Glycan Structural Elucidation On A Novel Quadrupole Dual Cell Linear Ion Trap Orbitrap Hybrid Mass Spectrometer

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Overview

Purpose: To demonstrate the use of HCD MS^3 for glycan structural elucidation on a novel hybrid mass spectrometer, based on mass resolving quadrupole, Thermo Scientific™ Orbitrap™ analyzer, collision cell, linear ion trap architecture.

Methods: HCD MS^2 and MS^3 spectra of permethylated chicken ovalbumin glycans were acquired on a Thermo Scientific™ Orbitrap Fusion™ Tribrid™ mass spectrometer. Structural elucidation was performed using SimGlycan® software

Results: The combination of permethylation, HCD MS^3 and SimGlycan software enabled successful identification and differentiation of structural isomers of chicken-ovalbumin-released glycans

Introduction

Glycans in glycoproteins are involved in a wide-range of biological and physiological processes including recognition and regulatory functions, cellular communication, gene expression, cellular immunity, growth and development. Mass spectrometry (MS) has emerged as a powerful tool for glycan structural elucidation. The use of permethylation in combination with multistage fragmentation (MS^n) is critical to the success of this approach. Only analysis by MS^n truly characterizes glycans as it allows identification of heterogeneity, branching, linkages and resolution of isobaric structures which are otherwise indistinguishable in MS^2 spectra. Traditionally, MS^n has been restricted to low-energy, collisional-induced dissociation (CID) in linear ion trap mass spectrometers, thereby, requiring multiple stages of fragmentation (MS^6, MS^7…) for structural elucidation. Here we demonstrate for the first time the use of higher-energy collisional dissociation (HCD) MS^n for glycan structural elucidation on a novel instrument, the Orbitrap Fusion™ Tribrid™ mass spectrometer based on a mass resolving quadrupole, Orbitrap analyzer, collision cell, linear ion trap (Q-OT-qIT) architecture (Figure 1). The primary advantage of HCD fragmentation is the production of glycosidic, cross-ring, and internal double cleavage ions at the MS^2 level, where branching, linkage and resolution of isobaric structures are derived from the latter two types of ions. The availability of HCD MS^n enables comprehensive glycan structural elucidation at much lower MS^n stages.

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 using an in-house protocol.

Mass Spectrometry

All MS experiments were performed using an Orbitrap Fusion Tribrid mass spectrometer via direct infusion into the nano-electrospray source. The mass spectrometer settings and SimGlycan software [1,2] version 4.50 (PREMIER Biosoft International) search parameters are listed in Tables 1 and 2.

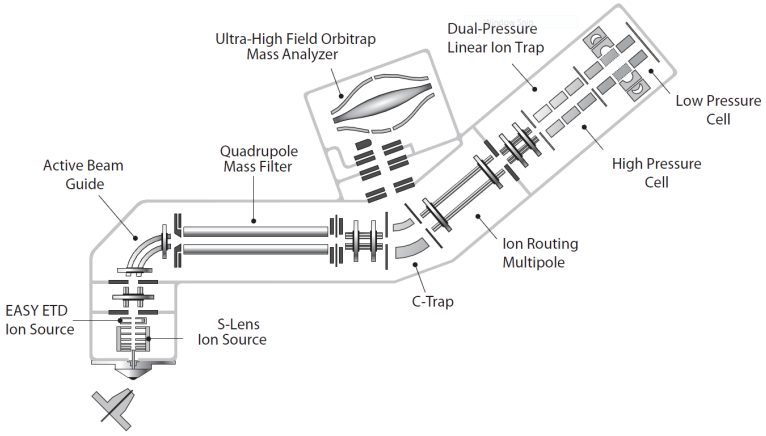
Table 1. Mass Spectrometer Settings

Source	HESI	Isolation Width	3
Capillary Temperature	250 °C	Collision Energy	35
S-lens RF Level	60 %		
Source voltage [kV]	3.8		
Full MS Mass Range	700-1600 (*m/z*)		
MS Resolution	60000 @ *m/z* 200		
MS/MS Resolution	60000 @ *m/z* 200		

Table 2. SimGlycan Software 4.50 Settings

Ion Mode	Positive	Class	Glycoprotein
Adducts	Sodium	SubClass	N-Glycan (All)
Precursor *m/z* Error Tolerance	10 ppm	Biological Source	Chicken, Ovalbumin
Spectrum *m/z* Error Tolerance	0.01 Da	Pathway	Unknown
Chemical Derivatization	Permethylated	Search Structure	All
Reducing Terminal	Free	Glycan Type	All

FIGURE 1. Schematic representation of the novel Orbitrap Fusion Tribrid mass spectrometer architecture.



Results

HCD fragmentation provides much more informative spectra for glycan analysis compared to low-energy CID fragmentation available on linear ion trap mass spectrometers, (Figure 2). Targeting glycans with HCD fragmentation results in the production of cross-ring fragment ions and internal double-cleavage ions which, even with permethylation, can be lacking with low energy CID fragmentation. The ability to generate these types of ions at the MS^2 level provides sufficient information in a lot of cases to successfully elucidate glycan structures.

Though HCD can produce much more informative fragmentation at the MS^2 level, differentiating structural isomers can be an issue as fragments from mixed spectrum can complicate spectral assignment. MS^n would still be required for differentiation of these isomers. Currently the acquisition of HCD fragmentation is still limited to MS^2 on commercial mass spectrometers. However, we have recently built a new quadrupole, dual cell linear ion trap, Orbitrap hybrid mass spectrometer that has a novel architecture and ion transfer path that enables HCD MS^n analysis.

Structural elucidation using HCD MS^n was initially tested on glycans released from chicken ovalbumin. Since the glycan content of ovalbumin has been characterized in depth,[3] it was an ideal system to examine the capabilities of the novel Orbitrap Fusion Tribrid mass spectrometer. Figure 3 shows the MS profile of permethylated glycans derived from ovalbumin acquired on this instrument. Data-dependent MS^2 spectra were acquired on all precursors with a charge state greater than two ($Z \geq 2$) and for each MS^2 spectra subsequent top 20 MS^3 spectra were acquired.

The MS^n data interpretation workflow is as follows. The acquired MS^2 data is brought into SimGlycan software for automatic compositional identification. Based on the criteria selected (Table 2), SimGlycan software searches its database for glycan matches. For example the MS^2 spectrum (Figure 4) for the glycan at *m/z* 1046.511 (Figure 2) is brought into SimGlycan for identification. Based on the MS^2 fragmentation pattern SimGlycan interprets the spectrum as most likely being a hybrid glycan with a bisecting GlcNAc, as this is the glycan ranked as the top structure in SimGlycan search result (Figure 5).

FIGURE 2. Comparison of CID MS² vs HCD MS² spectrum of glycan at *m/z* 1046.511 (+2).

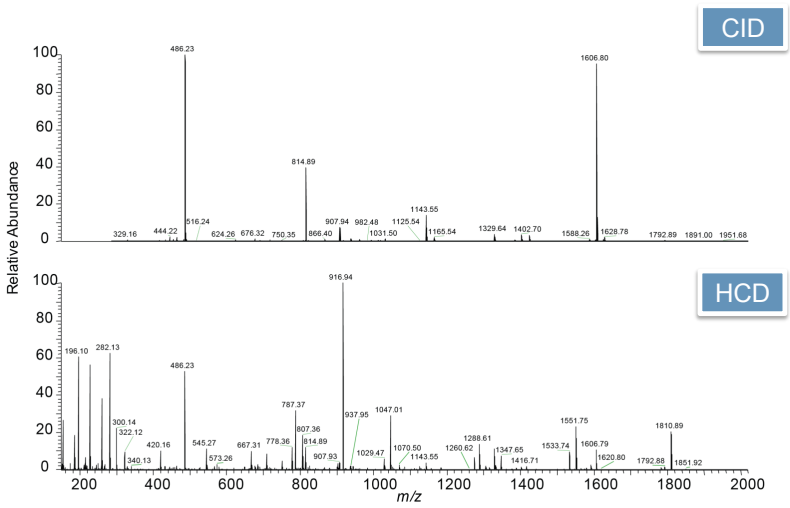


FIGURE 3. FT full scan mass spectrum of permethylated ovalbumin released glycans.

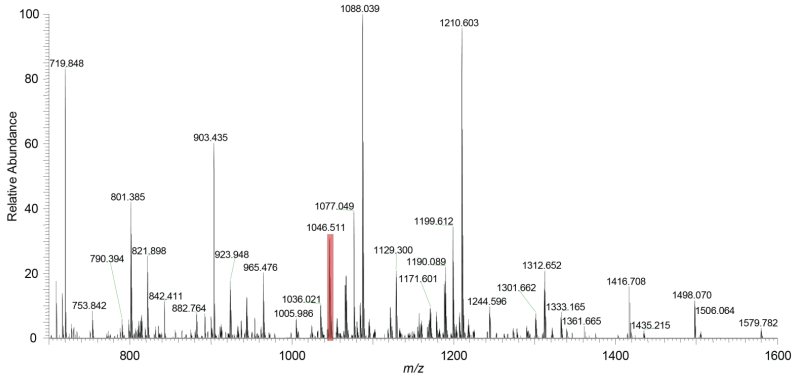


FIGURE 4. HCD MS² spectrum of the peak at *m/z* 1046.511 (+2). Peaks assigned correspond to the hybrid glycan with a bisecting GlcNAc, ranked 1 in the SimGlycan search.

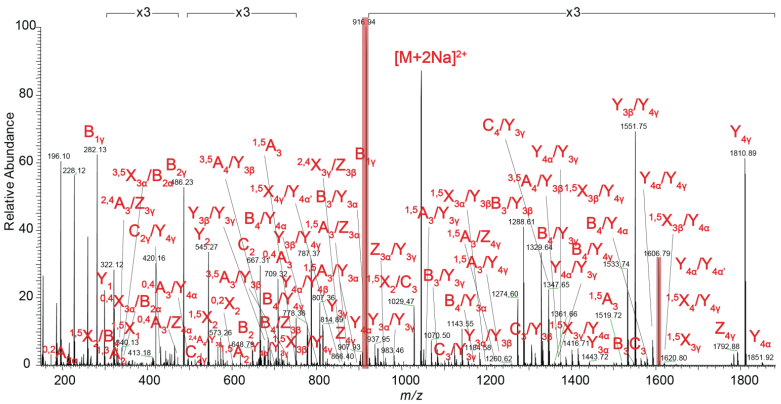


FIGURE 5. SimGlycan software search results for the HCD MS² spectrum of the precursor ion at *m/z* 1046.511 (+2). Symbolic representation of the top ranked and the two lower ranked glycan search results obtained from the SimGlycan software.

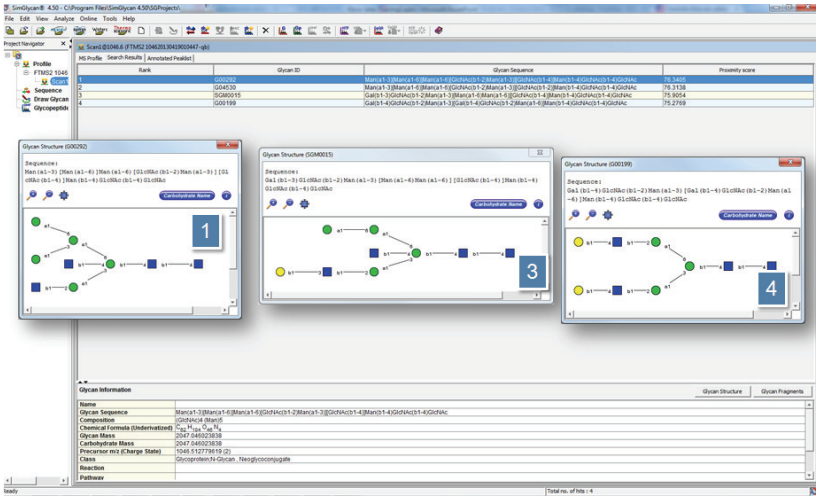
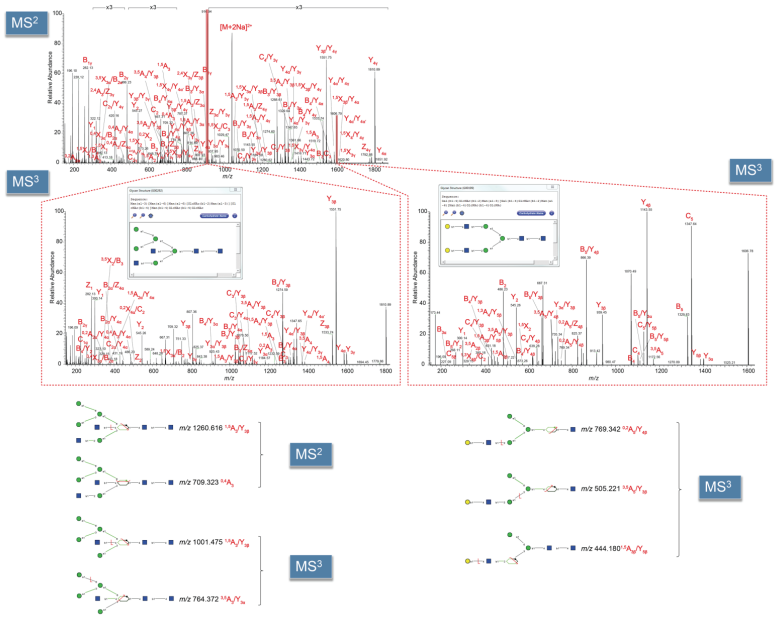


FIGURE 6. HCD MS² AND MS³ spectra acquired for permethylated ovalbumin released glycan at *m/z* 1046.511 (+2). Peaks are labeled according to nomenclature proposed by Domon and Costello [4]. Symbolic representation of the fragment ions at the MS² and MS³ level that aided in differentiating structural isomers are shown below the spectra.



Further examination of the glycan result reported by SimGlycan software for the MS² spectrum shows additional glycan compositions that possess the same mass but are ranked much lower. In order to ensure that we characterized all possible structural isomers. We incorporated MS³ spectra in our data analysis. Figure 6 shows that indeed the hybrid glycan with a bisecting GlcNAc is present at *m/z* 1046.511, as the combination of MS² and MS³ spectra show fragmentation specific to the hybrid glycan with a bisecting GlcNAc (rank 1) and not from other structures (Figure 6).

It should be pointed out that the HCD MS^2 and MS^3 spectra generated are information rich (Figure 6), containing informative glycosidic and cross-ring fragment ions. An additional advantage of HCD fragmentation is that the ions are measured within the Orbitrap mass analyzer with high-resolution, accurate-mass (HR/AM). This allows for differentiation of near mass fragment ions, which we observed to be very useful for correctly assigning branching and linkage ions.

Though we were able to confidentially identify the hybrid glycan with a bisecting GlcNAc (rank 1), there were additional structural isomers present. Closer examination of the MS^2 spectrum for the ion at *m/z* 1046.511 reveals numerous unidentified peaks that we could not attribute to the top ranked glycan. The acquisition of MS^3 spectra on these peaks enabled identification of additional isomers. For example, the fragment ion observed at *m/z* 1606.789 in the MS^2 spectrum (Figure 4) indicates the loss of Gal-GlcNAc, which is most likely to occur from the non-reducing end of either asialyl digalactosyl biantennary glycan (ranked 4) or the other hybrid glycan with bisecting GlcNAc (ranked 3). The acquisition of MS^3 spectrum for the ion at *m/z* 1606.789 provided additional fragment ions unique to the asialyl digalactosyl biantennary glycan that confirmed the presence of this structure at *m/z* 1046.511.

Overall, we were able to identify three structural isomers at *m/z* 1046.511. An additional hybrid glycan with the bisecting GlcNAc (ranked 3) was also identified. We had previously characterized these structural isomers on a Thermo Scientific™ Velos Pro™ mass spectrometer using CID MSn.[1] In order to differentiate the structural isomers, it required MS^7, MS^8 stages of fragmentation with CID MS^n.

Conclusion

- The novel Orbitrap Fusion Tribrid mass spectrometer based on mass resolving quadrupole, Orbitrap, collision cell, linear ion trap (Q-OT-qIT) architecture enables HCD MS^n.
- Additional glycan fragmentation pathways are accessible with this mass spectrometer as it has the ability to use any fragmentation mode, at any stage of MS^n analysis.
- The combination of permethylation, HCD MS^3 and SimGlycan software enabled successful identification and differentiation of structural isomers of chicken ovalbumin released glycans.

References

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2. Apte, A.; Meitei, N. S., Bioinformatics in glycomics: glycan characterization with mass spectrometric data using SimGlycan. *Methods Mol. Biol.* **2010**, 600, 269-281.
3. 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.
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