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Journal of Integrated OMICS

a methodological journal

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Journal of Integrated OMICS, JIOMICS, provides a forum for the publication of original research papers, preliminary communications, technical notes and critical reviews in all branches of pure and applied "-omics", such as genomics, proteomics, lipidomics, metabolomics or metallomics. The manuscripts must address methodological development. Contributions are evaluated based on established guidelines, including the fundamental nature of the study, scientific novelty, and substantial improvement or advantage over existing technology or method. Original research papers on fundamental studies, and novel sensor and instrumentation development, are especially encouraged. It is expected that improvements will also be demonstrated within the context of (or with regard to) a specific biological question; ability to promote the analysis of molecular mechanisms is of particular interest. Novel or improved applications in areas such as clinical, medicinal and biological chemistry, environmental analysis, pharmacology and materials science and engineering are welcome.

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Special Issue

Proceeding Abstracts of the 4th International Congress on Analytical Proteomics (ICAP 2015)

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SPECIAL ISSUE: PROCEEDING ABSTRACTS OF THE 4th INTERNATIONAL CONGRESS ON ANALYTICAL PROTEOMICS (ICAP 2015)

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Special Issue: Proceeding Abstracts of the 4th International Congress on Analytical Proteomics (ICAP 2015)

One gene, many changes: a long history involving soybean

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ABSTRACT

Exogenous genes are successfully introduced into plants since the 80´s, and that one, which confer the resistance of the soybeans to glyphosate, the most used herbicide in the world, the first reported in the literature [1]. Since then, genetically modified organisms (GMOs) are being exponentially produced, so that 170 million hectares is nowadays related to crops engineered in at least 28 countries [2]. This fact not only reinforce the importance of such organisms, but also shows a good appeal to sustainability once that these GMOs produce enhanced characteristics such as high amount of fibers, proteins, are resistant to pests and/or insects, among others [2].

In this way, our research group is deeply interested in studying, since long time ago, the effects that a transgenic modification has on the proteome, the metabolome, the ionome, besides others of an organism [3-5], also evaluating different generations of plants. In this way, enzymatic evaluation, proteomics, and bioaccessibility will be presented for investigating some aspects regarding soybean [*Glycine max* (L.) Merril] seeds and plants. Platforms like 2-D DIGE, ICP-MS, and ESI-MS/MS will be presented, as well as some results regarding stress production and the cultivation of this culture in the presence of nanoparticles.

Acknowledgements: FAPESP, CNPq, CAPES

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Proteomic profiling of neuromuscular disorders

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ABSTRACT

Purpose: Neuromuscular disorders are associated with a great variety of pathophysiological triggering mechanisms, including primary genetic defects, acquired autoimmune processes, traumatic tissue injury, drug-induced abnormalities, cytotoxic insults and disuse-related atrophy, as well as changes during natural aging. In order to establish robust biomarker signatures for developing improved diagnostic, prognostic and therapy-monitoring test systems that can differentiate between individual muscular diseases, systematic evaluations of proteome-wide changes in pathological muscle specimens have been carried out.

Experimental description: Mass spectrometry-based proteomic profiling was used to compare skeletal muscle samples from normal versus pathological samples. Both, animal models of common neuromuscular disorders and human biopsy material was used in large-scale comparative studies. Subcellular fractionation procedures, fluorescence two-dimensional gel electrophoresis and/or liquid chromatography were employed for the efficient separation of the assessable muscle proteome. The independent verification of proteomic findings was carried out by enzyme assays, protein binding experiments, immunoblotting surveys and immunofluorescence microscopy.

Results: The systematic profiling of pathological skeletal muscle specimens has resulted in the establishment of a variety of novel biomarker candidates for the detailed characterization of muscular dystrophy, motor neuron disease, myotonia, diabetes-associated muscle weakness and sarcopenia of old age. Identified proteins are associated with cellular signalling, ion homeostasis, excitation-contraction coupling, the contractile apparatus, the cytoskeleton, the extracellular matrix and the cellular stress response.

Conclusions: The newly identified proteomic biomarker candidates can now be further characterized and tested for their suitability as diagnostic, prognostic and therapy-monitoring indicator molecules in the field of neuromuscular pathology.

Keywords: biomarker, diabetes, motor neuron disease, muscular dystrophy, myotonia, sarcopenia of old age.

Acknowledgements: Research was funded by the Irish Higher Education Authority, the Deutsche Duchenne Stiftung aktion benni & co e.V., Muscular Dystrophy Ireland and the Hume scholarship programme of Maynooth University. The Programme for Research in Third Level Institutions PRTLI Cycle 5 is co-funded by the Irish Government and the European Union under Ireland's EU Structural Funds Programme 2007-2013.

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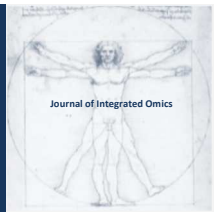

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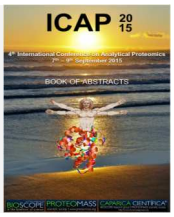

Special Issue: Proceeding Abstracts of the 4th International Congress on Analytical Proteomics (ICAP 2015)

The proteomic proficiency to identify and elucidate antimicrobial resistance bacteria

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Abstract

Purpose: To consider the contribution of proteomics to the study of antimicrobial-resistant (AMR) bacteria from clinical and environmental origin.

Experimental description: Proteomic approaches have been considerably improved during the past decade and have been used to overview and investigate the differences in protein expression profiles of cells grown under a broad spectrum of growth conditions and with different stress factors including antibiotics. The Functional Genomics and Proteomics Unit based at the University of Trás-os-Montes e Alto Douro (Vila Real, Portugal), using gel-based proteomic methodologies (e.g. 2D-GE, MALDI-TOF/MS, LC-MS/MS) has aimed to elucidate the distinctive characteristics of AMR bacteria from different sources, either clinical or environmental.

Results: From *Salmonella* Typhimurium and methicillin-resistant *Staphylococcus aureus* human clinical strains to *Enterococcus* spp. and *Escherichia coli* environmental strains recovered from wildlife species the work done so far has allowed to overview the proteome of several strains during induced stresses and to identify the most recurrent and deviations in the identified proteins associated biological processes. The research done allowed identifying antimicrobial proteins directly related with antimicrobial resistance or to bacterial virulence that complemented previously published genomic data where no specific mechanisms of resistance/virulence have been elucidated. Other identified proteins shown to be indirectly related with antimicrobial resistance or to bacterial virulence like chaperone proteins linked with aminoglycosides resistance, membranar proteins previously shown to increase the level of resistance to tetracycline or associated with increased virulence. Moreover, comparative proteomics studies presented an overview of the complex changes in expression and metabolism that occur when AMR bacteria is stressed with specific antibiotics. For instance, the abundance of chaperone, porin and export proteins showed to be particularly affected during antibiotic stressing conditions, which indicates that the stress response and transport functions are essential and directly influence the antibiotic resistance response of AMR strains.

Conclusions: The proteomic profiling allows to obtain an overview of the proteins present under specific stress conditions while the comparative proteomics methodologies allows to investigate differences in protein expression profiles of cells grown under different stress factors. Proteomic studies of stress-induced metabolism changes in bacterial strains from clinical and environmental origins might show how different strains are related and how different environments act as reservoirs.

Keywords: Proteomics, Gel-based Methodologies, Antimicrobial Resistance, Clinical Bacteria, Environmental Bacteria.

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Special Issue: Proceeding Abstracts of the 4th International Congress on Analytical Proteomics (ICAP 2015)

Protein of interests for serological diagnosis in Bird fancier's Lung

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Abstract

Purpose: Hypersensitivity pneumonitis (HP) is an inflammatory interstitial lung disease resulting from exposure to a wide variety of antigens contained in organic particles dust. Bird fancier's lung (BFL) is a form of HP caused by repeated inhalation of avian antigens, mainly located in droppings, blooms and feathers. Several candidate substances have been reported such as IgA, IgG and pigeon intestinal mucin, but none of them have been clearly identified. Diagnosis of HP requires an association of clinical, radiological and biological markers including serological test. However, immunological tests routinely used lack of specificity and standardization. The aim of this study was to investigate immunogenic proteins in pigeon dropping by comparative western blot analysis and to identify them by mass spectrometry.

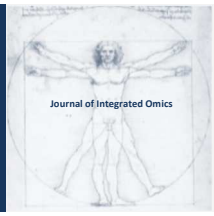
Experimental description: To evaluate antibody-mediated immunity, two-dimensional western blotting using *Columba livia* dropping were performed. The membranes were probed with sera from 10 patients with BFL, 2 healthy exposed controls and 2 controls without exposure to birds. 14 candidate proteins were excised from the two-dimensional-gel Electrophoresis and identified by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

Results: First of all, none of sera from control and exposed subjects presented specific antibodies against proteins from pigeon dropping extract. We identified 6 proteins which may be grouped into three categories based on their putative functions. The heavy chain V-III, the lambda chain and the polymeric receptor of immunoglobulin was respectively bound by antibodies from at least 90%, 80% and 80% of patient with BFL. Among the proteins of the immune system of birds, we also identified the mucin recognized by antibodies from at least 70% of patients. Pancreatic lipase and chymotrypsinogen, both digestive proteins secreted into the pancreatic fluid were also identified. These proteins were revealed by Western blotting for 80% of patients.

Conclusions: This study allowed the identification of 6 proteins of interest for serological diagnosis of BFL. These proteins related to immune and digestive systems of birds.Genes coding for these proteins were cloned and recombinant antigen are currently under development. The performance of these recombinant antigens will be evaluated by ELISA and may allow optimization of the serological diagnosis of BFL.

Keywords: Bird fancier's lung, mass spectrometry, immunoglobulin, western blotting, bird droppings.

Acknowledgements: This work was supported by the Besançon University Hospital through a project entitled APICHU D-PEO (Appel à Projet Interne de recherché Clinique ou biologique du Centre Hospitalier Universitaire). The authors thank breeders in the Franche-Comté region for their collaboration in this study.



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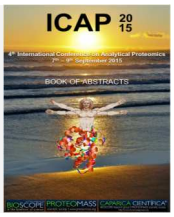

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A case report of cyclosporine complexed with human calcineurin remaining in the gut after 25 years of its consumption

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Abstract

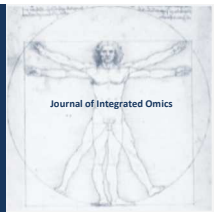
Purpose: To report on how residuals of cyclosporine, an extremely toxic immunosuppressant drug, can remain in the human gut after decades of its consumption and how a cleansing protocol of the colon can remove it.

Experimental description: in our Group we are in the way of implementing a leaning protocol aimed at promoting a beneficial modulation of the intestinal microbiota, increased physical activity and improved alimentary habits. The first part of this protocol is a 6-day intestinal cleansing program which includes: supplemented fasting, 1 daily transanal irrigation and consumption of psyllium (PSY), montmorillonite (MMT), prebiotics and probiotics. This program allows the collection of human intestinal mucus (HIM) samples in good quantity and quality as necessary for proteomic studies, a field we have been working on. Proteins from HIM were treated with a denaturalized buffer, and their separation was achieved by 2D-PAGE. Digested peptides were analyzed with a NanoAcquity UPLC chromatograph coupled to a HDMS spectrometer equipped with a NanoESI source.

Results: As a casual finding, the protein Chain-crystal-structure-of-human-calcineurin complexed-with-cyclosporin-A-and-human-cyclophilin was identified in the sample from a 40 years old woman. When asked about consumption of cyclosporine in the previous days to the cleansing program, this volunteer confirmed that she has taken it under medical prescription, but when she was only 15 y old, after she was diagnosed with Systemic lupus erythematosus (SLE). She remembered well the name of the drug, because she lost her hair and her nails at that time, as adverse effects to the drug. Calcineurin (CN) is a protein phosphatase that activates the nuclear factor of activated T cell cytoplasmic (NFATc), upregulating the expression of interleukin 2 (IL-2), which, in turn, stimulates the growth and differentiation of T cell response. Cyclosporine is an inhibitor of this protein and is commonly used in some autoimmune diseases.

Conclusions: 1. This finding confirm one of the most important functions of HIM as protection against pathobiota, metabolic waste and xenobiotics; 2. The proteomic study of HIM from an individual can be useful to know part of his/her clinical history as well as to help in the implementation of customized medicine; 3. Periodic cleansing of the gut can be advisable for persons that have orally consumed toxics, but probably also for people looking to improve their general well being. MMT, for instance is used in people diagnosed with or being suspected of intoxication with aflatoxins, as it can adsorb these toxins and help to remove them from the intestinal tract.

Keywords: cyclosporine, human intestinal mucus, transanal irrigation.



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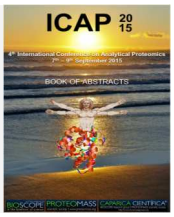

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Improved efficiency of *in situ* tryptic proteolysis for fresh frozen and FFPE tissue

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Abstract

Purpose: Despite improvements to on-tissue digestion efficiency, methods to further improve both reliability and number of proteins identified when using a bottom up proteomic approach remains. Current investigations focus on improving the yield of tryptic peptides from fresh frozen rat brain tissue and FFPE mouse brain tissue.

Experimental description: Fresh frozen tissue: Following wash steps of ethanol and chloroform, 20µg/mL trypsin solution containing either OcGlu or MEGA-81 was deposited using the SunCollect sprayer (SunChrom, Friedrichsdorf, Germany). Samples were incubated for 4 hours at 37°C.

FFPE tissue: Following tissue deparaffinization using xylene and wash steps of ethanol in increasing concentration, an antigen retrieval step was carried out. 20µg/mL trypsin solution containing either OcGlu or MEGA-8 was deposited using the SunCollect sprayer. Samples were incubated for overnight at 37°C.

MALDI analysis: α-cyano-4-hydroxycinnamic acid (CHCA) with aniline in acetonitrile:water:TFAaq was spotted (10mg/mL) onto the digest regions or sprayed using the SunCollect (5mg/mL) and subjected to MALDI-MSP or MSI (HDMS SYNAPTTM G2 system, Waters Corporation, Manchester) analysis.

Results: The study concluded that the incorporation of a detergent yielded several hundred peptide peaks with S/N ratio above 3.5 for the digestion of fresh frozen tissue. Most of the peaks were considered to be from the most abundant proteins in brain; the tentatively identified myelin basic protein (MBP). In fresh frozen tissue, peak intensities originating from the detergent MEGA-8 are clearly superior to those of OcGlu. Existing methodologies have been transferred onto the digestion of FFPE tissue to generate a more efficient proteolysis.

Conclusions: In conclusion this study shows that ion population, abundance and protein identification can be further improved, thus opening up the way to more reliable and efficient biomarker discovery and screening.

Keywords: MALDI, *in situ*, Proteolysis, Detergents, Fingermarks.

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Development and evaluation of method for metalloproteins detection involved in bipolar disorder

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Abstract

Purpose: Bipolar disorder (BD) is characterized as a chronic psychiatric illness, severe and highly debilitating, with recurrent mood disorders, presenting episodes of mania and depression separated by periods of normal behavior (Sussulini, *et al*., 2009;).The cause of bipolar disorder is unknown, although it is known that a variety of biochemical, genetic, and environmental factors can be involved in the process of developing of the disorder. To date, there is no biochemical test to confirm the disease, and the diagnosis is still based on experience and clinical judgment (Jesus, *et al*., 2015). The main focus of this work was to propose a metalloproteomic strategy for BD, since this area has been not carried out in the literature.

Experimental description: Metal and metalloid ion involved in this pathology were determined by ICP-MS, using a dynamic reaction cell (Perkin Elmer, ELAN DRC-e). For metalloproteomic evaluation, a mild procedure for removal of major abundant protein was applied. For this task, a high performance liquid chromatograph was used to separate the proteins, using a sequential detection of proteins (UV-Vis) and metal ions (hyphenated LC-ICP-MS).

Results: Considering the elemental determination, the DRC strategy was considered as an efficient method in the removal of interferences, since satisfactory results in terms of trueness and precision were obtained, using a certified reference material. Thus, an ionomic profile of each group was possible. After the chromatographic separation of proteins and metalloproteins, the removal of major abundant proteins is a main challenging step, since for a metallomic study, the information about tertiary structure must be preserved as intact as possible. The detection of proteins in UV-Vis and metalloproteins in LC-ICP-MS confirmed that our strategy was reliable for a metalloprotemic evaluation.

Conclusions: Differential levels of metal and metalloid ions were observed when comparing the three studied groups, as well as the detection of metalloproteins by LC-ICP-MS. Thus, our method can be considered as reliable for evaluation of BD disorder.

Keywords: bipolar disorder, metallomic, metalloprotein.

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Using protein biomarkers to understand the biological mechanisms underlying beef sensory qualities

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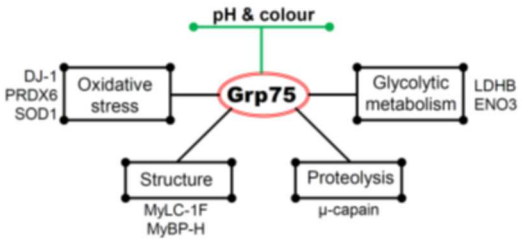
ABSTRACT

Purpose: Comparative proteomic analyses have revealed that numerous proteins are related to meat sensory qualities. They belong to myriad biological pathways: metabolism, structure, heat stress, oxidative stress, apoptosis, proteolysis and others[1]. These relationships suggest that these proteins may play key roles in the mechanisms underlying meat qualities. Therefore, they can be used, not only as biomarkers for meat and carcasses quality, but also to unravel these mechanisms. In this study, the central role of Grp75 (*HSPA9*), a member of the Hsp70 family, in the biological mechanisms involved in the conversion of muscle to meat is described.



Experimental description: *Longissimus thoracis* (LT, fast oxydo-glycolytic) muscles of 21 French Blonde d'Aquitaine young bulls were used in this study[2]. pH parameters at 45 min, 3h and 30h *p.m.* and CIE-*L*a*b** colour traits after 24h *p.m.* were determined. Protein biomarkers according to Picard *et al*[1] were quantified using Dot-Blot on muscles sampled 30 min after slaughter. Relationships between colour and pH traits and the biomarkers were determined by means of regression and Pearson correlation analyses using SAS 9.2 software.

Results: Grp75 protein was related to pH and colour parameters. It was also related to numerous biological pathways, likely glycolytic metabolism (Enolase, r = 0.63, $P < 0.01$ and LDHB, r = 0.42, $P < 0.05$), proteolysis (µ-calpain, a Ca^{2+} dependent protease, r = 0.54, $P < 0.05$), structure maintenance (MyLC1-F, r = 0.52, $P < 0.05$ and MyBP-H, r = 0.63, $P < 0.01$) and oxidative stress (Prdx6, SOD1 and DJ-1, r = 0.61-0.63, $P < 0.01$). Grp75 was earlier described as a multi-functional protein. It is a cytoprotective chaperone present in nearly all cellular compartments and assists in the import and folding of mitochondrial proteins and protects cells from glucose deprivation. It is a member of the Hsp70 protein family, and not induced by heat stress but by glucose deprivation, Ca^{2+} influx or agents perturbing glycolysis. At slaughter, after bleeding of the animal, lack of oxygen and nutriments will lead to accumulation of ROS in the mitochondria, the end of ATP production, acidification of the cytoplasm and a deregulation of Ca^{2+} in the muscle cells which may set off apoptosis[3]. In the present study, Grp75 content soon after slaughter was related to the glycolysis pathway and pH, suggesting that it plays a central role in the conversion of muscle to meat, and consequently, meat sensory qualities. In addition, Grp75 was also reported to link the inositol 1,4,5-trisphosphate receptor (IP3R) to voltage-dependent anion channel (VDAC), presumably enhancing Ca^{2+} trafficking from endoplasmic reticulum (ER) towards mitochondria and cytosol. Possibly, increased Ca^{2+} levels in the cytoplasm activate µ-calpain and/or regulate apoptosis. Our results indicate that Grp75 may play a central role in changes in the structural proteins in the muscle cell, and consequently in meat colour development.

Conclusions: This example illustrates the important role of analytical proteomics in improving knowledge on the determinism of meat sensory qualities.

Keywords: Beef meat; Colour; pH; Biomarkers; Validation; Prediction; Biological mechanisms.

Acknowledgements: This work was part of the EU ProSafeBeef project (FOODCT-2006-36241). A grant of the PROFAS B+ program given to M.G. is also gratefully acknowledged.

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Special Issue: Proceeding Abstracts of the 4th International Congress on Analytical Proteomics (ICAP 2015)

Some proteins of possible -clinical interest found in samples of human intestinal mucus (HIM)

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Abstract

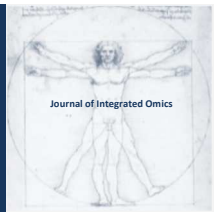
Purpose: To report the identification of some proteins isolated from samples of human intestinal mucus (HIM) with possible clinical interest.

Experimental description: HIM was collected from six volunteers admitted to a leaning program which included a colon cleansing protocol and a rectosigmoidoscopy. Protein extraction was achieved by a denaturalization buffer, separation was performed by gel electrophoresis both with SDS-PAGE and 2D-PAGE. Protein shotgun was done with solution desalinization by SPE and albumin depletion. Digested peptides were analysed using a NanoAcquity UPLC cromatograph coupled to a HDSM espectrometer, equiped with a NanoESI source. Samples were injected to a C_{18} precolumn with a flow of 5 µL/min water/acetonitrile (97:3) with 0,1% formica cid and then transfered to an analytical column C_{18} and eluted with a flow of 1 µL/min.

Partial results: 1. A methodology for the analysis of HIM from a single person is reported, from the preparation of the patients for the collection of the sample, till protein characterization of the mucus. This is done using mass spectrometry, using three different techniques: SDS -PAGE, 2D-PAGE y shotgun; 2. 14 proteins of clinical interest have been identified so far: Annexin A2, Calcium-activated chloride channel regulator, Chain crystal structure of human calcineurin complexed with cyclosporin A and human cyclophilin, Complement C3, Disulfide isomerase, Galectin-3 y 4, Inmunoglobulin programmed cell death 1 ligand, LAMTOR3, Muc2, Nuclear Protein 8, RuvB like 2, Tyrosine Protein phosphatase non receptor type 11 and Zymogen granule membrane protein 16.

Conclusion: The proteomic study of HIM can be a very useful tool for the study of different diseases, not only of those directly related to the digestive system, bus also many associated with the immune system.

Keywords: electrophoresis, human intestinal mucus, shotgun proteomics.



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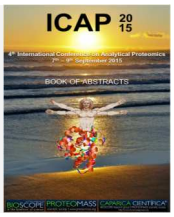

Special Issue: Proceeding Abstracts of the 4th International Congress on Analytical Proteomics (ICAP 2015)

Proteomic alterations in transgenic and non-transgenic soybean leaves after plant cultivation in the presence of silver nanoparticles

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Abstract

Purpose: The present study evaluates the proteomic map of transgenic (T) and non-transgenic (NT) soybean leaves after plant cultivation with silver nanoparticles (AgNPs) or silver nitrate ($AgNO_3$), at 50 mg kg^{-1} silver concentration.

Experimental description: The T and NT soybean plants were cultivated for 21 days in a growth chamber with controlled photoperiod (12 h) and temperature (27 ± 1 °C). For treated plants, $AgNO_3$ and AgNP solutions were added, resulting, in the end of the cultivation period, a 50 mg kg^{-1} of silver. The proteins were extracted from leaves with phenol, separated by two-dimensional difference gel electrophoresis (2-D DIGE) to evaluate possible changes in the proteomic map. The differential protein abundances were evaluated by a DeCyder® program, considering a threshold of 2.0 fold (100% variation).

Results: The protein content for control T soybeans leaves was higher than NT soybean leaves (*ca.* 18%). For NT plants exposed to $AgNO_3$ (NT-$AgNO_3$) and AgNP (NT-AgNP), the NT-$AgNO_3$ showed an increase of *ca.* 35% in terms of protein concentration in their leaves. No significant alterations in protein content for NT-AgNP in comparison with the control were observed. However, for T plants treated with $AgNO_3$ (T-$AgNO_3$) and AgNP (T-AgNP), the protein concentration in T soybean leaves was changed in both cases. A decrease of *ca.* 6% in T-$AgNO_3$ compared with control plants was observed, whereas for T-AgNP an increase in the protein content (*ca.* 28%) was observed. Regarding the evaluation of differential abundant proteins, 19 and 11 protein spots are found for NT *vs* NT-$AgNO_3$ and NT *vs* NT-AgNP, respectively, and 22 protein spots are differential for NT-$AgNO_3$ *vs* NT-AgNP group. Considering T *vs* T-$AgNO_3$ and T *vs* T-AgNP groups, 8 and 12 protein spots are differential abundant, respectively, while for T-$AgNO_3$ *vs* T-AgNP group, 9 protein spots are found. These results corroborates with those of protein concentration, suggesting that changes in the proteomic map were dependent on the genetic modification, as well as the form in which silver is added in the cultivation (AgNP or $AgNO_3$).

Conclusions: According to the results, the treatment with $AgNO_3$ and AgNP changes the proteomic map for both T and NT soybean leaves, once that protein spots with different abundances were found. Further experiments will be carried out for identification of these differential proteins by LC-MS/MS.

Keywords: transgenic soybeans; silver nanoparticles; proteomics; 2-D DIGE.

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Special Issue: Proceeding Abstracts of the 4th International Congress on Analytical Proteomics (ICAP 2015)

Rice Proteomics: Method Development of 2D-PAGE for Malaysian Upland Rice

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ABSTRACT

Purpose: Method development is the establishment of analytical method that will be applicable and copacetic for the target analysis. It is one of the critical determinants to ensure the successful of the experiment being carried out. The aim of this study is to develop a suitable 2D-PAGE method that could help in the proteomics analysis of Malaysian upland rice. Although 2D-PAGE for rice proteomics has been established for more than a decade ago, none has been reported for Malaysian upland rice and it is acknowledged no universal protocol ever exists as each plant behaves differently.

Experimental description: In this study, a cultivar of Malaysian upland rice named Wai with age ranging from 4-16 weeks were extracted by using modified TCA/Acetone based method. The isoelectric focusing (IEF) was performed using 18cm immobiline pH gradient strip, pH range 4-7 with the protocol: 1 h at 100 V (linear), 2h at 500 V (gradient), 12h at 8000 V (gradient), 2h at 8000 V (linear) and 10h at 100 V (slow). The strip was loaded on 12% acrylamide gel for the second phase and electrophoresis was carried out at 100 V for 16 hours and stained with comassie brilliant blue red 250. The protein spot was analyzed using PDQuest software version 8.0.

Results: The suitability of the method developed is determined based on the resolution of the protein spot on the gel and its pattern. The result shows that it is feasible to visualize numbers of protein spot with good intensity for all age range (4-16 weeks) of Wai cultivar which indicates the suitable method for 2D-PAGE analysis of Malaysian upland rice is developed.

Conclusions: This study accommodates new insights for upland rice protein separation, serving as platform that boosts the relevance and importance of the developed 2D-PAGE for further Malaysian upland rice proteomics analysis.

Keywords: Development, Proteomics, Upland Rice, 2D-PAGE.

Acknowledgements: We thank the Universiti Teknologi Malaysia (UTM) and Ministry of Education (MOE) for funding this project (Fundamental Research Grant Scheme, Vote: 4F366 and Research University Grant, Vote: 10J16) and Zamalah for sponsoring the graduate student scholarship.

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Effect of resistance inducers and *Hemileia vastatrix* infection on protein patterns of *Coffea arabica* leaves Title

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Abstract

Purpose: The coffee leaf rust (*Hemileia vastatrix*) disease causes serious losses in coffee production and quality, and if no chemical control measures are taken huge depreciation of marketing values will occur. For a sustainable coffee production there are increasing societal expectations to reduce pesticide treatments and make use of alternative strategies of plant protection, such as the application of resistance inducers. Using a comparative proteomic analysis of *Coffea arabica* leaves we aimed to understand the cellular mechanisms that are behind the disease control capacity of the resistance inducers Greenforce CuCa or Bion®, when applied prior to infection with *H. vastatrix*. These resistance inducers have shown efficiency in the control of the disease when compared to a standard fungicide[1]

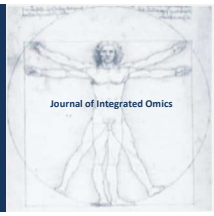
Experimental description: Leaves of *Coffea arabica* cv. Mundo Novo were sprayed with GreenForce CuCa (formulation based on coffee berry husk supplemented with cooper and calcium, developed in UFLA, Brazil) and Bion® (acibenzolar-S-methyl-ASM). Three days after treatment, leaves were sprayed with *H. vastratrix* urediniospores. Leaves treated with water or inoculated (without any previous treatment) were used as controls. Samples were collected at 3, 5 and 7 days after treatments, corresponding respectively to 0, 2 and 4 days after inoculation. Protein extracts were obtained by precipitation with trichloroacetic acid in acetone following purification with the 2D clean-up kit (GE Healthcare). After separation of proteins by 2-DE and staining with Colloidal Coomassie blue, the most abundant and visible polypeptides spots were removed from the gel and their identification was accomplished by mass spectrometry (MALDI/TOF/TOF MS) followed by homology search in ESTs coffee databases.

Results: We were able to identify a total of 204 leaf proteins which were mainly involved in photosynthesis, protein metabolism, biotic and abiotic stresses, redox reactions, glycolysis and secondary metabolism; of those proteins 65 changed in abundance between treated and/or infected leave samples. GreenForce CuCa affected mainly photosynthetic proteins, while Bion® treatment, affected mainly energy metabolism and stress. The protein profile of leaves treated with Bion® resembles more the profile of the healthy control than of the GreenForce CuCa treatment.

Conclusions: The results showed that the resistance inducers affected mainly proteins of the primary metabolism; this metabolic adjustment has contributed to an increased in the resistance of the coffee plant to the pathogen. [1]Monteiro *et al.* 2013. In: VIII Simpósio de Pesquisas dos Cafés do Brasil, Salvador, Bhaia, Brazil.

Keywords: Coffee Leaf Rust, Leaf proteome, 2-DE, MALDI-TOF/TOF-MS, GreenForce CuCa, Bion®

Acknowledgements: This work was supported by Portuguese Funds through FCT (Fundação para a Ciência e a Tecnologia), under the project PTDC/AGR-GPL/109990/2009 and the strategic project PEst-OE/EQB/LA0004/2011 and by a Pos-PhD grant and PhD grant of CNPq (Conselho Nacional de Desenvolvimento Cientifico e Tecnológico - Brazil) and INCT-Café (Instituto Nacional de Ciência e Tecnologia do Café, Brazil).



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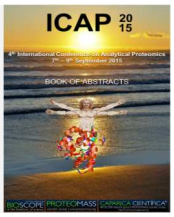

Special Issue: Proceeding Abstracts of the 4th International Congress on Analytical Proteomics (ICAP 2015)

A quest for innovative and robust biomarkers using MS-based proteomics

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Abstract

Purpose: The identification of novel biomarkers with proven clinical utility relies on a complex process composed of discovery, qualification, verification, and validation steps. Proteomic biomarker discovery has provided numerous candidates but only few of those have reached the validation stage and even less are used in today's routine clinical practice. Mass spectrometry (MS)-based proteomics is powerful enough to comprehensively characterize biological and clinical samples but offers limited sample throughput, mainly due to complex sample preparation upstream of MS. Proteomic biomarker discovery studies are therefore often performed with a too small number of samples, compromising study design. We have developed dedicated highly-automated workflows to facilitate, accelerate, and improve molecular phenotyping and translation of clinical proteomic discovery findings in human body fluids such as plasma and cerebrospinal fluid.

Experimental description: A highly automated MS-based proteomic discovery approach that comprises immuno-affinity depletion of abundant plasma proteins, buffer exchange, reduction/alkylation, tryptic digestion, isobaric labeling, sample purification and reversed-phase liquid chromatography tandem MS analysis was developed, characterized [1] and finally used to analyze cohorts of patients, ranging from 100 to 1'000 individuals [2].

Results: Analytical figures of merit, *e.g.*, precision and trueness, of our approach demonstrated increased robustness compared to to-date published manual or less automated workflows while ensuring sufficient throughput for large-scale studies. Application to clinical biomarker research programs in the fields of metabolic and brain health provided protein biomarker models for stratification of subjects and prediction of relevant clinical readouts.

Conclusions: Our results revealed that the analysis of large number of samples for biomarker discovery in clinical research using today's MS-based shotgun proteomics technology has become feasible. The analytical and biological consistency of our results regarding technical validation but also clinical research demonstrated the value of our approach.

Keywords: Biomarker; Body fluid; Blood; Clinical proteomics; Mass spectrometry; Obesity; Alzheimer's disease.

References:

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Special Issue: Proceeding Abstracts of the 4th International Congress on Analytical Proteomics (ICAP 2015)

Matrix metalloproteinases (MMPs) in buck blood chronically infected by arthritis encephalitis virus in Northeast of Brazil

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Abstract

The objective of this work was to investigate the activity of the MMPs (MMP-2 e MMP-9) in blood serum of chronically infected bucks by CAEV. The experiment was constituted by two groups (n = 5 each group). The first one was composed by five naturally infected bucks (4-5 years) and the second group constituted of five negative bucks (4-5 years) for CAE. Serology was performed using the Western Blotting (WB) and confirmed by Polymerase Chain Reaction (PCR). Blood samples were collected by puncturing the jugular vein from animals and evaluated by zymography (SDS-PAGE) using gelatin as substratum. The gel densitometry analysis showed higher activity ($P<0.05$) of the pro-MMP-2 and MMP-2 in seropositive group (Fig. 1). The higher activity of the MMPs (MMP-2 and MMP-9) in infected group to CAE suggests a role of these proteases in the chronic inflammatory process in goats, once the key role of MMPs molecules in inflammation are involved in patho-physiological remodeling of the vascular wall, even in animals without apparent disease.

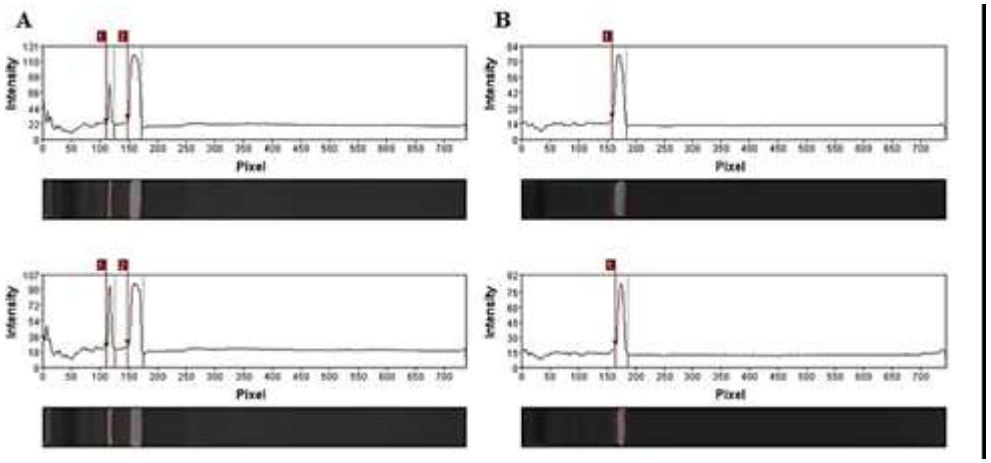

Fig.1 Densitometry of gels zymography showing the MMPs activity in seropositive (A) and seronegative (B) group for CAE.

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Identification of immunoreactive microbial proteins in the field of allergic diseases, and application for serodiagnosis

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Abstract

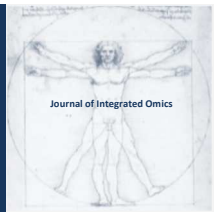
Purpose: Diagnosis of immunoallergenic pathologies due to environmental microorganisms includes detection of circulating specific antibodies. Immunoproteomics have proved to be useful for identifying the immunogenic proteins in several microorganisms linked to allergic diseases.

Experimental description: With this approach, the causative microorganisms are first isolated from the environment of patients. Then the proteins are separated by two-dimensional electrophoresis and revealed by Western blotting with sera of different patients suffering from the disease compared to sera of asymptomatic exposed controls. Immunoreactive proteins are identified by mass spectrometry. Identified immunoreactive proteins found to be specific markers for the disease could be subsequently produced as recombinant antigens using various expression systems to develop ELISA tests.

Results: Using recombinant antigens, standardized ELISA techniques can be developed, with sensitivity and specificity reaching 80% and 90%, respectively. Such techniques have been developed in our lab for diagnosis of Farmer Lung Disease (FLD), which is the most frequent form of occupational hypersensitivity pneumonia. Combinations of recombinant antigens from the 3 main micro-organisms involved in FLD (2 fungi and one actinomycete) are being tested at the moment in a multicenter study. We also have developed an ELISA test for diagnosis Machine Operator Lung, using 2 recombinant antigens from mycobacteria.

Conclusion: Immunoproteomics can be applied to any environmental microorganisms, with the aim of proposing panels of recombinant antigens able to improve the sensitivity and standardization of serologic diagnosis of hypersensitivity pneumonitis, but also other mold-induced allergic diseases such as allergic bronchopulmonary aspergillosis or asthma.

Keywords: allergy; immunoproteomics; serodiagnosis; recombinant antigens.



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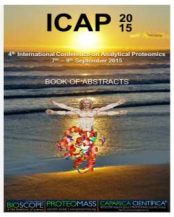

Special Issue: Proceeding Abstracts of the 4th International Congress on Analytical Proteomics (ICAP 2015)

Caenorhabditis elegans based proteomic analysis during bacterial infections

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Abstract

Purpose: Infectious disease caused by specific bacteria with increased resistance to available antibiotics raise alarm for better understanding of microbial infections and host susceptibility. Studying host-pathogen interaction is a complex process, the outcome can be defined using information from both host and pathogen perspective in different dimensions. Evolution of molecular tools have enabled to explore the bacterial virulence factors and host immune effector molecules providing in depth knowledge about infection and immune response. Further, suitability of model organisms and advancement of proteomics techniques shed light on host innate immune response against bacterial infections. *Caenorhabditis elegans* is a well-known model for studying bacterial infections for decades and enormous data has been generated using forward and reverse genetics in this model. Using *C. elegans* as a model, the host proteome changes against set of Gram negative pathogens including *Vibrio alginolyticus*, *Proteus mirabilis* and *Pseudomonas aeruginosa* were studied.

Experimental description: In our studies, regulation of *C. elegans* proteome was monitored against Gram negative bacterial infections using quantitative proteomic approach. Proteins were separated using two-dimensional differential gel electrophoresis (2D-DIGE) and differentially regulated proteins were identified using PMF and MALDI TOF-TOF analyses. The levels of expression of candidate proteins and their specific mRNA were validated using Western blot analysis and quantitative PCR, respectively. The interacting partners were shortlisted by bioinformatics tools and subsequently validated.

Results: Our results suggest that *C. elegans* displayed pathogen specific response by differentially regulating proteins against different pathogens. Identified proteins are found to be key regulators of essential pathways namely, unfolded protein response, MAP kinase and RAS signaling pathways.

Conclusions: For the first time our studies report the role of *C. elegans* proteins PDI-2, DAF-21 and EEF-2 in regulating host immune system against bacterial infections.

Keywords: Caenorhabditis elegans, Vibrio, Pseudomonas, Proteus, Proteomics.

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Quantitative analysis of the mitochondrial proteome and phosphoproteome in the yeast *Saccharomyces cerevisiae*

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Abstract

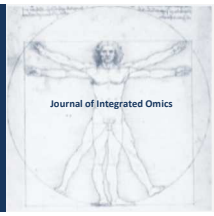
The mitochondrion is an organelle with multiple functions, the most important of these being to provide energy to the cell as a whole in the form of ATP, generated by oxidative phosphorylation catalyzed by the respiratory enzymes in the mitochondrial inner membrane. In humans, deregulation of mitochondrial functions, particularly with regard to the respiratory chain, is associated with several pathologies, including neurodegenerative diseases, neuromuscular diseases, type II diabetes and cancer. The activity of the respiratory enzymes may be modulated in response to metabolic demand and various types of stress. Several levels of regulation may be conceived, including changes in protein expression magnitudes, reversible interaction with effectors, and post-translational modifications, such as phosphorylation. The steadily increasing number of identified mitochondrial phosphoproteins suggests that reversible protein phosphorylation could be an important level of regulation in mitochondria. However, this hypothesis cannot be tested without quantitative data on variations in the abundance of mitochondrial proteins and their level of phosphorylation under different growth conditions. The yeast *Saccharomyces cerevisiae* is a powerful tool for studying various energetic and physiological states as it is a facultative aerobe which can grow on either fermentative or respiratory substrates.

We present for the first time a quantitative study of both protein abundance and phosphorylation levels in isolated yeast mitochondria under respiratory (lactate) and fermentative (glucose or galactose) conditions. To focus our analysis specifically on mitochondrial proteins, we performed a subcellular fractionation and used LC– MS/MS to overcome the limitations of 2D gel electrophoresis. Protein abundances were quantified using a label-free method. The phosphoproteome was analyzed quantitatively using the multiplex stable isotope dimethyl labeling procedure. For all quantified phosphopeptides, protein abundance was determined, allowing normalization of the data and permitting analysis of the specific variation of phosphorylation status independent of changes in protein abundance.

Label free quantitative analysis of protein accumulation revealed significant variation of 176 mitochondrial proteins including 108 proteins less accumulated in glucose medium than in lactate and galactose media. We also showed that the responses to galactose and glucose are not similar. Stable isotope dimethyl labeling allowed the quantitative comparison of phosphorylation levels between the different growth conditions. This study enlarges significantly the map of yeast mitochondrial phosphosites as 670 phosphorylation sites were identified, of which 214 were new and quantified. Above all, we showed that 90 phosphosites displayed a significant variation according to the medium and that variation of phosphorylation level is site-dependent.

This proteomic and phosphoproteomic study is the first extensive study providing quantitative data on phosphosites responses to different carbon substrates independent of the variations of protein quantities in the yeast *S. cerevisiae* mitochondria. The significant changes observed in the level of phosphorylation according to the carbon substrate open the way to the study of the regulation of mitochondrial proteins by phosphorylation in fermentative and respiratory media. In addition, the identification of a large number of newphosphorylation sites show that the characterization of mitochondrial phosphoproteome is not yet completed.

Keywords: Phosphoproteomic, Proteomic, Glucose, Galactose, Lactate, OXPHOS.



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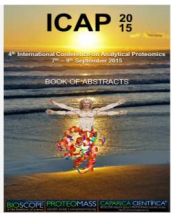

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Dynamism of protein expression in gametes and early mammalian embryos analysed by a holistic approach and multiplexed SRM

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Abstract

Purpose: Early embryogenesis is a highly critical period of mammalian development. Morphology of pre-implantation development has been well studied, but molecular processes, particularly at the level of proteins, are still poorly understood. To identify molecular key players during early development, we performed holistic proteome analyses using the bovine model, which shows crucial similarities to the human reproduction system. Using multiplexed SRM assays, the dynamism of protein expression during embryo development was precisely quantified during all characteristic stages from the oocyte to the expanded blastocysts.

Experimental description: In a holistic approach, four biological replicates of bovine embryos (100 per replicate) at the zygote, 2-cell and 4-cell stage and MII oocytes as a reference were quantitatively analysed by an iTRAQ LC-MS/MS approach on an Orbitrap mass spectrometer. Data processing and bioinformatics analysis comprised MASCOT v2.4, DAVID Bioinformatics Resources 6.7, Self-organizing tree algorithm (SOTA), Cytoscape 3.1.0 plug-ins ClueGO v2.0.8 and CluePedia v1.0.9. SRM based quantifications were performed using batches of 10 zygotes or embryos on a Q-Trap 5500 instrument. GraphPad Prism 6.04 was used for statistical analysis (multiple T-tests).

Results: Among 1072 proteins identified in the iTRAQ approach, 87 showed significant abundance differences in the four stages analysed. The proteomes of 2-cell and 4-cell embryos differed most from the reference MII oocyte. Relevant differences between transcript and protein abundance levels were detected, e.g., for WEE2. Especially affected during development are proteins of the p53 pathway, mitosis, energy and lipid metabolism, and members of "The unfolded protein response". By Principal Component Analysis (PCA), SRM quantifications comprising a panel of only five proteins were shown to discriminate between all four developmental stages analysed. Using an expanded 27-plex SRM assay, proteins were reliably quantified in nine developmental stages. Absolute protein contents were quantified for nine proteins (n=6).

Conclusions: Despite silence of transcription and translation, the proteome in zygotes and early embryonic stages is highly dynamic. SRM quantification in nine developmental stages revealed high-resolution profiles of the dynamic embryo proteome. Absolute quantification of nine developmentally relevant proteins by SRM revealed protein contents of low femtomole down to attomole range per embryo, and provides an essential data basis for systems biology oriented pathway modelling and prediction algorithms.

Keywords: embryo, oocyte, development, mass spectrometry, SRM, proteomics

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A new view of brain function: quantifying protein responses to learning and memory with reverse phase protein arrays

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Abstract

Purpose: Clinical trials with a diverse array of drugs designed to improve cognition in intellectual disability (ID) have been conducted, are in progress or are planned. Preclinical evaluations of these drugs are generally limited to the study of a single mouse model, use only male mice, and do not generate information on the molecular links between drug action and the functions of the genes causing ID. In addition, analysis of brain expression in learning/memory studies has been typically confined to screening of oligonucleotide arrays or measurement of fewer than 5-10 proteins. Use of quantitative proteomics in preclinical evaluation of drug responses would lead to understanding of the molecular basis of ID and its rescue with drug treatments and could contribute to design of more effective clinical trials.

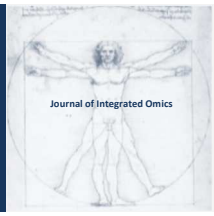
Experimental description: We use the technique of Reverse Phase Protein Arrays (RPPA) in which protein lysates are spotted robotically onto nitrocellulose coated microscope slides. A single slide can accommodate 4000 spots, sufficient for 200 samples in 3-4 replicates of a 5 point sample dilution series. Slides are screened with antibodies to proteins of interest in a protocol of blocking and washing similar to that used in Western blots. RPPA provides advantages of sensitivity, accuracy, throughput, and requirement of minimal sample volume. We investigate changes in protein expression in mouse models of neurological disorders. We generate protein profiles from multiple brain regions, in female and male animals, of different ages, with and without exposure to tests of learning/memory, with and without drug treatment. In a typical experiment, we measure levels of ~100 proteins/protein modifications, including 15 components of both MAPK and MTOR pathways, multiple subunits of glutamate receptors, apoptosis and immediate early gene proteins, in 20-100 mice. For this, RPPA is an ideal technique.

Results:(1) Molecular responses to tests of learning and memory and to drug treatments are more complex and involve a greater diversity of pathways and complexes than previously known. Responses do not propagate linearly within a pathway, likely reflecting the complexities of cross talk among pathways. (2) Gender differences are significant: in hippocampus of untreated control mice, levels of 40% of proteins are significantly higher in females than in males. (3) Gender differences are differentially perturbed in female and male mouse models of neurological disorders, e.g. in one mouse model, in female mice, 20% of hippocampal and 70% of cerebellar proteins are perturbed, but in male mice, only 40% and 20%, respectively, are perturbed. (4) A drug treatment that rescues a deficit in learning/memory does not normalize the protein profile, but instead creates a new pattern of protein expression compatible with successful learning.

Conclusions: RPPA facilitates accurate quantitation of several hundred proteins in hundreds of samples, thus capturing differences among many experimental variables, as well as identifying inter-individual variation. Preclinical evaluations of candidate drugs should include protein expression profiling, in multiple genetic models, in multiple tests of learning/memory, and most importantly, in both female and male mice. Such analyses could lead to identification of effective combinations of drugs with a greater probability of positive outcomes in human clinical trials.

Keywords: protein lysate arrays, antibody, MAP kinase, hippocampus, cerebellum, pathway analysis, mouse models

Acknowledgements: Funded by the National Institutes of Health (USA) and the Linda Crnic Institute for Down Syndrome (University of Colorado School of Medicine).



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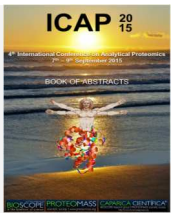

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Dysregulation of IFN alpha and IL-10 in central nervous system in transgenic porcine Huntington's disease model

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Abstract

Purpose: Huntington's disease (HD) is an inherited neurodegenerative disorder which is progressive and fatal. Any preventive or disease-modifying therapies are not available as yet, hence a better understanding of the earliest changes in brain as well as peripheral cells could be crucial for development of new therapeutic approaches aimed at elimination or repair of the impairing changes caused by huntingtin mutation.

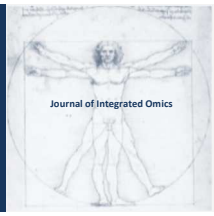
Experimental description: we utilised transgenic porcine HD model bearing an N-terminal fragment of human mutant huntingtin (mHTT) to profile cytokine levels in cerebrospinal fluid, serum and secretomes of microglial cells and blood monocytes in the course of HD using bead based multiplexing Luminex xMAP technology.

Results: among the most pronounced changes was the decline of IFNα in CSF of transgenic animals and this was observed from very early time intervals of HD. IFNα was also decreased in secretome of microglial cells but not blood monocyte in transgenic animals. In addition, IL-10 was lower in CSF as well as microglia secretome. On the contrary, elevated levels of pro-inflammatory IL-1β and IL-8 were produced by microglial cells of transgenic animals. We further demonstrated higher proportion of the mHTT related to endogenous HTT in microglial cells compared to blood monocytes in transgenic minipigs which may have a causative impact on cytokine production.

Conclusion: in this study, we revealed emerging role of IFNα and IL-10 in central nervous system inflammation and immune response imbalance in HD progression.

Keywords: porcine Huntington's disease model, IFNα, IL10, multiplexing, cerebrospinal fluid, microglial cells.

Acknowledgements: this work was supported by the CHDI Research Agreement A-8248, the Operational Program Research and Development for Innovations (EXAM; CZ.1.05/2.1.00/03.0124) and Institutional Research Concept RVO67985904 (IAPG, AS CR, v.v.i). Thermo Fisher Scientific partnered this study to further evaluate the Swine Cytokine Magnetic 7-plex Panel (Invitrogen ™ LSC0001M, Thermo Fisher Scientific Inc., formally LSC0001 from Life Technologies) as well as provided scientific, experimental and statistical analysis support including the algorithm used in this study for data evaluation.



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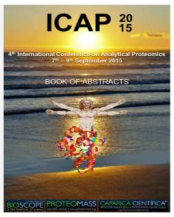

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Targeted quantitative proteomics in pharmaceutical sciences

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Abstract

Purpose: Drug metabolizing enzymes (DMEs) and drug transporters (DTs) are important determinants of drug efficacy and adverse effect. Liver is the major organ of drug disposition and hepatic cytochrome P450 enzymes (P450s), flavin-containing monooxygenases (FMOs), and sinusoidal membrane transporters (e.g., OATP1B1 and OCT1) have clinically significance in mediating drug-drug interactions. The goal of this study was to develop LC-MRM-based targeted proteomic methods for absolute quantification of P450s, FMOs, OATP1B1 and OCT1 in human liver samples and evaluate the protein quantification results with orthogonal assays.

Experimental description: Several panels of individual donor human liver microsomes (HLMs) were either prepared in house or purchased from commercial vendors and used for P450 and FMO quantification. Total membrane proteins were extracted from individual donor human liver tissues using the ProteoExtract Native Membrane Protein Extraction Kit. Signature (surrogate, proteotypic or AQUA) peptides were selected in silico based on previously reported criteria and at least two signature peptides were chosen for each protein. Protein samples were reduced, alkylated, and trypsin-digested using optimized protocols. Absolute protein quantification was achieved by using two types of calibration standards. One contained trypsin digests of recombinant proteins of known concentrations and the other contained synthetic signature peptides of known concentrations.

Results: LC-MRM-based targeted proteomic methods were developed for quantification of human hepatic DMEs (CYP3A4, CYP3A5, CYP3A7, CYP2C9, CYP2C19, CYP4F2, CYP4F3B, FMO1, FMO3, and FMO5) and transporters (OATP1B1, OATP1B3, OATP2B1, OCT1 and P-gp). The absolute protein concentrations of these proteins were determined using HLMs or total membrane proteins. CYP4F enzymes contributed significantly (~15%) to the overall P450 content in HLMs. Ethnic and age-dependent variability in the abundance of these proteins were also observed, e.g., fetal-adult developmental switch between CYP3A4 and CYP3A7, as well as between FMO3 and FMO1. Absolute quantification using protein digests as standards conferred good coherence between different signature peptides of the same protein, whereas signature peptide-dependent quantification was observed using synthetic peptide standards.

Conclusions: LC-MRM-based targeted proteomics provide a multiplexed quantification strategy for DMEs and DTs. Absolute protein quantification of these proteins are expected to improve our understanding of the underlying causes of interindividual variability of drug response. In addition, determination of absolute protein concentrations in human tissues and surrogate models (e.g., sandwich-cultured human hepatocytes and liver-on-a-chip) will enable physiologically-based pharmacokinetic (PBPK) modeling to predict drug exposure in humans prior to clinical trials.

Keywords: targeted proteomics, quantitative pharmacology, drug metabolizing enzymes, drug transporters, quantitative pharmacology

Acknowledgements: This work was supported in part by research grants from Eli Lilly & Co. and NIH.

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Quantitative mass spectrometric measurement of multiple serum apolipoproteins: ready for validation and clinical application

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ABSTRACT

Purpose: Direct High and Low Density Lipoprotein cholesterol (HDL-C and LDL-C) tests are currently used for cardiovascular disease (CVD) risk classification but suffer from serious drawbacks, especially in dyslipidemic patients [1]. Therefore, we aimed to develop an alternative mass spectrometry (MS) based method for absolute and direct quantitation of the well-defined, clinically relevant serum apolipoprotein counterparts. The MS-based method should have an analytical performance in line with quality specifications, derived from biological variation.

Experimental description: Sample preparation was performed using a modification of the protocol described earlier for apo A-I and B100 [2] using deoxycholate for protein denaturation. Two proteotypic peptides were selected for each of the apolipoproteins (apo) A-I, B100, C-I, C-II, C-III, E as well as additional peptides to distinguish the apo E2, E3 and E4 isoforms [3]. All peptides were detected by multiple reaction monitoring using a 1290 LC-system coupled to a 6490 triple quadrupole mass spectrometer (Agilent) [2,4]. Stable isotope labeled peptides were added before digestion for internal standardization. External calibration was performed using commutable, serum based calibrators. In case of apo A-I and B100, traceability to WHO-IFCC international reference standards is established (www.bipm.org/jctlm). The method is validated according to Clinical and Laboratory Standards Institute guidelines EP15-A and EP9-A2, and the different steps outlined in the EAS-EFLM framework [5]-

Results and Conclusions: Apo A-I and B100 concentrations can be measured by MS with imprecision below the minimal specifications of 5.0 and 5.3 %, respectively [2]. The apo C-II, C-III and E protein concentrations could be measured with imprecisions < 9.2%. LC-MS/MS quantitation of apo A-I, B100, C-II, C-III and E in 54 normo-and 46 hypertriglyceridemic (NTG and HTG) samples correlated well with immunoturbidimetric assays (R2 > 0.96). Good correlations were found between the two peptides of these proteins (R2 > 0.95), and apo E2 and E4 isoforms could be distinguished from the most common E3 isoform. Our method thus allows individual detection and quantitation of six apolipoproteins with additional apo E phenotyping. Using a Bravo workstation and a fully automated protocol for sample preparation about 80 specimens can be analysed in singlicate per day.

We conclude that our multiplexed apo method is ready for clinical application, both in routine patient care and in clinical or epidemiological studies. Its added value compared to conventional serum HDL-C/LDL-C tests for CVD risk assessment and/or treatment monitoring is currently subject of clinical validation studies.

Keywords: apolipoproteins, cardiovascular disease, traceability, multiple reaction monitoring

Acknowledgements: We thank Agilent Technologies for support and fruitful discussions .

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Proteomic analysis and μ-, δ- and κ-opioid receptor content in brain cortex of rats exposed to increasing doses of morphine for 10 days; comparison with animals sacrificed 20 days since the last dose of morphine

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Abstract

Purpose: The aim of this work was to detect the morphine-induced change of μ-, δ- and κ-OR in rat forebrain cortex as well as the alteration of the total spectrum of proteins by this drug when acting for long period of time.

Experimental description: Rats were exposed to increasing doses of morphine for 10 days (10-50 mg/kg) and sacrificed 24 hours (group +M10) or 20 days (group +M10/ - M20) after the last dose of this drug (in parallel with control animals: groups - M10 and - M10/ - M20). The post-nuclear supernatant fraction (PNS) was analyzed for the content of μ-, δ- and κ-opioid receptors by 1D-SDS-PAGE performed under the non-dissociated (- DTT) and dissociated (+DTT) conditions. The individual receptor subtypes were recognized by immunoblot detection with specific antibodies. PNS fraction was also resolved by 2D-ELFO and analyzed by MALDI-TOF MS/MS and *label-free* quantification using the MaxLFQ intensity determination and normalization procedure introduced by Cox et al. in 2014.

Results: (A) The total amount of μ-OR, δ-OR and κ-OR was unchanged by morphine when resolved by 1D-SDS-PAGE under the non-dissociated conditions (- DTT). However, the resolution under the dissociated conditions (+DTT) revealed the presence of multiple protein bands exhibiting the wide range of $M_r \approx$ 20-106 kDa. All these immunoblot signals were also unchanged with the exception of minor isoforms of μ-OR (Mr ≈ 20-32 kDa) and δ-OR (Mr ≈ 68 kDa, ± DTT) which were decreased in morphine-treated samples, $p<0.05$. Determination of Na,K-ATPase indicated no difference between control and morphine-treated samples of PNS. This result was verified by binding of selective inhibitor of this enzyme, [^{3}H]ouabain. Noticeably, our data indicated a significant increase of Caveolin-1, $p<0.05$, but no change of Caveolin-2. (B) Proteomic analysis of the total protein composition by MALDI-TOF MS/MS indicated the morphine-induced change of 27 proteins (+M10). Number of altered proteins was decreased to 14 in rats sacrificed 20 days since the last dose of morphine (+M10/ - M20). The high resolution by *label-free* quantification identified 118 altered proteins (+M10) and this number was decreased to 41 (+M10/ - M20).

Conclusions: The morphine-induced decrease of μ-OR and δ-OR in rat forebrain cortex was surprisingly small; κ-OR was unchanged. Contrarily, depending on sensitivity of method used for proteomic analysis, the 27 (MALDI-TOF MS/MS) or 118 (*label-free* quantification) proteins were altered after 10 days of morphine exposure. In rats sacrificed 20 days after the last dose of morphine, these numbers were decreased to 14 and 41, respectively.

Keywords: morphine, long-term exposure, rat forebrain cortex, post-nuclear supernatant fraction, μ-, δ- and κ-opioid receptors, 1D- and 2D-SDS-PAGE, MALDI-TOF MS/MS, label-free quantification, MaxLFQ.

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Advantages and disadvantages of chromatographic approaches to study bacterial metalloproteomes

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ABSTRACT

Purpose: Although it is now clear that at least a third of all proteins require a metal cofactor for function, it is still not straightforward to predict metal requirements and binding sites of proteins. This is especially true for the plethora of "hypothetical proteins" emerging from genome sequencing that have no biochemically characterised homologues. Indeed, a previous study concluded that "microbial metalloproteomes are largely uncharacterized" [1]. Analytical approaches are therefore needed to begin filling the gaps [2]. We are particularly interested in bacterial zinc proteomes [3], and have been exploring chromatographic separation techniques to fractionate metalloproteomes. This talk will briefly summarise the major conclusions from our work so far.

Experimental description: The marine cyanobacterium *Synechococcus* sp. WH8102 was cultured in artificial seawater. Cells were harvested and lysed, and lysates were fractionated by different chromatographies (size exclu-sion, anion exchange, IMAC). Fractions were analyzed by 1D SDS-PAGE, peptide mass fingerprinting, and ICP-MS.

Results: Separation by 2D orthogonal chromatography did not lead to the identification of major zinc-requiring proteins. Instead, most intracellular zinc was found in fractions with high levels of phycobilisome proteins. The latter are highly abundant in cyanobacteria, and not only caused problems with the detection of lower abundance proteins, but were also likely to lead to zinc re-distribution [4]. In contrast, similar approaches were able to detect intact Ni-superoxide dismutase [5], highlighting the differences in the bio-coordination chemistry of these two metal ions. Further experiments employing immobilised zinc affinity chromatography led to enrichment of several proteins with zinc-binding ability, including some unexpected, highly interesting new candidates.

Conclusions: Even the mildest separation conditions may disturb cellular zinc speciation, and care must be taken to avoid misinterpretation of analytical results.

Keywords: metalloproteomics, zinc, 2D-LC, ICP-MS.

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Mechanisms underlying metabolic disorders deciphered using quantitative proteomics of non-sequenced species

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ABSTRACT

Purpose: Since the last few years, there is growing evidence that important questions related to human health may simply not be answered by only studies in the mainstream of laboratory murine models. In this context, there is an increasing interest on wild animals as a source of new information. The aim is here to present examples where proteomics applied to exotic species can help understanding how certain animals do support environmental conditions that would be detrimental to human health.

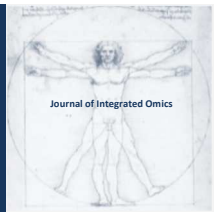
Experimental description: We examined the impact of different conditions (e.g. food scarcity, weightlessness and physical inactivity, high -fat diets) on the proteome of various tissues collected from laboratory rodents and also from wild penguins, space-flown mice, wild bears and selected lines of voles. Because no protein or even genomic sequence was available for most of these species, we performed both classical database searches and de novo sequencing followed by Blast searches to identify a maximum of proteins. Quantitative proteomics was then achieved using a combination of complementary approaches, including 2D-DIGE/MS and label-free MS-based proteomics (spectral counting and/or on the basis of XICs).

Results: In non-sequenced species, the use of de novo sequencing allowed identifying 20% more proteins in comparison with the use of only classical Mascot searches. To obtain this result, de novo sequencing was performed only from high quality spectra as filtered using the Recover module of our software suite (https://msda.unistra.fr/). In any studied species, hundreds to thousands of proteins were identified (FDR<1%) then quantified, and automatically classified according to their function. We notably found drastic differences in the plasma proteome of breeding penguins vs. that of laboratory rats in response to prolonged fasting, in the muscle proteome of mice after a 30-day space flight vs. that of bears after 5 months of physical inactivity, and in the brown adipose tissue (BAT) proteome of voles selected for their BAT activity level (high vs. low) in response to a high-fat diet. These proteomic data provided essential metabolic insights in mechanisms related to modern human pathologies, including undernutrition, muscle atrophy, obesity and diabetes.

Conclusions: Studying diversity of animal adaptations/responses to environmental constraints can provide new basic knowledge of importance for biomedicine. To this end, de novo sequencing and quantitative proteomics strategies offers unique possibilities to provide a number of consistent results.

Keywords: exotic species, de novo sequencing, quantitative proteomics, functional annotations, pathway analysis

Acknowledgements: This work was funded by the Centre National d'Etudes Spatiales (CNES), the CNRS and Strasbourg University (H2E project; IDEX UNISTRA) and the French Proteomic Infrastructure (ProFI; ANR-10-INSB-08-03).



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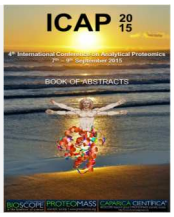

Special Issue: Proceeding Abstracts of the 4th International Congress on Analytical Proteomics (ICAP 2015)

Aptamers in cancer research

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Abstract

Introduction: Along with the generation of the term "magic bullet" by Ehrlich a great concept arises of a cancer treatment method, where only tumor cells would be killed by an ideal therapeutic agent.

The targeted therapy has become a main goal for conquer cancer. Most therapeutic agents in current clinical use, such as chemotherapy, radiotherapy, and immunotherapy are not specific for the molecules responsible for human disease, thus distribution of the drug in the organism results in unwanted side effects. Targeted treatment is based on specific ligand recognition.

Aptamers are short single-stranded nucleic acids with a defined three-dimensional shape that allows them to interact with high affinity with a target molecule. They have advantage that they do not induce immunological response. The low immunogenicity and cellular-targeting properties, together with the fact that they can be chemically synthesizes in a vast amounts might result in a suitable drugs with low side effects. They can be used for identification of cell-surface molecules associated with a pathogenic state of a cell.

Purpose: Identification of proteins targeted by aptamers in cancer cells.

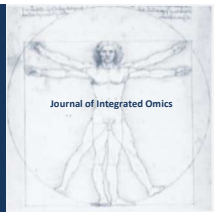
Experimental description: We introduced the proteomic studies of aptamers targets in cancer cell lines PC3 and MCF7. We applied biotinylated forms of aptamers characterized by the strong affinity to streptavidin to isolate target proteins. We have performed traditional bottom-up proteomic approach to analyze the group of proteins that bind to A26 and A33 aptamers that can serve as potential metastases molecular markers and targets for selective chemotherapy. We have found that cell surface receptors and proteins localized inside the cells are capable of binding A26 and A33.

Results: Molecular targets of aptamers in cancer cells were identified. Based on the presented research we indicate the importance of the applicability of aptamers in many technologies including selective diagnostics and therapeutics

Conclusions: Aptamers can be considered as effective tools, since they possess unique properties to aid in cancer diagnosis, prevention, and treatment.

Keywords: aptamer, proteomics, cancer.

Acknowledgements : This work was supported by the grant "META" No. 5/EuroNanoMed/2012



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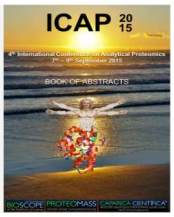

Special Issue: Proceeding Abstracts of the 4th International Congress on Analytical Proteomics (ICAP 2015)

Quantitative proteomics in human drug metabolism

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Abstract

Purpose: The aim of this study was to develop reproducible, accurate methods for determining the concentrations of enzymes and transporters involved in human drug metabolism.

Experimental description: We have developed QconCAT-based LC-MS/MS methodology for quantifying the cytochrome P450 and UGT enzymes and the drug transporters in human tissue. The QconCATs (artificial proteins consisting of labelled standard peptides, released by proteolysis) initially did not express in E. coli and methods for expressing recalcitrant QconCATs were developed as a part of the project. Specifically, we fused the two QconCATs (MetCAT and TransCAT which quantify enzymes and transporters respectively) to proteins with known characteristics. The MetCAT was fused to a QconCAT protein, known to express in inclusion bodies in high yield, whereas the TransCAT was fused to a soluble construct and expressed in cytosol.

The MetCAT has now been used to quantify the enzymes in a panel of 24 livers, and the TransCAT has been used to quantify transporters in four intestinal samples. We have also analysed three liver samples using label-free methodology.

Results: Cytochrome P450 and uridine 5'-diphospho-glucuronosyltransferase enzymes are responsible for much of the metabolism of drugs in the liver, intestine and other tissues. There is, however, substantial inter-individual variability in their concentrations, as well as variation between groups of individuals (ethnic groups, age groups). Transporter proteins are responsible for further variations in how a given dose of a drug may lead to different plasma and tissues in different people and groups.

Our results show wide variation in a population between the levels of the different enzymes, for example CYP3A4, which metabolizes many important drugs, is present with a range 10.4–262.1 pmol mg^{-1} protein. We determined several correlations between enzymes, including a positive correlation between levels of CYP3A4 and CYP2B6.

Most recently, label-free quantification has been shown to give broadly comparable results and, in addition, to give quantification data on other proteins in the samples.

Conclusions: Personalized medicine is crucially dependent on an understanding of the concentrations of drug-metabolizing enzymes and transporters in human tissue. Quantitative LC-MS/MS is a powerful tool for understanding these enzymes and transporters, and QconCAT and label-free quantification methods deliver complementary but consistent information.

Keywords: QconCAT, label-free, quantification (quantitation), cytochrome P450, UGTs.

Acknowledgements: Mass spectrometry was performed at the Michael Barber Centre for Mass Spectrometry, University of Manchester.

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Scorpion venom proteomics and transcriptomics

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ABSTRACT

Background: Scorpions (Arthropoda, Arachnida) occupy vast territory of the world and their ability to produce and deliver venoms is an essential factor in this success. Scorpion venom is a very complex mixture of substances, where peptides are the major constituents with various biological and pharmacological properties. Excluding the phenomena of intraspecific diversity, it is estimated that more than 150,000 different peptides/proteins expressed in the venom glands of 1500 different scorpion species.

Experimental description: During the last decade, the cutting edge OMIC technologies of proteomics and transcriptomics have been largely employed to deeply understand structure and molecular diversity of animal venoms from various species including scorpions.

Results: To date, using proteomic analyses, there are 5314 unique molecular masses have been identified from the venom of 27 different scorpions. Mass fingerprinting data clearly showed remarkable differences in the number of venom molecules (ranging from 60 to 665) between scorpion species. Few of the 5314 molecules were fully characterized which mainly belonging to neurotoxins, antimicrobial peptides and bradykinin potentiating peptides. Also, the venom gland proteome of 20 different scorpion species has been revealed using a combination of transcriptomic and bioinformatics methods and resulting in about 73 thousands ESTs. These sequences are corresponding to neurotoxins, antimicrobial peptides, housekeeping proteins, hypothetical proteins and a large number of unassigned types of scorpion venom peptides/proteins.

Conclusions: The main advantages of using transcriptomics in scorpion venomics are: (i) adding new insights about biological processes taking place in scorpion venom glands; (ii) facilitating the identification of various novel biologically active peptides and (iii) drawing an accurate image about interspecific and intraspecific venom diversity. Recent studies of scorpion venomics will be discussed, with special focusing on antimicrobial peptides derived from scorpion venoms.

Keywords: Scorpion venomics, Proteomics, Transcriptomics, Venom gland, cDNA library, Antimicrobial peptides.

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Comparative proteomic profiling of brain tissues of rat with different behavioral characteristics during emotional stress.

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ABSTRACT

Purpose: Evidence from animal studies has demonstrated that the limbico-reticular complex is initially involved in emotional responses. The amygdale and hippocampus are the most multifunctional structures of the limbic system. The reticular formation is directly connected with the emotions due to promote new and significant information to the different brain regions. To identify the differences of proteomic expression in rat brain caused by emotional stress we performed comparative proteomic analysis.

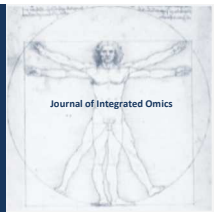
Experimental description: Wistar male rats were divided on 2 groups of behaviorally passive and active animals. Immobilization of rats during 12h (from 9p.m. till 9a.m.) served as a model of acute emotional stress. The protein expression profiles of hippocampus, amygdale, reticular formation and cortex were studied using by two-dimensional electrophoresis and MALDI-TOF.

Results: The proteomic analysis showed the down-regulated expression of GFAP, septine-5, calreticulin, alpha-synuclein, phosphatidylethanolamine-binding protein 1, thiomorpholine-carboxylate dehydrogenase, prohibitin, glutamine synthetase and up-regulated expression of lactate dehydrogenase, F-actin-capping protein subunit alpha-2 in different parts of brain depending on behavioral type of rats and the stage of stress.

Conclusions: Thus, individual behavioral features affect the specific pathway of organism response to the stress and determine an adaptive potential of the organism.

Keywords: brain, emotional stress, comparative proteomics, behavior

Acknowledgements: Prof. S.Pertsov from P.K.Anokhin Research Institute of Normal Physiology, Moscow, Russia.



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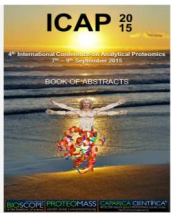

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Cell-free lysates for the production of difficult proteins

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Abstract

Purpose: Cell-free production of proteins and other biomolecules has emerged during the last decade as an important core technology in the new field of synthetic biology. Lysates of either eukaryotic or prokaryotic organisms can be employed for the synthesis of particularly difficult proteins such as membrane proteins, large multisubunit assemblies or highly oxidized proteins. Cell-free expression provides an open and accessible reaction environment. Additionally, functional properties of the lysate proteomes can easily be modulated in order to generate ideal conditions for the production of high quality samples of individual target proteins.

Experimental description: Protocols for the preparation of cell-free lysates have been developed and optimized. Libraries of additives have been screened for the manipulation and optimization of lysate proteomes.

Results: We present strategies for the manipulation of cell-free lysates and reaction conditions for the efficient production of difficult proteins such as G-protein coupled receptors or enzymes. We further exemplify the optimization of protein sample quality by directed fractionation of lysate proteomes and selective inactivation of particular enzyme classes and pathways. Examples focusing on the structural and functional characterization of membrane proteins will be discussed.

Conclusions: Lysate proteomes for the cell-free production of biomolecules can be adjusted according to the requirements of individual targets.

Keywords: cell-free expression, bacterial lysates, membrane protein environments, synthetic biology, artificial proteomes.

Acknowledgements: The work was funded by the Collaborative Research Center (SFB) 807 of the German Research Foundation (DFG) and by the German Ministry of Education and Science (BMBF). Support was further obtained by Instruct, part of the European Strategy Forum on Research Infrastructures (ESFRI).

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Linear Algebra Analysis and Quantification of Arginine Dimethylation in Histone Modification using Liquid Chromatography–Tandem Mass Spectrometry-Based Targeted Proteomics

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ABSTRACT

Purpose: Protein methylation at arginine residues is a prevalent post-translational modification in eukaryotic cells that has been implicated in processes from RNA-binding and transporting to protein sorting and transcription activation. Three main forms of methylarginine have been identified: NG-monomethylarginine (MMA), asymmetric NG,NG-dimethylarginine (aDMA), and symmetric NG,NG-dimethylarginine (sDMA). Current methods are able to predict and determine thousands of methylargnine sites, whereas stoichiometric distinction and quantification of these methylarginine forms, especially aDMA and sDMA are still challenging.

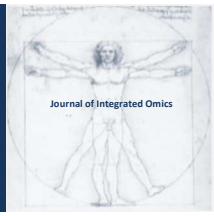
Experimental description: Liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS)-based targeted proteomics is emerging as a promising technique for site-specific quantification of protein modification using proteolytic peptides as surrogates of proteins. However, the routine targeted proteomics assay can not easily distinguish the contribution of aDMA and sDMA. In this study, linear algebra algorithms were employed as an add-on to targeted proteomics to retrieve information on individual dimethylarginine peptides.

Results: A LC–MS/MS-based targeted proteomics assay was first developed and validated for each dimethylarginine peptide. The linear algebra analysis of aDMA and sDMA was achieved from LC-MS/MS of their common spectra using an internal standard and a calculated response factor for each peptide. Finally, we applied this approach to determine the stoichiometry of histone methylation in breast cancer cells and tissue samples

Conclusions: LC –MS/MS-based targeted proteomics assay combined with linear algebra algorithms has a potential for simultaneously determining various methylated forms in protein modification.

Keywords: Protein methylation, aDMA, sDMA, histone, linear algebra, liquid chromatography–tandem mass spectrometry, targeted proteomics

Acknowledgements: The National Natural Science Fund (21175071), the project sponsored by SRF for ROCS, SEM (39), the Jiangsu Six-type Top Talents Program (D), and the Open Foundation of Nanjing University (SKLACLS1102) awarded to Dr. Chen.



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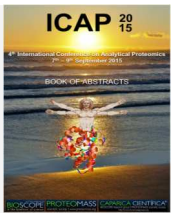

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Integrated Proteomic and Metabolomic Approaches Reveal Energy Disruption in Epilepsy

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Abstract

Purpose: Epilepsy affects ~ 1% of the human population and despite extensive study, the precise nature of epilepsy is poorly understood. Recently we have evaluated the levels and types of post-translational modifications (PTMs) of proteins in surgically removed epileptic brain tissue, which includes seizure onset, irritative and silent zones. The overall goal of this study was to identify enzymes and metabolites responsible for asynchronization of energy metabolism.

Experimental description: Tissue used included surgical resections from patients with epilepsy as well as a Kv1.1 KO mouse model of familial epilepsy. Non targeted lipid analysis was carried out on a Bruker Solarix FT-ICR by LC/MS. Amino acid analysis was performed using the aTRAQ method on an ABSciex 4000 Q-Trap. Proteome screening for oxidative modification (carbonylation) was performed using 2-D DIGE followed by peptide analysis on a Thermo Velos Pro LTQ. Adenosine energy metabolites were determined through etheno adduct formation and fluorescent detection by HPLC.

Results: Our data revealed tissue specific accumulation of oxidatively damaged proteins in the seizure onset zone. The most significant protein affected was identified as GAPDH, a key enzyme of energy metabolism interconnecting glycolysis (GP), pentose phosphate pathway (PPP) and tricarboxylic acid cycle (TCA). Moreover, higher levels of oxidative stress were observed in onset zones. Together with fact that epilepsies which respond well to the ketogenic diet (KD) have impaired glucose uptake our data suggests that higher level of oxidative stress impacts GAPDH activity, thus reducing levels of TCA intermediates and resulting in temporal asynchronization of GP, PPP and TCA, ultimately decreasing ATP levels. This imbalance can trigger an epileptic seizure. Seizures, on the other hand, can be prevented by supplying cells with TCA intermediates or their precursors such as ketone bodies in KD treatment.

Conclusions: Energy and redox balance is primarily controlled by GP, PPP and TCA. Under normal conditions glucose is transformed to pyruvate by GP, producing a small amount of ATP. The vast majority of ATP is generated in mitochondria through TCA and oxidative phosphorylation which utilizes pyruvate as a substrate. Under oxidative stress, glucose transformation is diverted from GP to PPP resulting in an increase in cellular reducing power through increased NADPH formation. Products of PPP are linked to GP through the enzyme GAPDH. Our data indicates that higher levels of oxidative stress partially inactivate GAPDH, a key enzyme interconnecting GP, PPP and TCA. This reduces the levels of metabolic intermediates available for TCA, leads to asynchronization of GP, PPP and TCA, and to a temporal decrease in ATP levels. Imbalance in the pathways involved in energy metabolism and redox balance, together with low ATP levels, result in neuronal network hyperexcitability. This phenotype can be reduced or prevented by supplying cells with TCA intermediates or their precursors, such as the case in KD treatment.

Keywords: Epilepsy, Energy metabolism, Oxidative damage.

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Methods for the preparation of acute myeloid leukemia patient samples for proteomic and phosphoproteomic analysis

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ABSTRACT

Purpose: The study of global protein expression in acute myeloid leukemia (AML) patients by mass spectrometry (MS) can help identifying differential expression and post-translational modifications of proteins that could represent disease-related biomarkers for early diagnosis or for improved prognostics and to predict the patient response to different therapeutics. To optimize the proteome and phosphoproteome coverage of samples from AML patients by LC-MS/MS analysis, we have tested several methods with different peptide fractionation and phosphopeptide enrichment strategies.

Experimental description: Peptide samples were prepared with an in-solution digestion protocol, using urea or guanidinium hydrochloride as denaturant, and with the filter aided sample preparation (FASP) methodology. Peptide fractionation was carried out with reverse phase/strong cation exchange (SCX) and SCX disks in a stage-tip. Techniques for phosphopeptide enrichment included metal oxide affinity chromatography (MOAC), immobilized metal affinity chromatography (IMAC) and sequential elution from IMAC (SIMAC).

Results: From an initial screening of the different samples on a Linear Trap Quadrupole (LTQ) Orbitrap Elite MS, we found the best proteome coverage with the sequential FASP method, with Lys-C and trypsin as digestion enzymes, which identified and quantified 3100 proteins from 20 µg of sample. With the same strategy, followed by a separate MOAC/TiO2-beads enrichment of the two peptide pools, we identified and quantified 2.900 phosphorylation sites from only 250 µg of AML patient sample. On a QExactive HF hybrid Quadrupole-Orbitrap MS, 5400 proteins and 4000 phosphosites were identified and quantified from these FASP-prepared samples.

Conclusions: To improve the proteome and phosphoproteome coverage of AML patient samples by MS analysis to discover new biomarkers, testing of different methods can be beneficial. Based on sample preparation optimization in our laboratory, we have chosen the FASP protocol to prepare AML patient samples for MS-based proteomic and phosphoproteomic studies.

Keywords: AML, proteomics, phosphoproteomics, sample preparation.

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Comparison of 2D proteomic maps revealed properties of *Ambrosia artemisiifolia* sub-pollen particles accounting for more severe asthma symptoms than its whole pollen grains

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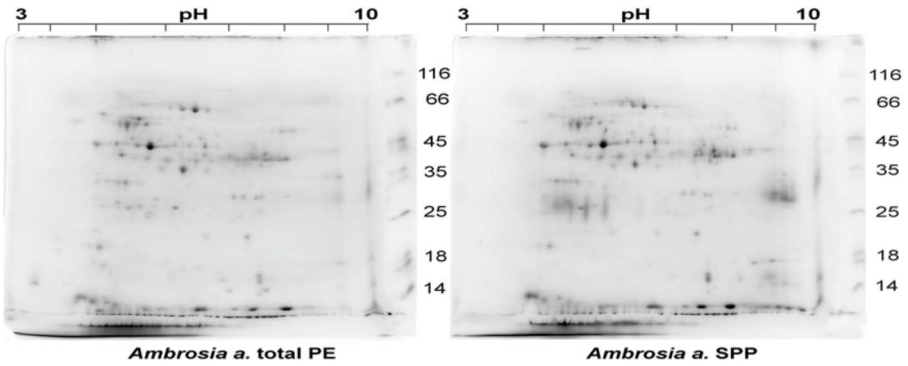
ABSTRACT

Aims and scopes: It is known that sub-pollen particles (SPP) cause more severe symptoms of asthma than its whole pollen grain counterparts, due to its smaller size and ability to penetrate deeper into the lungs. To reveal other possible causes of such more severe asthma symptoms induced by *Ambrosia artemisiifolia* SPP, its sub-pollen particle and pollen grain proteomes were characterized and compared.

Experimental description: Protein extract of short ragweed (*Ambrosia artemisiifolia)* pollen and its SPP were prepared and subjected to denaturing 2-D electrophoresis. Pollen proteome spots were excised after colloidal coomassie blue brillinat (cCBB) staining and in gel digested for liquid chromatography coupled with high resolution LTQ Orbitrap XL hybrid mass spectrometry. Parallel to that, cCBB stained gels were analyzed and quantified with laser scanner Typhoon 7000 series and Image 2D Master Platinum 7.0 software (GE Healthcare, USA).

Results: There is statistically significant difference between the contents of major allergen Amb a 1.05 subgroup in the ragweed whole pollen grains and SPP, the latter being richer in Amb a 1.05 (2 times), in major allergen Amb a 11 (5 times), in minor allergens Amb a 4 (7 times) and Amb a 6 (4 times). The 30 kDa basic antigen group in SPP (8 times more abundant) needs further investigation.



Conclusions: Beside its smaller size and hence easier penetrability, short ragweed SPP possess significantly higher load of major *Ambrosia artemisiifolia* allergens, Amb a 1.05 and Amb a 11, minor allergens Amb a 4 and Amb a 6 which could contribute to more severe asthma symptoms caused by SPP.

Keywords: Ambrosia pollen, sub-pollen particles, short ragweed, asthma, Amb allergens.

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Effect of a quorum sensing inhibitor on the Pseudomonas aeruginosa PAO1 proteome

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Abstract

Purpose: Research in the field of quorum sensing (QS) inhibition resulted in the identification of numerous QS inhibitors (QSIs). But their mode of action and impact on proteome of bacterial pathogens remains unclear. Hence, the aim of the present study is to find out the molecular mechanism of QSI and its effect on cytoplasmic proteome of *P. aeruginosa* PAO1.

Experimental description: Cytoplasmic proteins of *P. aeruginosa* PAO1 grown in the absence and presence of 5 µg of QSI were extracted by sonication. For 2DE, 600µg each of cytoplasmic proteins from control and treated samples were subjected to isoelectric focusing using immobilized pH gradient (IPG) strips (18 cm, pH 3-10 NL (GE Healthcare)) at 20°C using standard parameters for 18 h. Then the IPG strips were reduced and alkylated for 20 min. After this step, strips were placed on 12 to 15% gradient sodium dodecyl sulphate-polyacrylamide gels (Ettan DALT six, GE Healthcare) for the second dimension and electrophoresis was performed. Protein spots were visualized by colloidal Coomassie brilliant blue G-250. The gels were scanned with Image scanner III (GE Healthcare) and the raw images were analyzed using ImageMaster 2D Platinum 7. Experiments were performed in biological triplicates. More than two fold differentially regulated protein spots were selected for in-gel trypsin digestion and analyzed using MALDI TOF/TOF (AXIMA Performance, Shimadzu.Biotech). The proteins were identified by peptide mass fingerprinting using the software tool MS-Fit (http://prospector.ucsf.edu).

Results: Among the detected spots (613), 48 (7.8%) spots are downregulated and 31 (5%) spots are upregulated by more than two fold. Based on statistical significance (ANOVA 0.05), seventeen downregulated and five upregulated protein spots were selected for MALDI TOF/TOF analysis. Downregulated proteins are involved in iron transport, transcriptional regulation, antibiotic resistance, chemotaxis and two component signaling systems. QSI treatment upregulated the expression of histidine kinase, ABC-transporter protein, putative transcriptional regulator, hypothetical proteins G655_17540 and PSPA7_1308.

Conclusions: Since iron transport proteins, two-component response regulators and sensor are involved in the activation of protease, exotoxin A, and pyoverdin biosynthesis proteins in *P. aeruginosa,* downregulation of above said proteins by QSI could be a major event in attenuation of virulence factor production. In addition, QSI also blocked the expression of proteins involved in the pyoveridine and pyochelin synthesis proteins. Most notably, putative iron-sulfur proteins which are known to express highly in biofilms are significantly downregulated upon QSI treatment. In conclusion, QSI inhibits the virulence factor production by targeting the iron homeostasis, pyoveridine and pyochelin biosynthesis pathways.

Keywords: quorum sensing inhibitor; P. aeruginosa PAO1; quorum sensing.

Acknowledgements: The financial support from ICMR and CSIR is thankfully acknowledged. The Instrumentation Facility provided by DST (FIST & PURSE) and UGC (SAP-DRS1) and Computational/Bioinformatics Facility provided by DBT (BIF) are gratefully acknowledged.

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Special Issue: Proceeding Abstracts of the 4th International Congress on Analytical Proteomics (ICAP 2015)

Immune responses of silkworm, *Bombyx mori* against infection by an endoparasitoid

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Abstract

Purpose: In insects it is known that humoral and cellular immunity plays pivotal role in defending the body against microbial and macrobial infection. Humoral immunity is provided by activation of immune - associated genes and its transcripts whereas the cellular immunity is provided by the cells in the circulating haemolymph, the hemocytes. Recently we showed immuno - competence of the larval integument which forms the primary barrier between environment and internal milieu through proteomics approach. On the other hand, hemocytes spread over the parasitic surface and encapsulate it. However, the uzi fly infection induced toxicity in hemocytes leading to detoxification reactions. We thus elucidated differential immune responses of integumental epithelium and hemocytes induced through infestation by a dipteran endoparasitoid, Exorista bombycis in the economically important silk producing insect and a lepidopteran model, Bombyx mori.

Experimental description: Sub cellular responses of integumental epithelium and hemocytes were analyzed by transmission electron microscopy. Immune responses of integumental epithelium were analyzed by SDS-PAGE, 2D electrophoresis, in-gel digestion and mass spectrometry. Toxic and detoxification responses of hemocytes were analyzed by SDS-PAGE and mass spectrometry and transcriptome responses by microarray analysis. Gene expression was performed by Real – time PCR (qPCR) analysis. All data were analyzed statistically using ANOVA, PCA and regression analysis.

Results: Integumental epithelium showed signs of autophagy and apoptosis and differential expression of associated genes after the infestaton. Proteomic analysis revealed enhanced expression of innate immunity components of toll and melanization pathways, cytokines, signaling molecules, chaperones, and proteolytic enzymes demonstrating diverse host responses. qPCR analysis revealed upregulation of genes encoding cytokine, spatzle, BmToll, and NF kappa B transcription factors Dorsal and BmRel. NF kappa B inhibitor cactus showed diminished expression when Dorsal and BmRel were upregulated, revealing a negative correlation ($R^2 = 0.612$). The hemocytes showed signs of toxicity and activation of certain detoxification mechanisms. Microarray and qPCR showed up regulation of genes associated with detoxification mechanism.

Conclusions: Using Proteomic tools, we showed activation of immune proteins in integumental epithelium which is a first time report in any invertebrates. Moreover, endoparasitoid – induced toxicity and activation of detoxification mechanism in hemocytes could be revealed by mass spectrometry and microarray analysis, thereby exposed the differential responses of two tissues to macroparasitic infestation.

Keywords: immunity; detoxification; mass spectrometry; microarray; Bombyx mori; parasitism.

Acknowledgements: To Central Silk Board for permission and laboratory facilities. To Department of Biotechnology, Government of India for funding through research projects to ARP.

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Proteomic characterization of Colorectal Cancer tissues reveals tumor stage- and EGFR-specific CRC biomarkers

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Abstract

Purpose: To investigate the total membrane-enriched proteome derived from a cohort of paired tumorigenic and adjacent non-tumorigenic colon tissues from CRC patients, to identify deregulated proteins associated with CRC, the individual TNM stages as well as the EGFR status of the CRC tissues

Experimental description: Membrane proteins were extracted by Triton-X114 phase partitioning, separated by gel electrophoresis, in-gel trypsin digested into 10 fractions and subjected to label-free LC-MS/MS analysis by LTQ orbitrap XL. Identified proteins were analyzed by advanced pathway analysis to identify proteomes and the associated networks, based on tumor stages and EGFR expression

Results: Various cancer associated proteins, including CRC-specific proteins, were found to be significantly up- or down-regulated in CRC tumors relative to adjacent normal tissues. Advanced proteomic analysis identified networks and pathways such as, immunological diseases, cancer and cell to cell signalling to be up-regulated and cellular functions, movements and assembly to be down-regulated in CRC tumor.

We further employed a proteomics-driven approach to provide a comprehensive view of the CRC tissue proteomes and the associated networks, based on tumor stages and EGFR expression.

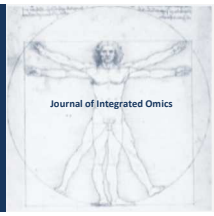
Among proteomic features distinguishing between early and late stage CRC tumors were Increased expression of several cytoskeletal proteins, including cytokeratins (KRT2, KRT18, and KRT19), tubulins (TUBB2A, TUBB2B and TUBB6), actins (ACTB and ACTBL2) and filamins (FLNA and FLNC), which were significantly overexpressed in late stage CRC tumors.

Of note was the up-regulation of the signature CRC metastasis-associated proteins, JUP, EPCAM and SDCBP, as well as cytoskeletal proteins associated with tumor metastasis (ACTB, KRT18, KRT19, TUBB2A, TUBB2B, TUBB6, DES, FLNA and FLNC), observed exclusively in late stage CRC tumors, independent of EGFR expression status. In $EGFR^+$ tumors, on the other hand, exclusive EGFR-dependent overexpression of cell adhesion molecules (CEACAM5, 6 and 7) was observed, exclusive of tumor stage.

A close association between EGFR expression status and tumor stage was also observed through similar expression patterns of certain cancer-specific proteins. Among these were the exclusive observation of ERBB2 and its interacting partners (GRB7 and SRC) in $EGFR^+$ and late stage tumors (absent in $EGFR^-$ and early stage tumors), as well as elevated expression of members of heat shock proteins (HSP90AB1, HSPA1A, HSPD1, HSPA8 and HSPD1) in $EGFR^+$ and late stage tumors. Similar expression profiles for cytoskeletal (SPTAN1, SPTBN1, TPM1, TPM3, CAPZA1, CAPZA2, CDH1 and LIM1) and cell adhesion proteins (CTNNA1, CTNNA2, DSG2 and LSGALs4) as well as several glycotransferases (FUT3, FUT8 and ST6GALNAC1), mucins (MUC2, MUC13 and FCGBP) and ribosomal proteins were observed in both $EGFR^+$ and early stage CRC tumors.

Conclusions: This work provides a detailed map of the altered proteome and associated protein pathways in CRC. Expression profiles of cancer-associated proteins, including several CRC-specific proteins, involved in tumor growth, progression and metastasis, influenced by EGFR expression status, tumor stage or both, were identified. The insights gained from this study may open avenues for a knowledge-driven search for candidate CRC protein markers.

Keywords: colorectal cancer, membrane-enriched proteome, label free shotgun proteomics, epidermal growth factor receptor (EGFR), TNM tumor stage .



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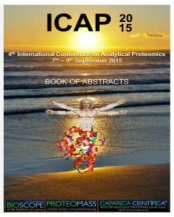

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Probing the structure and conformational changes in complex biological systems using zero-length crosslinking and mass spectrometry

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Abstract

Purpose: Chemical cross-linking and mass spectrometry (CX-MS) combined with molecular modeling is a powerful structural MS strategy. Zero-length cross-linkers are particularly useful for refining and confirming structural models because they provide the most precise distance constraints between cross-linked sites. However, zero-length cross-linked peptides are difficult to identify, and therefore this method has been used far less frequently than longer cross-linkers such as the popular amine-reactive homobifunctional reagents. Due the great value and under-utilization of zero-length cross-link analysis, we have developed methods to enhance its routine application to diverse biological problems.

Experimental description: Our group has greatly improved in-depth detection of zero-length cross-links by optimizing mass spectrometry data acquisition for this application, and by developing a software tool, ZXMiner, specifically for zero-length cross-link identification. The strategy utilizes label-free LC-MS/MS quantitative comparisons of tryptic digests from zero-length cross-linked samples and uncross-linked controls to identify putative cross-linked peptide precursors. Spectra from all precursors within 5 ppm of theoretical cross-linked peptides are compared to predicted cross-linked peptide theoretical spectra. Graphical tools facilitate verification of cross-link assignments. Resulting high-confidence peptide identifications are then employed to identify novel interactions and as distance restraints for molecular modeling experiments.

Results: We initially applied this method to large segments of the human red cell protein, spectrin using a Thermo Orbitrap XL mass spectrometer. The data acquisition method and scoring algorithm were subsequently optimized on a Thermo Q Exactive Plus mass spectrometer to achieve improved depth of analysis on complex samples. This method was applied to purified human red cell plasma membranes as a complex physiological model system. Using a 4-hour gradient without sample fractionation, 57 proteins could be identified by five or more peptides, which represents a total search space of almost 5 million Da of unique sequence. Representative results identified 65 cross-linked peptides at an FDR=0. Most cross-links were associated with the most abundant proteins, although 3 cross-links involved several of the least abundant proteins in the database. The most abundant proteins in the membrane are the anion exchanger (AE1) and spectrin, which contained 12 and 34 identified high confidence cross-links, respectively. These data have been used to develop detailed structural models and to map locations of interactions with other membrane proteins.

Conclusions: CX-MS using zero-length cross-linking can be applied to multi-million Da complexes to identify novel interactions and to provide precise distance constraints for molecular modeling and development of experimentally verified structures of large protein complexes.

Keywords: zero-length crosslinking, structural mass spectrometry, protein structure, macromolecular complexes.

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The O_2-Affinity and Cooperativity of Hemoglobin Are Regulated by the Effector-Linked 4D-Structural Changes (Protein Dynamics) rather than the Ligand-Linked 3D-Structural Changes

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ABSTRACT

Introduction: The current, widely-held mechanism of allostery of hemoglobin (Hb) has been that the changes from the R-quaternary/tertiary structures of oxy-Hb to the T-quaternary/tertiary structures of deoxy-Hb exert certain structural constraints on the coordination structure of the heme group, leading to a lower O_2-affinity of the hemes and thus that of Hb [1]. However, we found that there is no causal correlation between the static T/R-quaternary structures and the low/ high O_2-affinities of Hb [2]. We explore an alternative mechanism of the regulation of the ligand-affinity and cooperativity in Hb.

Results and Discussion: The O_2-affinities of free complexes Fe[II]- and Co[II]-protoporphyrins with axial nitrogenous bases in organic solvents are very low (P_{50} > 10^2 ~ 10^3 Torr), whereas the apparent O_2-affinities of these metalloporphyrins, which are incorporated in proteins such as apo-myoglobin, apo-Hb, serum albumin, etc., increase substantially to P_{50} < 10^{-1} ~ 10^4 Torr, though their coordination structures are apparently unchanged [3]. Such substantial increases in the apparent ligand-affinities of metalloporphyrin-containing proteins are accomplished by preventing/inteferring with the dissociation of the un-bonded ligand by protein matrix, since the interior of globin is nearly fully packed by protein matrix. In Hb and Co-substituted Hb (Co-Hb), the dissociation process of the un-bounded ligand proceeds through the "caged" state [4-6], which can be produced by cryogenic photolysis of the ligated-states at 4.2K and in which the metal-ligand bond is broken and the un-bonded ligand is trapped near the bonding site within the globin moietiy. This "caged" state has spectral features distinct from those of either deoxy- or ligated states of the respective hemoproteins. The apparent ligand-affinities (P_{50}, K_T, *and* K_R, where $P_{50} = 2/[K_T + K_R]$) of Hb and Co-Hb are regulated by heterotropic effectors, as observed by Bohr [7] without detectable changes in either static T-/R-quaternary/tertiary structures of the globin moiety or the coordination/electronic structures of the metalloporphyrin moiety and thus the ligand-affinity of the metalloporphyrins themselves [8-10]. The substantial reduction of the apparent ligand-affinities of Hb and Co-Hb by heterotropic effectors, may be caused by increase in the migration rate of ligands through globin matrix from the "caged" state to solvent, resulting from the effector-linked, enhanced high-frequency (>GHz) thermal fluctuations which increase the transparency of the globin matrix toward small diatomic ligands such as oxygen, CO, and NO [8-10], which may concomitantly reduce the rate of geminate-recombination of the ligand to the metalloporphyrins. Thus, we structurally identify Monod-Wyman-Changeux (MWC)'s *"T"-low-* and *"R"-high- affinity-* functional states [11] as high- and low-amplitude, high-frequency (>GHz) thermally fluctuating dynamic states of Hb, respectively, regardless of its static T-/R-quaternary/tertiary structures [10].

Conclusion: The ligand-affinity of Hb is regulated through the 4D structural changes (or protein dynamics) induced by heterotropic effectors [9], rather than the ligand-linked, static T-/R-quaternary/tertiary structural changes, as proposed by Perutz [1]. Thus, the "caged" state of Hb acts as a critical transition state in regulation of the affinity for small diatomic ligands in Hb and Co-Hb [10].

Keywords: Allostery, Protein Dynamics, Cooperativity, Hemoglobin

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Flagellar proteins and their key roles in human diseases: Once neglected, still underestimated

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Abstract

Organelle proteomics, such as the flagellum, has been one of the most suitable approaches used to identify novel components and modifications of a given cell, tissue, organ or even organism. Information is usually gained at a large scale and is very valuable to further understand biological processes of the given compartment. In this presentation I will talk about a particular subset of flagellar proteins, which happen to be amazingly related to protozoa virulence in Leishmaniases. As all eukaryotic flagella, the *Leishmania* flagellum is a protein nanomachine, but it has a particular way in being arranged to invade human macrophages. Yes, I just said to invade, rather than to be engulfed or phagocyted by macrophages. *Leishmania* flagellar apparatus turns the protozoan into an "infection motor" by means of complex, intricate surface and shallow subsurface features that provide major biological processes in this trypanosomatid survival and virulence, from motility and cell division, towards invasion and persistence in host cells. Using conventional proteome assays and computational molecular modeling coupled with atomic force microscopy (AFM), my research group has studied specific morphological data resulting from protein structural organization of *Leishmania spp.* flagellum, gathering information not yet explored neither in a nanostructured nor in a pathogenic approach for this species. AFM imaging allows detailed visualizations of the flagellum surface, as can be seen by Figure 1, which, in a 3D-view, shows an undulated surface made of two sided border-like elevations along all the elongated flagellum and the red arrow illustrates one of these; the inner region between these two elevations, a major groove, measured around 200nm in average (redline). Figure 1b also shows a prominent membrane surface of flagellar adhesion zone (FAZ) next to the cell body. The underlying hypothesis of all such studies is that polymerization and depolymerization of parasite actin and actin motor-associated proteins, during both processes of motility and host cell entry, might be key events for successful infection, including the parasite survival within phagosomes. Our reports have revealed an actin-polymerization *Leishmania* protein, profilin, and its partner, formin, as involved in axonemal assembly/disassembly, therefore, flagellar dynamics / remodeling, while *Leishmania* coronins and Arp2/3 complex are believed to participate in the phagosome formation. We must recall that *Leishmania* has differential levels of survival inside phagosomes, suggesting that the survival of parasites is linked to their ability to be targeted to tight/non-cidal and non-degradative compartments. To what extent these different killing pathways (delayed or not) are used in a given phagosome certainly depends on various cellular and environmental conditions (usually a plethora of macrophage receptors at variable proportions that may influence phagosome fate to variable degrees), including interactions with flagellar proteins here emphasized.

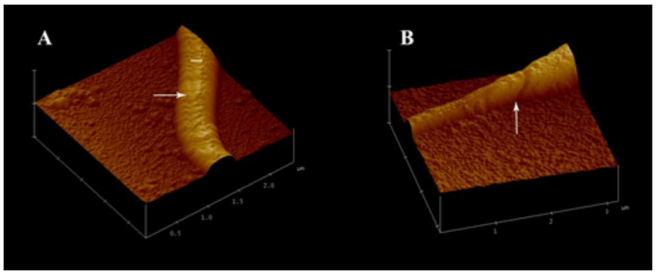
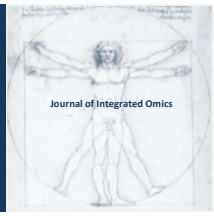


Figure 1. 3D-view images of L. chagasi flagellum. (A) Region within the furrow/groove. There is an undulated surface (arrow) made of two sided border-like elevations and a measurement of one groove/furrow whole extension (redline). (B) The adhesion zone (FAZ) to the cell body can be seen (arrow).

Keywords: flagellar proteins; organelle proteome; molecular modeling; atomic force microscopy (AFM); phagosome.



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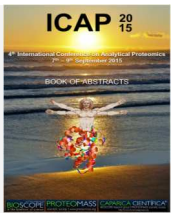

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Effects of heavy metals on *Cyanothece* sp. CCY 0110 growth, extracellular polymeric substances (EPS) production, ultrastructure and protein profiles

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Abstract

Purpose: The aim of this study was to evaluate the effects of several heavy metals on the growth/survival, EPS production, ultrastructure and protein profiles of the highly efficient extracellular polymeric substances (EPS)-producer cyanobacterium *Cyanothece* sp. CCY 0110.

Experimental description: After exposure to different concentrations of metals (Cu^{2+}, Pb^{2+}, Cd^{2+}, or Li^{+}), cell growth, production of total carbohydrates and RPS (released polysaccharides) and ultrastructural changes were investigated. Proteomes were compared by two 8-plex iTRAQ studies and results were validated with data from Western blots, O_2 evolution and ROS measurements and enzymatic assays.

Results: The heavy metals affected the cells differently, triggering distinctive responses. Concerning chronic exposure to the metals, the cells were more affected by Cu^{2+} followed by Pb^{2+}, Cd^{2+}, and Li^{+}. The presence of metals led to remarkable ultrastructural changes, mainly at the thylakoid level. The comparison of the proteomes allowed to follow the stress responses and to distinguish specific effects related to the time of exposure and/or the concentration of an essential (Cu^{2+}) and a non-essential (Cd^{2+}) metal. The majority of the proteins identified with significant fold changes were associated with photosynthesis, CO_2 fixation and carbohydrate metabolism, translation, and nitrogen and amino acid metabolism. Unexpectedly, the amount of released polysaccharides (RPS) was not enhanced by the presence of heavy metals.

Conclusions: This work shows the holistic effects of different heavy metals on the cyanobacterium *Cyanothece* sp. CCY 0110. Overall, the results suggest that during Cu^{2+} chronic exposure the cells adjust their metabolism to invest the spare energy in the activation of metal detoxification mechanisms. In contrast, the toxic effects of Cd^{2+} accumulate over time suggesting that cells might not have the same capacity to deal with this non-essential metal.

Keywords: Cyanobacteria; *Cyanothece*; extracellular polymeric substances (EPS); heavy metals; iTRAQ; proteome.

Acknowledgements: The work was funded by FEDER Funds through the Operational Competitiveness Programme – COMPETE and national funds through FCT – Fundação para a Ciência e a Tecnologia under the project FCOMP-01-0124-FEDER-028314 (PTDC/BIA-MIC/2889/2012) and the scholarships SFRH/BD/84914/2012 and SFRH/BDP/72400/2010. We thank Professor Lucas Stal for providing Cyanothece sp. CCY 0110. Sheffield acknowledges the EPSRC (EP/E036252/1).

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Brain ubiquitin mitochondrial subproteome and its changes induced by neurotoxins and neuroprotectors

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ABSTRACT

Ubiquitin is a 76-residue protein that tags proteins and thus regulates numerous intracellular processes. Incubation of the rat brain mitochondrial fraction with biotinylated ubiquitin *in vitro* followed by avidin-agarose chromatography and mass spectrometry (MS) detection revealed direct ubiquitination of extra- and intramitochondrial proteins. However, the proportion of directly ubiquitinated proteins represented not more than 20% of the total number of identified proteins that specifically bound to avidin-agarose. Profiling of endogenously ubiquitinated proteins of the mitochondrial compartment by means of affinity chromatography on the proteasome subunit S5a conjugated with agarose followed by their MS detection revealed 58 individual proteins identified by a characteristic diglycine fragment of their tryptic peptides. Administration of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) inducing symptoms of parkinsonism in mice had a minor effect on the total number of identified proteins (59) but caused qualitative changes in the proteomic profile. Pretreatment with a non-peptide neuroprotector, isatin, decreased MPTP-induced neurotoxicity and significantly reduced the total number of individual ubiquitinated proteins (to 36). Isatin (interacting with a wide range of isatin-binding proteins in the brain and regulating expression of isatin-sensitive genes) administered to control mice also decreased the total number of ubiquitinated proteins (to 30).

Conclusions: The qualitative changes in the ubiquitin mitochondrial subproteome suggest that the neuroprotector effect of isatin is associated with "metabolic immobilization of the brain" induced by interaction of isatin with isatin binding proteins and altered patterns of ubiquitinated proteins of the mitochondrial compartments and inhibition of monoamine oxidase (B) as well.

Keywords: mitochondrial subproteome, affinity-based profiling, ubiquitin, isatin, isatin-binding proteins, MPTP

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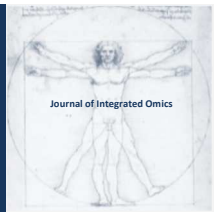

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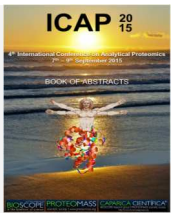

Special Issue: Proceeding Abstracts of the 4th International Congress on Analytical Proteomics (ICAP 2015)

The Resonant Recognition model to design short bioactive therapeutic peptides: does it really work?

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Abstract

In recent years, more focus has been placed on the role of small molecular weight peptides in clinical medicine, mostly for their ability to penetrate cellular membranes, and to interfere with enzymatic functions or protein-protein interactions. The ability to predict the 3D structures and functions of biological molecules would certainly be useful in designing therapeutic drugs. Hence, the Resonant Recognition Model (RRM), applied in this study, is a physico-mathematical model that incorporates signal processing methodology for structure-function analysis of proteins. The aim of our research is to design and investigate the biological effects of RRM bioactive peptides and their potential to be applied as therapeutic agents.

Experimental description: The RRM approach was used to design 18-22 mer bioactive peptide analogues of natural proteins known to possess cytotoxic effects on cancer cells including Myxoma virus (MV); mammalian interleukins (IL) and tumor necrosis factor (TNFα). The biological effects of RRM-MV; RRM-IL; and RRM-TNF were investigated on mammalian cancer and normal cell lines. The cytotoxic effects were evaluated by qualitative and quantitative cell survival methods. In addition non-bioactive peptides lacking the specific RRM frequencies or peptides with scrambled sequences were also tested as controls. Human apoptosis protein arrays were used to detect the expression levels of pro-apoptotic and anti-apoptotic proteins in treated versus non-treated cancer cells. Glycomic arrays and fluorescent microscopy were used to find the potential cellular targets of these peptides in cancer cells. Furthermore, we also applied the RRM to design short peptides with antimicrobial activity as analogues for known antimicrobial natural peptides like azurocidin (CAP37) and lactoferrins and evaluated their effect on *Escherichia coli*, and Staphylococci including a methicillin resistant *S. aureus* (MRSA) strain.

Results: The bioactive peptides RRM-MV, RRM-IL, and RRM-TNF produced significant apoptotic/necrotic effects on B16 mouse melanoma cells, human melanoma MM96L, and COLO16squamous cell carcinoma, PC3 prostate cancer and MCF7 breast cancer. However, no cytotoxic effects were detected on human red blood cells, normal skin fibroblasts; mouse macrophages, mouse fibroblasts, and Chinese hamster ovary cells (CHO). Yet the non-bioactive, control peptides did not cause any cytotoxic changes in any type of cancer or normal cells. The bioactive peptides were found to be located inside cytoplasmic components in treated cancer cells prior to necrosis stage. Possible cellular targets were predicted to be specific glycoproteins, however the bioactive peptides have a different binding specificity to glycans as Sialy Lewis X, gangliosides and mannoses. Furthermore, the azurocidin peptide analogue had a bacteriostatic antimicrobial effect on all tested bacteria including MRSA.

Conclusions: The RRM is a powerful computational tool to design bioactive peptides with specific biological functions. Our data indicates that each of the RRM designed peptides possessed the desired biological function which was conserved in its unique sequence.

Keywords: Resonant recognition model, RRM-designed therapeutic peptides, anticancer; antimicrobial.

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Knots in proteins – a tangled tale

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Abstract

Ever since Lord Kelvin has conjectured that atoms are composed of knots in the ether, these peculiar objects have stimulated the imagination of mathematicians and natural scientists alike. In recent years the field went through a renaissance and progressed considerably, spurred by the discovery of knotted DNA and proteins.

Even though protein knots are rare, they occur in all kingdoms of life and their topology is typically preserved amongst homologs. Nowadays, knotted protein structures can even be created artificially, and knotted designs could in the future be used in the context of protein engineering. In this talk I will provide an introductory overview to the field and evidence which indicates why the folding of knotted proteins may not be so difficult after all.

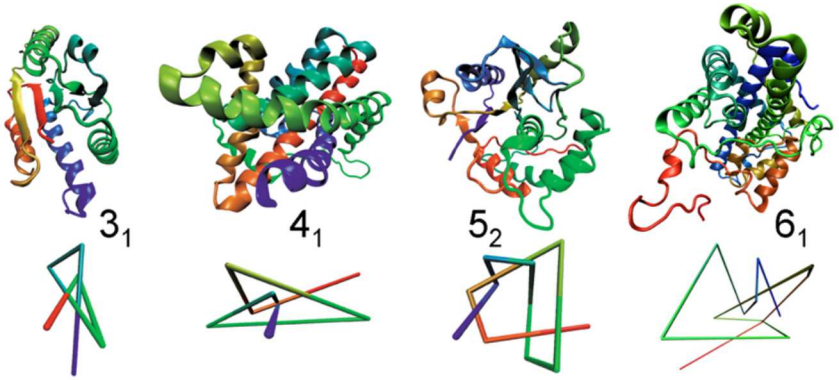

Figure 1. Knotted protein structures. From Bölinger et al, PLoS Comp. Biol. 6, e1000731 (2010).

Keywords: knots in proteins, computer simulations, folding.

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Comparative proteomic profile between transgenic and non-transgenic soybean seeds by 2D-PAGE

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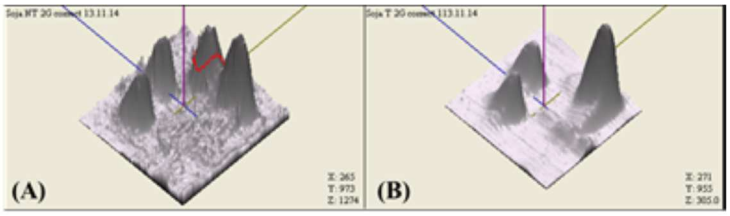
ABSTRACT

Purpose: Evaluate possible differences in proteomic map between transgenic and non-transgenic soybean seeds varieties (RR 7511 and 8200, respectively) due to change in its genome by the insertion of *cp4*EPSPS gene, which confers to the soybeans tolerance to glyphosate.

Experimental description: A proteomic approach based on 2D-PAGE separation to identify alterations between T and NT profile was used. The soybean seeds proteins were extracted using a buffer (50 mmol L^{-1} de Tris-HCl, 1,5 mmol L^{-1} KCl, 10 mmol L^{-1} DTT, 1,0 mmol L^{-1} PMSF e 0,1 % SDS (m/v)) and its precipitation performed with ammonium acetate 0,1 mol L^{-1}. The precipitate was solubilized in a specific buffer and quantified with a 2D Quant Kit (according to the manufacturer's instructions). A total of 500 µg of protein, was applied to immobilized pH gradient strips (pH range 4–7) and they were rehydrated at room temperature for at least 12 h. Isoelectric focusing was performed for a total of 14,300 Vh. The second-dimension separation was carried out at 25 mA per gel and 100 W on 1-mm 12% (w/v) SDS polyacrylamide gels. After separation, the gels were stained with colloidal Coomassie brilliant blue. The gels were scanned using an ImageScanner II and the images processed by ImageMaster 2D Platinum 6.0, which allowed the estimation of the number of spots, the pI values and molecular mass of the proteins.

Results: The proteomic profile of T and NT seeds is different, thereby more than 20% of concentration of total protein was found in T seeds. Additionally, throughout the 3D evaluation, 14 different spots were found in the T and only 3 in NT seeds, as exemplified in Figure 1. This indicates that protein synthesis was different between T and NT soybeans. Intensities of the differentials spots were also observed. For a more accurate assessment, a quantitative analysis by 2D-DIGE will be performed to assess these changes. As future prospects, we will also quantify and identify these alterations in the proteomic profile and then propose a biochemical pathway.



Conclusions: The insertion of gene *cp4*EPSPS in soybean may be changing not only their genome but also their proteomic profile, thus most probably affecting future generations of plant. Regarding to the human food safety, no case has been reported however bioaccessibility tests need to be performed.

Keywords: Soybean, Organism Genetically Modified, Transgenic, Proteins, Proteomics, Electrophoresis.

Acknowledgements: The authors thank to FAPESP, CAPES and CNPq for financial supporting.

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Comparative evaluation of extraction methods for UPLC-ESI-QTOF MS analysis of lipids from human blood serum

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Abstract

Purpose: The aim of this study was to perform a comparative evaluation of lipid extraction protocols from human blood serum using different chromatographic columns by UPLC-ESI-QTOF MS.

Experimental description: The evaluated extraction procedures included three liquid-liquid extraction techniques optimized for lipid profiling: i) modified Folch method, ii) Bligh and Dyer method, and iii) MTBE method. Modified Folch method consisted in adding 150 µL of methanol (MeOH) to 20 µL of serum sample and vortexing the mixture. Subsequently, 300 µL of chloroform (CHCl3) were added and the mixture was incubated for one hour in a shaker before we induced phase separation by adding 125 µL of an aqueous solution of NaCl 0.15 mol L-1. The mixture was centrifuged for 10 minutes at 8000 *g* at 10 °C and the lower (organic) phase was collected. For Bligh and Dyer extraction, 30 µL of serum were mixed with 360 µL of CHCl3 and 180 µL of MeOH. The phase separation was achieved by adding 120 µL of water, the mixture was centrifuged for 10 minutes at 8000 *g* at 10 °C and the lower (organic) phase was collected. MTBE method was performed by adding 150 µL of MeOH to 20 µL of serum sample and vortexing the mixture. Subsequently, 500 µL of methyl tert-butyl ether (MTBE) was added and the mixture was incubated for one hour in a shaker before we induced phase separation by adding 125 µL of water. The mixture was centrifuged for 10 minutes at 8000 *g* at 10 °C and the upper (organic) phase was collected. All collected phase in each extraction was dried to complete dryness with liquid nitrogen, and then reconstituted in the proper mobile phase for lipid profiling analysis. Additionally, the chromatographic separation of the lipids was performed with three different columns (C8, C18, and HILIC), using UPLC-ESI-QTOF MS in positive and negative ionization mode. For all the analyses, a 1290 UPLC (Agilent) and a 6650 QTOF mass spectrometer (Agilent) were used.

Results: The results of this study show which extraction method is more efficient and which chromatographic column is more selective in the lipid profiling analysis of serum samples. It was considered four criteria to compare the extraction protocols: overall extraction coverage by comparing the number of identified peaks and the sum of the peak intensities, reproducibility of lipid quantifications, similarity of metabolite profile based on multivariate statistics and the specificity of extraction methods by comparing the peak abundances for each lipid class.

Conclusions: With the results obtained by lipid profiling analysis, we could compare the efficiency of each type of extraction using different chromatographic columns by UPLC-ESI-QTOF MS. The results from this preliminary study will be further applied in the search for bipolar disorder potential lipid biomarkers.

Keywords: Extraction methods, Lipid profiling, Serum and UPLC-MS.

Acknowledgements: The authors are grateful to FAEPEX for their financial support. The authors also thank Célio F. F. Angolini for helping with the mass spectrometry experiments.

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Differential protein expression in the testes of ganders under different monochromatic light sources

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Abstract

Purpose: In modern poultry production, patterns of artificial illumination treatment such as light schedule, intensity, and color are important factors that influence productive performances. The purpose of this study was to investigate whether different monochromatic light sources affect the protein expression in the testes of ganders.

Experimental description: Six male White Roman ganders were used. The ganders were allocated into white or blue light groups. The lighting photoperiod of 7L:17D was applied for 6 weeks as an adjusting period. After the adjusting period, the photoperiod was changed to 9L:15D and maintained for 24 weeks. The ganders were sacrificed at the end of treatment to collect testis samples for proteomic analysis.

Results: The results of two-dimensional difference gel electrophoresis revealed that 37 protein spots were differentially expressed in the testes of ganders with different monochromatic light treatments. A total of 31 spots were identified by MALDI-TOF/TOF. Gene ontology analysis revealed that the differentially expressed proteins were mostly located in extracellular vesicular exosome (27%, 7 proteins), nucleus (11%, 3 proteins), and cytoplasm (11%, 3 proteins). Most of the proteins were with molecular function of protein binding (23%, 6 proteins), ion binding (23%, 6 proteins), carbohydrate binding (19%, 5 proteins), and heterocyclic compound binding (15%, 4 proteins). The majority of the proteins were participated in biological process of biological regulation (27%, 7 proteins) and cellular process (23%, 6 proteins). In addition, proteins related to sperm maturation and capacitation, including hemoglobin, apolipoprotein A-1, and phosphatidylethanolamine-binding protein 1, were downregulated after blue light treatment. The result suggested that blue light might disturb sperm functions and fertility on ganders.

Conclusions: A total of 37 protein spots were differentially expressed in the testes of ganders with blue and white light exposure. Proteins related to sperm maturation and capacitation were downregulated in the testes of blue light treated ganders. The exact role of the differentially expressed proteins needs further investigation.

Keywords: Testes, Monochromatic light sources, Protein expression, Ganders.

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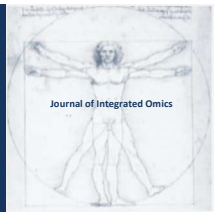

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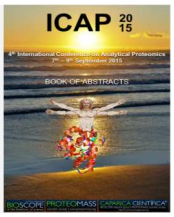

Special Issue: Proceeding Abstracts of the 4th International Congress on Analytical Proteomics (ICAP 2015)

Proteomic comparative study of smooth muscle cells isolated from systemic and pulmonary arteries

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ABSTRACT

Purpose: The cellular compositions of the systemic and pulmonary arterial walls are very similar. However the functional characteristics of these vessels show many differences. One of the most noticeable is in their reaction to hypoxia. We assume this difference can be consequence of the smooth muscle cells (SMC) protein composition of the peripheral arteries. Thus as we assume the definition of their proteomic differences can guide us to the important points with the potential to be a cause of their specific reactions.

Experimental description: We used 14 adult male Wistar rats (200-250g). Animals lived in normoxia (n=9) or were exposed to isobaric hypoxia (FiO_2=10%) in hypoxic chamber for 4 days (n=5). The rats were euthanased by the intraperitoneal injection of thiopental. Peripheral pulmonary and mesenteric arteries were dissected under the microscopic control. Smooth muscle cells were obtained by the enzymatic digestion of the vascular fragments and the isolated material was subjected to sonication in lysis buffer. Protein mixture was analyzed by one-dimensional gel SDS-polyacrylamide electrophoresis followed by two-dimensional gel SDS-polyacrylamide electrophoresis and identified using nLC-MS/MS. Proteins were identified by correlating tandem mass spectra to IPI and SwissProt databases.

Results: We detected the significant differences in the protein composition between the samples separated from the pulmonary and systemic smooth muscle cells and also between the pulmonary smooth muscle isolated from the rats living in normoxia and exposed to 4 days hypoxia. We detected proteins more abundant in pulmonary SMC (vinculin, annexins, 14-3-3 proteins) and in systemic SMC (disulfide-isomerase, aldehyde dehydrogenase) and we observed the significant increase of collagen VI quantity in the normoxic pulmonary SMC in comparison with animals exposed to hypoxia.

Conclusions: We observed the significant differences of the proteomic profiles between pulmonary and systemic smooth muscle cells and also the significant changes in the normoxic pulmonary smooth muscle cells in comparison with animals exposed to hypoxia. These results bring new findings about extracellular matrix remodeling during hypoxia.

Keywords: smooth muscle cells, pulmonary, systemic artery, proteome, hypertension

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Protein expression profiles of serum samples bipolar disorder patients mapped by 2-D DIGE coupled with nanoLC-MS/MS

J.R. Jesus*[1], R. M. Galazzi[1], B. K. Campos [1], A. Sussulini[1], C. E. M. Banzatto[2], J. L. C. Martínez[3], M. A. Z. Arruda[1]

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Abstract

Purpose: Bipolar Disorder (BD), which affects up to 3% of the worldwide population, is a mental disease with unclear connections to other similar disorder. This study had aim application approach comparative proteomic to identify potential biomarkers in patient serum samples with bipolar disorder.

Experimental description: This study was approved by the Ethics Committee of the Hospital das Clinicas (University of Campinas, Brazil). Fifty six serum samples (23-57 years old) were collected and classified into five groups: (A) healthy family control (n= 3), (B) healthy no-family control (n= 9), (C) BD patients under treatment with lithium (Li) (n= 14), (D) schizophrenia patients (n= 25) and (E) other diagnostic patients under treatment with Li (n=4). The participants did not have other concomitant diseases such as cancer, endocrinological or metabolic diseases. Abundant proteins (IgG and albumin) depletion were performed using ProteoMiner spin columns. The analysis was performed using two-dimensional fluorescence difference-in-gel electrophoresis (2-D DIGE) coupled with nanoliquid chromatography-tandem mass spectrometry (nanoLC-MS/MS). For the analysis of 2-D DIGE, the samples were compared. As follows: (i) group A vs group B; (ii) group A vs group E; (iii) group B vs group E; (iv) group C vs group E; (v) group E vs group D.

Results: Approximately 50 µg [(pH range 4-70) determined from the 2-D Quant Kit] of each sample and the corresponding amount of the internal pooled standard were labeled with 400 ρmol of CyDye DIGE Fluors minimal dyes. The ratios of protein abundance that increased or decreased more than 2.0 fold (t-test, $p<0.05$) were considered significant. Forty two protein spots were detected as significant difference among groups (41 increased and 1 decreased). In the experiments (i), (ii), (iii), (iv) and (v), were identified 4 protein spots (3 with higher abundance and 1 with lower abundance for B group), 8 (all protein spots with higher abundance for C group), 13 (all protein spots with higher abundance for C group), 9 (all protein spots with higher abundance for D group) and 8 (all protein spots with higher abundance for C group) protein spots presenting significant difference, respectively. All proteins spots presenting significant difference were selected for in-gel trypsin digest and nanoLC-MS/MS sequencing for protein identification. In the next step, the identification of proteins by nanoLC-MS/MS will be made.

Conclusions: In this study, we found that close forty protein spots shown significant differences among the groups compared using 2-D DIGE. As perspective, the proteins will be identified by nanoLC-MS/MS and some proteins will be selected to confirm the comparative proteomic data using immunoassay.

Keywords: Bipolar disorder, comparative proteomic, blood biomarker, 2-D DIGE, nanoLC-MS/MS.

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Western blotting - detection of a specific protein in MCF7 and MCF7-Z tissue

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Abstract

Purpose: In the contemporary world, breast cancer is one of the most common and dangerous types of cancer in females. Comparison between two different variants of MCF-7 (MCF-7 vs MCF-7-Z) human breast cancer cell lines used in subcutaneous xenograft tumor models, has shown different tumor growth characteristics in mice. Glycosylated proteins are among post-translational modifications one of the most important types of proteins involved in tumor progression. The aim of the research was to search for the presence of selected glycoproteins, based on quantitative and qualitative analysis in cancer progression.

Experimental description: For samples preparation we used the strategy utilizing western blotting and nanoLC-MS/MS analysis. After isolation of proteins from the tissue, glycoproteins were isolated by lectin affinity chromatography. Glycoproteins fractions were separated by the sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE), followed by two complementary analyses. One part of the gel was transferred onto PVDF membrane, and another part was CBB stained. Based on the images obtained after immunostaining, the bands were excised from the CBB-stained gel and analyzed by tandem mass spectrometry coupled to liquid chromatography (LC-MS/MS).

Results: Using LC-MS/MS technique we have succeeded to identify 3000 differentially expressed proteins between MCF7 and MCF7-Z. The selected proteins were associated with cancer metastasis, which plays a vital role in tumor progression, and are involved in cell structure organization. The presence of the selected proteins was confirmed using western blotting.

Conclusions: We have identified the potential biomarkers, which can be important in cancer progression.

Keywords: cell lines MCF-7, glycoproteins, tumor, breast cancer.

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The quantitative proteomic study of human saliva samples obtained from caries-free and caries-susceptible people

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Abstract

Purpose: The human teeth have been together with the saliva in the center of interest for many years, due to medical problems with them [1]. Only a minor part of the population (ca 10 % of people in the age at ca 30 years) is resistant to tooth caries. In saliva there were detected over 2000 proteins to these days, and important part of them are salivary defense proteins. Our study was aimed to proteomics of saliva and to find the differences in the abundances of the responsible proteins in these tissues between caries-resistant and caries-susceptible people..

Experimental description: The human saliva samples from adult patients (aged 17-35) were collected and divided into two groups with respect to the number of dental caries (caries-susceptible vs. caries-resistant people). The proteins of oral fluids were separated by two-dimensional electrophoresis or by difference gel electrophoresis and the resulting protein maps were quantitatively evaluated. Spots exhibiting statistically significant changes were excised, digested by trypsin, and analyzed by nano-liquid chromatography coupled to a MaXis Q-TOF mass spectrometer with high resolution.

Results: We detected more than several hundreds of proteins in each kind of sample (pulp, dentin, saliva) [2]. Then we compared the individual proteome maps of saliva samples by 2-DE and DIGE and indentified the changes in the protein abundances among the individuals. The observed significant differences between caries-resistant and caries-susceptible groups. Special emphasis was put on looking for the abundance changes of proteins that may be involved in formation of dental caries due to their biological functions.

Conclusion: This study comaring proteomes of whole saliva from carious-resistant and carious-susceptible people brings new findings to the saliva protection against tooth caries.

Keywords: human saliva, proteome, two-dimensional gel electrophoresis, DIGE.

Acknowledgements: This work was supported by the Ministry of Health of the Czech Republic Departmental Program for Research and Development (NT14324-3/2013), by the Czech Science Foundation (No. 13-172224S), and with support for long-term conceptual development of research organization RVO:67985823.

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The quantitative comparison of the human tooth pulp obtained from caries-free and caries-susceptible people

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ABSTRACT

Purpose: The majority of the human population worldwide experiences dental caries. Only a minor part of the population (ca. 10 % of people in the age at 30 years) is resistant to development of this oral disorder. The anti-caries resistance can be caused by overall immune system, nourishment, dental care efficiency and/or by many other causes, such as composition of saliva or teeth. Differences in the abundances of the responsible proteins in these tissues between caries-resistant and caries-susceptible people are expected and are in the center of our interest. The aim of our work was to investigate the proteomic profile of human tooth pulp from adult people and compare them mutually.

Experimental description: Sound human teeth extracted for clinical reasons from adult patients (aged 17-40) were divided into two groups with respect to the number of dental caries (caries-susceptible vs. caries-resistant people). The proteins from individual tooth pulp samples were extracted and processed by the method described previously [1]. The proteins were separated either by two-dimensional electrophoresis followed by Coomassie colloidal blue staining or by difference gel electrophoresis and the resulting protein maps were quantitatively evaluated. Spots exhibiting statistically significant changes were excised, digested by trypsin, and analyzed by nano-liquid chromatography coupled to a MaXis Q-TOF mass spectrometer with ultrahigh resolution.

Results: We detected more than several hundreds of spots in the proteome maps of each human tooth pulp sample. Individual proteome maps were compared by both methods (2-DE and DIGE) and changes in the protein abundances were identified among the individuals. The observed differences were statistically evaluated to find the significant changes. Some of these spots corresponded e.g. to peroxiredoxin 1, GTP-binding nuclear protein Ran, apolipoprotein A-I, glutathione S-transferase P, phosphoglycerate kinase 1 or retinal dehydrogenase. These proteins exhibits variety of biological functions: cell communication and signal transduction, transport or metabolism.

Conclusions: We determined some quantitative differences in the whole proteomes of human teeth pulp samples between carious-resistant and carious-susceptible people. Special emphasis will be engaged in further investigations of these proteins that may be involved in formation of dental caries due to their biological functions.

Keywords: human tooth pulp, proteome, two-dimensional gel electrophoresis.

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In vivo determination of the CYP4A and COX5A expression and catalytic activity in rat hepatic microsomes after drug administration

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Abstract

Purpose: Drug dependence influences cell functions and their biological pathways, but the processes do not seem to be clear and understood. Global analysis of liver proteome can contribute to the explanation of drugs' influence on the metabolic processes and may help to explain the whole problem. The rat liver microsomes were taken from control and drugs-addicted rats, and up- and down regulated proteins were evaluated.

Experimental description: Adult male Wistar rats were treated s.c. with either saline (control) or amphetamine, morphine, or cocaine in saline, for 15 days. Liver microsomes were prepared according to a standard protocol. The proteins were divided into up- and down regulated groups, based on LC-MS^n analyses, followed by the Mascot server (Matrix Science) identification, after SDS-PAGE separation. The expression of the selected proteins was determined by Western Blotting and the catalytic activity of cytochromes P450 was also determined.

Results: Two proteins were chosen from up- and down regulated proteins identified by LC-MS^n for Western Blotting, cytochrome P450 4A10 (CYP4A) and cytochrome c oxidase subunit Va (COX5A). The enzymatic activity of CYP4A was determined by LC-MS^n determination of ω-hydroxylation of lauric acid.

Conclusions: After administration of various drugs (amphetamine, morphine, and cocaine) to the rats, significant changes in liver proteome were observed, also including cytochromes P450 responsible for oxidative metabolism. The expression and catalytic activity of CYP4A and COX5A was determined.

Keywords: cytochrome P450, CYP4A, COX5A, microsomes, drug dependence.

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Label free proteomics from rat liver microsomes in drug dependence

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ABSTRACT

Purpose: The drug dependence has a significant impact on the cells' functions and biological processes in mammalian body. In many cases, the mechanism of drug dependence is not fully understood and the knowledge on its influence on the human body is not complete. The presented work shows application of the label free methodology in proteome analysis in drug dependence.

Experimental description: Microsomes were prepared from the Wistar rats after administration of a drug for 15 days. Four groups of animals were analyzed: amphetamine-, morphine-, cocaine-dependent, and control. Proteins were separated by SDS-PAGE and the molecules within the range of 46-58 kDa were trypsin digested and analyzed by LC-MS^n. Peptide maps were separated by the reversed phase capillary system, and detected by an Amazon ETD mass spectrometer (Bruker Daltonics, Bremen, Germany). Data were processed by the Profile Analysis (label free approach) and Proteinscape softwares form Bruker Daltonics. Finally, the GO analysis for up-, and down regulated proteins were performed using the PANTHER Gene List Analysis.

Results: Identification of peptides was performed on the basis of CID fragmentation. MS data allowed for proteins identification by the Mascot server (Matrix Science). After label free analyses, the proteins identified in rat microsomes (control and drug dependent) were divided into up-, and down regulated groups. The GO Biological Functions and GO Biological Processes for up and down regulated proteins were defined.

Conclusions: The project presents application of the label free proteomic research in drug addiction with the focus on cytochromes P450 (CYP450), mainly responsible for the metabolic processes in liver. Also, the results show comparison of an influence of different drugs (amphetamine, morphine and cocaine) on proteins present in liver microsomes.

Keywords: label free proteomics, microsomes, cytochromes P450, drug dependence

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Sodium effect on agonist binding to δ-opioid receptors in isolated plasma membranes from forebrain cortex of rats exposed to increasing doses of morphine (10 - 50 mg/kg) for 10 days

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Abstract

Purpose: The aim of this work was to determine the effect of sodium cations, representing the specific allosteric modulators of ligand binding site of opioid receptors (OR), on agonist binding to δ-opioid receptors (δ-OR) in plasma membranes isolated from forebrain cortex of rats exposed to morphine for prolonged period of time.

Experimental description: Rats were exposed to increasing doses of morphine (10 - 50 mg/kg) for 10 days and sacrificed 24 hours (group +M10) or 20 days (group +M10/−M20) after the last dose. Control animals were sacrificed in parallel with morphine-treated (groups −M10 and (−M10/−M20)). Plasma membrane enriched fraction (PM) was isolated from rat brain cortex (+M10 and -M10) and analyzed by specific radioligand binding. δ-OR were characterized by saturation binding studies with δ-OR agonists [^{3}H]DADLE and [^{3}H]DPDPE in the presence or absence of 100 mM NaCl. Percoll-purified PM (from all groups) were also resolved by 1D-SDS-PAGE and 2D-ELFO. Subsequently, immunoblot detection with specific antibodies and proteomic analysis was performed.

Results: Maximum number of [^{3}H]DADLE binding sites in PM isolated from morphine-treated rats was 1.4-fold higher than in PM isolated from control rats. Surprisingly, 100 mM sodium chloride had no effect on [^{3}H]DADLE binding in PM prepared from morphine-treated animals (+M10), but, as expected, it did inhibit agonist binding to PM isolated from control animals (-M10). Morphine-induced increase of ligand binding to δ-OR was substantially higher when more specific ligand, [^{3}H]DPDPE, was used for determination of δ-OR in brain membranes. Maximum number of [^{3}H]DPDPE binding sites in morphine-treated membranes was 2.1-fold higher than in control membranes. Thus, the inhibition of agonist binding by sodium was detected in control membranes only. Our results indicated a specific up-regulation of adenylyl cyclases I (8-fold) and II (2.5-fold) and significant increase of proteins functionally related to oxidative stress and apoptosis.

Conclusions: Sodium ions have been described as efficient inhibitors of agonist binding to numerous GPCR including OR, causing the shift of receptor molecules from active (R*) to inactive (R) conformation and the uncoupling of receptor from the cognate G protein, i.e. inverse agonist effect. The low sensitivity of δ-OR to inhibitory effect of sodium in morphine-treated samples of forebrain cortex may be interpreted as disturbance of equilibrium between the active and non-active forms of δ-OR molecules.

Keywords: sodium, morphine, rat forebrain cortex, opioid receptors.

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Do or do not. There is no try

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ABSTRACT

Protein biomarker discovery for medical diagnostic or for pharmacological purposes is an issue of primary importance for the proteomic community. In proteomics, the traditional sample treatments for protein identification through trypsination are tedious and time-consuming. Nowadays, ultrasound energy is gaining momentum to speed up sample digestion as well as different steps of the sample treatment for protein identification. Thus, gel washing, enzymatic digestion and peptide extraction from gels are speeded using USE. However, there is still a lack in the number of samples that can be treated at once1,2,3,4. Herein, we propose the use of a new ultrasonic technology, the Microplate Horn Assembly, to reduce the complexity of classical protocols to identify proteins as well as to increase the number of samples treated a once to an unprecedented level.

Keywords: Proteomics, protein identification, ultrasound energy, protein biomarker discovery.

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Proteomics analysis of the peritoneal dialysis effluent: a longitudinal study

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ABSTRACT

Purpose: Long-term peritoneal dialysis (PD) leads to morphological and functional alterations in the peritoneum, reducing the lifespan of this dialysis up to five years, and forcing the replacement of PD by other renal replacement therapies. Peritoneum failure does not occur in every patient in the same sequence and to the same extent. This work aims to develop longitudinal studies to unravel the evolution of the proteome of the peritoneal dialysate with time, so biomarkers and molecular profiles for diagnosis and prognosis can be obtained.

Experimental description/ Results: Peritoneal dialysis effluent (PDE) samples from anonymous patients, already at different stages of dialysis were taken. The PDE from the patients was analyzed using 2D gel electrophoresis[1]. Gel comparison and statistical analysis using Progenesis SameSpots indicated the gel spots differentially expressed for each patient (P01 (6 proteins overexpressed (↑), 3 underexpressed (↓)); P02 (7(↑), 2(↓)); P03 (12 (↑), 19 (↓)); P04 (4 proteins (↓)); P05 (5 proteins (↑), 2 (↓)). All the detected spots were excised digested and identified by MALDI-MS.

Conclusion: All the identified proteins were studied for their biochemical function. Identification of specific molecular changes can be particularly interesting for the understanding and early detection of long-term peritoneum alterations as well as for the development of new therapies to increase the lifespan of the peritoneal dialysis.

Keywords: Peritoneal dialysis, Peritoneal dialysate effluent, MALDI-TOF-MS, 2D-SDS-PAGE, long-term PD.

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Differential expression of egg white proteins in different breeds of chickens and their association with physical characteristics of hard-boiled eggs

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Abstract

Purpose: The purpose of this study was to explore the differential expression of egg white proteins in different breeds of chickens and their association with physical characteristics of hard-boiled eggs.

Experimental description: Fresh eggs collected from 31-wk-old ISA Brown, L2 strain Taiwan country chickens (L2 strain TCCs), and Quemoy chickens, were stored at room temperature (25-26°C) for 3 days before further analysis. The eggs were subjected to measuring general traits and physical characteristics of hard-boiled eggs evaluation, including texture profile analysis, stress relaxation, breaking test, and puncture test. The egg white were used for proteomic analysis.

Results: The general egg traits showed that eggs from ISA Brown had higher egg weight, egg length, and egg width than those of L2 strain TCCs and Quemoy chickens ($P < 0.05$). The egg shape index, yolk weight, albumin height, and Haugh unit did not differ among breeds. The physical characteristics of hard-boiled eggs in L2 strain TCCs showed higher hardness, fracturability, gumminess, chewiness, puncture value, viscoelasticity, and toughness than those of the other two breeds ($P < 0.05$). The levels of 16 out of 124 quantified protein spots differed significantly among the three breeds ($P < 0.05$). Fifteen of the differed protein spots were successfully identified by peptide mass fingerprinting and represented 7 distinct proteins, including Hep21 protein precursor, ovoinhibitor, ovalbumin related protein X, ovalbumin, ovotransferrin, serum albumin, and phosphoglucomutase-1. The levels of one and three ovotransferrin spots in L2 strain TCCs were significantly higher than those in ISA Brown and Quemoy chickens. Pearson correlation further showed that the level of ovotransferrin was highly positive-correlated with most of the physical characteristics of hard-boiled eggs.

Conclusions: The result of this study suggested that the reason why the eggs of L2 strain TCCs have better physical characteristics of hard-boiled eggs may result from the higher level of ovotransferrin in their egg white.

Keywords: Chicken eggs, Egg white proteins, Physical characteristics, Hard-boiled eggs.

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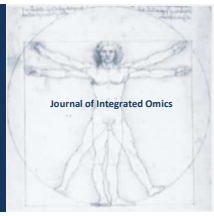

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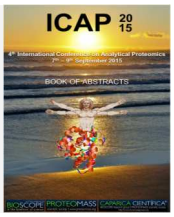

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Methods for the preparation of acute myeloid leukemia patient samples for proteomic and phosphoproteomic analysis

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Abstract

Purpose: The study of global protein expression in acute myeloid leukemia (AML) patients by mass spectrometry (MS) can help identifying differential expression and post-translational modifications of proteins that could represent disease-related biomarkers for early diagnosis or for improved prognostics and to predict the patient response to different therapeutics. To optimize the proteome and phosphoproteome coverage of samples from AML patients by LC-MS/MS analysis, we have tested several methods with different peptide fractionation and phosphopeptide enrichment strategies.

Experimental description: Peptide samples were prepared with an in-solution digestion protocol, using urea or guanidinium hydrochloride as denaturant, and with the filter aided sample preparation (FASP) methodology. Peptide fractionation was carried out with reverse phase/strong cation exchange (SCX) and SCX disks in a stage-tip. Techniques for phosphopeptide enrichment included metal oxide affinity chromatography (MOAC), immobilized metal affinity chromatography (IMAC) and sequential elution from IMAC (SIMAC).

Results: From an initial screening of the different samples on a Linear Trap Quadrupole (LTQ) Orbitrap Elite MS, we found the best proteome coverage with the sequential FASP method, with Lys-C and trypsin as digestion enzymes, which identified and quantified 3100 proteins from 20 µg of sample. With the same strategy, followed by a separate MOAC/TiO_2-beads enrichment of the two peptide pools, we identified and quantified 2.900 phosphorylation sites from only 250 µg of AML patient sample. On a QExactive HF hybrid Quadrupole-Orbitrap MS, 5400 proteins and 4000 phosphosites were identified and quantified from these FASP-prepared samples.

Conclusions: To improve the proteome and phosphoproteome coverage of AML patient samples by MS analysis to discover new biomarkers, testing of different methods can be beneficial. Based on sample preparation optimization in our laboratory, we have chosen the FASP protocol to prepare AML patient samples for MS-based proteomic and phosphoproteomic studies.

Keywords: AML, proteomics, phosphoproteomics, sample preparation.

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Proteomics analysis of the peritoneal dialysate effluent reveals the presence of calcium-regulation proteins and acute inflammatory response

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ABSTRACT

Purpose: Peritoneal dialysis (PD) is a form of renal replacement used for advanced chronic kidney disease. PD effluent holds a great potential for biomarker discovery for diagnosis and prognosis. In this study a novel approach to unraveling the proteome of PD effluent based-on dithiothreitol depletion followed by 2D-SDS-PAGE and protein identification using tandem mass spectrometry is proposed.

Experimental description: In the present study, samples of peritoneal dialysate effluent were collect from six patients from the Complexo Hospitalario Universitario de Ourense (CHUO). Before proteomics analysis the six samples of peritoneal fluid were depleted with DTT and quantified using a Bradford protein assay. In order to analyze the differences among the patients, 2D gels were carried out by triplicate for each patient. A total of 100 µg of protein was loaded onto pH 3–10 strips, and then proteins were visualized with CBB. Gel spots were excised and subjected to in-gel digestion and MALDI-TOF/TOF analysis.

Results: A total of 49 spots were analyzed revealing 25 proteins differentially expressed, among them many proteins involved in calcium regulation.

Conclusions: Through this work it has been demonstrated that the proteome of the peritoneal dialysis effluent is far from being well established. A total of 25 proteins differentially expressed have been identified among 6 patients with PD. It is concluded that studies dealing with PDE are difficult to compare if the methodology used to treat the samples and to identify the proteins are not the same. It has been shown that the DTT-based depletion method is a cheap alternative to be considered to other expensive approaches. Remarkably, a group of proteins dealing with calcium metabolism and calcium regulation has been found to be lost through peritoneal dialysate effluent. This finding might be directly linked to the calcification of soft tissues, in patients subjected to PD, and opens new insights into the potential use of PDE as a sample for diagnosis and prognosis of patients with renal failure.

Keywords: Peritoneal dialysis effluent, 2D-Gel Electrophoresis, Protein identification, Proteomics.

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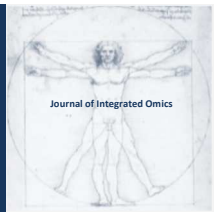

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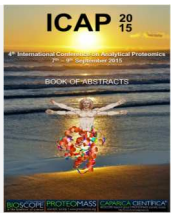

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Evaluation of alternative strategies depletion proteins for biomarker discovery in patients serum with bipolar disorder

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Abstract

Purpose: Bipolar Disorder (BD) is a mental disease with unclear connections to other similar disorder that affect hundreds of thousand of individuals in the world. Serum may be utilized as a rich source of biomarker of disease. However, the large number of proteins in serum makes the analysis very difficult because high abundant proteins (albumin and Immunoglobulin) tend to mask of those of lower abundance. Depletion of abundant serum proteins may help in the discovery and detection proteins with potential biomarker of diseases, such as bipolar disorder. Thus, this study had aim evaluate alternative procedure depletion abundant proteins to biomarkers discovery in patient serum samples with bipolar disorder.

Experimental description: A variety of depletion methods for removal of high abundant proteins from serum have been related in the literature. This work shows the potential of chemical protein depletion, using dithiothreitol (DTT), acetonitrile (ACN), and tandem depletion (DTT/ACN). For protein depletion with DTT, approximately 2.0 µL of DTT (500mM) was added to 20 µL (> 10µg μL^{-1}) of serum human. Following, the mixture was incubated for 60 min at 37 °C until a white precipite come to light, which is pellet by centrifugation at 13,000 rpm for 40 min. The supernatant was transferred into microtubes and stored at -20 °C until analysis by SDS-PAGE. For protein depletion with ACN, 20 µL (> 10µg μL^{-1}) of serum human was diluted to about 60 µL with Milli-Q water. Then, acetonitrile was added drop by drop to a final concentration of 60% (v/v). Then, samples were vortexed and sonicated in an ultrasonic bath for 20 min. Supernatant was collected after centrifugation at 13,000 rpm for 20 min, and evaporated to dryness in SpeedVac. For tandem depletion, the sera were subjected to a sequential depletion protocol involving the combination of the two precipitation steps previous: protein depletion was first performed with DTT and the supernatant was treated with ACN in order. Following, the depleted protein were analyzed by SDS-PAGE and all proteic bands were, digested (trypsin) and analyzed by nanoLC-MS/MS.

Results: A total de 17 proteins were identified for DTT depletion. Of these, 9 belonged to the group of the major proteins (APOA1, IGHG1, A1AT, C4-A, IGKC, CERUM, APOB, HPT, A1AG1). It was found that this method reduce the presence of albumin in serum human sample. For ACN depletion, it was found that this method reduces the presence of protein above 75 KDa, for example apoliproteins. The number of proteins identified in the depleted serum by ACN strategies was 16, of which 6 (HPT, A1AG1, ALBU, CERUM, IGKC, A1AT) belonged to the group of the major proteins. 4 (A1AG1, A1AT, CERUM, HPT) were the abundant proteins identified using tandem depletion. Thus, in this way the relative protein in-gel load of other less-abundant proteins was increased.

Conclusions: In this study, we evaluated the application of DTT, ACN and tandem depletion as an alternative procedure of protein major depletion for the search of disease biomarker in serum and accordingly tandem depletion present as an efficient tool.

Keywords: Bipolar disorder, depletion protein, serum human, SDS-PAGE, nanoLC-MS/MS.

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Evaluation of proteome alterations induced by cadmium stress in sunflower (*Helianthus annuus L.*) cultures using 2-D DIGE and nESI-Q-Tof

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Abstract

Purpose: The present study evaluates, at a proteomic level, changes in the proteomic map sunflower leaves in the absence or presence (at 50 or 700 mg) of cadmium (as $CdCl_2$). Experimental description: At the end of the cultivation period (45 days), proteins are extracted from leaves with phenol, separated by two-dimensional difference gel electrophoresis (2-D DIGE), and excised from the gels. The differential protein abundances (for proteins differing by more than 1.8 fold, which corresponds to 90% variation) are characterized using nESI-Q-Tof. After the experiments LC-MS/MS, the identified proteins were evaluated using the String 9.1 program.

Results: The protein content decreases by approximately 41% in plants treated with 700 mg Cd compared with control plants. By comparing all groups of plants evaluated in this study (Control vs Cd-lower, Control vs Cd-higher and Cd-lower vs Cd-higher), 39 proteins are found differential and 18 accurately identified; the control vs Cdhigher treatment is that presenting the most differential proteins. From identified proteins, those involved in energy and disease/defense (including stress), are the ribulose bisphosphate carboxylase large chain, transketolase, and heat shock proteins are the most differential abundant proteins. The analysis of the identified proteins using String program revealed that the major physiological processes of the plant affected by cadmium stress were as follows: primary metabolic process, cellular component organization, organic substance metabolic process and metabolic process, cellular process and response to stimulus.

Conclusions: According to the results, photosynthesis is the main process affected by the presence of high Cd concentrations in sunflowers, although the plants are highly tolerant to Cd exposure, as only minor changes were observed in their physiological processes. Therefore, proteomic results from this study are in agreement with those from our ionomic approaches (Lopes Júnior et al., Environ Exp Bot, 107, 2014, 180-186).

Keywords: sunflower; cadmium; proteomics; 2-D DIGE; LC-MS/MS.

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