische Universität München, Freising, Bavaria, Germany.

E-mail: hock@wzw.tum.de

Initial steps of antibody (ab) engineering two decades ago revolutionized the technology of ab production, particularly in the area of immunotherapy and diagnostics. However, this field has been extended only hesitantly to other applications. This contribution is addressed to recombinant ab technology focusing on ab libraries for pesticides and antibiotics. The first library was constructed by cloning triazine-specific subsets of murine ab repertoires into phagemids utilizing a commercial vector system. The second library was based on ab genes derived from immunized rabbits. For this purpose, a new phagemid vector was developed for cloning rabbit ab genes. Both libraries were functionally expressed as ab fragments on the surface of filamentous phages. Recombinant virions displaying specific ab fragments were then selected by analyte-coated surfaces. Subsequently, soluble ab fragments were expressed from individual binding clones and kinetically characterized revealing affinities for the corresponding target structures in the nanomolar range. The most efficient strategy for the *in vitro* evolutionary optimization of ab binding properties turned out to be a combination of randomized point mutations and sequential recombination of variable ab gene repertoires employing the analyte-selective library. This strategy resulted in a shift of the equilibrium dissociation constant  $K_D$  from  $1.3 \times 10^{-8}$  M of the template ab to  $7.5 \times 10^{-10}$  M of the optimized variant. DNA sequencing revealed that the distribution of the ab sequence alterations of these clones mirrored those mutations observed during ab affinity maturation *in vivo*. An example for the application of the optimized ab variant is given for the analysis of atrazine contaminations in soil samples. The results closely match corresponding HPLC data.

### L3-002

#### Creating novel proteins for therapeutic applications

R. Rudolph<sup>1</sup>, H. Lilie<sup>1</sup> and U. Fiedler<sup>2</sup>

<sup>1</sup>Institute of Biotechnology, Martin-Luther-Universität Halle-Wittenberg, Halle, Germany, <sup>2</sup>Scil Proteins GmbH, Halle, Germany. E-mail: rudolph@biochemtech.uni-halle.de

Recombinant proteins are by now widely used for the treatment of various diseases. For some therapeutic applications (e.g. cancer treatment) natural proteins offer only limited strategies for therapeutic intervention. For these applications, we designed novel modular proteins. We used polyionic adaptor peptides containing cysteine residues as highly specific heterodimerization motifs. Fusion of these polyionic peptides to both a protein module recognizing specific cell surface epitopes, and another module inducing cell death resulted in disulfide-bonded immunotoxins, which possess a very high and specific cytotoxicity. The polyionic adaptor motif was also used to specifically modify virus like

particles. As modular delivery systems, composed of a specific recognition domain, *in vitro* assembled capsids, and the therapeutic cargo (e.g. DNA) specific cell transformation could be achieved. Finally, novel binding molecules were created. For this purpose, a very stable protein from the human eye lens,  $\gamma$ -crystallin, was randomized in a solvent-exposed beta-sheet area. Applying suitable display and screening techniques specifically binding molecules can be isolated for both low molecular weight substances and globular proteins.

### L3-003

#### Revival of a classical resource for thermophiles and thermophilic enzymes

T. Oshima<sup>1,2</sup> and T. Moriya<sup>1,2</sup>

<sup>1</sup>Tokyo University of Pharmacy and Life Science, Tokyo, Japan,

<sup>2</sup>Institute of Environmental Microbiology, Tokyo, Japan.

E-mail: toshima@ls.toyaku.ac.jp

Compost was most important source for isolating thermophiles until middle of 20th century. Inside of compost was hot due to heat liberated during fermentation processes. The maximum temperature inside has been reported to be up to 80°C and many moderate thermophiles belonging to genus *Geobacillus* or *Clostridium* were isolated from compost soil. However, recently we found that in some composting industry the inside temperature is much hotter than those reported before; 95°C or even higher. We isolated new extreme thermophiles from soil samples collected from “high temperature compost”. The isolates differ from thermophiles belonging to *Geobacillus* in cell surface structure and can be classified into a new genus. 16S rDNA sequence comparison supported our speculation. We also noticed that thermophiles, which are responsible for the “high temperature compost”, are so to speak “unculturable” microorganisms. In some cases, we isolated DNA coding for a desirable enzyme directly from compost soil and in other cases we isolated enzyme protein directly from compost soil. “High temperature compost” seems to be a new promising source for isolation of enzymes of industrial interest.

### L3-004

#### The impact of recombinant proteins on the pharmaceutical sector

C. R. Noe

Department of Medicinal Chemistry, University of Vienna, Vienna, Austria. E-mail: christian.noe@univie.ac.at

The huge potential of gene technology for the pharmaceutical sector was recognized immediately with the discovery and development of techniques of molecular biology. The expectations set in the new opportunities are well reflected by the rapid growth of the American biotech industry about 25 years ago. During the first heroic phase, paradigmatic and radical changes in the modalities of treatment of diseases were predicted, usually referring to the year 2000, when conventional drug therapy would be replaced by gene therapy and other novel therapeutic approaches,

such as tumor vaccines or advanced immunotherapy. Recombinant proteins marketed as drugs, sometimes just creating superior access to established proteins, were then considered to be just a first step in this direction. Twenty-five years later, recombinant proteins make up a significant, but by no means dominant part of the "treasure of medicines". Some of them have become generic in the meantime, by that fact generating seemingly trivial problems, such as defining criteria how to show bioequivalence of a recombinant generic product. Today, some of the novel therapeutic approaches suffer from failed attempts of inventors and investors to reach the market or at least the clinical phase of development as soon as possible, without taking care of established rules in drug development, e.g. the study of the LADME-parameters. Gene therapy (somatic and antisense), tumor vaccines and other fascinating concepts have undergone phases, when they were considered to be "out". Nevertheless fundamentally novel therapeutic options remain great hopes in drug discovery. In the lecture an attempt will be made to present an overview analysis on the main lines of development of pharmaceutical biotech research, focusing on application of recombinant proteins. Apart from therapeutic applications, they are important elements of the rapidly growing diagnostic field. Finally, it will be shown that recombinant proteins are nowadays indispensable tools in rational drug research. In a fascinating manner, this research aims at understanding the genetic basis of the etiology of diseases, finding molecular targets for the action of drugs, finding novel lead compounds, and finally optimizing and developing them into successful new drugs.

### L3-005

#### Levels of expressed Cry1Ab toxin in genetically modified corn DK-440-BTY (YIELDGARD) and stubble

A. Szekacs, J. Juracek, L. A. Polgar and B. Darvas  
Department of Ecotoxicology and Environmental Analysis, Plant Protection Institute, Hungarian Academy of Sciences, Budapest, Hungary. E-mail: aszek@nki.hu

The production of the *Bacillus thuringiensis*-originated Cry1Ab toxin (*Bt*-toxin) in the genetically modified (GM) DK-440-BTY corn was analyzed in a 3-year period using immunoanalytical detection. By biochemical analysis of the transgenic proteins with specific antibodies, the content of the three-domain  $\delta$ -endotoxin was monitored throughout each entire vegetation period and was compared to that detected in isogenic corn line. Toxin content was found to be in the order: leaves > anther wall > root > stem > seed > pollen. Relative toxin content in the leaves increased until June, and slightly dropped afterwards. The toxin level produced on the plantation area was calculated to be 1500–2000 times higher than toxin dosage corresponding to registered applications of the *Bt*-toxin-based biopesticide *DIPEL*. Thus, the GM corn represents a 1500–2000 times higher environmental toxin load than registered non-biotechnological toxin applications. Nearly 84% of the toxin content was produced in the leaves, the stem, seed, roots and the tassel represented 8, 4, 3 and 1%, respectively. Alarming that about 8% of the produced toxin remains on the area after harvest, and a significant part was found to persist in the stubble after 11 months. Although corn pollen contains low amounts of toxin, cross-pollination occurs on non-*Bt*-corn already in the first vegetation period, and seeds of the subsequent intraspecific hybrids contained one-third of the toxin content in DK-440-BTY.

**Acknowledgments:** This research was supported by the Hungarian Ministry of Education (BIO-00042/2000) and the Ministry of Environment (K-36-01-00017/2002).

### L3-006

#### Characterization of antigens for an optimal TB vaccine

J. M. Nagy, A. Tsolaki and B. Robertson  
Flowers Building, CMMI, Imperial College, London, UK.  
E-mail: j.nagy@imperial.ac.uk

Tuberculosis (TB) is the leading infectious disease in the developing world and with one-third of the world population being infected, it affects every country. Currently the most effective treatment involves a long and costly chemotherapy regime. BCG, the only available vaccine, provides minimal and inconsistent protection against the most common and infectious form of TB in adults. Consequently a new and improved vaccine is needed to improve the immune status of the adolescent population in TB endemic areas. Several live and subunit vaccines candidates have been identified with improved efficacy compared to BCG. In addition to these new vaccine candidates, new protein and non-protein antigens have been discovered which are now being characterized and evaluated for an optimal TB vaccine. The majority of tested vaccine antigens are expressed in various strains of the common laboratory organism *Escherichia coli*, in spite of the fact that the protein folding and post-translational modification mechanism in the pathogen might be very different. Several protective antigens have been isolated from culture supernatants of *M. tuberculosis*. Three of these, ESAT6, Antigen85B and TB10.4 were selected to construct two hybrid fusions which have been shown to induce a strong dose dependant immune response when administered to mice in the adjuvant combination dimethyl dioctadecylammonium bromide-monophosphoryl lipid A (Olsen AW et al. *Infection and Immunity* 2001). To determine the effects of post-translational modification on recombinant vaccine antigens two hybrid TB antigen constructs, Hyb1 (Antigen85B/ESAT6) and Hyvac4 (Antigen85B/TB10.4) were cloned into the recently developed tetracycline inducible plasmid system (Blokpoel M et al. 2004) and pSHK (Wiles S et al. 2004). The new constructs were transformed into *M. smegmatis* mc2155 and BCG-Pasteur and the over-expressing proteins purified. The purified proteins were characterized using 1D and 2D gel electrophoresis and investigated for post-translational modifications. All investigations were carried out in comparison with the same hybrids expressed in *E. coli* DH5a previously (Vinther Skjot RL et al. *Infection and Immunity* 2000). The modified antigens will be tested in the murine mode and the immunogenicity evaluated. They will be also used for screening for the closest possible immune response to that induced by natural infection which combined with the most effective vaccine delivery system should prove a rationally optimized TB vaccine. The modified antigens will be tested in the murine mode and the immunogenicity evaluated. They will be also used for screening for the closest possible immune response to that induced by natural infection which combined with the most effective vaccine delivery system should prove a rationally optimized TB vaccine.

### L3-007P

#### Purification and investigation of the antimycobacterial activity of Syringomycin E

E. Buber<sup>1</sup>, A. Alp<sup>2</sup> and N. L. Acan<sup>1</sup>  
<sup>1</sup>Department of Biochemistry, Faculty of Medicine, Hacettepe University, Ankara, Turkey, <sup>2</sup>Department of Microbiology, Faculty of Medicine, Hacettepe University, Ankara, Turkey.  
E-mail: nla@hacettepe.edu.tr

Syringomycin E is a cyclic lipodepsipeptid produced by several *Pseudomonas syringae* pv. *syringae* strains. Besides being a strong antifungal agent, our studies with partially purified lipopeptides

produced by *P. syringae* pv. *syringae* B359 strain showed that it also might be a potential antimycobacterial agent. Due to the enormous increase in mycobacterial infections in recent years, there is an urgent need to develop new and potent antimycobacterial drugs. Therefore, in this study we aimed to purify syringomycin E produced by *P. syringae* pv. *syringae* B359 strain and investigate antimycobacterial properties of it in more detail. For the purification, ammonium sulphate precipitation, acetone extraction, cation exchange chromatography and reverse phase HPLC chromatography on C18 column were used. By this way syringomycin E, syringopeptin 22 and some other syringomycins which are produced at low concentrations were isolated. Minimal inhibitory concentrations of purified syringomycins and syringopeptins for *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* were investigated. MIC value of syringomycin E for *M. smegmatis* was found as 4.3 µg/ml. It was also observed that the isolates have antimycobacterial activity towards *M. tuberculosis*. Minimal bactericidal concentrations for *M. smegmatis* was also investigated and found out that the effect was bactericidal.

**Acknowledgment:** This study was supported by Hacettepe University Research Unit, Project No. 0102101019.

### L3-008P

#### Cloning and expression of DNA POL1 gene from *Bacillus thermoleovorans* in *Escherichia coli*

A. Akhmaloka<sup>1</sup>, H. Pramono<sup>2</sup>, I. N. Tika<sup>1</sup>, L. Ambarsari<sup>1</sup>, M. R. Moes<sup>3</sup> and F. Madayanti<sup>1</sup>

<sup>1</sup>Biochemistry, Chemistry, Institut Teknologi Bandung, Bandung, West Java Indonesia, <sup>2</sup>Biochemistry, Biology, Unsoed, Purwokerto, Central Java Indonesia, <sup>3</sup>Molecular Biology, Biology, Institut Teknologi Bandung, Bandung, West Java Indonesia.  
E-mail: loka@chem.itb.ac.id

A few thermophilic bacteria were isolated from a hot spring at Cimanggu, Bandung, West Java, Indonesia. One of them has been identified using ribotyping analysis as *Bacillus thermoleovorans*. DNA polI gene from the isolate was cloned through *in vitro* amplification (PCR) method. Two sets of primers were constructed to amplify the gene. The first primers amplified 0.6-kb fragment lied on the upstream of the gene while the second primers amplified the whole coding region of the gene. Nucleotide sequence analysis showed that the fragment was a highly homology to all DNA polI, with the highest homology to DNA polI of *Bacillus caldotenax*. Further comparison to the *Bacillus caldotenax* polI gene showed that one amino acid deletion, a few amino acid substitution and a few silent mutations. Expression of the gene was performed in *E. coli* using pTRX-Fus expression vector. SDS-PAGE analysis of the crude extract showed that the gene was highly expressed up to 35% of total protein. The crude extract still showed polymerase activity using nucleotide radiolabelable incorporation. The heterolog protein was purified by cation exchange chromatography.

### L3-009P

#### Production of human LBK-type alkaline phosphatase in the milk of transgenic rabbits

L. Bodrogi<sup>1</sup>, W. Raaben<sup>2</sup>, D. Fiechter<sup>2</sup>, B. Bender<sup>1</sup>, R. Brands<sup>2</sup>, W. Seinen<sup>2</sup> and Z. Bösze<sup>1</sup>

<sup>1</sup>*In vivo gene expression and regulation group, Department of Animal Biology, Agricultural Biotechnology Center, Gödöllő, Hungary, <sup>2</sup>IRAS, University of Utrecht, Utrecht, The Netherlands.*  
E-mail: bodrogi@abc.hu

The idea for using the mammary gland as a bioreactor has been developed to the point that pharmaceuticals derived from the milk

of transgenic farm animals are currently in advanced stages of clinical trials. Gram negative sepsis, an lipopolysaccharide (LPS)-mediated disease is a major cause of mortality in hospitals. It has been shown that alkaline phosphatase (AP) dephosphorylates and thereby detoxifies LPS at physiological pH levels [1]. Animal experiments have proven this effect in mice and piglets [2, 3]. The aim of the project is to produce transgenic (tr.) rabbits expressing large amount of human liver bone kidney type (LBK) AP in their milk. The gene construct contains a 2.4-kb long fragment of the mouse whey acidic protein (WAP) promoter and the LBK AP minigene, which harbours the first intron and the cDNA. Two tr. lines were produced by pronuclear microinjection (#932 and #949). Milk samples were taken from both founder females and from a hemizygous #932 female and transgene expression on the protein level has been evaluated. AP activity was measured with a PNPP based method after Triton-X100 extraction of the milk to release the potential AP from the fat globules. High AP activity (about 170 IU/ml) was detected in the milk of the #932 founder, while in the milk of the #949 founder female lower, but significant activity (10.5 IU/ml) was revealed. Experiments are underway to purify the recombinant AP from rabbit milk and evaluate its biological activity in Gram negative sepsis animal models.

#### References

1. Poelstra K *et al.* *Am J Pathol* 1997; **151**: 1163–1169.
2. Beumer C *et al.* *J Pharmacol Exp* 2003; **307**: 737–744.
3. Verweij WR *et al.* *Shock* 2004; **22**: 174–179.

### L3-010P

#### Expression of low-phenylalanine kappa-casein in the milk of transgenic rabbits

M. Baranyi<sup>1</sup>, A. P. Catunda Lemos<sup>1</sup>, L. Bodrogi<sup>1</sup>, B. Bender<sup>1</sup>, L. Szabó<sup>2</sup>, L. Hiripi<sup>1</sup> and Z. Bösze<sup>1</sup>

<sup>1</sup>*In vivo gene expression and regulation group, Institute of Animal Biology, Agricultural Biotechnology Center, Gödöllő, Hungary, <sup>2</sup>Electron microscope laboratory, Institute of Environmental Biosafety, Agricultural Biotechnology Center, Gödöllő, Hungary.*  
E-mail: catunda@abc.hu

Phenylketonuria (PKU) is a genetic disorder that is characterized by an inability of the body to utilize the essential amino acid phenylalanine (Phe). Chronically high levels of phenylalanine and some of its breakdown products in the blood which usually cause significant brain problems like mental retardation and seizures can only be avoided by a low-Phe diet. Our ultimate aim is to produce milk proteins with low or no Phe content and to develop a milk product consumable also by people with PKU. As a first step toward that aim we created transgenic rabbits expressing low-Phe kappa-casein (κ-CN). The endogenous rabbit κ-CN has been modified. A gene construct was developed in which all Phe codons – except for the one responsible for chymosin digestion and milk clotting – have been replaced with other amino acid codons. Transgenic rabbits were created by the pronuclear microinjection method [1]. Two transgenic rabbit lines (#63 and #82) were established. Transgene copy numbers determined by real-time PCR were 3 in #63 and 6 in #82. Production of recombinant κ-CN in the milk of the animals was confirmed by Western-blot analysis using specific antibodies and N-terminal sequencing. Recombinant protein levels in the transgenic rabbit lines were equivalent to 26% (#82 heterozygote), 36.6% (#82 homozygote) and 17.5% (#63 heterozygote) of the endogenous κ-CN. Altered milk composition did not influence the lactation performance or overall health of the transgenic females. Casein micelle sizes were significantly reduced in the milk of the higher expressing #82 rabbits indicating that the recombinant κ-CN is functional.

#### Reference

1. Hiripi *et al.* *DNA Cell Biol* 2003; **22**: 41–45.

**L3-011P****Improving of bacterial expression of recombinant non-structural plant viral proteins for raising antibodies**

T. Pecenkova, M. Filigarova and N. Cerovska

*Laboratory of Virology, Institute of Experimental Botany CAS, Prague, Czech Republic. E-mail: cerovska@ueb.cas.cz*

In order to obtain strong bacterial expression of proteins that seem to be hard to express in bacteria or are highly toxic for bacteria, it is possible to create various shortened variants of original construct, either by standard digestion with suitable restriction enzymes, or by exonucleaseIII/S1 nuclease digestion. The first approach is limited by the restriction map of the cloned fragment, and only few possibilities could be exploited, while the second approach can give rise to the palette of similar constructs, differing only by several nucleotides, gradually deleted from the one end of full-length clone. We utilized these two procedures to produce in bacteria one of potato mop-top virus (PMTV) movement proteins, namely triple gene block protein 1 (TGBp1) which was very hard to express in its original length. The cloned gene carrying the 6xHis tag on its 5' end was deleted on its 3' end of the gene in two ways. Additionally, we tested whether the deletion of N terminus could improve the expression of this protein. The 5' terminus of cloned fragment was gradually deleted with exonucleaseIII, leaving 6xHis tag intact. The SDS-page and Western blot of bacterial culture lysate with anti-His antibodies confirmed that in both cases we have achieved the efficient expression of these shortened variants of 6xHis tagged TGBp1 protein. We selected one of these shortened variants, which was produced in high range in bacteria. The protein was used as immunogen and the rabbit polyclonal antibodies were obtained.

**Acknowledgment:** This study was supported by grant No. 522/04/1329 of the Grant Agency of Czech Republic.

**L3-012P****Proteins of isolated cell nuclei from *Secale cereale* L. and *Nicotiana tabacum* L. during genome expression and modification**

V. V. Chyzyk and V. N. Reshetnikov

*Department of Plant Biochemistry and Biotechnology, National Academy of Sciences, Central Botanical Garden, Minsk, Belarus.**E-mail: alisa67@hotmail.ru*

Isolated interphase cell nuclei from winter rye dry seeds and 24-, 72-h seedlings as well as leaves and calluses of control and transgenic (ipt) plants of tobacco were the object of our investigations. On the base of using solutions with different ionic power,  $Mg^{++}$  concentration and detergents, we have divided interphase cell proteins on five main fractions: karyoplasm, euchromatin-I, euchromatin-II, heterochromatin, nuclear matrix. During the natural expression of winter rye genome (germination) the common nuclear protein heterogeneity is increased on 50%. Nuclei in active state are characterized by increasing protein containing (from its common quantity) in karyoplasm and skeletal structures (matrix); by decreasing of protein containing in euchromatin fraction due to increasing of nucleic acids portion; by increasing of low-molecular polypeptides quantity (10–25 kD), by raising of proteolytic activity in karyoplasm and in labile and compact chromatin. The hypothesis of unregular and specific structural organization of deoxyribonucleoprotein complex in interphase cell nucleus volume due to species-specific DNA-connecting proteins has confirmed. During the ipt-gene involving into DNA, we can see a polypeptides heterogeneity

changes in skeletal structures and in karyoplasms. The investigated transgenic plants had low indexes of pigments synthesis and higher reaction on light in culture *in vitro*. So the ipt-gene involving in nucleous influences not only on growth intensity, but also on a number of accompanying biosynthetic and physiological processes. The results of our work creates the theoretical base for biotechnological work and optimization of growth regulating substances exploiting.

**L3-013P****Functional expression of mammalian receptors and membrane channels in different cells**N. Eifler<sup>1</sup>, P. J. Werten<sup>1</sup>, M. Duckely<sup>1</sup>, A. Lüthi<sup>2</sup> and A. Engel<sup>1</sup><sup>1</sup>*M.E. Müller-Institute for Microscopy, Biozentrum, University of Basel, Basel, Switzerland,* <sup>2</sup>*Division Pharmacology/Neurobiology, Biozentrum, University of Basel, Basel, Switzerland.**E-mail: nora.eifler@unibas.ch*

The majority of medically important membrane proteins is present in native tissues only at very low concentrations, making their overexpression in heterologous systems a prerequisite for structural studies. Lipid environment, particular chaperones and post-translational modifications all play an important role in the production of structurally intact and functional membrane proteins. Since the influence of such conditions is difficult to predict, expression of membrane proteins is still an exploratory art. In this study we therefore compare the commonly used eukaryotic expression systems yeast, baculovirus/Sf9 and various cell lines transfected by Semliki Forest virus for the expression of a wide range of membrane proteins from different classes. Whereas the SFV system should provide a better environment for mammalian membrane proteins, the two other expression systems allow an easy handling and are cheaper for large-scale production.

**L3-014P****Proteinases propeptides effective to nematode and insect-pests**E. L. Z. Figueira<sup>1</sup>, F. B. Silva<sup>1,2</sup>, B. M. Marra<sup>1,3</sup>,R. P. Del Sarto<sup>1,3</sup>, O. B. Oliveira Neto<sup>1</sup>, J. A. N. Batista<sup>1</sup> and M. F. Grossi de Sá<sup>1,2</sup><sup>1</sup>*Molecular Plant-Pest Interaction, Biotechnology, Embrapa Recursos Genéticos e Biotecnologia, Brasília, DF Brazil,* <sup>2</sup>*Universidade Católica de Brasília, Brasília, DF Brazil,* <sup>3</sup>*Universidade de Brasília, Brasília, DF Brazil. E-mail: luiz@cenargen.embrapa.br*

Plant-parasitic insects and nematodes represent an economically important group of pests worldwide that can devastate a wide range of crop plants, causing annually billions of dollars in yield losses. The proteolytic enzymes are essential to the feeding process of plant-parasitic nematodes and insects, being potential targets to the control of these pests. Based on studies, which indicate that prodomain peptide of various proteinase zymogens can inhibit their corresponding enzymes, a 16.3 kDa cysteine proteinase propeptide from *Heterodera glycines* (PHGCP) and a 11.0 kDa cysteine proteinase propeptide from *Acanthoscelides obtectus* (PCPAO) were cloned and expressed in *Escherichia coli* cells. Both recombinants propeptide were capable to inhibit their cognate enzymes. Recombinant PCPAO inhibited 96% of the cognate proteinase (CPAO) activity, while the recombinant PHGCP inhibited 98% of the cognate enzyme. The *H. glycines* bioassay in transformed soybean roots expressing PHGCP resulted in a significative decrease in the amount of *H. glycines* females (36%), eggs position (58%) and female development (20%). Novel *in vitro* assays are carried out to evaluate the

potential use of the PCPAO and PHGCP against important economically pests control. Our data suggest that propeptides of cysteine proteinases represent a new strategy to be used as bioinsecticide/nematicide, and could be an effective tool to apply in plant genetic improvement programs.

**Acknowledgments:** This work was supported by EMBRAPA, CNPq and CAPES.

### L3-015P

#### Anti-HIV antibody detection by allosteric beta-galactosidase sensors

R. M. Ferraz<sup>1</sup>, G. Álvaro<sup>2</sup>, G. González<sup>2</sup>, J. López-Santín<sup>2</sup>, A. Villaverde<sup>1</sup> and A. Arís<sup>1</sup>

<sup>1</sup>Microbiología aplicada, Institut de Biotecnologia i de Biomedicina and Departament de Genètica i de Microbiologia, Universitat Autònoma de Barcelona, Bellaterra, Spain, <sup>2</sup>Departament de Enginyeria Química, Universitat Autònoma de Barcelona, Bellaterra, Spain.

E-mail: rferraz@servet.uab.es

Allosteric biosensors are based on enzymatic responses to specific analytes. In this work, we developed a recombinant *Escherichia coli* beta-galactosidase that responds enzymatically to human antibodies raised against human immunodeficiency virus. A viral antigenic peptide, conveniently displayed in the vicinity of the active site of the enzyme, acts as a receptor. The allosteric response has been deeply studied. The comparison of different enzyme and substrate concentrations reveals some conditions that dramatically improve the allosteric response. We have also explored the performance of five colorimetric, fluorescent and luminescent substrates of beta-galactosidase to know their influence on the enzyme activation factor. Interestingly, the used substrate determines the dynamic range of the signal and significantly influences the sensitivity of the sensing reaction. Ortho-nitrophenyl beta-D-galactopyranoside substrate (ONPG) allows the detection of 0.024 ng/μl of a model anti-gp41 monoclonal antibody being the most sensitive of all of them. Phenol red beta-D-galactopyranoside substrate (CPRG) offers the most dynamic response, with signal/background ratios higher than 12-fold. In conclusion, we consider that chromogenic substrates are the best option for detection in allosteric response. Finally, since it would be desirable the development of a solid-phase biosensor version that could be used by non-skilled personnel in a very simple and reliable way, we have immobilized the enzyme in an agarose support and compared the results obtained with ONPG and CPRG chromogenic substrates.

### L3-016P

#### Molecular characterization of the PPZ1 gene from *Candida albicans*

I. Farkas<sup>1</sup>, C. Ádám<sup>1</sup>, G. Molnár<sup>1</sup>, L. Kovács<sup>1</sup>, L. Majoros<sup>2</sup>, J. Aradi<sup>3</sup> and V. Dombrádi<sup>1</sup>

<sup>1</sup>Department of Medical Chemistry, Medical and Health Science Center, University of Debrecen, Debrecen, Hungary, <sup>2</sup>Department of Medical Microbiology, Medical and Health Science Center, University of Debrecen, Debrecen, Hungary, <sup>3</sup>Department of Biochemistry and Molecular Biology, Medical and Health Science Center, University of Debrecen, Debrecen, Hungary.  
E-mail: farkas@jaguar.unideb.hu

According to recent statistics, the number of fungal infections has increased steadily in the last decade. In 60–70% of the cases *Candida albicans* was found as the pathogenic agent. Because of the frequent side effects and the increasing resistance against traditional fungicides our goal was to find new potential therapeutic targets for novel antifungal treatments to control *C. albicans* infection. According to the literature and sequence databases,

protein phosphatase Z (PPZ) can be found only in the fungal kingdom and plays important roles in the cell wall synthesis, osmotic stability, and cell cycle regulation. That is why we analyzed the occurrence and structure of PPZ gene(s) in *C. albicans*. In Southern blots (using a *S. cerevisiae* PPZ1 DNA probe) we confirmed that *C. albicans* has a single gene coding for PPZ that was termed CaPPZ1. We amplified and cloned a 2700 bp genomic fragment containing the gene from a standard ATCC 10231 *C. albicans* strain and from clinical isolates by PCR. The sequence analysis of several independent clones revealed an unexpectedly high level of polymorphism within the non-coding and coding regions of the CaPPZ1 gene. We also isolated and cloned the gene from fungal colonies generated from single *C. albicans* cells. In the latter case we found two distinct sequences corresponding to the two alleles of the diploid organism. We suggest that the obligatory vegetative life cycle of *C. albicans* resulted in two divergent alleles within a single cell during evolution. In addition, a large number of individual mutations contributed to the genetic variability of this pathogenic organism. For the characterization of the gene product we isolated the cDNA of CaPPZ1 by the combination of two methods. First we obtained several partial cDNAs from a pSCGAL10-SNC. *albicans* library by PCR. The longest PCR clone contains 1178 bp of the coding sequence. The missing 5'- and 3'-ends were amplified in RACE experiments. A 1010 bp portion of the 5'-end as well as a 933 bp fragment of the 3'-end was determined in this way. From the three overlapping polynucleotide segments the full cDNA sequence was reconstructed. Our results indicate that the CaPPZ1 gene has no introns in the coding region. The cDNA sequences also confirm the genetic polymorphism mentioned earlier. Based on the gene and mRNA sequences, we selected some highly conserved sequence regions and initiated the synthesis of chemically modified oligonucleotides in order to reduce the expression of CaPPZ1 in *C. albicans*.

**Acknowledgment:** This work was supported by the OMFB BIO00088/2002 grant.

### L3-017P

#### Molecular evolution of alpha-amylase inhibitor: screening for cotton boll weevil, *Anthonomus grandis*

M. F. Grossi-de-Sa<sup>1,2</sup>, E. L. Z. Figueira<sup>1</sup>, R. P. Del Sarto<sup>1,3</sup>, O. B. Oliveira-Neto<sup>1</sup>, G. R. Oliveira<sup>1,4</sup>, P. S. F. Brunetta<sup>1,2</sup>, E. Romano<sup>1</sup> and M. C. M. Silva<sup>1</sup>

<sup>1</sup>Molecular Interaction Plant-Pests, Biotechnology, EMBRAPA Recursos Genéticos e Biotecnologia, Brasília, DF Brazil,

<sup>2</sup>Universidade Católica de Brasília, Brasília, DF Brazil,

<sup>3</sup>Universidade de Brasília, Brasília, DF Brazil, <sup>4</sup>Universidade

Federal do Rio Grande do Sul, Porto Alegre, RS Brazil.

E-mail: fatimasa@cenargen.embrapa.br

The cotton boll weevil *Anthonomus grandis* is an important insect-pest, causing severe damage in cotton floral fruits and buds. Its control is difficult due its endophytic larvae development, occurring into the cotton floral structure. As well as other insect-pests, the cotton boll weevil secretes a high level of  $\alpha$ -amylases and proteinases in the midgut lumen, which are required for digestion of carbohydrates and proteins. Thus, the  $\alpha$ -amylase inhibitors (AIs) represent a powerful tool to be applied on the control of insect pests. This research focused on the phage-display screening of  $\alpha$ -amylase inhibitors by using a  $10^6$  recombinant  $\alpha$ -amylase inhibitors library produced by *in vitro* recombination of  $\alpha A11$  and  $\alpha A12$  (from *Phaseolus vulgaris*) homologous genes. The search for high-affinity ligands among phage-displayed random  $\alpha$ -amylases inhibitor mutants yielded enrichment after four cycles, and the activities of the mutant inhibitors selected were determined. While the wild type inhibitors used to create the shuffled library did not

inhibit the assayed  $\alpha$ -amylases, 95% of the screened recombinant proteins evaluated showed variable inhibitory activities, from 3 to 100 AIU, to cotton boll weevil  $\alpha$ -amylases. A large number of mutations in the amino acid sequences were found for the different mutant inhibitors screened, which were evaluated for the amylases specificities. Our data indicate that the new screened AIs present potential for use on the cotton boll weevil control and documents that DNA shuffling and phage-display combined techniques are important strategies allowing both, to generate large number of mutants *in vitro* and to achieve new molecules with improved activities towards insect-pests.

**Acknowledgments:** This work was supported by EMBRAPA, CAPES, CNPq, Facual, Fialgo.

### L3-018P

#### Computer modeling of protein adsorption to a biomaterials surface: integrated software for fabrication of nanobiotechnology systems

A. H. Goldstein, A. N. Cormack and R. J. Lewis

Biomaterials Engineering, Alfred University, Alfred, NY, USA.

E-mail: fgoldste@alfred.edu

Development of modeling systems with atomic-scale resolution is universally recognized as essential to optimized fabrication of nanoengineered biomaterials. Modeling of biomolecular-materials composites offers both the greatest challenge and the greatest range of potential applications. The ability to simulate is essential the ability to fabricate. Implanted biomedical devices require that biomaterials come into direct contact with the body or body fluids, while optimization of biotechnology tools such as DNA/protein microarrays and microfluidics also require a mechanistic understanding of how biological macromolecules interact with materials surfaces. Interfacial phenomena, especially protein adsorption, control essential biomaterials properties such as biocompatibility/bioreactivity, and will control the efficiency of bioengineered nano/micro devices. Currently, no unified biomaterials simulation software exists to serve the needs of the community. We have integrated widely available, biopolymer and materials modeling programs and have adapted this software to a multi-component system in which a hydrated protein encounters a materials surface in solution. This work lays the foundation for standardized, biomaterials modeling software that will allow researchers to compare predictions of adsorption and experimental observations across a wide range of laboratories and biomaterials systems (*J Phys Chem B* 2004; **108**: 20408–20418). We will report on further validation of our software, using data on differential protein adsorption to the surface of a nanoporous silica powder. This material binds a thermostable DNA polymerase (AmpliTaq<sup>®</sup>), but does not bind bovine serum albumin (BSA), even at 10,000-fold molar excess of BSA:AmpliTaq<sup>®</sup>. Performance of the simulation vs. *in vitro* data will be reported.

### L3-019P

#### Biochemical properties of chitosanase A from *Streptomyces coelicolor* A3(2), the first member of GH46 family secreted by the TAT pathway

M. G. Ghinet<sup>1</sup>, H. Li<sup>2</sup>, P. É. Jacques<sup>1</sup>, M. È. Lacombe-Harvey<sup>1</sup>, R. Morosoli<sup>2</sup> and R. Brzezinski<sup>1</sup>

<sup>1</sup>Department of biology, University of Sherbrooke, Sherbrooke, Québec, Canada, <sup>2</sup>INRS-Institut Armand-Frappier, University of Québec, Laval, Québec, Canada.

E-mail: mariana.ghinet@USherbrooke.ca

Chitosan (a deacetylated form of chitin) as well as the products derived from its hydrolysis received much attention because of

many potential applications in biomedical, agricultural and environmental technologies. Enzymatic hydrolysis represents an adequate way to control the molecular weight of chitosan derivatives. To date, the chitosanase from *Streptomyces* sp. N174 have been used for the large scale hydrolysis of chitosan. It is necessary to continue to study the chitosanases, to make the industrial process economically accessible. The genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2) revealed a whole array of genes/proteins dedicated to the metabolism of chitin and its derivatives. Among these, SCO0677 (*csnA*) encodes a chitosanase belonging to the family 46 of glycoside hydrolases. *S. coelicolor* A3(2) did not produce detectable amounts of chitosanase when grown on chitosan and this chitosanase could be studied only after its expression as a recombinant protein. Studies on the secretion mode of this protein revealed that CsnA is secreted more efficiently through the TAT pathway than the SEC pathway. For expression, the mature part-encoding segment of CsnA was cloned in a derivative of pIAF907C vector, in which the SEC-dependent signal peptide sequence of cellulase A was replaced by the TAT-dependent signal peptide sequence of SCO0624. The resulting plasmid was introduced into *Streptomyces lividans* 10–164 by protoplast transformation. Here we present the biochemical and biophysical properties of this protein. These studies have shown that CsnA chitosanase is distinct from other well studied members of family 46 of glycoside hydrolases (such as the chitosanase from *Streptomyces* sp. N174).

### L3-020P

#### Metalloproteins screening in orange juice using a multidimensional metallomic approach based on 2D-PAGE, ICP-Q-MS and mass spectrometry (Q-TOF-MS)

J. L. Gómez-Ariza, M. J. Villegas-Portero and V. Bernal-Daza

Laboratory of Environ Anal and Bioanalysis, Department of Chemistry, Faculty of Experimental Science, University of Huelva, Huelva, Spain. E-mail: ariza@uhu.es

Orange juice is perhaps the most usual fruit juice consumed over the world elaborated with sweet oranges (*Citrus sinensis*). Quality and authenticity of this food is usually checked on the profile analysis of specific compounds, e.g., of sugars, amino acids, organic acids, carotenoids, flavor components, limonoids, flavonoids and phenols. The increasing information available about chemical composition of orange juice has allowed more refined methods of adulteration. Therefore, more powerful analytical methodologies are necessary for unequivocal characterization of the orange juice quality. The protein content of oranges provides very specific information about the type of fruit and the changes that it can suffer: diseases, maturity, origin, and others. The traditional methods reported in literature for protein presence evaluation in complex matrices are based on second dimension-polyacrylamide gel electrophoresis (2D-PAGE), followed by spot scissions and their tryptic digestion. The peptide mixture obtained is usually evaluated by mass spectrometry (MS) to achieve the protein identification. Besides, the use of ICP-MS can be used to detect the presence of metallo-proteins. In the present study, 2D-PAGE followed by spots scissions and ICP-MS metal analysis after acid digestion (HClO<sub>4</sub>:HNO<sub>3</sub>:HCl) was used for fast metalloproteins identification. Latterly, a nLC-ESI-Q-TOC-MS system was used for the characterization of metalloproteins in this metal-tagged spots after tryptic digestion. This metallomic approach [1, 2] has been applied to protein appraisal in fresh hand-squeezed orange juice.

#### References

- Gómez-Ariza JL, García-Barrera T, Lorenzo F, Bernal V, Villegas MJ, Oliveira V. *Analytica Chimica Acta* 2004; **524**: 15.
- Szpunar J. *Anal Bioanal Chem* 2004; **378**: 54–56.



**L3-021P****Substitution of Ala-183 to Thr in 5-enolpyruvylshikimate 3-phosphate synthase of *E. coli* (k12) and transformation of this construct to rapeseed (*Brassica napus*) for reducing of Glyphosate affinity**D. Kahrizi<sup>1</sup>, A. H. Salmanian<sup>2</sup>, A. Afshari<sup>2</sup>, A. Mousavi<sup>2</sup>, A. Moieni<sup>1</sup> and G. Karimzadeh<sup>1</sup><sup>1</sup>Laboratory of Molecular Genetics, Department of Plant Breeding, Tarbiat Modarres University, Tehran, Iran, <sup>2</sup>Laboratory of Molecular Genetics, Department of Plant Molecular Genetics, National Institute for Genetic Engineering and Biotechnology, Tehran, Iran. E-mail: dkahrizi@yahoo.com

Glyphosate (Roundup) is a non-selective broad-spectrum herbicide that inhibits 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS), a key enzyme in the aromatic amino acid biosynthesis in microorganisms and plants. The manipulation of bacterial EPSPS gene in order to reduce its affinity to glyphosate and transformation of it to plants is one of the most effective methods for production of glyphosate tolerant plants. In this research, we study on alanine183 of *E. coli* (k12) EPSPS enzyme. This amino acid is an important residue for EPSPS-Glyphosate configuration. We used site directed mutagenesis (SDM) method to inducing a point mutation in *E. coli* EPSPS gene to convert alanine183 to threonine (Ala-183 to Thr). The manipulated EPSPS gene was cloned in pUC18 as a universal cloning vector and pBI121 as a plant expression vector. The results of molecular analysis and sequencing showed that the manipulated genes has been correctly changed and cloned in correct orientation in both plasmids. Recombinant pBI121 containing altered EPSPS gene was transferred to rapeseed (*B. napus*) via *Agrobacterium tumefaciens* mediated transformation method. The positive plants were screened in 25 mg/l kanamycin containing media. Presence and copy numbers of construct in plants were confirmed with PCR and southern blotting techniques respectively. Transgenic plants were assayed for glyphosate resistance by spraying them with Roundup at a commercial recommended dose equivalent to 1.8 g/l. One week after the first application, no damage appeared in any transgenic plants. After spraying a second time, all transgenic plants survived and produced new leaves. Non-transgenic control plants were completely killed 5 days after the first spraying. Then Ala-183 to Thr Substitution influenced on EPSPS-Glyphosate interaction.

**L3-022P****SPINE in York**

V. M. Levnikov, E. V. Blagova, J. A. Brannigan, M. J. Fogg, O. V. Moroz, M. Boyle, R. Grenha, A. Muller, J. Wade, A. J. Wilkinson and K. S. Wilson

York Structural Biology Laboratory, Department of Chemistry, University of York, York, UK. E-mail: levnikov@ysbl.york.ac.uk

The EC project Structural Proteomics In Europe (SPINE) targets proteins of bio-medical importance. The results of High Throughput technologies (predominantly for "Wet Lab" applications) will be presented. We have solved 17 protein structures (by X-ray crystallography and NMR) from three human pathogenic organisms, *Bacillus anthracis*, the causative agent of anthrax, *Campylobacter jejuni*, a food borne pathogen and *Mycobacterium tuberculosis*, the cause of tuberculosis. Additionally we have solved structures from *Bacillus subtilis*, a non-pathogenic relative of *B. anthracis*. Target protein identification utilized automated bioinformatic tools as well as functional criteria. Gene cloning was carried out using pET-YSBLIC vector [developed in York using the pET-28a cloning vector, modified to allow Ligation Independent Cloning (LIC)] with protein over-expression in BL21 strains. Protein

purification was accomplished with an ÄKTA 3D protein purification system, using Hi-Trap Ni chelating columns and gel filtration. Initial crystallization conditions were screened with 96 well (nanolitre) crystallization screens, set up using TTP Labtech Mosquito robot (York). Optimization of crystal growth conditions was accomplished by the Mosquito robot or by hand. The proteins were crystallized with non-cleavable 6xHis Tags and NMR data were collected (Utrecht) with similar constructs. Crystals used for diffraction data were obtained directly from nanolitre scale 96 well plates or manual microlitre scale drops. Crystals were tested for diffraction in-house; most diffraction data were obtained using synchrotron radiation sources at ESRF Grenoble or at CCLRC Daresbury Laboratory and the NMR data at Utrecht.

**Acknowledgments:** This project is funded by the EC contract, SPINE, No. QLG2-CT-2002-00988.

**L3-023P****Preliminary characterization of a virulent staphylococcal bacteriophage of broad host specificity**M. Lewandowska<sup>1,2</sup>, B. Weber-Dąbrowska<sup>2</sup>, E. Sadowy<sup>3</sup>, J. Krzysztoń-Rusjan<sup>3</sup>, A. Górski<sup>2</sup> and M. Lobočka<sup>1</sup><sup>1</sup>Department of Microbial Biochemistry, Institute of Biochemistry and Biophysics, PAS, Warsaw, Poland, <sup>2</sup>Institute of Immunology and Experimental Therapy, PAS, Wrocław, Poland, <sup>3</sup>National Institute of Public Health, Warsaw, Poland. E-mail: Lmi@ibb.waw.pl

An increasing number of staphylococcal infections by antibiotic resistant strains creates a need to look for new anti-staphylococcal agents. Naturally occurring, virulent bacteriophages that infect and kill a wide range of staphylococcal strains encode numerous proteins lethal for Staphylococci. A5 is a member of a family of polyvalent staphylococcal phages that infect several isolates of *S. aureus* and *S. carnosus*. We characterized one of its derivatives A5W, which appeared to be especially effective in killing methicillin resistant *S. aureus* strains. A5W virions consist of an icosahedral head of about 100 nm in diameter and a 250 nm long, contractile tail, which ends with a large base plate. The major virion components are 50, 65, and 70 kD proteins that apparently represent capsid, tail tube and tail sheath subunits. Virions contain linear double-stranded DNA molecules that are about 145 kD long. They can be digested by the majority of restriction enzymes, but some that recognize GC-rich sequences or the GATC motif, which is the substrate for the *S. aureus* restriction enzyme Sau3AI. The genomic sequence of A5W appears to be highly homologous to that of the staphylococcal bacteriophage K. However, significant differences between both phages reflect their different evolutionary history. Analysis of selected genome fragments of A5W and their comparison with fragments of K genome reveals genes whose products may serve as prototypes for design of new bacteriotoxic agents directed specifically against Staphylococci, or new biotechnological tools.

**L3-024P*****Yarrowia lipolytica*, a yeast expression system adapted to the genetic engineering of complex proteins: directed mutagenesis of a fungal laccase**C. Madzak<sup>1</sup>, C. Jolival<sup>2</sup>, M.-C. Mimmi<sup>3</sup>, A. Brault<sup>3</sup>, E. Caminade<sup>3</sup>, C. Mougin<sup>3</sup> and J.-M. Beckerich<sup>1</sup><sup>1</sup>UMR1238 Microbiologie et Génétique Moléculaire, INRA/CNRS/INA-PG, Thiverval-Grignon, France, <sup>2</sup>UMR7573 Synthèse Sélective Organique et Produits Naturels, CNRS/ENSCP, Paris, France, <sup>3</sup>UR258 Phytopharmacie et Médiateurs Chimiques, INRA, Versailles, France. E-mail: madzak@grignon.inra.fr

Numerous heterologous proteins have been successfully produced in *Y. lipolytica*, a dimorphic yeast with high secreting capacities

(Madzak J. *Biotechnol* 2004; **109**: 63), including complex metallo-proteins such as laccases (Jolivald *AMB* 2005; **66**: 450; Madzak *FEMS* 2005; in press). Laccases are multicopper oxidases used in industrial oxidative processes, with potential applications in depollution (Mougin. *Environ Chem Lett* 2003; **1**: 145). The design of recombinant laccases fully adapted to industrial applications will be possible using genetic engineering. *Y. lipolytica* expression system enables high transformation efficiency, as well as control of both copy number and integration locus of transformants. The successful production of active *Trametes versicolor* laccase (Jolivald, 2005) has been a preliminary step towards engineering this enzyme for environmental applications. Crystal structure of *T. versicolor* laccase (Bertrand. *Biochem* 2002; **41**: 7325) enlightened the interaction of amino acid 206 (Aspartate) with the substrate. This Aspartate is conserved among laccases from basidiomycetes. We tested the effects of its replacement by Glutamate (conserved among ascomycetes), Asparagine (conserved among plants), or Alanine. Mutated recombinant laccases were expressed in *Y. lipolytica*, using an expression/secretion vector which allows the precise targeting of monocopy integration events at a docking platform into the recipient strain genome (Madzak *JMMB* 2000; **2**: 207). This system reproducibly provides transformants carrying a unique expression cassette, integrated at a precisely known site. We were thus able to analyze the consequences of each mutation on laccase activity on various substrates. This work exemplifies the potential of *Y. lipolytica* expression system for the genetic engineering of complex proteins.

### L3-025P

#### Biological activity of recombinant 2A protease from CVB3

N. Maghsoudi<sup>1</sup>, M. Zeinoddini<sup>2</sup>, M. A. Nasiri<sup>3</sup> and A. A. Deldar<sup>2</sup>  
<sup>1</sup>Molecular Biology, Neuroscience, Shaheed Beheshti, Tehran, Iran,  
<sup>2</sup>Genetic Engineering, Mau, Tehran, Iran, <sup>3</sup>Biochemistry, Mau, Tehran, Iran, E-mail: maghsoudi@yahoo.com

2A protease is a critical enzyme for coxsackievirus B3 (CVB3) reproduction. Cleavage of P220 (the eukaryotic translation initiation factor 4G-eIF4G) in CVB3 infected cells occurs by 2Apro activity which leads to host cell translational system shut-off. Biological activity of recombinant 2Apro that was previously produced and purified, was investigated by developing a specific method. For this, polyclonal antibodies against antigenic sites of P220 was prepared by synthesizing small peptides, corresponding to antigenic site of P220 and coupled to KLH which was injected subcutaneously to rabbit. Then, the enzyme and its substrate (HeLa cells lysate that contain P220) incubated together for different times intervals. P220 cleavage by 2Apro was assessed by SDS-PAGE and western blot analysis. Cleavage of P220 by r2Apro was prominent after 24 h. This system can be used as a standard model for assaying biological activity of r2Apro and also detecting presence of CVB3 in live samples. Key words: 2A protease, p220, antibody, activity.

### L3-026P

#### Prediction of protein retention time in hydrophobic interaction chromatography using automated docking

A. Mahn<sup>1</sup>, G. Navarro<sup>1</sup> and G. Zapata<sup>2</sup>

<sup>1</sup>Chemical Engineering, University of Chile, Santiago, Santiago Chile, <sup>2</sup>Millennium Institute for Advanced Studies in Cell Biology and Biotechnology, University of Chile, Santiago, Santiago Chile. E-mail: amahn\_2000@yahoo.es

Hydrophobic Interaction Chromatography (HIC) is a powerful technique for protein purification. The main protein property

determining retention in HIC is hydrophobicity, which can be estimated as 'surface hydrophobicity' starting from the protein 3D-structure data. This concept has been used to predict protein retention time in HIC, with a reasonable success. However, this methodology was not able to explain the chromatographic behavior of several proteins, because it does not consider the effect of surface hydrophobicity distribution. In previous work, we demonstrated that this property has a very important effect on protein retention in HIC. Based on a classical thermodynamic model, the contact area between a hydrophobic matrix and the protein when adsorbed was determined. This parameter correlated extremely well with protein retention time, however many experiments are necessary to determine it. In order to overcome this difficulty, we have used Molecular Docking to identify the zone on a protein's surface that most probably interacts with hydrophobic resins used in HIC. In this work, we performed molecular docking simulations to investigate the interaction of different proteins and the hydrophobic moiety used in Phenyl Sepharose<sup>TM</sup>, using the program AUTODOCK 3.0. The interacting amino acids in each protein were identified. A conformation selection methodology was proposed, based on quantitative and qualitative considerations. Using this novel methodology, the surface hydrophobicity of the interfacial zone (Local Hydrophobicity, LH) was determined. The LH showed a high correlation level with protein retention time. It was concluded that it is possible to identify the zones on the protein surface involved in protein retention in HIC, using molecular docking simulations. Given the good correlation found between LH and protein retention, this constitutes a new approach to predict protein behavior in HIC, avoiding experimental work.

### L3-027P

#### Mycobacterial haloalkane dehalogenases

M. Monincová<sup>1</sup>, A. Jesenská<sup>1</sup>, M. Pavlová<sup>1</sup>, M. Strouhal<sup>1</sup>, I. Tišínská<sup>1</sup>, R. Chaloupková<sup>1</sup>, Z. Prokop<sup>1</sup>, M. Bartoš<sup>2</sup>, I. Pavlík<sup>2</sup>, I. Rychlík<sup>2</sup>, P. Möbius<sup>3</sup>, Y. Nagata<sup>4</sup> and J. Damborský<sup>1</sup>

<sup>1</sup>National Centre for Biomolecular Research, Faculty of Science, Masaryk University, Brno, Czech Republic, <sup>2</sup>Veterinary Research Institute, Brno, Czech Republic, <sup>3</sup>Federal Research Institute of Animal Health, Jena, Germany, <sup>4</sup>Department of Environmental Life Sciences, Graduate School of Life Sciences, Tohoku University, Sendai, Japan. E-mail: martam@chemi.muni.cz

Haloalkane dehalogenases are enzymes capable to convert halogenated compounds to corresponding alcohols and have primarily been isolated from bacterial strains living in soil contaminated with halogenated aliphatic compounds. The genes coding for putative haloalkane dehalogenases were searched in genomes of *Mycobacterium* spp. including saprophytic, animal and human tissue-colonizing strains. Open reading frames aal17946, map0345c and map2057 from *Mycobacterium smegmatis* MC2 155 and *Mycobacterium avium* subsp. *paratuberculosis* K10, as well as rv2579 and rv2296 from pathogenic *Mycobacterium tuberculosis* H37Rv, can potentially code for haloalkane dehalogenases genes. The study was mainly focused on rv2579 (later designed dmbA) because of its 68% sequence identity with linB. An active site and entrance tunnel of Rv2579 (DmbA) was re-constructed in LinB using site-directed mutagenesis. Final six-fold mutant was presumed to have an active site and an entrance tunnel of DmbA and exhibited dehalogenase activity with the haloalkanes tested, confirming that DmbA is a member of the haloalkane dehalogenase family [1]. Consequently the dmbA gene was cloned from *Mycobacterium bovis* 5032/66 into *Escherichia coli*. DmbA was overproduced and purified to homogeneity. Recombinant DmbA shows hydrolytic dehalogenating activity, further confirming the conclusions based on the site-directed mutagenesis study. Temperature optimum of DmbA is at 45°C and the double pH optimum at pH

5.5 and pH 9.0. DmbA is capable to catalyse relatively broad spectrum of halogenated substrates, degrading 23 out of 30 tested halogenated compounds with various physico-chemical properties. Mycobacterial haloalkane dehalogenases are interesting proteins due to the fact that their function especially tissue-colonizing mycobacteria is not clear. It is speculated about their role in immune reactions, but experimental evidences are lacking. Further studies will be aimed to clarify origin, evolution and function of mycobacterial dehalogenases.

#### Reference

1. Nagata Y, Prokop Z, Marvanova S, Sykorova J, Monincova M, Tsuda M, Damborsky J. (2003) *Applied and Environmental Microbiology* 2003; **69**: 2349–2355.

### L3-028P

#### Efficient import of a recombinant form of mammalian cytochrome P450<sub>scc</sub> into yeast mitochondria

A. N. Minenko, I. E. Kovaleva and V. N. Lusikov  
Laboratory of Protein Topogenesis, A.N.Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow, Russian Federation. E-mail: minenkoa@mail.ru

Cytochrome P450<sub>scc</sub> (CYP11A1) is a key enzyme of mammalian steroidogenesis system that transforms cholesterol into pregnenolone. This protein is located to the inner membrane of adrenocortical mitochondria. Its arrangement in the membrane as well as the mechanism of insertion into the membrane are still debatable. In particular, the import of CYP11A1 into mitochondria was found to be tissue-specific. However, it has earlier been shown that CYP11A1 with its own targeting sequence can be imported into yeast mitochondria, although the import is ineffective in the sense that the protein failed to be inserted into the inner mitochondrial membrane. The imported protein mostly accumulated in the matrix where it either aggregated or suffered degradation. A recombinant version of CYP11A1 with a targeting signal ensured its insertion into the membrane from the matrix through the "re-export" mechanism was membrane-bound but enzymatically inactive. In the present work we have constructed a fusion protein where CYP11A1 sequence was preceded by the full-size sequence of the ATP/ADP carrier. The AAC-mCYP11A1 version expressed in the *S. cerevisiae* yeast was shown to be inserted into the inner mitochondrial membrane from the intermembrane space. The recombinant protein proved to be catalytically active despite the presence of the bulky AAC domain at the N-terminal end of the CYP11A1 polypeptide chain. By comparing the activities of mitoplasts and inverted submitochondrial particles we have shown that there are two pools of AAC-mCYP11A1 molecules with different orientation of mCYP11A1 active sites relative to the inner membrane sides, with one of them being exposed to the mitochondrial matrix similarly with the situation in adrenocortical mitochondria.

### L3-029P

#### Isolation and cloning of gene and promoter of KCS enzyme for production of erucic acid in rapeseed and sense construct design

A. R. Zebajadi<sup>1</sup>, M. J. Javaran<sup>1</sup>, A. H. Salmanian<sup>2</sup>, G. Karimzadeh<sup>1</sup> and A. Mousavi<sup>2</sup>

<sup>1</sup>Molecular Biology and Genetic Engineering, Department of Plant Breeding, Faculty of Agriculture Tarbiat Modarres University, Tehran, Iran, <sup>2</sup>National Research Center for Genetic Engineering and Biotechnology, Tehran, Iran. E-mail: m\_jalali@modares.ac.ir

The saturated and unsaturated fatty acids biosynthesis pathway has many different stages which are controlled by various

enzymes. One of the methods for improve of oil quality is change of fatty acids profile.  $\beta$ -ketoacyl-CoA synthase (KCS) is the key enzyme in erucic acid biosynthesis. This enzyme is involved in producing of eicosanoic (C20:1) and erucic acids (C22:1) from C18 fatty acids. Classic and new methods use for change in erucic acid content. In this study, the gene isolation of this enzyme using specific promoter was the first step for changing of erucic acid content in rapeseed. Specific primers were used to amplify the gene and its promoter from genomic DNA by PCR technique. Digestion by restriction enzymes was used for insuring of correction of these fragments. The putative gene and promoter from *Brassica napus* were sequenced for further analysis. Then gene and its promoter was cloned into pSK vector and supply sense construct. This construct was cloned into expression vector, pBI121, and molecular analysis is done. Recombinant vector transferred to rapeseed plant via *Agrobacterium*. This construct can be used to engineer plants for modified oil composition.

### L3-030P

#### Random oligonucleotide mutagenesis of *Escherichia coli* argA gene and selection of high active N-acetylglutamate synthetase mutants

L. R. Ptitsyn, S. V. Smirnov, V. A. Kotlyarova and I. B. Altman  
Ajinomoto-Genetika Research Institute, Moscow, Russian Federation. E-mail: leonid\_ptitsyn@agri.ru

The biosynthesis of arginine in *Escherichia coli* is initiated by the acetylation of glutamate by N-acetylglutamate synthetase (NAGS) encoded by argA. This enzymatic step is regulated by feedback inhibition of NAGS by arginine. Some feedback-resistant (fbr) NAGSes were obtained by chemical mutagenesis (point mutations H15Y, Y19C, S54N, R58H, G287S, and Q432R). The random oligonucleotide mutagenesis is the method to incorporate random mutations, encoded on a synthetic oligonucleotide, into a specific region of a gene. This allows targeting of mutagenesis into an area likely to have an effect on desired properties and explores a large amount of sequence space within the given region. We constructed the combinatorial library of mutant argA genes by using the full randomization of nucleotide sequence at as few as five positions of protein sequence (from 15th to 19th amino acid residues). The selection of *E. coli* TG1 cells carrying plasmids pKK-argA-random was carried out on LB agar plates. The delay of cell growth of some mutant clones was observed and this effect was supposed to correlate with production of the active fbr NAGS mutants. It was found that mutant NAGSes possessed different activity levels. For few of them it was higher than that of derepressed wild-type (wt) NAGS. The wt NAGS, mutant NAGS carrying the single substitution Y19C (ArgA-4) and two mutant NAGSes with numerous substitutions in mutated region (ArgA-11 with sequence -Val15Val16Trp17Arg18Ala19-, and ArgA-13 with sequence -Ser15Ala16Ala17Ser18-Arg19-) were purified and some kinetic parameters of these enzymes were studied. The enzyme activity of wt NAGS and ArgA-4 was reduced to about 10% and 50% of initial in presence of arginine (20 mM), while was not changed for ArgA-11 and was increased twofold for ArgA-13. These data confirmed that the random oligonucleotide mutagenesis allows to construct mutant proteins with restored near to natural (or even increased) levels of their enzymatic activity.

**L3-031P****The comparison of folding of the recombinant rotavirus VP2 expressed in *E. coli* and eukaryotic cells by electron microscopy**F. Pourasgari<sup>1</sup>, S. Ahmadian<sup>1</sup> and A. H. Salmanian<sup>2</sup><sup>1</sup>Institute of Biochemistry and Biophysics, Tehran University, Tehran, Iran, <sup>2</sup>National Institute of Genetic Engineering and Biotechnology, Tehran, Iran. E-mail: pourasgari@ibb.ut.ac.ir

Rotavirus is a member of the Reoviridae family and is the leading cause of severe diarrhea in new-borns worldwide. These viruses are triple-layered viruses surrounding a genome with all segments of double-stranded monocistronic RNA. Inner layer is composed of three viral proteins (VP): VP1, VP2 and VP3. The rotavirus core is composed of VP2 which is the most abundant protein of the central of the RNA polymerase complex. Endogenous transcription is a common and key molecular process that initiates gene expression in segmented double-stranded RNAs viruses. In this study VP2 cDNA was cloned in an eukaryotic expression vector and in an prokaryotic expression vector, separately. Then, expression and folding of VP2 was detected by transmission electron microscopy. The expression of VP2 in eukaryotic cells results in the production of particles, called pseudocores that have the same geometry as viral cores but in *E. coli* it was different, although some tendencies to production of pseudocores are seen in *E. coli*.

**L3-032P****Expression and activity identification of the melittin gene of *Polistes hebraeus* in insect expression system**W.-J. Shi<sup>1</sup>, C.-X. Zhang<sup>2</sup> and J.-A. Cheng<sup>3</sup><sup>1</sup>Environmental Biotechnology, Resources & Environmental Science, Shanghai Jiaotong, Shanghai, PR China, <sup>2</sup>Insect Molecular biology, Institute of Applied Entomology, Zhejiang, Hangzhou, PR China, <sup>3</sup>Insect Ecology, Institute of Applied Entomology, Zhejiang, Hangzhou, PR China. E-mail: shiwanjun@yahoo.com

Melittin, a peptide constituting about 50% of its dry venom, is the main toxin of *Apis mellifera* venom. It has important biological activities. It causes the lysis of erythrocytes and the release of marker ions from liposomes and of histamine from mast cells. Its amphiphilic structure-a large apolar portion and a very basic polar end-is ideal for promoting interactions of melittin with lipid membranes. In this study the restriction fragment of GSTPhM was obtained by PCR from the recombinant plasmid pGEX-PhM and inserted into the multiple cloning site of the pBacFastHTb to construct recombinant donor plasmid, pBacHT-GSTPhM, which was used to transposed to the target Bacmid in *E. coli* (DH10) by Tn7 transposition function. Then the recombinant Bacmids were transfected into Tn-5B1-4 cells of the cabbage looper, *Trichoplusia ni*, mediated by Lipofectin. The expressed protein of about 34 kDa was detected by western blot and triple antibody sandwich ELISA, indicating that the recombinant protein is the fusion protein of GST-PhM. The cytopathic effect of Tn cells after infection of Bacmid-GSTPhM at different times was similar to that of Tn cells infection with Bacmid. Purified and recovered recombinant melittin of *Polistes hebraeus* showed bioactivity in activating rabbit platelets to aggregate.

**L3-033P****Chemically engineered intramolecular FRET mutants of GFP for visualization of versatile cellular events**M. Suzuki<sup>1</sup>, Y. Ito<sup>2</sup>, Y. Oki<sup>1</sup>, Y. Husimi<sup>1</sup> and K. T. Douglas<sup>3</sup><sup>1</sup>Department of Functional Materials Science, Saitama University, Saitama, Japan, <sup>2</sup>Department of Biotechnology, Osaka, University, Osaka, Japan, <sup>3</sup>School of Pharmacy and Pharmaceutical Sciences, University of Manchester, Manchester, UK. E-mail: miho@fms.saitama-u.ac.jp

A sequence of cellular events consists of many biological reactions in various types. For example, on apoptotic procedures we observe some proteolysis, kinations and DNA digestions etc. We have developed molecules to sense protease and DNase activities for imaging of their dynamics *in vivo* or *in vitro* as bio-probes highly applicable for detection of multi-types of cellular processes but simply designed. We have prepared novel GFP constructs by replacing cysteines by non-thiolic amino-acids, and introducing new cysteine at specific, surface positions designed to be suitable for site-specific chemical modification by organic compounds based fluorescence labels. These dye moieties gave excellent chemical FRET in partnership with native GFP fluorophore. These GFP mutants were further engineered with trypsin, caspase-3 sensitive sequences inserted close to the dyes-bearing cysteine. On proteolysis the FRET was destroyed, as the partners became located on separate molecules. We have highly detected caspase-3 activity in apoptosis induced cell lysate and visualized it during cell death by introduction of probes into cells with a protein delivery system. We expanded the protease sensitive molecules into nuclease responsive ones by conjugation with dye labeled DNA instead of thiol reactive dye. Thus, a simple molecular design process provides efficient intramolecular FRET systems, easily arranged to control their emission properties through enzyme-sensitive sequences or attached DNA. This intramolecular FRET system would be extended to other color variants of GFP chemically derivatized with fluorescent reagent or fluorescent labeled other biomaterials and then provide sensing molecules for various types of enzymatic reactions such as protein phosphorylation/dephosphorylation. We could simultaneously monitor a series of cellular processes using these versatile probes with different fluorescent properties in cells and *in vitro* with protein chip.

**L3-034P****Cloning and expression of K<sup>+</sup> channels blocker scorpion toxins**E. G. Stehling<sup>1</sup>, N. I. Zanchin<sup>1</sup> and A. Spisni<sup>1,2</sup><sup>1</sup>NMR Laboratory, Center of Molecular Structural Biology, Brazilian Synchrotron Light Laboratory, Campinas, São Paulo Brazil, <sup>2</sup>Section of Chemistry and Structural Biochemistry, Department of Experimental Medicine, University of Parma, Parma, Italy. E-mail: eliana@lnls.br

Scorpion venoms contain toxic peptides that recognize K<sup>+</sup> channels of excitable and non-excitable cells. These toxins comprise three structurally distinct groups, designed as alpha-KTx, beta-KTx and gamma-KTx. The alpha-KTx group include peptides 28–40 residues long, with three or four disulfide bridges. In this work, five alpha-KTx toxins (OsK2, Tc32, PbTx1, BmKK3 and TdK1) were cloned into the pBUF5 vector (pET21d plasmid modified) in fusion with the D protein (major capsid of phage lambda) and transformed into *Escherichia coli* BL21 (DE3) cells. The fusion protein contains a hexa-histidine tag to facilitate

purification by metal-chelating chromatography. Out of these five toxins, only TdK1 turned out to be expressed in an insoluble form. OsK2 and Tc32 that were expressed in a soluble form, were purified by affinity chromatography using a Ni-NTA super-flow column and, subsequently, an ion exchange chromatography method utilizing the HiTrap-Q column. The fractions containing the purified fusion proteins were treated with the TEV protease to remove it and the resulting toxins analyzed by MALDI TOF Mass Spectrometry. The spectra show a mass of 3041.0 Da for OsK2 and 3666.4 Da for Tc32 in agreement with the value expected for the presence of two disulfide bridges in OsK2 and three disulfide bridges in Tc32.

### L3-035P

#### ***In vivo* bioinsecticidal activity to *Ceratitis capitata* and *Callosobruchus maculatus* and *in vitro* bioinsecticidal activity towards different orders of insect pests of a trypsin inhibitor purified from Tamarind tree (*Tamarindus indica*) seeds**

C. L. Araújo<sup>1</sup>, J. M. S. L. L. Fook<sup>2</sup>, A. E. A. d. D. Barbosa<sup>1</sup>, F. M. Teixeira<sup>1</sup>, C. E. M. Gomes<sup>1</sup>, L. L. P. Macedo<sup>1</sup>, G. E. M. d. D. Moura<sup>1</sup>, R. d. M. Moura<sup>3</sup>, A. S. Oliveira<sup>1,4</sup>, A. F. S. Queiroz<sup>3</sup>, E. A. Santos<sup>1</sup>, O. L. Franco<sup>5</sup>, C. Bloch-Jr<sup>6</sup>, F. P. Macedo<sup>7</sup> and M. P. Sales<sup>1</sup>

<sup>1</sup>Laboratório de Química e Função de Proteínas Bioativas, Departamento de Bioquímica, Universidade Federal do Rio Grande do Norte, Natal, RN Brazil, <sup>2</sup>Departamento de Morfologia, Universidade Federal do Rio Grande do Norte, Natal, RN Brazil,

<sup>3</sup>Laboratório de Biofísica Molecular, Departamento de Biofísica, Universidade Federal do Rio Grande do Norte, Natal, RN Brazil,

<sup>4</sup>Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Ceará, Fortaleza, CE Brazil, <sup>5</sup>Universidade Católica de Brasília, Brasília, DF Brazil, <sup>6</sup>Centro Nacional de Recursos Genéticos e Biotecnologia/EMBRAPA, Brasília, DF Brazil, <sup>7</sup>Laboratório de Moscas das Frutas, Departamento de Biologia Celular e Genética, Universidade Federal do Rio Grande do Norte, Natal, RN Brazil. E-mail: msales@cb.ufrn.br/mpsales@uol.com.br

A proteinaceous inhibitor with high activity against trypsin-like serine proteinases was purified from seeds of tamarind tree (*Tamarindus indica*) by gel filtration on Sephacryl S-200 followed by a Reverse-Phase HPLC Vidac C18 TP. The Inhibitor named TTI (*Tamarindus Trypsin Inhibitor*) showed a Mr of 21.42 kDa by mass spectrometry analysis. TTI is a non-competitive inhibitor with a  $K_i$  value of  $1.7 \times 10^{-7}$  M. *In vitro* bioinsecticidal activity against digestive insect enzymes from different orders showed that TTI had remarkable activity against enzymes from Coleopteran, *Anthonomus grandis* (63.8%), *Rhyzopertha dominica* (76.6%) and *Callosobruchus maculatus* (92.2%), and to Lepidopteron, *Alabama argillacea* (54.2%) and *Spodoptera frugiperda* (97.1%). Also, digestive enzymes from Diptera, *Ceratitis capitata* (fruit fly) are inhibited (52.9%). *In vivo* bioinsecticidal assays towards *C. capitata* and *C. maculatus* larvae were developed. When TTI was added at a level of 4.0% in artificial seeds produced ca. 44.5% mortality to *C. maculatus* and affected the weight larvae in 50.0% at level of 3.2% (w/w). Furthermore, the weight *C. capitata* larvae was affected in 47.4% and larvae survival in 38.5% at level of 4.0% of TTI incorporated in artificial diets. This occurrence associated with high inhibitory *in vitro* activity of TTI was an indicative to use of TTI as potential insecticidal agent. The insect resistance to transgenic plant is happening and the pest control using proteinase inhibitors, in the future, can be assigned in association of different types of inhibitors to different target enzymes in a concerted manner.

### L3-036P

#### **Prokaryotic and eukaryotic *in vitro* translation systems as valuable tools for protein research**

W. Stiege, M. Gerrits, S. Kubick, H. Merk and J. Strey  
RiNA GmbH Netzwerk RNA-Technologien, Berlin, Germany.  
E-mail: stiege@rna-network.com

An essential demand of the post-genome era is the development of efficient tools for protein production and screening. To address this challenge we have designed highly productive cell-free translation systems of prokaryotic and eukaryotic origin. Cell-free translation is an attractive alternative to *in vivo* expression systems, especially because of the possibility to easily adapt the reaction conditions to the requirements of the target protein (e.g. by addition/coexpression of folding catalysts or adjustment of the redox milieu). Furthermore proteins can be labeled or modified by incorporation of unnatural amino acids in a random or even site-specific manner. With our prokaryotic system based on *E. coli* up to 700 µg/ml of soluble protein can be synthesized in <1 h. To speed up template generation the Expression PCR (E-PCR) was developed [1]. Starting from various genetic sources like cDNA or mRNA coding regions can be equipped with all necessary elements for efficient transcription, translation, stabilization and protein purification by application of gene specific and extension primers in a single tube PCR. These "ready to express" templates can be used directly for cell-free protein production without the need of time-consuming cloning procedures. Both methods, PCR-based template generation and cell-free transcription/translation, may be combined in robot-assisted high throughput systems, enabling isolation and expression of several hundred genes including subsequent protein purification in only 1–2 days. Furthermore we could demonstrate the efficient site-directed introduction of certain unnatural amino acids, e.g. spin- or biotin-labeled ones into target proteins by means of amber suppressor tRNAs, that have been chemically aminoacylated. Though the prokaryotic *in vitro* translation system is highly valuable in terms of productivity and velocity it is still limited to proteins lacking complex post-translational modifications such as glycosylation. For this reason we have recently developed a cell-free system from insect (Sf21) cells [2]. In this system glycosylation of various human proteins (e.g. orosomucoid, erythropoietin, L-selectin) could be demonstrated.

#### **References**

1. Merk H, Meschkat D, Stiege W. Expression-PCR: from gene pools to purified proteins within 1 day. In: Swartz JR (Ed.). *Cell-free Protein Expression*. Springer, Berlin, Heidelberg, New York, 2003; pp. 15–23.
2. Kubick S, Schacherl J, Fleischer-Notter H, Royall E, Roberts LO, Stiege W. *In vitro* translation in an insect-based cell-free system. In: Swartz JR (Ed.). *Cell-free Protein Expression*. Springer, Berlin, Heidelberg, New York, 2003; pp. 209–217.

### L3-037P

#### **Functionalizing human amylin (20–29) fibrils**

V. L. Sedman<sup>1</sup>, S. Allen<sup>1</sup>, W. C. Chan<sup>2</sup>, B. Kellam<sup>2</sup> and S. J. Tendler<sup>1</sup>

<sup>1</sup>Laboratory of Biophysics and Surface Analysis, School of Pharmacy, University of Nottingham, Nottingham, UK,

<sup>2</sup>Medicinal Chemistry and Structural Biology, School of Pharmacy, University of Nottingham, Nottingham, UK.

E-mail: paxvls2@nottingham.ac.uk

Amylin is a 37 amino acid polypeptide secreted by the pancreatic islets beta-cells [1]. In the human disease state type-II diabetes mellitus, amylin is found aggregated into fibrillar structures termed amyloid [2], which associate to form toxic plaques within the cells. A core region of human amylin corresponding to amino

acid residues 20–29 has been found to be intrinsically amyloidogenic in nature [3]. We have used the atomic force microscope to investigate the morphology [4] and growth of the fibrillar structures formed by the peptide fragment of human amylin (20–29). Using solid phase peptide synthesis we have introduced two functional moieties to the human amylin (20–29) sequence. We show that with these functional groups, amylin (20–29) is still capable of forming fibrillar structures. We present atomic force microscopy images demonstrating that these fibrillar structures are similar in morphology to fibrils formed by non-functionalized amylin (20–29) peptides. The ability to decorate such fibrils allows for additional chemistry modifications of these stable, rigid structures, and highlights their potential for use in the nanotechnology industry.

#### References

1. Cooper GJS, Willis AC, Clark A, Turner RC, Sim RB, Reid K.B.M (1987) *Proc Nat Acad Sci*. **84**:8628–8632.
2. Kahn SE, Andrikopoulos S, Verchere CB. *Diabetes* 1999; **48**(2): 640–645.
3. Westermark P, Engstrom U, Johnson KH, Westermark GT, Betsholtz C. *Proc Nat Acad Sci* 1990; **87**: 5036–5040.
4. Sedman VL, Allen S, Chan WC, Davies MC, Roberts CJ, Tendler SJB, Williams PM. *Protein Peptide Lett* 2005; **12**: 79–83.

#### L3-038P

##### Cloning and expression of novel somatotropin gene in bacterial (*E. coli*) and yeast (*Pichia pastoris*) expression systems

S. Sadaf<sup>1,2</sup>, L. M. Damasceno<sup>3</sup>, D. B. Wilson<sup>2</sup> and M. W. Akhtar<sup>4</sup>

<sup>1</sup>Institute of Biochemistry and Biotechnology, University of the Punjab, Lahore, Pakistan, <sup>2</sup>Department of Molecular Biology and Genetics, Cornell University, Ithaca, NY, USA, <sup>3</sup>Field of Microbiology, Cornell University, Ithaca, NY, USA, <sup>4</sup>School of Biological Sciences, University of the Punjab, Lahore, Pakistan.  
E-mail: sasadaf@hotmail.com

Somatotropin (ST), a protein of 191 amino acids with a molecular mass of 22 kDa, is synthesized and secreted by somatotrophs of anterior pituitary gland under hypothalamic control. The protein has been isolated and characterized from range of vertebrate species including bovine, ovine, caprine, equine, canine, porcine and human and sequence information are available at protein and/or nucleic acid level. Sequence of Pakistani Nili-Ravi buffalo (*Bubalus bubalis* – one of the best dairy breeds of water buffalo in the world) somatotropin gene, so far, has not been worked out. Present study is the first report about isolation of ST gene of indigenous bubaline breeds, its characterization by nucleotide sequencing and optimized expression in prokaryotic and eukaryotic systems. Total RNA was isolated from pituitary tissues of animal using QIAgen RNeasy kit and quantified by measuring absorbance at A260. Using specially designed gene specific primers, isolated RNA was RT-PCR amplified and cloned in pGEM T-easy vector according to standard molecular biology protocols. Protein–protein BLAST and nucleotide sequencing of the cloned gene revealed several silent and couple of interesting point mutations in the putative amino acid sequence, one of which was in highly conserved receptor binding region. Further, the gene was expressed in *E. coli* BL21 (DE3) under the control of T7-lac promoter to translocate its secretion in periplasmic space for efficient purification in subsequent steps. Two-step chromatographic purification scheme, however, yielded only 20 mg/l protein from clarified supernatant. Because of poor yield, bubaline ST gene was further expressed in *Pichia pastoris* – a methylotrophic yeast considered as one of the best eukaryotic system for gene expression.

pPICZa-bbST expression construct was generated to express ST under the control of strong AOX-I promoter which is methanol inducible. *Pichia pastoris* KM71H (Muts) strain was chosen to transform pPICZa-bbST. Maximum expression of ST protein was achieved 120 h post-methanol induction and was several times greater than that achieved with *E. coli*.

#### L3-039P

##### Interaction of immobilized betulinic acid and phospholipase A2

H.-C. Tseng and Y.-C. Liu

Department of Life Science, National Tsing-Hua University, Hsin-Chu, Taiwan ROC. E-mail: ycliu@life.nthu.edu.tw

Betulinic acid (BA), a triterpenoid of white birch (*Betula alba*), exhibits anti-viral, anti-malarial, anti-tumor and anti-inflammatory activities. Although it has been shown to be an inhibitor of bovine pancreatic phospholipase 2 (bp-PLA2), the identity of its cellular targets remain uncertain. We found that BA could inhibit bp-PLA2 enzymatic activity and cytotoxicity of N-PLA2 (PLA2 of *Naja nigricollis*). The interaction between BA and the PLA2 seemed to be mediated by electrostatic interaction and hydrophobic interaction. As an effort of identifying the cellular target of BA, the BA immobilized matrix was made and the utility of the immobilized column was studied. As expected, the BA-immobilized column exhibited a strong affinity with bp-PLA2 or N-PLA2. Surprisingly, the column also binds cardiotoxin  $\gamma$ , which does not resemble to PLA2 in tertiary structure. Thus, BA-immobilized affinity column appears to be useful to explore the cellular targets of BA.

#### L3-040P

##### Recombinant Enterobacter cloacae P99 Amp C beta-lactamase and its mutants: overexpression in *Bacillus subtilis*, purification and characterization

M. W. Tsang and Y. C. Leung

Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hong Kong, SAR PR China.  
E-mail: 02900488r@polyu.edu.hk

With the recent rapid spread of Amp C beta-lactamase-mediated antibiotic resistance, studies on structure–function relationship of Amp C beta-lactamase is becoming an important task in the clinical and pharmacological aspects for drug improvement and rational inhibitor design. In our study, new strategies have developed in expressing recombinant Enterobacter cloacae P99 Amp C beta-lactamase in *Bacillus subtilis*. With the utilization of a thermo-inducible phi 105 MU331 system, functionally active Amp C beta-lactamase was successfully expressed in *Bacillus subtilis*, either intracellularly with N-terminal histidine-tag or extracellularly as a native enzyme. Our results indicated that higher enzyme expression level was accomplished by producing the enzyme as an intracellular histidine-tagged protein rather than as a native extracellular protein. Moreover, with the use of the intracellular histidine-tagged expression strategy, more than 95% pure histidine-tagged P99 wild-type, K315C and Y150C mutants were obtained by a single step of nickel affinity chromatography with a yield of 29 mg/l of culture, 6 mg/l of culture and 66 mg/l of culture respectively. In addition, the roles of the conserved residues, K315 and Y150, in the Amp C beta-lactamase were examined. The observation of a drastic impairment of the catalytic efficiency of the two mutants on nitrocefin, a cephalosporin-type

beta-lactam antibiotic, was consistent with the proposed catalytic roles of these two residues. On the contrary, as revealed by the far-UV CD analysis, replacement of cysteine at positions 315 and 150 did not cause any significant structural perturbation of the enzyme. This implied that these two residues are not involved in the structural architecture of the Amp C enzyme.

### L3-041P

#### Spontaneously generated active truncated forms of CP4 EPSPS protein

S. Stuchlik, S. Natarajan and J. Turna

*Molecular Biology, Comenius University, Bratislava, Slovakia.*

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

Several studies to determine possible horizontal gene transfer (HGT) into microbial flora there have been published recently. We have studied this possible phenomenon too and we developed a rescue system based on the complementation of CP4 EPSPS gene (5-enolpyruvylshikimate-3-phosphate synthase). During our study of HGT of the CP4 EPSPS gene from genetically modified feed through gastrointestinal tract to bacteria living in animal gut we observed functional truncated EPSPS protein forms present in bacteria. We have investigated truncated forms of EPSPS more in detail and compare their properties in respect to full length EPSPS. All forms of EPSPS were expressed in *E. coli* under T7 expression system. We have tested properties of EPSPS forms *in vivo* by complementation test on M9 medium and LB medium with glyphosate, and by *in vitro* enzyme assays with partially purified proteins. Finally we can conclude that some truncated forms of EPSPS can confer full function of native protein and these findings also should be taken into account in risk assessment of possible HGT from GM food/feed.

### L3-042P

#### Expression and identification of the protein and cDNA markers of resistance or susceptibility of *Solanum tuberosum* to potato virus X- and potato leafroll virus on the basis of the experimental model of nearly isogenic potato clones

N. B. Vlasava, A. N. Palilova and E. V. Spiridovich

*Department of Plant Biochemistry and Biotechnology, National Academy of Sciences, Central Botanical Gardens, Minsk, Belarus.*

*E-mail: nastasia\_vlasova@nm.ru*

Virus infections still cause serious losses of the potato crop. Potato virus X- (PVX) and potato leafroll virus (PLRV) are the most widespread and serious viruses of potato in the Eastern Europe. The research aimed at identifying host factors involved in either viral resistance or viral pathogenicity are encouraged by use of model system of relatively isogenic clones of potato, which are contrast on the resistance to PVX and/ or PLRV. The data base on the protein markers of the state of resistance of potato to PVX and/ or PLRV was created. Previous results testify the role of a number of proteins in the formation of the reactions of resistance or susceptibility to named viruses. Created model of nearly isogenic clones allow to isolate genes, specifically involved in the realization of the resistance/susceptibility states and estimate the effect of the expression of these genes. RT-PCR of the clones, distinct on the resistance/susceptibility to PVX and /or PLRV revealed the different expression of the several genome regions. Fragment 500 bp was quantitatively more expressed at resistant to PLRV in comparison with resistant to PVX clone of Loshitski cv. and was not determined at susceptible to PLRV and both viruses clones. Random primers allowed to mark the

resistance to PLRV: fragment ~800 bp was detected at cDNA amplification with a mix of OPV-08 and OPK-08 primers only at X + L- clones with genotypes of Loshitski and Javar cv. Further cloning of revealed fragments on the basis of preliminary protein data base will allow to determine the sequence and structure of putative genes and proteins, which are equipped in the reactions of resistance and susceptibility to this viruses.

### L3-043P

#### On the track for a hypothetical new cardosin?

T. Cardoso<sup>1,3</sup>, R. Ribeiro<sup>1</sup>, R. Vilar<sup>4</sup>, N. Rosa<sup>4</sup>, H. Fonseca<sup>1</sup>, E. Pires<sup>2</sup> and M. Barros<sup>3,4</sup>

<sup>1</sup>Department of Biology, University of Aveiro, Aveiro, Portugal,

<sup>2</sup>Centre for Neurosciences and Cell Biology, University of Coimbra, Coimbra, Portugal, <sup>3</sup>Department of Biology – Centre for Cell Biology, University of Aveiro, Aveiro, Portugal, <sup>4</sup>Laboratory of Protein Chemistry, School of Dentistry, Portuguese Catholic

University, Viseu, Portugal. *E-mail: rita.vilar@sapo.pt*

The role of plant aspartic proteinases in protein processing or degradation under different conditions and in different stages of plant development suggests some functional specialization. Cardosins are aspartic proteinases, extracted from flowers of *Cynara cardunculus* L. These plants are traditionally used in Portugal for cheese making. Two different cardosins have been isolated, cardosins A and B, which share 73% similarity. Cardosin A has two chains with 31 and 15 KDa and cardosin B does also have two chains of 34 and 14 KDa. Cardosin A has been suggested to be involved in RGD – mediated proteolytic mechanisms and appears to be similar to chymosin. Cardosin B was proposed to be involved both in defensive mechanisms against pathogens and pistil development and/or in pollen–pistil interactions and closely resembles pepsin. After making modifications to the purification procedure of these proteins previously established by Verissimo, an unknown protein was found with a slightly different apparent molecular weight. The aim of this work is to characterize the protein found. The zymography results show caseinolytic activity. A screening of several harvesting years of the cardoon species used, has always shown the presence of this protein, but in different cardosin B/unknown protein ratios. The recognition of the unknown protein by an antibody, produced against cardosin A and that recognizes cardosin B, does not seem to be as specific as for the cardosin B itself. At the present moment, the results seem to point to an isoform of cardosin B, yet with at least differences in apparent molecular weight and probably in sequence.

**Acknowledgment:** The authors wish to acknowledge FCT for supporting Cardoso T (SFRH/BD/6406/2001).

### L3-044P

#### Production of tetrameric single-chain antibodies using enzymatic biotinylation

V. Virág<sup>1</sup>, J. Prechl<sup>2</sup> and A. Erdei<sup>1,2</sup>

<sup>1</sup>Laboratory of Molecular Biology, Department of Immunology, University of Eötvös Loránd, Budapest, Hungary, <sup>2</sup>Laboratory of Molecular Biology, Immunology Research Group, Hungarian Academy of Sciences, Budapest, Hungary.

*E-mail: viragpalanta@ludens.elte.hu*

Single-chain antibody fragments contain the variable region of the heavy and light chains of the Ig molecule joined together by a flexible region. The valency of the scFv molecule, which is capable of binding a single antigenic epitope, needs to be enhanced for several applications. One possible method is through enzymatic biotinylation, which enables a site-specific integration of biotin. BirA ligase is capable of binding a biotin to the lysine of the following amino-acid sequence: GLNDIFEAQKIEWHE

(Biotin Acceptor Domain, BAD). We inserted the appropriate coding DNA sequence into a PET3d vector which we use for scFv expression. The domain was ligated either to the C or the N terminus of the scFv in the following order: BAD scFv myc-, his-tag, or scFv BAD his-tag. The ligation reaction was verified by PCR amplification, sequencing and enzymatic digestion. *Escherichia coli* wraps the single chain antibody into inclusion bodies which accumulate in the cytoplasm; therefore it was purified from the cytoplasmic extract using Ni-NTA affinity chromatography. The purity and quality of the protein was confirmed by SDS-PAGE, Western blot and ELISA. We verified that the bacteria were producing the recombinant protein with the same efficiency as the original single chain antibody fragment. A small fraction of the protein was already biotinylated upon purification possibly due to BirA activity in the cells. Following biotinylation, the purified scFv was tetramerized using 1:4 molar ratio of streptavidin and scFv, thereby creating the so called streptabody (Stab). Tetramerization was conformed and molar ratio optimized using 4–15% SDS-PAGE. Flow-cytometry was used to characterize the binding capabilities of the tetramer form. In summary, we created a pET vector-based expression cassette for the production of BAD fused scFv. Using this system we enzymatically biotinylated the otherwise sensitive, precipitation-prone molecules and generated tetramers of them.

### L3-045P

#### **Selenoproteins screening in formula and cow milk by the combined use of 2-D-PAGE, ICP-MS and nLC-Q-TOF-MS**

J. L. Gomez-Ariza, V. Bernal-Daza and M. J. Villegas-Portero  
*Laboratory of Environ Analysis and Bioanalysis, Department of Chemistry; Faculty of Experimental Sciences, University of Huelva, Huelva, Spain. E-mail: mariajose.villegas@ono.com*

Food quality and authenticity are priorities to assure a safe and healthy feeding. Milk is an important food from birth, and its quality is strongly dependent on the presence of essential biomolecules such as proteins. Caseins and whey proteins ( $\beta$ -lactalbumin,  $\beta$ -lactoglobulin and serum albumin) represent almost the entire protein mass of sweet and acid whey, but other minor proteins and metalloproteins are also very important for health. Many times the element in metalloproteins plays an important role on the protein function, and suitable analytical tools characterize the metal bound to the protein moiety are necessary. This metal-tagged analytical approach for proteins identification and characterization (metallomics) [1, 2] require the combined use of both atomic and mass detectors as well as separation techniques, integrates in multidimensional instrumental arrangements. In this study two-dimensional polyacrylamide gel electrophoresis has been selected for milk protein separation. Fat was eliminated by centrifugation and proteins precipitated with trichloroacetic acid.

The spots were isolated from the gel and digested with  $\text{HClO}_4\text{:HNO}_3\text{:HCl}$  acid mixture for metals release. ICP-MS was used to perform the metal profile characterization, the spots in which selenium was present were selected for further study. Selenium tagged spots were digested with trypsin and the resulting peptide mixture analyzed using a nLC-ESI-Q-TOF-MS system. This peptide mass fingerprinting was used for minor selenium proteins identification in the milk.

#### **References**

1. Gomez-Ariza JL, Garcia-Barrera T, Lorenzo F, Bernal V, Villegas MJ, Oliveira V. *Analytica Chimica Acta* 2004; **524**: 15.
2. Szpunar J. *Anal Bioanal Chem* 2004; **378**: 54–56.

### L3-046P

#### **Cloning and high level expression of wild-type and mutated bacterial EPSP synthase and analysis its second structures**

K. Zakikhan<sup>1</sup>, A. H. Salmanian<sup>1</sup> and K. Khageh<sup>2</sup>

<sup>1</sup>Laboratory of Plant Biotechnology, Department of Cell and Molecular Biology, University of Khatam, Tehran, Iran, <sup>2</sup>Laboratory of Biochemistry, Department of Biochemistry, University of Modares, Tehran, Iran. E-mail: ezakikhan@yahoo.com

The 5-enolpyruvyl shikimate-3-phosphate (EPSP) synthase is a key enzyme in the biosynthesis of aromatic amino acids in shikimate pathway. Biosynthesis of aromatic compounds occurs in prokaryotes and plants but not in mammalian or other eukaryotes. EPSP synthase is the target of a wide-spectrum herbicide, glyphosate (N-phosphonomethyl glycine). Interactions between glyphosate and EPSPS have been studied extensively. It has been shown that the mutated enzymes are insensitive to glyphosate. Previously we isolated the EPSPS gene from *E. coli* (K12) and introduced two mutations (Ala183 to Thr, Gly 96 to Ala) on it by using site directed mutagenesis method and PCR technique. For analysis of the second structure of two target molecules, the wild-type and one of the mutated EPSPS enzymes (Ala183 to Thr) were cloned under the control of chemically inducible T7promoter (PET system, Novagen). The enzymes were over expressed by induction in *E. coli* BL21 DE3 – pLYSs with different concentration of IPTG and bacteria ( $\text{OD}_{600} = 0.4\text{--}1$ ) in 37°C for 2, 4, 6 and 24 h. The expressed proteins were analyzed electrophoretically on 12% SDS-PAGE. For comparing two protein structures with CD and Fluorescence, the purification of recombinant enzymes were carried out with Ion exchange chromatography (DEAE sephacryl and Mono Q). Our data shows that the PET system in combination with appropriate host could express the mutated and wild-type bacterial EPSP synthase genes in the presence of 1 mM IPTG at  $\text{OD}_{600} = 0.4$  in 37°C and after 4 h induction. After the purification the CD and Fluorescence analysis show that there are no any changes in the structures of two target enzymes after this mutation.