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Xylose Migration During Tandem Mass Spectrometry of *N*-Linked Glycans

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Abstract

Understanding the rearrangement of gas-phase ions via tandem mass spectrometry is critical to improving manual and automated interpretation of complex datasets. *N*-glycan analysis may be carried out under collision induced (CID) or higher energy collision dissociation (HCD), which favors cleavage at the glycosidic bond. However, fucose migration has been observed in tandem MS, leading to the formation of new bonds over four saccharide units away. In the following work, we report the second instance of saccharide migration ever to occur for *N*-glycans. Using horseradish peroxidase as a standard, the beta-1,2 xylose was observed to migrate from a hexose to a glucosamine residue on the (Xyl)Man₃GlcNac₂ glycan. This investigation was followed up in a complex *N*-linked glycan mixture derived from stem differentiating xylem tissue, and the rearranged product ion was observed for 75% of the glycans. Rearrangement was not favored in isomeric glycans with a core or antennae fucose and unobserved in glycans predicted to have a permanent core-fucose modification. As the first empirical observation of this rearrangement, this work warrants dissemination so it may be searched in *de novo* sequencing glycan workflows.

Graphical Abstract

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Introduction

Tandem mass spectrometry and MS^N have been successfully used to determine the structure of oligosaccharides. In general, the dissociation of glycans conforms to the rules outlined by Domon and Costello (1988) [1], producing a set of cohesive guidelines for manual and automated software for glycomics sequencing [2–6]. For example, in the GlycoWorkbench platform, the number and type (X, Y, A, and B) of cleavages may be user-defined, and then combinatorically applied to produce a theoretical fragmentation database [7–9]. While cross-ring cleavages (X, Y) are rarer in collision cells compared to electron transfer dissociation (ETD) [10], they may still be observed with significant abundance.

Theoretical information may be complemented by experimental results, which can yield unique transitions for specific glycans. For example, 1,6 versus 2,6 sialic acids give rise to X and Y ions with reproducibly different frequencies [11, 12] and this dissociation is not predictable by glycan fragmentation rules. Likewise, rearrangement of the fucose on glycan structures has been experimentally observed, but is not predicted by conventional rules [10, 13]. In a mechanism similar to methyl-migration in peptides, fucoses have been shown to migrate up to four saccharides away, and this process is energetically supported by computer simulations [12, 14, 15]. Permethylation, which changes the chemistry of these bonds, precludes this migration from occurring during tandem MS [15].

While the MS/MS spectra for complex mammalian glycans are well-documented, studies on xylose-containing species are lacking. Xyloses are primarily incorporated into the glycomes of plants, and across this field, there has been a renewed interest to investigate the relationship between glycosylation and biological processes [16, 17]. Our group has extensively studied *Populus trichocarpa* to model flux through the monolignol biosynthetic pathway, which is important for the pulp and paper and biofuels industries [18–20]. We

recently showed that approximately 50% of the enzymes involved in the monolignol pathway are glycosylated with occupancy levels between 10–100%, and work is ongoing to better define the role of glycosylation in regards to how it modulates enzymatic activity [21].

In the following study, we highlight for the first time the ability of xylose to migrate during HCD and CID. We initially explored this phenomenon with a structurally characterized glycan released from horseradish peroxidase (HRP) [22], validating the rearranged product ion's identity using MS³. We then demonstrated the relevance of this observation for the analysis of biological samples from *P. trichocarpa*, where xylose rearrangement is discussed in terms of its relationship with glycan structure.

Methods

Materials

All materials were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. All solvents were of HPLC-grade and obtained from Honeywell Burdick & Jackson (Muskegon, MI).

N-Glycan Purification and Preparation

Extraction of glycans from crude xylem protein (250 µg) [21] and HRP (50 µg) [23] was performed as previously described. For the glycan preparation, the “spike-in” protocol described by Hecht, McCord, and Muddiman was applied [23]. Glycan hydrazide tagging at the reducing terminus was performed using the 4-phenethyl-benzohydrazide reagent (GTK-1000, Cambridge Isotope Labs, Andover, MA) and tagged *N*-glycans were resuspended in 30 µl or 50 µl of H₂O for the plant and HRP sample, respectively. Equimolar mixtures of native (NAT) and ¹³C₆ stable isotope labeled (SIL) glycans were combined for LC-MS analysis.

LC-MS/MS Analysis and Bioinformatics

Tagged glycans were loaded (2 µl plant, 5 µl HRP) onto a 25 cm × 75 µm column packed with C18 2.6 µm, 100 Å resin (Phenomenex, Torrance, CA) at 600 bar. Samples were analyzed using an Easy nLC-1000 configured to a Q Exactive™ High Field mass spectrometer (Thermo Scientific, San Jose, CA). A 60 min. gradient was run at 300 nL/min with mobile phases A (98:2: water:acetonitrile, 0.1% formic acid) and B (98:2 acetonitrile:water, 0.1% formic acid): 5–30%B (1 min), 30–40%B (40 min), 40–63%B (5 min), 63–90%B (1 min), 90%B (8 min), 90–5%B (1 min), 5%B (10 min). Glycans were ionized according to these MS¹ conditions: 600–1900 *m/z*, 60,000 resolution, 5×10⁵ AGC, 64 ms injection time, 65 RF S-lens, 325 °C capillary, and 1.75 kV spray voltage and MS² conditions: 15,000 or 60,000 resolution for lignin or HRP experiments, respectively, 5×10⁴ AGC, 100 ms injection time, 1% underfill ratio, 1.4 Th window, 125 Th fixed first mass, top 12 data dependent acquisition, 15 s exclusion window, peptide match preferred and 10/20/30 stepped normalized collision energy (NCE). For targeted selected ion monitoring (t-SIM) experiments, the isolation window was set to 0.7 Th.

For MS³ experiments, HRP was injected onto a 15 cm × 75 μm C18 column interfaced to an Orbitrap Elite (Thermo Scientific, San Jose, CA) using the same ionization parameters described and an expedited gradient: 5% B (1 min), 5–22% B (1 min), 22–45% B (10 min), 45–90% B (1 min). The MS analysis was performed in the ion trap at a NCE of 30 % at the MS² level (m/z 1279.50924), and 15% at the MS³ level (m/z 336.12946). To isolate the 336 ion, the activation quad potential was changed to 0.23.

All RAW files were manually annotated using the XCalibur Qual browser. Glycan composition identifications made in lignin were based on MS¹ and MS/MS data and defined by their hexose (Hex, H), *N*-acetyl hexosamine (HexNac, N), xylose (Xyl, X), and fucose (Fuc, F) subunits. The structure of HRP was confirmed by tandem MS and, based on the literature, assigned as Man α 1–3(Man α 1–6) (Xyl β 1–2)Man β 1–4 GlcNac β 1–4 GlcNac, where Man and GlcNac are mannose and *N*-acetyl glucosamine, respectively.

Results and Discussion

Evaluation of HRP MS/MS Spectra for Xylose Fragments

HRP may be used as a model system as its glycan structures have been thoroughly characterized in the literature using NMR and MS^N, and it may be well-resolved by liquid chromatography [22]. These studies document the location of the xylose to the primary mannose with a β 1,2 linkage (Figure 1). The (Xyl)Man₃GlcNac₂ glycan was isolated in a t-SIM experiment, well resolved at the MS¹ level (Figure 2A), and fragmented over a range of normalized collision energies (Figure 2B). Product ions were observed in good abundance, with at least one isotopic species, and with high mass accuracy (< 5 ppm) at 70,000 resolving power (Figure 2C). The generation of the GlcNac₁Xyl₁ ion, produced by a xylose rearrangement, and the standard Man₁Xyl₁ oxonium fragment were compared. The abundance of the migration-generated species was 3–10% of the Man₁Xyl₁ species and diminished below detection at a NCE greater than 25% (Figure 2B). The optimal collisional energy for both species was similar, at ~20%. A linear ion trap was used to validate the identity of the 336 m/z MS² ion from (Xyl)Man₃GlcNac₂ and to determine if the rearrangement could occur during CID. The collision energy required to generate sufficient fragmentation was significantly higher under CID (30 vs. 20%), and the ion of interest was observed at low levels. Isolation of the 336 ion for MS³ analysis showed that the sole product ion produced corresponded to 204.09 m/z , the oxonium ion of *N*-acetyl glucosamine (Figure 3). This observation supports the neutral loss of a xylose and provides evidence for the proposed rearrangement (Figure 1).

Production of Xylose-Migration Fragments Across *P. trichocarpa* Tissue Glycans

Investigation into the exact mechanism by which xylose migration occurs is limited by a lack of suitable standards. However, insight can be gained by monitoring the production of this fragment across a wide array of glycans. The glycome of *P. trichocarpa* has recently been defined and includes 27 glycans, of which 48% are xylosylated. Excluding H₅N₄F₁X₁, which did not have MS/MS spectra, 75% of the xylosylated species produced an ion corresponding to N₁X₁ (Table S1). Interestingly, the only four glycans, H₅N₄F₂X₁, H₅N₄F₃X₁, H₃N₂F₁X₁, and H₂N₂F₁X₁, that did not undergo a xylose-rearrangement

contained a core fucose. To try to explore if the presence of antennae fucoses influenced the degree of rearrangement, the amount of N_1X_1 observed was calculated as percentage of the primary hexose oxonium ion (m/z 163.06), which served as an internal standard. For one $H_3N_4F_1X_1$ and two $H_4N_3F_1X_1$ MS² spectra, the N_1X_1 fragment was completely missing, and a minimum abundance value of 1000 was substituted to calculate the ratio. The H_1/N_1X_1 ratios were 12.9 and 5.6 for $H_3N_3F_1X_1$ and $H_3N_3F_0X_1$, respectively; 1.1 and 0.9 for $H_3N_4F_1X_1$ and $H_3N_4F_0X_1$, respectively; 5.6 and 5.8 for $H_4N_3F_1X_1$ and $H_4N_3F_0X_1$, respectively. This data suggests a trend in which core fucoses completely prevent rearrangement and fucoses present on the antennae limit the migration rate. With the advent of xylose-containing glycan standards, future work may explore the precise energetics of this rearrangement.

Conclusions

Here, we provide the first data that supports the migration of xylose during HCD and CID tandem mass spectrometry and demonstrate the impact of glycan structure on the rate of migration. Though a lack of standards limits the mechanistic information that may be explored, knowledge of this rearrangement can benefit MS/MS elucidation, software development, and accurate identification.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
Suggested mechanism to generate the Man₁Xyl₁ and GlcNAc₁Xyl₁ species from (Xyl)Man₃GlcNAc₂ during CID or HCD. The hydrazide tag quickly dissociates during tandem MS, making the penultimate or terminal GlcNAc available to migrating species.

Figure 2.

(A) Profile of the co-eluting NAT and SIL (Xyl)Man₃GlcNac₂ species from HRP. (B) Average abundance of the Man₁Xyl₁ and GlcNac₁Xyl₁ product ions of interest as a function of normalized collision energy on a Q Exactive HF. (C) Annotated HCD MS/MS spectra of H₃N₂X₁, with the region containing fragments of interest enlarged.

Figure 3.

The rearranged N_1X_1 ion was isolated in a linear ion trap from $H_3N_2X_1$. CID analysis (NCE = 15%) showed the parent ion (336.13) and the neutral loss of a xylose, yielding the 204.09 ion.