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Complementary structural information of positive- and negative-ion MSⁿ spectra of glycopeptides with neutral and sialylated N-glycans

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Abstract: Positive- and negative-ion MSⁿ spectra of chicken egg yolk glycopeptides binding a neutral and a sialylated N-glycan were acquired by using electrospray linear-ion trap time-of-flight mass spectrometry (ESI-LIT-TOFMS) and collision-induced dissociation (CID) with He as a collision gas. Several characteristic differences were observed between the positive- and negative-ion CID MSⁿ (n=2,3) spectra. In the positive-ion MS² spectra, the peptide moiety was presumably stable, but the neutral N-glycan moiety caused several B-type fragmentations and the sialylated N-glycan almost lost sialic acid(s). In contrast, in the negative-ion MS² spectra, the peptide moiety caused several side-chain and N-glycan residue (e.g., *N*-acetylglucosamine (GlcNAc) residue) fragmentations in addition to backbone cleavages, but the N-glycan moieties were relatively stable. The positive-ion MS³ spectra derived from the protonated peptide ion containing GlcNAc residue (203.1 Da) provided enough information to determine the peptide amino-acid sequence including the glycosylation site, while the negative-ion MS³ spectra derived from the deprotonated peptide containing a ^{0,2}X₁-type cross-ring cleavage (83.1Da) complicated the peptide sequence analysis due to side-chain and the ^{0,2}X₁ residue related fragmentations. However, for the structural information of the N-glycan moiety of the glycopeptides, the negative-ion CID MS³ spectra derived from the deprotonated ^{2,4}A₆-type cross-ring cleavage ion (neutral N-glycan) or the doubly deprotonated B₆-type fragment ion (sialylated N-glycan) is more informative than are those of the corresponding positive-ion CID MS³ spectra. Thus, the positive-ion mode of CID is useful for the analyses of

peptide amino-acid sequences including the glycosylation site. The negative-ion mode of CID is especially useful for sialylated N-glycan structural analysis. Therefore, in the structural analysis of N-glycopeptides, their roles are complementary.

Introduction

Glycosylation is the most common of post-translational modifications and its characterization is an important topic in glycoproteomics.¹ Mass spectrometry (MS), most predominantly featuring matrix-assisted laser desorption/ionization (MALDI^{2,3}) and electrospray ionization (ESI^{4,5}), play important roles in the rapid and sensitive structural analysis of proteins and post-translational modified proteins. Since the first attempt of Biemann and Martin in 1987,⁶ positively charged parent and product ions have been used for analyzing peptide amino-acid sequences, but negatively charged ions has been paid little attention. Database searching, e.g., by MASCOT⁷ after MS analysis is also based on only positively charged ions. As mentioned by Bowie *et al.* in their recent review article,⁸ because fragment (product) ion spectra derived from negative parent ions of peptides are relatively complex due to several types of side chain cleavages and reactions, peptide amino-acid sequences are sometimes difficult to analyze. Thus, it appears that only negative fragment ions have been discussed with regard to the MS fragmentation mechanism⁸ or the sensitive MS detection of acidic peptides.⁹

In this study, we present MSⁿ spectra of chicken egg yolk glycopeptides with a neutral (asialo) or a sialylated N-glycans (hereafter called neutral N-glycopeptide and sialylated N-glycopeptide). The MSⁿ spectra were acquired by using a nanoelectrospray linear-ion trap time-of-flight mass spectrometry (nanoESI-LIT-TOFMS) in the positive- and negative-ion modes and by collision-induced dissociation (CID) with He as a collision gas. Several characteristic differences were observed in the positive- and negative-ion CID MSⁿ (n=2,3) spectra of the neutral and sialylated N-glycopeptides. Here, we first compare the positive-ion and negative-ion CID MSⁿ (n=2,3) spectra of the neutral N-glycopeptides. Then, the MSⁿ spectral comparison is applied to the sialylated N-glycopeptide. Although many attempts at targeting a direct analysis of peptide amino-acid sequences and glycan structures of glycopeptides have recently been reported,¹⁰⁻²¹ to the best of our knowledge, negative-ion CID MSⁿ (n=2,3) spectra of N-glycopeptides have neither been reported nor discussed. This work first demonstrates that negative-ion CID MSⁿ spectra of N-glycopeptides are useful for the direct structural analysis of the N-glycan moiety of, especially, sialylated N-glycopeptides, which is difficult in the positive-ion mode due to the easy occurrence of the neutral loss of sialic acid(s), in other words, a complete loss of its linkage and position information occurred. However, positive-ion CID MSⁿ spectra are definitely useful for determining the peptide amino-acid sequence including the glycosylation site of N-glycopeptides, which was difficult in negative-ion

CID MSⁿ spectra investigated in this work. In this sense, the positive- and negative-ion CID MSⁿ spectra play complementary roles for structural analysis of N-glycopeptides.

EXPERIMENTAL

Chemicals and samples.

Methanol (HPLC/MS-grade), acetonitrile (HPLC/MS-grade), water, and ammonium acetate were purchased from Wako Chemical (Tokyo, Japan). A sample preparation of chicken egg yolk is described in the reference.²² The procedure we did was simply as follows. Unfertilized eggs were diluted with an equal volume water; the diluted egg yolk was mixed with 1/10 volume of phenol/water (9:1, w/w) and stirred for 2 hours. After centrifuging at 6,000 r.p.m. for 30 min, the supernatant was concentrated under the reduced pressure. Then, the sample was separated by a GPC (Sephadex G-50) column and sialylated N-glycopeptide was fraction collected. A portion of the collected sample was desialylated at 90°C for 1 hour with 0.01 M HCl (pH 2.0) and then neutralized with 1.0 M ammonium bicarbonate buffer. Scheme 1 illustrates the peptide amino-acid sequence and N-glycan structures of the chicken egg yolk N-glycopeptides.

NanoHPLC/ESI-IT TOF MS analysis.

The HPLC/MS systems used was a NanoFrontier L (Hitachi High-Technologies, Tokyo, Japan), which consists of a capillary HPLC system and an ESI-Linear IT-TOF mass spectrometer.²³⁻²⁵ The sample was directly infused into the ESI source through a SilicaTip electrosprayer (tip diameter 10 μm) (New Objective, Woburn, MA). The ESI-LIT-TOFMS conditions were as follows. The ESI voltage was 1.5-1.7 kV, the curtain (nitrogen) gas was used at a flow rate of 0.8-1.0 L/min without heating, the scan mass range (m/z) was 100-2000, and the mass accuracy and resolution were within ± 50 ppm and more than 8000 FWHM, respectively. The CID-related parameters in the positive- and negative-ion modes were: CID gas (He) flow rate, 1 mL/min; isolation time, 5 ms; isolation width, 15 (MS^2) and 8 (MS^3); and CID gain, 1.2 (MS^2) and 1.0 (MS^3).

RESULTS AND DISCUSSION

MS^n spectra of neutral N-glycopeptide from chicken egg yolk.

MS^n ($n=1-3$) spectra were acquired by using the nanoHPLC/ESI-LIT-TOFMS under the analytical conditions described in the Experimental section. Figure 1A shows the positive-ion MS^2 spectrum derived from the precursor ion ($[\text{M}+\text{H}+\text{Na}]^{2+}$, m/z 1153.1), which appeared as a base peak ion in the MS^1 spectrum (not shown). The protonated peptide ion (m/z 863.5) with GlcNAc residue (203.1 Da) and the sodiated B_5 -type (Domon and Costello's nomenclature²⁶ is used hereafter) fragmented N-glycan ion (m/z 1442.4) were observed in the positive-ion MS^2 spectrum accompanying the several

protonated and sodiated B-type fragment ions (e.g., m/z 366.1(H^+), m/z 388.1(Na^+), m/z 550.2(Na^+)). Figure 1B shows the negative-ion MS^2 spectrum derived from the precursor ion ($[M-2H]^{2-}$, m/z 1139.1) in the MS^1 spectrum (not shown). The deprotonated peptide ion (m/z 741.5) with $^{0,2}X_1$ -type cross-ring cleavage of the GlcNAc residue (83.1 Da) and the deprotonated $^{2,4}A_6$ -type cross-ring cleavage fragment ion (m/z 1479.6) from the N-glycan moiety were relatively abundant. Interestingly, there was no other fragment ion derived from the N-glycan moiety in the lower m/z region and, instead, several fragment ions (e.g., m/z 314.2, m/z 382.2, m/z 426.2, m/z 641.4, m/z 697.5) from the peptide moiety were seen in Figure 1B. This seems to be one of the characteristic differences between the positive- and negative-ion MS^2 spectra of the neutral N-glycopeptide.

Figures 2A and 2B show the positive- and negative-ion MS^3 spectra which were acquired by selecting each of the peptide fragment ions (m/z 863.5 and m/z 741.5) as a precursor ion, respectively. In the positive-ion MS^2 spectrum (Figure 2A), b/y-type (Roepstorff's nomenclature ²⁷ is used hereafter) fragment ions could easily be assigned. Then, the peptide amino-acid sequence (KVANKT) including the binding site (N: Asparagine) of the GlcNAc residue was determined from these fragment ion series. In contrast, the fragment ions in the negative-ion MS^2 spectrum (Figure 2B) were difficult to assign because of side-chain and the $^{0,2}X_1$ residue (83.1 Da) related fragmentations rather than the usual backbone cleavage, which is necessary for peptide amino-acid sequence analysis. The fragment ions in Figure 2B were assigned by referring to the results of Bowie *et al.* ⁸ The

numbers in parenthesis in Figure 2B correspond to the neutral losses of side-chain fragments (17 Da: NH₃ (Asn); 44 Da: MeCHO (Thr)/ CO₂) suggested by Bowie *et al.*⁸, and the neutral loss of MeCHO (44 Da) from the ^{0,2}X₁-type cross ring cleavage of the GlcNAc residue (83.1 Da). In addition, a neutral loss of threonine (Thr) seems to reduce the mass value by 100.1 Da instead of the usually expected 101.1 Da. It might be due to that an additional migration of hydrogen (H) occurred during the fragmentation pathway of the neutral loss of Thr. Or, it might be due to that a ring-imide formation occurred at Asn (-100 Da) (anonymous reviewer's comment). The details are still unclear.

The positive- and negative-ion MS³ spectra, which were derived from the B₅-type (*m/z* 1442.4) and ^{2,4}A₆-type (*m/z* 1479.6) fragment ions observed in Figures 1A and 1B, respectively, are also quite different from each other. In the positive-ion MS³ spectrum (Figure 3A), only B-type fragment series ions generated by one and two glycoside-bond cleavages were easily observed. The annotations in Figure 3A show only one type of fragment ions of the corresponding two cleavages of the glycoside bonds. In contrast, in the negative-ion MS³ spectrum (Figure 3B), various (A, B, C, and D) types of fragment ions were observed.^{28, 29} Their annotations (i.e., ^{1,3}A₃, C₂, ^{2,4}A₆/Y₃, ^{2,4}A₆/Y₄) are shown in the inset without distinguishing between the upper (α1,6) or lower (α1,3) branch (antennae). Such a characteristic difference between the positive- and negative-fragment ions of these N-glycans has been previously discussed.³⁰⁻³²

MSⁿ spectra of sialylated N-glycopeptide from chicken egg yolk.

Figure 4 shows positive-ion MS¹ (A) and MS² (B-D) spectra of sialylated N-glycopeptide from chicken egg yolk. Here, zn (n=2, 3) indicates a number of charges. The MS² spectra in Figure 4B-D were derived from the precursor ions [M+2H+Na]³⁺ (*m/z* 963.0), [M+2H]²⁺ (*m/z* 1433.5), and [M+H+Na]²⁺ (*m/z* 1444.5) in Figure 4A, respectively. A neutral loss of sialic acid(s) was commonly observed in these MS² spectra and in the MS¹ spectrum (i.e., *m/z* 1142.5, *m/z* 1288.5) as well. This means that the important information regarding binding position and linkage type of sialic acid(s) has been completely lost in the positive-ion mode.

Figure 5 shows negative-ion MS¹ (A) and MS² (B,C) spectra of the same sialylated N-glycopeptide from chicken egg yolk. The MS² spectra in Figures 5B and 5C were derived from the precursor ions [M-3H]³⁻ (*m/z* 953.8) and [M-2H]²⁻ (*m/z* 1431.7) in Figure 5A, respectively. Although the neutral loss of sialic acid (*m/z* 1286.2) was observed in these MS² spectra as well, the MS² spectrum (Figure 5B) derived from the triply deprotonated precursor ion (*m/z* 953.8) shows the deprotonated peptide ion (*m/z* 741.5) containing the ^{0,2}X₁-type cross-ring cleavage of the GlcNAc residue (83.1 Da) and the two deprotonated N-glycan fragment ions B₆ (*m/z* 1000.4) and ^{0,2}A₇(z2) (*m/z* 1060.5) without a loss of sialic acid. However, the MS² spectrum in Figure 5C derived from the doubly deprotonated precursor ion (*m/z* 1431.7) caused a dominantly neutral loss of sialic acid (*m/z* 1286.2). The MS³ spectrum was derived from this peptide ion (*m/z* 741.5). It was almost the same as that of Figure 2B

(data not shown). This indicates that these negative-ion CID MS³ spectra of the peptide residue are very reproducible. In the lower m/z region in Figure 5B, several fragment ions derived from the peptide residue are observed (see Figure 2B), in addition to B₁-, B₃-, and C₄-type fragment ions indicated in the insert.

Figure 6 shows MS³ spectra of the two deprotonated N-glycan fragment ions (m/z 1000.4 and 1060.5). Figure 6A indicates that the ^{0,2}A₇ (z₂)-type cross-ring cleavage ion (m/z 1060.5) readily changes to the B₆(z₂)-type fragment ion (m/z 1000.4). The MS³ spectrum of the N-glycan residue in Figure 6B was then driven from the B₆(z₂)-type fragment ion (m/z 1000.4). The annotations of fragment ions in the inset do not distinguish between the upper (α 1,6) or lower (α 1,3) branch. Several characteristic fragment ions useful for the structural analysis of N-glycan are observed in Figure 6B. In particular, D-type ions and A-type cross-ring cleavage ions are known to be useful for distinguishing the branch and linkage positions of N-glycans.^{28, 29} The intensity patterns of the ions are also useful for distinguishing isomeric neutral and sialylated N-glycans, as discussed previously.³⁰⁻³² The N-glycan structural analysis based on MSⁿ spectral matching with the PA N-glycan standards will be reported separately.

CONCLUSIONS

Positive- and negative-ion MSⁿ spectra of chicken egg yolk glycopeptides binding neutral and sialylated N-glycans (N-glycopeptides) were acquired by using electrospray linear-ion trap time-of-flight mass spectrometry (ESI-LIT-TOF MS) and collision-induced dissociation (CID) with He as a collision gas. Several characteristic differences between positive- and negative-ion CID MS² spectra were shown and discussed. In the positive-ion mode, the peptide moiety was presumably stable, but the neutral N-glycan moiety caused several B-type fragmentations and the sialylated N-glycan almost lost sialic acid(s). Therefore, the positive-ion MS³ spectrum derived from the protonated peptide containing GlcNAc residue (203 Da), which appeared in the MS² spectrum of neutral N-glycopeptide, provided the peptide amino-acid sequence information including the glycosylation site. In contrast, in the negative-ion mode, the peptide moiety caused several fragmentations, but both the neutral and sialylated N-glycan moieties were relatively stable. Thus, the negative-ion MS³ spectra derived from both the singly deprotonated ^{2,4}A₆-type cross-ring cleavage ion of neutral N-glycans and doubly deprotonated B₆-type fragment ions of sialylated N-glycans provided informative structural information of the N-glycan moieties of both the neutral and sialylated N-glycopeptides. Thus, it is concluded that both the positive- and negative-ion modes provide complementary structural information of N-glycopeptides.

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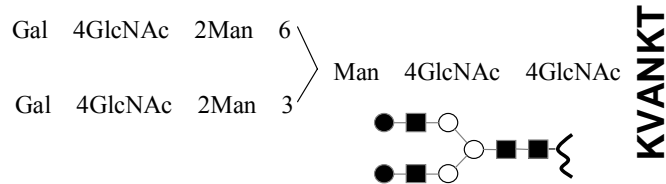
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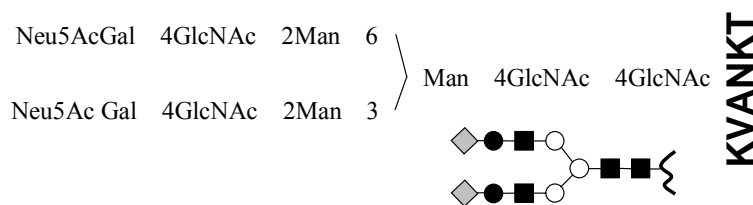
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SCHEME, FIGURES, and CAPTIONS

Neutral N-glycopeptide



Sialylated N-glycopeptide



Man : mannose (○); GlcNAc: *N*-acetyl-glucosamine (■);
 Gal : galactose (●); Neu5Ac : *N*-acetyl-neuramic acid (◆); Peptide: ⌞

Scheme 1: Structures of neutral and sialylated N-glycopeptides from chicken egg yolk

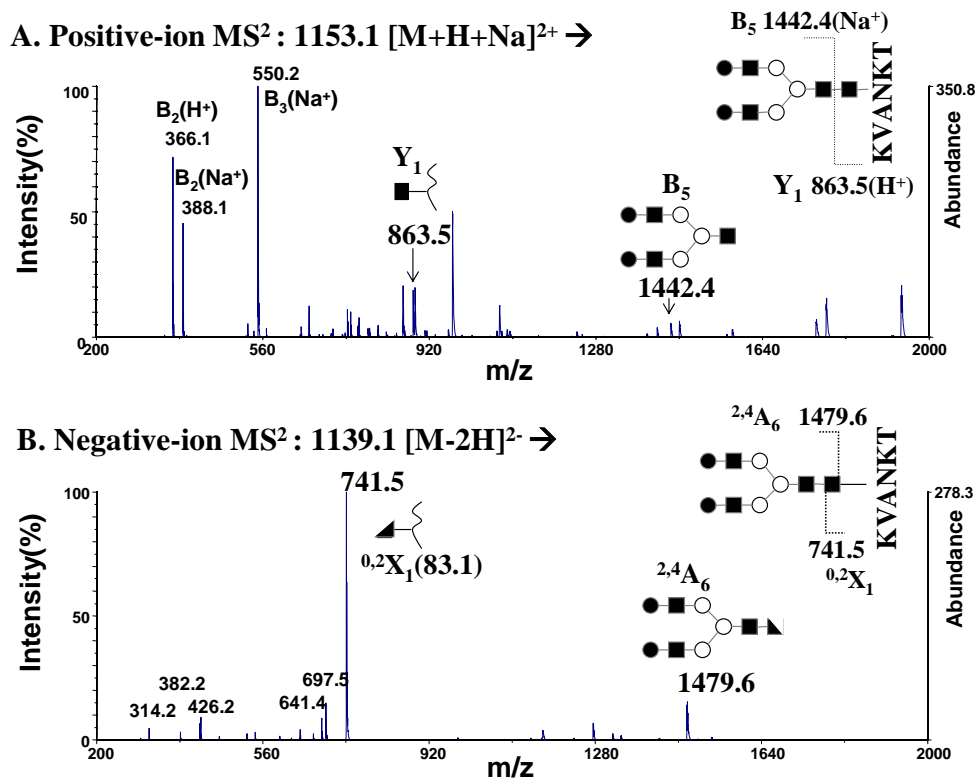


Figure 1. Comparison of positive- and negative-ion MS² spectra of neutral N-glycopeptide from chicken egg yolk.

(A): positive-ion MS² spectrum derived from the precursor ion ($[M+H+Na]^{2+}$, m/z 1153.1); (B) negative-ion MS² spectrum derived from the precursor ion ($[M-2H]^{2-}$, m/z 1139.1).

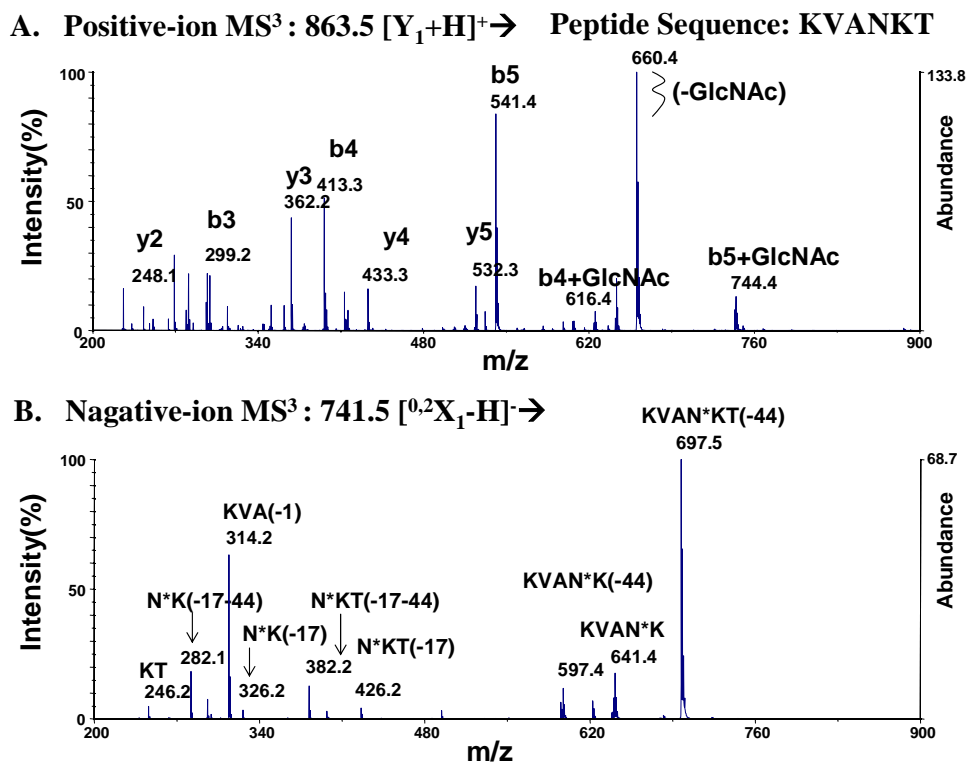
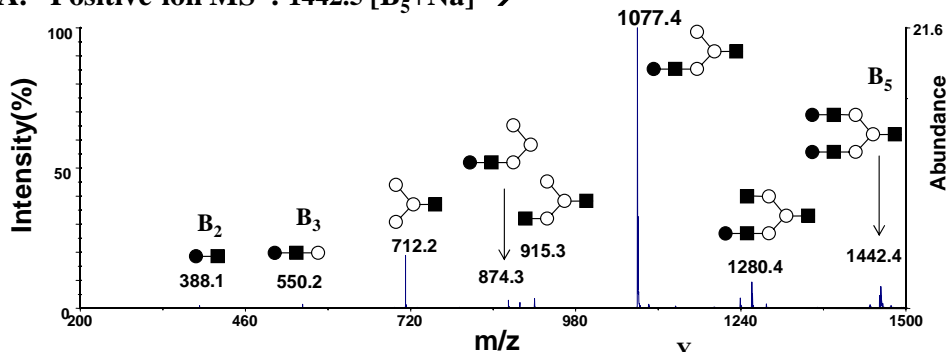


Figure 2. Comparison of positive- and negative-ion MS³ spectra derived from the peptide residue ions in Figure 1.

(A): positive-ion MS³ spectrum derived from the protonated peptide fragment ion (m/z 863.5) in Figure 1A; (B) negative-ion MS³ spectrum derived from the deprotonated peptide fragment ion (m/z 741.5) in Figure 1B. The peptide amino-acid sequence (KVANKT) including the binding site (N: Asparagine) of GlcNAc residue was determined from the fragment ion series in (A). The annotations in (B) were manually performed. N* means Asparagine with some fragment of GlcNAc residue. The numbers in parenthesis correspond to 17 Da: NH₃ (Asn); 44 Da: MeCHO (Thr)/ CO₂ /MeCHO(GlcNAc).

A. Positive-ion MS³ : 1442.5 [B₅+Na]⁺ →



B. Negative-ion MS³ : 1479.6 [2,4A₆-H]⁻ →

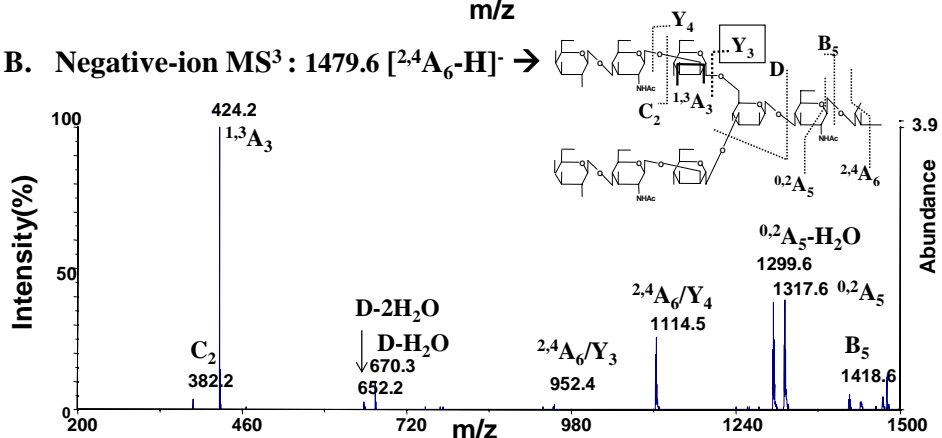


Figure 3. Comparison of positive- and negative-ion MS³ spectra derived from the neutral N-glycan residue ions in Figure 1.

(A): positive-ion MS³ spectrum derived from the B₅-type fragment ion (*m/z* 1442.5) in Figure 1A;

(B): negative-ion MS³ spectrum derived from the 2,4A₆-type cross-ring cleavage ion (*m/z* 1479.6) in

Figure 1B. Annotations show only one type of fragment ions of the two glycoside-bond cleavages and do not distinguish between the upper (α1,6) or lower (α1,3) branch.

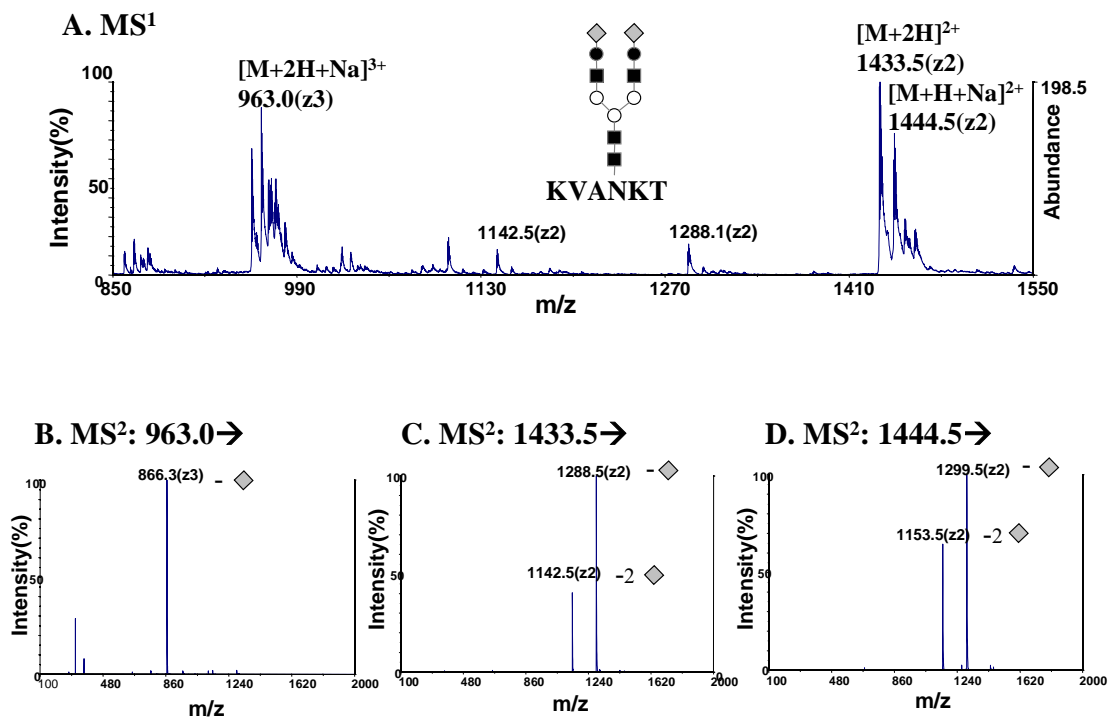


Figure 4. Positive-ion MS¹ (A) and MS² (B-D) spectra of sialylated N-glycopeptide from chicken egg yolk.

MS² spectra (B-D) were derived from the precursor ions [M+2H+Na]³⁺ (*m/z* 936.0), [M+2H]²⁺ (*m/z* 1433.5), and [M+H+Na]²⁺ (*m/z* 1444.5), respectively. Fragment ions of *m/z* 1142.5 and *m/z* 1288.5 correspond to a neutral loss of sialic acid(s).

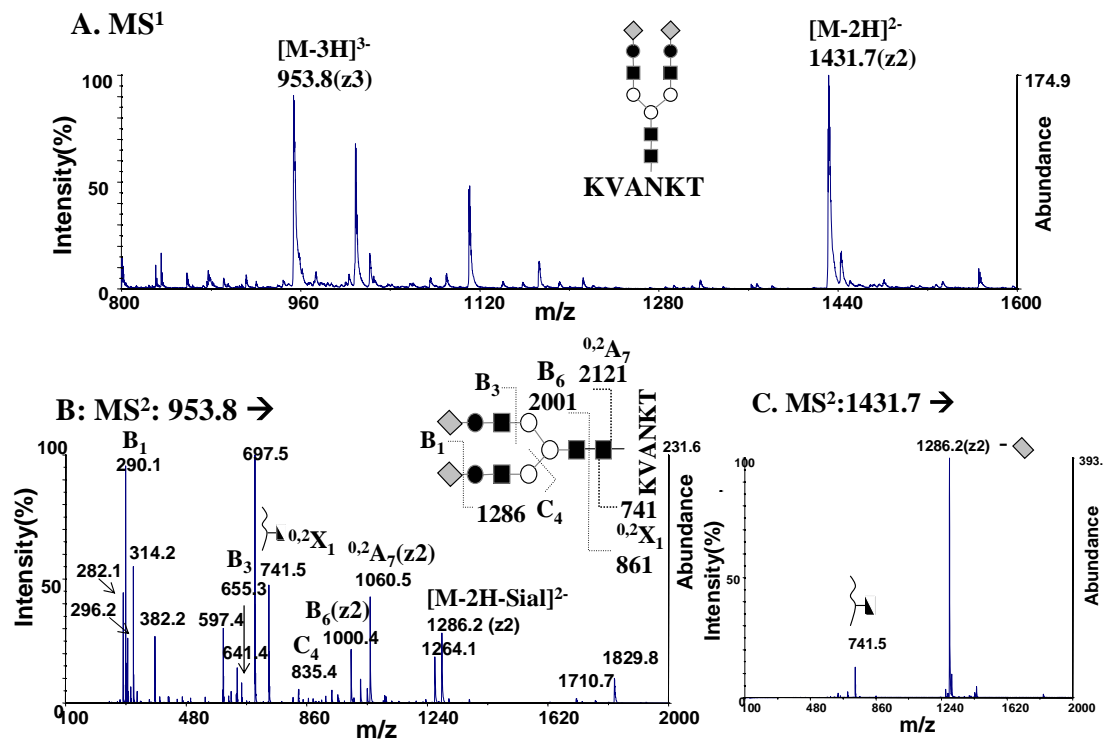


Figure 5. Negative-ion MS¹ (A) and MS² (B,C) spectra of sialylated N-glycopeptide from chicken egg yolk.

MS² spectra (B) and (C) were derived from the precursor ions [M-3H]³⁻ (*m/z* 953.8) and [M-2H]²⁻ (*m/z* 1431.7), respectively. A neutral loss of sialic acid (*m/z* 1286.2) is observed, especially in the MS² spectrum (C) derived from the doubly deprotonated precursor ion (*m/z* 1431.7).

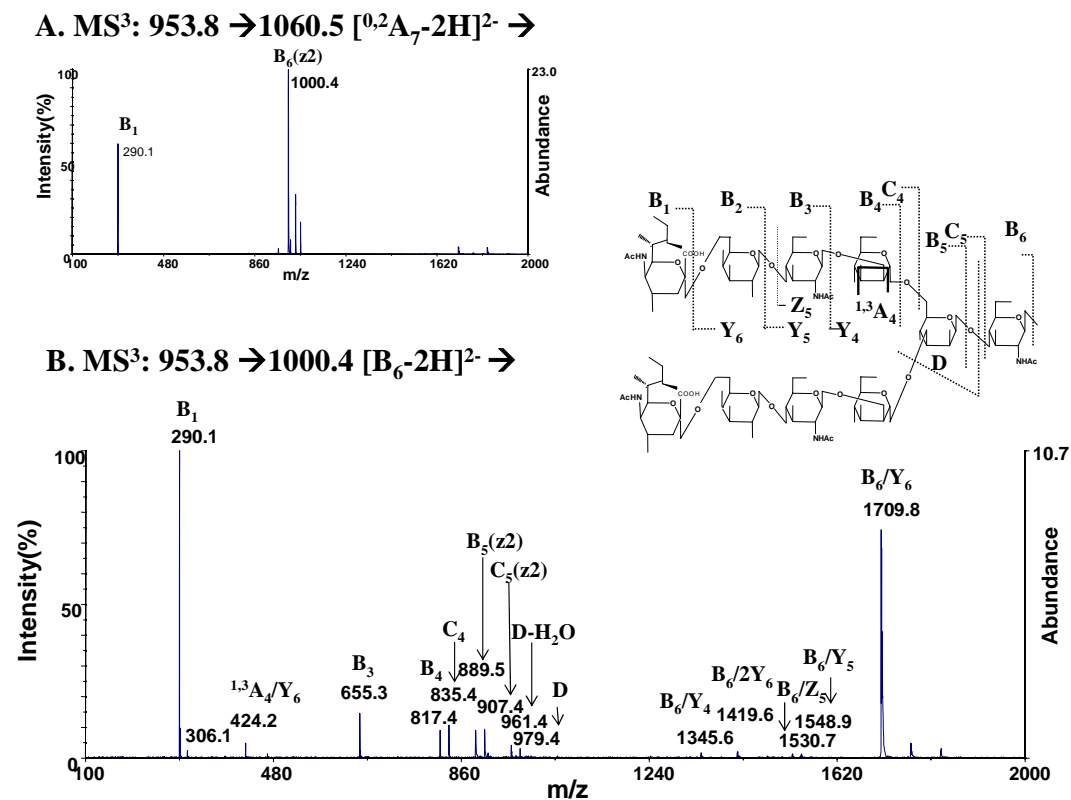


Figure 6. Negative-ion MS³ spectra derived from the sialylated N-glycan residues in Figure 5.

(A): MS³ spectrum derived from the ^{0,2}A₆ (z2)-type cross-ring cleavage ion (*m/z* 1060.5); (B) MS³ spectrum derived from the B₆(z2)-type fragment ion (*m/z* 1000.4). Annotations of fragment ions do not distinguish between the upper (α 1,6) or lower (α 1,3) branch of N-glycan.