5th Norwegian National Proteomics Meeting
ABSTRACT BOOK

26th - 27th October 2009
VilVite, University of Bergen

5th Norwegian National Proteomics Meeting

Invited speakers:
Jesper V. Olsen, Novo Nordisk Foundation Center for Protein Research, Copenhagen
Hasmik Keshishian, Broad Institute of MIT and Harvard

Local organizing committee for Norwegian Proteomics Society:
Ann Kristin Frøyset, Dept. Molecular Biology
Geir Olav Løken, Dept. Biomedicine
Frode Berven, PROBE, Dept. Biomedicine
Olav Mjaavatn, PROBE, Dept. Biomedicine
Kari E. Fladmark, Dept. Molecular Biology

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Monday October 26th

0800-0950 Registration, poster and exhibition mounting

Session 1: Chaired by Kari Fladmark and Frode S. Berven

0950-1000 Welcome by Kari Fladmark

1000-1045 **Hasmik Keshishian**, Broad Institute of MIT and Harvard: "Verification of Candidate Biomarkers by Stable Isotope Dilution Mass Spectrometry"

1045-1105 **Therese Solstad**, University of Oslo: "A proteomic approach to identify biomarkers for naturally occurring regulatory T cells"

1105-1125 **Frode S. Berven**, University of Bergen: "Discovery of novel diagnostic biomarker candidates for multiple sclerosis through clinical proteomics"

1125-1145 **Coffee Break**

1145-1215 **Volker Kruft**, Applied Biosystems: "New workflows for the quantitation of protein biomarkers using hybrid triple quadrupole/linear ion trap mass spectrometry".

1215-1245 **Christine Carapito**, Institut Pluridisciplinaire Hubert Curien: "Taking advantage of the high sensitivity and high reproducibility of the LC-Chip/QQQ system for SRM-based quantitative proteomics"

1245-14 00 **Lunch with poster session**

Session 2: Chaired by Morten Skaugen

1400-1420 **Stefania Gudrun Bjarnadottir**, Nofima Mat: "Cloned pigs show larger variation in protein expression than siblings".

1420-1440 **Alvhild Alette Bjørkum**, Bergen University College: "Human Serum Protein Profile and Protein Identification after Sleep Restriction".

1440-1500 **Katarina Alenäs**, Denator AB: "Heat Stabilization of the Proteome for improved sample preparation within proteomics"

1500-1530 **Madalina Oppermann**: Thermo Fisher Scientific "Comprehensive mapping of the human seminal plasma proteome by a novel, iterative LC-MS/MS analysis and database search workflow"

1530-1600 **NPF Annual meeting and election**.

1830 **Dinner at Nøsteboden (see map)**

Pub at Nøsteboden opens at 1700

Tuesday October 27th

Session 3: Chaired by Frode Selheim

0900-0945 **Jesper Olsen**, University of Copenhagen: "Global analysis of cell signaling networks by quantitative proteomics"

0945-1005 **Gustavo de Souza**, University of Bergen: "Merging closely related protein databases to improve proteomic characterization of prokaryotic microbes"

1005-1025 **Ralf Kellmann**, University of Bergen: "Differential protein expression of human neuroblastoma cells in response to azaspiracid-1"

1025-1100 **Coffee Break**

1100-1120 **Vibeke Hervik Bull**, University of Oslo: "A temporal proteomic study of sorafenib induced human neuroblastoma cells".

1120-1140 **Even Birkeland**, University of Bergen: "Epac-induced changes in the proteome of SH-SY5Y neuroblastoma cells".

1140-1330 **Lunch with poster session**

Session 4: Chaired by Lars Hagen

1330-1350 **Thomas Arnesen**, University of Bergen: "Proteomics reveals the evolutionary conservation and divergence of N-terminal acetyltransferases from yeast and humans"

1350-1410 **Astrid E. V Tutturen**, University of Oslo:"Two Novel Strategies for a targeted proteomic analysis of protein citrullination"

1410-1430 **Siri Dørum**, University of Oslo: "Identification of the best gluten substrates for transglutaminase 2 in proteolytic digest of whole gluten".

1430-1450 **Jesper Dyekær**, Invitrogen: "The next generation in protein profiling

Click-iT™ AHA for nascent protein synthesis and Click-iT™ glycoprotein

labeling reagents for Glycoprotein Detection"

1450 **Conclusive remarks**

Bergen INFO

A buss (free of charge) shuttles between VilVite and Bergen centre every 10 minutes.

Location dinner Monday evening:

"Nøsteboden" Nøstegaten 32, Bergen

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Verification of Candidate Biomarkers by Stable Isotope Dilution Mass Spectrometry

Hasmik Keshishian

Broad Institute of MIT and Harvard, Cambridge, MA

Candidate-based biomarker verification relies upon specific, quantitative assays optimized for selective detection of target proteins, and is increasingly viewed as a critical step in the biomarker discovery pipeline that bridges unbiased discovery to pre-clinical validation. The primary objective of verification is to screen potential biomarkers to ensure that only the highest quality candidates from the discovery phase are taken forward into pre-clinical validation. Multiple reaction monitoring (MRM) coupled with stable isotope dilution mass spectrometry (SID-MS) has recently been shown to be well suited for direct quantification of proteins in plasma due to the high specificity, sensitivity, reproducibility, and precision of the quantitative measurements. In addition, MRM assays can be highly multiplexed such that many candidate proteins can be simultaneously targeted and measured in large numbers of patient samples required to show sufficient sensitivity and specificity toward the targeted disease. However, sensitivity for unambiguous detection and quantification of proteins by MS-based assays is often constrained by sample complexity particularly when the measurements are being made in plasma. Plasma can be made significantly less complex by employing fractionation and peptide antibody enrichment strategies prior to SID-MRM-MS. Using such approaches, we have recently demonstrated that MS-based assays can be configured for a number of known markers of cardiovascular disease and, more importantly, can be deployed to measure their concentrations in clinical samples.

A proteomic approach to identify biomarkers for naturally occurring regulatory T cells

T Solstad, SJ Bains, B Thiede, K Taskén, and KM Torgersen

Biotechnology Centre of Oslo and Centre for Molecular Medicine Norway, Nordic EMBL Partnership, University of Oslo, Norway

Introduction

Regulatory T cells (Tregs) maintain peripheral tolerance and immune homeostasis by their ability to suppress activation of other immune cells. So far the mechanisms behind this critical function are relatively poorly understood. To define a protein expression profile and identify potential candidates involved in immune suppression, we compared the protein expression profiles in Tregs vs. effector T cells (Teffs) from human blood. Candidate proteins were identified in several subcellular compartments and subjected to phenotypical verification and functional testing in Treg suppression assays.

Methods

Tregs and Teffs were pre-enriched from human buffy coats by enriching for CD4+ cells. To ensure highest possible purity of cells, Tregs and Teffs were isolated by FACS (Fluorescence Activated Cell Sorting*)* prior to subcellular fractionation, SDS-PAGE, in-gel trypsination, chemical labeling, sample preparation and analysis by LC-LTQ-Orbitrap MS (Fig. 1). Mass spectrometric data were searched against the Swiss Prot human proteome database using MASCOT as the search engine. Quantitative analysis was performed using MSQuant. Candidate proteins were further validated by RT-qPCR, flow cytometry, western blot analysis and functional analysis by the Treg suppression assay (2).

Results

We have identified approx. 30 proteins that seem to be uniquely or highly expressed in the Treg population as compared to Teffs. Our mass spectrometric data were confirmed by flow cytometry and western blot analysis on a selected set of proteins, in addition to RT-qPCR on all our candidates. Interestingly, most of the identified proteins revealed only marginal differences on mRNA expression level, thus confirming the importance of a proteomic approach rather than a microarray/RNA-based approach in the search for Treg biomarkers.
Several of our candidates were identified as transmembrane proteins defining so far uncharacterized Treg subsets. Furthermore, functional studies on these subsets suggest that one of these membrane proteins splits the Treg population into two functionally distinct populations. Cells expressing the protein at a high level were highly suppressive, while the low expressing cells displayed little or no suppressive ability.

Innovative aspects

- Purification and isolation of primary T cells by FACS prior to subcellular fractionation ensures highest possible purity of cells and reduces complexity prior to proteomic analysis
- Identification of novel Treg biomarker whose cell surface expression correlates highly with immune suppression
- Identification of novel markers for Treg subpopulations

References

(1) Wildin, R.S., et al., *X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy.* Nat Genet, 2001. **27**(1): p. 18-20

(2) Torheim, E.A., et al., *Interleukin-10-secreting T cells define a suppressive subset within the HIV-1-specific T-cell population.* Eur J Immunol, 2009.

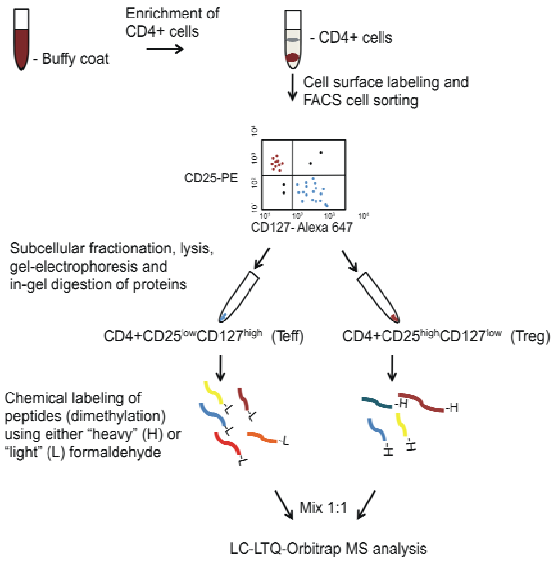

Figure 1. Work flow of the strategy applied to identify Treg biomarkers

Discovery of novel diagnostic biomarker candidates for multiple sclerosis through clinical proteomics

Berven FS[1,2], Kroksveen AC[2], Myhr KM[3,4], Vedeler C[3], Ulvik RJ[2].

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Multiple sclerosis is a chronic inflammatory disease with an insidious and unpredictable clinical course. Treatment is only effective at an early stage of the disease. It is very difficult to reliably diagnose multiple sclerosis at an early stage with current laboratory tests, and the diagnosis is often delayed by several years. Due to the unpredictable clinical course, it is also very difficult to give optimized treatment for these patients. In recent years proteomics based on mass-spectrometry and sophisticated computational software has been introduced to clinical medicine as a promising analytical technique to improve biochemical diagnostics. By comparing the protein content of a particular body fluid like plasma or cerebrospinal fluid (CSF) in disease and health, one may detect disease specific protein biomarkers that will be of high clinical value. There are generally four different phases in the discovery of new biomarkers; the discovery phase, the qualification phase, the verification phase and the validation phase. We have used state of the art proteomics approaches to discover novel disease specific biomarkers candidates in CSF from patients with multiple sclerosis versus patients with other neurological diseases. Our extensive quantitative biomarker discovery effort was to a large degree based on using global shotgun proteomics with iTRAQ labeling (isobaric tag for relative and absolute quantification), where the goal was to do an unbiased identification and quantification of as many proteins in CSF as possible. So far, this approach has materialized in a total of eleven very promising biomarker candidates from more than 1000 identified and quantified proteins in CSF. Three of these proteins had significantly higher abundance in CSF of multiple sclerosis patients, whereas the other eight had significantly lower abundance. The eleven proteins have suggested functions that are in line with the expected pathogenesis of multiple sclerosis. We are now moving towards the qualification and verification phase for these findings in a larger set of samples using novel mass-spectrometry-based targeted protein quantification technology.

New workflows for the quantitation of protein biomarkers using hybrid triple quadrupole/linear ion trap mass spectrometry

Volker Kruft

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Hybrid Triple Quadrupole/Linear Ion Trap mass spectrometers (QqLIT or QTRAP® Systems) were commercially introduced in 2002. Since then QTRAP® Systems have fast become one of the most versatile and successful mass spectrometer types. In 2003 and 2005, second-generation instruments were launched with improved performance specifications. We have recently presented a newly developed third generation QqLIT mass spectrometer, the QTRAP® 5500 LC/MS/MS System. Due to the completely re-designed instrument hardware and electronics, significant improvements in almost all aspects of performance and robustness have been achieved. We will discuss the hardware and software improvements made for the use of the QTRAP® 5500 System for the quantitation of protein biomarkers in complex biological samples. These improvements have enabled new workflows such as the development of peptide/protein MRMs directly from biological samples, and the use of Multiple Reaction Monitoring Cubed (or MRM3) for biomarker quantitation in background of high complexity. Principles and examples for both workflows will be presented.

Taking advantage of the high sensitivity and high reproducibility of the LC-Chip/QQQ system for SRM-based quantitative proteomics

Carapito C., Colas C., Schaeffer C., Van Dorsselaer A.

Laboratoire de Spectrométrie de Masse Bio-Organique, IPHC-DSA, ULP, CNRS, UMR7178, Strasbourg, France.

Selected reaction monitoring (SRM)-based proteomics is emerging as the technology that ideally complements the discovery capabilities of shotgun proteomics by its unique potential for reliable quantification of the analytes.
The specificity of the technology is provided by the measurement of specific pairs of precursor and fragment ions on triple quadrupole-like instruments. High sensitivity and high reproducibility are two other absolute requirements in order to confidently quantify low abundant analytes in very complex mixtures.
In this context, we have performed an extensive evaluation of the LC-Chip/QQQ system (Agilent Technologies) for reliable quantification of low abundant peptides in very complex protein digests. We have established key parameters that have to be optimized in order to get the highest quality data from the system. The optimized methods have been successfully applied for the quantification of peptides from standard protein digests, a whole yeast cell lysate and a total rat tissue proteome. Reliable quantification was obtained on low femtomol amounts of peptides and over at least 3 orders of magnitude. The dynamic SRM-functioning allowed us to follow up to several hundred transitions in a single analysis.

Cloned pigs show larger variation in protein expression than siblings

Bjarnadottir, Stefania Gudrun

Nofima Mat, UMB

Cloned animals are of potentially great benefit to biological science, since they have identical genomes. For biomedical research, cloning promised to create a large set of littermates, all having the same genetic makeup. Pigs are excellent model organism for characterizing human diseases and cloning of pigs has therefore gained much resent interest. However, there is need for more understanding of the detailed health effects of cloning on the molecular, cellular and epigenetic level. We therefore used Q-star Elite LC/MS/MS instrument to characterize the variations in the proteomes of muscle and liver tissues, in both a litter of cloned piglets and a litter of normal siblings. We have observed that amongst cloned littermates, which in principle should be regarded as genetically identical, there is larger inter-animal divergence in proteome expression, than amongst normal siblings/littermates.

Human serum protein profile and protein identification after sleep restriction

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Sleep restriction (SR) might lead to cell stress. Humans (n=6-8) was subjected to 3 or 6 hours of SR and blood were sampled before, during and after the SR-night at the same time points (16, 48 h). Seldi-Tof-MS (Ciphergen), Maldi-Tof-MS (AutoFlex, Bruker Daltonics) and an Hsp70 ELISA-kit (EKS-700 Stressgen biotechnologies) were used to detect changes in the human blood serum proteome. Protein profile changes after SR were searched for by building a decision tree from the information gain in the m/z spectrum. Principal component analysis (PCA, Sirius 7.0-PRS), support vector machine-, decision three-models were also used analyzing the mass spectrometry data. A protein of 71 kDa was decreased in the blood (serum) 0h, 3h and 9h after 3h SR. Similarly Hsp-70 with molecular weight around 70 kDa was also reduced 0h, 3h and 9h after 3h SR by the Stressgen-kit measurement. sIgA showed a changed profile after 3 and 6 h SR and the day after the SR night. The protein profile from the Seldi-Tof-MS (2.5 - 100 kDa, n=3) measurements also showed changed expression for several proteins. Proteins highly expressed at basal level seem to be reduced after SR (not to basal level) the day after or night before. The protein profile from the Maldi-Tof-MS (0.4 - 15 kDa, n=7) also showed changed expression for several proteins. Several proteins (2.5 - 100 kDa) where differentially expressed after 3 and 6 hours of sleep restriction, specifically Hsp-70 was reduced after 3 hours of sleep restriction. One of several changed proteins were identified as Inter-alpha-trypsin-inhibitor-family heavy-chain-related protein and verified by Q-Tof-MS of the synthesised protein (by Bejing SBS Genetech Co, Ltd, www.sbsbio.com) and are now being explored with the curated database GeneGo (www.genego.com). The decrease of many proteins as Hsp-70 in the blood during sleep restriction is in line with what has been observed in obstructive sleep apnoeas. Immune parameters as sIgA might reflect a weakened immune response after 3 and 6 h SR and is in line with earlier studies.
Keywords; Proteins, sleep restriction, information gain, decision tree

Heat stabilization of the proteome for improved sample preparation within proteomics

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Removal of a sample from its natural surrounding leads to major disturbance of the tissue homeostasis. The action of proteases and other protein-modifying enzymes rapidly can change the composition of the proteome and post translational modifications (1, 2). Subsequent analytical results reflect a mix of *in vivo* proteome and degradation products. In this experiment a novel stabilization system was used to treat fresh and frozen tissue samples in an attempt to stop degradation and preserve the *in vivo* proteome by inactivation early in the sample preparation chain.
The system utilizes rapid heat inactivation to eliminate enzymatic activity in tissue and thereby enable detection of a stabilized proteome including proteins, endogenous peptides, and preserved protein phosphorylations.
The stabilizing effect of the rapid heat inactivation system was assessed by nano-LC-MS, MALDI and western blotting on samples from brain, heart muscle, and liver. The inactivation method should preserve the proteome and enable detection of endogenous peptides and monitoring of important post-translational modifications, such as phosphorylation of peptides and proteins.
Inadequate sample handling normally cause an increase in degradation fragments which has been shown in these experiments. However, after immediate sample stabilization, no protein degradation fragments were detectable. Proteases, phosphatases and other enzymes were effectively inactivated. The mass spectrometrically identified peptide peaks in the stabilized samples consisted of several known neuropeptides, endogenous peptides, and novel potentially biologically active peptides. Most peaks detected in an untreated group originate from proteins such as hemoglobin, cyclophilin, NADH dehydrogenase, synuclein and other highly expressed proteins. The levels of phosphorylated forms of CREB, GSK and MAPK remained unchanged after 2 hours in room temperature after stabilization treatment as the levels of the same proteins in untreated tissue decreased in only 10 minutes.

References

[1] K. Sköld, M. Svensson, M. Norrman et al., *Proteomics*, **7** (24), 4445-4456 (2007)
[2] M. Svensson, M. Borén, K. Sköld et al., *J Proteome Res,* **8** (2), 974-981(2009)

Comprehensive mapping of the human seminal plasma proteome by a novel, iterative LC-MS/MS analysis and database search workflow

Oppermann, Madalina

Thermo Fisher Scientific

Few studies have been performed to investigate the protein composition of seminal plasma. Several proteins were identified in the seminal plasma of healthy donors using a 2D gel-mass spectrometry approach. More recent studies were performed using a combination of 1D gel electrophoresis and LC-MS/MS analysis. In 2006, 923 proteins were identified by Mann and co-workers using this strategy with mass analysis performed by an LTQ FT instrument. However, this study failed to identify known markers in the seminal plasma such as epididymis-specific defensins that may have been lost during the fractionation step, demonstrating the complexity of this biological fluid. Mass spectrometers have a limited dynamic range where peptide analyte ions of interest can be fragmented efficiently for confident identification by database search. Due to sample preparation, chromatographic, and mass spectrometric constraints, a limited number of peptides can be analyzed in any given LC-MS/MS experiment. On-the-fly strategies involve the selection of the most intense peptides for fragmentation in a data-dependent manner. Very complex samples like seminal plasma thus require an optimized data acquisition strategy to achieve a thorough analysis of the sample. Here we describe a method for extensive protein identification in complex samples that involves iterative nanoflow LC-MS/MS analysis, exclusion list generation, and iterative database searching with Proteome Discoverer software, leading to the identification of numerous low-copy-number proteins in the seminal plasma proteome.

Global analysis of cell signaling networks by quantitative proteomics

Jesper V. Olsen

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Abstract: Mass spectrometry-based proteomics is a powerful tool for analyzing endogenous proteins and post-translational modifications on a global scale. Recent technical [Olsen JV et al, MCP 2005] and computational advances [Cox J, Mann M, Nat Biotech 2008] in high-resolution mass spectrometry (MS) based quantitative proteomics and phosphoproteomics [Macek B, Mann M, Olsen JV, Ann Rev Tox 2009] have enabled new ways of analyzing cell signaling pathways to an unprecedented depth in an unbiased manner. We have developed a quantitative phosphoproteomics technology that combines stable isotope labeling by amino acids in cell culture (SILAC) [Ong SE et al, MCP 2002] for quantitation with phosphopeptide enrichment and high-performance mass spectrometry. We have recently reported the time-resolved analysis of changes in the phosphoproteome of HeLa cells stimulated with EGF and quantified more than 6,000 phosphosites [Olsen JV et al, Cell 2006] and presented the first complete eukaryotic proteome quantified by mass spectrometry [De Godoy LMF, Olsen JV et al, Nature 2008]. Our approach is completely generic for identification of key phosphorylation events as well as proteome changes in signaling pathways on a systems-wide scale and is applicable to any cell culture system, tissue or organism that can be SILAC or chemically isotope labeled. We have now extended our methodology to analyze important disease signaling networks in inflammation (TNF-alpha) and leukemia (FLT3). Our quantitative proteomics technology provides an unbiased and powerful tool to study time-resolved changes of the proteome and phosphoproteome of signaling pathways at a systems wide level.

Merging closely related protein databases to improve proteomic characterization of prokaryotic microbes

Magnus Ø Arntzen[1#], Gustavo A. de Souza[1#] and Harald G. Wiker[1,2*]

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High-throughput genomics has increased the number of completed genomic sequences to over 800 bacterial species. In proteomics, the use of a correct dataset determines the outcome of an experiment. However, the use of different bioinformatic approaches to annotate a gene or open reading frame (ORF) results in divergent gene annotations, even for data generated from the same genomic sequences. There are also examples of observed proteins that were not annotated. Characterization of clinical isolates with many specific genetic variations is suboptimal if they are not represented in the selected database. Therefore, it is crucial to provide protein databases that can cover annotation errors and genetic variations among closely related organisms. We developed a software called MSMSpdbb (Multi-Strain Mass Spectrometry Prokaryotic DataBase Builder) that can merge protein databases from several sources and be applied on any prokaryotic organism. The clustering is performed so that sequences sharing high similarity are reported only once, and sequence differences resulting from mutations or different translational start choices are appended to the primary sequence. We tested our software through generating a database for Mycobacterium tuberculosis complex (including 3 strains of M. bovis and 4 of M. tuberculosis), and analyzing several M. tuberculosis H37Rv (lab strain) data collected in our group. Surprisingly, in M. tuberculosis H37Rv a major protein was identified that indicates an error in the original genomic sequence. Multiple peptides were observed matching MT2420, an ESAT-6-like protein, only described in the M. tuberculosis CDC1551 and M. tuberculosis H37Ra genomes. Recent research had shown several differences when comparing the original genomic sequence of M. tuberculosis H37Rv with selected parts of properly obtained M. tuberculosis H37Rv ATCC27294 and argued that the strain used for the original genomic sequence was different from the ATCC27294 strain. Our data provides further evidence to support this conclusion. Finally, multi-strain proteomic databases allows for identification of sequence variations between strains such as SNPs, insertions/deletions and divergent translational start sites. The MSMSpdbb software and several databases are freely available at http://services.cbu.uib.no/software/prokaryote_databases/.

Differential protein expression of human neuroblastoma cells in response to azaspiracid-1

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Azaspiracid-1 is a novel algal toxin, which causes an instantaneous rise of intracellular messengers, and an irreversible disarrangement of the actin cytoskeleton. Little is known regarding the molecular mechanisms that are involved in azaspiracid-1 toxicity. This study investigated global changes in protein expression by stable-isotope labelling with amino acids in culture and mass spectrometry, following exposure of human neuroblastoma cells to azaspiracid-1. After 12 hours of azaspiracid-1 treatment, the most highly upregulated proteins were involved in cellular energy metabolism, followed by cytoskeleton regulating proteins. The majority of downregulated proteins were involved in transcription, translation and protein modification. In addition, component of oligomeric Golgi complex 5 and ras-related protein RAB1, which are involved in maintenance of the Golgi complex and vesicle transport, respectively, were downregulated. Electron microscopy revealed a disruption of the Golgi complex by azaspiracid-1, and an accumulation of vesicles. In this study, the differential protein expression was examined prior to changes of the cytoskeleton structure in order to capture the primary effects of azaspiracid-1, however the observed changes were of unexpected complexity. Azaspiracid-1 caused a pronounced, but temporary depletion of ATP, which may be the reason for the observed complexity of cellular changes. In a follow-up experiment, global protein expression changes were investigated after 2 hours of exposure to azaspiracid-1. A preliminary interpretation of the new data will be presented.

A temporal proteomic study of sorafenib induced human neuroblastoma cells

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Sorafenib is a new multikinase inhibitor that was first approved for use against renal carcinoma cancer, but has recently been used also against primary liver cancer. It has been proposed that sorafenib induce ER stress resulting in apoptosis in human leukemia cells (U937). ER-stress and ER-stress induced apoptosis is known to contribute in a variety of diseases including diabetes, ischemia-reperfusion injury, Alzheimer disease, and Parkinson disease. The exact mechanism of sorafenib induced cell death is not clear, however, and to study sorafenib`s effects in human neuroblastoma cells (SH-SY5Y), we performed a quantitative proteomic study using stable metabolic labeling (SILAC) in combination with mass spectrometry. Statistical analysis of four replicates resulted in detection of more than 250 proteins that were significantly altered upon sorafenib exposure. We further analyzed the these proteins using data analysis software such as DAVID (http://david.abcc.ncifcrf.gov), FunCoup (http://funcoup.sbc.su.se) and Cytoscape (http://www.cytoscape.org) to determine which cellular processes and protein pathways were significantly affected by sorafenib treatment, as well as to detect protein interactions between our regulated proteins. We found that cell cycle regulators, especially proteins that are necessary for G1 phase entry; mitochondrial ribosome proteins that are responsible for protein translation in mitochondria; as well as proteins involved in oxidative phosphorylation, that is, the process responsible for ATP production in mitochondria were significantly affected by sorafenib treatment. Notably, oxidative phosphorylation is also responsible for most of the production of reactive oxygen species (ROS) in eukaryotes, which can induce oxidative stress and subsequently apoptosis when deregulated. Identification of new proteins or cellular pathways that contribute to apoptotic signaling has the potential to improve conventional chemotherapeutic drugs.

Epac-induced changes in the proteome of SH-SY5Y neuroblastoma cells

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The stage of neuroblastoma tumor cell differentiation is known to influence patient outcome, with a high differentiation stage correlating to favourable prognosis. To elucidate the effects of cAMP in SH-SY5Y neuroblastoma differentiation we used selective cAMP analogs to activate Epac and PKA. We found that activation of Epac induced actin and tubulin polymerization and neurite outgrowth, whereas PKA activation did not. The effects of the Epac stimulation were abolished by knock down of Epac1 with shRNA. Stable isotope labelling with amino acids in cell culture (SILAC) and mass spectrometry were used to disclose the long-time effects of Epac activation on the SH-SY5Y proteome. Upregulated proteins were in general associated with neuronal cell differentiation and adhesion, whereas downregulated proteins typically were involved in RNA processing. We conclude that cAMP-induced morphological and biochemical neuronal differentiation of human neuroblastoma SH-SY5Y cells are mediated by Epac.

Proteomics reveals the evolutionary conservation and divergence of N-terminal acetyltransferases from yeast and humans

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Protein acetylation occurs both at lysine residues within proteins and at the N-terminus of proteins, catalyzed by lysine acetyltransferases (KATs/HATs) and N-terminal acetyltransferases (NATs), respectively. N-terminal acetylation is one of the most common protein modifications in eukaryotes, but the functional role of this modification remains elusive. The COmbined FRActional DIagonal Chromatography (COFRADIC) proteomics technology that can be specifically used to isolate N-terminal peptides was used to determine the N-terminal acetylation status of 742 human and 379 yeast protein N-termini, representing the largest eukaryotic dataset of N-terminal acetylation. The major N-terminal acetyltransferase (NAT), NatA, acts on subclasses of proteins with Ser-, Ala-, Thr-, Gly-, Cys- and Val- N-termini. NatA is composed of subunits encoded by yARD1 and yNAT1 in yeast and hARD1 and hNAT1 in humans. A yeast ard1-Δ nat1-Δ strain was phenotypically complemented by hARD1 hNAT1, suggesting that yNatA and hNatA are similar. Proteomics of a yeast ard1-Δ nat1-Δ strain expressing hNatA demonstrated that hNatA acts on nearly the same set of yeast proteins as yNatA, further revealing that NatA from humans and yeast have identical or nearly identical specificities. Nevertheless, all NatA substrates in yeast were only partially N-acetylated, whereas the corresponding NatA substrates in HeLa cells were mainly completely N-acetylated. Overall, we observed a higher proportion of N-terminally acetylated proteins in humans (84%) as compared with yeast (57%). N-acetylation occurred on approximately one-half of the human proteins with Met-Lys- termini, but did not occur on yeast proteins with such termini. Thus, although we revealed different N-acetylation patterns in yeast and humans, the major NAT, NatA, acetylates the same substrates in both species.

Two novel strategies for a targeted proteomic analysis of protein citrullination

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Introduction Protein citrullination results from enzymatic deimination of peptidylarginine. Despite its important role both in health and disease, the identity and function of citrullinated proteins in vivo remain widely unknown. Proteomic studies of protein citrullination will require tools for a targeted analysis of citrullinated polypeptides which are currently not available. Here we describe two novel strategies for such analyses which are based on chemical modification of citrulline: (i) signature ion based identification of citrullinated peptides; (ii) specific enrichment of citrullinated peptides. Methods Signature ion based analysis: Digests of citrullinated proteins were incubated with 2,3-butanedione and antipyrine at low pH. After work-up by SCX and RP chromatography, samples were analyzed on an HCTultra ion trap instrument using alternating collision induced dissociation (CID) and electron transfer dissociation (ETD). Enrichment of citrullinated peptides: Glyoxal derivatives were covalently attached to beads via a base labile linker. These citrulline reactive beads (CRB) were incubated with digests of citrullinated proteins at low pH resulting in immobilization of citrullinated peptides. After extensive washing, bound peptides were cleaved off at high pH and analyzed by MALDI-TOF/TOF. For data evaluation, variable modifications of arginine were implemented in the search engine. Results Citrulline residues can be specifically derivatized by either 2,3-butanedione/antipyrine or by glyoxal derivatives. No other amino acid side chain is modified. CID of butanedione/antipyrine-modified citrulline generates an intense fragment ion at m/z 201.1028 which serves as a signature ion for monitoring of citrullinated peptides in LC-MS/MS. In ETD, the modification remains intact and MS/MS spectra show nearly complete sequence coverage. Therefore, LC-MS/MS with alternating CID and ETD allows targeted identification of protein citrullination sites, as shown by the analysis of myelin basic protein (MBP) which was deiminated in vitro (1). Two variants of CRB were prepared, carrying either in-house synthesized 4-glyoxalbenzoic acid or commercial 4-hydroxyphenylglyoxal. Both CRB allowed a highly specific enrichment of synthetic citrullinated peptides added to protein digests. CRB were applied to enrich and identify citrullinated peptides from digests of enzymatically deiminated proteins (e.g. MBP) (2). Further, a soluble and tagged glyoxal derivative was synthesized and applied to enrich citrullinated peptides from synovial fluid of rheumatoid arthritis patients. Innovative aspects • Specific chemical modification of citrulline • Signature ion based detection and identification of citrullinated peptides • Specific enrichment of citrullinated peptides by beads functionalized with glyoxal derivatives References (1) Stensland M et al, Targeted analysis of protein citrullination using chemical modification and tandem mass spectrometry; Rapid Commun. Mass Spectrom. 2009; 23: 2754-2762 (2) Tutturen AEV et al, A novel bead-based technique for the specific enrichment of citrullinated peptides; Journal of Proteomics (Submitted)

Identification of the best gluten substrates for Transglutaminase 2 in proteolytic digest of whole gluten

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Celiac disease (CD) is a chronic inflammatory disorder of the gut caused by an inappropriate immune response to dietary gluten of wheat. The disease has a strong genetic association with HLA-DQ2 [1] and the enzyme transglutaminase 2 (TG2) plays a central role by specifically deamidating gluten peptides generating T cell epitopes. TG2 catalyzes transamidation (cross-linking) or deamidation of specific glutamine residues in peptides and proteins [2]. The in vivo deamidation of gluten peptides introduces negative charges to the peptides what increases their binding affinity to the CD-associated HLA-molecule. During the recent years, several gluten-derived epitopes have been identified which becomes deamidated by TG2. The rate by which these epitopes becomes deamidated can differ considerably [3]. However, it remains to be elucidated whether these gluten epitopes are identical with the preferred peptide substrates for TG2 when the enzyme is present in a highly heterogeneous proteolytic digest of whole gluten. To address this, we established a method for enrichment of gluten peptides targeted by TG2. The enrichment step is based on the transamidation reaction mediated by TG2. Small biotinylated amines are cross-linked to distinct glutamine residues in the targeted peptides, which thereby become biotinylated. These transamidated peptides are specifically enriched for using streptavidin beads and analyzed by LC-MS/MS (LTQ Orbitrap XL, microTof-Q). To identify the peptides and their modification sites, the obtained MS/MS-data are searched against a tailored database containing all known protein sequences from wheat gluten (Triticum aestivum). Results obtained so far show that the known gluten T cell epitopes are among the identified preferred substrates of TG2. Those newly identified TG2 peptide substrates which are expected to bind to HLA-DQ2 will be tested for their T cell stimulatory capacity. Results will put new insight into the role of TG2 in the selection of T cell epitopes in CD. [1] L. M. Sollid, Annu. Rev. Immunol. 18, 53 (2000) [2] J. E. Folk, Adv. Enzymol. Relat Areas Mol. Biol. 54, 1 (1983) [3] S. Dorum, S. W. Qiao, L. M. Sollid, B. Fleckenstein, J. Proteome. Res. 8, 1748 (2009)

The next generation in protein profiling
Click-iT™ AHA for nascent protein synthesis and Click-iT™ glycoprotein labeling reagents for Glycoprotein Detection

Jens Dyekær

Lifetech, Invitrogen

L-AHA is an amino acid analog of methionine containing a very small modification: an azido moiety. Click-iT™ AHA is fed to cultured cells and incorporated into proteins during active protein synthesis. Detection of the amino acid is carried out through a chemoselective ligation, or "click" reaction, between an azide and an alkyne; the azido-modified protein is detected with TAMRA, Dapoxyl®, or biotin alkyne

Four different Click-iT™ glycoprotein labeling reagents are available, each providing an azide-modified sugar for biosynthetic incorporation into a specific subclass of protein glycan structures: Click-iT™ GalNAz,
a tetraacetylated azido galactosamine for in vivo labeling of O-linked glycoproteins, Click-iT™ ManNAz, a tetraacetylated azido mannosamine for in vivo labeling of cellsurface sialic acid–modified glycoproteins, Click-iT™ GlcNAz, a tetraacetylated azido glucosamine for in vivo labeling of intracellular O-linked N-acetylglucosamine (O-GlcNAc)–modified glycoproteins, and Click-iT™ O-GlcNAc, an azido galactose for in vitro labeling of O-GlcNAc–modified glycoproteins.

Posters

Inactivation of tissue samples stabilizes the in vivo levels of proteins, peptides and phosphorylations

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Upon sampling, protein-modifying enzymes change proteome composition (1). Subsequent analytical results reflect a mix of in vivo proteome and degradation products. In this way information about the ”pre-sampling” state may be distorted or destroyed, leading to reduced reproducibility and even faulty conclusions. We address this problem by rapid sample inactivation prior to storing and/or analysis. A novel tissue stabilization system was used to halt post-sampling modifications by enzyme denaturation. The technology is based on rapid and reproducible heat transfer to the sample. The stabilizing system processes solid tissues without the limitations associated with other enzyme inactivation technologies, e.g. chemical inhibitors. After inactivation the samples from different tissues were analyzed with downstream techniques such as western blotting, MALDI-MS or Nano-LC-MS. After immediate sample stabilization the identified peptide peaks consist of known neuropeptides, endogenous peptides and small proteins. Most peaks detected in untreated tissue were degradation fragments originating from proteins such as hemoglobin, cyclophilin, NADH dehydrogenase, synuclein and other highly expressed proteins. After stabilization the levels of phosphorylated CREB, GSK and MAPK were maintained for up to 2 hours in room temperature after treatment whilst the levels in untreated tissue decreased. References: (1) K. Sköld et al, The significance of biochemical and molecular sample integrity in brain proteomics and peptidomics: Stathmin 2-20 and peptides as sample quality indicators; Proteomics. 2007, 24, 4445-56

Proteomic and genomic changes after routine magnetic resonance imaging (MRI) in children. A Pilot Study

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Objective: To identify genomic changes in white blood cells from girls after routine 3T MR examination.
Material and methods: After Regional Ethical Committee approval six healthy females aged 6-10 years subjected to a routine MR-scan were sampled for 2.5ml blood immediately before and after an unenhanced 3T MR examination. The blood samples were collected in PAXgene Blood RNA Tubes and total RNA was extracted and all microarray experiments were performed using the Applied Biosystems 1700 Expression Array system. The AB human microarray contains 31700 probes against 27868 genes, and around 1000 control probes. The chemiluminescent signal detection, image acquisition and image analysis of the microarrays were performed on the Applied Biosystems 1700 Chemiluminescent Microarray Analyzer.
Also protein profile changes in blood serum (after MRI) after Maldi-Tof-MS (AutoFlex, Bruker Daltonics)-runs were searched for by building a decision tree from the information gain in the m/z spectrum. Principal component analysis (PCA, Sirius 7.0-PRS), support vector machine-, decision three-models were also used analyzing the mass spectrometry data. Then ESI-Q-Tof-MS-runs and MASCOT-search where done to try to identify possible changed fragments of peptides and proteins.

Results: Changes were seen in 205 of approximately 14 000 genes in all 6 girls. The five most commonly involved functional biological processes were: 1) cell cycle (G1-S Growth factor regulation and mitosis), 2) immune (antigen presentation and TCR signaling), 3) inflammation (IgE signaling and NK cell cytotoxicity), 4) development (skeletal muscle) and 5) cell adhesion (leucocyte chemotaxis and cell junctions). Similarly, the microarray screening showed distinct changes in gene groups defined as members of specific gene functional pathways (canonical pathways), of which the top five pathways were: 1) G-Protein mediated regulation p38 and JNK signaling, 2) calcium signaling, 3) antigen presentation by MHC class II, 4) ICOC-ICOSL pathway in T-helper cell, 5) NFAT in immune response. The changes were consistent using two different software packages for analysis and visualization of microarray data (J-express Pro and GeneGo). The microarray results should be confirmed using RT-PCR for the top-five of these differentially expressed genes.
In addition we are in the process of analyzing proteomic data from the same data set. So far we have identified a change in a prothrombin precursor that seemed to be fragmented in a systematic fashion by one amino acid thus resulting in peptide-fragments that only differed from each other by one amino sequence. Further investigation will be to synthesize the peptide and look for fragmentation and also try to determine the quantity. We will also explore the result system biology-vice with the curated database GeneGo (www.genego.com).

Conclusion: Our pilot suggests changes involving canonical pathways for cell cycle and immune response, amongst others. Similar changes have been reported in patients with colorectal, prostate and breast neoplasms, as well as in patients with arthritis or hepatitis. Also protein changes seem possible in children after routine MR.

Proteomic analysis for diagnostic microbiology. Applying tandem-MS to identify bacterial clinical isolates, expressed proteins and specific peptide sequences.

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Bacteriological examination of samples from patients with infections involves characterization of different phenotypic and genotypic features. This provides a basis for determining the species and eventually of which group the species of microbe belongs to. In addition, this is an important foundation to say anything about pathogenicity and choice of treatment strategy. Precise and correct species identification is an important basis for this project which aims to explore opportunities that the most recent developments of mass spectrometry methods, which had opened up to improve microbiologic diagnostics in a completely fundamental way. Study of proteins expressed by microbes is basically a phenotypic characterization, but modern proteomics also provides exact sequence information so that MS-based proteomics can be said to involve a unique combination of phenotypic and genotypic characterization.

Our main objective is to identify peptides in microbes as candidates for identification of microbial species and possibly the strain, efficient discovery of expressed virulent factors and efficient discovery of resistance mechanisms against antibiotics, which could be used in a "high throughput" diagnostic platform in the microbiology routine laboratory. In this project we have chosen to study the following bacteria: *Borrelia* species, *E. coli* species with and without ESBL, *Staphylococcus aureus* including strains with methicillin resistance (MRSA), group A *Streptococcus* and *Neisseria meningitidis* group B. To achieve that, we intend to characterize in depth the peptides, the abundance of expressed proteins, and specific genetic features of the proteins as the presence of single nucleotide polymorphisms (SNP) in all those strains. This will be performed by using proteomic-friendly protein databases generated by our group, and that includes non-redundant protein information of all sequenced genomes of a certain species. Using these databases for *Mycobacterium tuberculosis*, for example, we were able to identify strain-specific variations and not predicted gene insertions in clinical and laboratory strains, respectively.

What a difference melphalan makes!
Differential protein expression in melphalan sensitive and resistant multiple myeloma cells revealed by SILAC-based quantitative proteomics

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Multiple myeloma is a cancer characterised by abnormal proliferation of malignant plasma cells in the bone marrow. The bifunctional alkylating agent melphalan is widely used in treatment of multiply myeloma, however virtually all patients develop resistance to the drug and mean survival time is around 3 years. Although many pathways, including modulated drug transport and metabolism, enhanced DNA repair and decreased apoptotic signalling have been suggested to be involved in melphalan resistance, major determinants are still not known.
SILAC-based quantitative proteomic profiling has remarkable potential to enhance our understanding of processes involved in developing melphalan resistance and to identify potential biomarkers. Here, we subjected multiply myeloma cell line RPMI 8226 (sensitive) and its derivative RPMI 8226-LR5 (adapted to growth in melphalan) to differential labelling using SILAC, and identified differentially expressed proteins subsequent to 1D-PAGE and OrbiTrap analysis of tryptic peptides.
In summary, 2827 proteins were identified and 1163 quantified using Mascot Distiller and Maxquant software. Only differentially expressed proteins were taken into consideration and preliminary analysis shows that many of them are involved in DNA replication, recombination and repair, in cell cycle regulation as well as in cancer- and cell death pathways. Validation of the data by western analysis of selected candidate proteins is now underway.

Potential protein marker for meat tenderness in bovine longissimus thoracis muscle

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Norway Biopsies from the longissimus thoracis muscle of 26 Norwegian Red bulls were analysed by 2-DE and western blotting. The samples were chosen from a selection of very tender or tough muscles. From the 2-DE experiments four proteins, stress-70 protein, protein DJ-1, peroxiredoxin-6 and malate dehydrogenase were changed in abundance, but only peroxiredoxin-6 was confirmed by Western blot quantification. Peroxiredoxin-6 is identified as three spots of the same molecular weight but with different pI on the gels. Only one spot was increased in abundance in the biopsies from the tender group. In addition, we analysed the abundance of peroxiredoxin-6 in samples collected 1 h post mortem from the same animals and muscles by Western blotting. In the post mortem samples the same spot of peroxiredoxin-6 was increased in abundance in the tender group. In addition one of the other peroxiredoxin-6 spots was also increased in abundance. In conclusion, peroxiredoxin-6 is a potential protein marker correlated to meat tenderness. However, more animal samples are needed to verify the findings of the present study.

Characterization the effect of an lactate dehydrogenase deficiency in Enterococcus faecalis V583

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In order to get a better understanding of regulation and control of central metabolic processes in Enterococcus faecalis V583, we constructed three mutants deficient in lactate dehydrogenase. One part of the study was a detailed metabolic characterization of the strains. To follow up these experiments, we also analyzed the differences in proteome/transcriptome between the ldh 1, 2-negative mutant and the wild type V583. The strains were grown in chemically defined medium. The metabolic end products from wild type and ldh 1,2-knockout cells at OD600 = 0, 6 were analyzed using HPLC and headspace GC. The samples were also analyzed using 2-D polyacrylamide gel electrophoresis and differentially expressed proteins identified by MALDI TOF and the transcriptome analysed with microarrays. Results show that E. faecalis uses a mixed acid fermentation pattern during rapid growth in batch, and that the ldh knockout copes with the lack of lactate production by increasing the amounts of acetoin, acetate, ethanol and formate. Regulation seems to be both at a transcriptional level and at a translational level. The sugar utilization genes are among those most heavily differentially expressed, and the corresponding proteins show a similar pattern.

Comprehensive proteomics based biomarker discovery study for multiple sclerosis

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Multiple sclerosis is a chronic demyelinating inflammatory disease affecting the central nervous system, causing axonal injury and irreversible neurological damage. The cause of multiple sclerosis is unknown, but it is believed that the disease is a result of environmental factors acting on genetically susceptible individuals. Patients suffering from multiple sclerosis will benefit from early treatment, but the tests used today are not specific enough to make the diagnosis at an early stage. It may therefore take several months to years before the diagnosis is confirmed or disconfirmed. There is evidence that neurodegeneration and demyelination takes place before the first symptoms occurs and early treatment will slow down the degeneration process and the disease progress. It is therefore important to diagnose multiple sclerosis at an earlier stage than what is possible with the techniques used today. In the recent years, different proteomic techniques have been used for biomarker discovery where the goal is to detect proteins and peptides that can be used as diagnostic markers for a disease. Today, no such markers are known for multiple sclerosis. We have searched for biomarkers in cerebrospinal fluid (CSF) in order to find candidate markers that can be used to make an earlier diagnosis of multiple sclerosis. By studying the low molecular weight CSF proteome using MALDI-TOF and chemometric data analysis, we have been able to find a biomarker pattern that can distinguish patients with multiple sclerosis from healthy patients and patients with other neurological diseases (OND). The second approach we have been using is quantitative analysis using iTRAQ (isobaric tag for relative and absolute quantification) and Orbitrap mass spectrometry, comparing patients with multiple sclerosis, patients with OND and patients with other inflammatory neurological disease. This approach resulted in more than 1000 identified proteins and identification of 11 biomarker candidates, all with clinical relevance for multiple sclerosis. A label-free approach using FT-MS have also been performed, showing promising biomarker candidates for early diagnosis of multiple sclerosis.

From SELDI-TOF to protein identification

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The aim of many proteomic studies is a detailed study of a complex biological tissue/fluid, with the discovery of novel biomarkers. SELDI-TOF MS has shown its potential as a ‘gel-free’ biomarker discovery tool within a wide range of research fields. This technology is attractive because it is rapid and requires low sample volumes. In addition, the technology is optimal for the low molecular mass range, which holds great potential as a source of diagnostic biomarkers. The principle of the technology is a selective protein extraction and retention on chromatographic chip surfaces followed by analysis in desorption/ionization mass spectrometer. A major limitation using SELDI-TOF MS is that a direct identification of the potential biomarker is not possible. As a consequence, protein peaks of interest have to be purified and analyzed by a mass spectrometer with higher resolution. The purification of proteins can be a cumbersome process which requires a certain amount of sample, and it is uncertain whether the purified protein is truly the protein peak observed on SELDI-TOF. This study has therefore focused on the possibilities for direct trypsin digestion of the peptides/proteins bound to the ProteinChip, either by (a) on-chip digestion where the peptides/proteins are digested while they are bound to the chip surface or by (b) in-solution digestion of the selected peptides/proteins where the peptides/proteins are ‘eluted’ from the spot by use of pH, and then digested by trypsin in solution. Identification of proteins can then be done by use of LC-MS/MS. Preliminary results show that it is possible to identify the proteins in a known protein-mix bound to the surface. The combination of ProteinChip surfaces, SELDI-TOF and LC-MS/MS can possibly provide a complete experimental platform for sensitive detection and identification of biomarker candidates, with minimized sample loss.

Crowding induces changes in muscle and blood plasma proteomes in Atlantic salmon (Salmo salar)

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Norway Salmon aquaculture has become a major industry for Norway, and today the production of Atlantic salmon in Norway accounts for more than twice the sum of all other farmed meat products. It is well known that stressful handling and crowding prior to slaughter negatively affects the fillet quality of salmon, and the main objective of this study was to examine the stress-related changes in muscle and blood plasma of salmon. One group of the salmon was carefully handled prior to slaughter, while another group was crowded to a density of >200kg fish/m3 for a 40-min period prior to slaughter. Immediately after slaughter, blood and muscle samples were taken from all fish and frozen in liquid nitrogen. Muscle samples were extracted in two ways, 1) as a whole muscle homogenate (predominantly structural proteins), and 2) proteins soluble in a tris-EDTA-sucrose buffer (mainly metabolic enzymes). The muscle extracts and blood plasma samples were all separated on IPG-strip covering the pH-area of 4-7, followed by 12.5% SDS-PAGE. All protein changes found due to crowding are involved in different secondary and tertiary stress responses, including altered energy metabolism, osmotic regulation and immune function.