NEW APPROACHES FOR QUANTITATIVE ANALYSIS OF GLYCOPEPTIDE USING LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

by

YINING HUANG

(Under the Direction of Ron Orlando)

ABSTRACT

The analysis of protein glycosylation is necessary to improve our understanding of various biological processes, as well as to facilitate correlation of glycan structures with healthy and disease states. In contrast to released glycan analysis, the key attractive feature of glycopeptide-based anlaysis is its ability of characterize the glycan population at each glycosylation site on. This dissertation focuses on the development of new approaches for separating, detecting and quantitating glycopeptides using liquid chromatography (LC)-mass spectrometry (MS).

Chapter 1 is an introduction of glycosylation and its biological significant. Also included are description of the two commonly used approaches for glycomic study: released glycan analysis and glycopeptide analysis.

Chapter 2 evaluates hydrophilic interaction chromatography (HILIC) for the separation of isomeric glycopeptide mixtures. The interaction between HILIC and glycopepitdes permit glycopeptides to be resolved based on differences in their amino acid sequences and/or their attached glycans. The separations of glycans in HILIC is sufficient to permit resolution of

isomeric N-glycan structures, such as sialylated N-glycan isomers differing in α2-3 vs α2-6 linkages while these glycans remain attached to peptides.

Chapter 3 investigates the kinetics of glycan release by PNGase F from glycopeptides of IgGs. LC-selective reaction monitoring (SRM) analysis of the glycopeptides from IgGs revealed that slight differences in amino acid sequences were not found to cause a statistically different deglycosylation rate. However, significant differences in the rate constant associated with this reaction were observed between glycopeptides with differing only in glycan structure (i.e. non-fucosylated, fucosylated, bisecting-GlcNAc, sialylated, etc.).

Chapter 4 investigates the PNGase F catalyzed release of glycans from a variety of standard glycoproteins (Ribonuclease B, Fetuin, and Transferrin) to determine if there are correlation between glycan structure, peptide sequence and the rate of glycan release. The experimental results revealed that significant differences in the deglycosylation rate constants were found between glycopeptides with very different peptides sequences but the same glycan structure, as well as between glycopeptides differing only in glycan structures. We predict the differences in release kinetics can lead to significant quantitative variations.

INDEX WORDS: Mass Spectrometry, Liquid Chromatography, Glycopeptide, Hydrophilic Interaction Liquid Chromatography, Selected Reaction Monitoring, Isomeric Separation, Sialic Acid Linkage, PNGase F, Kinetics

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DEDICATION

This dissertation is dedicated to my grandmother, Meihua Lv, my parents Mingjiu Huang and Nanfei Xu, and all my family members for their unconditional love and support. I love you all.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1. Glycosylation and biological significance

Glycosylation is one of the most important types of protein posttranslational modifications [1]. Glycoprotein are known to involve in many important biological process at both cellular (e.g. cell growth [2], cell-cell adhesion [3], fertilization [4], immune system [5], degradation of blood clots [6], viral replication [7] and inflammation [8]) and protein levels (e.g. protein-protein binding and protein molecular stability). Glycosylation is also known to change with diseases [9-11], such as cancers [115], Alzheimer ’s disease [116], and rheumatoid arthritis [117]. Consequently, the analysis of protein glycosylation is necessary to improve our understanding of various biological processes, as well as to facilitate correlation of glycan structures with healthy and disease states.

Glycosylation is very structurally complex, which introduces great challenges to the analysis of protein glycosylation. Glycosylation of protein can be divided into two O-linked type and N-linked type depend on the glycan attachment site. N-linked glycosylation occur at asparagine residues within Asn-X-Thr/Ser consensus sequence, and O-linked glycan attach to serine or threonine residues [12]. There is no specific sequence required for O-linked glycosylation such as in the case of N-linked glycan. In addition, the glycan structures attach to each potential glycosylation site (N-linked or O-linked) are very heterogeneous. The monosaccharides as shown in table 1.1 that constitute mammalian glycans are including: fucose, galactose (Gal), mannose (Man), N-acetylglucosamine, N-acetylgalactosamine (GlcNAc), and sialic acid (NueAc

and NueGc). The variability in the glycan's monosaccharide sequence order, branching pattern and length result in highly complex glycan's structures. Therefore, the development of methodologies and technologies of glycosylation analysis is very crucial to the success of glycobiology.

Table 1.1 Common monosaccharides in N-linked glycans

Symbol	Abbreviation	Name
▲	Fuc	Fucose
●	Gal	Galactose
■	GlcNAc	N-acetylglucosamine
●	Man	Mannose
◆	NeuAc	N-acetylneuraminic acid
◇	NeuGc	N-glycolylneuraminic acid

This study primarily focused on the analysis of N-linked glycans from mammalian glycoproteins. In N-linked glycosylation, carbohydrates are covalently linked to the asparagine residue in the consensus sequence Asn-X-Thr/Ser, where X is any amino acid except proline [12]. All N-glycans share a common core pentasacchraide consisiting, Manα1-6(Manα1-3) Manβ1-4GlcNAcβ1-4GlcNAcβ1. N-linked glycans are classified into three types: (1) high mannose, in which the core is extended with only additional mannose residues; (2) complex, in which "antennae" are initiated by addition of GlcNAc to the core; and (3) hybrid, in which only mannoses are attached to the Manα1-6 arm of the core and while the Manα1-3 arm contains one

or two antennae that are initiated by the attachment of a GlcNAc[83]. An example of each N-glycan type is illustrated in Figure 1.1.

N-linked glycans are very hydrophilic, and have relatively large molecular weight comparing with other common protein modifications such as phosphorylation. The molecular weights of N-glycans range from 1884 Da, for a high-mannose carbohydrate ($GlcNAc_2Man_5$), to 2851 Da, for a sialylated trianternarry complex saccharide ($GlcNAc_2Man_3GlcNAc_3Gal_3NeuAc_3$).

The N-linked glycan potentially has a strong influence on the structure of protein which it covalently binds to [84-86]. N-glycans mainly affect protein structure in two ways. First, N-glycan can impact or facilitate the protein-folding process. Second, the carbohydrate can stabilize the mature protein [87].

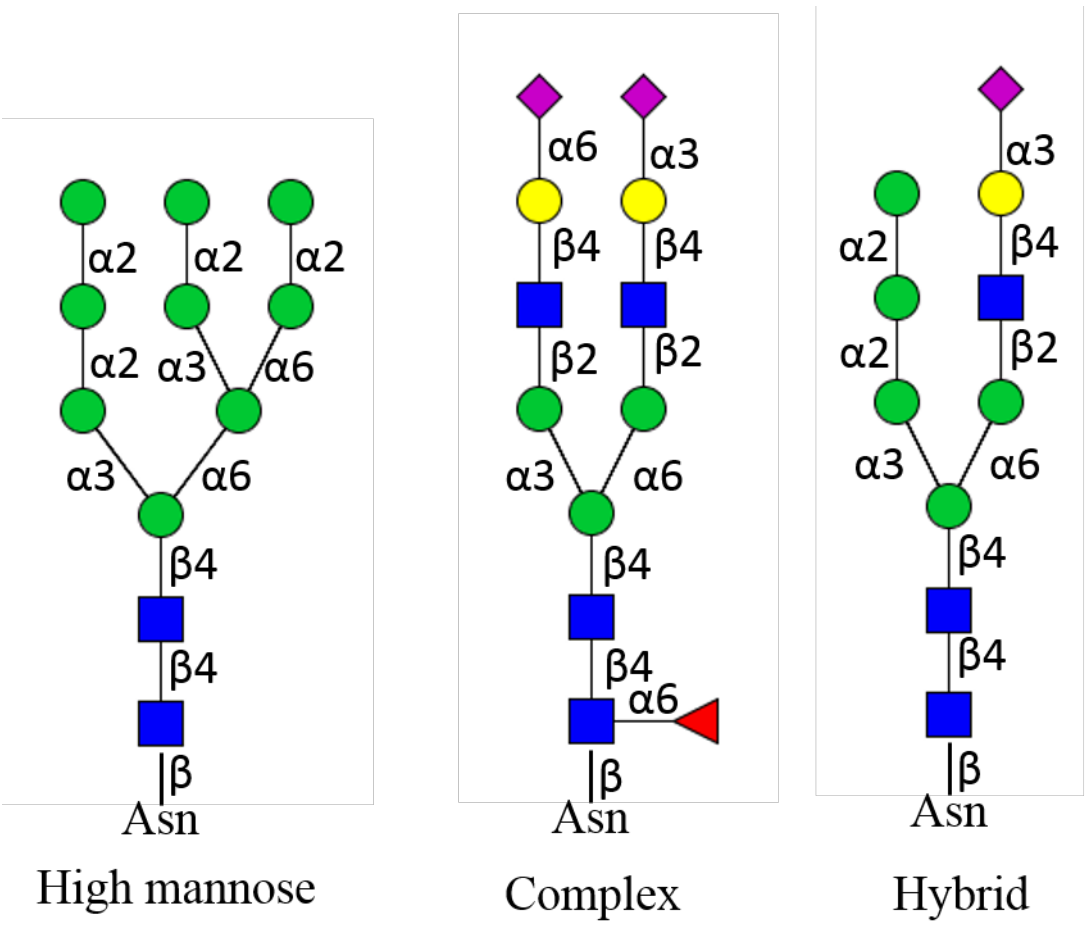


Figure 1.1. Three types of N-linked glycans.

2. Analytical strategies of N-linked glycosylation

2.1. Overall

Liquid chromatography (LC) coupled with mass spectrometry (MS) has been an enabling technology for the analysis of protein glycosylation. MS has developed into a valuable tool for glycan analysis because of its high sensitivity, selectivity, and throughput [13]. LC complements MS analysis since it can separate isomeric glycans/glycopeptides, and thus the combination of

LC/MS offers the ability to characterize and quantitate individual glycoforms in complex mixtures [88].

There are two general approaches to characterize N-glycans using LC/MS: glycoprofiling of liberated glycans or analysis of glycopeptides from glycoprotein. The first approach comprise three steps: the glycans are released from the glycoprotein enzymatically or chemically; secondly the released glycans are usually modified to enhance the ionization efficiency for MS analysis, such as permethylation or reductive amination labeling; thirdly the glycan are analyzed by LC-MS approach [13]. Liberating the glycans before analysis simplifies the analysis; however it causes the loss of information; specifically, information on the sites of glycan attachment and the quantities of each glycan at individual glycosylation sites is lost when the glycans are released when more than one glycosylation site is present in the analyte. A second approach is to analyze the glycans present at each location by resolution of the intact glycopeptides produced by proteolysis of the glycoprotein, without release of the glycans [14-16]. In the following section, a brief introduction and literature review has been done for analysis of released N-linked glycans; a more detailed introduction and literature review has been done for analysis of glycopeptides.

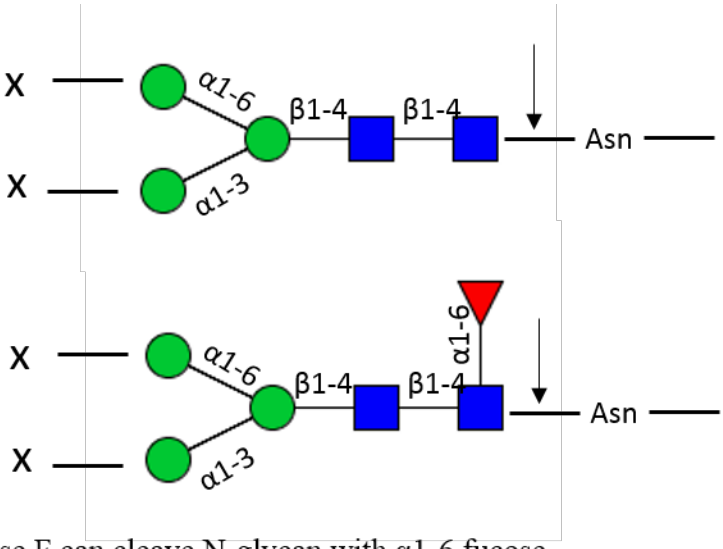
2.2 Analysis of released N-linked glycans

2.2.1 Releasing of N-linked glycans

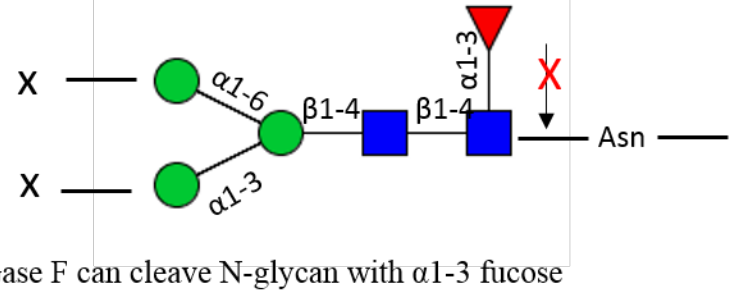
Intact N-glycans can be released from their peptide backbone both chemically and enzymatically. A number of chemical techniques for releasing N-glycans have been developed and utilized for several years but mostly commonly used are hydrazinolysis and alkali/reducing

conditions (β-elimination) [89, 90]. However, due to the problem of the destruction of the peptide backbone and the potential to degrade the oligosaccharides, the chemical hydrolysis release of N-glycans is not as widely used as enzymatically releasing. The most widely used enzyme for releasing intact N-glycans is Peptide N-glycosidase F (PNGase F). PNGase Fcleaves the linkage between the asparagines residue and innermost GlcNAc of N-linked glycans as illustrated in Figure 1.2, except those containing a 3-linked fucose attached to the reducing terminal GlcNAc residue [17]. However, α 1-3 linked fucose usually only exist in plants, and peptide N-Glycosidase A (PNGase A) can be used to release those glycans with 1, 3-linked core fucose residues [18]. In contrast to PNGase F, which can cleave N-glycans from both glycoprotein and glycopeptides, PNGase A requires glycopeptides as substrates [18]. Endoglycosidases that cleave the glyosidic bond between the GlcNAcs residues within the chitobiose core can also be used. These endoglycosidase leave the innermost GlcNAc attach to the asparagine, which enables the identification of glycosylation site after releasing glycans [93]. However, the endoglycosidase that cleave between the chitobiose core are not as general as PNGase F, meaning that each enzyme only releases a subset of N-linked glycans. Endoglycosidase H (Endo H), which is probably the most widely used endoglycosidase, only releases high-mannose type and most of the hybrid type of N-glycans [92]. Endoglycosidase F1, F_2 and F_3 (Endo F1, F2, F3) are more suitable to release N-glycans from non-denatured proteins, which are less sensitive to protein conformation than PNGase F [94]. Endo F1 has specificity to cleave asparagine-linked or free oligomannose and hybrid type N-glycans, but not complex. The rate of releasing by Endo F1 will be reduce to more than 50-fold when core fucose exist in hybrid structures [94]. Endo F2 has specificity to release asparagine-linked or free oligomannose, and biantennary complex type N-glycans [94]. However, the Endo F2 releasing rate of

oligomannose structures are 20-fold slower than biantennary complex type N-glycans. Asparagine-linked biantennary and triantennary complex type N-glycans can be cleaved by Endo F3 [94]. Therefore, each type of releasing technique has its own merits, and the choice of technique will depend on the nature of the sample and the purpose of the study.



PNGase F can cleave N-glycan with α1-6 fucose



PNGase F can cleave N-glycan with α1-3 fucose

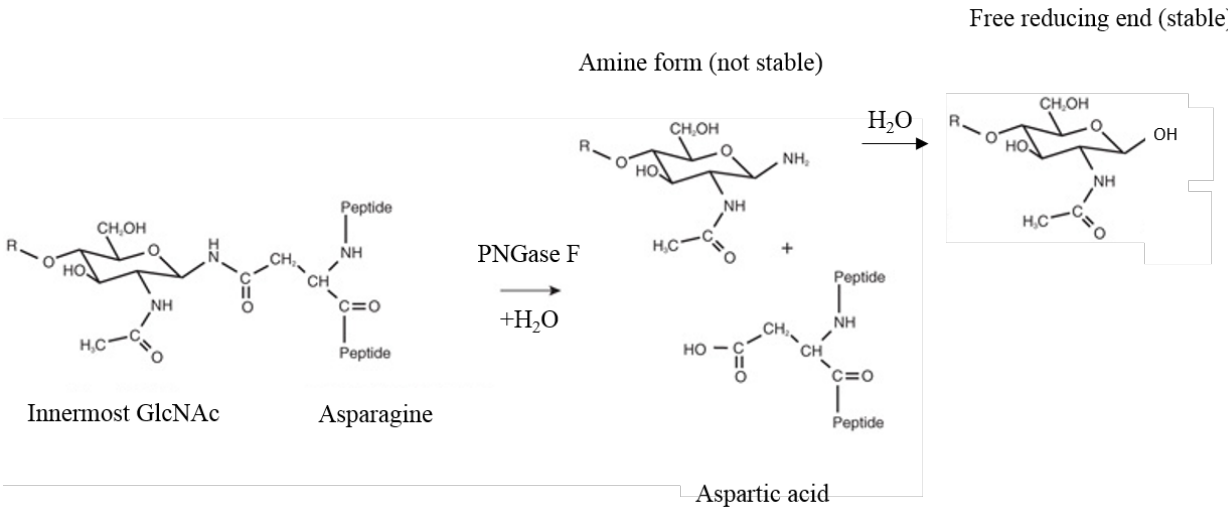


Figure 1.2. PNGase F releasing of N-linked glycans

2.2.2 Derivatization of N-linked Glycans

Released glycans are usually derivatized to make them more amenable for analysis. Two most commonly used derivatization methods for released N-glycans are permethylation and reductive amination.

One common derivatization process is permethylation of glycan, which replaces the hydrogen atom of hydroxyl and amide groups to methyl groups as illustrated in Figure 1.3 [19]. After permethylation, the glycans are more stable than native glycans which minimize the in-source or post-source decay of the labile sialic acid residues in acidic oligosaccharides. Permethylation also make the glycan more hydrophobic which enhance the ionization efficiency for ESI and MALDI [20]. Permethylated glycans are changed to be neural, so both natural and acidic glycans can be detected under positive mode [19, 20]. For structure characterization, permethlyated glycans produce more informative tandem mass spectra. Except the glyosidic

bond all other hydroxyl groups are permethylated, thus cleavage during tandem MS gives underivatized sites (glyosidic bond) as "scars", which reflects the linkage positions. The tandem mass spectra of permethylated glycans have a higher abundance of cross-ring fragments, which allows a higher probability of structure assignment for branching and linkage isomers [21-23]. Permethylation was used to think not compatible with some glycan modification groups such as sulfate, but some works have been done to make permetlylation work on sulfated glycans [118]. However, there're also some concerns about permethylation. The conversion of permethylation is hard to achieve 100% yield and variable permethylation efficiency toward different glycan structures [134]. Sample losses also occur during the derivatization workflow, which usually makes it incompatible for small-scale samples. However, solid-phase permethylation has been developed by Mechref's group, which was applied to deal with small-scale samples [119]. Overall, permethylation is an important derivatization method for released glycan, which makes native glycan more amenable for MS analysis.

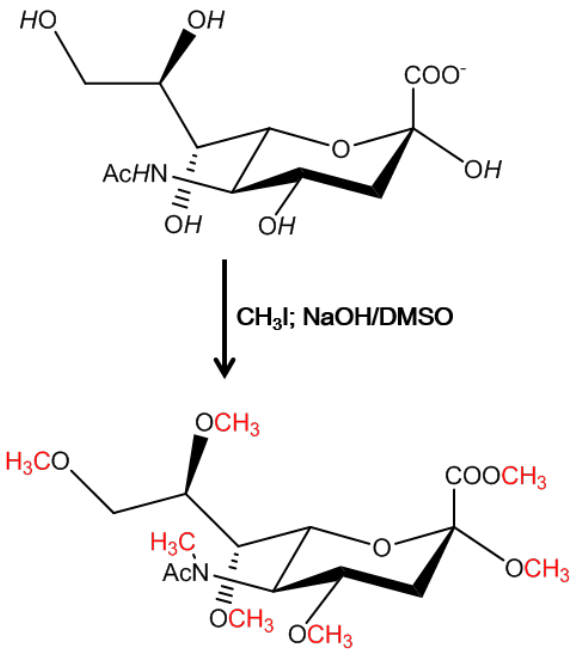


Figure 1.3. Permethylation of oligosacchraides

Reductive amination is another widely-used derivatization method for reducing glycans. As shown in Figure 1.4, the open-ring aldehyde react with a primary amine to form a glycosyl imine (also known as a Schiff base), and then imine is reduced to a secondary amine to increase its stability [24]. The advantages of reductive amination is that the reaction is reliable and can be used to attach a chromophore, or fluorophore to a single position of a glycan, which make the glycans are detectable with UV or fluorescence [25, 26]. For mass spectrometric detection, reductively aminated glycans with hydrophobic groups produce significantly stronger signals than native glycans [27]. Various amines have been applied to labeling glycans via the reaction of reductive amination, such as 2-aminobenzamide (2-AB) [120], 2-aminobenzoic acid (2-AA) [121], 2-aminopyridine (PA) [122], 2-aminonaphthalene trisulfonic acid (ANTS) [123], 1-aminopyrene-3,6,8-trisulfonic acid (APTS) [124] and procainamide (PA) [30]. Among those, the 2-AB labeling has been studied most thoroughly and comprehensive databases have been developed for 2-AB labeled glycans in HPLC separation [38].

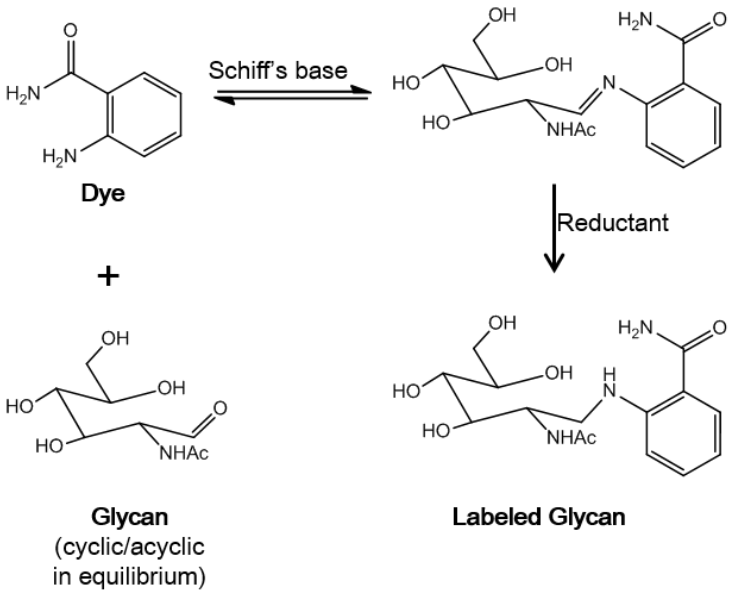


Figure 1.4. Reductive animation of oligosaccride

2.2.3 On-line LC separation of released N-linked glycans

The LC separation of glycans is usually utilized prior to MS analysis due to the chromatographic enrichment of low abundant glycoforms to significantly reduce the ionization suppression of other present ions. Moreover, the LC separation offers greatly potential for isomeric structure characterization and quantitation. Various LC strategies have demonstrated the ability to analyze either native or derivatized glycans, including porous graphitized carbon (PGC) chromatography [28], reversed-phase (RP) chromatography [29], hydrophilic interaction chromatography (HILIC) [30], and anion-exchange (AE) chromatography [105].

The outstanding separation ability of PGC on non-derivatized glycans was reported about 20 years ago [31]. The separation mechanism of PGC is relatively complex comparing with RP and HILIC. It is accepted that the separation on PGC stationary phase is driven by the co-function of hydrophobic, polar and ionic interaction. PGC-LC has high shape-discrimination capability toward isomeric and isobaric glycan structures. It was applied to successfully separated isomers of high- mannose glycans from RNAse B [33, 34], isomers of core/antenna fucosylated glycans [34] and α2, 3 and α2, 6 linkage isomers of sialyated glycans [35]. Due to the high structure-discrimination capability of PGC-LC, a structure- retention time correlation system based on reference glycans has established [32]. The nature of the organic modifier, the column temperature and redox state of PGC materials were all indicated as important parameters impacting retention of oligosaccharides [95, 96]. Although PGC showed remarkable separation power towards isomeric structures of glycan, there're also some concerns about the performance of PGC. As their ability to strongly adsorb a variety of substances may lead to increasing back pressure and unstable retention times [97], hence the PGC columns are suggested to be washed

by backflush regularly. The relatively complex retention mechanism of PGC also makes the retention time less predictable [98].

RP is usually used to separate derivatized glycans. Stationary phase of RP can be any inert non-polar substance that archives sufficient packing. The most popular reverse stationary phase is an octadecyl chain (C-18)-bounded silica [99]. The mobile phase of RP is mixture of aqueous buffer and organic solvents, where the organic solvents must be miscible with water. The most common organic solvents are methanol, acetonitrile and 2-propanaol. RP-LC has been applied to separate some bio-molecules with hydrophobic characters such as proteins, peptides and nucleic acid. However, native glycans are very hydrophilic molecules, so native glycans can hardly adsorb to RP phase. RP-LC become a sensible option only with derivatized glycans, such as reductive animated glycans and permethylated glycans. The separation of permethylated glycans by C18 column was firstly conducted by Delaney and Vouros [100]. More recently, Novotny and Mechref have developed the method that apply chip-based RP microfluidic liquid chromatography to separate permethylated N-glycans for the discovery of cancer biomarkers [101]. The 2-AB labeled N-glycans from recombinant immunoglobulin G were separated and analyzed by RP-LC-MS by Chen and Flynn [102]. The increasing of temperature was found to improve the performance of C18 separation of derivatized glycans [103]. The mobile phase of RP-LC with high concentration of organic solvent makes it very compatible with MS analysis, but its ability for isomer separation may need further evidence.

HILIC separations of released N-glycans are developing rapidly. It has been widely accepted that the mechanism of HILIC separation involve a portioning of analytes between the organic mobile phase and a water layer adsorbed on the HILIC phase in contrast to the traditional adsorption chromatography on normal phase materials [36]. The most commonly used mobile-

phases for HILIC are acetonitrile (ACN) and water. The pH and ionic strength the mobile phase is usually modified by adding volatile additives such as ammonium acetate and ammonium formate. In general, the retention of glycan on HILIC material depends on the number of polar groups, which leads to highly predictable and intuitively elution patterns [37]. With the HILIC separation, a database approach for analysis of the structures of oligosaccharids with 2-AB labeled glycans has been established [38], which is a mass-retention time matching system based on standard glycans. The ability of HILIC to separate structural isomers makes HILIC a valuable tool for structure analysis of oligosaccharides. For instance, α2-3/2-6 sialic acid (SA) linkage isomers of N-glycans can be resolved by zwitterionic type of HILIC [104] or HILIC [30].

AE chromatography separates analytes based on their charges using an ion-exchange resin containing positively charged groups. In solution, the positively charged resin will interact with negatively charged counter-ions (anions). The separation order of analytes on AE column is determined by the strength of the negative charge of the analytes. Anion exchange chromatography has been widely used to separate native glycans at high pH levels [105-109]. Hardy and Townsend firstly used 22mM NaOH as column effluent to separate underivatized monosaccharides such as fucose, galatosamine, galacose, glucose and mannose by AE chromatography followed by pulsed amperometric detection [106]. Later on, Hermentin and Witzel developed a strategy for mapping N-glycans using high-pH AE chromatography with pulsed amperometric detection [105]. Since mobile phase of AE chromatography has high concentration of salts, the most commonly used detector coupled with AE is pulsed amperometric detection rather than mass spectrometer. In order to use mass spectrometer, a desalter is usually applied between AE-LC and mass spectrometer. For example, oligosaccharide analysis by capillary-scale high-pH AE chromatography with on-line ion-trap mass spectrometry

(IT-MS) was conducted by Bruggink and Wuhrer [109]. In their study, the routine separation of isomeric oligosaccharides was accomplish by high –pH AE chromatography coupled with IT-MS. The information on monosaccharide sequence and linkage positions of isomeric glycoform is characterized by MS/MS fragmentation using the IT-MS [109].

2.3. Analysis of glcopeptides

2.3.1 Overall

In contrast to released glycan analysis, the key attractive feature of glycopeptide-based anlaysis is its ability of link glycosylation information to exact locations (glycosylation site) on glycoproteins. Often the identification of the site of glycan attachment is critical for biological activities. For instance with therapeutic antibodies, the glycan located in the variable domain influence the serum clearance rate while glycans in constant domain affect the activity [110].

2.3.2 Digestion strategy of glycopeptides

A digestion strategy should be designed before the glycopeptides analysis. Several proteolysis enzymes were utilized including trypsin chymotrypsin [64], proteinase K [65], and Glu-C [66]. Trypsin is commonly used as the only enzyme in most cases. Peptide chains are cleaved by trypsin at the C-terminal to arginine or lysine residue, except when either is followed by proline [135]. However, sometimes when the size of tryptic glycopeptide is too large or multiple glycosylation sites are present at a single tryptic glycopeptide sequence, pairing trypsin with a second enzyme is necessary to cut glycopeptide into smaller pieces or into peptide with single glycosylation site. For instance, using Glu-C with trypsin together to digest EPO made the digested glycopeptides of EPO are easier to be analyzed [66], where Glu-C selectively cleaves

peptide chain at the carboxyl side of glutamic acid residues [136]. In conclusion, the digestion strategy have to be designed by the nature of glycoprotein that to be analyzed.

2.3.3 Sample enrichment of glycopeptides

Off-line enrichment of glycopeptides is usually necessary prior to LC-MS anlysis. Since glycopeptides present at relatively low concentration comparing to non-glycosylated peptides, in order to reduce the ion suppression effect and enhancing the MS signal of glycopeptides, removal of any non-glycosylated peptides is beneficial [71-74]. On 2004, the group of Wada et al. firstly demonstrated a simple enrichment approach of glycopeptides using Sepharose beads [72]. Glycopeptides was enriched by affinity separation that partition with cellulose or Sepharose [72]. More recently, a strategy that using HILIC stationary phase has been described [75]. Glycopeptides from Fetuin are enriched by magnetic bead-based zwitterionic HILIC (ZIC-HILIC) with high recovery yield (95-100%). Size exclusion chromatography [125] and boronate affinity monolith [126] also have been reported to successfully provide a significant enrichment of N-linked glycopeptides.

2.3.4 LC separation of glycopeptides

Various on-line LC strategies have demonstrated the ability to separate glycopeptides, including reversed-phase (RP) chromatography [77-79], hydrophilic interaction chromatography (HILIC) [104], porous graphitized carbon (PGC) chromatography [81]

RP-LC/MS is one of the most widely utilized methods to separate and analyze glycopeptides [77-79]. With RP-LC, retention of glycopeptides is predominantly driven by the hydrophobic character of the peptide portion, and as a result, glycans attached to peptides with different sequences typically elute at different times. However, because the glycan does not

interact with the RP-LC stationary phase, the glycans have a very limited effect on the separation, and thus all glycopeptides with the same peptide backbone are poorly resolved. Most of the glycofroms containing the same peptide sequence co-elute. This co-elution may benefit the data analysis, because it's easier to find all the glycoforms at a given glycosylation site are. The disadvantage of co-elution is the ion suppression caused by that abundant glycofroms suppress the signal of less abundant glycoforms. Therefore, RP usually has limited power to resolve different glycoforms of glycopeptides.

HILIC is a separation strategy that enable to separate glycopeptides depend on both peptide sequence and glycan structures. The hydrophilic moieties on both the glycan and the peptide will interact with the hydrophilic stationary phase of HILIC. Thus changes to either of these moieties can alter the chromatographic behavior of a glycopeptide. Moreover, the retention of glycopeptides on HILIC column depends on a variety of glycan features such as size, linkage, charge composition, and branching [80], which makes HILIC capable of resolving isomeric glycoforms on glycpeptides.

PGC is another useful strategy that could be employed to separate glycopeptide. The separation mechanism of PGC is relatively complex comparing with RP and HILIC. It is accepted that the separation on PGC stationary phase is driven by the co-function of hydrophobic, polar and ionic interaction. Therefore, on contrast to RP, both the carbohydrate moiety and amino acid chain of glycopeptides influence on the retention of PGC. The group of Fan et al. firstly utilized PGC to separate glycopeptides in applying acetonitrile-water and NH_4OH [127]. In addition, PGC was also proved to have the ability to separate isomeric glycoforms of glycopeptides. Lebrilla and co-workers [81] separated isomeric glycofroms of glycopeptides from RNase B by PGC. In this study, glycopeptides of RNase B that typically

shows 5 different compositions has been resolved to 13 peaks. However, due to the complex separation mechanism of PGC, the retention times of glycopeptides on PGC are less predictable than RP and HILIC.

2.4. Mass spectrometric analysis of glycomics

In the following sections, mass spectrometric analysis is discussed generally for both released N-glycan and glycopeptide.

2.4.1. Ionization Method

In order to conduct mass spectrometric analysis, the first basic decision is about the choice of ionization methods of matrix-assisted laser desorption/ionization (MALDI) or electrospray ionization (ESI).

MALID is a soft ionization technique used in mass spectrometry. By using MALDI, first the sample of analyte is mixed with a UV-absorbing weak organic acid matrix compound such as 2, 5-dihydroxybenzoic acid. A small volume of the mixture (1–2 μl) of analyte and matrix is dried on a metal plate, allowing crystals to form. Second the matrix, which carries the analyte with it, is irradiated by a pulsed laser. The matrix absorbs the most of the laser energy, which is usually in the UV range, and transfers the energy to the analyte. Finally, the matrix acts as a proton donor or receptor to ionize the analyte into either positive or negative ions, and can then analyzed by mass spectrometer[128, 129]. MALDI is a highly suitable method to analyze neutral and even negatively charged glycans [39-43]. It appears that stachyose, a phytochemical terasaccharide was the first carbohydrate examined by MALDI [130]. Profiling permethylated glycans by MALDI is intensively used as analysis method of released glycans [44-49]. Since sialylated, sulfated, or phosphorylated carbohydrates possibly undergo in-source fragmentation during MALDI ionization, permethylation can greatly protect glycans from fragmentation during

the MALDI process. In addition, MALDI predominantly produces singly charged ions, which significantly simplified the interpretation of spectrum. Since MALDI is usually not very compatible with on-line LC separation, the major drawback of MALDI is that the sample should be carefully purified before the MS analysis.

Another soft ionization technique is ESI, which ions are produced by applying high voltage to electrospray and result in an aerosol. In the ionization process of ESI, first a solution containing the analyte is sprayed through a needle, to where the electronic potential is applied. Very fine charged droplets are formed by the electronic potential. Charged droplets move toward the mass spectrometer orifice while undergoing solvent evaporation. With the evaporation of solvent, the charged droplets are shrunk and become unstable due to high charge density. Eventually, the droplets reach Rayleigh limit to break apart and form Coulomb fission [50-52]. Since a lot of solvent evaporations involved in the ionization process of ESI, samples are usually dissolved in the solvent of water mixing with volatile organic solvent such as methanol and acetonitrile [51]. Acids such as acetic acid and formic acid are usually added to the solution to provide the sources of protons [51]. For large-flow electrospray, a heated inert gas such as nitrogen can be applied to the ESI source to aid the declustering and evaporation process of ionization [131], In contrast to MALDI, ESI generates multiple charged gas phase ions [53], which makes high molecular ions possible to be detected at relatively low mass to charge ratio range. ESI is compatible with many types of mass spectrometers and is particularly suitable with online liquid chromatography.

2.4.2. Mass analyzers

Mass analyzer is used to separate ions based their mass to charger (m/z) ratios though electric or magnetic field. The most relevant features of a mass analyzer are sensitivity, mass accuracy,

resolution, speed of data acquisition and the possibility of performing single or even multi-stage MS/MS. Resolution of mass analyzers is calculated by dividing the peak m/z value by the peak width at half its height (FWHM). For example, the resolution of a peak at m/z 400 and 1m/z wide at FWHM yields is 400. There are several different types of mass analyzers; most widely used are quadrupole, time-of-flight (TOF), ion trap and orbitrap. Each mass analyzer has its own advantages and limitations. Choosing proper mass analyzer in different projects should be based on the individual application purpose.

2.4.2.1.Quadrupole mass analyzer

Quadrupole mass analyzer can selectively stabilize the ions' pathways by changing the radio frequency field between four parallel metal rods [111,132] as illustrated in Figure 1.5. For those four parallel metal rods, one rod is connected with the other rod in the opposing direction electrically. A hyperbolic field is created between four parallel metal rods by applying a RF voltage with direct current (DC) voltage. Under particular ratio of RF and DC voltage, only ions with certain m/z value can obtain a stable trajectory long the center of the field, transmitted through the hyperbolic field and reach to the detector; other ions with unstable trajectory will collide on the rods. The operation of a quadrupole mass analyzer is usually illustrated in terms of stability diagram. For a given mass m, a qualitative representation of stability diagram is shown in Figure 1.6. The "a" (y-axis) equals to kU/m; and "q" (x-axis) equals to kV/m. The triangle area in the diagram is stable region. A san lines with certain slope are plotted in the diagram. Changing the slope of the scan line will change the resolution. The scan line with steeper slope has less overlap (the region at the apex region that is bounded by the scan line) with stable region, which results in smaller number of ions that reach the detector and higher resolution; whereas the scan line with smaller slope lead to lower resolution. The principal of

operation of quadruple allows it work as a mass filter with a particular ratio of RF/DC to selectively scan ions of certain m/z value, or permits it work at full scan mode by continuously varying the applied voltage ratio of RF/DC to scan a range of m/z values. Since quadrupole is a scanning instrument, the detector of it must capable of detecting ions one m/z at a time. Therefore, the signal of quadrupole has a linear dynamic range, which makes quadrupole an ideal instrument to do quantitation.

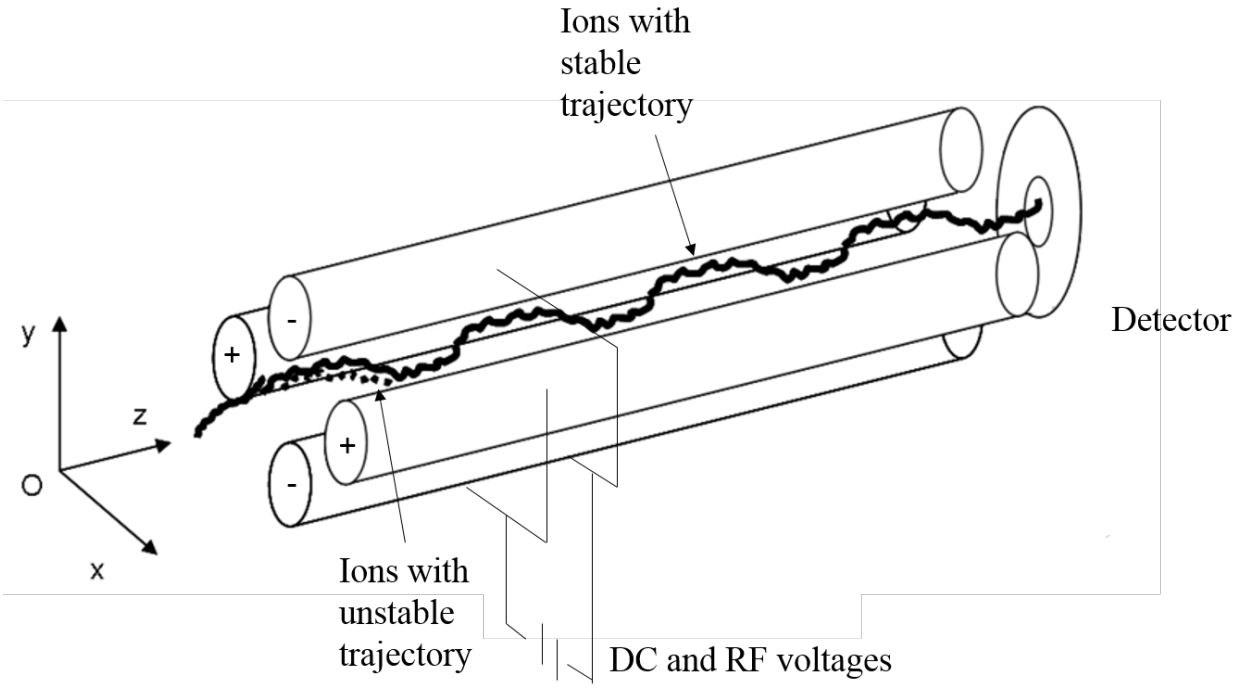


Figure 1.5. Diagram of quadrupole mass analyzer

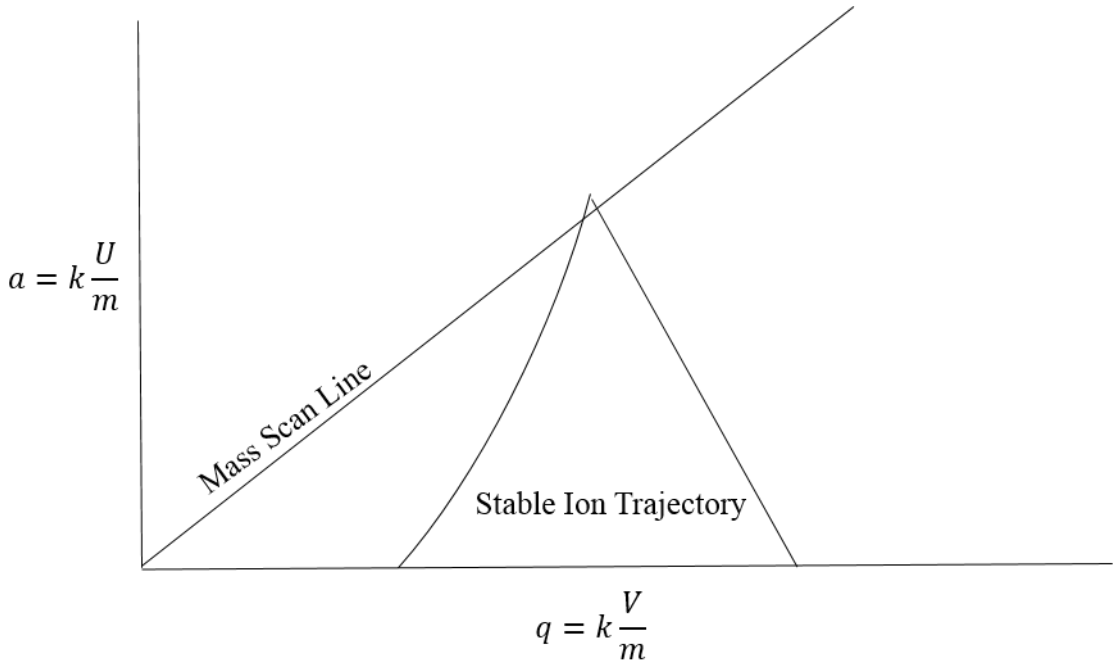


Figure 1.6. Stability diagram of quadrupole

2.4.2.2. Linear ion trap (LIT) mass analyzer

The linear ion trap consists with a set of quadrupole rods and two end electrodes. The set of quadrupole produce a two-dimensional RF field, which confines ions radially. The static electrical potential applied on the two end electrodes traps ions axially. The resonance frequency of ions can match the resonance applied to the trap by change the RF and DC voltage at the electrodes, where ions will be excited, become unstable and ejected out to the detector [113]. Comparing with 3D trap, LIT usually has higher trapping efficiency, trap more ions and has strong focusing of ions long the centerline. [133]. Moreover, LIT is not only able to operate as stand-alone mass spectrometers, but also can be combined with other mass analyzers into hybrid instruments. They can be used to isolate ions of selected mass to charge ratios, and to perform tandem mass spectrometry experiments [114].

2.4.2.3.Time-of flight (TOF) mass analyzer

TOF mass spectrometry is a method that the ion's m/z value is determined by measuring the flight time. Ions are accelerated by a fixed strength of electric field (2-25 kV). This acceleration results in that all the ions obtain the same kinetic energy, since all the ions travel through the same distance by the same force. The kinetic energy associates with any mass is expressed as following the equation: $KE=1/2\ m/v^2$. The potential energy of a charged particle in an electric field is related to the charge of the particle (z) and to the strength of the electronic field (U) as PE=zU. When the potential energy is converted to kinetic energy, then the equation expressed as $KE=PE=1/2\ m/v^2=zU$, which shows that the square-root of its m/z value is inversely proportional to velocity of an ion. Therefore, larger m/z ions need more time to fly to the detector. The traditional TOF has a relatively low resolution of only around 500 units [54]. With the developing of technologies, there are two major techniques largely increase the TOF's resolution. The first one is "Delayed Extraction" [55]. Delayed extraction, which refers to an operation mode that the applied accelerating voltage is postponed some short time (200-500 ns) delay after the ionization event, is usually used with MALDI or laser desorption/ionization (LDI) ion sources. Ions with greater initial kinetic energy result in a higher velocity. They are closer to the extraction electrode before the accelerating voltage is applied. After a certain time, the delayed extraction pulse is added to compensate for the spread in kinetic energies. Finally, the ions with the same *m/z* will reach the detector at the same time. The resolution can also be improved using a reflectron [56, 57], where the kinetic energy distribution in the direction of ion flight can be corrected. A reflectron, as shown in Figure 1.6, uses an electrostatic “mirror” which reflects the ions towards the detector. The ions with higher initial kinetic energy penetrate deeper into the reflectron, while lower kinetic ions of the same m/z travel a shorter distance. The result

is that ions of same *m/z* but with slightly different initial kinetic energies will arrive at the detector at the same time. With those technologies, TOF instrument currently can achieve a resolving power of more than 10,000 units.

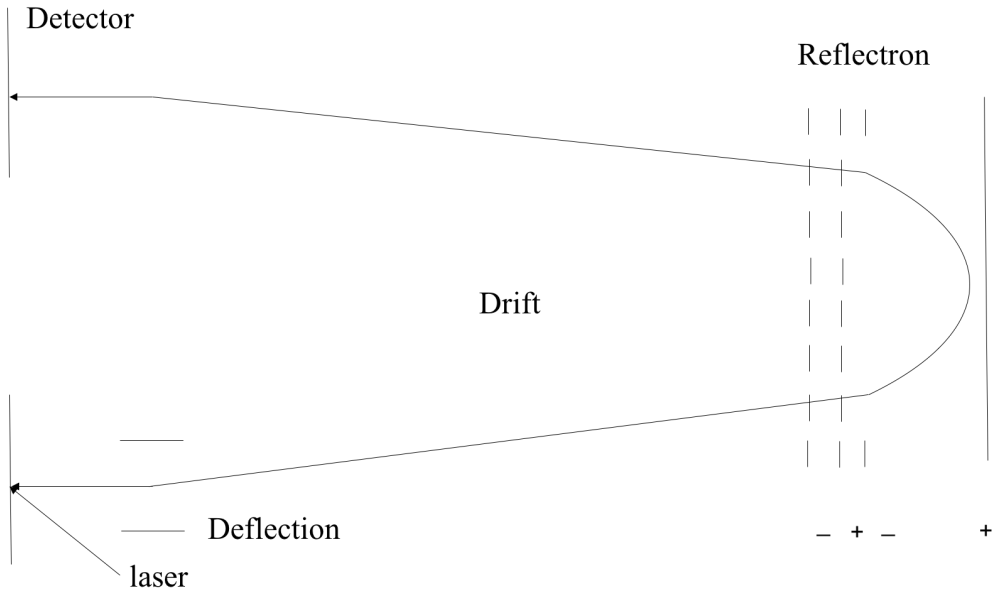


Figure1.6. The diagram of a reflection TOF-MS with a laser ionization source

2.4.2.4.Triple Quadrupole (3Q)

The combination of triple quadrupole MS and LIT technology is known as Q-Trap (AB Sciex). Various operation modes can be conducted by Q-Trap. As the system is based on a triple quadruple platform as illustrated in Figure 1.7 where Q3 can be operated either in the normal RF/DC mode or in the LIT mode [58], it retains the classical triple quadrupole scan functions such as selected reaction monitoring (SRM), neutral loss (NL), and precursor ion scan, as well as providing access to sensitive ion trap experiments [59].

Selected reaction monitoring (SRM), also called multiple reaction monitoring (MRM), is a principle technique that is utilized to detect and quantitate analytes in complex mixtures with

excellent specificity and sensitivity [60]. SRM mode employs a two-stages mass filtering strategy. At the first stage, instead of obtaining full scan of precursor ions, where all the precursor ions are analyzed in Q1, only ions of interest (precursor ions) are selectively scanned in quadrupole 1 (Q1). Then, the precursor ions are induced to fragments by collisional excitation in the collision cell (Q2). At the second stage, only a small number of specific transition ions (fragment ions) are selectively analyzed in Q3. The selective-analyzing characteristic of SRM mode greatly enhances the lower detection limit for target analysts by one or two orders of magnitude compared with conventional "full scan" techniques. Moreover, it results in a linear response over a wide dynamic range up to five orders of magnitude, which is very important for quantitative studies with low abundant analytes in highly complex mixtures. Overall, selectivity, sensitivity, dynamic range and adaptation to the sample complexity make SRM technique a promising candidate for quantitative glycomics.

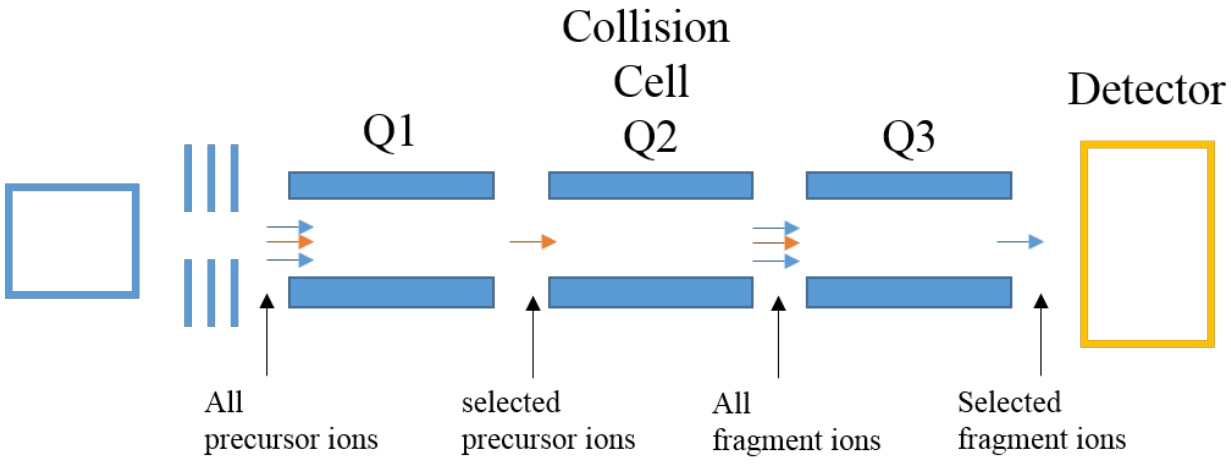


Figure 1.7. The diagram of triple quadrupole and selected monitoring mode

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CHAPTER 2

RESOLVING ISOMERIC GLYCOPEPTIDE GLYCOFORMS WITH HYDROPHILIC INTERACTION CHROMATOGRAPHY (HILIC)

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Abstract

The ability to resolve glycans while attached to tryptic peptides would greatly facilitate glycoproteomics since this would enable site specific glycan characterization. Peptide/glycopeptide separations are typically performed using reversed phase liquid chromatography (RPLC) where retention is driven by hydrophobic interaction. Because the hydrophilic glycans do not interact significantly with the RPLC stationary phase, it is difficult to resolve glycopeptides that differ only in their glycan structure, even when these differences are large. Alternatively, glycans interact extensively with the stationary phases used in hydrophilic interaction chromatography (HILIC), and consequently, differences in glycan structure have profound chromatographic shifts in this chromatographic mode. Here, we evaluate HILIC for the separation of isomeric glycopeptide mixtures, which have the same peptide backbone but isomeric glycans. Hydrophilic functional groups on both the peptide and the glycan interact with the HILIC stationary phase, and thus changes to either of these moieties can alter the chromatographic behavior of a glycopeptide. The interactive processes permit glycopeptides to be resolved from each other based on differences in their amino acid sequences and/or their attached glycans. The separations of glycans in HILIC is sufficient to permit resolution of isomeric N-glycan structures, such as sialylated N-glycan isomers differing in α2-3 and α2-6 linkages while these glycans remain attached to peptides.

1. Introduction

Protein glycosylation is a common post-translational modification which is known to affect many cellular processes such as cell adhesion, receptor activation, and signal transduction [1-5]. Alteration of glycosylation is associated with various human pathologies, such as cancers [6], Alzheimer 's disease [7], and rheumatoid arthritis [8]. Consequently, the analysis of protein glycosylation is necessary to improve our understanding of various biological processes, as well as to facilitate correlation of glycan structures with healthy and disease states.

Liquid chromatography (LC) coupled with mass spectrometry (MS) has been an enabling technology for the analysis of protein glycosylation. MS has developed into a valuable tool for glycan analysis because of its high sensitivity, selectivity, and throughput [9]. LC complements MS analysis since it can separate isomeric glycans/glycopeptides, and thus the combination of LC/MS offers the ability to characterize and quantitate individual glycoforms in complex mixtures.

There are two general approaches to characterize glycans using LC/MS. In the first of these, the glycans are released from the glycoprotein enzymatically or chemically before LC/MS analysis [10]. Various LC strategies have demonstrated the ability to analyze either native or derivatized glycans, including porous graphitized carbon (PGC) [11], reversed-phase (RP) [12] and hydrophilic interaction (HILIC) [13]. Such techniques have been utilized to accomplish separation, identification, quantification and occasionally isomeric structure analysis of glycans [13, 14]. For instance, α2-3/2-6 sialic acid (SA) linkage isomers of N-glycans can be resolved by HILIC [13]. The solvents/buffers used in each of these separation approaches are MS compatible, and thus, all of these have been used for LC/MS analysis of glycans.

Liberating the glycans before analysis simplifies the analysis; however it causes the loss of information; specifically, information on the sites of glycan attachment and the quantities of each glycan at individual glycosylation sites is lost when the glycans are released when more than one glycosylation site is present in the analyte. A second approach is to analyze the glycans present at each location by resolution of the intact glycopeptides produced by proteolysis of the glycoprotein, without release of the glycans [15-17]. When examining glycopeptides, the peptide sequence analysis can enable identification of the site of glycan attachment. Often site occupancy by the relevant glycan is critical for biological activity. For instance, with therapeutic antibodies, the glycans located in the variable domain region influence the serum clearance rate, while glycans in the constant domain region affect the activity [18]. Consequently, the comprehensive characterization of protein glycosylation microheterogeneity -- the identification and quantitation of isomeric glycan occupancy at potentially all glycosylation sites on a protein -- is a significantly more difficult challenge than glycomic profiling and requires the development of new approaches to overcome these analytical challenges.

RPLC/MS is one of the most widely utilized methods to separate and analyze glycopeptides [19-21]. With RPLC, retention of glycopeptides is predominantly driven by the hydrophobic character of the peptide portion, and as a result, glycans attached to peptides with different sequences typically elute at different times. However, because the glycan does not interact with the RPLC stationary phase, the glycans have a very limited effect on the separation, and thus all glycopeptides with the same peptide backbone are poorly resolved. Consequently, RPLC does not separate isomeric glycans attached to the same glycosylation sites, and offers limited selectivity, even for significant differences in glycan composition.

HILIC is a commonly used approach for the analysis of released glycans and is capable of resolving released glycans. This ability led us to investigate if HILIC would be capable resolving the glycoforms of a glycopeptide. In this manuscript, we demonstrate that HILIC is capable of resolving isomeric glycoforms of glycopeptides and that the separations obtained on glycopeptides are similar to those obtained by HILIC of released glycans, with high separation selectivity differences based on composition, and even on branch position, for complex glycans. Additionally, because the hydrophilic amino acid side chains of the glycopeptide also interact with the stationary phase, glycopeptides with different peptide sequences can also be separated. The ability to separate glycopeptides based on both the glycan and the peptide makes HILIC a useful tool in the glycoproteomic toolbox.

2. Experiment Section:

2.1. Materials:

Fetuin, human serum, trypsin (TPCK treated), DL-dithiothreitol (DTT), idoacetamide (IDA), ammonium bicarbonate, ammonium formate, and formic acid (for LC-MS) were purchased from Sigma-Aldrich. Acetonitrile (ACN, HPLC grade) was purchased from Fisher. Octadecyl (C18) disposable extraction columns were purchased from J.T. Baker. All reagents were analytical grade.

2.2. Trypsin Digestion:

Bovine fetuin (200µg) was dissolved in 100 µl of 50 mM ammonium bicarbonate. For human serum, a 50 µl aliquot (70ug/ul protein) was mixed with 50µl of 100 mM ammonium bicarbonate. Five µl of 200mM DTT was added to both sample solutions to reduce the disulfide bonds. Sulfhydryl alkylation was carried out by adding 4µl of 1M IDA to the sample, and the excess IDA was neutralized by adding 20µl of 200mM DTT solution. Trypsin digestion was

carried out at 37^{o}C overnight with the enzyme amount adjusted to establish the ratio of trypsin to sample protein was 1:20 (w/w). The trypsin digested peptide and glycopeptide mixture was separated from the undigested protein, trypsin and other impurities (DTT and IDA) by reversed-phase C18 solid phase extraction (SPE) column. The trypsin digests were loaded onto a C18 SPE column, which had been pre-equilibrated in 5% acetic acid. The peptides and glycopeptides were eluted with 5 mL of 65% acetonitrile in 5% acetic acid, which was collected and dried in the SpeedVac.

2.3.C18 Separation of N-glycopeptides of Fetuin:

The study utilized a Nexera UFLC (Shimadzu) LC system and Agilent C18 columns (Eclipse XDB-C18, 4.6 mm×15cm, 5μm particle size). The separation was carried out at a flow rate of 0.4 mL/min at room temperature with a mobile phase A consisting of 99.9% H_2O with 0.1% formic acid and mobile phase B of 99.9% ACN with 0.1% formic acid. A linear gradient of 5% mobile phase B to 40% mobile phase B in 60 min was utilized. Samples were dispersed in 0.1% formic acid in water and maintained at room temperature in the autosampler until analysis.

2.4.HILIC Separation of N-glycopeptides:

The study utilized a Nexera UFLC (Shimadzu) LC system and Halo Penta-HILIC columns (Advanced Materials Technology, 2.1 mm×15cm, 2.7μm particle size, Wilmington, DE). The separation was carried out at a flow rate of 0.4 mL/min at 60^{o}C with a mobile phase A consisting of 95% H_2O/ACN with 50 mM ammonium formate (adjusted to pH 4.4 with formic acid) and mobile phase B being pure ACN.

For glycopeptides of fetuin, a linear gradient of 85% mobile phase B to 48% mobile phase B in 75 min was utilized. For glycopeptides of IgGs in human serum, a segmented linear gradients were employed; 1) 62% mobile phase B to 61.2% mobile phase B in 9 min, 2) 61.2%

mobile phase B to 60.2% mobile phase B in 10 min, and 3) 60.2% mobile phase B to 58% mobile phase B in 11 min was utilized. In each case, the column was flushed at 25% mobile phase for 5 minutes, before returning to the starting mobile phase composition.

2.5.SRM Detection:

MS analysis was performed using selected reaction monitoring (SRM) mode on a 4000 Q-Trap (AB SCIEX) mass spectrometer. In SRM mode, two stages of mass filtering are employed in the triple quadrupole mass spectrometer. In the first stage, ions of interest (precursor ions) are selected in quadrupole 1 (Q1) and induced to fragment by collisional excitation with neutral gas in a collision cell (q2). In the second stage, instead of obtaining full scan ms/ms, where all the possible fragment ions derived from the precursor are mass analyzed in Q3, only a small number of specific fragment ions (transition ions) are analyzed in Q3. This targeted MS analysis using SRM enhances the lower detection limit for target analysts. The masses of glycopeptides in Fetuin and the human serum IgGs (precursor masses) were predicted by adding the masses of targeted N-glycans to the masses of targeted tryptic peptides containing the N-glycosylation sites of interest. The m/z values used as the precursor ions used in the SRM experiments for the Fetuin glycopeptides are listed in Table S2.1 of the Supporting Information. For example, the m/z value of 1218.8 corresponds to the triply charged glycopeptide with peptide backbone of LCPDCPLLAPLNDSR (GP15) and biantennary N-glycan with two SAs (Bi-2SA), which is abbreviated as GP15-Bi-2SA. The m/z values used as precursors in the SRM experiments performed on the glycopeptide of the human serum IgGs are listed in Table S2.2. MS/MS experiments performed on the various glycopeptides revealed that each produced two intense fragment ions. The common fragment at m/z 365.7 corresponds to the oxonium ion of hexose-N-acetylhexoseamine (Hex-HexNAc). The other intense fragment ion corresponds to the

complete peptide backbone for the selected precursor, combined with a single N-acetylglucosamine (GlcNAc) attached. These two fragments ions were used in the SRM experiment for all the glycopeptides as Q3 transition ions, and significantly reduced the possibility of false positives. A collision energy (CE) of 70 V and declustering potential (DP) of 40 V was selected as an appropriate compromise between selected ion intensity and background current. The dwell time for data collection was set at 100 ms, and unit resolution was used in both Q1 and Q3.

3. Result and Discussion:

Both RP and HILIC were used to analyze N-linked glycopeptides from fetuin, and the results were compared to demonstrate the features of these two separation modes for resolving N-glycopeptide mixtures. Fetuin is a standard glycoprotein that has three well-characterized N-glycosylation sites, occupied by N-glycans consisting predominantly of bi-, tri-, and tetra-antennary complex structures, possessing variable degrees of sialylation [22-24]. For this paper, the fetuin glycans are named using the following common nomenclature; the number of antenna (Bi-, Tri- Tetra-) followed by the number of sialic acid residues (1-SA, etc.). The RP separation of N-glycopeptides of Fetuin is shown in Figure 2.1A. The extracted ion chromatograms (EIC) from full ms scans obtained for the glycoforms from one of the fetuin glycopeptides (Figure 1A inset) illustrates that GP15-Bi-2SA and the GP15-Tri-3SA have virtually identical elution profile. GP15-Tri-4SA is partially resolved from GP15-Bi-2SA and GP15-Tri-3SA. These ion traces show that the glycoforms attached to this peptide essentially co-elute by RPLC, and is consistent with the RP separation mechanism being driven by the hydrophobic interactions of the stationary phase with the peptide backbone. The hydrophilic glycan has minimal interaction with the stationary phase and thus has little effect on the RP chromatographic behavior. Hence,

even dramatic changes in glycan structures (e.g. bi- vs. tri-antennary N-glycans) only leads to minimal shifts in retention of glycopeptide glycoforms. A consequence caused by all of a glycopeptide's glycoforms co-eluting is the inability to differentiate between glycoforms that are authentically present in the sample, from ions corresponding to the m/z value of smaller glycoforms that result from in-source fragmentation of a larger glycoform(s). The presence of in-source generated fragment ions can also cause an error in glycoform quantitation since a signal can correspond to both an authentic glycoform and coincident fragments from a larger glycoform. Thus, while RP separations offer some benefits for glycopeptide analysis, development of alternative strategies could lead to greater certainty for accurate identification or quantitation of isomeric glycopeptide glycoforms.

The HILIC separation of targeted glycopeptides is shown in Figure 2.1B. The EIC of the same glycopeptide glycoforms discussed above (Figure 1B inset) demonstrates that the glycoforms of GP15 (GP15-Bi-2SA, GP15-Tri-3SA, and GP15-Tri-4SA) are baseline resolved from each other. Comparison of N-glycopeptides separation with RP and HILIC chromatography confirms that the attached glycans have a significant contribution to HILIC retention, compared to RP, and that the retention processes generate sufficient selectivity to enable HILIC to resolve different glycopeptide glycoforms.

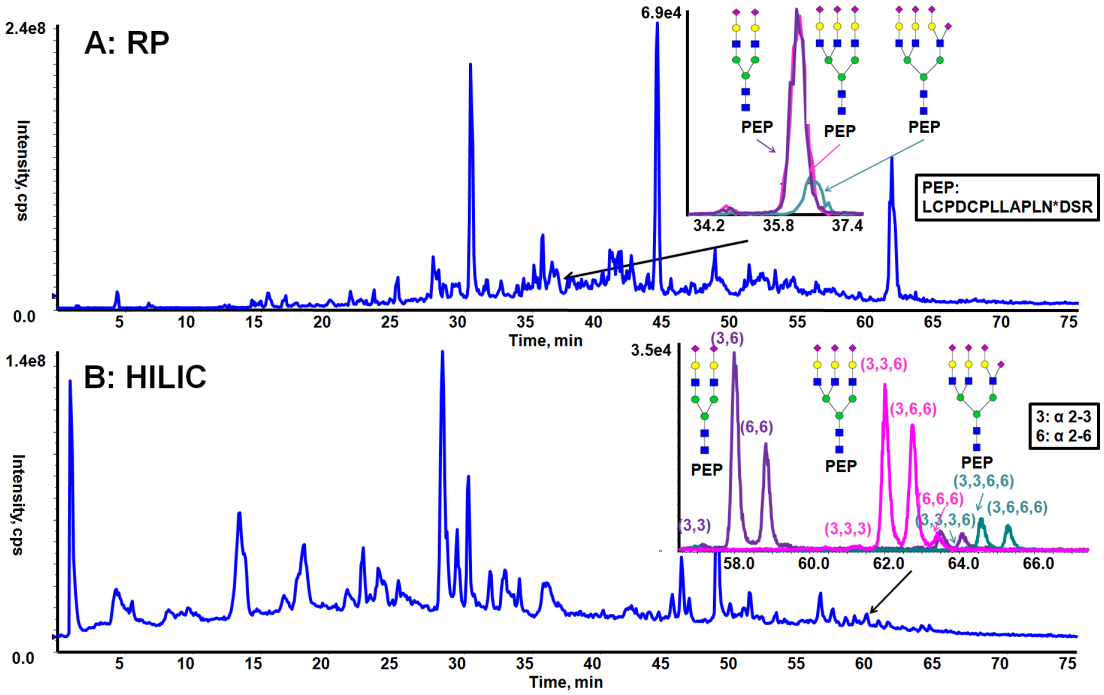

Figure 2.1. Behavior of tryptic glycopeptides of Fetuin during RP and HILIC Chromatography. (A) the TIC (blue trace) and EIC (insert graph: purple trace of GP15-Bi-2SA, pink trace of GP15-Tri-3SA, and green trace of GP15-Tri-4SA) from the RP separation and (B) the TIC (blue trace) and EIC (insert graph: purple trace of GP15-Bi-2SA, pink trace of GP15-Tri-3SA, and green trace of GP15-Tri-4SA) of HILIC separation of trypsin digested Fetuin.

The multiple peaks observed in the HILIC chromatogram for fetuin glycopeptides with the same mass suggests that these multiple peaks result from chromatographic resolution of isomeric glycoforms. Such elution profiles are similar to the HILIC separation of isomeric released glycans wherein the SA α2-3/6 linkage isomers can be resolved. The potential ability to separate isomeric glycans while still attached to peptides led us to compare the HILIC separation of released N-glycans of Fetuin with the glycopeptides of Fetuin. The HILIC separation of the procainamide (ProA) labeled N-glycans from fetuin has been studied by exoglycosidase digestion, and revealed that baseline separation was achieved for N-glycan isomers with α2-3/α2-6 SA linkages [13]. As illustrated in Figure 2A, isomeric glycans are being resolved based

on the ratio of α2-3-to-α2-6 SA linkages present in the glycoform, with the α2-3 linked isomers eluting before the α2-6 glycoforms. In these experiments, the same LC gradient was used to analyze the ProA-labeled N-glycans and glycopeptides of Fetuin. By comparing the retention time of glycopeptides (Figure 2.2B) and ProA-glycans (Figure 2.2A), it is clear that the presence of peptide backbone caused the glycopeptide to be retained longer in the HILIC separation than the ProA-glycans. However, the presence of the peptide did not significantly alter the relative retention time differences between the glycoforms, as shown by the similarity of the chromatographic profiles of the glycopeptides and the ProA-glycans. Analogous HILIC separation qualities were observed with the other N-linked glycopeptides of fetuin (GP30 and GP27).

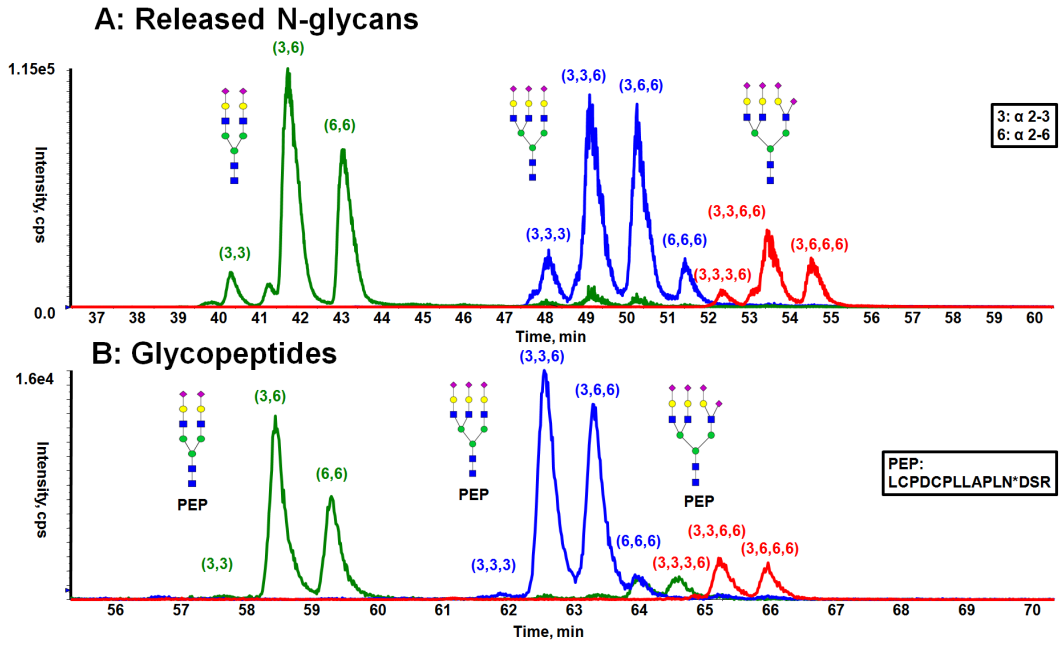

Figure 2.2. Comparison of the separation obtained from released N-glycans and glycopeptides of Fetuin by HILIC. (A) HILIC separation of procainamide labeled released N-glycans. (B) HILIC separation of glycopeptides with the same peptide backbone.

The chromatographic resolution between adjacent α2-3/ α2-6 sialic acid linkage isomers was determined for several of the glycoforms using Equation 1. The results from these calculations (Table 2.1) demonstrates that the HILIC resolution for glycopeptides is similar to that obtained for the released and derivatized N-glycans (Figure 2.2A) while using the same gradient conditions. Therefore, HILIC appears to be equally capable of resolving N-linked glycopeptide glycoforms as well as this approach resolves the tagged N-glycans.

$$Resolution = \frac{Retention\ time(Peak\ 2) - Retention\ time(Peak\ 1)}{\frac{1}{2}[Peak\ width(Peak\ 2) + Peak\ width(Peak\ 1)]} \qquad \text{Equation 1}$$

Table 2.1. The chromatographic resolution between adjacent α2-3/ α2-6 sialic acid linkage isomers obtained from the analysis of glycopeptides and released glycans using the identical LC gradient conditions.

Peak1	Peak2	Resolution	
		Glycans	Glycopeptides
Bi-2SA(3,3)	**Bi-2SA(3,6)**	4.56	3.80
Bi-2SA(3,6)	**Bi-2SA(6,6)**	4.09	3.67
Tri-3SA(3,3,3)	**Tri-3SA(3,3,6)**	3.41	2.30
Tri-3SA(3,3,6)	**Tri-3SA(3,6,6)**	3.53	2.79
Tri-3SA(3,6,6)	**Tri-3SA(6,6,6)**	4.02	2.75
Tri-4SA(3,3,3)	**Tri-4SA(3,3,6,6)**	4.09	2.56
Tri-4SA(3,3,6,6)	**Tri-4SA(3,6,6,6)**	3.49	2.12

The ability of HILIC to resolve linkage glycoforms of glycopeptides led us to investigate the HILIC separation of a more complex glycopeptide mixture, those obtained by tryptic digestion of

human serum IgGs. Human IgGs occur in four subclasses (IgG1, IgG2, IgG3, and IgG4), all of which carry a single N-glycosylation site at asparagine (Asn) 297 in the conserved domain of the crystallizable fragment (Fc) region. After trypsin digestion, the amino acid sequences of glycopeptides from IgG1, IgG2/3 and IgG4 are relatively similar (Table S2.2, Supporting Information). The N-glycans attached to Asn-297 are predominantly complex biantennary structure with core fucosylation, and there are small populations of glycans carrying terminal α2-6 linked sialic acids or a bisecting GlcNAc. Consequently, this sample consists of glycopeptide mixtures with closely related peptide sequences in addition to glycan heterogeneity. The predicted m/z values of all the known IgG glycopeptides are listed in Table S2.2 (Supporting Information). The HILIC separation of several selected major N-glycopeptides from IgG1, IgG2/3 and IgG4, are displayed in Figure 3. The retention order of the glycopeptides with different peptide sequences can be rationalized by the hydrophilicity of the peptide backbones. The amino acid sequences of IgG 2/3 (EEQFNSTFR), IgG 4 (EEQFNSTYR) and IgG 1 (EEQYNSTYR) differ by a phenylalanine (F) to tyrosine (Y) substitution at one or two locations. Since tyrosine is more hydrophilic than phenylalanine, IgG 2/3 has the more hydrophobic amino acid sequence and glycopeptides with this backbone elute earliest. IgG 4 has the intermediate hydrophobicity, and IgG 1 is the most hydrophilic, all of these are consistent with their elution order (Figure 2.3). Varying glycans on the same peptide sequence exhibits greater HILIC retention for the species with larger glycans. Within these assemblies of glycans, separation of isomeric glycoforms is observed, as is shown by the two peaks detected for each glycopeptide possessing the N-glycan with a composition of Hex4HexNAc4Fuc1 (G1F). This glycan structure has two isomers resulting from attachment of the galactose to each of the two antennae.

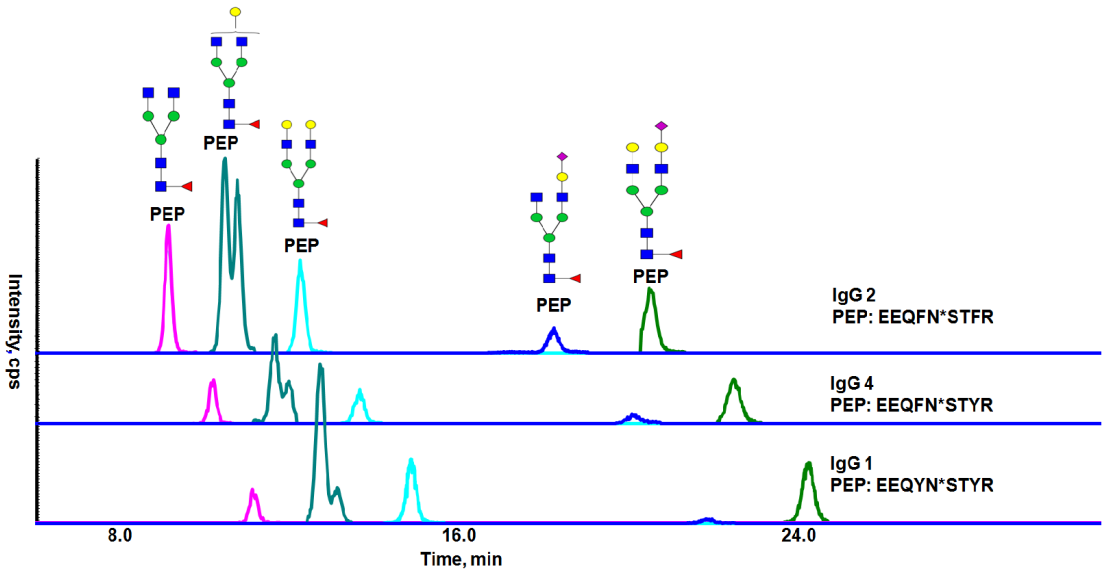

Figure 2.3. The HILIC separation of major N-glycopeptides in IgG1, IgG2 and IgG4.

A scheduled SRM approach was developed to enable detection of the lower abundance glycoforms and thus better evaluate the HILIC retention behavior from each of the expected glycoforms of the IgG glycopeptides. The glycan and peptide sequence heterogeneity of the IgG glycopeptides combines to yield a total 78 distinct precursor masses. Given this sample complexity, and thus the high sensitivity and a high dynamic range required to identify the potential glycopeptide structures that could be resolved by the 30 minute long LC/MS acquisition, parameters for SRM scheduling required knowledge of estimated retention times. A retention time was found experimentally for each glycopeptide glycoform, and these are listed in Table S2.3. The standard deviations (SD) of retention times from ten LC runs ranged from 0.104 to 0.383min indicating good reproducibility of the HILIC separation. The detection window set for each transition is 3 min., selected for peak widths observed to be approximately 50 s. Under these conditions, numerous glycopeptides of human serum IgGs are detected and are well resolved as illustrated in Figure 2.4.

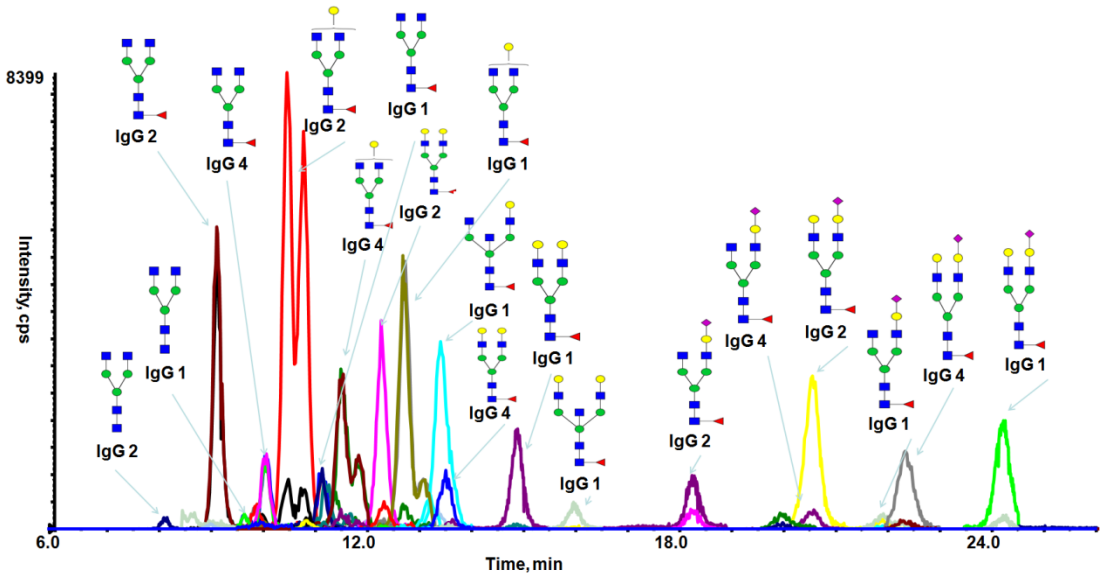

Figure 2.4. HILIC SRM analysis of human serum IgGs demonstrating the ability to resolve isomeric glycopeptide glycoforms.

4. Conclusion:

Hydrophilic functional groups on both the peptide and the glycan interact with the HILIC stationary phase, and thus both peptide and glycan structure determine retention of a glycopeptide. This characteristic enables the resolution of mixtures of closely related glycopeptides. The combination of glycopeptide amino acid sequence and glycan composition permits the resolution of glycoforms, which can extend to even resolving isomeric and positional glycan variants. Thus, LC/MS/MS can provide the site-specific glycans profiles at individual sequence sites from complex mixtures of glycoproteins.

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CHAPTER 3

STUDYING THE KINETICS OF N-GLYCAN RELEASE BY PNGASE F WITH SRM QUANTITATION OF THE GLYCOPEPTIDES FROM HUMAN SERUM IMMUNOGLOBULIN G

To be submitted to Journal of Analytical Chemistry.

Abstract:

It is widely accepted that the biological activity of IgGs is modulated by its Fc N-glycosylation. The decreased galactosylation is restricted to Fc N-linked oligosaccharides. Since the glycosylation plays a critical role in IgGs effecter function, the analysis of glycosylation of IgGs has been and is still extensively developed. One of the most common approaches to analyze glycosylation of IgGs is to study the released N-glycans of IgGs. The most widely used enzyme for releasing intact N-glycans is Peptide N-glycosidase F (PNGase F), which is an endoglycosidase that cleaves the linkage between the asparagines residue and innermost GlcNAc of N-linked glycans, except those containing a 3-linked fucose attached to the reducing terminal GlcNAc residue. While performing glycomic studies, we have determined that the PNGase F release introduces the largest source of quantitative variation, and led us to conduct a more detailed study of this enzyme system. Here we investigated the kinetics of glycan release from glycopeptides of IgGs, to determine the effect of glycan structure and amino acid sequence on the rate of glycan release from glycopeptide of IgGs. With the method of SRM Quantitation of the glycopeptides from human serum IgGs, it revealed that slight differences in amino acid sequences were not found to cause a statistically different deglycosylation rate; significant differences in the deglycosylation rate constants were observed between glycopeptides differing only in glycan structure (i.e. non-fucosylated, fucosylated, bisecting-GlcNAc, sialylated, etc.). For example, a single sialic acid residue was found to decrease the rate by a factor of 3. Similar reductions in rate were associated with the presence of a bisecting-GlcNAc. We predict the differences in release kinetics can lead to significant in quantitative variations of glycosylation study of IgGs.

1. Introduction:

Immunoglobulin (Igs) are the major class of serum glycoprotein that is used by the adaptive immune system to identify and neutralize pathogens. Immunoglobulin G (IgG) is one of the five distinct classes (IgG, IgM, IgA, IgE, and IgD) that have been identified in human. IgG is a glycoprotein complex built from four peptide chains that are two identical class γ heavy chains (HCs) of about 50 kDa and two identical light chains (LCs) of about 25 kDa arranged in Y-shape typical of antibody monomers. There're fours subclasses of IgGs (IgG1, IgG2, IgG3, IgG4), which are based on the conserved domain of their polypeptide chains. However, all of them carry a single N-glycosylation site at asparagine (Asn) 297 in the conserved Cγ2 domain of the crystallizable fragment (Fc) part of the HCs. The N-glycans found on IgG are predominantly core-fucosylated complex biantennary structure. The biantennary, core-fucoyslated structure carrying tow, one, or no galacose residue are called G2, G1 and G0 (Figure 1). Some of the glycans carry terminal α2-6 linked sialic acids (SA) and/or a bisecting N-acetyglucosamine (GlcNAc).

It is widely accepted that the biological activity of IgGs is modulated by its Fc N-glycosylation. The decreased galactosylation is restricted to Fc N-linked oligosaccharides. The portion of G0 glycoform ("agalactosyl" IgG) is particularly large in patients with various chronic inflammatory and infectious diseases including rheumatoid arthritis, juvenile chronic arthritis , active Crohn's disease, tuberculosis, Lyme disease and sarcoidosis [1, 2]. Many reports have shown that the absence of core-fucose on Fc N-glycans of IgG1 may lead to dramatic enhancement of antibody-depend cellular cytotoxicity (ADCC) [3-6]. The anti-inflammatory properties of intravenous immunoglobulin (IVIG) are depend on sialylation of the Fc N-glycans

[7]. In healthy individuals, age, gender, and pregnancy are reflected by IgG glycosylation feature. For example, the amount of galactose on Fc N-glycans depend on the age. In childhood and in elderly people, IgG is less galactosylated. The percentage of G0 glycofroms increases in people older than 50 [8]. Since the glycosylation plays a critical role in IgGs effecter function, the analysis of glycosylation of IgGs has been and is still extensively developed [1].

One of the most common approaches to analyze glycosylation of IgGs is to study the released N-glycans of IgGs. Starting from purified IgG, glycans are released either enzymatically or chemically. However, due to the problem of the destruction of the peptide backbone and the potential to degrade the oligosaccharides, the chemical hydrolysis release of N-glycans is not widely used. The most widely used enzyme for releasing intact N-glycans is Peptide N-glycosidase F (PNGase F) [9-22], which is an endoglycosidase that cleaves the linkage between the asparagines residue and innermost GlcNAc of N-linked glycans, except those containing a 3-linked fucose attached to the reducing terminal GlcNAc residue [23]. Because this enzyme release all mammalian N-linked glycans, it is widely used in the analysis of glycoprotein. By using PNGase F to release N-glycan and further to characterize and especially quantitate released N-glycan, it's usually assumed that all the N-glycans are cleaved at 100% yield. However, this assumption hasn't been proved yet. In addition, while performing glycomic studies, we have determined that the PNGase F release introduces the largest source of quantitative variation, and led us to conduct a more detailed study of this enzyme system. Here we investigated the kinetics of glycan release from glycopeptides of IgGs, to determine the effect of glycan structure and amino acid sequence on the rate of glycan release from glycopeptide of IgGs, as this would be a reasonable explanation for the observed quantitative variations.

2. Experiment Section:

The overall experimental procedure is illustrated in Figure 3.1. The details of the experimental procedure are described in the following paragraphs.

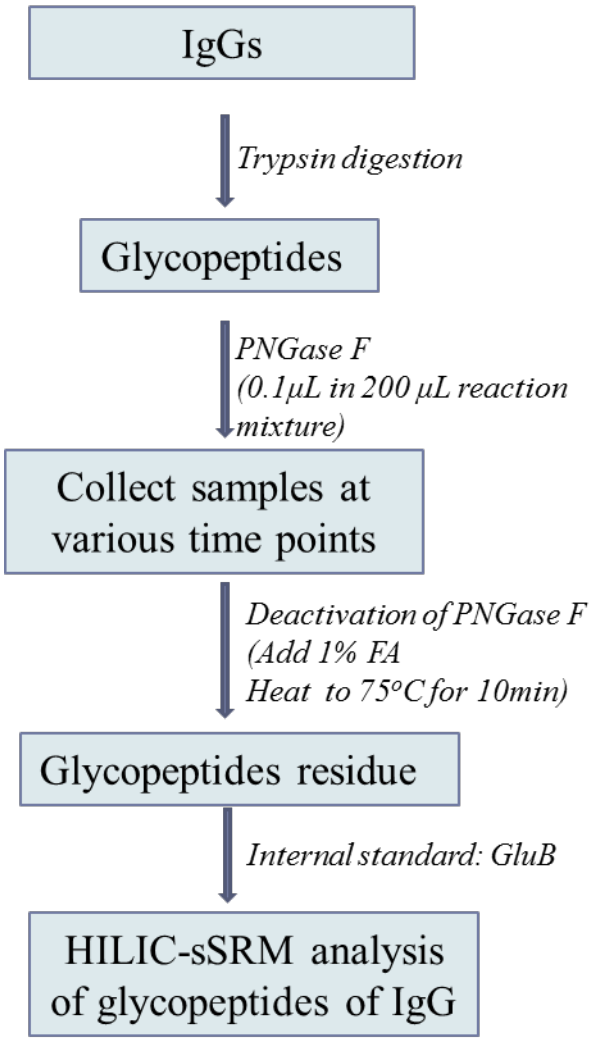

Figure 3.1. The scheme of the experiment

2.1.Materials:

Human serum, trypsin (TPCK treated), DL-dithiothreitol (DTT), idoacetamide (IDA), ammonium bicarbonate, ammonium formate, and formic acid (for LC-MS) were purchased from Sigma Aldrich. PNGase F (Glycerol free) was purchased from New England Biolabs (NEB)

Acetonitrile (ACN, HPLC grade) was purchased from Fisher. Octadecyl (C18) disposable extraction columns were purchased from J.T. Baker. Other reagents were analytical grade.

2.2.Trypsin digestion:

200 µl of human serum was lyophilized to dry (dried human serum weighted as 14mg) and resuspended with 200 µl of 50mM ammonium bicarbonate. The sample solution was added 5µl of 200mM DTT to reduce the disulfide bond. Sulfhydryl alkylation was carried out by adding 4µl of 1M iodoacetamide stock to the sample and neutralize the remaining iodoacetamide by adding 20µl of DTT stock. Trypsin digestion was carried out at 37°C overnight with adjusted enzyme amount (the ratio of trypsin to sample 1:20). The glycopeptides were enriched from the trypsin digested peptide mixture by reversed-phase C18 SPE column. The trypsin digests were loaded onto a C18-Sep-Pac, which had been pre-equilibrated in 5% acetic acid. The sample was eluted with 5 mL of 65% acetonitrile in 5% acetic acid; the glycopeptides were present in the flow-through, which was collected, frozen, and lyophilized to dryness.

2.3.PNGase F digestion:

Human serum was subjected to a standard trypsin digestion protocol, followed by PNGase F digestions. Dried tryptic peptides/glycopeptides were dissolved by 300 µl of 50Mm ammonium bicarbonate, and then treated with 500 unit (0.1 µl) of PNGase. Aliquots of the digestion mixture were taken at every 10 minutes and quenched by lowering the pH and quickly heating to 75°C. [Glu1]-Fibrinopeptide B (GluB) was spiked into the samples and served as an internal standard for quantization.

2.4.LC-MS analysis:

These aliquots collected from PNGase F digestion mixture were analyzed by LC-MS using a 2.1 mm ID Penta-HILIC column (AMT) on Shimadzu Nexera LC system interfaced to a Q-trap 4000 MS analyzer (ABSciex). The separation was carried out at a flow rate of 0.4 mL/min at 60^{o}C with a mobile phase A consisting of 95% H_2O/CAN with 50mM ammonium formate (adjusted to pH 4.4 with formic acid) and mobile phase B being pure ACN. A segmented linear gradients were employed; 1) 62% mobile phase B to 61.2% mobile phase B in 9 min, 2) 61.2% mobile phase B to 60.2% mobile phase B in 10 min, and 3) 60.2% mobile phase B to 58% mobile phase B in 11 min was utilized. In each case, the column was flushed at 25% mobile phase for 5 minutes, before returning to the starting mobile phase composition.
Scheduled selected reaction monitoring (SRM) mode on a Q-tap 4000 mass analyzer were utilized to quantitate every known glycoform of each tryptic peptide from the IgGs and for the deglycosylated form of these peptides. In SRM mode, two stages of mass filtering are employed in the triple quadrupole mass spectrometer. In the first stage, ions of interest (precursor ions) are selected in quadrupole 1 (Q1) and induced to fragment by collisional excitation with neutral gas in a collision cell (Q2). In the second stage, instead of obtaining full scan ms/ms, where all the possible fragment ions derived from the precursor are mass analyzed in Q3, only a small number of specific fragment ions (transition ions) are analyzed in Q3. This targeted MS analysis using SRM enhances the lower detection limit for target analysts. The masses of glycopeptides of human serum IgGs (precursor masses) were predicted by adding the masses of targeted N-glycans to the masses of targeted tryptic peptides containing the N-glycosylation sites of interest. The m/z values used as precursors (Q1 ions) in the SRM experiments performed on the glycopeptide of the human serum IgGs are listed in Table S-1. MS/MS experiments performed

on the various glycopeptides revealed that each produced two intense fragment ions. The common fragment at m/z 365.7 corresponds to the oxonium ion of hexose-N-acetylhexoseamine (Hex-HexNAc). The other intense fragment ion corresponds to the complete peptide backbone for the selected precursor, combined with a single N-acetylglucosamine (GlcNAc) attached. These two fragments ions were used in the SRM experiment for all the glycopeptides as Q3 transition ions, and significantly reduced the possibility of false positives. Retention times was found experimentally for all the glycopeptides, and these are listed in Table S-2. The detection window set for each transition is 3 min. A collision energy (CE) of 70 V and declustering potential (DP) of 40 V was selected as an appropriate compromise between selected ion intensity and background current. The dwell time for data collection was set at 100 ms, and unit resolution was used in both Q1 and Q3.

3. Results and Discussion:

3.1. Pseudo First Order Reaction:

PNGase F releases N-linked glycans from the peptide backbone by hydrolyzing the amide group of the asparagine side chain as shown in reaction equation (I). Because PNGase F is an enzyme, which is not consumed during the reaction and therefore its concentration stays relatively constant. The concentration of H_2O is much greater than the concentration of glycopeptide, so the concentration of H_2O can be treated as constant. Therefore, this third order reaction can be predicted to be modeled by pseudo first order process, reaction equation (II). The differential rate equation describing a pseudo first order kinetics is given in equation (III), and its integrated form is shown in equation (IV). In both of these equations, k is the rate constant.

$$Glycopeptide + H_2O + PNGase\ F \rightarrow N\text{-}Glycan + Peptide + PNGaseF \quad (I)$$

$$Glycopeptide \rightarrow N\text{-}Glycan + Peptide \quad (II)$$

$$-d[GP]/dt = k[GP] \quad (III)$$

$$ln[GP] = -kt + ln[GP_0] \quad (IV)$$

The chromatograms of peptide backbone without glycans appeared and glycopeptide residues left in the sample at different time points are shown in Figure 3.2. The peaks of glycopeptides were gradually disappearing, as well as the deglycosylated peptide backbones were gradually appearing.

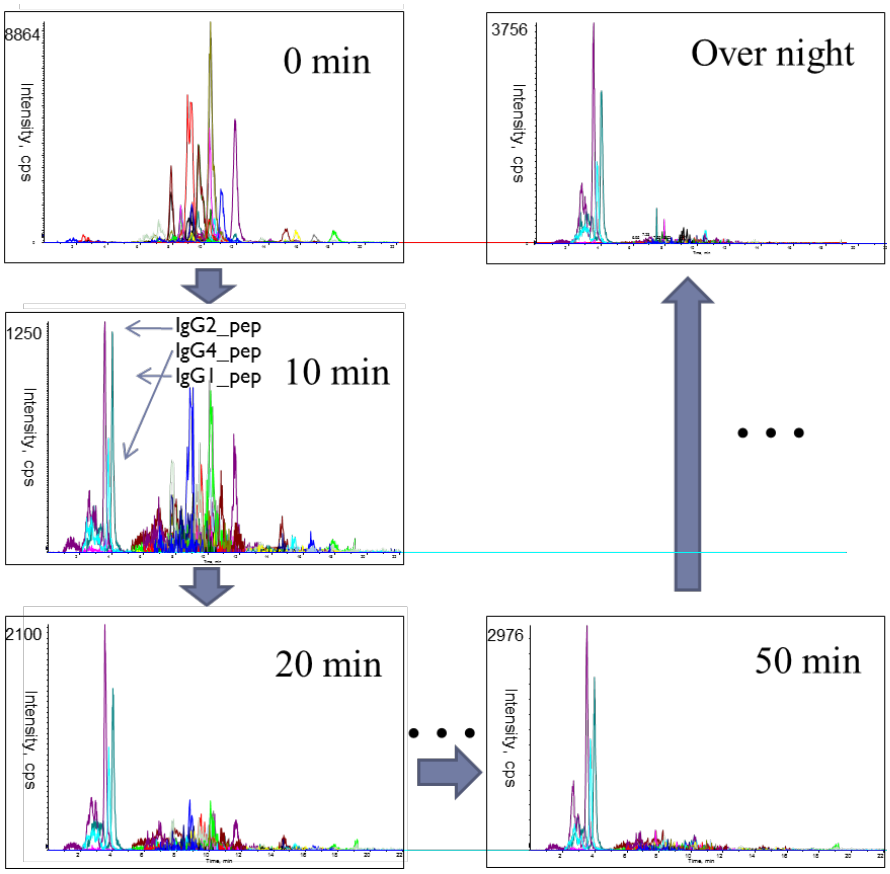

Figure 3.2. The chromatograms of appearance of deglycoslyated peptide backbone and disappearance of glycopeptide residues in the sample from 0 min to over night

The ‘concentration of glycopeptide’ ([GP]) from each aliquot collected at different time point was calculated by the integrated peak area of glycopeptide divided by the integrated peak area of internal standard—GluB. The plot of [GP] versus time is illustrated in Figure 3.3. Plotting natural log transformation of ‘concentration of glycopeptide’ (ln[GP]) with respect to time, as shown in Figure 3.4, gives a straight line, which confirms the PNGase F releasing reaction can be modeled as a pseudo first order reaction. Because of the linear relationship between ln[GP] and reaction time, the deglycosylation process follows a pseudo first order reaction model allowing the rate constants of glycan release to be determined by the slope of ln[GP] vs time plots.

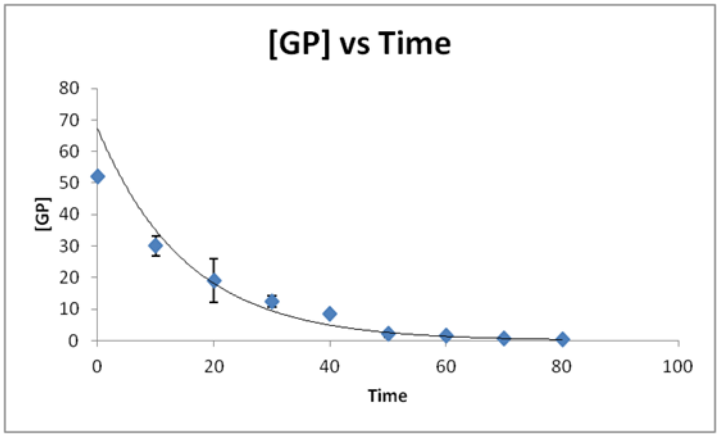

Figure 3.3. The plot of ‘concentration of glycopeptide’ ([GP]) versus time

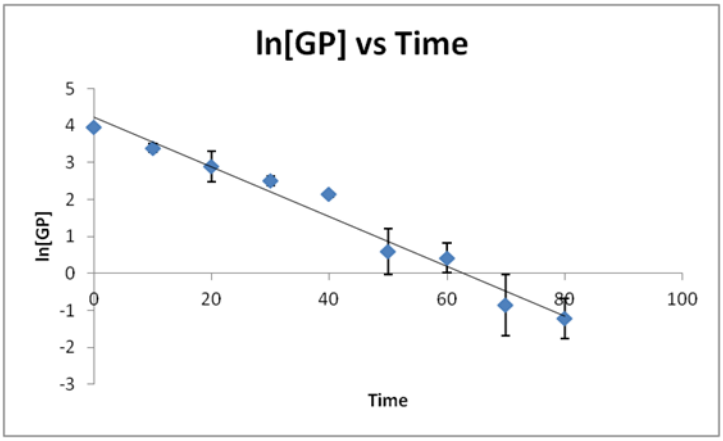

Figure3.4. The plot of natural log transformation of 'concentration of glycopeptide' (ln [GP]) versus time

3.2.Effect of Glycan Structures and Amimo Acid Sequence of Peptides on the Rate of Glycan Release:

Two potential effects on the rate of glycan release are considered here: (1) glycan structures and (2) amino acid sequence of the peptides. The heterogeneity of glycan structures and peptide sequences of IgGs glycosylation makes it an ideal model to study the effect of glycan structure and amino acid sequence of peptides on the rate of glycan release. The glycan structures of all the glycopeptides detected on IgGs were categorized into four groups: fucosylated, non-fucosylated, bisecting-GlcNAc, sialylated as shown in Figure3.5.B. The fucosylated and non-fucosylated glycoforms are only differ by a core fucose, so the comparison between releasing rate of fucosylated and non fucosylated glycofroms can reveal the effect of core fucose on PNGase F releasing rate. The same can be applied to the comparisons between fucosylated, bisecting-GlcNAc and sialylated glycoforms. The effect of bisecting-GlcNAc can be found by comparison between the releasing rate of fucosylated and bisecting-GlcNAc glycofroms, as well as the effect of terminal sialic acid can be studied by comparing releasing

rate of fucosylated and sialylated glycpeptides. The amino acid sequence of the peptides are different among the IgG subclasses (IgG1, IgG2/3, and IgG4). The sequences of IgG 2/3 (EEQFNSTFR), IgG 4 (EEQFNSTYR) and IgG 1 (EEQYNSTYR) differ by a phenylalanine (F) to tyrosine (Y) substitution at one or two locations as shown in Figure 3.5.A. The effect of peptide sequence on PNGase F releasing will be revealed by comparing the deglycosylation rates of glycopeptides with same glycoforms but different peptide backbones.

A. Amino Acid Sequence:

IgG1: EEQYNSTYR
IgG2/3: EEQFNSTFR
IgG4: EEQFNSTYR

B. Glycan Structure:

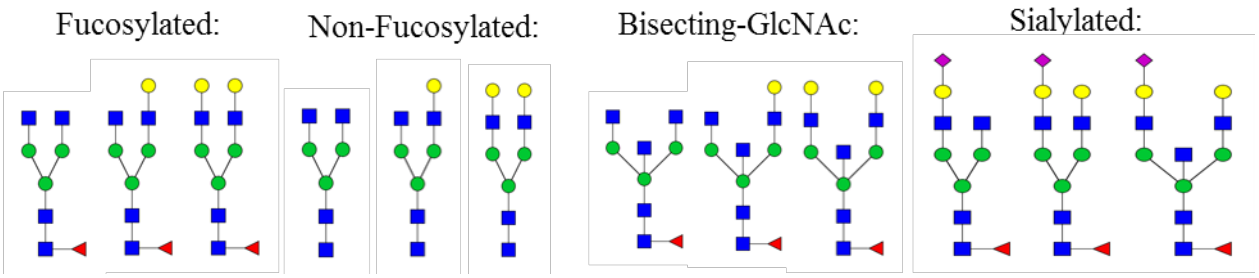

Figure3.5. A.The amino acid sequences of tryptic glycopeptide of IgG1, IgG2/3 and IgG4. B. The glycan structures of all the glycopeptides detected on IgGs were categorized into four groups: fucosylated, non-fucosylated, bisecting-GlcNAc, sialylated.

The changes of ln[GP] of each categorical glycofroms (fucosylated, non-fucosylated, bisecting-GlcNAc and sialylatd) with difference peptide backbones are plotted as functions of time in Figure3.6 to Figure3.9 respectively. The slopes of the linear lines (ln[GP] vs time), which are the rate constant of PNGase F releasing, illustrated in Figure 3.6 to Figure3.9 are summarized in Table 1. From the slopes of linear lines of ln[GP] vs time, the glycopeptides with same

categorical glycoform but different peptide backbones seem to have similar slopes which means their deglycosylation rates by PNGase F are about the same. For glycopeptides with same peptide backbones but different categorical glycofroms, fucosylated glycopeptides seem to have the highest glycosylation rate (steepest slope) comparing to other categorical glycopeptides.

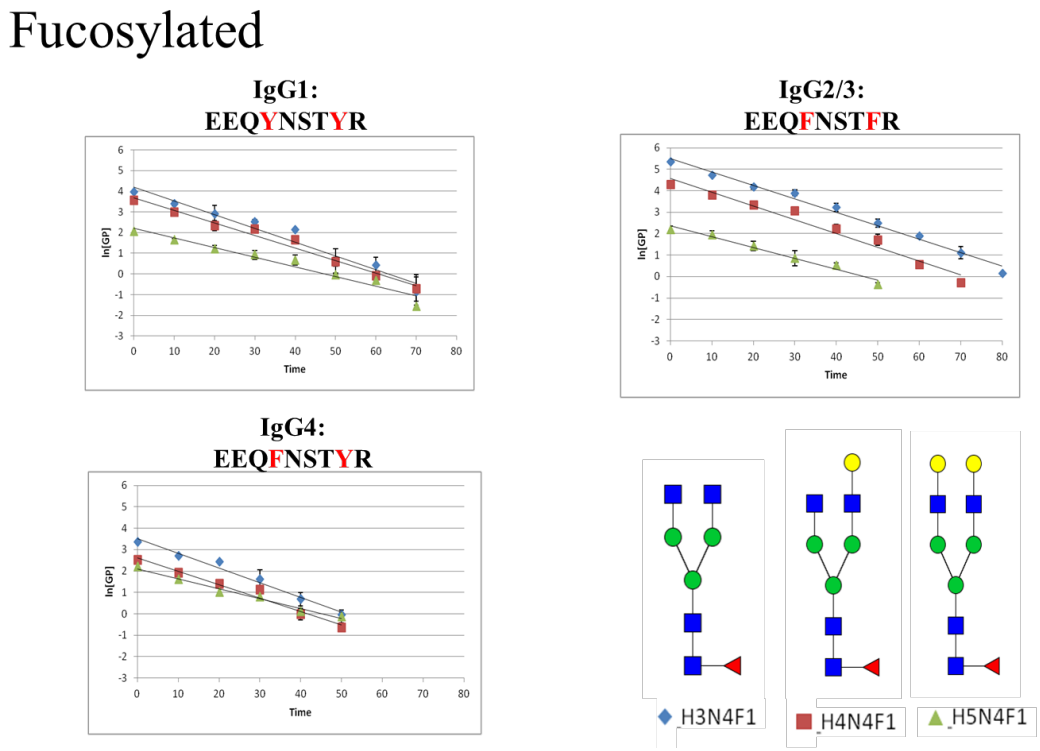

Figure3.6. The change of ln[GP] of fucosylated glycopeptides as function of time

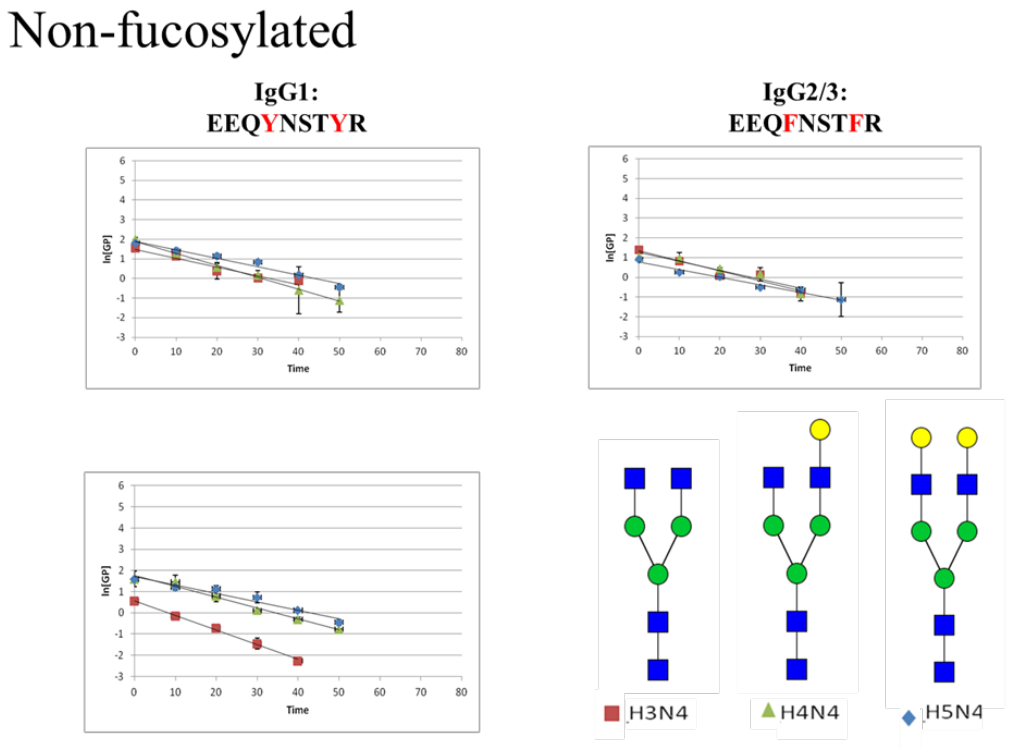

Figure3.7. The change of ln[GP] of non-fucosylated glycopeptides as function of time

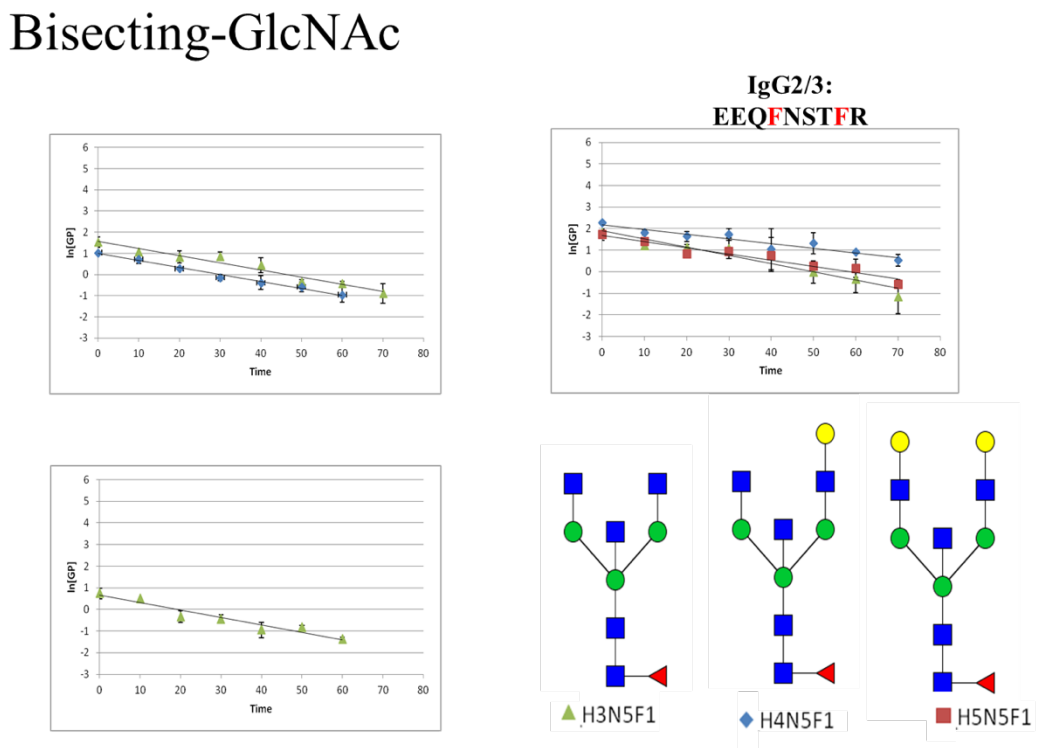

Figure3.8. The change of ln[GP] of glycopeptides with bisecting-GlcNAc as function of time

Sialylated

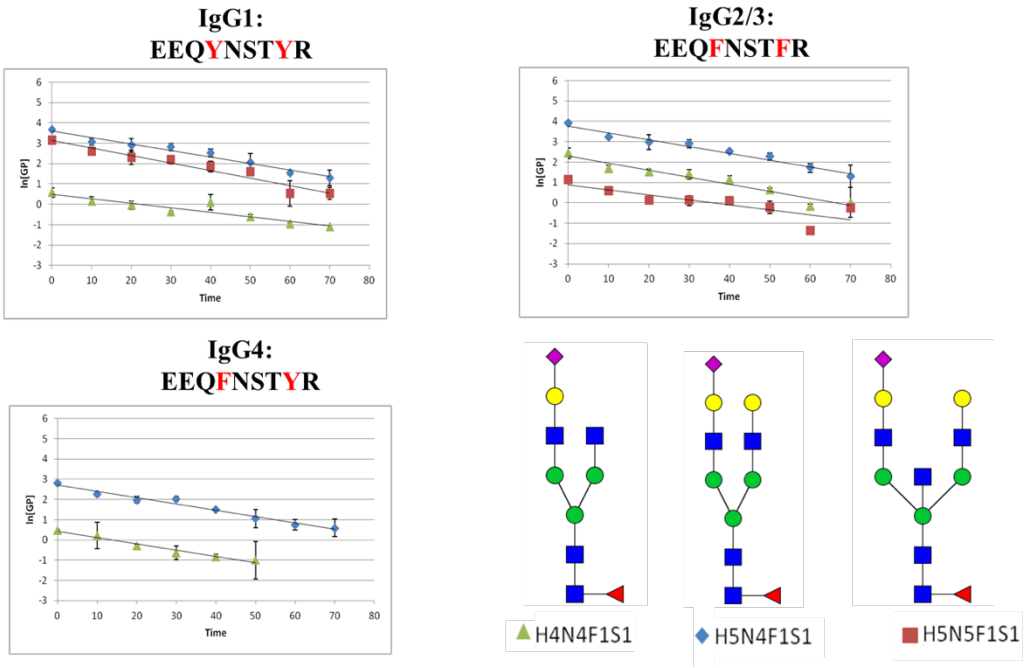

Figure3.9. The change of ln[GP] of sialylated glycopeptides as function of time

Table 4. Summary of Rate Constant

	Rate constant (k)	IgG1	IgG2/3	IgG4
Fucosylated	H3N4F1	0.0665	0.0626	0.0682
	H4N4F1	0.0609	0.0641	0.0626
	H5N4F1	0.0466	0.0502	0.0467
Nonfucosylatd	H3N4	0.0448	0.0516	0.0693
	H4N4	0.0614	0.0455	0.051
	H5N4	0.0435	0.0385	0.0396
Bisecting	H3N5F	NA	0.0383	NA
	H4N5F	0.0330	0.0220	0.0344
	H5N5F1	0.0336	0.0290	NA
Sialylated	H4N4F1S1	0.0221	0.0247	0.0309
	H5N4F1S1	0.0322	0.0335	0.0314
	H5N5F1S1	0.0370	0.0345	NA

3.3.Statistics test:

Mann-Whitney test was utilized here to investigate if the differences in the rate constants between different categorical glycan structures and peptide backbones are statistically significant. Mann-Whitney test is a nonparametric test of the null hypothesis that two samples have equal sample mean against an alternative hypothesis that the sample means from two samples are not equal. Unlike the two sample *t*-test, it does not require the assumption of normal distributions. The results are listed in Table 3. The results from every glycoform of IgG1 and IgG2/3 were compared and not found to be statistically different. However, the rate constants of glycans from different categories are found to be significantly different. Compared to nonfucosylated, bisecting-GlcNAc and sialylated glycans, fucosylated glycans are released from IgGs glycopeptides at higher rate. In other works, both bisecting-GlcNAc and terminal sialic acid have a negative effect on the PNGase F releasing rate. For example, a single sialic acid residue was found to decrease the rate by a factor of 3.

Table 3. Results of Mann-Whitney Test

Comparison	p-value	Result
k(IgG1) vs k(IgG2/3)	0.83	k(IgG1)=k(IgG2)
k(Fuc.) vs k(non-Fuc.)	0.002	k(non-Fuc.)<k(Fuc.)
k(Fuc.) vs k(Bisec.)	<0.001	k(Bisec.)<k(Fuc.)
K(Fuc.) vs k(Sia.)	<0.001	k(Sia.)<k(Fuc.)

4. Conclusion:

Slight differences in amino acid sequences were not found to cause a statistically different deglycosylation rate. In the future, we plan to investigate if larger changes in the amino acid sequence are associated with alteration of glycan release rate.Significant differences in the deglycosylation rate constants were observed between glycopeptides differing only in glycan structure (i.e. non-fucosylated, fucosylated, bisecting-GlcNAc, sialylated, etc.). For example, a single sialic acid residue was found to decrease the rate by a factor of 3. Similar reductions in rate were associated with the presence of a bisecting-GlcNAc. We predict the differences in release kinetics can lead to significant in quantitative variations of glycosylation study of IgGs.

5. Acknowledgements:

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CHAPTER 4

STUDYING THE KINETICS OF N-GLYCAN RELEASE BY PNGASE F WITH SRM QUANTITATION OF THE GLYCOPEPTIDES FROM STANDARD GLYCOPROTEINS

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Abstract

Glycosylation is one of the most important types of protein posttranslational modifications. Among all the biologically important proteins, over half of them are glycosylated. The analysis of protein glycosylation is necessary to improve our understanding of various biological processes, as well as to facilitate correlation of glycan structures with healthy and disease states. One of general approaches to characterize N-glycans using LC/MS is glycoprofiling of liberated glycans from glycoprotein. The most widely used enzyme for releasing intact N-glycans is Peptide N-glycosidase F (PNGase F), which is an endoglycosidase that cleaves the linkage between the asparagine residue and innermost GlcNAc of N-linked glycans, except those containing a 3-linked fucose attached to the reducing terminal GlcNAc residue. In chapter 3, we investigated the kinetics of glycan release from glycopeptides of Immunoglobulin G (IgGs), to determine the effect of glycan structure and amino acid sequence on the rate of glycan release from glycopeptides of the human serum IgG subclasses (i.e., IgG1, IgG2/3, and IgG4). The experimental rate constants were statistically indistinguishable for release of the same N-linked glycan structure from peptides with slightly different amino acid sequences. However, the rate constants of N-glycopeptides with different glycan structures (i.e. non-fucosylated, fucosylated, bisecting-GlcNAc, sialylated, etc.) and the same peptide sequence were found to be significantly different. For example, fucosylated N-glycans are released twice as fast as N-glycans with a bisecting-GlcNAc. Here, we investigate the glycoforms of the other standard glycoproteins (Ribonuclease B, Fetuin, and Transferrin) to determine if there are other correlations between glycan structure, peptide sequence and the rate of glycan release. The experimental results revealed that significant differences in the deglycosylation rate constants were found between glycopeptides with very different peptides sequences but the same glycan structure, as well as between glycopeptides differing only in glycan structures.

1. Introduction:

Glycosylation is one of the most important types of protein posttranslational modifications. Among all the biologically important proteins, over half of them are glycosylated [1]. Glycoprotein are known to involve in many important biological process including cell growth [2], cell-cell adhesion [3], fertilization [4], immune system [5], degradation of blood clots [6], viral replication [7] and inflammation [8]. Glycosylation is also found to be varied with diseases such as cancers [9], Alzheimer 's disease [10], and rheumatoid arthritis [11], which leads to the manipulation of glycosylation may alter the properties of glycoproteins and result in beneficial therapeutic results. Consequently, the analysis of protein glycosylation is necessary to improve our understanding of various biological processes, as well as to facilitate correlation of glycan structures with healthy and disease states.

In human, one of most prevalent glycosylation sites occur at asparagine residues in the sequence containing Asn-X-S/T, where X corresponds to any amino acid except for proline. Here, the glycans attach to asparagine residues are called N-linked glycans.

One of general approaches to characterize N-glycans using LC/MS is glycoprofiling of liberated glycans from glycoprotein. It comprise three steps: firstly the glycans are released from the glycoprotein enzymatically or chemically; secondly the released glycans are modified by permethylation or reductive amination labeling; thirdly the glycan profiling is established by LC-MS based method [12]. Usually, in the first step, intact N-glycans can be released from their peptide backbone both chemically and enzymatically. However, due to the problem of the destruction of the peptide backbone and the potential to degrade the oligosaccharides, the chemical hydrolysis release of N-glycans is not widely used. The most widely used enzyme for releasing intact N-glycans is Peptide N-glycosidase F (PNGase F) [14-22], which is an endoglycosidase that cleaves the linkage between the asparagine residue and innermost GlcNAc of N-linked glycans, except those containing a 3-linked fucose attached to the reducing terminal GlcNAc residue [13]. Because this enzyme release all mammalian N-linked glycans, it is widely used in the analysis of glycoprotein. By using PNGase F to release N-glycan and further to

characterize and especially quantitate released N-glycan, it's usually assumed that all the N-glycans are cleaved at 100% yield. However, this assumption hasn't been proved yet. In addition, while performing glycomic studies, we have determined that the PNGase F release introduces the largest source of quantitative variation, and led us to conduct a more detailed study of this enzyme system. In chapter 3, we investigated the kinetics of glycan release from glycopeptides of Immunoglobulin G (IgGs), to determine the effect of glycan structure and amino acid sequence on the rate of glycan release from glycopeptides of the human serum IgG subclasses (i.e., IgG1, IgG2/3, and IgG4). The experimental rate constants were statistically indistinguishable for release of the same N-linked glycan structure from peptides with slightly different amino acid sequences. However, the rate constants of N-glycopeptides with different glycan structures (i.e. non-fucosylated, fucosylated, bisecting-GlcNAc, sialylated, etc.) and the same peptide sequence were found to be significantly different. For example, fucosylated N-glycans are released twice as fast as N-glycans with a bisecting-GlcNAc. Here, we investigate the glycoforms of the other standard glycoproteins (Ribonuclease B, Fetuin, and Transferrin) to determine if there are other correlations between glycan structure, peptide sequence and the rate of glycan release.

2. Experiment Section:

The overall experimental procedure is illustrated in Figure 4.1. The details of the experimental procedure are described in the following paragraphs.

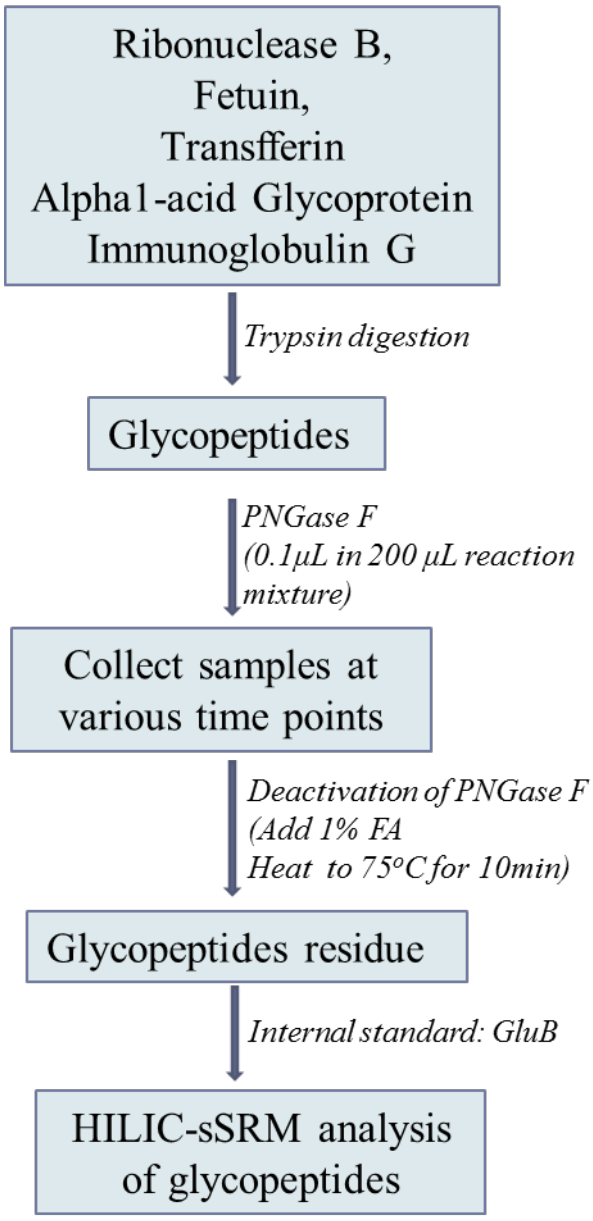


Figure 4.1. The scheme of the experiment

2.1.Materials:

Human serum, Ribonuclease B (RB) from bovine pancreases, Fetuin from fetal bovine serum, purified IgGs from human serum, trypsin (TPCK treated), DL-dithiothreitol (DTT), idoacetamide (IDA), ammonium bicarbonate, ammonium formate, and formic acid (for LC-MS) were purchased from Sigma Aldrich. PNGase F (Glycerol free) was purchased from New England Biolabs (NEB) Acetonitrile (ACN, HPLC grade) was purchased from Fisher. Octadecyl (C18) disposable extraction columns were purchased from J.T. Baker. Other reagents were analytical grade.

2.2.Trypsin digestion:

200 µg of RB, 200 ug of Fetuin and 200 ug of purified IgGs were mixed and dissolved with 200µl of 50mM ammonium bicarbonate. 200 µl of human serum was lyophilized to dry (dried human serum weighted as 14mg) and resuspended with 200 µl of 50mM ammonium bicarbonate. Both sample solutions were added 5µl of 200mM DTT to reduce the disulfide bond. Sulfhydryl alkylation was carried out by adding 4µl of 1M iodoacetamide stock to the samples and neutralize the remaining iodoacetamide by adding 20µl of DTT stock. Trypsin digestions were carried out at 37°C overnight with adjusted enzyme amount (the ratio of trypsin to sample was 1:20). The glycopeptides were enriched from the trypsin digested peptide mixture by reversed-phase C18 SPE column. The trypsin digests were loaded onto a C18-Sep-Pac, which had been pre-equilibrated in 5% acetic acid. The sample was eluted with 5 mL of 65% acetonitrile in 5% acetic acid; the glycopeptides were present in the flow-through, which was collected, frozen, and lyophilized to dryness.

2.3.PNGase F digestion:

Human serum was subjected to a standard trypsin digestion protocol, followed by PNGase F digestions. Dried tryptic peptides/glycopeptides from human serum and mixture of RB, Fetuin and purified IgGs were dissolved by 300 µl of 50Mm ammonium bicarbonate, and then treated with 500 unit (0.1 µl) of PNGase separately. Aliquots of the digestion mixture were taken from both samples at every 10 minutes and quenched by lowering the pH and quickly heating to 75^{o}C. [Glu1]-Fibrinopeptide B (GluB) was spiked into the samples and served as an internal standard for quantization.

2.4.LC-MS analysis:

These aliquots collected from PNGase F digestion mixture were analyzed by LC-MS using a 2.1 mm ID Penta-HILIC column (AMT) on Shimadzu Nexera LC system interfaced to a Q-trap 4000 MS analyzer (ABSciex). The separation was carried out at a flow rate of 0.4 mL/min at 60^{o}C with a mobile phase A consisting of 95% H_2O/CAN with 50mM ammonium formate (adjusted to pH 4.4 with formic acid) and mobile phase B being pure ACN. A segmented linear gradients were employed; 1) 62% mobile phase B to 61.2% mobile phase B in 9 min, 2) 61.2% mobile phase B to 60.2% mobile phase B in 10 min, and 3) 60.2% mobile phase B to 58% mobile phase B in 11 min was utilized. In each case, the column was flushed at 25% mobile phase for 5 minutes, before returning to the starting mobile phase composition.
Selected reaction monitoring (SRM) mode on a Q-tap 4000 mass analyzer were utilized to quantitate every known glycoform of each tryptic peptide from the Fetuin, RB, IgGs, Transferrin and alpha 1-acid glycoprotein. In SRM mode, two stages of mass filtering are employed in the triple quadrupole mass spectrometer. In the first stage, ions of interest (precursor ions) are

selected in quadrupole 1 (Q1) and induced to fragment by collisional excitation with neutral gas in a collision cell (Q2). In the second stage, instead of obtaining full scan MS/MS, where all the possible fragment ions derived from the precursor are mass analyzed in Q3, only a small number of specific fragment ions (transition ions) are analyzed in Q3. This targeted MS analysis using SRM enhances the lower detection limit for target analysts. The masses of glycopeptides of Fetuin, RB, IgGs, Transferrin and alpha 1-acid glycoprotein (precursor masses) were predicted by adding the masses of targeted N-glycans to the masses of targeted tryptic peptides containing the N-glycosylation sites of interest. The m/z values used as precursors (Q1 ions) in the SRM experiments performed on the glycopeptide of Fetuin, RB, IgGs, Transferrin and alpha 1-acid glycoprotein are listed in Table S4.1. MS/MS experiments performed on the various glycopeptides revealed that each produced two intense fragment ions. The common fragment at m/z 365.7 corresponds to the oxonium ion of hexose-N-acetylhexoseamine (Hex-HexNAc). The other intense fragment ion corresponds to the complete peptide backbone for the selected precursor, combined with a single N-acetylglucosamine (GlcNAc) attached. These two fragments ions were used in the SRM experiment for all the glycopeptides as Q3 transition ions, and significantly reduced the possibility of false positives. Retention times was found experimentally for all the glycopeptides. The detection window set for each transition is 3 min. A collision energy (CE) of 70 V and declustering potential (DP) of 40 V was selected as an appropriate compromise between selected ion intensity and background current. The dwell time for data collection was set at 100 ms, and unit resolution was used in both Q1 and Q3.

3. Results and Discussions:

3.1.Pseudo First Order Reaction:

PNGase F releases N-linked glycans from the peptide backbone by hydrolyzing the amide group of the asparagine side chain as shown in reaction equation (I). Because PNGase F is an enzyme, which is not consumed during the reaction and therefore its concentration stays relatively constant. The concentration of H_2O is much greater than the concentration of glycopeptide, so the concentration of H_2O can be treated as constant. Therefore, this third order reaction can be predicted to be modeled by pseudo first order process, reaction equation (II). The differential rate equation describing a pseudo first order kinetics is given in equation (III), and its integrated form is shown in equation (IV). In both of these equations, k is the rate constant.

$$Glycopeptide + H_2O + PNGase\ F \rightarrow N\text{-}Glycan + Peptide + PNGaseF \quad (I)$$

$$Glycopeptide \rightarrow N\text{-}Glycan + Peptide \quad (II)$$

$$-d[GP]/dt = k[GP] \quad (III)$$

$$ln[GP] = -kt + ln[GP_0] \quad (IV)$$

The 'concentration of glycopeptide' ([GP]) from each aliquot collected at different time point was calculated by the integrated peak area of glycopeptide divided by the integrated peak area of internal standard—GluB as illustrated in Figure S4.1. The plot of [GP] versus time is illustrated in Figure 4.2. Plotting natural log transformation of 'concentration of glycopeptide' (ln[GP]) with respect to time, as shown in Figure 4.3, gives a straight line, which confirms the PNGase F releasing reaction can be modeled as a pseudo first order reaction. Because of the linear relationship between ln[GP] and reaction time, the deglycosylation process follows a pseudo first order reaction model allowing the rate constants of glycan release to be determined by the slope of ln[GP] vs time plots.

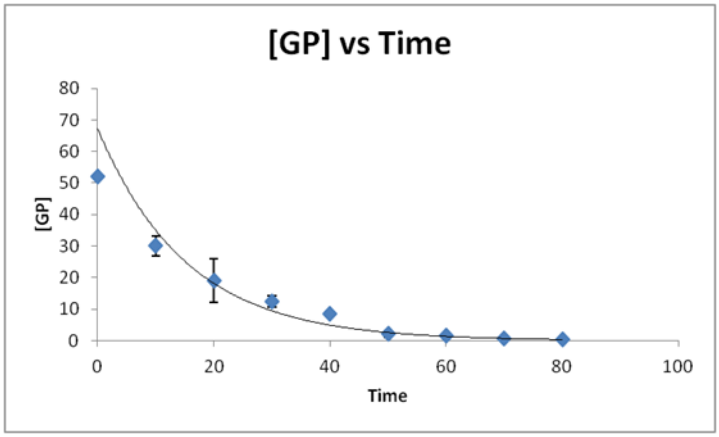


Figure 4.2. The plot of 'concentration of glycopeptide' ([GP]) versus time

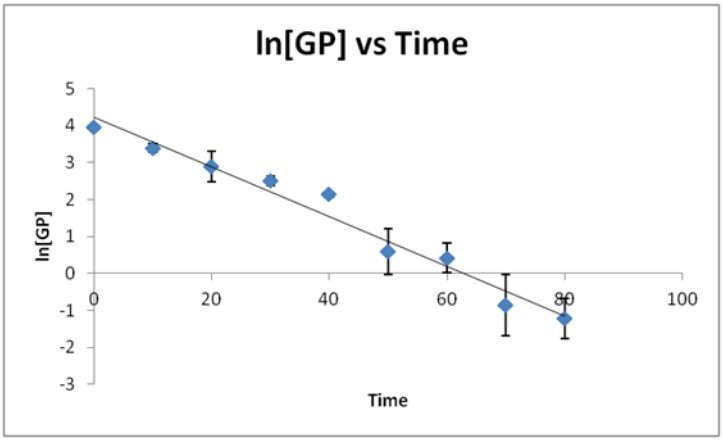


Figure 4.3. The plot of natural log transformation of 'concentration of glycopeptide' (ln [GP]) versus time.

The experiments were conducted separately for two different samples. The first sample is the mixture of glycopeptides from Fetuin, RB and IgGs (sample1); the second sample is trypsin digested human serum (sample2), where three major glycoproteins in human serum: Transferrin, Alpha 1-acid glycoprotein and IgGs were studied. These two samples share one same glycoprotein, which is IgGs, thus, the final results from these two different system were calibrated and compared together.

The glycofroms and peptide backbones of glycopeptides from RB, Fetuin, IgGs and Transferrin are highly heterogeneous (as shown in Figure S4.2). RB bears one glycosylation site, which is primarily occupied with high mannose N-glycans from Man5 to Man9. After trypsin digestion, the resulted tryptic peptide carrying the glycosylation site only has four amino acid (NLTK). Three glycosylation sites of Fetuin are well characterized by previous studies. N-glycans attach on the glycosylation sites of Fetuin are predominantly highly sialylated bi- and tri-antennary complex structures. There're fours subclasses of IgGs: IgG1, IgG2, IgG3, IgG4, However, all of them carry a single N-glycosylation site. The N-glycans found on IgG are predominantly core-fucosylated complex biantennary structure. Some of the glycans carry terminal α2-6 linked sialic acids (SA) and/or a bisecting N-acetyglucosamine (GlcNAc). After trypsin digestion, the glycopeptide backbone sequences of IgG 2/3 (EEQFNSTFR), IgG 4 (EEQFNSTYR) and IgG 1 (EEQYNSTYR) differ by a phenylalanine (F) to tyrosine (Y) substitution at one or two locations. Apo-transferrin from human serum has two well-known glycosylation sites, which generally have bi-antennary complex glycan with one or two terminal sialic acid attached to. Among all the glycopeptides from RB, Fetuin, Trasferrin and IgGs, several of them share same or similar glycoforms but very different peptide backbones, which makes it possible to study the effect of peptide backbone on the PNGase F releasing rate.

The chromatograms of some representative glycopeptide residues left in the sample1 from 0 to 50min are shown in Figure 4.2. The peaks of glycopeptides from Fetuin disappear quickest and have mostly disappeared in an hour. In contrast, the peaks of RB glycopeptides are fairly resistant to deglycosylation and these persist even with an overnight digestion. The disappearance of representative glycopeptide residues of Transferrin (peaks under red arrows), Alpa-1-acid glycoprotein (peaks under blue arrows) and IgGs (all the other peaks besides

Transferrin and Alpa-1-acid glycoprotein) in the sample2 from 0 min to overnight are illustrated in Figure 4.3. The glycopeptides of IgGs were deglycosylated at the highest rate, which completed in less than 1 hour. The deglycosylation of glycopeptides of Alpha-1-acid glycoprotein was accomplish after 4 hours, but non-deglycosylated glycopeptides of Transferrin were still observed in the sample.

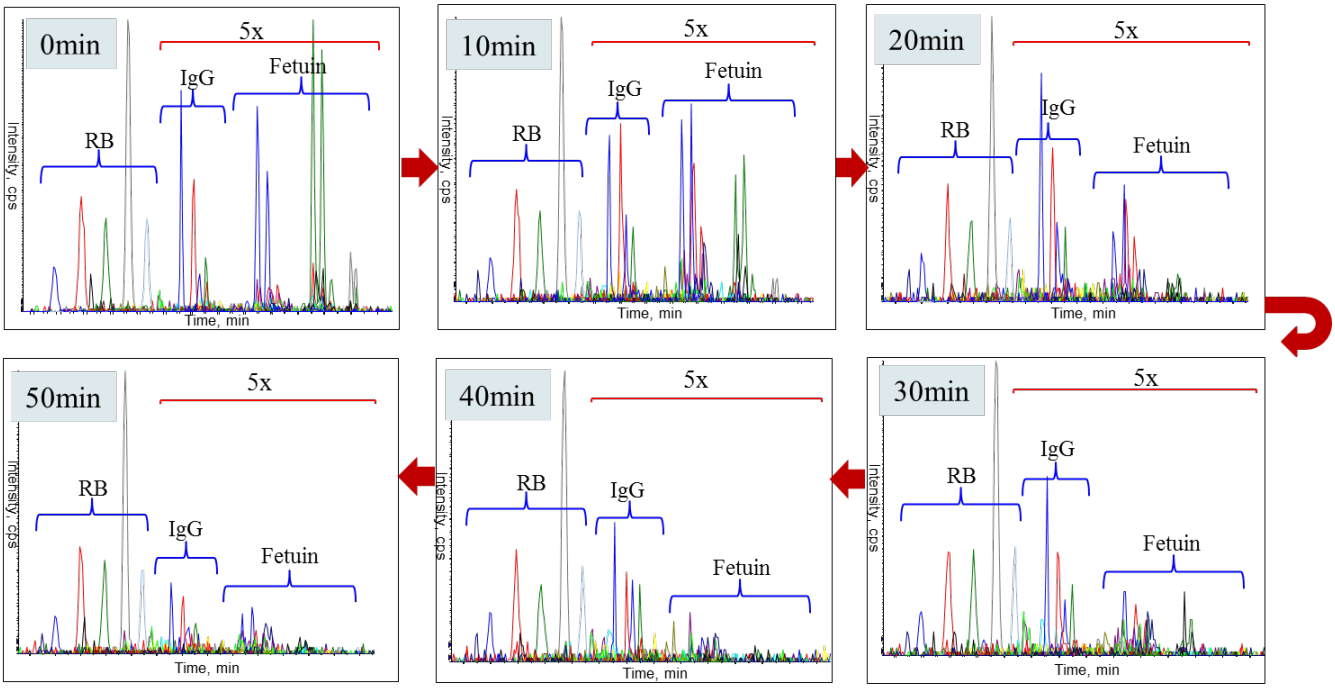


Figure 4.4. The chromatograms of disappearance of representative glycopeptide residues of RB, IgG and Fetuin in the sample from 0 min to 50min.

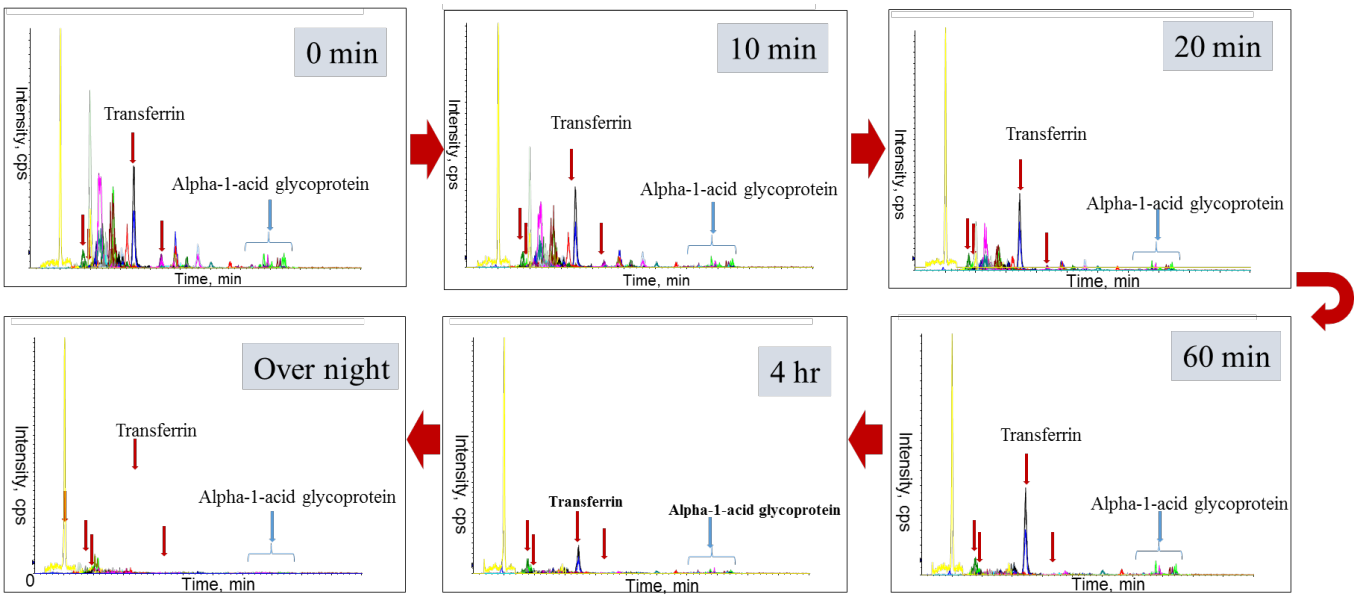


Figure 4.5. The chromatograms of disappearance of representative glycopeptide residues of Transferrin (peaks under red arrows), Alpa-1-acid glycoprotein (peaks under blue arrows) and IgGs (all the other peaks besides Transferrin and Alpa-1-acid glycoprotein) in the sample from 0 min to overnight.

The relative change of the natural log transformation ($\ln[GP]_t/\ln[GP]_0$) of abundance of representative glycopeptides form RNase B, IgG and Fetuin as functions of time during PNGase F digestion are shown in Figure 4.6. The slopes of the three linear lines are obviously very different. The slop of the blue line (representative glycopeptide of RNase B) almost equal to zero, which means the amount of glycopeptide was stay constant after treated with PNGase F for 150 min. The green line (representative glycopeptide of Fetuin) has the steepest slope, while the red line (representative glycopeptide of IgGs) has the moderate slope.

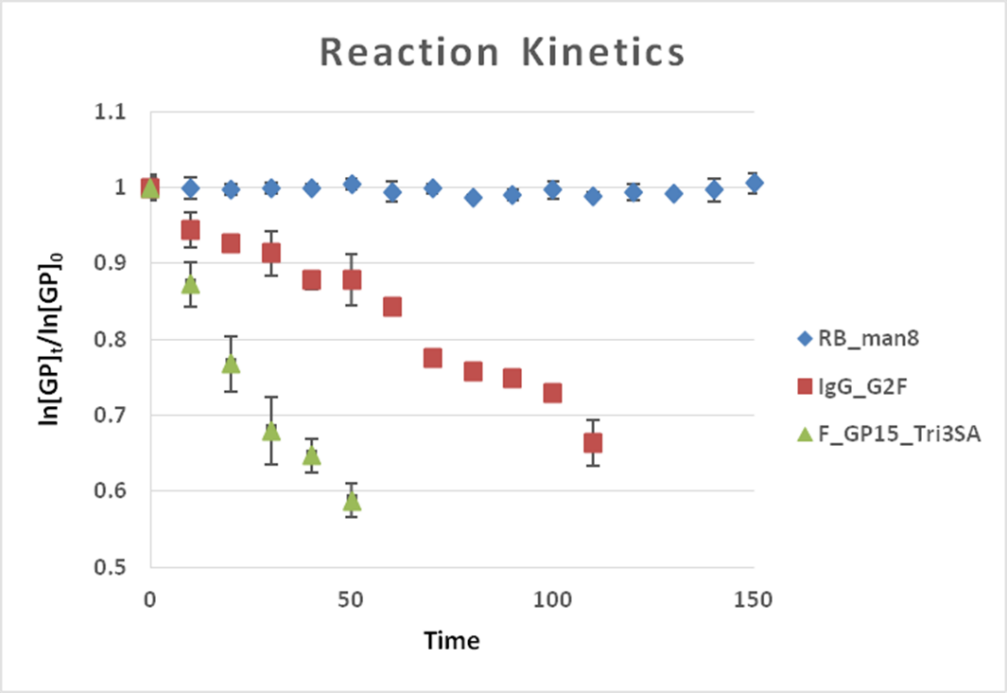
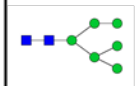
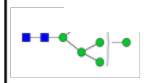
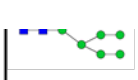


Figure 4.6. The relative change of the natural log transformation ($ln[GP]_t/ln[GP]_0$]) of abundance of representative glycopeptides form RNase B, IgG and Fetuin as functions of time during PNGase F digestion

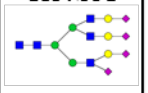
The changes of ln[GP] of glycopeptides from Fetuin, Ribonuclease B and IgGs are plotted as functions of time in FigureS4.3 to Figure4.5 respectively. The slopes of the linear lines (ln[GP] vs time), which are the rate constant of PNGase F releasing, are summarized in Table 4.1. From the slopes of linear lines of ln[GP] vs time, generally the glycopeptides from different glycoproteins seem to have very different slopes, which means their deglycosylation rates by PNGase F are quite different. For glycopeptides with same peptide backbones but different categorical glycofroms have been studied by the model of glycopeptides from IgGs, where fucosylated glycopeptides seem to have the highest glycosylation rate (steepest slope) comparing

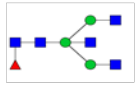
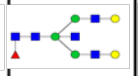
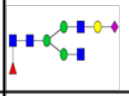
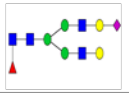
to other categorical glycopeptides. For glycopeptides with same or similar glycoforms but very different peptide backbones, the amino acid sequence flanking the glycosylation site influences the release rate as can be seen by the glycopeptides (in red) from Transferrin and Fetuin which share the same glycan (Bi2SA) but have different peptide sequences, where the PNGase F releasing rate of glycopeptide of Transferrin is 60 times slower than the glycopeptide of Feutin, as well as the glycopeptides (in blue) from IgGs and Transferrin which share the similar glycans (Bi2SA and Bi2SAF) but very different peptide sequences.

Table 4.1. Summary of rate constants

RNase B	Man5 	Man6 	M 		
NLTK	0.00072	0.00292	0.00047	0.00014	0.00096

Transferrin	BiSA 	Bi2SA 
CGLVPVLAENYNK	0.00169	0.00072
QQQHLFGSNVTDCSGNFCLFR	0.00378	NA

Fetuin	Bi2SA 	Tri3SA 	Tri4SA 
DVRKLCPDCPLLAPLNDSR	0.04508	0.07887	0.12543
PTGEVYDIEIDTLETTCHVLDPTPLANCSVR	NA	0.03862	NA

	Fucosylated			Nonfucosylated			Bisecting			Sialylated		
IgG	G0F 	G1F 	G2F 	G0 	G1 	G2 	G0FN 	G1FN 	G2FN 	G1FS 	G2FS 	G2FNS 
EEQYNSTYR	0.01672	0.01805	0.01949	0.01400	0.01919	0.01359	NA	0.01031	0.01050	0.00691	0.01006	0.01156
EEQFNSTFR	0.01956	0.02003	0.01569	0.01613	0.01422	0.01203	0.01197	0.00688	0.00906	0.00772	0.01047	0.01078
EEQFNSTYR	0.02131	0.01956	0.01459	0.02166	0.01594	0.01238	NA	0.01075	NA	0.00966	0.00981	NA

3.2.Statistics test:

Mann-Whitney test was utilized here to investigate if the differences in the rate constants between glycopeptides with different glycoforms and peptide backbones are statistically significant. Mann-Whitney test is a nonparametric test of the null hypothesis that two samples have equal sample mean against an alternative hypothesis that the sample means from two samples are not equal. Unlike the two sample *t*-test, it does not require the assumption of normal distributions and equal variance, which is more appropriate in this case. The calculation process of test statistic and p-value of Mann-Whitney test is illustrated in Appendix C.

The results between glycopeptides of RNase B, Transferrin, IgG and Fetuin were compared by Mann-Whitney test and found to be significantly different (listed in Table 4.2). The order of the PNGase F releasing rate from quirkiest to slowest is Fetuin, IgGs, Transferrin and RB. The results of Man-Whitney test for IgG glycopeptides are listed in Table 4.3. The results from every glycoform of IgG1 and IgG2/3 were compared and not found to be statistically different. However, the rate constants of glycans from different categories are found to be significantly different. Compared to nonfucosylated, bisecting-GlcNAc and sialylated glycans, fucosylated glycans are released from IgGs glycopeptides at higher rate. In other works, both bisecting-GlcNAc and terminal sialic acid have a negative effect on the PNGase F releasing rate. For example, a single sialic acid residue was found to decrease the rate by a factor of 3.

Table 4.2. The results of Mann-Whitney test between glycopeptides of RNase B, Transferrin, IgG and Fetuin

Comparison	p-value	Result
k(RB) vs k(Transferrin)	<0.001	k(RB)<k(Transferrin)
k(Transferrin) vs k(IgG)	<0.001	k(Transferrin)<k(IgG)
k(IgG) vs k(Fetuin)	<0.001	k(IgG)<k(Fetuin)
k(RB)<k(Transferrin) <k(IgG)<k(Fetuin)		

Table 4.3. The results of Man-Whitney test for IgG glycopeptides

Comparison	p-value	Result
k(IgG1) vs k(IgG2/3)	0.83	k(IgG1)=k(IgG2)
k(Fuc.) vs k(non-Fuc.)	0.002	k(non-Fuc.)<k(Fuc.)
k(Fuc.) vs k(Bisec.)	<0.001	k(Bisec.)<k(Fuc.)
k(Fuc.) vs k(Sia.)	<0.001	k(Sia.)<k(Fuc.)

4. Conclusion:

The PNGase F releasing rate of glycopeptide with different peptide backbones and glycoforms are significantly different. Slight differences in amino acid sequences were not found to cause a statistically different deglycosylation rate (i.e. IgG subclasses). Significant differences in the deglycosylation rate constants were found between glycopeptides with very different peptides sequences but the same glycan structure. Significant differences in the deglycosylation

rate constants were observed between glycopeptides differing only in glycan structure. We predict the differences in release kinetics can lead to significant in quantitative variations.

5. Acknowledgements:

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CHAPTER 5

CONCLUSION

Glycosylation is one of the most important types of protein posttranslational modifications. Among all the biologically important proteins, over half of them are glycosylated. The analysis of protein glycosylation is necessary to improve our understanding of various biological processes, as well as to facilitate correlation of glycan structures with healthy and disease states.

There are two general approaches to characterize N-glycans using LC/MS: glycoprofiling of liberated glycans or glycopeptides from glycoprotein. In contrast to released glycan analysis, the key attractive feature of glycopeptide-based anlaysis is its ability of link glycosylation information to exact locations (glycosylation site) on glycoproteins. Often the identification of the site of glycan attachment is critical for biological activities.

Hydrophilic functional groups on both the peptide and the glycan interact with the HILIC stationary phase, and thus both peptide and glycan structure determine retention of a glycopeptide. This characteristic enables the resolution of mixtures of closely related glycopeptides. The combination of glycopeptide amino acid sequence and glycan composition permits the resolution of glycoforms, which can extend to even resolving isomeric and positional glycan variants. Thus, LC/MS/MS can provide the site-specific glycans profiles at individual sequence sites from complex mixtures of glycoproteins.

The other general approach to characterize N-glycans using LC/MS is glycoprofiling of liberated glycans from glycoprotein. The most widely used enzyme for releasing intact N-

glycans is Peptide N-glycosidase F (PNGase F). With the method of SRM Quantitation of the glycopeptides from human serum IgGs, it revealed that slight differences in amino acid sequences were not found to cause a statistically different deglycosylation rate; significant differences in the deglycosylation rate constants were observed between glycopeptides differing only in glycan structure (i.e. non-fucosylated, fucosylated, bisecting-GlcNAc, sialylated, etc.). For example, a single sialic acid residue was found to decrease the rate by a factor of 3. Similar reductions in rate were associated with the presence of a bisecting-GlcNAc.

In order to determine if there are other correlations between glycan structure, peptide sequence and the rate of glycan release, we investigate the glycoforms of the other standard glycoproteins (Ribonuclease B, Fetuin, Transferrin and Alpha 1- acid glycoprotein). The experimental results revealed that significant differences in the deglycosylation rate constants were found between glycopeptides with very different peptides sequences but the same glycan structure, as well as between glycopeptides differing only in glycan strucutures. We predict the differences in release kinetics can lead to significant in quantitative variations.

APPENDIX A

SUPPLEMENTAL INFORMATION TO CHAPTER 2:

RESOLVING ISOMERIC GLYCOPEPTIDE GLYCOFORMS WITH HYDROPHILIC INTERACTION CHROMATOGRAPHY (HILIC)

Table S2.1. Predicted m/z values of glycopeptides of Fetuin

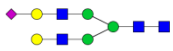
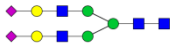
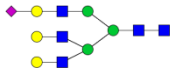
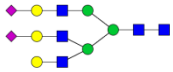
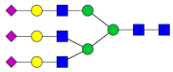
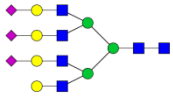
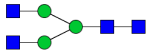
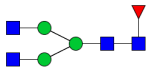
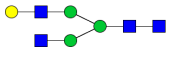
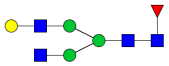
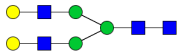
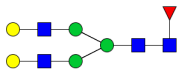
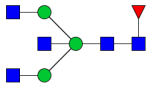
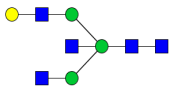
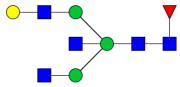
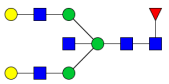
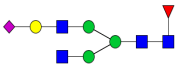
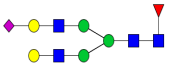
N-Glycan	N-Glycan Structure	LCPDCPLLAPLN*DSR (GP15)	RPGEVYDIEIDTLETTCHVLDPTPLAN*CSR (GP30)	VVHAVEVALATFNAESN*GSYQLVEISR (GP27)
Bi-SA		1218.84(z=3)	1397.11(z=4)	1233.31(z=4)
Bi-2SA		1315.87(z=3)	1469.89(z=4)	1306.09(z=4)
Tri-SA		1340.55(z=3)	1488.40(z=4)	1324.60(z=4)
Tri-2SA		1437.58(z=3)	1249.14(z=5)	1397.37(z=5)
Tri-3SA		1151.21(z=4)	1307.35(z=5)	1470.14(z=5)
Tri-4SA		1253.99(z=4)	1365.57(z=5)	1542.92(z=5)

Table S2.2. Predicted m/z values of glycopeptides of IgGs

N-Glycan	N-Glycan Structure	IgG1 EEQYN*STYR	IgG2/3 EEQFN*STFR	IgG4 EEQFN*STYR
H3N4		1244.65(z=2)	1228.60(z=2)	1236.55(z=2)
H3N4F1		1317.70(z=2)	1301.65(z=2)	1309.60(z=2)
H4N4		1325.65(z=2)	1309.60(z=2)	1317.55(z=2)
H4N4F1		1398.70(z=2)	1382.65(z=2)	1390.60(z=2)
H5N4		1406.65(z=2)	1390.60(z=2)	1398.55(z=2)
H5N4F1		1479.70(z=2)	1463.65(z=2)	1471.75(z=2)
H3N5F1		1419.25(z=2)	1403.20(z=2)	1411.15(z=2)
H4N5		1427.05(z=2)	1411.00(z=2)	1418.95(z=2)
H4N5F1		1000.50(z=3)	1484.35(z=2)	1492.15(z=2)
H5N5F1		1054.50(z=3)	1043.80(z=3)	1049.1(z=3)
H4N4F1S1		1029.80(z=3)	1019.10(z=2)	1024.50(z=3)
H5N4F1S1		1083.80(z=3)	1073.10(z=2)	1078.40(z=3)

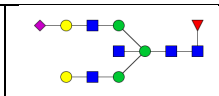
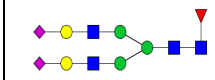
H5N5F1S1		1151.50(z=3)	1140.80(z=2)	1146.10(z=3)
H5N4F1S2		1180.80(z=3)	1170.10(z=2)	1175.40(z=3)

Table S2.3. Retention times of HILIC separation of glycopeptides of IgGs

Glycan composition	**Retention time(min)**					
	IgG 1	**SD(n=10)**	**IgG 2/3**	**SD(n=10)**	**IgG 4**	**SD(n=10)**
H3N4	10.03	0.104	8.14	0.171	8.98	0.142
H3N4F1	11.18	0.189	9.13	0.116	10.04	0.159
H4N4	11.50	0.196	10.09	0.138	11.14	0.167
H4N4F1	12.78	0.170	10.49	0.154	11.51	0.156
H5N4	13.49	0.226	11.57	0.146	12.73	0.174
H3N5F1	12.18	0.220	9.87	0.104	10.89	0.182
H4N5	12.35	0.215	10.91	0.188	N/A	N/A
H5N4F1	14.97	0.240	12.33	0.176	13.54	0.188
H4N5F1	13.49	0.200	11.25	0.140	12.39	0.174
H4N4F1S1	21.80	0.304	18.39	0.268	20.10	0.315
H5N5F1	16.02	0.233	13.26	0.212	N/A	N/A
H5N4F1S1	24.00	0.272	20.66	0.302	22.48	0.318
H4N5F1S1	27.47	0.383	N/A	N/A	N/A	N/A
H5N5F1S1	20.17	0.303	22.07	0.327	N/A	N/A
H5N4F1S2	19.41	0.273	23.38	0.369	N/A	N/A

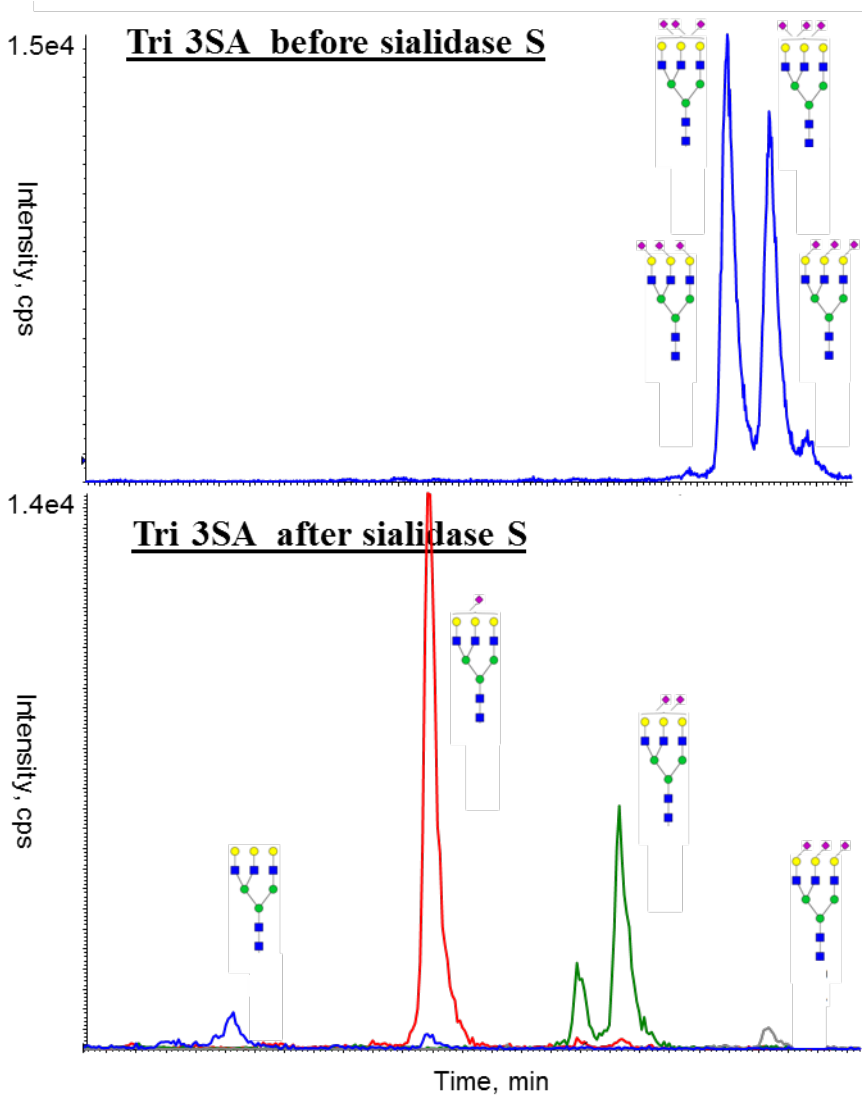


Figure S2.1. Sialidase S digestion of glcopeptide Tri_3SA of Fetuin

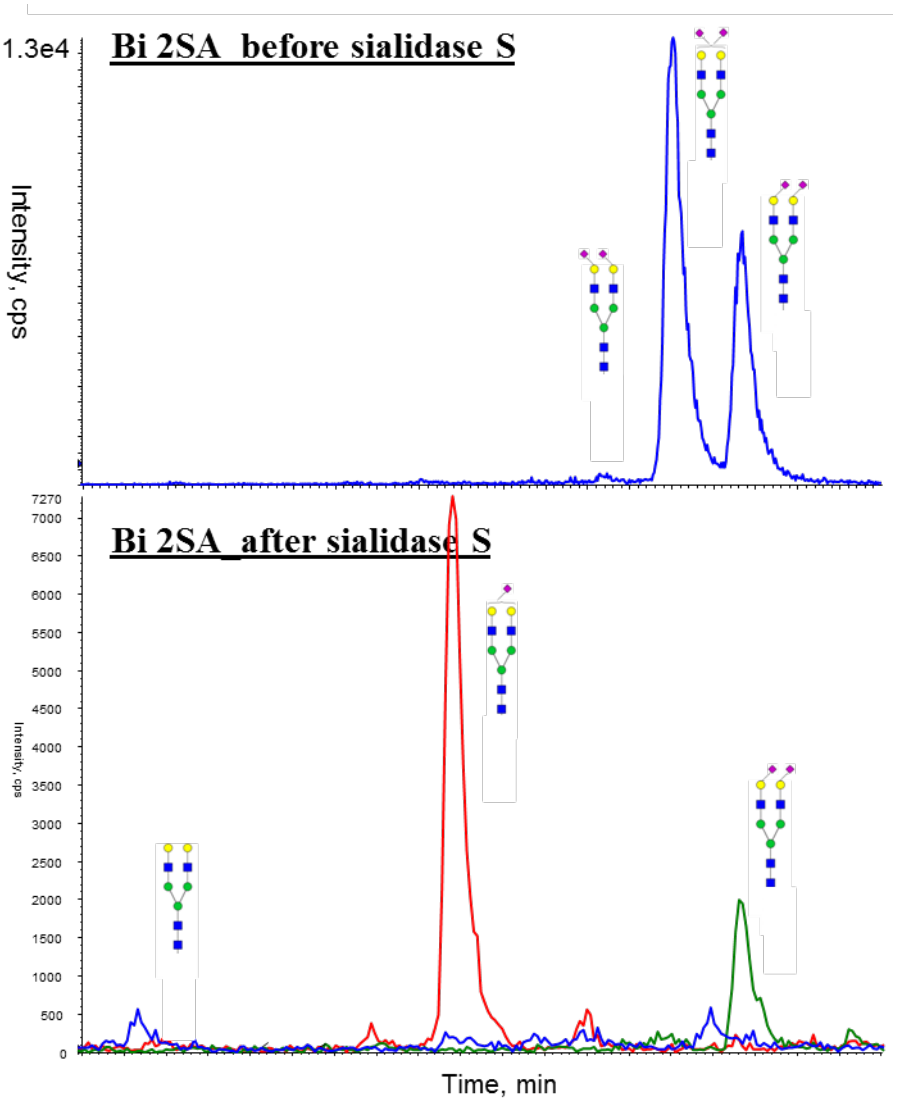


Figure S2.2. Sialidase S digestion of glcopeptide Bi_2SA of Fetuin

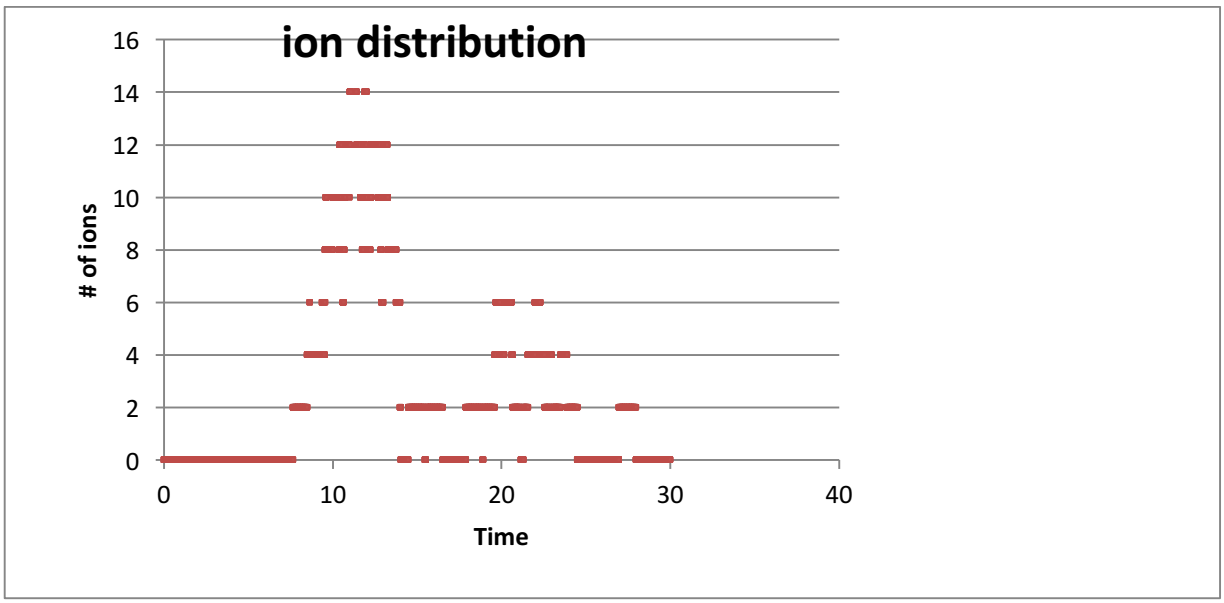


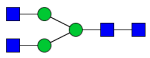
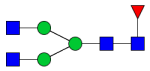
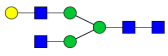
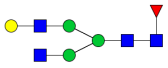
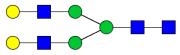
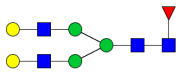
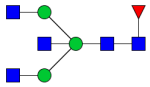
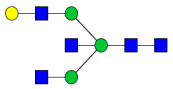
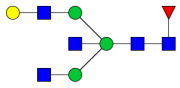
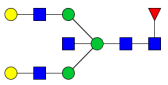
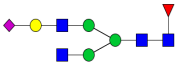
Figure S2.3. Ion distribution of scheduled SRM for detection of glycopeptides of IgGs

APPENDIX B

SUPPLEMENTAL INFORMATION TO CHAPTER 3:

STUDYING THE KINETICS OF N-GLYCAN RELEASE BY PNGASE F WITH SRM QUANTITATION OF THE GLYCOPEPTIDES FROM HUMAN SERUM IMMUNOGLOBULIN G

Table S3.1. Predicted m/z values of glycopeptides of IgGs

N-Glycan	N-Glycan Structure	IgG1 EEQYN*STYR	IgG2/3 EEQFN*STFR	IgG4 EEQFN*STYR
H3N4		1244.65(z=2)	1228.60(z=2)	1236.55(z=2)
H3N4F1		1317.70(z=2)	1301.65(z=2)	1309.60(z=2)
H4N4		1325.65(z=2)	1309.60(z=2)	1317.55(z=2)
H4N4F1		1398.70(z=2)	1382.65(z=2)	1390.60(z=2)
H5N4		1406.65(z=2)	1390.60(z=2)	1398.55(z=2)
H5N4F1		1479.70(z=2)	1463.65(z=2)	1471.75(z=2)
H3N5F1		1419.25(z=2)	1403.20(z=2)	1411.15(z=2)
H4N5		1427.05(z=2)	1411.00(z=2)	1418.95(z=2)
H4N5F1		1000.50(z=3)	1484.35(z=2)	1492.15(z=2)
H5N5F1		1054.50(z=3)	1043.80(z=3)	1049.1(z=3)
H4N4F1S1		1029.80(z=3)	1019.10(z=2)	1024.50(z=3)

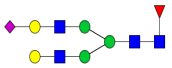
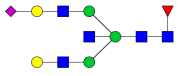
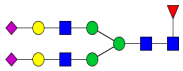
H5N4F1S1		1083.80(z=3)	1073.10(z=2)	1078.40(z=3)
H5N5F1S1		1151.50(z=3)	1140.80(z=2)	1146.10(z=3)
H5N4F1S2		1180.80(z=3)	1170.10(z=2)	1175.40(z=3)

Table S3.2. Retention times of HILIC separation of glycopeptides of IgGs

Glycan composition	**Retention time(min)**					
	IgG 1	**SD(n=10)**	**IgG 2/3**	**SD(n=10)**	**IgG 4**	**SD(n=10)**
H3N4	10.03	0.104	8.14	0.171	8.98	0.142
H3N4F1	11.18	0.189	9.13	0.116	10.04	0.159
H4N4	11.50	0.196	10.09	0.138	11.14	0.167
H4N4F1	12.78	0.170	10.49	0.154	11.51	0.156
H5N4	13.49	0.226	11.57	0.146	12.73	0.174
H3N5F1	12.18	0.220	9.87	0.104	10.89	0.182
H4N5	12.35	0.215	10.91	0.188	N/A	N/A
H5N4F1	14.97	0.240	12.33	0.176	13.54	0.188
H4N5F1	13.49	0.200	11.25	0.140	12.39	0.174
H4N4F1S1	21.80	0.304	18.39	0.268	20.10	0.315
H5N5F1	16.02	0.233	13.26	0.212	N/A	N/A
H5N4F1S1	24.00	0.272	20.66	0.302	22.48	0.318
H4N5F1S1	27.47	0.383	N/A	N/A	N/A	N/A
H5N5F1S1	20.17	0.303	22.07	0.327	N/A	N/A
H5N4F1S2	19.41	0.273	23.38	0.369	N/A	N/A

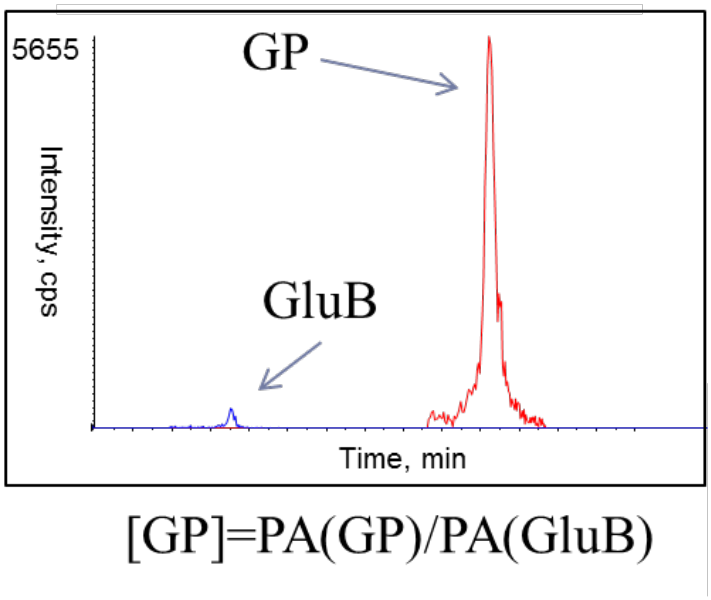


Figure S3.1. The 'concentration of glycopeptide' ([GP]) from each aliquot collected at different time point was calculated by the integrated peak area of glycopeptide divided by the integrated peak area of internal standard—GluB.

Mann-Whitney Test: test differences between two populations based on ranks

Two random samples:

Sample 1: $X_1, X_2, \ldots X_n$ of size n

Smaple 2: $Y_1, Y_2, \ldots Y_n$ of size m

N=n+m

Sample1	Sample2	Rank
0.0755		1
0.0775		2
	0.083	3
0.0903		4
	0.0982	5
	0.1045	6

Test Statistic: $T = \sum_{i=1}^{n} R(Xi)$

T'=n(N+1)-T

Two-Tailed Test:$H_{0:}$ E(X)=E(Y)

$H_{a:}$ E(X)≠E(Y)

$$p-value = 2 \times p\left(z \le \frac{T(or\,T') + \frac{1}{2} - n\frac{N+1}{2}}{\sqrt{\frac{nm(N+1)}{12}}} \right)$$

Where, use smaller T or T'

At level of $\alpha = 0.05$, H_0 is rejected when p-value < 0.05

If H_0 is rejected, then compare μ(X) and μ(Y) to determine big or small.

APPENDIX C

SUPPLEMENTAL INFORMATION TO CHAPTER 4:

STUDYING THE KINETICS OF N-GLYCAN RELEASE BY PNGASE F WITH SRM QUANTITATION OF THE GLYCOPEPTIDES FROM STANDARD GLYCOPROTEINS

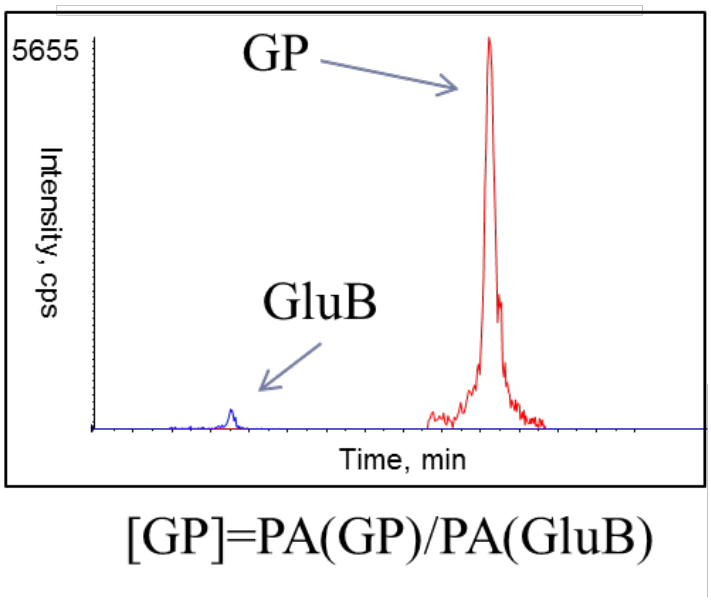
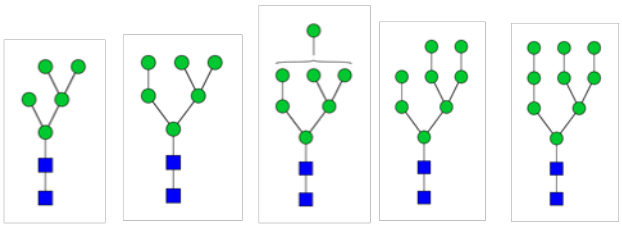


Figure S4.1. The 'concentration of glycopeptide' ([GP]) from each aliquot collected at different time point was calculated by the integrated peak area of glycopeptide divided by the integrated peak area of internal standard—GluB.

Ribonuclease B:

NLTK

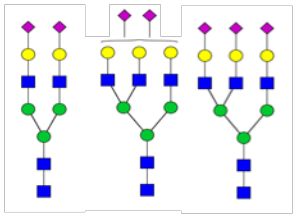


Fetuin:

DVRKLCPDCPLLAPLNDSR

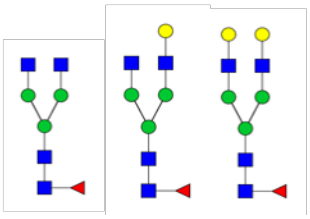
PTGEVYDIEIDTLETTCHVLDPTPLANCSVR

VVHAVEVALATFNAESNGSYLQLVEISR



IgG1:

EEQYNSTYR



Transferrin:

CGLVPVLAENYNK

QQQHLFGSNVIDCSGNFCLFR

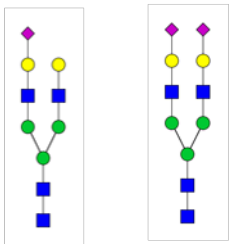


Figure S4.2. Tryptic peptide backbones containing glycosylation sites and glycoforms of glycopeptides from Ribonuclease B, Fetuin, IgG1 and Transferrin

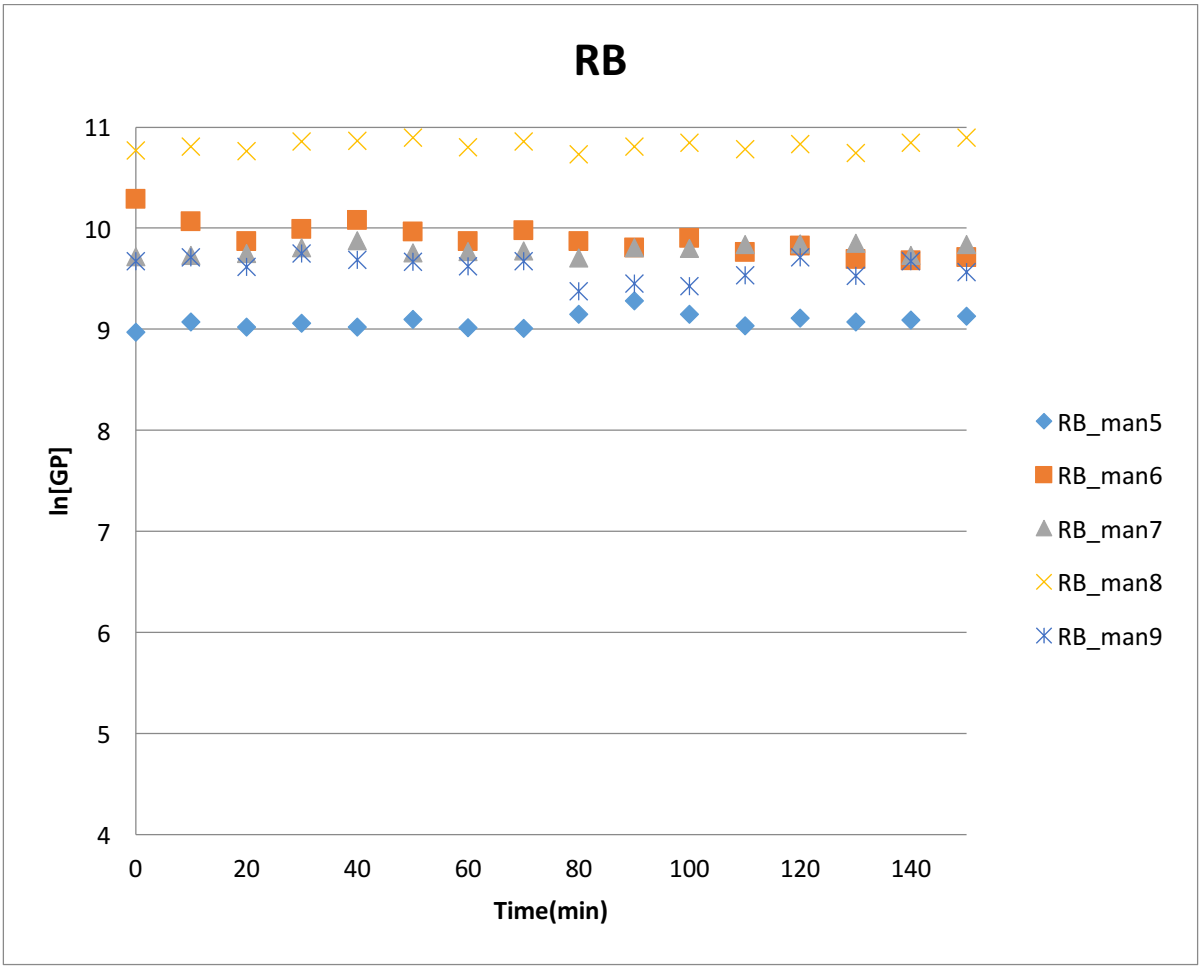


Figure S4.3. The changes of ln[GP] of glycopeptides from Ribonuclease B as function of time

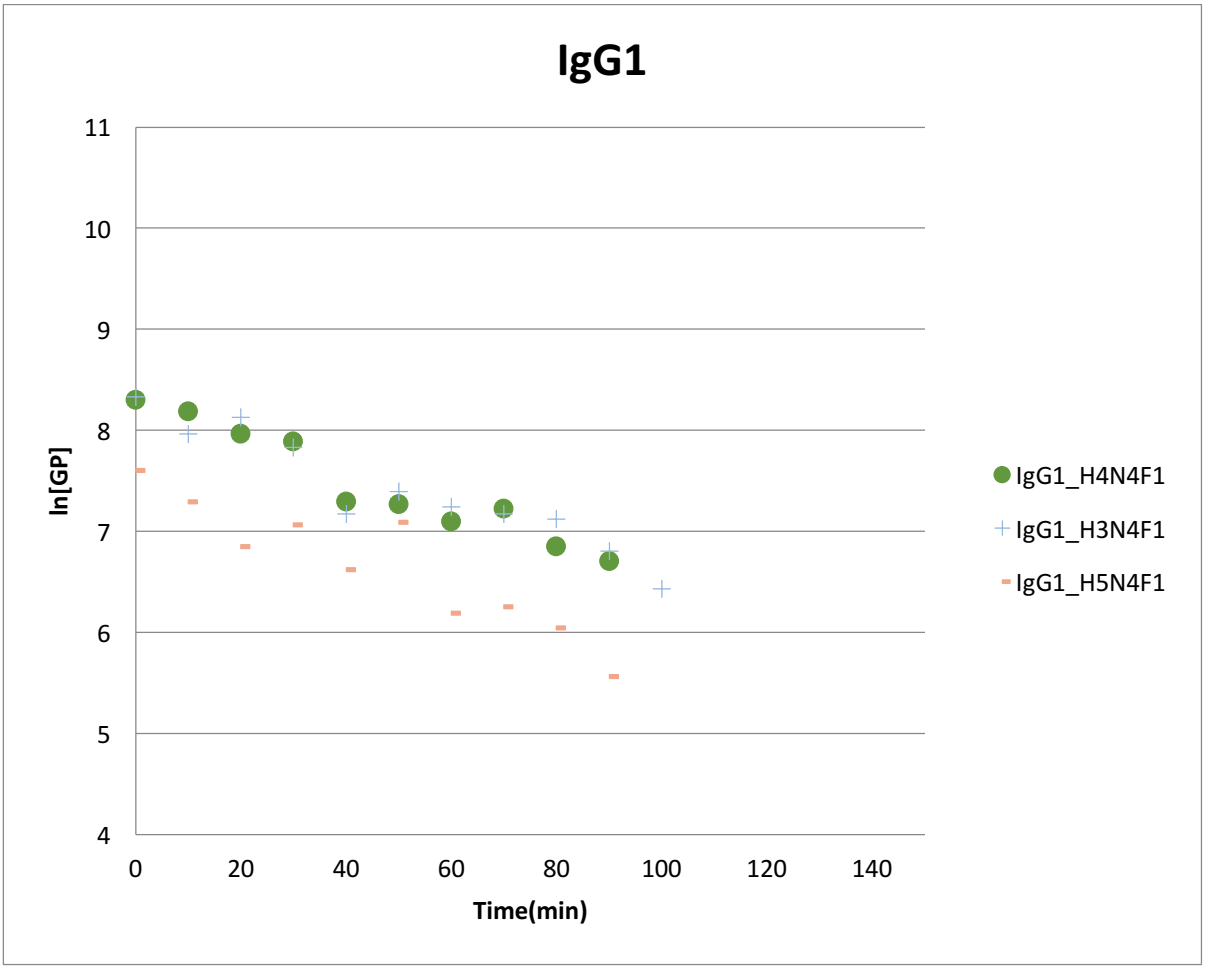


Figure S4.4. The changes of ln[GP] of glycopeptides from IgG1 as function of time

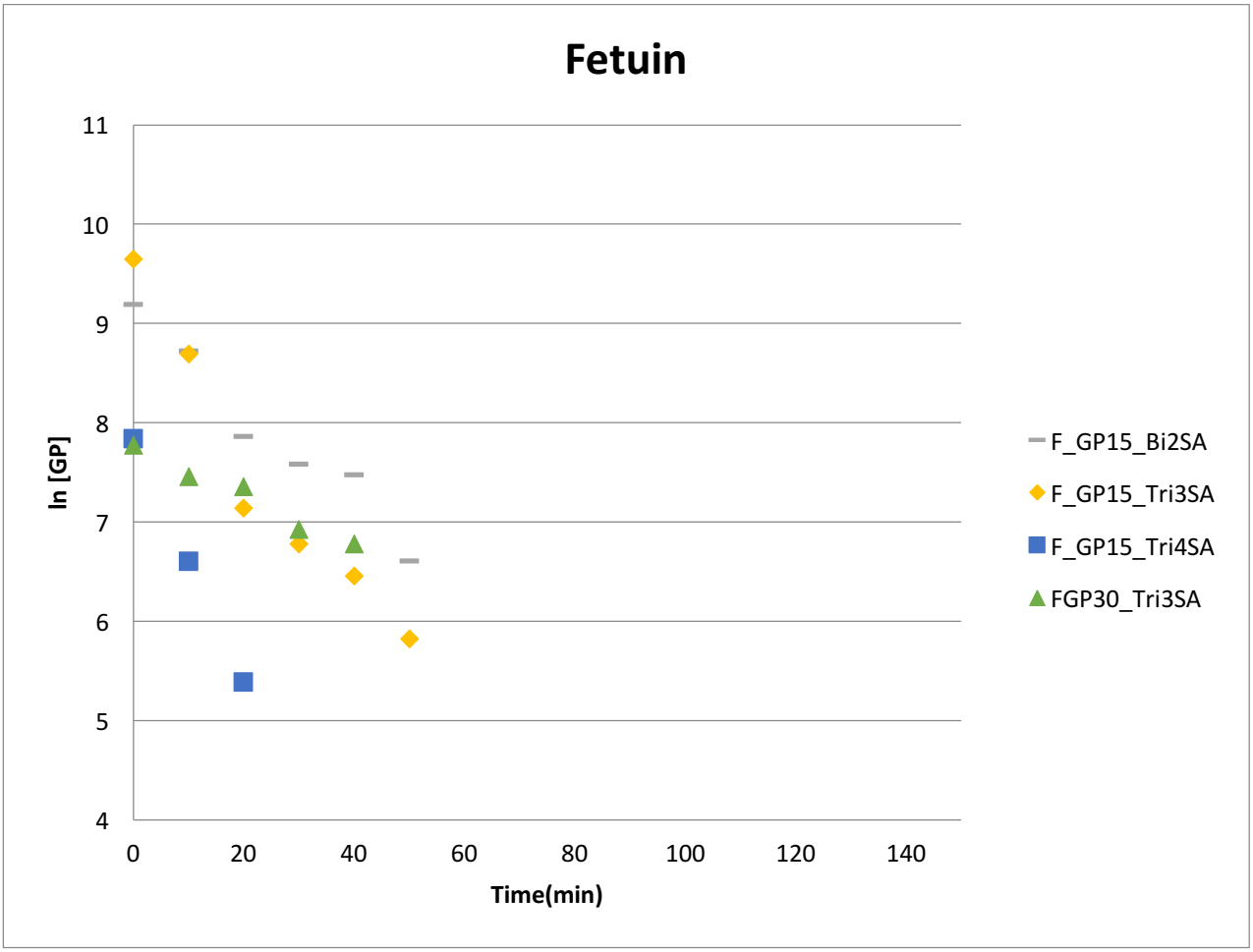


Figure S4.5. The changes of ln[GP] of glycopeptides from Fetuin as function of time

Mann-Whitney Test: test differences between two populations based on ranks

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Smaple 2: $Y_1, Y_2, \ldots Y_n$ of size m

N=n+m

Sample1	Sample2	Rank
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	0.083	3
0.0903		4
	0.0982	5
	0.1045	6

Test Statistic: $T = \sum_{i=1}^{n} R(Xi)$

T'=n(N+1)-T

Two-Tailed Test:$H_{0:}$ E(X)=E(Y)

$H_{a:}$ E(X)≠E(Y)

$$p - value = 2 \times p\left(z \le \frac{T(orT') + \frac{1}{2} - n\frac{N+1}{2}}{\sqrt{\frac{nm(N+1)}{12}}} \right)$$

Where, use smaller T or T'

At level of $\alpha = 0.05$, H_0 is rejected when p-value < 0.05

If H_0 is rejected, then compare $\mu(X)$ and $\mu(Y)$ to determine big or small.