บทความวิชาการ

ไกลโคโปรติโอมิคส์

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บทคัดย่อ

ไกลโคโปรติโอมิคส์ (Glycoproteomics) คือ การศึกษาการปรับแต่งโปรตีนด้วยการเติมโมเลกุลคาร์โบไฮเดรตเป็นสาขาหนึ่งของโปรติโอมิคส์ (Proteomics) โดยงานด้านโปรติโอมิคส์มีขอบเขตที่กว้าง เนื่องจากเป็นการศึกษาทั้งในเชิงโครงสร้างและหน้าที่ของโปรตีนภายในเซลล์ เนื้อเยื่อ หรือร่างกายของสิ่งมีชีวิต และครอบคลุมการศึกษาคุณสมบัติด้านต่างๆ ของโปรตีน รวมไปถึงกระบวนการปรับแต่งหลังแปรรหัสทางพันธุกรรม (post-translational modifications, PTM) ในการสังเคราะห์โปรตีนด้วย ซึ่งการปรับแต่งโปรตีนที่เกิดขึ้นมักจะมีความสำคัญต่อหน้าที่ของโปรตีนในกระบวนทางชีวภาพ โดยการปรับแต่งโครงสร้างของโปรตีนเกิดขึ้นได้หลายแบบ ตัวอย่างเช่น การเติมหมู่เมทิล (methylation) และการเติมหมู่ฟอสเฟต (phosphorylation) ซึ่งในการปรับแต่งโปรตีนแบบต่างๆ พบว่าการเติมโมเลกุลคาร์โบไฮเดรต (glycosylation) ได้รับความสนใจในการศึกษาวิจัยเป็นพิเศษ ทั้งนี้เนื่องจากคาร์โบไฮเดรตเป็นกลุ่มสารประกอบที่มีความหลากหลายของโครงสร้างที่พบในธรรมชาติมากที่สุด และเนื่องจากความหลากหลายของโครงสร้างนี้เองทำให้การศึกษาคาร์โบไฮเดรตเป็นงานที่ท้าทายอย่างมาก นอกจากนี้ยังมีการรายงานอย่างแพร่หลายว่าคาร์โบไฮเดรตมีบทบาทสำคัญในระบบต่างๆ ของสิ่งมีชีวิต เช่น การเจริญเติบโตของเซลล์และระบบภูมิคุ้มกันของร่างกาย เป็นต้น ซึ่งบทความฉบับนี้ได้ทำการรวบรวมและนำเสนอกระบวนการศึกษาเกี่ยวกับไกลโคโปรติโอมิคส์ โดยมีการใช้เทคนิคการตรวจวัดมวล (mass spectrometry) เป็นเครื่องมือหลักในการวิเคราะห์เพื่อให้ได้ข้อมูลที่สามารถใช้ในการอธิบายความสำคัญของการเติมโมเลกุลคาร์โบไฮเดรตหรือตอบคำถามเกี่ยวกับตำแหน่งและโครงสร้างของโมเลกุลคาร์โบไฮเดรตบนโปรตีนได้

คำสำคัญ: ไกลโคโปรติโอมิคส์ คาร์โบไฮเดรต ไกลโคซิเลชัน แมสสเปกโทรเมทรี

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Glycoproteomics

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ABSTRACT

Glycoproteomics, the study of protein glycosylation, is a branch of proteomics which covers a wide range of protein study including the study of structures and functions of proteins within a cell, tissue or organism. In addition, post-translational modifications (PTMs) are also included. The modifications of proteins play fundamental roles in controlling various biological processes. Since many different protein modifications have been presented, the specific terms of each PTM were proposed such as hydroxylation, methylation and phosphorylation. Among PTMs, glycosylation is of particular interest, because carbohydrates are the compounds which have the highest diversity of structures found in nature, with their enormous structural diversity making a comprehensive study hugely challenging. In addition, there are many evidences showing that glycans play important roles in many systems such as cell growth and immune recognition/response etc. This paper reviews glycoproteomics using mass spectrometry-based techniques to answer a wide range of biological questions such as an importance of glycosylation on certain glycoproteins, or position and structure of carbohydrates attached to the glycoproteins.

Keywords: glycoproteomics, carbohydrate, glycosylation, mass spectrometry

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1. Introduction

A progress in life sciences such as biology or medicine has a great impact on human life. However, it would not have been possible without basic knowledge and tools developed by analytical chemists. On the contrary, several analytical techniques have been developed because of the questions raised by other scientific fields. Protein is one of major macromolecules, alongside nucleic acid (DNA), carbohydrate, and lipid, and plays a critical role in various biological processes of living organisms. Therefore, the study of proteins is very important for the explanation of life's system. Proteome, proteins expressed by a genome, was first coined by Wilkins *et al.* in 1995. The term "Proteomice" is considered to be the study of structures and functions of proteins within a cell, tissue or organism. The field of proteomics continues to be rapidly expanding with many thousand proteomic studies published to date. A research in proteomics provides so many crucial knowledges, and also creates several novel fields related to protein study. The analysis of PTMs, chemical modifications playing a role in functional proteomics, is one of important aspects in proteomics. It is estimated that there are more than 200 types of post-translational modifications of proteins [1]. Protein glycosylation is one of the major PTMs, and perhaps the most common PTM in mammals, found to have more than 50% of all proteins glycosylated [2]. Carbohydrates attached to proteins can modify the intrinsic properties of the proteins, and are necessary for protein functions, such as the effects on protein folding, conformation, stability, activity and distribution. Moreover, much evidence has shown that glycans also play important roles in many systems, such as cell growth and development [3, 4], tumor growth and metastasis [5, 6], anticoagulation [7], immune recognition/response [8, 9], cell-cell communication [10, 11], and microbial pathogenesis [12, 13]. Glycosylation is not only important for proteins to function normally, but also used as a marker to monitor several diseases in which changes occur to the glycosylation of proteins. Carbohydrates are the compounds which have the highest diversity of structures found in nature. As with DNA and protein, carbohydrate monomers can be polymerized to form long chains. However, they can be highly branched molecules with many different possible linkage positions through which they are connected to one another, which is different from other macromolecules. For carbohydrate analysis, glycomics is a term that refers to the comprehensive study of the entirety of carbohydrates in an organism. Since the roles of carbohydrate on protein are varied depending on the protein to which it is attached, a field of glycoproteomics has been established to study glycosylation in term of the structure and function of the entire glycoprotein. Glycoproteins are proteins covalently attached to carbohydrate chains which are normally oligosaccharides. In mammalian glycosylation, there are three types of glycan attachments which are N-linked glycans, O-linked glycans, and glycosylphosphatidylinositol (GPI) lipid anchors. In N-glycosylation,

the chains of oligosaccharides are attached to the amide nitrogen on the asparagine side chain in the consensus sequence (Asn-X-(Ser or Thr)), where X is any amino acid except proline. In O-glycosylation, the chains of oligosaccharides are linked to the hydroxyl oxygen in the side chain of serine or threonine. GPI anchors are glycolipids which can be linked to the C-terminus of certain proteins by a phosphodiester linkage during post-translational modification. Then the phosphoinositol residue is connected (anchored) to the cell membrane by another phosphodiester linking it to a lipid.

Although the protein analysis is routinely performed in proteomics laboratories, the work on glycoproteins remains challenging. Glycans of glycoprotein consist of a heterogeneous mixture of structure which derives from their non-template driven biosynthesis. The wide range of glycan structures, with both positional monosaccharide, and anomeric isoforms makes their full structure analysis very demanding. Moreover, the small amounts of available glycan material from biological systems increase further the demands of glycan analysis. Therefore, structural characterization of carbohydrates is a challenge for the analytical biochemist in term of both functional structures and analytical methodology development [14-16]. Figure 1 illustrates the general strategy for an integrated glycoproteomic analysis. This review primarily focuses on sample preparation methods of glyco-peptides and-proteins, and their analysis using MS-based techniques. Nowadays, MS is the most widely used approaches for glycoprotein analysis. MS is a feasible and powerful tool for structure analysis of carbohydrates and protein sequencing, because it provides very high sensitivity, high throughput, and analytical versatility. In addition, a range of separation approaches, which are compatible with proteomic and glycoproteomic analyses, in combination with analytical systems to improve analysis scale and sensitivity are also presented herein.

2. Glycoprotein/glycopeptide purification and separation

In practical, the protocols of glycoprotein or glycopeptide isolation and analysis are similar in which electrophoretic and chromatographic methods followed by mass spectrometry (MS) have been intensively exploited. In standard approach, protein analysis is performed by the separation of the proteome using slab-gel electrophoresis to primarily reduce complexity of the protein mixture. Although the glycoproteins of interest can be isolated, they however contain different glycoforms resulting in difficulty to resolve. Therefore, the proteins are generally digested by trypsin or other proteases via in-gel protocol. The digested (glyco) proteins are then directly analyzed by mass spectrometry with soft ionization such as matrix-assisted laser desorption ionization (MALDI), or subjected to separation approach prior to MS for fingerprint analysis and glycan structural characterization. In the case of glycoproteomics, separation and

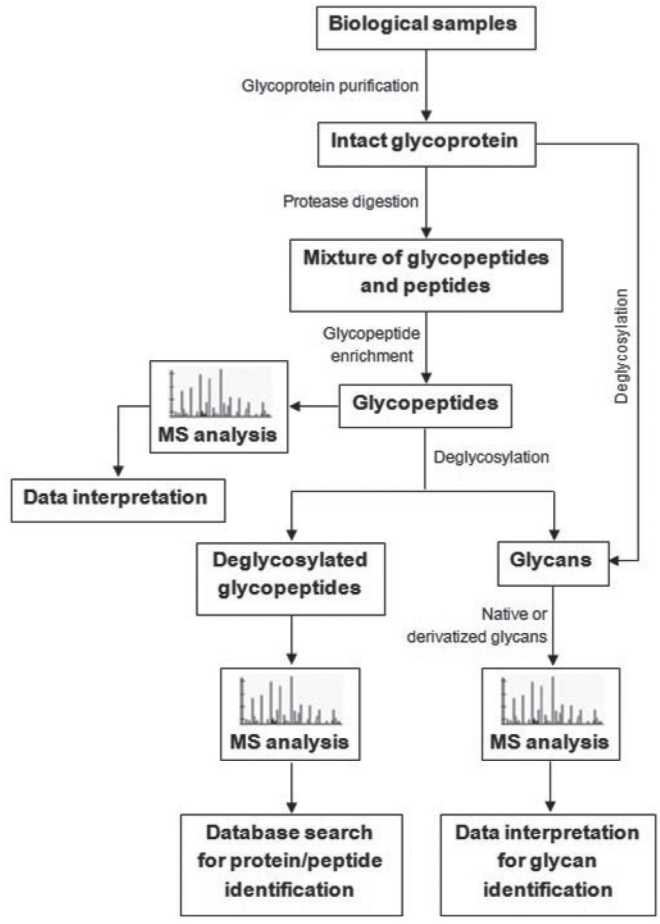

Figure 1. The strategies of mass spectrometry based glycoproteomic analysis.

enrichment systems of glycopeptides and glycans are critical, because the levels of modification are considerably less than non-modified peptides in the digest mixture. Therefore, purification and separation are very important steps in proteomics and glycoproteomics.

2.1 Gel electrophoresis

In the case of protein analysis, one of the most widely-used protein separation techniques is sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gel electrophoresis is generally the first choice for protein analysis in a biology laboratory. Not only purification or separation but also enrichment is achieved by employing SDS-PAGE. One dimensional (1D) PAGE and two dimensional (2D) PAGE are generally used in protein study. In many study [17-20], the separation of (glyco)protein samples can be accomplished in 1D-PAGE. However, since proteome contains a number of proteins, therefore higher separation efficiency is required to resolve such complex protein mixture. 2D-PAGE is the method of

choice for the protein separation at which the first dimension separates according to their charge (isoelectric point, pI) using isoelectric focusing approach (IEF), and the second dimension separates by mass using ordinary 1D-PAGE. Profiling proteomics, profiling the proteins expressed differently between two or more different samples, can be performed. Comparative analysis of multiple samples is achieved using a single 1D format, while 2D format is exploited to resolve multiple component of a single sample simultaneously. Protein visualization following 1D- or 2D-gel electrophorsis separation is critical step for profiling protein or quantitative proteome analysis [21], since different methods provide different limit of detection, dynamic range, and compatibility with detection system such as MS. Among staining methods, fluorescence labeling such as SYPRO Ruby [22] has gained increased popularity because it provides high sensitivity in a range of nanogram amounts of protein, a wide dynamic range, and high reproducibility. In addition, SYPRO Ruby can be used to monitor protein modifications such as metalloproteins, lipoproteins, and glycoproteins.

Apart from tagging with fluorescent or chemiluminescent substrates, several methods can be exploited for detecting glycoprotein in gel. Silver staining has been widely used for detection of protein, but it is generally not suitable for glycoproteins because of steric interference by the carbohydrate moieties with the binding of silver ions. However, Møller and Poulsen (1995) developed the method to be at least ten times more sensitive than previously publish methods [23]. In addition, periodic acid Schiff's reagent (PAS) procedure, involving initial oxidation of carbohydrates by periodic acid and subsequent staining with Schiff's reagent, is used to label all kinds of glycoproteins containing cis-vicinal diols on carbohydrates. The method becomes much more sensitive by coupling the reaction with fluorescent substrate such as Pro-Q Emerald, a fluorescent dye specific for glycoprotein detection [24]. Glycosylation of protein is able to be monitored by the change of molecular weight and/or pI of a protein to which position of the protein spot on the proteomic pattern is shifted. Furthermore, comparative analysis of protein spot locations between native protein and deglycosylated protein using glycan-specific enzyme can also be exploited [25-27].

Gel electrophoresis is a very simple and relatively economical separation tool compared to other techniques such as high performance liquid chromatography (HPLC) and capillary electrophoresis (CE). However, it suffers several limitations including low throughput, limited solubility of hydrophobic and membrane proteins, limited dynamic detection range, difficulties in analyzing highly basic and acidic proteins, poor reproducibility, poor sensitivity, and poor quantitation. In addition, separated glycoprotein bands are usually broad owing to the heterogeneity of carbohydrates attached to individual polypeptides. Therefore, complete separation of different glycoforms is very difficult using SDS-PAGE.

2.2 Chromatographic methods for glycopeptide and glycan separations

Nowadays, one of the main challenges for the bioanalytical chemist includes the development of integrated approaches which are compatible with proteomic and glycoproteomic analyses, especially in terms of analysis scale and sensitivity. With several limitations of slab-gel electrophoresis including not amenable to automation, aqueous-based separation techniques such as LC and CE are ideal with higher sensitivity, less sample amounts required, possess a large number of separation mechanisms, amenable to automation, and higher sample throughput. Particularly, HPLC techniques are widely used combined with mass spectrometry to allow detail information for structural analysis of glycans and glycoproteins to be obtained with high sensitivity.

In general, various forms of HPLC such as reversed phase chromatography [28, 29], normal phase chromatography [30] affinity chromatography [31, 32], size exclusion [33], ion exchange [34-36], and hydrophilic interaction [37], can be used in glycoprotein and glycopeptide fractionations. In addition, graphitized carbon also demonstrated good separation for glycopeptides analysis. [38, 39].

In glycoproteomics, chromatographic methods are not only used for glycoprotein and glycopeptides isolation, but also for glycan separation. Glycan profiling is a one of the main questions in this area of study. There are several methods for releasing glycans from the glycoprotein, and those are discussed in Section 3. Structural analysis of glycans is very challenging because of the extreme variety of possible glycan structures. In addition, most glycoproteins contain more than one site of glycosylation (macroheterogeneity), and most glycosylation sites bear a multitude of glycoforms (microheterogeneity). Therefore, even though only one glycoprotein is isolated, a complex mixture of glycoforms still occurs, resulting in a requirement of separation apprpoaches to reduce glycan complexity prior to structural characterization. There are several types of HPLC applied to glycan analysis works. Glycans are naturally very polar compound. They exhibit poor retention on reversed phases. However, several derivatization protocols have been exploited to improve the retention of glycans on reversed-phase materials [40-41]. Underivatized glycans have been separated by a combination of normal-phase (NP) LC linked to ESI-ion trap MS [42]. However, derivatized glycans such as 2-aminobenzamide (2-AB) derivatives [43] were also separated by using normal phase. Graphitized carbon columns (GCC) have been used for oligosaccharide separation since the early nineties [44]. This type of column has a unique ability to resolve isomeric and closely related compounds. Together with NP and GCC chromatography for underivatized carbohydrate separations, one of the most widely used LC methods for these analytes is a high performance anion exchange chromatography (HPAEC) using alkali hydroxide and alkali acetate-based

eluents [45, 46]. The preferred detection system for this kind of LC rather than MS is pulsed amperometric detection (PAD), because it is compatible with high ionic strength of the eluents from HPAEC techniques. However, a desalting device has now been exploited to remove the salt. Therefore, the coupling of HPAEC with MS using a desalting membrane is possible, although certainly not ideal for on-line analysis [46-48].

Monoliths are a group of chromatographic materials [49]. They show several advantages over traditional particle-supported chromatography such as better mass transfer properties and the low back pressure at high flow rates. The same as traditional particle-supported materials, monolithic materials can carry many types of ligands or functional groups to perform any separation mode. However, only a few works published on using monolithic column for glycoproteomics. Nevertheless, a recent review [34] suggests that monolithic columns are likely to be exploited intensively for protein and glycoprotein analysis. In addition to separation purposes, monolithic support material can be used as an enzyme reactor for glycan mapping [50]. New strategies of HPLC have been developed to enhance the performance of analyte separations by improving the column efficiency and also developing new sorption materials. Hydrophilic interaction chromatography is a relatively new HPLC mode for highly polar molecule analyses [51] including glycans. Churms observed the applications of HILIC for glycan separations and reported that glycans can be separated on unmodified silica, but the modified material produced by chemical bonding of suitable functional groups can perform more effectively [52]. Naturally, glycans themselves lack chromophores for UV detection, and they are not sensitive MS analytes. Therefore derivatization or labeling techniques are often used to enhance detection for the glycan analyses. Glycans modified with fluorescent probes such as fluorophore 2-amino benzamide (2-AB) and 2-anthranilic acid (2-AA) for HILIC separation has been presented [53], and more developments in HILIC application to glycans are detailed in reviews by Churms [52] and Novotny [54]. In the analysis of glycosylation, HILIC shows an ability to be an effective tool for PTM determinations. Several publications have presented the successful use of HILIC to enrich glycopeptides, followed by enzymatic deglycosylation to identify glycosylation sites and also characterize glycan structures using mass spectrometric methods [55-58]. Released glycans can also be separated by using capillary HILIC column coupled to MS for on-line analysis [59].

2.3 Glycopeptide enrichment

Before MS analysis of glycoprotein, proteome of an organism is generally digested with protease enzyme to generate a mixture of (glyco)peptides rather than subjecting intact glycoproteins to MS. In the mixture, analysis of glycopeptides is very difficult, because of a relative low abundance of glycosylated peptides compared to unglycosylated peptides. In

addition, so many different glycoforms are generally occurred in glycosylation of proteins. Therefore, enrichment step of glycopeptides is very important to reduce complexity of analytes in the total peptide pool, to ensure that glycoproteomic information is not suppressed by much higher amounts of proteomic data. Solid phase extraction approaches which contain hydrophilic interaction, graphitized carbon, cellulose, or lectin materials are often required. Multidimentional separations either on-line or off-line analysis prior to MS are also performed for further separating enriched (glyco)peptides of very complex analytes such as serum samples [60].

Hydrophilic interaction liquid chromatography (HILIC) is a relatively new HPLC mode for highly polar molecule analyses. It can be successfully used for glycopeptides purification. As presented in figure 2A and 2B, HILIC-solid phase extraction was applied for trypsin-digested fetuin separation. Figure 2A shows the MALDI-mass spectrum of the fetuin tryptic digest components that bound to HILIC-SPE, and figure 2B shows the spectrum of the fetuin tryptic digest components that bound to HILIC-SPE and then treated with Peptide *N*-glycosidase F (PNGase F). Owing to the very large glycopeptides produced on tryptic digestion of fetuin, they were not observed within the range of detection of MALDI-MS, while a few peptides were detected (Fig. 2A). However, after PNGase F treatment, the de-*N*-glycosylated peptides were within the *m/z* range for detection (Fig. 2B). Four signals from de-*N*-glycosylated peptides (Fig. 2B) were detected at *m/z* 1741.8, 1869.9, 3017.5, and 3672.8 with a few of non-glycosylated peptides [61].

Lectin affinity chromatography has been widely used in glycoprotein studies [62]. The interaction between lectin and carbohydrates is strong enough to be utilized for glycoprotein or glycopeptide isolation. Both relatively broad specificity such as concanavalin A (conA) [60, 63-65] and more specific lectin for certain glycan structures such as *Vicia villosa* lectin (VVL) [66] were presented. Several lectin approaches were presented in glycoproteome studies: single lectin [67], serial lectin [68] and multi-lectin affinity chromatography (M-LAC) [69-70]. As affinity chromatography using lectins depends on the substrate specificity of each lectin, it is therefore a limitation for glycopeptides with an unknown variety of glycan forms. However, M-LAC showed a great performance for glycoprotein isolation, since the M-LAC column contains different agrose immobilized lectins, which are specific for different types of glycan structure. A combination of lectin affinity, conA, as the first dimension and reversed-phase HPLC as the second dimension connected to ESI-MS for glycosylation characterization was presented [60].

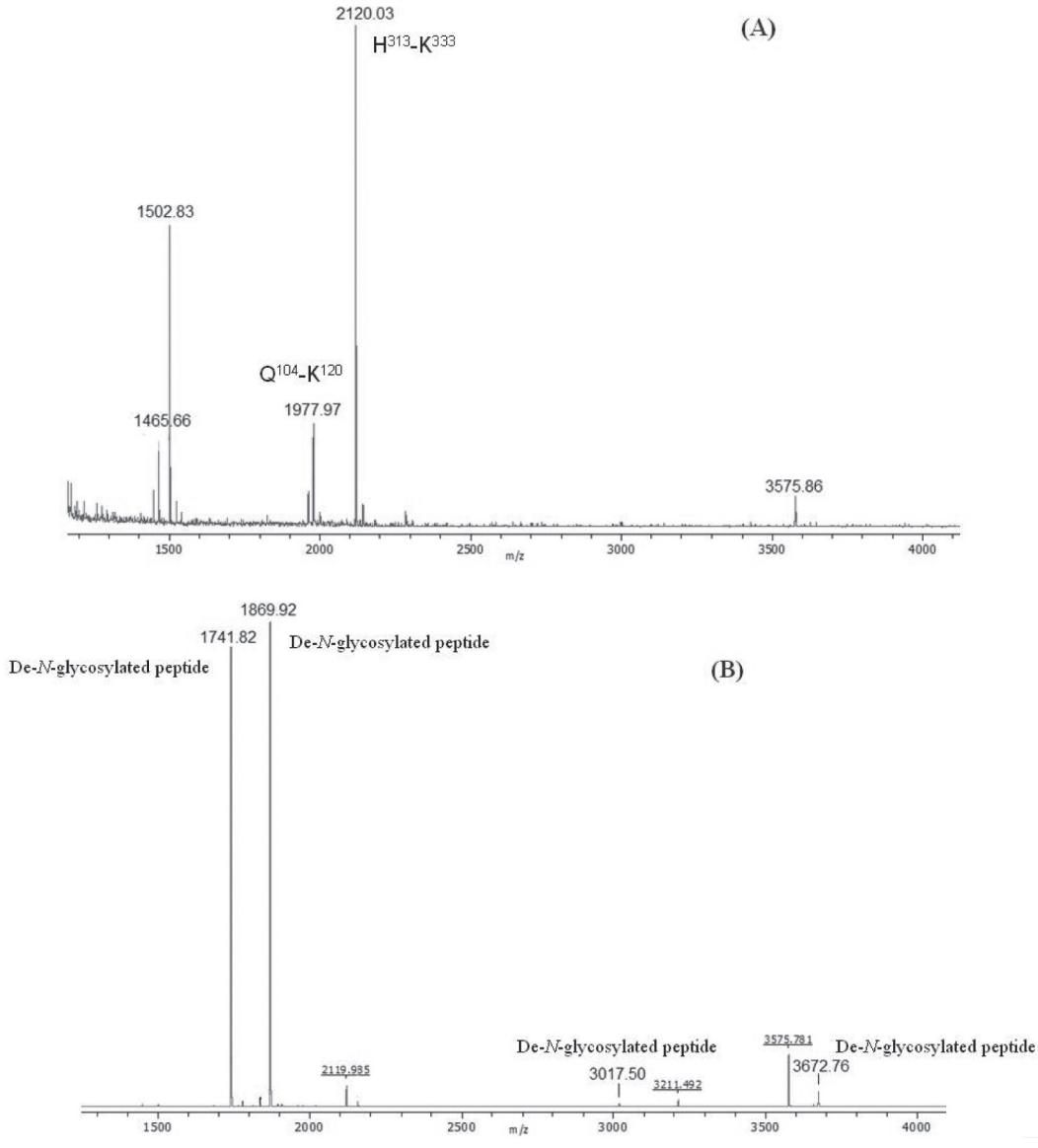

Figure 2 MALDI mass spectra of (A) HILIC-bound peptides from trypsin-digested fetuin and (B) HILIC-bound peptides from trypsin-digested fetuin following PNGase F treatment [61].

Another effective method for *N*-glycopeptide isolation is the use of hydrazide functionalized beads [71-72]. It is a chemical immobilization method by conjugating glycopeptides to a solid support using hydrazide chemistry. Glycopeptides are covalently captured on the hydrazide resin, while the non-glycosylated peptides are removed. The formerly *N*-linked glycosylated peptides are then released from the resin by treatment with PNGase F for *N*-glycosylation site identification. However, information on the glycan structure can not be achieved.

3. Glycans released from glycoproteins

Biologically functional glycans are mostly glycoconjugate molecules. Thus, glycans are generally released from the complex molecules prior to characterizing their structures. Then the glycans are separated from the digest to be characterized by either NMR or MS. In the case of glyco-proteins and -peptides, both *N*- and *O*-glycans can be released by either chemical or enzymatic release protocols. Each of these protocols offers different advantages and disadvantages. Chemical releases may cause partial or complete destruction of the polypeptide backbone. Both *N*- and *O*-linked glycans can be released from glycoproteins by hydrazine hydrolysis reaction [73]. However, this protocol has several disadvantages; because the reagent cleaves the amide linkage between asparagine and the *N*-glycans, other amidic bonds are also cleaved causing protein sample destruction which results in losing information on the site of glycosylation. In addition, under the reaction conditions the acetyl groups of *N*-acetylamino sugars and sialic acids are also hydrolyzed. β-Elimination is another chemical release commonly used for the release of *O*-linked glycans [74], but under different reaction conditions *N*-linked glycans can also be cleaved. In alkaline conditions isomerization and degradation by “peeling reactions” can also occur. To prevent these side reactions, a strong reducing agent such as sodium borohydride is incorporated in the reaction [54]. Consequently, the formation of alditols (reduced form) of the glycans occurs. There are some disadvantages of this protocol. The alditol glycans do not undergo reductive amination which can be used for the attachment of a chromophore allowing UV detection [75]. However, derivatization such as methylation and acetylation prior to mass spectrometric detection can improve sensitivity. Another drawback of this protocol is the excessive amounts of salts, which cause a problem for mass detection. As a typical β-elimination protocol has several disadvantages, Thomas-Oates et al. (1998) presented a modified β-elimination method by using ammonium hydroxide at room temperature to release glycans instead of sodium hydroxide. With these mild conditions, the released glycans still have intact reducing termini, and also do not undergo peeling reactions. This approach is compatible with MS detection too, because easily volatile reagents are

employed in the reaction [76].

Enzymatic procedures show an advantage of yielding intact glycans and proteins or peptides, but not all glycan types are liberated. It depends on specificity of enzyme to glycan structures. There are several enzymes available commercially for *N*-glycan release from glycopteins or glycopeptides. Peptide *N*-glycosidase F (PNGase F) is the most effective and widely used, because the released glycans are intact and the deglycosylated proteins/peptides can be further analyzed to identify the site of glycosylation. The enzyme cleaves the linkage between the glycan and the asparagine (N, mass = 114 Da) attachment site, which is then converted to aspartic acid (D, mass = 115 Da) after being deglycosylated. The mass increment of 1 mass unit is a marking of occupied glycosylation sites. As shown in Figure 3A and 3B, deglycosylated progranulin peptides by using PNGase F were characterized by ESI-MS/MS. Observation of both the amidated and deamidated species of asparagine residues is consistent with partial occupation of *N*-glycan on this site [18]. The enzyme is specific for all *N*-glycans except those bearing fucose α (1-3)-linked to the reducing terminal *N*-acetylglucosamine (GlcNAc), which can be released enzymatically by PNGase A [77]. However, PNGase A is ineffective with *N*-linked glycans containing sialic acid. Endoglycosidases such as endoglycosidase H and endoglycosidase F series (F_1, F_2, and F_3) are also commonly used. They cleave the linkage between the two GlcNAc residues of the *N*-glycan core and leave one GlcNAc residue attached to the asparagine which leads to the possibility of losing the information related to the presence of fucose on the reducing-terminal GlcNAc. Enzyme for *O*-linked glycan release is much more limited, because there is no effective enzyme for releasing all the intact *O*-linked glycans. Only endo-α-*N*-acetylgalactosaminidase has been reported to release the unsubstituted Gal-*β*(1-3)-GalNAc core structure; modification to the core structure blocks *O*-glycosidase activity [78]. In the case of Gal-*β*(1-3)-GalNAc with other modifications, exoglycosidases are applied to sequentially hydrolyse monosaccharides until only the core structure remains. Limitations of the *O*-glycosidase are one reason why *N*-glycan analysis has far more progress than *O*-glycan.

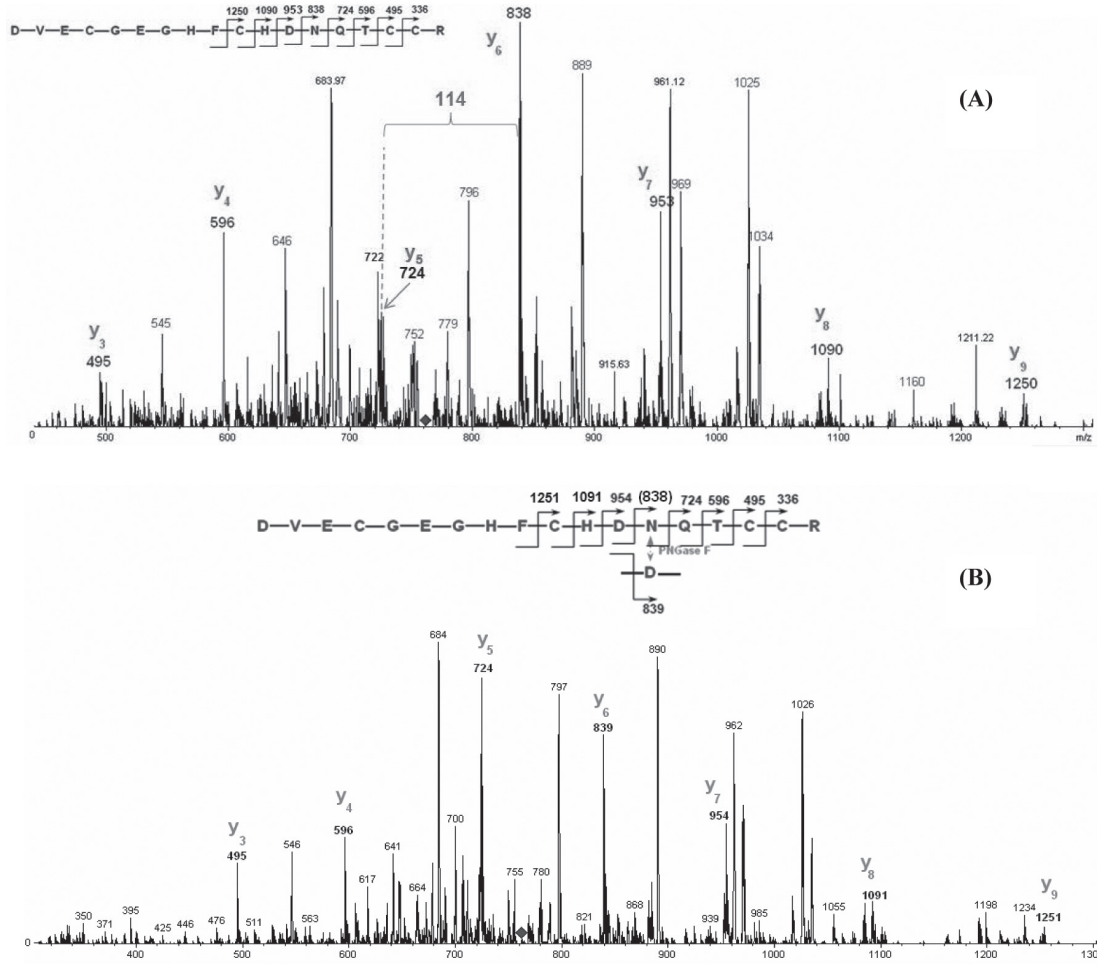

Figure 3. CID product ion spectrum and fragmentation schemes for progranulin peptide containing site N^{530}, precursor with *m/z* = 761.4 and 761.8, $[M+3H]^{3+}$, respectively. (A) non-glycosylated N^{530}-and (B) glycosylated N^{530}. On the fragmentation schemes, *m/z* value noted in parentheses is the one expected for the alternative structure, but not detected [18].

4. Mass spectrometry for glycoproteomics

4.1 General strategy for glycoprotein analysis

After glycoprotein isolation using either gel electrophoresis or chromatography, the glycoproteins are then characterized using the chosen methods according to the specific research question such as sites of glycosylation, glycan structures, and the linkages of glycan. In many cases, the glycoproteins are digested with specific endoproteinases, such as trypsin, chrymotrypsin, Glu-C, Asp-N, and Lys-C, to generate a mixture of peptides and glycopeptides

which have promising sizes able to be detected in a working rage of the detector such as MS. In the case that glycoprotein is isolated using either 1D- or 2D-gel electrophoresis, in-gel digestion can also be successfully applied to the glycoprotein [79]. Following these protocols, glycopeptides bearing individual glycosylation sites are obtained. Since glycopeptides generally have relatively low MS signal intensities compared to non-glycosylated peptides because of lower abundance and lower ionization efficiency, enrichment or separation step is required. However, sometimes specific enzymes produce very large glycopeptides that are difficult to detect by widely available MS. An alternative approach for site specific characterization of glycosylation using a non-specific protease such as pronase is applied. The non-specific enzyme pronase can hydrolyze all peptide bonds. However, steric hindrance around carbohydrate moieties or other modifications can restrict access to nearby peptide bonds, resulting in the release of glycopeptides with short peptides. This enables them to be more readily detected by mass spectrometry.

4.2. Characterization of glycans

N- and *O*-glycosylations are the two main types of protein glycosylation. While all *N*-glycans share a common pentasaccharide core which consists of two molecules of *N*-acetylglucosamine (GlcNAc) and three molecules of mannose (Man), $Man_3GlcNAc_2$, *O*-glycan do not share a distinct core structure. Prediction of the sites of *N*- and *O*-glycosylations is very difficult and not totally reliable, although the consensus sequence for *N*-glycosylation have been well known, but not all of the consensus sequences can be glycosylated. The most widely used strategy for monitoring site of *N*-glycosylation is enzymatic treatment with PNGase F. The glycosylation sites on the polypeptide backbone can be identified, because the asparagine residue that was glycosylated is changed to an aspartic acid on glycan release. The same as protease, PNGase F is able to perform in in-gel procedure successfully [18,80]. In addition, as tripeptide linked with glycan at asparagine as a central residue is a minimum substrate for PNGase F to be active, therefore the enzyme is applicable for both glyco-protein and -peptide. Moreover, if the sequence of the protein is known, masses of *O*-linked glycan can be differentiated from *N*-linked glycan, after treating the protein with PNGase F [81]. For the purposes of characterization of glycans and (glyco)peptides, the approaches of HPLC technique with either offline or online MS detection are widely used because of providing high sensitivity and excellent performance.

In the specific area of glycoprotein analysis, strategies based on one or more of enzymatic digestion, NMR and MS are the most widely-used approaches. Mass spectrometry is a powerful analytical technique employed for many purposes, namely, compound identification,

quantification, and structural characterization. Before the 1980s, the use of mass spectrometry for glycan analysis was quite limited, because intact glycans have low volatility, so that derivatizations with appropriate reagents were required to impart volatility. Since then, methodologies for ionization and analyzer systems have been developed continuously, and they will be improved further in the future. Now mass spectrometry is intensively used for structural study. Electron ionization (EI), the earliest means of ionization, is still used, but it is only applicable to smaller molecules not for macromolecules as protein, most of which require derivatization. Because of an advance of technology and a progress in the field of macromolecule studies, Fast Atom Bombardment (FAB), soft ionization technique, was developed. Nowadays, FAB has been superseded by the new generation of mass spectrometry ionization techniques that provide more advantages. Therefore, this technique has been replaced by the newer ionization systems of MALDI and electrospray ionization (ESI).

4.3 Mass spectrometric fragmentation of glycoprotein/glycopeptide

Although the efficiency of the soft ionization techniques such as FAB, MALDI, and ESI may provide sufficient internal energy resulting in some fragmentations, additional dissociation process is required to get reliable fragmentation and generate structural information. There are several techniques to fragment intact molecule, a precursor, such as collision-induced dissociation (CID), sustained off-resonance irradiation collision-induced dissociation (SORI-CID), electron-capture dissociation (ECD), and electron-transfer dissociation (ETD). Each of these fragmentation techniques has different process and mechanism of generating fragmented species. Therefore, different techniques of fragmentation suit with different types of mass spectrometer. CID is commonly used and commercially available on most instruments. It has been successfully applied to characterize a wide range of samples. For glycoproteomics, CID has been intensively exploited to obtain proteomic and glycomic information as presented in a large number of both original and review papers [18, 82-85]. All of the proteomic strategies discussed thus far utilise MS and MS/MS for protein identification and quantitation. When peptides are fragmented by low energy CID, it is the peptide bond along the backbone that predominantly cleaves. Cleavage of the peptide bond predominantly gives rise to ‘b-ions’ and ‘y-ions’. If the charge remains on the C-terminal portion of the peptide the ions are termed ‘y-ions’, and if the charge is retained by the N-terminal portion the ions are termed ‘b-ions’. The number relates to the number of amino acids in each fragment. Additional ions (a, c, x and z ions) can also be formed, if the peptide fragments as indicated in figure 4.

In the case of glycan fragmentation, it can generally be classified into two pathways. Glycosidic cleavages result from the breaking of a bond between two monosaccharide rings.

Second, cross-ring cleave involves the breaking of two bonds within a monosaccharide ring. The glycosidic cleavage pathway mainly provides information on sequence and branching, while the cross-ring cleavage may give information on linkage [86-88]. As illustrated in figure 5, showing the nomenclature for describing the major fragment ions from carbohydrates, ions formed from the carbohydrate portions that retain charge on the non-reducing terminal portion of the chain are designated A_i, B_i, and C_i, where *i* represents the number of the cleaved glycosidic bond numbering from the non-reducing terminus. Those ions that contain the reducing terminus are labeled X_j, Y_j, and Z_j, where *j* is the number of the interglycosidic bond numbering from the reducing terminus. A and X ions are the products of cross-ring cleavages, while the others are from glycosidic cleavages. Y_0 and Z_0 derive from fragmentation of the glycosidic bond linking to the aglycone. The cross-ring cleavages are given a superscript number that shows the position of the cleaved bonds, followed by the alphabet letter showing the type of ion. MS addressed with CID unit can be exploited both positive and negative modes for glycan analysis, and different information is achieved from different modes of operation. In positive mode, cleavage positions are mainly at the glycosidic bonds, while negative mode gave predominantly cross-ring fragmentation, which is more complicated for interpretation, but provides useful branching information, as demonstrated by several reports [89-91].

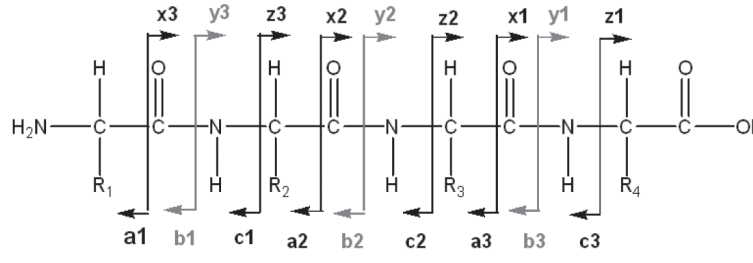

Figure 4 Peptide fragmentation nomenclature; b- and y-ions are predominantly produced when CID is used to fragment peptides.

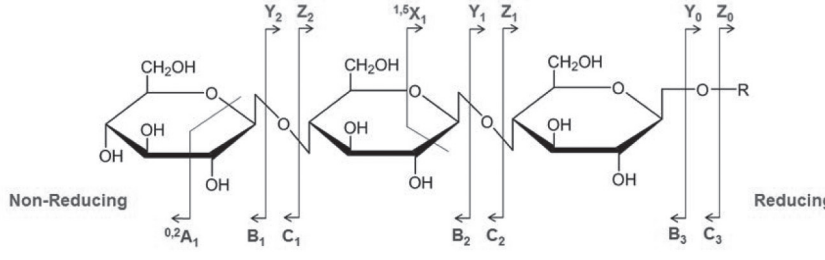

Figure 5 Scheme illustrating the nomenclature for the carbohydrate fragment ions. (Modified from Domon and Costello [92]).

However, CID fragmentation of glycopeptides is dominated by the B-type and Y-type fragmented species of glycosidic linkages without or only few detectable peptide fragmentations [18, 93, 94]. A degree of glycosidic and peptide backbone fragmentation by CID depends on the mass analyzer used, as QTOF type instruments normally generate more diversity of fragmented species, product ions, than those of ion-trapped instrument in an single stage of dissociation [95]. SORI-CID [96] and infrared multiproton dissociation (IRMPD) [97, 98] are dissociation techniques, so called slow-heating techniques, most often used in Fourier transform ion cyclotron resonance (FT-ICR) MS. Their feature is similar to CID in which cleavage of glycosidic bonds is preferable rather than peptide backbones [99, 100]. Interestingly, the unique behavior in IRMPD for high-mannose type glycopeptides was reported, which peptide linkages are fragmented effectively with glycosidic bond cleavage [101]. ECD [102] is another dissociation technique used in FT-ICR MS. An advantage of ECD over CID is that post-translational modifications are left intact during ECD, as there is no time for energy randomization, so it is not necessarily that the weakest bonds are cleaved. For glycopeptide, the peptide backbone is preferentially fragmented, while the glycosylation is retained on the backbone with only minor, or without, fragmentation. Therefore, a combination of CID or IRMPD and ECD dissociations is very useful for glycoprotein study including other PTMs, since both peptide sequence and glycan profile can be achieved [18, 93, 94]. Although ECD provides the great amount of information, it is carried out only an FT-ICR MS, the most expensive and therefore one of the least common MS used. The dissociation technique called ETD was than developed [103-104]. ETD gives similar mechanism of fragmentation to ECD, but can be operated in the instruments that are more widely accessible. Fragmentation process of ETD is non-ergodic and so energy randomisation does not occur, which is the same as ECD. This means that as soon as the radical cation is formed, fragmentation occurs at the adjacent bond. As with ECD, PTMs tend to remain intact after peptide fragmentation by ETD, with c and $z^{\cdot}$ ions being predominantly produced [93, 105, 106]. Figure 6 shows CID and ETD MS/MS spectra of the triply protonated ribonuclease B tryptic glycopeptides bearing five molecules of mannose and two molecules of *N*-acetylglucosamine (Man5GlcNAc2) [93]. CID (Fig. 6A) provides intense fragmentation signals from the glycan cleaved at the glycosidic bonds without peptide fragmentation, while ETD (Fig. 6B) generates fragmentation of the peptide without glycan fragmentation observed.

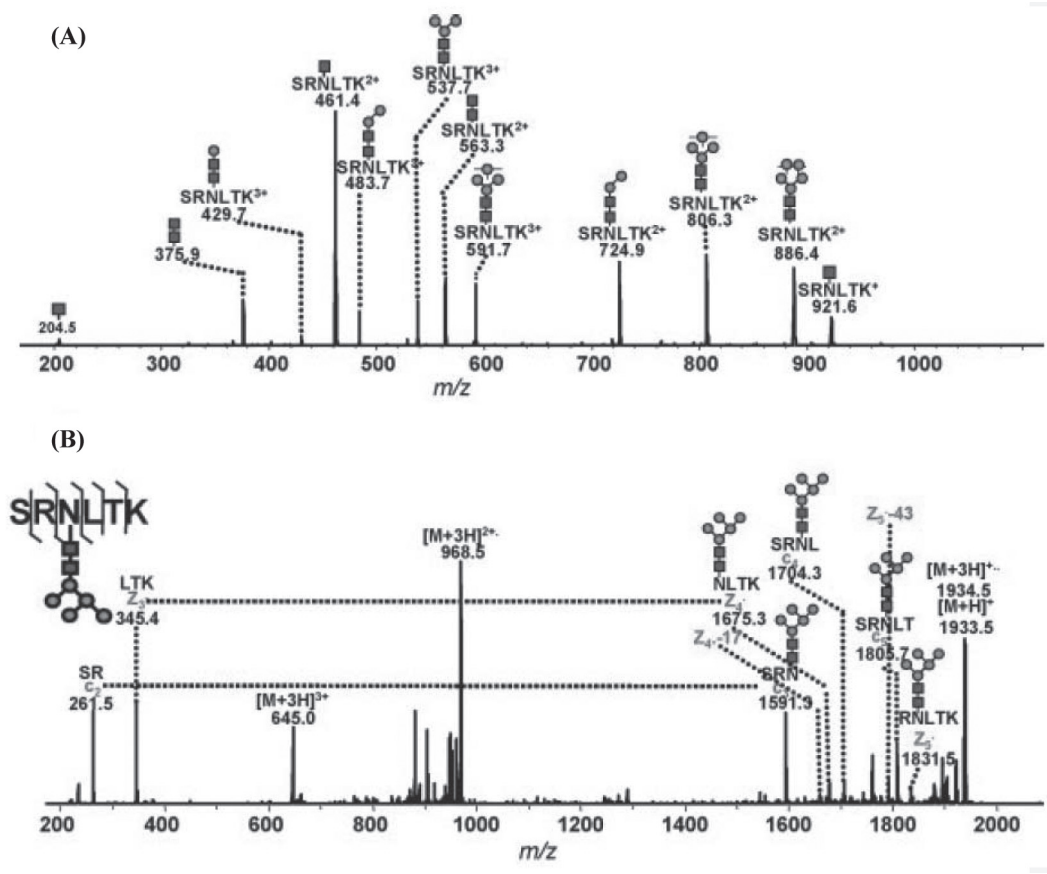

Figure 6 CID (A) and ETD (B) fragmentation spectra of the triply charged Man5 tryptic glycopeptides derived from bovine ribonuclease B. Symbols: square = *N*-acetylglucosamine and circle = mannose [93].

4.4 Databases and bioinformatic tools for glycoprotein analysis

In many cases, unknown glycoproteins have been analyzed. The identification of the protein is based on both the peptide mass and sequence ion data using MS and MS/MS experiments, respectively, which allow the peptides to be matched to the database protein sequence predicted from the known DNA sequence. Genomics and protein databases are very important in protein identification, and there are several databases used for proteomics searches, for example Swiss-Prot, NCBInr, and TrEMBL. Several available search engines such as MASCOT [107] and SEQUEST [108] are then used to identify the protein from which the peptides have originated. These searching programs compare observe MS or MS/MS spectra to predicted spectra for candidate peptides from the database. MASCOT is a probability based search engine which implements the MOlecular Weight SEarch (MOWSE) algorithm [109]. Experimentally obtained peptide masses are compared to calculated peptide masses for each

entry in the protein database, and when a calculated mass value falls within a given range of the experimental mass value, a 'match' is obtained. A statistical weighting is applied to each match, and an overall score is determined. These search engines can be set to consider the modifications, fixed and variable, on peptides. For deglycosylation using PNGase F, Asn is converted to Asp. Therefore, deamidation as a variable modification is monitored in order to allow identification of the sites of *N*-glycosylation, and the possible oxidation of methionine and carbamidomethylation of cysteines is decided as a fixed modification [18]. The databases for glycan characterization [110-114] have been now developing for fully automation assignment as same as proteomics software tools. Although there is no such master tool that complete structural information consisting of composition, sequence, branching, linkage and anomericity, can be achieved now, the databases and bioinformatic tools for glycans are in a great progress [115].

5. Conclusions

A research field of glycoproteomics is not yet mature and growing rapidly. The advances in glycoproteomics have a significant impact on life science, because glycosylation involves many biological processes including mutation and disease progression. Glycoproteomics shows great progresses in terms of analysis development and novel knowledge. In order to achieve as much information as possible from biological samples available in very limited amounts, systematic analysis workflows are needed, and all steps in the workflows remain a significant challenge, which can be more developed. An integration of analytical techniques enables to resolve biological questions effectively. Traditionally, a range of protein isolation approaches has been used, from simple gel visualization to mutidimensional chromatography. Either chemical or enzymatic digestion is then performed to produce (glyco)peptides or to release glycans from glyco-protein or -peptides. Owing to very low abundance of generated glycopeptides or released glycans, enrichment approach is required. In addition, the complexity of the sample can also be reduced. Regarding to an advance in MS technologies in term of both hardware and software, MS has been a main tool for a rage of studies including glycoprotein characterization. Recently, the development of complementary fragmentation techniques in tandem MS provide great information of glycosylation modifications. However, the assignment and interpretation of glycopeptide spectra generated from MS methods are still time-consuming and labor intensive for analytical biochemists. Although proteomics databases for the fully automated assignment of the MS and MS/MS spectra of unknown peptides are widely exploited, the database for the characterization of glycans is still limited with an attempt to make it more available and more popular.

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