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Comparison of Glycopeptide Fragmentation by Collision Induced Dissociation and Ultraviolet Photodissociation

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Abstract

A comparison of the fragmentation pathways of both protonated and deprotonated O-linked glycopeptides from fetuin and κ -casein obtained upon collision induced dissociation (CID) and 193 nm ultraviolet photodissociation (UVPD) in a linear ion trap is presented. A strategy using non-specific pronase digestion, zwitterionic hydrophilic interaction liquid chromatography (ZIC-HILIC) solid phase extraction (SPE) enrichment, and nano-liquid chromatography (nano-LC) is employed. UVPD of deprotonated glycopeptides generally produced the greatest array of fragment ions, thus affording the most diagnostic information about both glycan structure and peptide sequence. In addition, UVPD generated unique fragment ion such as Y-type ions arising from cleavage at the N-terminus of proline. CID and UVPD of protonated glycopeptides produced fragment ions solely from glycan cleavages.

Introduction

Glycosylation is the most abundant post-translational modification and contributes to the functional roles of proteins that are related to cell adhesion, signaling, immune response, and inflammation.¹⁻³ In addition, there have been significant inroads in understanding the impact of the glycosylation status of proteins on human diseases such as cancer and inflammatory diseases.⁴⁻⁷ Despite the prevalence of glycosylation, it is estimated that only ~10% of glycoproteins have been characterized.⁸ In order to understand the role of glycosylation, systematic characterization of this modification is required. Tandem mass spectrometry has become one of the most versatile approaches for elucidating the glycoproteome, typically via sequencing the glycan and protein portions via bottom-up strategies.^{9,10}

Glycosylation analysis is especially challenging due to the complexity that arises from the heterogeneity of glycosylation, including the formation of branched glycan structures (unlike the linear sequences of proteins and nucleic acids). Moreover, variations in glycosylation sites and occupancy, as well as differences stemming from two dominant types of glycosylation (comprising N- and O-linkages), further complicate glycosylation

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characterization.^{2,11} Although cells may produce the same protein sequences with the same glycosylation sites, even then the glycans can be attached via different branching patterns and intersaccharide linkages.^{3,11} With respect to the glycan attachment sites, N-linked glycosylation occurs at specific sequences: Asp-Xxx-Ser/Thr (where the Asp side chain is the glycosylation site, and Xxx can be any amino acid except proline). In contrast, O-linked glycans may be attached to serine or threonine without specific sequons to help identify their locations in proteins. The differences in the attachment sites and constituent complexity of N-glycans versus O-glycans has motivated the development of different tandem mass spectrometric methods for their analysis.³ For example, N-linked glycans are typically substantially larger than O-linked glycans and can be selectively cleaved from proteins with peptide-N-glycosidase F (PNGase F).³ In addition, the known N-X-S/T amino acid sequon streamlines the mapping of the N-linked glycans because they are restricted to attachment at a well-defined amino acid sequence. For these reasons, N-glycosylation analysis has generally focused more heavily on elucidating the glycan structures, not the glycosylation sites. In contrast, the smaller size of O-glycans and less specific sites of attachment (in fact, conceivably over 10% of residues can be O-glycosylated based on the frequency of Thr and Ser amino acids in proteins) means that identifying the O-glycosylation sites is usually the greater challenge.

Collision-induced dissociation (CID) has been the most popular MS/MS technique for glycopeptide analysis,¹¹⁻¹⁷ but it typically causes only glycosidic cleavages that are useful for assigning the glycan portions but not for sequencing the peptide portions or pinpointing the glycosylation sites. CID also produces some reporter ions such as m/z 204 ([HexNAc + H]⁺), 292 ([NeuNAc + H]⁺), and 366 ([Hex-HexNAc + H]⁺) which are diagnostic for the presence of glycan modifications. These fragments are dominant because CID preferentially produces Y- and B-type glycosidic cleavage ions for glycopeptides¹⁰ in which B-type ions contain the non-reducing end of the glycan and Y-type ions contain the reducing end. The peptide sequences of the glycopeptides may be deduced after CID cleavage of the glycan portion, albeit requiring more elaborate multi-step MS³ strategies.¹⁸ Electron-based dissociation techniques including electron transfer dissociation (ETD)^{13,19-25} and electron capture dissociation (ECD)²⁶⁻³⁰ have also been employed to analyze glycopeptides. Interestingly, the electron-based methods predominantly result in formation of peptide sequence ions, not the glycan ions created by CID. In addition, retention of the glycan moieties by the c-type ions allows facile determination of the glycosylation sites of the peptides.^{30,31} Although more data intensive, pairing CID with electron-based MS/MS methods provides a compelling route for obtaining both glycan and peptide information.^{23,28,32} Photon-based activation methods including infrared multiphoton dissociation (IRMPD)^{12,26-28} and ultraviolet photodissociation (UVPD)³³ also have shown promising results for characterization of glycopeptides. For example, Lebrilla *et al.* reported the use of IRMPD for analysis of N-linked glycopeptides from mucin-type glycoproteins, generating spectra that displayed peptide fragments in addition to products from glycosidic bond cleavages.³² Reilly and coworker utilized UVPD using 157 nm photons to obtain both glycan and peptide sequence information from rich spectra containing x-, v-, w-, and y-type peptide fragment ions that retained the glycan as well as both glycosidic fragments and cross-ring cleavage products from N-linked glycopeptides.³³ We demonstrated 193 nm

UVPD for the characterization of acidic O-linked glycopeptide anions produced upon trypsin/Glu-C digestion of O-glycoproteins.³¹

The MS/MS methods used for protein identification have most commonly been applied for positively charged ions because trypsin is the most frequently used protease and yields Arg- or Lys-terminated peptides amenable to protonation. However, trypsin digestion is not as favorable for glycoproteins in general because the bulky glycans may hinder proteolysis and the low distribution of basic residues around the glycan site may lead to production of overly large peptides, impeding bottom-up strategies. Moreover, acidic glycans, such as those containing sialic acid, may suppress protonation. For these reasons, several groups have explored the characterization of glycopeptides in the negative mode.^{12,14,18,31,34-36} For example, Lebrilla and coworkers reported that IRMPD of deprotonated O-glycopeptide ions resulted in Ser- and Thr-specific side-chain losses that proved useful for pinpointing glycosylation sites.^{12,34} Another effort to overcome the shortcomings of trypsin entails the use of pronase which is a non-specific protease.^{12,32,34,36,37} Pronase is a mixture of several proteases that leads to more extensive digestion of proteins into smaller peptides containing fewer amino acids. Because of the non-specificity of proteolytic cleavage using pronase, there is no expected amino acid terminus or length of peptide after pronase digestion. The Lebrilla group has reported the use of immobilized pronase to decrease the sizes of the glycopeptide sequences and correspondingly increase their dissociation efficiencies while reducing the array of confounding non-glycosylated peptides.^{12,32,34}

Our group has developed 193 nm UVPD for structural characterization of a variety of biologically-relevant molecules, including proteins³⁸⁻⁴⁰, N-linked glycans⁴¹, lipids⁴², and nucleic acids.⁴³ Several other groups have explored the development and application of UVPD (either using 157 nm or 193 nm photons) as an activation method^{33,44-50}, and the high energy deposited by UV photons makes it an attractive option for biological molecules. UVPD produces rich fragmentation patterns that enhance the confident application of database search algorithms³⁹ and provide diagnostic ions that allow differentiation of subtle modifications of biopolymers. The recent promising results obtained upon the elucidation of glycan anions by UVPD (193 nm),⁴¹ N-linked glycopeptides by UVPD (157 nm),³³ and O-linked glycopeptides by UVPD (193 nm)³¹ motivated our interest in extending this methodology to O-linked glycopeptides created upon pronase digestion of glycoproteins. We report a systematic comparison of CID and UVPD for both positively- and negatively-charged O-linked glycopeptides from fetuin and κ -casein in the present study.

Experimental Section

Reagents

Fetuin, κ -casein, and pronase were purchased from Sigma (St. Louis, MO). ZIC-HILIC SPE column material was obtained from Sequant (Southboro, MA). All other chemicals were purchased from Fisher Scientific (Pittsburgh, PA) and used without any further purification.

Pronase Digestion

The general experimental strategy is illustrated in Figure 1. Each glycoprotein was digested with pronase to cause non-specific digestion and creating a greater array of sizes of peptides.

The usage of pronase instead of more specific proteases such as trypsin and Glu-C was justified based on the frequencies and locations of basic (R,K) and acidic (D,E) residues in each protein. For example, bovine κ -casein has many acidic residues around the O-linked glycosylation sites. In contrast, bovine fetuin has only a limited number of basic and acidic residues around the O-glycosylation sites. The low frequency of cleavable residues result in overly large glycopeptides, hampering their efficient dissociation by conventional ion activation methods. In order to overcome this factor, pronase was used as an alternative to trypsin or Glu-C.

Fetuin and κ -casein were reduced by adding 200 mM dithiothreitol (DTT) in 100 mM of NH_4HCO_3 . These solutions were incubated for 10 min in boiling water for denaturation. Then the proteins were alkylated with 300 mM of iodoacetamide solution for 1 hour at room temperature in the dark. For pronase digestion, pronase (1 mg/mL) was added at ratio of 1:50 (pronase/protein w/w) followed by incubation at 37°C for overnight.

ZIC-HILIC SPE

The resulting glycopeptides were enriched using a ZIC-HILIC SPE column prior to separation via C_{18} reversed phase nanoHPLC and then analysis by CID and UVPD in both the positive and negative modes. A self-packed zwitterionic hydrophilic interaction liquid chromatography (ZIC-HILIC) SPE column (25 mg of packing material) was used, following the manufacturer's protocol was followed. The column was washed with 2×1 mL of water, and the washed column was primed with 1 mL of 80% acetonitrile/15% water/5% formic acid. 20 μg of dried glycopeptides were dissolved in 1 mL of 80% acetonitrile/15% water/5% formic acid, and loaded onto the primed column. The column was washed with 2×1 mL of 80% acetonitrile/15% water/5% formic acid. Finally, the glycopeptides were eluted with 500 μL of water. The enriched glycopeptides were dried prior to LC-MS analysis.

Liquid Chromatography and Mass Spectrometry

The glycopeptides were separated by using a Dionex Acclaim PepMap RSLC C_{18} analytical column ($75 \mu\text{m} \times 15$ cm, $2 \mu\text{m}$ particle size). The mobile phases consisted of solvent A (0.05% acetic acid in water) and B (0.05% acetic acid in acetonitrile) applied as a gradient from 5% to 30% solvent B at a flow rate of 0.3 $\mu\text{L}/\text{min}$. Prior to separation on the C_{18} column, the glycopeptides were pre-concentrated with a Dionex Acclaim PepMap100 C_{18} preconcentration column ($75 \mu\text{m} \times 2$ cm, $3 \mu\text{m}$ particle size) at a volume of 1 μL . The preconcentration was done with 2% acetonitrile/0.05% acetic acid for 3 min at a flow rate of 5 $\mu\text{L}/\text{min}$.

All MS/MS experiments were undertaken using a Thermo Fisher Scientific LTQ Velos dual linear ion trap mass spectrometer (San Jose, CA) equipped with a Coherent ExciStar XS excimer laser (Santa Clara, CA) producing 193 nm photons at 2 mJ/pulse. The laser setup was the same as previously described.³⁸ For CID, a 30 ms activation time and 0.25 q_z value were used in both negative and positive modes. For UVPD, eight laser pulses per spectrum were used in a total activation period of 16 msec. The q_z value for UVPD was fixed at 0.1. Data-dependent nanoLC-MS/MS was performed by first acquiring an ESI mass spectrum

(m/z 400-2000), then 10 MS/MS events were performed on the ten most abundant peaks in the ESI survey spectrum.

Results and Discussion

Glycopeptides analyses with CID and UVPD

To demonstrate the performance of UVPD for characterization of O-linked glycopeptides, two well-known glycoproteins were digested and used as model proteins. Bovine fetuin has three N-linked glycosylation and four O-linked glycosylation sites. Eight different O-linked glycans have been previously reported for bovine fetuin based on a nano-LC-QTOF-CID strategy in the positive mode,³⁴ and most of O-linked glycans were found to be sialylated at the ends. In the present study, the fragmentation patterns of the fetuin glycopeptides were characterized by analysis of their corresponding deprotonated anions and protonated cations by both CID (for comparative purposes) and UVPD. Figure 2 illustrates examples of the MS/MS spectra for doubly protonated and doubly deprotonated AAGPT(HexNAc + Hex + Neu5Ac)PS from fetuin by CID and UVPD. Upon CID of $(M + 2H)^{2+}$, glycan reporter ions were detected including $([Neu5Ac - H_2O + H]^+)$ (m/z 274), $([Neu5Ac + H]^+)$ (m/z 292), and $([HexNAc + Hex + H]^+)$ (m/z 366). These reporter ions confirmed that the precursors contained glycan moieties, thus confirming them as glycopeptides. In addition, the most dominant product ions upon CID arose from glycan cleavages (i.e. loss of sugars from the glycopeptide). Also observed were two low abundance complementary sequence ions, b_5 and y_2 , that resulted from the characteristic cleavage at the N-terminus of proline. Most of the ions arising from glycan cleavages at glycosidic bonds provide some feedback about the glycan composition, albeit only the composition of the constituent monosaccharides. For example, the two ions of m/z 600 and 657 stem from loss of the entire glycan and the resulting free glycan which in fact is composed of HexNAc, Hex, and Neu5Ac. Other examples of the CID spectra of protonated glycopeptides are shown in Supplemental Figure 1. The two glycopeptides have different glycan structures but similar peptide sequences. As mentioned above, each doubly protonated glycopeptide yielded the complementary glycan cleavage products due to cleavage of the glycan from the glycopeptide (m/z 948 (glycan) and 856 (peptide) in Supplemental Fig. 1A and m/z 656 (glycan) and 785 (peptide) in Supplemental Fig. 1B). In addition, both glycopeptides underwent key proline-mediated cleavages to generate the b_7 and b_6/y_2 ions as seen in Supplemental Figures 1A and 1B, respectively. These characteristic glycan and proline-directed cleavage products are informative and consistent upon CID of protonated glycopeptides; however the glycosylation site cannot be extracted nor are there sufficient b/y ions to sequence the peptide. Particularly in the case of O-glycan analysis, the most challenging task is pinpointing the glycosylation sites because of the large number of possible glycosylation sites. Also, the O-glycosylations typically occur in serine/threonine rich regions which further confounds assignment of the specific glycosylation site. CID of the deprotonated glycopeptides did not yield any substantial new information (Figure 2B). Product ions due to cleavage of the glycan remained dominant. In comparison to CID of the protonated glycopeptides, CID of the glycopeptide anions yielded even fewer diagnostic product ions and lacked the sequence ions arising from peptide backbone cleavage N-terminal of proline mentioned above.

193 nm UVPD of the protonated glycopeptides yielded spectra similar to those described upon CID in addition to a few low abundance sequence and less predictable ions such as a_5+1 , b_4^{2+} and P/b_4 (where P represents the peptide after glycan loss and the specific peptide portion is represented by the b_n or y_n ion listed after the slash mark) (Figure 2C). The most informative spectrum was obtained upon UVPD of the deprotonated glycopeptide (Figure 2D). The most abundant and common ions were a/x -type peptide sequence ions which have been noted previously upon UVPD of deprotonated peptides.³⁹ Although the series of a - and x -type ions is not complete for the glycopeptide AAGPTPS in Figure 2, some of these a/x ions retain the entire glycan moiety. For example, the product ions a_5 , a_6 and x_3 , x_5 , x_6 all contained the NAcHex/Hex/Neu5Ac moiety, but a_2 does not. These product ions enable the confident determination of the specific glycosylation site. UVPD also promoted the proline-specific cleavages likewise noted upon CID. In Figure 2D, Y_2 and Y_4 ions are proline-specific fragments which have been noted in our previous UVPD study of peptides.³⁹ These additional Y ions provide confirmation of the peptide sequence and help verify the glycosylation sites. Additional noteworthy products upon UVPD of the glycopeptide anions arise from extensive (but uninformative) neutral losses of carbon dioxide (-44 Da/-CO₂), methoxyl radical (-31 Da/31), water (-18 Da/-H₂O), and ammonia (-17 Da/-NH₃) of as well as glycan cleavages, the latter also noted upon CID. Similar to the product ions created by the other MS/MS methods, negative UVPD also yielded glycan cleavage ions (i.e. losses of one or more sugars plus formation of free glycans). Moreover, B- and C-type saccharide ions were generated, in addition to reporter ions of the following types: B ion from Neu5Ac (m/z 290), C ion from Neu5Ac (m/z 308), and B ion from Neu5Ac-Hex (m/z 468).

In order to examine the charge state dependence of UVPD for glycopeptides, doubly and triply deprotonated glycopeptide anions were analyzed. Figure 3 illustrates the resulting fragmentation pattern of two different charge states of the AEAPSAVPD glycopeptide. Both charge state anions underwent the same type of extensive fragmentation noted above, such as a/x -type cleavages and glycan cleavages. In general, UVPD yielded an even greater array of fragment ions for the glycopeptide in the higher charge state. For instance, a_3 , a_4 , x_3 and x_4 ions in the lower m/z region appear in Figure 3B but not in Figure 3A. These additional product ions provide some supporting evidence for the glycosylation site. However, more neutral losses are also observed upon UVPD of the more highly charged glycopeptides, as well as a more abundant intact charge reduced ion. These trends are noted for other glycopeptides as well.

Another comparison of the CID and UVPD spectra is displayed in Figure 4 for a glycopeptide, AVEST(NAcHex + Hex +Neu5Ac)VAT, from bovine κ -casein, and the results mirror those summarized above for the fetuin glycopeptides. First, CID generated only glycan-specific fragments, similar to observations noted in previous reports. Two peptide sequence ions, b_7^{2+} and x_7^{2+} , were produced. (see Figure 4A and 4B, respectively) These types of peptide sequence ions are not frequently observed upon CID of glycopeptides, instead glycan cleavages are preferential. Extensive water losses in conjunction with glycan cleavages were also observed upon CID of the protonated glycopeptide. Those set of fragments are B- and C-type ions resulting from glycosidic cleavage. UVPD of the κ -casein glycopeptides resulted in spectra that were similar to the

corresponding CID spectra. The main difference between CID and UVPD of the protonated glycopeptides was the dissociation efficiency and the B-type ions from glycosidic cleavage (noted as loss of H₂O) in the CID spectra. UVPD of the deprotonated glycopeptide again resulted in the most informative spectra and confirmed the ability to obtain both glycan sequence and peptide information using a single MS/MS method. UVPD produced extensive *a*-/*x*-type ions that retained the whole glycan, thus ensuring the ability to pinpoint the exact location of the O-glycan. Glycosidic cleavage ions which are assigned using the circle, square, and diamond labels in the spectra were also observed.

In addition to sialylated glycopeptides, UVPD allows characterization of the glycopeptides which do not contain sialic acid at the end. Figure 5 is an example of the UVPD spectra acquired for the protonated and deprotonated glycopeptide AVEST(glycan)VAT. Five fragments from glycan specific cleavages and only one peptide sequence ion, z_6^{2+} , were generated upon UVPD of the protonated glycopeptide (Figure 5A). In contrast, UVPD of the same deprotonated glycopeptide (Figure 5B) resulted in almost a complete series of peptide sequence ions and glycan loss ions. In comparison to the sialylated glycopeptide in Figure 4D, Figure 5B yields better peptide sequence coverage including formation of key *b*₅, *y*₄ and *y*₅ ions.

Absorption of even a single 193 nm photon (~6.4 eV) by an ion can access excited electronic states. The photoactivated glycopeptides may dissociate directly from the excited states, leading to new fragmentation pathways not observed upon low energy collisional activation, or may undergo internal conversion and vibrational re-distribution, leading to more CID-like products. Moreover, electron photodetachment is a prominent process upon UV photoactivation of deprotonated peptides. Electron photodetachment results in the formation of charge-reduced radical ions; these ions may dissociate into different product ions than those generated directly from the fragmentation of the doubly charged peptide anions. It is anticipated that this combination of processes is what accounts for the more diverse, and in many cases more informative, fragmentation patterns upon UVPD of the glycopeptide anions. Specific mechanisms have not been deciphered and will require high mass accuracy measurements to confirm exact chemical formulas of the various product ions. However, the fact that UVPD of deprotonated glycopeptides, but not the analogous protonated species, leads to the more diagnostic mixture of peptide and glycan cleavages suggests that radical-mediated pathways (such as those occurring after electron photodetachment) may be key.

Conclusions

UVPD (193 nm) of deprotonated O-glycopeptides from fetuin and κ -casein produced greater arrays of diagnostic fragments evolving from both glycan and peptide backbone cleavages compared to CID of protonated or deprotonated glycopeptides and UVPD of protonated glycopeptides. These latter MS/MS methods primarily only provided significant glycan information. In terms of peptide sequence ions, CID and UVPD of protonated glycopeptides resulted in peptide backbone cleavage only at the N-terminus of proline; however UVPD of the corresponding deprotonated glycopeptides generated *a*-/*x*-type peptide backbone ions which facilitated assignment of the O-linked glycosylation sites. UVPD also resulted in

several unique fragmentation pathways, such as production of Y-type ions at the N-terminus of proline.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

UVPD and CID spectra were compared for protonated and deprotonated glycopeptides.

UVPD of protonated peptides resulted in primarily glycan cleavages.

UVPD of deprotonated peptides generated a/x-type ions and glycan cleavages.

Figure 1.
Scheme of experimental procedure for glycoprotein digestion, purification, and mass spectrometric analyses.

Figure 2.

MS/MS spectra of the doubly protonated and deprotonated fetuin glycopeptide AAGPT(glycan)PS: (A) CID (2+), (B) CID (2-), (C) UVPD (2+), and (D) UVPD (2-). The sugars are represented by circle (Hex), square (NAcHex), and diamond (Neu5Ac). Double cleavage products are labeled using a slash mark. Products that result from dehydration are denoted with '.

Figure 3.

UVPD spectra of AEAPS(glycan)AVPD peptide: (A) doubly deprotonated and (B) triply deprotonated. Products that result from dehydration are denoted with '.

Figure 4. MS/MS spectra of the doubly protonated and deprotonated κ -casein glycopeptide AVEST(glycan)VAT: (A) CID (2+), (B) CID (2-), (C) UVPD (2+), and (D) UVPD (2-). Products that result from dehydration are denoted with '.

Figure 5. MS/MS spectra of the doubly protonated and deprotonated fetuin glycopeptide AVEST(glycan)VAT: (A) UVPD (2+) and (B) UVPD (2-)