Chapter 10

Collision-Induced Dissociation Tandem Mass Spectrometry for Structural Elucidation of Glycans

Bensheng Li, Hyun Joo An, Jerry L. Hedrick, and Carlito B. Lebrilla

Summary

The complexity of glycans poses a major challenge for structure elucidation. Tandem mass spectrometry is currently an efficient and powerful technique for the structural characterization of glycans. Collision-induced dissociation (CID) is most commonly used, and involves first isolating the glycan ions of interest, translationally exciting them, and then striking them with inert target gas to fragment the precursor ions. The structural information of the glycan can be obtained from the fragment ions of the tandem MS spectra.

In this chapter, sustained off-resonance irradiation-collision-induced dissociation (SORI-CID) implemented with matrix-assisted laser desorption/ionization Fourier transform ion cyclotron resonance mass spectrometry (MALDI FT ICR MS) is demonstrated to be a useful analysis tool for structural elucidation of mucin-type *O*-glycans released from mucin glycoproteins. The mechanisms by which the glycans undergo fragmentations in the tandem mass analysis are also discussed.

Key words: *O*-Linked glycans, Glycoproteins, MALDI, FTICR MS, SORI-CID, Tandem mass spectrometry.

1. Introduction

As one of the most widespread posttranslational modifications of proteins in eukaryotes, glycosylation of proteins is recognized for biological versatility and indispensability. Glycans attached to a glycoprotein play important roles in many biological processes including the proper folding of proteins, molecular recognition involved in specific inter- and intracellular interactions, and cellular adhesion *(1–4)*. To better understand the roles of oligosaccharides in glycoproteins, it is important to elucidate their structures

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and distributions in glycoproteins. Unlike proteins and nucleic acids where the macromolecular chains are linear, the oligosaccharides can be connected in many ways and branched with as many as four carbohydrate residues linked to a central monosaccharide.

The *O*-glycosylation of proteins can result in the formation of mucin-type macromolecules. Mucins are *O*-glycosylated proteins mostly found on the cell surface or in the secretions of cells *(5)*. The *O*-glycans have specific functions, such as protecting underlying proteins as well as epithelial cell surfaces from pathogen attack, involving in sperm–egg recognition during fertilization, participating in the immune system, and in clotting blood *(6, 7)*. Recently the roles played by *O*-glycans in biology, physiology and immunology of cancer have become more and more realized, thus the investigation on *O*-glycans has been one of the most attractive areas in oncology and clinical study of cancers. *(6–10)*.

The structural complexity and variety of *O*-linked glycans have posed a challenge for structural elucidation. Traditional structure analysis of glycans by NMR has some limitations in that it requires a relatively large amount of sample and it is time consuming. Mass spectrometric analysis of *O*-glycans has proved to be a very powerful and efficient tool not only in profiling the structural distribution of glycans *(11–13)* but for specific structure elucidations as well *(14–20)*, due to its high sensitivity and potentially high throughput.

Collision-induced dissociation (CID, also called collision-activated dissociation, CAD) tandem mass spectrometry has been the most widely employed technique for structural elucidation and continues to play a prominent role in the analyses of oligosaccharides and other molecules *(21–26)*.

The advantage of FTICR (and ion traps in general) is that CID is temporally rather than spatially resolved. Furthermore, it is easily implemented with either MALDI- or ESI-produced ions. CID in FTICR is performed by isolating the desired ion. This procedure involves the resonance excitation of all other ions to the point where they are ejected from the cell or collide with the cell walls. In the past, ejection of unwanted ions was performed with selective resonance ejection of individual ions. More sophisticated methods have been developed using arbitrary waveform generators that can be programmed for the retention of desired masses *(27)*.

There are various CID techniques that vary not only in collision energy but also in the amount of internal energy deposited in precursor ions upon collision, the collision number prior to fragmentation, and the time scale between collision activation and detection. They can be broadly grouped into two categories based on the translational energy (collision energy) possessed by the precursor ions just prior to collision with the target inert

gas molecules: low (1–300 eV) and high (1–25 keV) collision energy CIDs.

With FTICR MS, CID generally involves low collision energy. In the ICR cell the ions of interest can be excited on-resonance, i.e., at a frequency equal to the ions' cyclotron frequency. The event can increase the translation energy to about 100 eV. In this method, the ions are translationally excited to a larger cyclotron orbit. The precursor ions can also be periodically excited by the application of a sustained (typically 600 ms) off-resonance irradiation (SORI) of alternating electric field pulse with a frequency slightly offset from the ions' natural cyclotron resonance frequency. A constant RF level is applied to the excite electrodes throughout the CID event. As a consequence, the ions undergo acceleration–deceleration cycles and thus a sequential activation of ions by multiple collisions of low translational energy (<10 eV) with the target gas throughout the duration of the electric field pulse. Spatially, the ions experience the corresponding cycles of being excited to a small radius away from the center of ICR cell, then relaxing back to the center. The precursor and fragment ions hover near the center of the cell allowing additional stages of CID and stronger intensities during detection. SORI-CID can facilitate the lowest energy pathway of fragmentation of precursor ions as only small increments of internal energy are deposited on the ions throughout the duration of the event *(28, 29)*. In this way, the SORI-CID is an analog to infrared multiphoton dissociation (IRMPD), which is discussed in the Chapter 2 *(30–43)*.

2. Materials

2.1. Release and Purification of Oligosaccharides

1. Release solution: 1.0 M $NaBH_4$ in 0.1 M NaOH, prepared freshly.
2. Lyopholized glycoprotein (e. g. egg jelly coat of *Xenopus tropicalis* (*see* **Note 1**).
3. Heating facility (42°C).
4. Neutralizing solution: 1.0 M HCl.
5. Solid phase extraction (SPE) graphitized carbon cartridges (150 mg, 4 mL) from Alltech Associates, Inc. (Deerfield, IL).
6. Activation solution: nano pure water and 80% acetonitrile (AcN) in 0.1% trifluoroacetic acid (TFA) (v/v).
7. Washing solution: nano pure water.
8. Elution solutions: 10% and 20% AcN in H_2O and 40% AcN with 0.05% TFA in H_2O.

9. Purification with HPLC as described in the Chapter 2 "Infrared Multiphoton Dissociation Mass Spectrometry for Structural Elucidation of Oligosaccharides."

2.2. MALDI-FTICR Analyses of Oligosaccharides

1. MALDI-FT mass spectrometer (IonSpec, Irvine, CA).
2. Released and/or HPLC fractionated oligosaccharides (1–6 μL) (*see* the Chapter 2 "Infrared Multiphoton Dissociation Mass Spectrometry for Structural Elucidation of Oligosaccharides").
3. MALDI matrix solution: 0.4 M matrix, 2,5-dihydroxy benzoic acid (DHB), in 50:50 H_2O/AcN.
4. Positive mode dopant: 0.1 M NaCl in 50:50 H_2O/AcN.

3. Methods

3.1. Release and Purification of Oligosaccharides (*See* Note 2)

1. Freshly prepared release solution is added into a 15 mL plastic tube containing the lyophilized glycoprotein to a volume of 267 μL release solution/mg of glycoprotein *(44)*. The solution is gently vortexed to mix well.
2. The tube is wrapped with parafilm and placed at 42°C for 16–20 h. The release of *O*-linked glycans from glycoproteins is a β-elimination reaction under strongly basic conditions (*see* **Note 3**).
3. After incubation, the excess $NaBH_4$ is slowly neutralized with 1.0 M HCl acid on ice until the solution is acidic (pH 2–5).
4. The salts and deglycosylated proteins are removed from the solution by solid phase extraction (SPE) with graphitized carbon cartridge. The cartridges are washed sequentially with nano pure water, 80% acetonitrile (AcN) in 0.1% trifluoroacetic acid (TFA) (v/v) and then pure water.
5. The flow rate during the SPE extraction should be kept at 200 μL/min after the sample loading to maximize the interaction of oligosaccharides with the stationary phase of the cartridge.
6. The subsequent wash with nano pure water is made 5–6 times (one full column each time) to remove salts and deglycosylated proteins.
7. After the wash, the glycans are eluted with 10%, 20% AcN in H_2O and 40% AcN with 0.05% TFA in H_2O, respectively. The collected glycan solution is dried and reconstituted in 10–30 μL of nano pure water. Oligosaccharides were further fractionated using HPLC, as described in this book (the Chapter 2

"Infrared Multiphoton Dissociation Mass Spectrometry for Structural Elucidation of Oligosaccharides").

3.2. MALDI-FTICR Analyses of Oligosaccharides

A commercial MALDI-FT mass spectrometer (IonSpec, Irvine, CA) with an external ion source was used to perform the analysis. The instrument is equipped with a 7.0-T shielded, superconducting magnet and an Nd:YAG laser at 355 nm. MALDI sample was prepared by loading 1–6 μL of analyte and 1 μL of MALDI matrix solution on a stainless steel target plate. For the positive mode analyses, 1 μL of 0.1 M NaCl in 50:50 H_2O/AcN was applied to the spot to enrich the Na^+ concentration and thus produce primarily sodiated species. The plate was placed in ambient air to dry the sample spots before insertion into the ion source.

3.3. Sustained Off-Resonance Irradiation-Collision-Induced Dissociation Tandem Mass Spectrometry for Structure Elucidation of Glycans

A typical pulse sequence for SORI-CID in an FTCIR mass spectrometer is shown in **Fig. 1**. A desired ion is readily selected in the analyzer with the use of an arbitrary waveform generator and a frequency synthesizer. All CID experiments were performed at +1,000 Hz off-resonance from the cyclotron frequency of the precursor ion. The CID excitation time was 1,000 milliseconds (ms). Two pulses of argon were introduced into analyzer chamber at 0 and 500 ms for collisions. The excitation voltages ranged from 2.5 to 8.0 V (base-to-peak) depending on the desired level of fragmentation and the size of oligosaccharides.

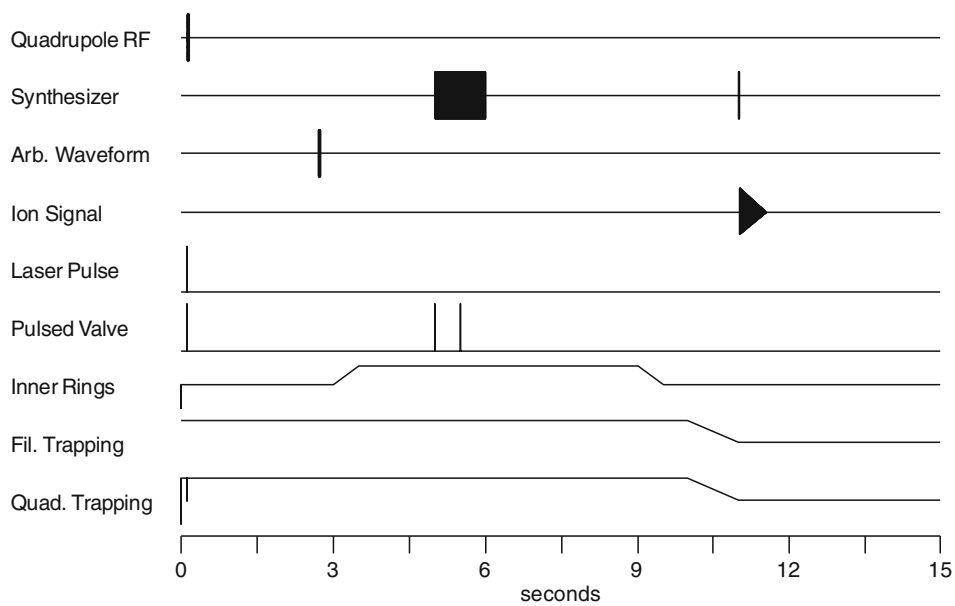


Fig. 1. A typical pulse sequence employed for sustained off-resonance irradiation collision-induced dissociation (SORI-CID) in an FTICR mass spectrometer.

3.4. Interpretation of SORI-CID Spectra of O-Linked Oligosaccharides

Manual interpretation of SORI-CID spectra involves knowledge of fragmentation pathways as well as understanding of the biosynthetic pathway of oligosaccharides. In addition, tables of masses of common monosaccharide building blocks are essential in order to work out the monosaccharide sequence of unknown structures. (for tables, *see* the Chapter 1 "Analysis of *N*- and *O*-Linked Glycans from Glycoproteins Using Maldi-TOF Mass Spectrometry"). Here, two examples are given of the interpretation and the logical reasoning for the assessment of an unknown structure. The first example of an *O*-linked oligosaccharide being probed by SORI-CID is shown in **Fig. 2**. The MS/MS spectrum of an *O*-linked glycan, *m/z* 1,268.463 ($[M + Na]^+$) is shown in **Fig. 2A**. The sodium cation allows a fine balance between abundant quasimolecular ions and CID fragment ions. The sequence of the glycan can be elucidated based on the CID spectra. The glycan consists of 2 hexoses (Hex), 2 deoxyhexoses (dHex), and 3 *N*-acetyl hexosamines (HexNAc) based on the accurate mass (experimental mass 1,268.463 Da, theoretical mass 1,268.475 Da, $\Delta m = 0.012$ or 9 ppm). **Figure 2B** corresponds to the MS^3 spectrum (1,268 to 1,122), while **Fig. 2C** corresponds to MS^4 spectrum (1,268 to 1,122 to 976). **Figure 2A, B** indicate that two dHex residues are readily lost from quasimolecular ion (*m/z* 1,268) to give the base peaks at *m/z* 1,122 and 976, respectively. The ion at *m/z* 1,268 loses a dHex (*m/z* 1,122) as the major loss, a Hex (*m/z* 1,106) as a minor, and a HexNAc (*m/z* 1,065) also minor, suggesting that the three residues are non-reducing termini (**Fig. 2A**). The ion (*m/z* 1,122) loses a dHex (*m/z* 976), a Hex (*m/z* 960), and a HexNAc (*m/z* 919). The latter two ions subsequently lose a HexNAc and a Hex, respectively, to yield *m/z* 757. The resulting ion further loses the reducing end GalNAc-ol to yield *m/z* 534 $[1dHex + 1Hex + 1HexNAc + Na]^+$. The fragmentation pattern suggests that the reducing terminus, GalNAc-ol, is linked to both HexNAc-Hex and Hex-HexNAc. The presence of the ion *m/z* 388 (**Fig. 2C**) corresponds to a $[HexNAc\text{-}Hex + Na]^+$ species while the absence of *m/z* 347 and *m/z* 429 which would correspond to a $[Hex\text{-}Hex + Na]^+$ and a $[HexNAc\text{-}HexNAc + Na]^+$, respectively, confirm the structural assignment. The position of the dHex is likely to be on an internal residue as the fragment ions indicate the presence of terminal Hex and HexNAc. In addition, dHex is almost never found on the core residue. Based on these considerations, the structure for the *m/z* 1,268 is proposed as shown in the inset of **Fig. 2C.**

As shown in **Fig. 2**, *O*-glycan alditols fragment to yield only B ions and Y ions under the SORI-CID conditions (*see* **Note 4**). This observation has been confirmed by extensive CID studies of many *O*-linked oligosaccharides *(45)*. The final residue is often the alditol core (*m/z* 246), which suggests that the reduced core or the alditol binds the most strongly to the sodium ion.

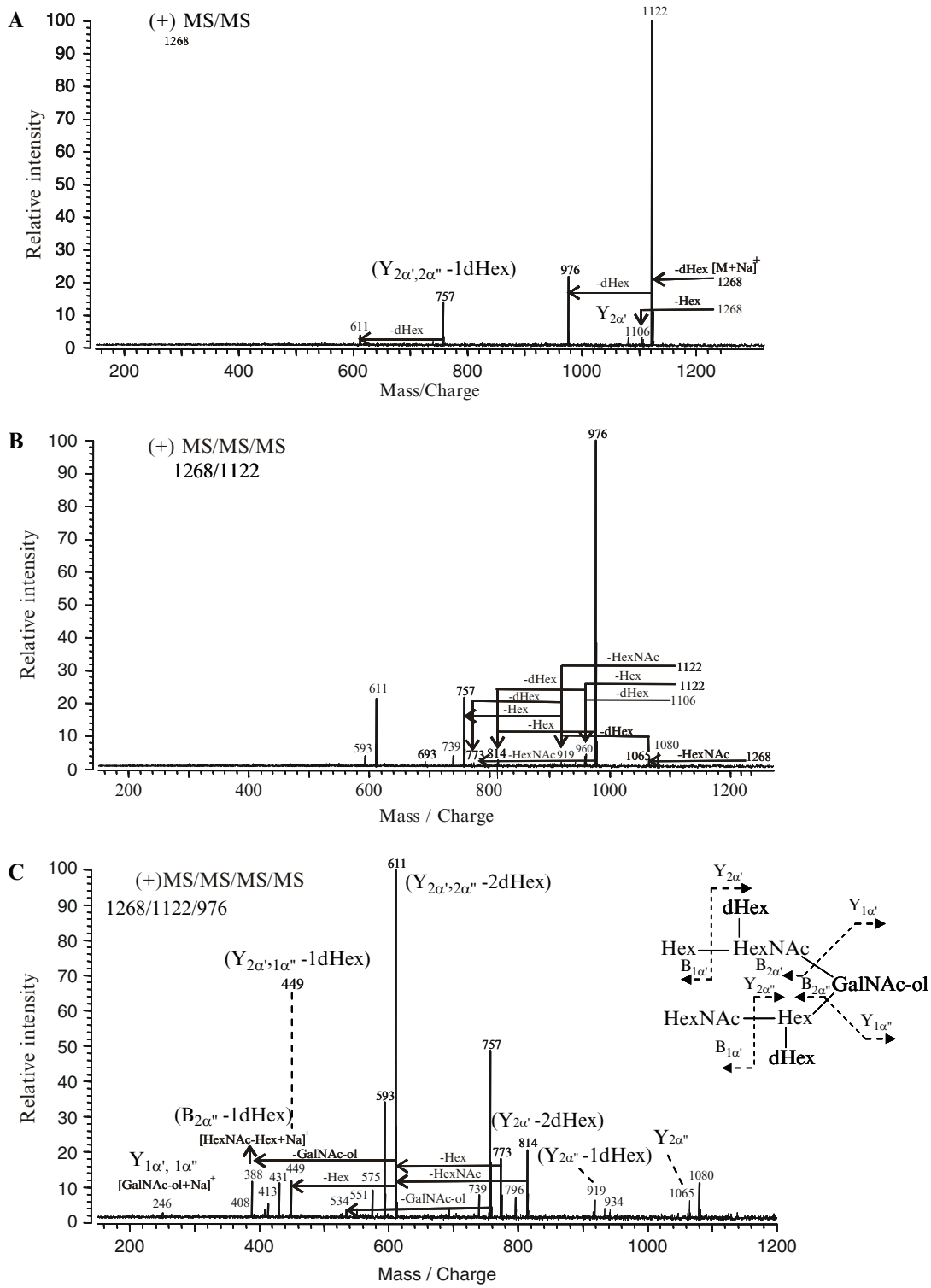


Fig. 2. MALDI SORI-CID tandem mass spectra of an *O*-linked glycan, *m/z* 1,268 ([M + Na]$^+$), from egg jelly glycoprotein of *Xenopus tropicalis* in positive mode. (**A**) MS^2 spectrum of the precursor ion, *m/z* 1,268. (**B**) MS^3 spectrum of a product ion (*m/z* 1,122) from MS^2. (**C**) MS^4 spectrum of a product ion (*m/z* 976) from MS^3. Based on the fragmentation patterns of the tandem mass spectra, the glycan structure of the quasimolecular ion, *m/z* 1,268, has been elucidated and shown in inset of the figure (systematic nomenclatures of some fragments are also included).

The second example of a CID analysis of an *O*-linked oligosaccharide is shown in **Fig. 3**. An oligosaccharide was found with *m/z* 1,065.405 ($[M + Na]^+$) consisting of 2 dHex, 2 Hex, and 2 HexNAc. The MS^2 spectrum is shown in **Fig. 3A**. The quasimolecular ion loses one dHex (*m/z* 919, the base peak) and one Hex (*m/z* 903) indicating that a dHex and a Hex are located at the non-reducing termini. The ion at *m/z* 919 further loses one dHex (*m/z* 773), one Hex (*m/z* 757) or one HexNAc (*m/z* 716). The loss of the HexNAc from *m/z* 919 indicates that the location of the first dHex is on the HexNAc. The simultaneous losses of Hex, HexNAc and dHex further indicate that the Hex and the dHex are bound to either the remaining Hex or the GalNAc-ol. However, it is seldom that a dHex is connected to the

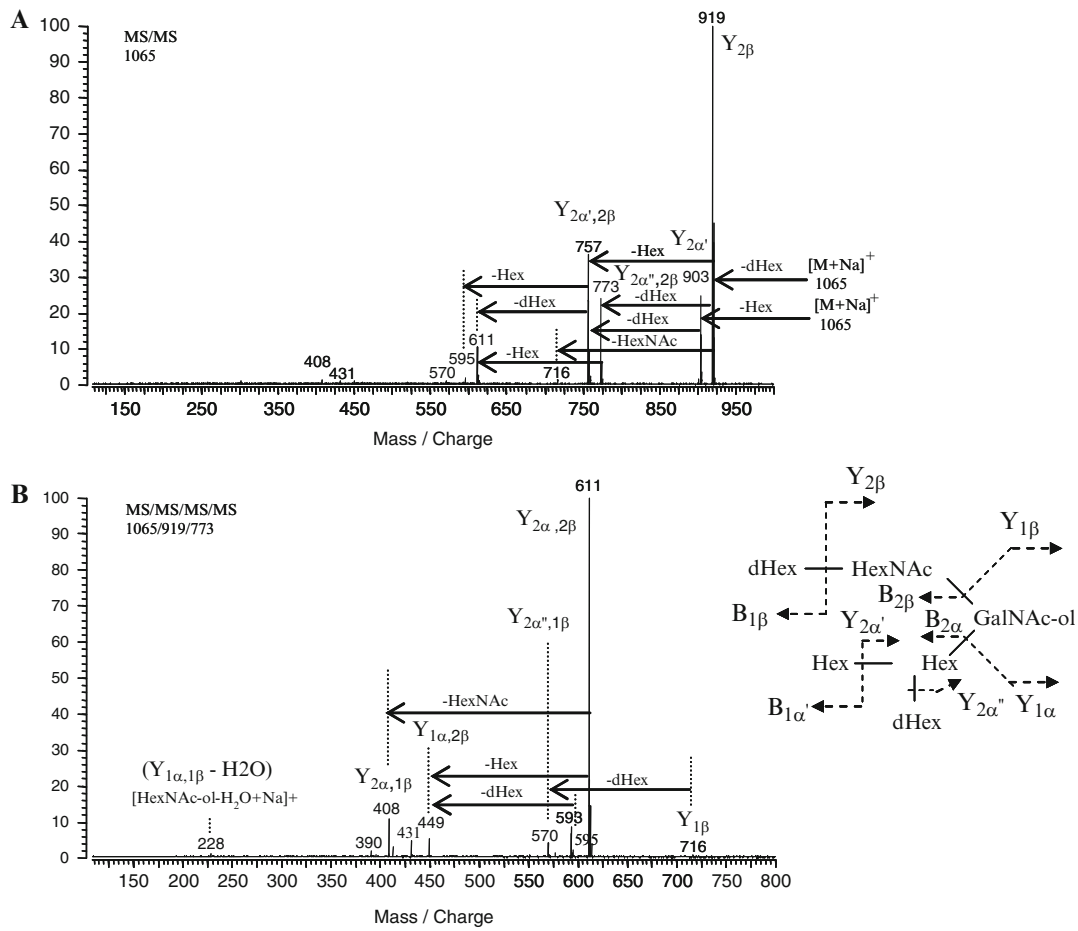


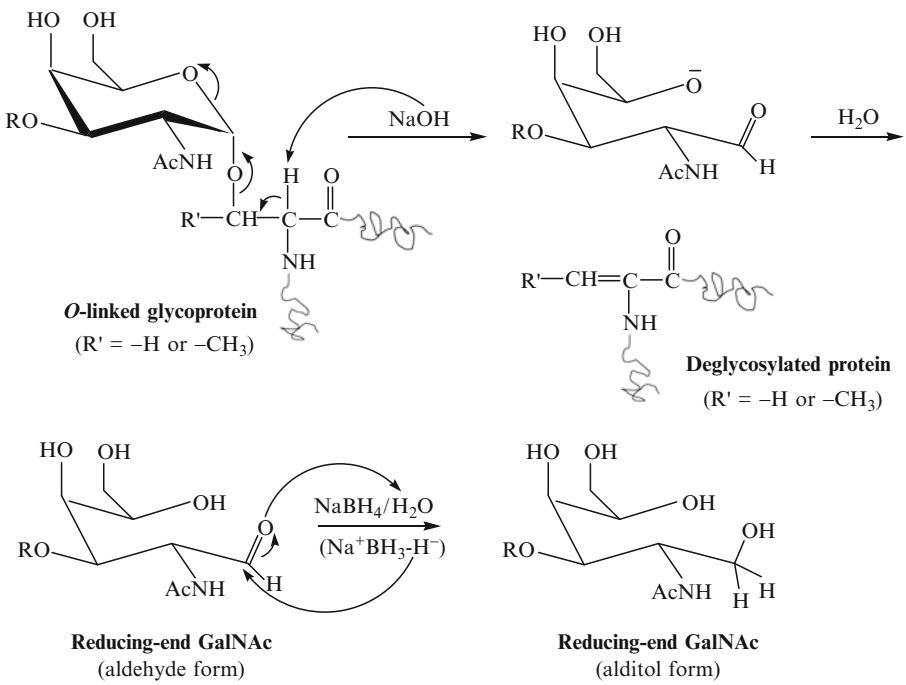
Fig. 3. MALDI SORI-CID tandem mass spectra of an *O*-linked glycan, *m/z* 1,065 ($[M + Na]^+$), from egg jelly glycoprotein of *Xenopus laevis* in positive mode. (**A**) MS^2 spectrum of the precursor ion, *m/z* 1,065. (**B**) MS^4 spectrum of a product ion (*m/z* 773). Based on the fragmentation patterns of the tandem mass spectra, the glycan structure of the quasimolecular ion, *m/z* 1,065, has been elucidated and shown in inset of the figure (systematic nomenclatures of some fragments are also included).

core GalNAc-ol, therefore it follows that Hex and dHex are connected to the same Hex. The ion at *m/z* 611 corresponds to one HexNAc, one Hex and one GalNAc-ol, and can further fragment one Hex (*m/z* 449) to yield [HexNAc-GalNAc-ol + Na]$^+$ (**Fig. 3B**). It can also lose one HexNAc (*m/z* 408) to yield the fragment ion [Hex-GalNAc-ol + Na]$^+$ suggesting that the reducing end is branched. The structure assigned for this ion is shown in the inset of **Fig. 3B**, while the other peaks in the tandem MS spectra (**Fig. 3A, B**) confirm the assignment.

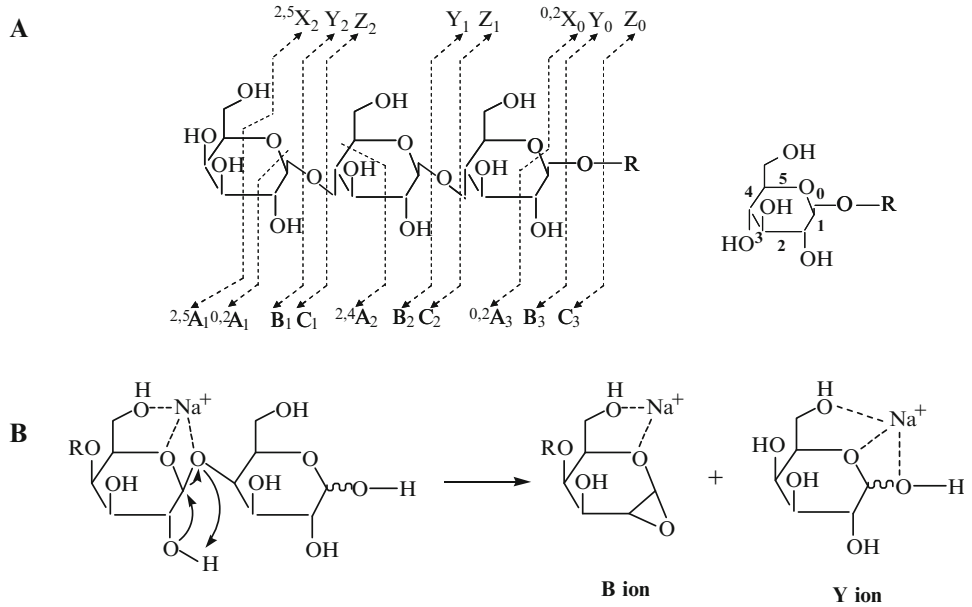
In both examples, glycosidic bond cleavages are abundant, while cross-ring cleavages are absent in the conditions used in the study.

4. Notes

1. Oligosaccharides were obtained from the lyophilized glycoproteins by the procedure described in earlier publications *(14–18)*.
2. To obtain clean and informative tandem mass spectra with high signal/noise, the glycan release reaction and sample preparations are crucial.



Scheme 1. The mechanism for reductive β-elimination under alkaline condition to release *O*-linked glycans from glycoprotein. ~~ represents protein chain.



Scheme 2 **(A)**. A systematic nomenclature for carbohydrate fragmentations. (**B**) A general mechanism for B/Y ion formation in positive mode.

3. The mechanism for reductive β-elimination is shown in **Scheme 1**. First the acidic proton at α carbon of a serine or threonine residue is abstracted by existing base (OH^-) followed by the cleavage of C_β-O bond linking the glycan to the serine or threonine residue, thus removing the glycan portion from the β carbon of the specific residue in protein. The nascent sugar aldehyde is reduced by BH_4^- ion to its corresponding alditol. The alditol prevents the peeling reaction that otherwise further degrades the oligosaccharide chain. From the standpoint of the MS, the alditol has the further advantage of designating the reducing end.
4. The ion fragments of glycans follow a systematic nomenclature coined by Domon and Costello *(46)*. The mechanism for B/Y ion formation in the positive mode is shown in **Scheme 2** *(47, 48)*.

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