Identification of *N*-linked carbohydrates by negative ion CID

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Introduction

We have recently developed a new, rapid method for the analysis of *N*-linked carbohydrates (those attached to asparagine) based on negative ion fragmentation using a Q-Tof mass spectrometer with nanospray ionization. The method allows the analysis of complex mixtures of released *N*-linked carbohydrates to be accomplished in only a few hours. The proposed fragmentation mechanisms occurring in the negative ion spectra were presented in a poster last year and have recently been published (1-3). In this poster, we present improvements to the method and applications to the analysis of carbohydrates from several biomedical projects. Further structural details were obtained by MS^3 fragmentation using a tandem linear ion-trap/orbitrap mass spectrometer. The high resolution capability of this instrument enabled elemental compositions of both parent and fragment ions to be determined.

Methods

Most *N*-linked carbohydrates were released from SDS-PAGE-separated glycoproteins with protein *N*-glycosidase F (PNGase F). IgY and ovalbumin glycans were released by hydrazinolysis. IgY glycans were additionally converted into 2-amino-benzamide (2-AB) derivatives by reductive amination. Underivatized glycans were purified with a Nafion 117™ membrane and examined by MALDI (positive ion) from 2,5-dihydroxybenzoic acid (DHB) with a Waters-Micromass TofSpec reflectron-TOF mass spectrometer (Waters, Manchester, UK) and by negative ion nanospray with a Waters-Micromass Q-Tof Ultima Global instrument. Nanospray samples were infused with Proxeon capillaries from water:methanol (1:1, v:v) containing either ammonium nitrate or phosphate. CID was performed with argon as the collision gas with the collision energy set to be appropriate to the mass of the glycans. MS^3 spectra were acquired with a Thermo-Electron Orbitrap mass spectrometer (Thermo-Electron Corporation, Bremen, Germany) with nanospray sample introduction from water:methanol (1:1).

Results and Discussion

Electrospray ionization with ammonium nitrate-spiked solvents infused at 5 μL/min was used in earlier work to examine hydrazine-released glycans whereas, for the work reported here, glycans were mainly released from within SDS-PAGE gels and II were ionized by nanospray. The negative ion spectra generally contained several ionic species involving adduction with the added ammonium nitrate or endogenous chloride or phosphate. However, all adducts produced similar CID fragmentation. Nanospray sample introduction from typically 8 μL of solution produced long infusion times (typically about 100 mins.) that allowed CID spectra to be collected from most carbohydrates of complex mixtures, including those present in less than 1% total glycans. Unlike the positive ion CID spectra, these negative ion spectra contained specific information such as the individual antenna composition, the substitution position of fucose and the occurrence of 'bisecting' GlcNAc residues (Figure 1, Table 1).

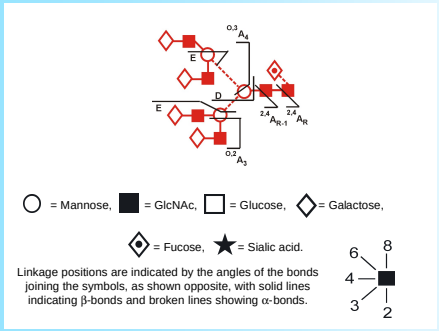

Figure 1, Some diagnostic negative ion fragmentations of *N*-linked glycans and key to structural diagrams for all figures

Table 1. Ions defining structural features in the negative ion spectra of *N*-linked glycans

Structural feature	Ion
Composition	Molecular ion mass
Antenna sequence	C Ions
Fucose at 6-position of reducing terminal GlcNAc	$[M\text{-}X\text{-}307]^-$ ($^{2,4}A_R$ ion) (X = Anionic adduct or H)
No fucose on reducing terminal GlcNAc	$[M\text{-}X\text{-}161]^-$ ($^{2,4}A_R$ ion)
Composition of 6-antenna	D and $[D\text{-}18]^-$ ions (e.g. Gal-GlcNAc-Man = *m/z* 688, 670; Man_3 = 647, 629) $^{0,3}A_{R-2}$ $^{0,4}A_{R-2}$
Composition of 3- and 6-antenna	Substituents plus 101 u from mannose (E ion) E ion contains substituents from 2- and 4-positions, therefore, branched 3- and 6-antenna can be differentiated. e.g. $Gal_2GlcNAc_2$ in 3-antenna gives *m/z* 831
Presence of bisecting GlcNAc	No, or weak D ion, strong D-221. e.g. Man_5 in 6-antenna, *m/z* 629, Gal-GlcNAc-Man, *m/z* 670
Presence of sialic acid	*m/z* 290.1
α2? 6-linked sialic acid	Additional *m/z* 306.1
Gal-GlcNAc in antenna	*m/z* 424.1 (Gal-GlcNAc+59)
α-galactose on antenna	*m/z* 586 (Gal-Gal-GlcNAc+59)
GalNAc-GlcNAc in antenna	*m/z* 465 (GalNAc-GlcNAc+59)
Gal-(Fuc)GlcNAc in antenna	*m/z* 570 (Gal-(Fuc)GlcNAc+59)

The fragmentations were initiated by removal of specific hydroxylic protons that catalysed diagnostic reactions (Figure 2). An example of a typical fragmentation spectrum is shown in Figure 3.

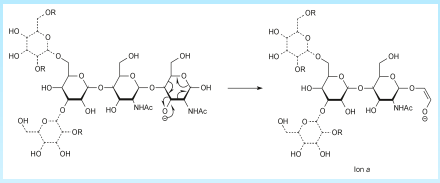

Figure 2. Proposed fragmentation scheme for the formation of $^{2,4}A_R$ ions from the reducing terminal GlcNAc of *N*-linked glycans. Core chitobiose residue in bold. R in $^{2,4}A_R$ = reducing terminus

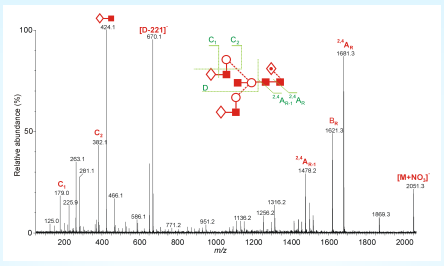

Figure 3. MS/MS spectrum of a biantennary *N*-linked glycan with a bisecting GlcNAc residue and fucose substituted on the reducing-terminal GlcNAc.

N-Linked glycans from the SARS virus

N-glycans were obtained from SARS virus glycoproteins expressed in African green monkey cells (VEro6). Figure 4 shows a MALDI profile annotated with structures from negative ion MS/MS. A representative MS/MS spectrum is shown in Figure 5.

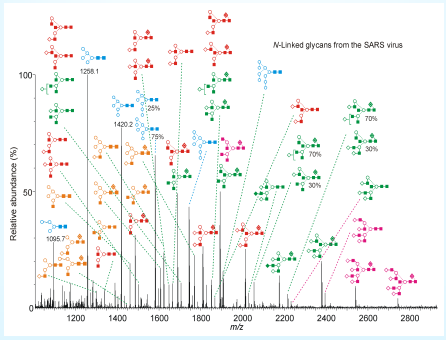

Figure 4. Positive ion MALDI mass spectrum of *N*-linked glycans from the SARS virus annotated with structures deduced from the negative ion fragmentation spectra.

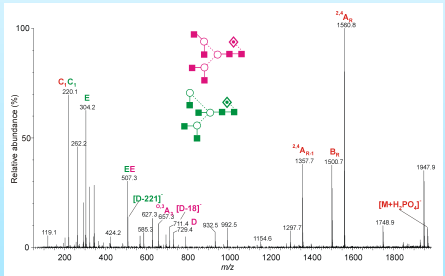

Figure 5. MS/MS spectrum of two isomeric compounds from SARS virus *N*-glycans at *m/z* 1965. Colours as in Figure 6.

N-Linked glycans from chicken IgY

Figure 6 shows the MS spectrum of the 2-AB derivatives of *N*-linked glycans released from chicken IgY. The structures of all of the annotated peaks were determined by MS/MS (see Figure 1 for the key to structural symbols). Fragmentation of the 2-AB derivatives was comparable to that of the underivatized compounds except that the $^{2,4}A_R$ ion was missing because of the open nature of the derivatized GlcNAc residue. However, the presence of substituents (e.g. fucose) on the core GlcNAc residues could still be detected by the mass of the $^{2,4}A_{R-1}$ ion. The fragmentation of the high-mannose glycan, $Hex_{10}GlcNAc_2$-2AB is shown in Figure 7.

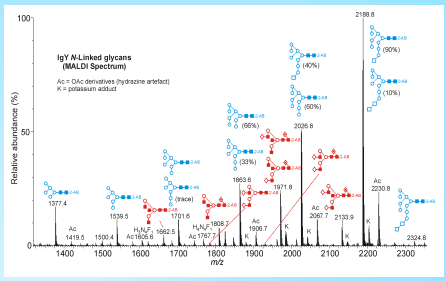

Figure 6. MALDI (DHB) MS spectrum of the 2-AB derivatives of *N*-linked glycans released from chicken IgY.

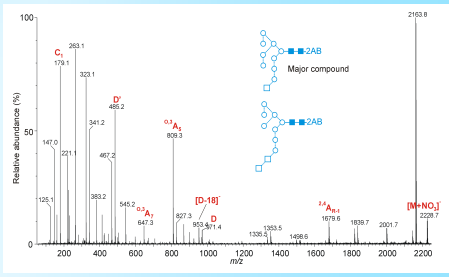

Figure 7. MS/MS spectrum of the 2-AB derivatives of *N*-linked glycans from chicken IgY having the composition of $Hex_{10}HexNAc_2$. D, $[D\text{-}18]^-$ and $^{2,4}A_6$ ions at *m/z* 971, 953 and 809 respectively, confirm the structure of the major glycan. Ions 162 mass units less show the presence of the second glycan.

Further structural studies were made with MS^3 spectra using the Thermo Orbitrap instrument. Figure 5 (top) shows the MS/MS spectrum of *N*-glycans released from chicken ovalbumin with an expansion of the ion at *m/z* 1702 showing a resolution of about 20,000. Figure 5 (centre) shows the MS^2 spectrum of this ion and Figure 3 (bottom) shows the MS^3 spectrum of the D ion (*m/z* 629).

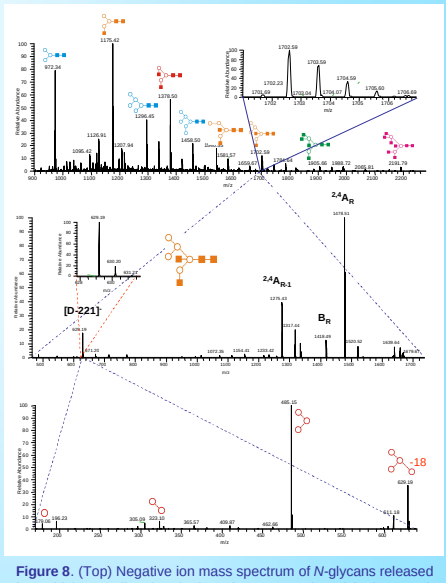

Figure 8. (Top) Negative ion mass spectrum of *N*-glycans released from chicken ovalbumin. The inset is an expansion of the ion at *m/z* 1702 (resolution about 20,000). (Centre) MS^2 spectrum of *m/z* 1702 with expansion of *m/z* 629 at 30,000 resolution. (Bottom) MS^3 spectrum of the D ion (*m/z* 629).

Conclusions

Negative ion nanospray ionization coupled with MS^2 and MS^3 fragmentation provides a powerful method for rapid identification of *N*-linked glycans. Identifiable structural features include antenna composition, presence of bisecting GlcNAc and position of fucose.

References

1. D. J. Harvey, Fragmentation of negative ions from carbohydrates: Part 1; Use of nitrate and other anionic adducts for the production of negative ion electrospray spectra from *N*-linked carbohydrates, *J. Am. Soc. Mass Spectrom.*, 2005, **16**, 622-630.
2. D. J. Harvey, Fragmentation of negative ions from carbohydrates: Part 2, Fragmentation of high-mannose *N*-linked glycans, *J. Am. Soc. Mass Spectrom.*, 2005, **16**, 631-646.
3. D. J. Harvey, Fragmentation of negative ions from carbohydrates: Part 3, Fragmentation of hybrid and complex *N*-linked glycans, *J. Am. Soc. Mass Spectrom.*, 2005, **16**, 647-659.