Automated Glycan Structural Isomer Differentiation Using A Bioinformatics Tool

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Overview

Purpose: To demonstrate the use of SimGlycan software for automated structural elucidation of glycan MS^n spectra acquired on Thermo Scientific mass spectrometers.

Methods: Sequential MS^n spectra of permethylated chicken ovalbumin glycans were acquired on an ion trap mass spectrometer. Structural elucidation was performed using SimGlycan software.

Results: The combination of permethylation, MS^n and SimGlycan software enabled successful identification and differentiation of the various structural isomers of chicken-ovalbumin-released glycans.

Introduction

Mass spectrometry (MS) has emerged as a powerful tool for the structural elucidation of glycans. The use of permethylation in combination with multistage fragmentation (MS^n) is a critical step in glycan structural characterization. Because it enables the identification of branching patterns, linkages and the resolution of isobaric structures which are otherwise indistinguishable in MS/MS spectra, MS^n is need to fully characterize glycan structure. However, MS^n analysis is complicated by the large number of spectra generated for each structure. Typically MS^6 or MS^7 level fragmentation must be acquired in order to differentiate potential glycan structural isomers. Here we present the use of SimGlycan software, a bioinformatics tool, for glycan structural isomer differentiation using MS^n data.

Methods

Sample Preparation

Ovalbumin (1 mg, Sigma) was reduced, alkylated and digested overnight with trypsin in of 25 mM ammonium bicarbonate buffer (pH~8) at 37 ºC. PNGase F solution (3 µL, Roche) was added to 200 µL of digested sample and the mixture was incubated for another 16 hours at 37 ºC. The released glycans were separated from the peptides using a Sep-Pak® C18 cartridge (Waters). The Sep-Pak C18 was conditioned by washing with acetonitrile, followed by water. PNGaseF digested sample was loaded onto the cartridge and the released glycans were eluted with 1% ethanol while the peptides remained bound to the Sep-Pak C18. The released ovalbumin oligosaccharides were first purified using a porous graphite carbon column (PhyNexus) and then permethylated as described previously.[1]

Mass Spectrometry

All MS^n experiments were performed using a Thermo Scientific Velos Pro linear ion trap mass spectrometer via direct infusion into the nanoelectrospray source. The mass spectrometer settings and SimGlycan software version 2.92 (PREMIER Biosoft International) search parameters are listed in Tables 1 and 2.

Table 1. Mass Spectrometer Settings

Source	nano-ESI	Isolation Width	3
Capillary Temperature	200 ºC	Collision Energy	30
S-lens RF Level	50 %	Activation Time	10 ms
Source voltage [kV]	1.3	Predictive AGC Enabled	Yes
Full MS Mass Range	150-2000 (*m/z*)	No. Microscans for Full MS	5
Scan Rate	Enhanced	Target Value Full MS	3e4
Maximum Injection Time	Full MS 50 ms	Target Value MS^n	3e4
MS^n	50 ms		

Table 2. SimGlycan Software 2.92 Search Parameters

Ion Mode	Positive	Class	Glycoprotein
Adducts	Sodium	SubClass	N-Glycan
Precursor *m/z* Error Tolerance	0.8 Da	Biological Source	Chicken
Spectrum *m/z* Error Tolerance	0.8 Da	Pathway	Unknown
Chemical Derivatization	Permethylated	Search Structure	All
Reducing Terminal	Reduced	Glycan Type	All

Results

Automated structural interpretation of MS^n glycan spectra was tested on glycans released from chicken ovalbumin (Figure 1). Because the glycan content of ovalbumin has been characterized in depth,[2] it is an ideal system to use to examine the capabilities of SimGlycan® software. In parallel, we manually interpreted the MS^n spectra and compared them with previously presented data, thus providing a perfect control.[3]

Figure 2a shows the MS profile of permethylated glycans derived from ovalbumin acquired on a Velos Pro™ mass spectrometer, an ideal instrument for this experiment. The Velos Pro mass system's dual-pressure ion trap and S-Lens ion optics provide increased ion transmission along with better trapping and fragmentation efficiency. Both of these features are critical for performing MS^n experiments. Table 3 shows all the glycans identified in this study. Figure 2b shows the MS/MS spectrum for a peak at *m/z* 1054.68 (+2). This peak was selected for the software evaluation because it has been interrogated previously.[3] In order to fully characterize the glycan structure, sequential MS^n fragmentation was applied to this precursor. The Velos Pro system was operated in "Enhanced Scan" profile mode for all MS experiments. The enhanced scan mode allows charge stage determination of precursors and fragment ions.

FIGURE 1. Workflow for automated structural interpretation of MS^n glycan spectra.

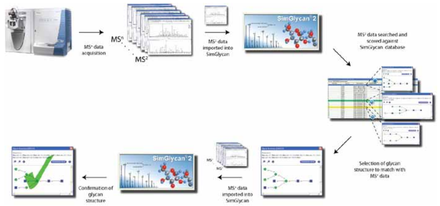


FIGURE 2. (a) Ion trap full-scan mass spectrum of permethylated ovalbumin released glycans (labeled peaks correspond to Table 3). (b) Ion trap MS/MS of the peak at *m/z* 1054.68. (c) Set of sequential MS^n spectra acquired for peak at *m/z* 1054.68 (+2).

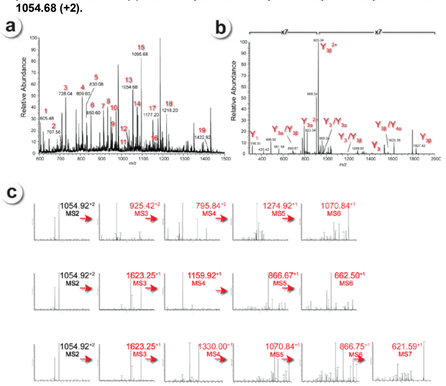
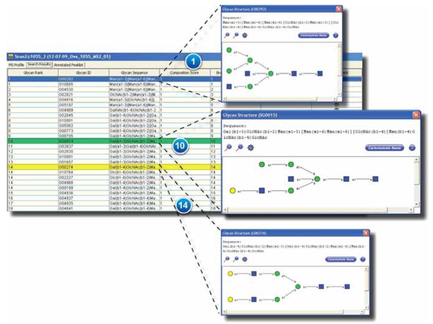


Figure 2c shows sets of MS^n spectra acquired for this precursor which are then imported into SimGlycan software for analysis. The MS^n data-interpretation workflow is as follows. The user submits the MS^2 spectrum for automatic compositional identification. Based on the criteria selected, SimGlycan software searches its database for matches.

If the user strictly relies on MS^2 data, the MS/MS fragmentation pattern for *m/z* 1054.68 (+2) could be interpreted as a hybrid glycan with a bisecting GlcNAc based on the top ranked glycan in the search results (Figure 3). Examination of the glycan list reported by SimGlycan software for the MS^2 spectrum submitted shows additional glycan compositions that posses the same mass but that are ranked much lower. These glycans, though reported to have a much lower probability of matching the submitted MS^2 spectrum, could represent additional isomers because not every major fragment in the spectra is assigned (Figure 2b).

In order to determine whether these glycans are additional isomers, SimGlycan software was used to determine if any of the lower ranked glycan structures matched the MS^n fragmentation pathway. Using the list generated by SimGlycan software, the user can select specific structures to compare with the MS^n fragmentation pathway. Each successive level of fragmentation can be brought in to match with the specific precursor selected for fragmentation in the previous level of the MS^n spectrum.

FIGURE 3. SimGlycan software search results for the ion trap MS/MS spectrum of the precursor ion at *m/z* 1054.68 (+2). Symbolic representation of the top-ranked and the two lower-ranked glycan search results obtained from the SimGlycan software.



For example, we selected the asialyl digalactosyl biantennary glycan from our list to confirm or deny as a potential isomer. It is ranked much lower based on the MS^2 data, but has the same precursor mass as the top match (Figure 3, ranked 14 on the list). In the MS^2 spectrum (Figure 2b) of *m/z* 1054.68 (+2), we selected the fragment ion at *m/z* 1623.25 (+1) for further fragmentation. Detection of this ion indicates the loss of Gal-GlcNAc from the non-reducing end of the asialyl digalactosyl biantennary glycan. Figure 2c shows the MS^3 spectra for this ion. Of particular interest in the MS^3 spectrum is the fragment ion at *m/z* 1159.93 (+1) which corresponds to the additional loss of Gal-GlcNAc. This loss is only possible from the selected asialyl digalactosyl biantennary glycan structure because the additional loss is possible from the non-reducing end.

Figure 4a shows the overall sequential fragmentation pathway for the proposed structure, and how it is only compatible with the selected structure and the set of sequential MS^n spectra acquired in Figure 2c (1054.68? 662.50). Figure 4b shows sequential (1054.68? 925.42 as in Figure 2c) fragmentation pathway for the hybrid glycan with the bisecting GlcNAc. This further confirms that this structure is also present in precursor at *m/z* 1054.68 (+2). An additional hybrid glycan is also identified for this precursor in Figure 4c (1054.68? 621.59 as in Figure 2c). As illustrated in Figures 4a-c, the SimGlycan software was able to resolve isobaric oligosaccharides and perform detailed characterization of selected structures.

FIGURE 4. Symbolic representation of Y-type glycosidic fragments are shown. MS^n fragmentation pathway for (b) $(Gal)_2(Man)_3(GlcNAc)_4$ (c) $(Man)_5(GlcNAc)_4$ and (d) $(Gal)(Man)_4(GlcNAc)_4$.

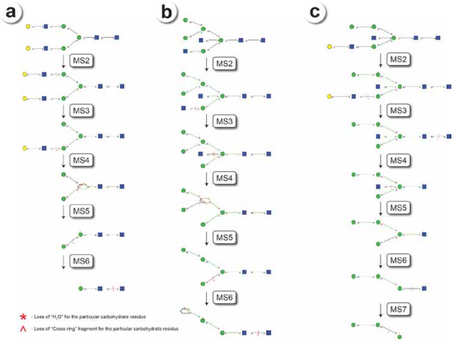


TABLE 3. Structures of chicken ovalbumin *N*-linked released glycans identified in this study (structures drawn using GlycoWorkbench).

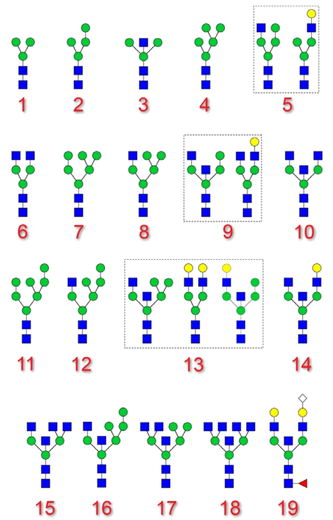
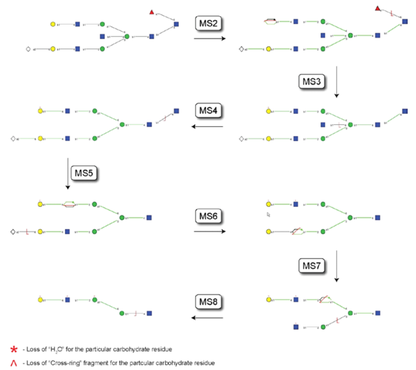


Table 3 shows two other glycan structural isomers (labeled as 5 and 9) that were identified by the SimGlycan software using the approach described here. In addition to differentiating structural isomers, MS^n can be used to elucidate correct glycan structures with confidence when insufficient fragmentation is generated at the MS^2 level. For example, the peak at *m/z* 1422.92(+2) represents a single glycan structure. However, the MS^2 spectrum does not provide enough information to clearly elucidate the correct structure. In fact, when the MS^2 data is submitted to the SimGlycan software, an incorrect structure is ranked number one due to the absence of key fragment ions. The correct structure is shown in Table 3 (labeled as 19). Figure 5 highlights the MS^n sequential fragmentation pathway required to identify this glycan and how SimGlycan software can be used to interpret the MS^n spectra.

FIGURE 5. Ion trap MS^n fragmentation pathway for precursor at *m/z* 1422.92 (+2).



Conclusion

- The combination of permethylation, MS^n and SimGlycan software enables successful identification and differentiation of the various structural isomers of chicken-ovalbumin-released glycans.
- The overall analysis time was reduced to matter of minutes. SimGlycan software enables truly automated, high-throughput data analysis.
- SimGlycan software simplifies data analysis by providing comprehensive support for MS^n experiments performed on Thermo Scientific ion trap and ion trap-Orbitrap hybrid mass spectrometers.

References

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2. Harvey, D. J.; Wing, D. R.; Kuster, B.; Wilson, I. B., Composition of N-linked carbohydrates from ovalbumin and co-purified glycoproteins. *J Am Soc Mass Spectrom* **2000,** 11, (6), 564-71.
3. Ashline, D. J.; Fournier, J.; Cernisenco, C.; Second, T., Software-assisted peak annotation and isomer detection for oligosaccharide mass spectra: A case study. ASMS 2010 poster, ThP13.