11.1

Mass Spectrometry Investigation of Human Plasma Derived and Recombinant Blood Coagulation Factor IX: A Comparison Using Off-line LC MALDI TOF MS

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The prevalence of the blood disorder Hemophilia B is about one in 30,000 persons. In patients suffering from this disorder, Human Factor IX (FIX) is either absent or non functional and must be substituted by repeated infusion. Therapeutic concentrates of FIX are derived either from human plasma or prepared using recombinant DNA technology. Mature FIX is known to be extensively post-translationally modified, incorporating γ-carboxylation, β-hydroxylation, deamidation, *N*- and *O*-glycosylation, sulphation and phosphorylation. In this study we directly compare FIX derived from human plasma with that derived from recombinant DNA. A sample of each was enzymatically digested and separated by reverse phase micro HPLC using an LC2010 (Shimadzu, Japan) configured with an on-line Accuspot (Shimadzu, Japan) for automatic spotting of the eluate onto the MALDI sample plate. Samples were then analysed by MALDI TOF mass spectrometry using an Axima CFRplus and Axima QIT TOF (Shimadzu Biotech, UK). This investigation considers both the native and de-N-gycosylated form of the glycoprotein. The comparison of plasma derived and recombinant FIX is of interest as it is a good example of heavily post-translationally modified proteins being used routinely in a therapeutic context. The comparison was mainly focused on these post-translational modifications. Sequence parts of the so-called light chain of both proteins were compared and differences were observed on the GLA domain (area containing γ-carboxyglutamic acids) as well as in the glycosylation pattern.

Metabolism, gene and protein synthesis, which is consistent with the high proliferation and differentiation characters of the fetal liver.

11.2

Electron Capture Dissociation and Infrared Multiphoton Dissociation FTICR-MS Under Liquid Chromatography Conditions

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Introduction: Electron capture dissociation (ECD) and infrared multiphoton dissociation (IRMPD) are emerging methods for the characterization of proteins and peptides in Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS). Often, data shown are extracted from tens or even hundreds of scans, as this can be required to generate spectra of sufficient quality. As such, these methods are considered to be incompatible with chromatographic techniques, and, as with many emerging methods, prejudices can exist since sample amounts and time are often limited. Here we demonstrate that through optimized implementation of these methods on a high-performance mass spectrometer it is possible to perform ECD and IRMPD analysis with accurate mass determination of the fragment ions from samples eluting from an HPLC.

Methods: ECD and IRMPD are implemented on a hybrid ion trap/Fourier transform ICR mass spectrometer (Finnigan LTQ-FT). Single scan data as well as multiple scans averaged from direct infusion and HPLC runs are evaluated for peptides, phosphorylated peptides and proteins. The results from ECD and IRMPD are compared to those generated by CID in the ion trap mass spectrometer. The analyses are performed under computer control, allowing for unattended data dependent analysis.

Preliminary Results: Single scan ECD and IRMPD spectra have a quality (based on signal to noise and information content) that is comparable to spectra generated with CID using the ion trap mass spectrometer. Smaller peptides (5 to 20 amino acids) in particular show up to 100% of the possible ECD cleavages. In many cases, the information content from a single scan is only slightly less than the information gained from the average of dozens of scans. As results can be complimentary, cycling between ECD, IRMPD and CID is beneficial for many analyses, especially since ECD has unique features, like conservation of modifications, e.g., sites of phosphorylation and glycosilation. The cleavage coverage of the different fragmentation methods is compared for standard peptides as well as peptide mixtures.

11.3

Parallel Processing on an FT-ICR Mass Spectrometer—The Ideal Tool for Proteomics

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Identifying proteins and detecting any kind of modifications or structure changes is of increasing interest to the different fields of proteomics. Since modified proteins are often present only in minor concentration the demand for more powerful mass spectrometers and a clear need for accurate mass determination and MS/MS both compatible with the chromatographic time constraints is high and increasing.

The knowledge of the accurate mass of an MS/MS precursor ion allows for a significantly higher reliability of protein identification resulting in less false positive hits, and increased database search speed. FTICR-MS is the most suitable way to achieve the required mass accuracy.

The Finnigan LTQ FT is a hybrid system with two analyzers, a linear ion trap and a FT ICR MS. The linear ion trap is characterized by a high ion storage capacity combined with a high scan rate and high MS/MS sensitivity while the FTICR- MS routinely achieves high mass resolution and excellent mass accuracy with external calibration.

The resolution of an ICR analysis is directly dependent on the time used to acquire the transient signal. To achieve a resolution of 100,000 the transient needs to be detected for more than 700 ms in the LTQ-FT. However, The Finnigan LTQ FT can use this time productively by operating the linear ion trap fully in parallel to the ICR detector acquisition. This allows for example the acquisition of several MS/MS scans concurrent to the acquisition of a HR MS spectrum fully automated through *data dependent* scanning.

This presentation will give an overview of the potentials of parallel processing in proteomics with real life samples under different LC conditions.

Comparing analysis with long (slow) to short (fast) gradients will demonstrate the advantage of high scan repetition rate under high resolution condition in the ICR part.

With the combination of the accurate mass of the peptide precursor and the sequence information of its MS/MS spectrum the data base search yield in faster protein identifications with higher coverage.

11.4

SIMPL: A Novel Technology for Protein Quantification Based on Isotopic Labelling

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Proteomics has emerged as a new experimental approach mostly because of several important innovations in mass spectrometry. Although MS is unparalleled in its ability to characterize proteins, it is a purely qualitative method and requires coupling to other analytical methods for quantitative profiling of the proteome.

We have developed a technology for quantitative proteomics based on isotopic labeling of peptides. Our labels contain a Cys-reacting group or a group reacting with the terminal amine, a group containing light or heavy stable isotopes, a cleavable group and a group that covalently binds to a solid matrix. Mixtures of denatured and reduced proteins modified by the light and heavy version of our reagents are combined and treated with proteolytic enzymes. The labelled peptides are covalently captured on the solid matrix and after rigorous washing to eliminate non-specifically bound molecules, are released by chemical cleavage. Identification and quantification of proteins are performed by ηLC-MS/MS and using Spectrum Mill software.

We demonstrated that our labels rapidly and selectively modify all SH-groups in both peptides and denatured proteins. Matrix purification allows complete elimination of peptides without cysteine. The use of our labels for the quantification of mixtures containing several purified proteins demonstrated excellent correlation between expected and measured ratios of proteins. We conclude that our technology can provide a reliable quantification of proteins in comparative proteomics analysis.

11.5

Combination of Field Asymmetric Ion Mobility Spectrometry (FAIMS) with FT ICR Mass Spectrometry for Proteomics Research

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The large variation in the relative abundances of proteins in biological systems presents a major challenge for proteomics. Although FT ICR mass spectrometry is the most powerful tool in the bottom-up and top-down proteomics, each improvements in mass accuracy and dynamic range opens new research avenues previously inaccessible. These parameters strongly depend on the composition of the ion beams introduced into the mass spectrometer. Different kinds of ion preselection techniques have been implemented with FT ICR to manipulate the ion beam composition in order to avoid influence of ions from most abundant species on the mass measurement quality for minor species in the context of high throughput proteomics research.

In this presentation we characterize the atmospheric pressure FAIMS for ion preselection after electrospray ionization source and before entering the FT ICR mass spectrometer interface.

Different protein and peptide mixtures were investigated. It was shown that FAIMS permits to prevent predominant peptides and proteins from penetrating into the mass spectrometer. Besides we observed significant decrease of chemical noise in mass spectra obtained using FAIMS separation. Attempts were undertaken to use FAIMS in combination with capillary electrophoresis to separate ampholytes from the analytes.

11.6

Bradykinin Potentiating Peptides in *Lachesis muta* Snake Venom: Identification of New Peptides Using Mass Spectrometry Technologies and cDNA Cloning

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Lachesis muta (Bushmaster) is the largest Crotalinae snake in the world. It is distributed in the tropical rain forests of Central and South America, including Brazil. As a consequence of envenomation, there are several predominant symptoms: intense local pain, nausea, abdominal colic, diarrhea, edema, hemostatic abnormalities, hypotension and neurotoxic signs. Most of this signs are due to the presence of proteins and peptides in the venom. One important application of MALDI TOF/MS is the direct analysis of complex samples allowing identification of low mass peptides and small proteins in a single step. In fact, this tool has been successfully used for the identification of a great number of peptides in crude venoms. The aim of this work is to use the MALDI-TOF/MS approach to obtain a mass pattern of the major peptides ($<$10 kDa) present in *Lachesis muta* crude venom. The samples were analyzed, using CHCA and SA matrix in the mass range 800–10.000 m/z with the linear mode. We identify in the MS spectra several peptides with molecular mass $<$ 2000 Da and only one with 2211 m/z. Some of these peaks were submitted to MALDI TOF/TOF and *de novo* sequencing was preformed by precursor ion fragmentation using N2 and/or air collision to induced dissociation. Collision cell pressure was kept at 2.8×10^{-6} torr. The sequence of four peptides was determined and shows high similarity with the BPPs (bradykinin potentiator peptides) described in the others snake venoms as *B. jararaca* and *B. jararacussu*. Parallel cDNA library sequencing was performed and resulted in the identification of the BPP precursor, of 1700 pb, highly expressed and encoding also the natriuretic peptide. Comparing the peptide masses from MALDI TOF, four of them matched with peptides deduced from the precursor and at least three of the derived sequences obtained by MALDI TOF/TOF perfectly matched the BPP precursor sequence. The combined proteomic and genomic represents powerful approaches that identified BPP peptides in the *Lachesis muta* venom, some of them being new uncharacterized ones.

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11.7

A Sequence Tag Approach to Identify Multiple Peptide Modifications from Tandem Mass Spectra

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Identification of protein modifications is important to understand biological functions of proteins. However, the current interpretation techniques for tandem MS spectra are limited in identifying multiple peptide modifications. Here we present an efficient way to interpret a tandem mass spectrum of a peptide having multiple post-translational modifications (PTMs). The methodology consists of five steps: peak selection, tag listing, peptide search, tag alignment, and PTM localization. In the peak selection step, we select peaks with relatively high intensities. These not only include those peaks with globally high intensities but also the peaks with locally high intensities. In the tag listing step, we first identify all the tags (partial amino acid sequences that do not contain PTMs) from the mass spectrum via de novo sequencing only using the set of selected peaks, and then make a list of the identified tags together with the locations of peaks used to generate the tag. In the peptide search step, we search the peptide database for the peptide sequences that have the identified tags. It must be noted that the peptide DB we use does not contain PTMs, thus avoiding the combinatorial explosion problem. In the tag alignment step, for each candidate peptide, we align the tags to each candidate peptide sequence to generate a chain of tags and in-between gaps. When we generate a chain of tags and in-between gaps, we require that the tags be aligned to the peptide sequence in appropriate locations. In the last step, these gaps are filled with partial sequences including PTMs. To fill each gap, we find a sequence with PTMs that best explains the corresponding part of mass spectrum. We do this by performing a branch-and-bound search over the space of possible sequences with PTMs and find the most similar one. By taking a hybrid approach, combining de novo sequencing and DB search, multiple modifications are identified effectively.

11.8

Methodology for Sample Handling and Separation of Human Plasma for Differential Expression Analysis in Serial Samples from Patients with Myocardial Infarction

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Novel Aspects: New analytical technology for multidimensional protein fractionation and semi-quantitative differential measurement for identification of plasma proteins in human cardiac disease.

Summary: Myocardial infarction (MI) is a major cause of morbidity and mortality worldwide. Proteomics may be used to identify both improved diagnostic markers and pathways triggered during myocardial injury that might serve as therapeutic targets. We have a developed a sample handling and multi dimensional separations methodology that utilizes top-down and bottom-up analysis. By separating plasma at the protein level using ion exchange chromatography we are able to avoid the need for depletion of high-abundance proteins thereby allowing us to interrogate both the proteome and peptidome in the same workflow. After initial separation at the protein level the methodology follows two paths. In the first, the protein fractions are analyzed using a top-down approach using a hybrid FTMS (LTQ-FT, Thermo Electron Corp., reviewed elsewhere at this meeting by Sutton *et al.*) Bottom-up analysis of digested fractions was done in triplicate using a nanoflow multidimensional liquid chromatograph (MDLC, GE Healthcare) coupled to a linear ion trap MS (LTQ, Thermo Electron Corp.) to assess quantitative changes over the course of the longitudinal experiment. Preliminary results from this model have allowed us to quantify and identify known biomarkers of cardiac disease (e.g. C-reactive protein, CRP) as well as several biologically relevant low-level soluble factors with potential applications as biomarkers.

11.9

Mass Spectrometry Is Essential for Modification Specific Proteomics

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The first annotation of the human genome placed the number of protein coding genes at approximately 30,000–40,000, i.e., only approximately twice as many as that of the fly or worm and five times that of yeast. This strongly implies that protein complexity and regulation in higher eukaryotes is governed by co- and post-translational modifications and splicing events. It is becoming increasing obvious in biological studies that numerous proteins and protein complexes are regulated by post-translational modifications. Several protein modifications have been documented and methods to systematically identify post-translational modifications during protein identification studies are being developed. We have initiated an approach, which we term Modification Specific Proteomics, for specific detection of various types of protein modifications in proteomics. The detection of PTM's is very challenging because these are often transient and highly heterogeneous in terms of modified site (e.g. phosphorylation) as well as the modifying group (e.g. glycosylation). Our concept is based on specific detection of PTM's in the 2D-gel, by specific "pull-out" of modified proteins/peptides, or by selective detection of the specific type of PTM in the mass spectrometer. It will be illustrated by our recent attempts to study protein phosphorylation, glycosylation and acylation. These types of modifications are found in more that 50% of all proteins in higher organisms. Due to their implications on protein structure and function, studies of biological phenomena must include detailed elucidation of the protein modifications on a level compatible with proteomics studies.

11.10

Mass Spectrometric Analysis of Posttranslational Modifications of a Carrot Extracellular Glycoprotein

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A soybean 4-kDa hormone-like peptide (4-kDa peptide) stimulates the growth and differentiation of carrot callus, suggesting that a 4-kDa-peptide-binding protein is present in carrot. The proteins, released into suspension culture medium from three-week-old carrot callus, were collected and subjected to surface plasmon resonance (SPR) analysis. A 4-kDa peptide-binding protein was detected and identified as an extracellular dermal glycoprotein (EDGP), which also binds insulin *in vitro*. The amino acid sequence alignment showed that the EDGP shared significant homology with proteins from legumes, tomato, *Arabidopsis*, and cotton. These proteins are involved in signal transduction or stress response systems. Most of the Cys residues in these proteins are conserved, suggesting that they share similar tertiary structures. The SPR analysis showed that reduction of the EDGP decreased its binding activity 20 fold, implying that disulfide bonds are important for its function. Therefore, we investigated the disulfide bond pattern in EDGP using mass spectrometry. Six disulfide bonds in EDGP were identified: Cys^{70}-Cys^{158}, Cys^{84}-Cys^{89}, Cys^{97}-Cys^{113}, Cys^{100}-Cys^{108}, Cys^{201}-Cys^{426}, and Cys^{332}-Cys^{378}. In addition, the N-terminal glutamine was cyclized into pyroglutamic acid. All four putative glycosylation sites were occupied by N-linked glycans, which have similar masses of *m/z* 1171. Finally, measuring the mass of the native protein showed that the posttranslational modifications of EDGP involved only disulfide bonds, N-terminal modification, and glycosylation.

11.11

Hydrogen/Deuterium Exchange Experiments at High Spatial Resolution: A Topological Study Nn the HET-s$^{218–289}$ Prion Protein

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In a search for an improved resolution of hydrogen/deuterium (H/D) exchange experiments analyzed by mass spectrometry (HXMS), we have evaluated two methodologies for a detailed structural study of solvent accessibility in the case of HET-s$^{218–289}$ prion protein from *Podospora anserina*. For the first approach, after incubation in the deuterated solvent, aggregated HET-s$^{218–289}$ was digested with pepsin and the generated peptides were analysed by nanospray mass spectrometry in an ion trap, with and without collision-induced dissociation. We have compared deuterium incorporation in peptides as determined on peptide pseudo-molecular ions and on b and y fragments produced by longer peptides under CID conditions. For both b and y fragment ions, an extensive H/D scrambling phenomenon was observed. Thus, the spatial resolution of HXMS experiments could not be improved by means of MS/MS data. In a second approach, the incorporation of deuterium was analysed by MS for 76 peptides of HET-s$^{218–289}$ peptide mass fingerprint, and the use of shared boundaries among peptic peptides allowed us to determine deuteration levels of small regions ranging from 1 to 4 amino acids. This methodology led to the evidence short regions along the HET-s$^{218–295}$ sequence that display a strongly reduced solvent accessibility.

11.12

Parallel LC-Coupling with ESI-Ion Trap and MALDI-TOF/TOF for Most Efficient Information Readout of Human Serum Samples

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Liquid chromatography (LC) coupled to Electrospray mass spectrometers, mainly ion traps or QTOFs, is a widely used tool for comprehensive proteome analysis. In addition to this online combination, offline LC-coupling with MALDI-TOF/TOF became a promising alternative. While in LC/ESI-MS/MS the duty cycle of the mass analyzer is usually a critical factor (in particular for complex proteome samples), there are no temporal constraints for an LC/MALDI-TOF/TOF analysis.

In a parallel approach for coupling capLC or with both techniques, the majority of medium and high abundant proteins are identified by fast online LC/MS/MS in the trap. Using a T-split in front of the ESI source, the LC run is fractionated on a MALDI target at the same time for further analysis. Controlled by an intelligent software tool for the entire workflow, the database search results from the ESI-trap run were used for MALDI TOF/TOF precursor ion selection in order to selectively fragment only those ions that either gave poor fragment spectra or were not fragmented at all due to time constraints. Thus, low abundant proteins in a proteome which are usually represented by just one or two peptides in an observed peptide mass fingerprint (PMF) are identified with a highly increased success rate.

Due to the different ionization behaviour, the parallel use of both MS techniques results as well in a generally increased sequence coverage to account for as much of the protein's structure and PTMs.

Digested human serum samples of high complexity and dynamic range were separated by capillary LC. A post column split of the LC flow was used with one half being directly analyzed by an ESI-ion trap MS, whereas the other half of the flow was spotted onto a 600 μm AnchorChip MALDI-target for offline MS and MS/MS. The eluent was deposited on a thin layer of HCCA matrix in 10–30 sec fractions.

Online MS and MS/MS spectra were obtained using a high capacity ESI-ion trap with a scan rate of 5–10 spectra in 3 sec. The database search results from Mascot were utilized for subsequent MALDI-MS/MS analysis so that the acquisition or redundant information was avoided. All MS and MS/MS data sets acquired by both MS techniques were finally merged for a joint database search. An intelligent software controlled the entire, complex workflow and handled the results.

Even though the scan rates of the ESI ion trap provided a very fast acquisition of MS/MS spectra, the ability to select all precursor ions for MS/MS analysis was limited by the chromatographic peak width and the complexity of the mixtures. As ESI and MALDI analyses typically provided complementary information, i.e., some peptides showed up in ESI and others in MALDI spectra only, complementary sequence information was obtained.

The combination of both techniques indeed lead to more identified proteins with higher sequence coverage for all investigated samples. Also when data sets of repeated experiments of the same sample acquired by only one single MS technique were combined, the results of the LC ESI/LC MALDI approach could not be achieved.

11.13

Role of Sample Preparation in Quantitation of Exogenous Peptides in Biologic Samples by LC/MS

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During the measurement of exogenous peptides in biologic samples, an effective method of sample preparation was badly needed. The Technology based on liquid chromatography-mass spectrometry with electrospray ionization (LC-ESI-MS) provided a powerful tool for quantitation of proteomic profiles. But the linearity of intensity versus analyte concentration and reproducibility of chromatographic processing made up the obstacles to the successful determination. In the study, for evaluating and optimizing a favorite method of sample preparation, four peptides with different molecular weight (>3000 Da) and pI were recruited in three sample preparation strategies including protein precipitation (PP) by organic solvents (methanol, ethanol, and acetonitrile), solid-phase extraction (SPE), and ultrafiltration (UF). The efficiency of preparation methods including the distribution of molecular weight of extracts, suppression effect of ionization, absolute recovery, linearity, physical absorption onto containers and reproducibility were discussed by the means of quantitative LC-ESI-MS. All peptides were separated without enzymatic digest and their multiple charged ions were monitored directly in specific selective reaction monitor (SRM) mode. The Results showed that, SPE was more general and reproducible for peptide extractions and showed a good linearity, relative high extraction recovery (20~60% for different peptides) and low suppression effect of ionization; methanol and acetonitrile PP also showed a various recovery (9~51% for different peptides) but the stability was easy to be disturbed by plasma matrices due to the characteristics of non-specific precipitate reaction. Ethanol and acetonitrile were with similar precipitation effect to both high M.W. proteins and low M.W. peptides while methanol PP is more effective to proteins. UF showed a clean-up background and an effect of enrichment, but usually accompanied with poor recoveries. In general, SPE is more practical, but further optimizations were still needed to gain higher reproducibility for off-line procedure.

11.14

Integrated LC-ESI-MALDI-MS/MS on a Disposable MALDI Target Platform

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The sensitive detection of PTMs from protein digests requires achieving high protein sequence coverage, a high sensitivity of the detection system and for MALDI-TOF a low probability of cross-contaminations between LC-MALDI runs for the detection of low abundant peptide species. In an integrated approach a capLC system (Agilent 1100) was coupled using a 1:1 post column split to a fast scanning ESI-ion trap mass spectrometer and to a robot for fraction spotting onto a MALDI target. After real time LC-MS/MS analysis on the trap and database searching, off line LC-MALDI-TOF/TOF analysis were performed in a data dependent fashion. MALDI was used 1) to provide additional peptide identifications that were not observed in previous ESI analysis, 2) to validate unsafe ESI matches, under conditions of full automation. The goal of these experiments is to approach 100% sequence coverage by MS and MS/MS from isolated proteins, such as the ones from 2D gels. That level of detail is required for unambiguous identification of splice variants, modifications (PTMs) or polymorphisms or other sequence variations in proteins. It is therefore essential for detailed protein structure elucidation. As several PTMs such as sulfation or phosphorylation can be present at sub-stoichiometric levels, the detection of low abundant peaks for the downstream acquisition of MS/MS spectra must safely exclude the possibility of MALDI target contaminations from previous runs. Disposable AnchorChip MALDI targets (Bruker) were used here. For the first time plastic sample plates were used that fulfill the demanding requirements of surface flatness and electrical conductivity for colinear MALDI. In addition, prespotted a-cyano-4-hydroxycinnamic acid matrix anchors (700 μm) allowed the direct application of the LC-eluate onto the target. One hundred amol detection sensitivity was achieved on these matrix anchors as they concentrate the analyte from the larger droplets that were deposited. Data dependent MALDI-MS/MS analysis subsequent to online-ESI-MS/MS provided a significant increase in sequence coverage. As an LC-run is immobilized on a MALDI target, the deposited fractions were available for re-analysis even after several days if careful data analysis suggested some to be particularly important to the final result. The disposable targets are particularly useful for extensive sample archival and the influence of archival time vs. data quality was evaluated.

11.15

Top-Down Analysis of Low Molecular Weight Human Plasma Proteome Using Hybrid Ion Trap-Fourier Transform Mass Spectrometry

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Novel Aspects: New analytical methodology and computer algorithms for top-down measurement and identification of intact low molecular weight plasma proteins.

Summary: Identification of the plasma proteome is a daunting analytical task due to the complex nature of the sample resulting from an extremely large dynamic range of protein components. Several different analytical approaches have been incorporated to identify proteins belonging to the low molecular weight (LMW) plasma proteome, including various enrichment schemes and numerous bottom-up mass spectrometric analyses. In this report we employ a novel top-down approach for identifying the LMW proteome and plasma peptideome that encompasses the use of Finnigan hybrid linear ion trap-Fourier transform mass spectrometer (LTQ-FTMS, Thermo Electron). Enrichment of the LMW fraction is achieved by ultrafiltration as previously reported by Harper *et al*. Plasma peptides are captured and concentrated using reversed-phase (RP) batch processed on a magnetic bead particle. Enriched peptides and LMW proteins are separated and detected using an RP-HPLC/μESI MS. Monoisotopic mass measurements are acquired in both full and MS^n mode at a resolution of 100,000 with a 1 ppm mass measurement. ProSlight (https://prosightptm.scs.uiuc.edu/), SEQUEST, and other algorithms are used for database acquisitions. Preliminary results demonstrate the effectiveness of this top-down methodology to detect and identify both intact LMW plasma proteins and peptides belonging to the plasma peptideome in an enzymatic free protocol.

11.16

A New Electroblotting Protein Chip for Proteome Analysis

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The direct blotting of separated peptides and proteins after SDS-polyacrylamide gel electrophoresis onto the surface of newly developed protein chip for mass spectrometry has an advantage over the 2D-MS procedure most commonly used. It could greatly shorten the period of total proteome analysis and improve the sensitivity and reproducibility of peak intensity. [Method and Materials] The model proteins mixture, rat serum, tissue extracts of mouse and cell extracts from human U937 cell line treated by phorbol 12-myristate 13-acetate (PMA) and dibutyryladenosine 3′:5-cyclic monophosphate were subjected to electrophoresis on SDS-polyacrylamide gel and then electroblotted to the new protein chip. In order to maximize the sensitivity by MALDI TOF-MS analysis alpha-ciano-4-hydroxy cinnamic acid (CHCA) was added for peptides and sinapic acid (SPA) for proteins as matrix. [Results] The new methodology using new protein chip allowed completing the entire procedure less than 4 hours. The CV values of relative intensities from 3000 to 20000 of M/z of rat serum were below 30% independently of M/z. The number of peptides and proteins detected by this method were more than 1700 in rat serum. The differential analysis using U937 cell extracts showed that all the up- or down- regulated peptides and proteins caused by stimuli were easily detected at one try. [Discussion] The new methodology was concluded to be useful for rapid and exhaustive proteome analysis of body fluids and tissue extracts in order to find biomarker molecules.

11.17

A Novel LC-MS Instrument Set-Up Used for the Identification of Phosphorylation Sites in Proteins

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The analysis of post translational modifications is of great interest within the field of proteomics. As shown by the ABRF-PRG03 analysis, such studies are not yet to be considered routine (Arnott *et al.*). In studies of protein phosphorylation, sensitivity is a major challenge. In most cases only a fraction of the proteins are phosphorylated. Additionally, the ionization efficiency is lower for phosphorylated peptides, compared to non-phosphorylated peptides. Also, because the phosphorylated peptides are more hydrophilic than their non-phosphorylated counterparts, chromatography conditions must be carefully selected.

Results show that the choice of MDLC system design is vital for the end result, when MDLC systems are used as a part of the sample preparation process. While phosphorylated peptides may be chromatographically enriched by the use of complexation with metal ions (Ueda *et al.*), contact with those metals in the rest of the LC-MS fluid pathway is detrimental to sensitivity. To obtain high sensitivity, a novel biocompatible MDLC system has been tested in combination with an ESI-LTQ-MS in this study.

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11.18

A Novel Strategy for Determining Protein Ubiquitination by Mass Spectrometry

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Protein ubiquitination plays an essential role in the degradation and functional regulation of cellular proteins in organisms ranging from yeasts to mammals. Technique advance in MS sequencing is needed for characterization of this type of protein modifications. Trypsin digestion of ubiquitin conjugated proteins produces branched peptide(s) in which the C-terminal Gly-Gly fragment of ubiquitin is attached to the ϵ-amino group of a modified lysine residue within the peptide. This provides a platform for mapping ubiquitination sites using mass spectrometry. Here we report the development of a novel strategy for determining post-traslational protein ubiquitination based on the N-terminal sulfonation of diglycine branched peptides. In contrast to conventional tandem MS spectra of native tryptic peptides, MALDI MS/MS analysis of a sulfonated tryptic peptide containing diglycine branch generates a unique spectrum composing of a signature portion and a sequence portion. The signature portion of the spectrum consists of several intense ions resulting from the elimination of the tags, the N-terminal residues at the peptide and the branch, and their combination. This unique ion distribution pattern can distinguish ubiquitination modified peptides from others. The sequencing portion consists of an exclusive series of y type ions that can directly reveals the amino acid sequence of the peptide and the precise location of the ubiquitination site. The technique is demonstrated on a series of synthetic peptides and is validated by an ubiquitinated protein, tetra-ubiquitin. Using this method, we have identified previously unknown ubiquitination sites from an ubiquitin modified protein, C-terminal Hsc70-interacting protein (CHIP). These results demonstrates that the MS/MS analysis of sulfonated tryptic peptides can provide a highly effective method for the determination of ubiquitination substrates, ubiquitination sites on protein targets and the modification sites on ubiquitins themselves.

11.19

Ion Suppression in Proteomics Analysis with ESI and MALDI/Mass Spectrometry

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The advent of soft ionization mass spectrometric techniques such as electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) has made the analysis of proteins feasible, which has become a powerful tool for the analysis of proteomics. However, many factors influence the result of detection such as charged side chains, the presence of aromatic amino acids, peptide hydrophobicity, molecular size, the potential to form stable secondary structures and ion suppression effects. Among of them, the ion suppression effects which make the loss of sensitivity of detection and "dirty" spectra is the major challenge for mass spectrometric identification of proteins. Several methods have been used to decrease ion suppression effectively from our experience and references, which are as follows: 1. The chromatographic separation is needed prior to identification of proteins implemented in conjunction with on-line ESI-MS/MS or off-line MALDI. 2. The continuous deposition of sample onto a track on the MALDI target, which is then placed into the MALDI-TOF-MS instrument. 3. An assay coupling of fast performance liquid chromatography (FPLC) pre-fractionation of protein extract and subsequent two-dimensional liquid chromatography/mass spectrometry. The method provided a perfect means to reduce ion suppression by co-eluting peptides. 4. The negative ion mode is a surprisingly underutilized tool in the analysis of proteins and protein digests. Data has already been shown that the negative ion mode can produce less matrix interference than the positive mode. It should be expected then, that by developing more new approaches minimized the effects of matrix components, the high sensitivity and high quality spectra from the analysis of protein could be obtained.

11.20

Direct Analysis of Nonsmall Cell Lung Cancer on Tissue Sections by MALDI Imaging Mass Spectrometry

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MALDI (matrix-assisted laser desorption/ionization) imaging mass spectrometry (IMS) has becoming into a new technology which generates molecular profiles of peptide and protein signals directly from the surface of thin tissue sections. The high sensitivity of this technique (low-femtomole to attomole levels for proteins and peptides) allows the study of organized biochemical processes occurring in, for example, mammalian tissue sections. Specific information can be obtained on the relative abundance and spatial distribution of proteins in normal and diseased lung cells. Different matrixes have been tested on the standard proteins and the results shows that CHCA has offered the best performance in mass-to-charge range from 1000 Da to over 50 kDa. Therefore, CHCA has been selected in identification the tissue slices on the glass slides. Here, 4700 Proteomics Analyzer mass spectrometry (ABI, USA) has been used to complete the proteins and peptides profile in nonsmall cell lung cancer sections. As the normal imaging mass technique, the results from liner mode can not offer the accurate mass to charge value when large proteins have been found, therefore, we developed some new methods to accurately find the target protein. Firstly, these important proteins, which might be correlative to the lung cancer, were directly selected as precursor ions to get their partial sequence when the molecular weight of these proteins was smaller than 10 kDa. Secondly, an on-plate enzyme digestion has been performed and the formed peptides were investigated to distinguish the minor difference between normal and diseased lung cell. More important information was obtained due to the small peptides have higher sensitivity than the large proteins. Thirdly, these target protein could be accurately identified by MS/MS spectra of these "target" peptides.

11.21

Study of Phosphorylation Sites of Phosphopeptides Using a MALDI QIT-TOF Mass Spectrometer

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Recently MALDI-MS or MALDI-MS/MS has been widely used for investigating post-translational modifications. However it is quite difficult to detect phosphorylated amino acid itself because of low efficiency of ionization. Here we report identification of phosphorylation site of phosphopeptide derived by a protease digest of beta-casein, ovalbumin and human Stathmin, by using MALDI QIT-TOFMS. In the case of monophosphopeptide, its molecular ion was detected as the most intense peak, accompanied with dephosphorylated ion which intensity was less than 30% of the molecular ion. Furthermore, we could observe a molecular ion of tetraphosphopeptide predominantly as well as in case of monophosphopeptide. By using a phosphopeptide of ovalbumin at 100 fmol from a protease digest it was found that the S/N ratios of spectrum obtained in MALDI QIT-TOFMS were improved by 5 times over those obtained in reflector-mode of MALDI TOFMS. In MS/MS spectrum of 100 fmol monophosphopeptide, we could observe the specific product ions which preserved their phosphate. Other product ions caused by neutral loss of the phosphate group were also observed. However most of their intensities were below 50% in comparison with phosphorylated ions. These results indicate that MALDI QIT-TOFMS has sufficient ion-cooling effect and sensitivity to detect not only Mw of phosphopeptide but also phosphorylated amino acid. These MALDI QIT-TOFMS performances has been applied for Stathmin, which is a 19 kDa cytosolic protein having four phosphorylation sites. The signal corresponding to phosphorylated peptide Ser27-Lys40 was found at m/z 1621.8. Of three Ser residues in the peptide, Ser38 was revealed to be phosphorylated on the observation of specific +80 shifted product ions by subsequent MS/MS analysis. The result is completely consistent with a previous immunoblot analysis for the protein.

11.22

Use of Mesoporous Material for MALDI-TOFMS

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The organic matrixes in MALDI can suffer from some problems which make them not always effective. As an alternative to organic matrix, inorganic material has been drawing considerable attention to develop mainly on its analytical sensitivity and spectra quality. This presentation will focus the investigation on highly-ordered mesoporous material. The inorganic powder was suspended in water/methanol (1:1, v/v) and sonicated for 5 min. The analytes were dissolved in water. 1 μl of the suspension was pipetted onto the target plate and then 1 μl of analyte was deposited on them. The samples were dried with air at room temperature before MALDI analysis.

Special experiments showed that mesoporous material has a stronger tolerance towards potassium salts than CHCA. A serial of reproducible spectra with reasonable S/N at pico-mol level were easily obtained using the mesoporous matrix, which represents a significant improvement compared with the nano-mol detection level previously reported. Experiments with mesoporous matrix as matrix were performed on a model protein system (MYO tryptic digest, 15 pmol), and the protein searched from SwissProt database is consistent with the standard MYO with a sequence coverage of 68%. Experiments also show that the mesopore is suitable as an nano-enzymatic-reactor. The mesoporous material has the capability to unfold protein partially prior to the proteolysis process.

11.23

Identification of Protein Phosphorylation Site by Peptide Enrichment of a Fe^{3+} Modified Nanozeolite Material and Direct MALDI-TOF-MS/MS Analysis

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Protein phosphorylation is one of the most important known posttranslational modifications. Tandem mass spectrometry has become an important tool for mapping out the phosphorylation sites. However, direct analysis of phosphopeptides in enzyme-digested complex mixtures is problematic due to the "ion suppression" effect and the low abundance of phosphopeptides. Enrichment of phosphopeptides from a digest mixture is desirable and often a critical step for MS/MS based site determination. In this work, using tryptic digest of bovine β-casein as the model, we report a rapid and effective enrichment of phosphorylated peptide by novel Fe^{3+} modified nanozeolite materials. It is shown that the matix-assisted laser desorption/ionization (MALDI) time-of-flight tandem mass peak of the monophosphorylated peptide (m/z 2062) is greatly improved compared to that without enrichment. The signal-to-noise ratios are much higher and the MOWSE (MOlecular Weight SEarch) MS/MS ion score can reach 80. More importantly, the phosphorylated peptides adsorbed on the nanozeolites can be directly analyzed by MALDI-TOF-MS due to zeolite's nanoscale patical size and high dispersibility, which avoids possible sample loss during the elution process.

11.24

Determination of Biomarker Proteins for Parkinson's Disease Using Differential Quantitative Proteomic Analysis with cICAT and Two-dimensional Chromatography on a Nanoelectrospray Linear-Ion Trap LC/MS System

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Diagnosis of most neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease, and Huntington's disease, is largely based on clinical symptoms and confident diagnosis can be very challenging. It is hoped that identification of disease biomarkers will provide a useful tool to assist in clinical diagnoses of these diseases and monitoring of disease progression. Characterization of proteins involved in these diseases will likely yield a better understanding of the molecular mechanisms of disease, which is poorly understood now, and yield promising target proteins and models for drug development efforts. In this study, we investigated the brain proteome of patients with Parkinson's disease, and compared them to age-matched controls. Nigral tissue proteins were digested, labeled with cleavable isotope-coded affinity tag (cICAT) and analyzed with two-dimensional liquid chromatography tandem mass spectrometry.

Data were analyzed using TurboSEQUEST. The results demonstrated hundreds of proteins identified with very high probability and reproducible quantitation. Many proteins were identified which appear to be up- and down-regulated in disease patients relative to controls.

11.25

A Parallel Column Based Proteomic Analysis by Multidimensional Chromatography and Tandem Mass Spectrometry

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Since shotgun proteomic approach developed by Yates *et al.*, proteome analysis based on multidimensional chromatography coupled with mass spectrometry has been widely applied in the past few years. In our previous work, a parallel column array based comprehensive two-dimensional chromatographic system and hyphenated with tandem MALDI-TOF/TOF MS was developed. Such a system using 10 to 20 reverse phase columns in parallel as the second dimension leaded to a total analysis time of the two dimensional system reduced to 2 to 3 hrs. Based on the two-dimensional chromatographic method, a three-dimensional chromatographic system coupled with tandem MS were further developed by using a size exclusion chromatography as the first dimension. The system provides more than ten-thousands peak capability. Analytical time is usually within 20 hrs.

A second array based multi-dimensional separation system consisted of a reverse phase chromatography as the first dimension, and 60 capillaries in parallel for capillary isoelectric focusing as the second dimension. Such a RPLC-array-CIEF system also provides more than ten-thousands peak capacity. The CIEF array separation was monitored by a home-made whole column image system with laser induced fluorescence. The CIEF-array system was further coupled to MALDI-MS for protein identifications. On-plate tryptic digestion of proteins and removal of ampholytes were studied for protein identifications. Mouse liver proteome was analyzed and demonstrated for their performance of the above two array-based multi-dimensional systems.

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11.26

The HUPO Proteomics Standard Initiative-Mass Spectrometry Standardization Efforts

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The HUPO Proteomics Standard Initiative-Mass Spectrometry (PSI-MS) working group, a loosely formed consortium, with participants from academics and industries, is working towards coming up a set of community standards: mzData, a standard format to represent processed peaklists from mass spectrometry instruments; mzProtID, a standard format to represent peptide/protein identifications from search engines; and controlled vocabularies used in capturing MS-related data. The main goal of this standardization effort is to develop new tools and to automate workflows by making the data accessible. Please visit psidev.sourceforge.net/ms/index.html for more information of our activities, and the current status of the project. Some of the instrument and software vendors have started to support mzData by implementing it into their products. We have also been working very closely with various HUPO tissue groups for them to start to use mzData as their data exchange formats. Along with these efforts, a proof-of-concept central repository to capture proteomics data will be presented in this conference.