7.1

Plasma Glycoproteome Analysis Using Lectins

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Human plasma proteome can represent physiological status. Therefore, mapping of plasma proteome may provide valuable basic information for finding disease marker proteins. Especially almost plasma proteins are glycoproteins and because glycosylation patterns are also dramatically changed dependent on physiological status, mapping and profiling glycosylation patterns of plasma proteome are also necessary. In this study, lectins were used to map glycoproteome of plasma obtained from normal human. This is the first attempt to map human glycoproteome using lectins and in the future, this map will be used as reference to plasma obtained from patients to detecting disease marker proteins.

7.2

Mass Spectrometry Developments to Facilitate Glycoproteomics

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Glycobiology presents special challenges because of the complex mixtures of closely-related structures and the temporal, developmental or degenerative changes in the glycoform distributions that occur in each organ/tissue/fluid of individuals within a set and among different species. The likely presence of branching and/or labile substituents, and the chance that changes in biological activity originate from minor or transient components add to the difficulty of the analysis. However, our rapidly expanding ability to unravel these complexities now allows us to define important structural details that are key to elucidating the structure/function relationships among carbohydrates and between carbohydrates and proteins. The increasing array of mass spectrometry-related approaches now provides powerful tools for this mission; ingenuity should sustain the growth over the coming years. Our laboratories focus on enhancing several facets of glycoproteomics: (1) improved approaches to microscale separation, purification and derivatization, (2) mild ionization conditions that preserve labile substituents, (3) high performance mass analysis and tandem mass spectrometry, with emphasis on novel FTMS methods, (4) data processing and design of libraries and search strategies optimized for glycan analysis. Our overall approaches will be discussed, with illustrations from ongoing glycomic studies.

7.3

A New Reagent for the Selective Detection of Glycoproteins on 2D Gel

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Glycosylation is one of the most common and complex post-translational modifications. This modification results in thousands of unique, bioactive glycoproteins that can be circulating, membrane bound, or confined to the cytoplasm. Glycoprotein detection is currently performed by modified Periodic Acid Schiff (PAS) methods. Following SDS-PAGE proteins are fixed in the gel. The carbohydrates on the proteins are oxidized to aldehydes by periodic acid. Colored dye is reacted then with the aldehyde functions leading to magenta bands with a light pink or colorless background. These classical methods for in-gel carbohydrate detection are very selective, but lack sensitivity (detection limit of 25–100 ng of carbohydrate). In order to improve sensibility, a fluorescent dye can be used. Although this staining procedure is quite selective for glycoproteins, some non-specific protein staining occurs which is more pronounced in some gel formulations. We have developed a new reagent for the selective detection of glycoproteins in polyacrylamide gels. This strategy is based on the well known reaction between the sugar vicinal diols and boronic acid leading to the formation of cyclic borate. More precisely, we have synthesized a molecule constituted by a phenylboronic acid for the selective reaction with glycoproteins, coupled via a linker to a fluorescent dye based on a ruthenium complex. This allows for the specific, sensitive detection of glycoproteins directly in gels. This new reagent was first tested on SDS PAGE with glycoproteins standards and/or molecular weight markers. This new fluorescent performed well on these proteins as the observed detection limit was 5–10 times lower than those observed with the Periodic Acid-Schiff base reagent. The next step currently under investigation in the laboratory is to apply this new fluorescent detection on more complex proteic samples from HCT 116 cells separated on 2D gel electrophoresis.

7.4

Expression of the Glycoprotein D of Herpes Simplex Virus Type-2 in Insect Cells

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Herpes simplex virus type 2 (HSV2) is the primary cause of genital herpes. It is highly prevalent in human population worldwide. Since the most HSV infections are subclinical, the development of an effective vaccine would provide a powerful control tool. In the present study the recombinant glycoprotein D of HSV2 was expressed in insect cells. Iranian isolate of HSV2 was propagated in HeLa cell line. The viral DNA was extracted and used as template for polymerase chain reaction using specific primers. The amplified gD2 DNA was cloned into *EcoR*I and *Xho*I sites of pFastBacHTc (Invitrogen) plasmid and transformed into DH5á cells. The gD2 recombinant plasmid was purified and transformed into *E. coli* DH10Bac cells (Invitrogen) for the sitespecific transposition of the gD2 DNA from pFastBacHTc to a bacmid DNA, a baculovirus shuttle vector, which is contained in the host cells. The recombinant bacmid was confirmed by PCR using M13/pUC primers and gD2 gene specific primers. The gD2 recombinant bacmid was transfected into Sf21 cells using cellfectin (Invitrogen), cultured in serum-free Grace's medium at 27OC and screened daily for observation cytopathic effects. After 48 h, the recombinant baculovirus was harvested from the cell culture medium and centrifuged to remove cell debries and named the gD2-recombinant baculovirus. To identify the protein expression, the gD2-recombinant baculovirus was examined by IFA test using monoclonal antibody against gD2 (Biodesign). The protein was detected in the nuclei and cytoplasm of baculovirus infected cells by 72 h.p.i. The recombinant gD2 could be used as a subunit vaccine as well as prime-boost immunization strategies.

7.5

A System for Rapid Identification of Oligosaccharide on Glycoprotein Using an Observational MSn Database of Human Glycans

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Glycosylation is the most wide-spread post-translational modification (PTM) in eukaryotes, however the role of oligosaccharides attached to proteins has been studied little because of the lack of a sensitive and easy analytical method for oligosaccharide structures. Recently tandem mass spectrometric techniques have been revealing that oligosaccharides might have characteristic signal intensity profiles. These facts prompted us to develop a practical and systemized technology, aiming at the glycan structure analysis of human tissue and serum, which is based on a combination of MSn data and computer technology. We describe here a strategy for the rapid identification of the oligosaccharide structures on glycoproteins using only mass spectrometry. It is based on a comparison of the signal intensity profiles of MSn spectra between the analyte and a library in an observational database which is built up by acquiring MSn spectra of a large variety of structurally defined oligosaccharides. We have cloned and characterized many human glycogenes in the past several years. Unlike in previous trials by others, our strategy utilizes a large variety of structurally defined oligosaccharides which can be obtained not only from natural sources but also by an effortless and rapid enzymatic preparation using a library of glycosyltransferases directed by the glycogenes which have been accumulated in our laboratory. Using this strategy we were able to identify the structure of N-linked oligosaccharides in transferrin and immunoglobulin G as examples. This work was supported by the New Energy and Industrial Technology Development Organization (NEDO).

7.6

The Carbohydrate Sequence Markup Language (CabosML): A XML Description of Carbohydrate Structures

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Bioinformatic resources for genomics and proteomics are widely available for molecular biologists, but this situation has not been achieved yet for glycobiologists. The complexity of carbohydrate sequences makes it difficult to define a common language to represent carbohydrate sequences and to develop bioinformatics tools for glycomics. In this study, we developed a carbohydrate sequence markup language (CabosML), a XML (extensible markup language) description of carbohydrate structures, and implemented a structural database of carbohydrates with the XML format. We also developed graphical user interfaces for editing and searching the carbohydarate structures, by which the depicted structures can be automatically converted into the XML formats. The XML description for carbohydrates described here will greatly contribute to the progress of informatics tools for glycomics.

7.7

Sequencing of N-linked Oligosaccharides of 1D-PAGE Separated Glycoproteins: In-gel Deglycosylation Followed by Fragmentation of 3-APH-derivatized Oligosacchardies with MALDI-TOF/TOF

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The fragmentation characteristics of native and 3-aminophthalic hydrizide (3-APH)-derivatized oligosaccharides using a matrix–assisted laser desorption/ionization (MALDI) time-of-flight/time-of-flight tandem mass spectrometer are described. 3-APH-derivatized maltoheptaose showed a 50-fold improvement in sensitivity over the underivatized one and could be detected at a level of 10 fmol. The extensive fragmentation including glycosidic cleavages and cross-ring cleavages is shown to facilitate the detailed structural characterization of N-linked oligosaccharides released from standard glycoproteins. Furthermore, it was demonstrated that the technique could be used to the characterization of glycan moieties extracted by in-gel PNGase F treatemnt of glycoproteins on one-dimensional sodium dodecyl sulfate-polyacryamide gel electrophoresis (SDS-PAGE).

7.8

Development of Analysis Method for Glycopeptides Using Cellulose Column Chromatography

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To improve glycoprotein analysis for glycomics with MALDI-TOF MS, separation of glycopeptides and non-glycopeptides from protease-digests is needed. That's because ionizing efficiencies of glycopeptides and non-glycopeptides are quite different in MALDI-TOF MS. Purification of glycopeptides from fetuin: Protease-digested fetuin was separated through reverse phase HPLC. Tri-antennary glycopeptides fractions were collected and digested with sialidase. Tryptic digestion of proteins: Transferrin (Tf) and asialofetuin (ASF) were both reductive-alkylated with DTT and Iodoacetamide, and then were trypsinized. Separation of glycopeptides and non-glycopeptides: protease-digested glycoproteins are subjected to cellulose cartridge equibrated with 60% buthanol. Binding fractions were eluted stepwise with elution buffer containing different concentration of Ethanol. 1. Fetuin-derived glycopetide (GP3) was approx. 10 times less ionized compared to the corresponding naked peptide (P3). 2. Glycopeptides and non-glycopeptides were efficiently separated from tryptic digests of Tf and ASF through cellulose column. 3. Cellulose column could be successfully applied to proteome analysis of mouse tissue. Cellulose column separation has been suggested to be strong tool for sample preparation for MALDI-TOF MS analysis in Proteomics/Glycoproteomics.

7.9

Characterization of Glycoproteins from Plasma Using Two-step Proteolytic Digestion Combined with Sequential Micro-Columns and Mass Spectrometry

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Protein glycosylation is involved in conformational stability, protection against degradation, molecular and cellular recognition etc. Due to the fact that glycosylated proteins may have a vide variety of glycan structures attached and that different sites may be only partially glycosylated, glycosylated proteins are difficult to characterize. Therefore, a combination of complementary techniques is needed to fully characterize a glycoprotein, decreasing the overall sensitivity. Here we describe a method, which allow characterization of small amount (low pmol level) of N-linked glycoproteins, including identification, glycosylation site mapping and partial structure analysis of the attached glycans. The gel-separated glycoprotein is submitted to in-gel digestion using trypsin followed by proteinase K digestion of the resulting peptides. Remaining peptides are removed by a micro-column packed with Poros R2 RP material. The glycopeptides passes through the R2 micro-column and can be purified on a graphite micro-column, and analyzed by MALDI MS and MSMS to provide a molecular weight of the peptide and the glycan sequence.

The strategy is illustrated by the analysis of the glycoprotein ovalbumin and glycoproteins contaminating the commercial available ovalbumin (ovomucoid and ovoglycoprotein). Preliminary result on characterization of glycoproteins in human plasma enriched by lectin affinity will be given.

7.10

Mass Spectrometric Sequencing of Protein-derived Carbohydrates Using MALDI-TOF/TOF Mass Spectrometry

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Among the most abundant post-translational modifications glycosylations comprise a highly complex group of carbohydrate modifications to the polypeptide backbone of proteins. The two most common forms, N- and O-glycosylation, are both characterized by branched three-dimensional structures which are highly diverse in form and size. Upon appearance of various mass spectrometric techniques from the proteomics sector also glycan analysis profits from the recent advances in instrumental design. Here we describe MALDI-TOF/TOF sequencing of protein-derived carbohydrates in respect to various matrices and system parameters. Oligosaccharides were obtained from various sources; partially model compounds (Sigma) and partially obtained by glycosidase treatment from glycoproteins. Standard dried droplet preparation on stainless steel MALDI targets was employed, DHB was the typical matrix but other matrices such as sDHB and THAP were also evaluated. MS and LIFT-TOF/TOF MS/MS spectra were obtained on an Ultraflex TOF/TOF (Bruker Daltonics) either under conditions of unimolecular decomposition (LID) or high energy CID (heCID) using primarily Ar as collision gas [1]. MS/MS spectra analysis was supported by new software tools. HeCID resulted in an enhanced occurrence of analytically valuable cross-ring fragmentations (A- and X-ion series) in addition to the commonly observed interglycosidic cleavages (B- and Y-ions). This offers the opportunity for fast initial sequencing of carbohydrate structures with the possibility to differentiate between structural linkage isomers. During the experimental approach we examined the influence of various system parameters including different matrices, LID vs. heCID and sample purity. It turned out that complex carbohydrate samples resulted in significantly different MS/MS spectra as compared to highly purified oligosaccharides, possibly due to significantly increased laser fluence settings. Another important aspect in this work was the development of an analytic strategy that included sample preparation, the fragmentation regime and spectra interpretation as well as structure visualization.

7.11

Construction of a Human Glycogene Library. Systematic Synthesis of Glycans Using This Glycogene Library and Supplying the Glycans to Rapid Identification of Glycan Structure Using an Observational MS^n Database

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Eighteen years have passed after the first mammalian glycosyltransferase was cloned. At the beginning of April, 2001, 110 genes for human glycosyltransferases, including modifying enzymes for carbohydrate chains such as sulfotransferases, had been cloned and analyzed. We started the Glycogene Project (GG project) in April 2001, a comprehensive study on human glycogenes with the aid of bioinformatic technology. The term glycogene includes the genes for glycosyltransferases, sulfotransferases adding sulfate to carbohydrates and sugar-nucleotide transporters, etc. Firstly, as many novel genes, which are the candidates for glycogenes, as possible were searched using bioinformatic technology in databases. They were then cloned and expressed in various expression systems to detect the activity for carbohydrate synthesis. Their substrate specificity was determined using various acceptors.

Second, it is possible for systematic preparation of oligosaccharide and glycopeptide libraries using a library of recombinant enzymes. For the synthesis of *O*-glycan peptide, the basic structure was synthesized chemically. Extension of *O*-glycans was achieved by sequential addition of each enzyme in a single tube. For the synthesis of *N*-glycans, the basic structures were commercially obtained. Further extension of *N*-glycans was achieved using a variety of the enzymes.

Third, these glycan libraries were supplied to MS^n analysis as structurally defined glycans. An observational database by acquiring MS^n spectra of a large variety of structurally defined oligosaccharides was built up and utilized for the rapid identification of glycan structures using only mass spectrometry. By this strategy we were able to identify the structure of *N*-linked oligosaccharides in transferrin and immunoglobulin G as examples.

This work was supported by New Energy and Industrial Technology Development Organization (NEDO) of Japanese Government.

7.12

Monolithic Columns Dedicated to the Specific Isolation of Glycopeptides

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Analysis of post-translational modifications in protein is an important step in understanding the changed functional properties of many proteins upon such a modification. Consequently, there is a need for novel analytical tools dedicated to such studies. We describe here novel chromatographic devices for the specific isolation of glycopeptides before their MS analysis. The columns rely on an acid boronic immobilized phase that is prepared on a polymer monolithic support. Monolithic supports were chosen for their versatility in terms of porosity and functionality and for their compatibility with the downscaling of the devices for their integration in a microfluidic system for instance. Polymer monolithic phases dedicated to glycopeptides isolation were successfully prepared in a capillary format (i.d. 75 μm). The resulting phases were first tested on a model, rutin, which is well-known glycosylated polyphenol. The column performed very well: rutin is selectively trapped by the phase and then eluted by a slightly basic solvent. We are investigating the column performances on a model digest of chicken ovalbumin: the retention of glycopeptides on the phase, the strength of their interaction, the strength of the elution conditions. The same phase polymerised in 30 x 10 μm microfabricated channels made from SU-8 resin by microelectronics technics are currently tested under similar conditions.

7.13

Hydrophilic Affinity Isolation and MALDI MS of Glycopeptides for Elucidation of Site-specific Glycan Structures of Glycoprotein

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In glycoproteomics, key structural issues, protein identification, locations of glycosylation sites, and evaluation of the glycan heterogeneity, should be evaluated in a large number of glycoproteins, while mass spectrometry (MS) provides substantial information about individual purified glycoproteins. Considering that these structural issues are elucidated by studying glycopeptides and that the tandem MS of a single tryptic peptide is usually enough for protein identification, construction of an MS-based method handling tryptic glycopeptides would be of enormous benefit in research. To this end, a simple and efficient method, utilizing hydrophilic binding of carbohydrate matrices such as Sepharose to oligosaccharides, was successfully applied to the isolation of tryptic glycopeptides. The recovery or specificity was improved by the presence of Mn^{2+} or acetate, respectively, in the binding solution. Both N- and O-glycosylated glycopeptides could be recovered. The glycan structures including sialylation of each collected glycopeptide were outlined by linear time-of-flight (TOF) measurement of the ions generated by matrix-assisted laser desorption/ionization (MALDI). Both peptide and oligosaccharide structures were elucidated by multiple-stage tandem MS of the ions from MALDI, as follows. The MALDI ion trap mass spectrum of a tryptic glycopeptide mixture was comprised of the $[M+H]^+$ ions of component glycopeptides. Collision-induced dissociation (CID) of the glycopeptide $[M+H]^+$ ion generated saccharide-spaced peaks and their fragment ions corresponding to the peptide and peptide + N-acetylglucosamine (GlcNAc) species in the MS/MS spectrum. The saccharide-spaced ladder served to outline oligosaccharide structures, which were then selected as precursors for subsequent multiple-stage CID measurements if further characterization was necessary. The peptide or peptide + GlcNAc ions in the MS/MS spectrum or the corresponding ions abundant in the MS spectrum were subjected to CID for determination of peptide sequences, to identify proteins and their glycosylation sites. The strategy, isolation of glycopeptides followed by MALDI linear TOF MS and multiple tandem MS analyses, efficiently characterized the difference of site-specific N-glycan structures between plasma and cellular fibronectins.