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Title	Direct structural assignment of neutral and sialylated N-glycans of glycopeptides using collision-induced dissociation MS spectral matching.
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Citation	Rapid Communications in Mass Spectrometry, 20(23), 3557-3565 https://doi.org/10.1002/rcm.2761
Issue Date	2006
Doc URL	http://hdl.handle.net/2115/17086
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Type	article (author version)
File Information	RCMS20-23.pdf

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Direct structural assignment of neutral and sialylated N-glycans of glycopeptides using collision-induced dissociation MS^n spectral matching[†]

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Abstract

Mass spectrometry analyses of various N-glycans binding to proteins and peptides are highly desirable for elucidating their biological roles. An approach based on collision-induced dissociation (CID) MS^n spectra acquired by ESI-Linear IT TOF MS in the positive- and negative-ion modes has been proposed as a direct method of assigning N-glycans without releasing them from N-glycopeptides. In the positive-ion mode of this approach, the MS^2 spectrum of N-glycopeptide was acquired so that a glycoside-bond cleavage occurs in the chitobiose residue (i.e., GlcNAcβ1-4GlcNAc, GlcNAc: N-acetylglucosamine) attached to asparagine (N), and two charges on the $[M+H+Na]^{2+}$ precursor ion are shared with both of the resulting fragments. These fragments are sodiated B_n-type fragment ions of oligosaccharide (N-glycan) and a protonated peptide ion retaining one GlcNAc residue on the asparagines (N) residue. The structure of N-glycan was assigned by comparing MS^3 spectra derived from both the sodiated B_n-type fragment ions of N-glycopeptide and the PA (2-aminopyridine) N-glycan standard (i.e., MS^n spectral matching). In a similar manner, the structural assignment of sialylated N-glycan was performed by employing the negative-ion CID MS^n spectra of deprotonated B_n-type fragment ions of N-glycopeptide and the PA N-glycan standard. The efficacy of this approach was tested with chicken egg yolk glycopeptides with a neutral and a sialylated N-glycan, and human serum IgG glycopeptides with neutral N-glycan isomers. These

results suggest that the approach based on MS^n spectral matching is useful for the direct and simple structural assignment of neutral and sialylated N-glycans of glycopeptides.

Introduction

Glycosylation is one of the most common post-translational modifications of proteins and is related to numerous biological processes (e.g., fertilization, infection, inflammation, and cell-cell adhesion). [1] To better understand the roles of glycoproteins, methods based on mass spectrometry (MS) have consistently played important roles in their rapid and sensitive analysis. At present, a variety of tandem or multistage tandem MS (MS^n) techniques is becoming indispensable in the glycomics and glycoproteomics research fields. [2-8] However, many difficulties and challenges remain due to the site heterogeneity of the oligosaccharides attached to protein and the structural diversity of the oligosaccharides, as well as the lack of an adequate database of oligosaccharides (although several attempts to develop such a database have been reported in recent years [9-16]).

The multistage tandem MS (MS^n) methods proposed to date and developed in the field of glycoproteomics can be roughly classified into two types. The first type consists of methods to analyze peptides and oligosaccharides separately after the oligosaccharides are released from the peptides by enzymatic digestion (e.g., peptide-N-glycosidase F (PNGase F) [4, 6] and end-β-N-acetylglucosaminidases (End D/H) [17]) for N-linked oligosaccharides (N-glycans) or by

chemical treatment (e.g., hydrazinolysis and β-elimination) for O-linked oligosaccharides (O-glycans). [6, 10] The second type consists of methods to directly analyze glycopeptides without releasing the oligosaccharides by optimizing collision-induced dissociation (CID) conditions for the peptides and oligosaccharides. [18-23] The second method is also achieved by adjusting laser power and irradiation time of MALDI [24-28] or by combining electron capture dissociation (ECD) or electron-transfer dissociation (ETD) for peptides and infrared-multiphoton dissociation (IRMPD) or CID for oligosaccharides.[29-36] This method is much simpler and more straightforward than is the first method. However, most discussions of the second method have focused on the peptide amino acid sequence, the composition of the oligosaccharides, and the determination of the glycosylation sites. Little attention has been given to the structural assignment of the oligosaccharides.

Recently, we reported that the positive-ion and negative-ion CID MS^{n} spectra play complementary roles for analyzing the peptide sequence and assigning the N-glycan moiety of N-glycopeptide.[37] In the present study, we focus on the structural assignment of the N-glycan moiety employing MS^{n} spectral matching with the PA N-glycan standards.[38-41] Briefly, the procedures in the positive-ion mode are as follows. (1) A $[M+H+Na]^{2+}$ precursor ion of glycopeptide is selected on the MS^{1} spectrum; (2) the MS^{2} spectrum is acquired by adjusting the CID parameters so that a glycoside-bond cleavage occurs between the two GlcNAc in chitobiose (i.e., GlcNAcβ1-4GlcNAc, GlcNAc: N-acetylglucosamine) attached to asparagine (N) and, also, two charges (H^{+} and Na^{+}) on the

precursor ion are shared with both the resulting fragments, i.e., the sodiated B_n-type (Domon and Costello's nomenclature [42]) fragment ion of N-glycan and the protonated Y_1-type peptide ion with a GlcNAc residue attached to asparagine (N); (3) the MS^3 spectra are derived from these fragment ions. The assignment of the B_n-type fragmented N-glycan residue is performed by employing MS^n spectral matching with the MS^3 spectra of the PA N-glycan standard. In a similar manner, negative-ion CID MS^n spectra can be used for the structural assignment of N-glycans.

To assess its efficacy, this approach was applied to chicken egg yolk glycopeptides carrying a neutral and a sialylated N-glycan, and to human serum IgG glycopeptides carrying a neutral N-glycan and its isomer. Both the positive- and negative-ion CID MS^3 spectra were successfully employed for the structural assignment of B_n-type fragmented N-glycan moieties in the neutral N-glycopeptides. The negative-ion mode was especially useful for the structural assignment of sialylated N-glycopeptide, which easily causes a neutral loss of sialic acid(s) in the positive-ion mode and makes it difficult to assign.[37] Although the samples tested are still limited, we note that the approach based on MS^n spectral matching has the potential to become a useful tool for the direct and simple structural assignment of neutral and sialylated N-glycans of glycopeptides without their release from the peptides.

EXPERIMENTAL

Materials.

Acetonitrile (HPLC/MS grade), ammonium acetate, ammonium bicarbonate, hydrogen chloride, and trypsin were purchased from Wako Pure Chemical Industries (Osaka, Japan). Water was purified by Milli-Q (Millipore Co., Milford, MA). IgG from human serum was purchased from Sigma-Aldrich Co. (St. Louis, MO). A ZIC-HILIC column (2.1 mm i.d. x 150 mm long, particle size 3.5 μm, pore size 20 nm) was purchased from SeQuant (Umeå, Sweden).

Sample preparation.

N-glycopeptides, shown in Scheme 1 with the nomenclature (200.4, 210.2, 210.3, 210.4, 2A1-200.4) proposed by Takahashi et al [43], are from human serum IgG and chicken egg yolk. They were prepared as follows. Human serum IgG (1 mg) was digested with 20 μg of trypsin at 37°C overnight in 100 μL of 50 mM ammonium bicarbonate buffer (pH 7.8). To terminate the digestion and to release the sialic acids, the digested mixture was heated at 90°C for 1 hour with 0.01 M HCl (pH 2.0). The reaction was then quenched by adding 1.0 M ammonium bicarbonate buffer. After evaporation, the sample was dissolved in 20 μL of water and centrifuged (20,400 g, 15 min, 4°C). A part of the supernatant liquid (4 μL) was diluted with acetonitrile (16 μL). Then, the sample was injected into a HPLC system using the ZIC-HILIC column; peptides with/without N-glycans were well separated; and the glycopeptides for this study were fraction corrected.[44, 45] Chicken egg yolk

glycopeptides were prepared by following the procedures described in the reference [46] and were then purified by the ZIC-HILIC column. Desialylation was performed in the same manner as that of human IgG peptides.

ESI-Linear IT TOF MS analysis.

Experiments on the structural assignment of the N-glycopeptides were performed by using a NanoFrontier (L) system (Hitachi High-Technologies, Tokyo, Japan) consisting of a capillary HPLC system based on a nanoGR generator [47, 48] and an ESI-Linear IT TOFMS.[49] An uncoated SilicaTips™ (tip i.d. 10 μm; New Objective, Woburn, MA) was used as a nanoelectrospray (nanoESI) tip. Samples dissolved in water (about 40 μM) were diluted by the addition of an equal volume of acetonitrile and infused into the nanoESI source with a syringe pump (Harvard Apparatus, Holliston, MA) at a flow rate of 200 nL/min. The conditions of the nanoESI-Linear IT TOF MS were: capillary voltage, +1.5-1.8 kV; flow rate of curtain gas (nitrogen), 0.8-1.2 L/min; scan mass range m/z, 200-2000; mass accuracy, ±50 ppm; and mass resolution, 8000 FWHM. The CID-related parameters used for MS^2 and MS^3 spectral acquisition were: CID gas (He) flow rates, 1 mL/min; isolation time, 5 ms; isolation width, 15 (MS^2) and 20 (MS^3); and optimized CID gain positive- and negative-ion modes, 1.1 (MS^2) and 1.8 (MS^3).

Optimization of the CID gain was achieved by using egg yolk glycopeptide (Egg Yolk (200.4) in Scheme 1) prior to MS^2 spectral acquisitions. Although the data are not shown, the precursor ion ($[M+H+Na]^{2+}$, *m/z* 1153.1) almost disappears at CID gain 1.0 in both the positive- and negative-ion modes. Then, a CID gain 1.1 was selected for all MS^2 spectral acquisitions in the positive- and negative-ion modes. Similarly, a CID gain of 1.8 was selected for positive- and negative-ion MS^3 spectral acquisitions.

RESULTS

Chicken egg yolk glycopeptide with a neutral N-glycan.

The first test sample is the chicken egg yolk glycopeptide binding the complex-type neutral N-glycan (Egg Yolk (200.4)) in Scheme 1. Figure 1 shows MS^n (n=2,3) spectra acquired by the nanoESI-Linear IT TOF MS in the positive-ion mode. The MS^2 spectrum (Figure 1A) was derived from the $[M+H+Na]^{2+}$ precursor ion (*m/z* 1153.1) of the egg yolk (200.4) in the MS^1 spectrum (not shown). Only the interesting B_n- and Y_n-type fragment ions of the N-glycan moiety, e.g., Y_n-type peptide ions with/without the GlcNAc residue (*m/z* 863.5(H^+)/660.3(H^+)) and sodiated B_5-type fragment ion of N-glycan (*m/z* 1442.4(Na^+)), are denoted with their *m/z* values. Table 1A summarizes the observed B_n-type fragment ions of the N-glycan moiety. These B_n-type fragment ions can be assigned independently of the peptide moiety. This has merit, namely, even if the peptide amino acid

sequence is unknown, the protonated peptide fragment ion (m/z 863.5 (H$^+$) in this case) can be readily found from the precursor ion (m/z 1153.1 (z2)) and the largest B$_n$-type fragment ion (the sodiated B$_5$-type fragment ion (m/z 1442.4(Na$^+$) in this case) in the MS^2 spectrum (i.e., 863.8 = 2x1153.1 – 1442.4). The oligosaccharide residue (GlcNAc in this case) that remained on the peptide moiety can be confirmed in the MS^3 spectrum (not shown) derived from the protonated peptide fragment ion (m/z 863.5 (H$^+$)), as reported previously.[37] Note that the protonated peptide ion with a GlcNAc residue (m/z 863.5(H$^+$)) attached to asparagine (N) and the sodiated B$_5$-type fragment ion (m/z 1442.4(Na$^+$)) of the N-glycan moiety, which were generated by a glycoside-bond cleavage in chitobiose (i.e., GlcNAcβ1-4GlcNAc) sharing two charges (H$^+$ and Na$^+$), are observed, although their abundances are relatively low.

Figure 1B is the MS^2 spectrum derived from the [M+H+Na]$^{2+}$ precursor ion (m/z 871.3) of the standard PA N-glycan 200.4 in the MS^1 spectrum (not shown). Y$_1$-type fragment ion with a GlcNAc residue (m/z 300.1(H$^+$)) and sodiated B$_5$-type fragment ion of N-glycan (m/z 1442.4(Na$^+$)) are observed. That is, this indicates that a glycoside-bond cleavage in chitobiose (i.e., GlcNAcβ1-4GlcNAc) sharing two charges (H$^+$ and Na$^+$) of the PA residue and the B$_5$-type fragment ion of N-glycan occurred as well. Figure 1C and 1D are the MS^3 spectra obtained from the B$_5$-type fragment ions (m/z 1442.4(Na$^+$)) in Figure 1 A and 1B, respectively. These MS^3 spectra are very similar and the spectral matching (i.e., correlation coefficient) value, which was calculated by

considering the annotated seven fragment ion intensities [40], is 0.985. This implies that the MS^3 spectra of the sodiated B_5-type fragment ions (m/z 1442.4(Na^+)) of N-glycans are likely to coincide well regardless of origin (i.e., PA N-glycan or N-glycopeptide).

Figure 2 shows the MS^n (n=2,3) spectra acquired in the negative-ion mode. The MS^2 spectra of Egg Yolk (200.4) and the standard PA N-glycan 200.4 (Figure 2A and 2B) were derived from the $[M-2H]^{2-}$ precursor ions (m/z 1139.1 and 858.4) in their MS^1 spectra (not shown), respectively. Only the interesting N-glycan-related fragment ions are denoted with their m/z values (see Table 1B, which summarizes the observed A_n-, B_n-, C_n-, and D-type fragment ions of the N-glycan moiety). Note that the deprotonated B_5-type fragment ion (m/z 1418.6) of the N-glycan moiety is observed in small abundance in addition to the deprotonated peptide ion with the cross-ring cleavage $^{0,2}X_0$ of the GlcNAc residue (m/z 741.5) and the cross-ring cleavage $^{2,4}A_6$-type fragment ion (m/z 1479.6) of the N-glycan moiety. [37] In contrast, the MS^2 spectrum of the standard N-glycan PA 200.4 (Figure 2B) shows the deprotonated Y_1-type fragment ion (PA-GlcNac, m/z 298.1) and the deprotonated B_5-type fragment ion (m/z 1418.6) of the N-glycan moiety. The cross-ring cleavage $^{2,4}A_6$-type fragment ion (m/z 1479.6) of the N-glycan moiety is not observed. This is probably because the GlcNAc binding to PA has an open ring. Thus, the MS^3 spectra in Figure 2C and 2D were derived from the deprotonated B_5-type fragment ions (m/z 1418.6), respectively. The MS^3 spectra obtained are very similar and the spectral matching (i.e., correlation coefficient) value between them is 0.993. Thus, the MS^3 spectra of

the deprotonated B_5-type fragment ions (m/z 1418.6) are likely to coincide well regardless of origin, as well as the case of the sodiated B_5-type fragment ions (m/z 1442.4(Na^+)) discussed above.

Chicken egg yolk glycopeptide with a sialylated N-glycan.

A sialic acid (*N*-acetyl-neuramic acid: Neu5Ac) is easily lost in positive-ion MS^2 spectra and its structural information (linkage type and position) cannot be obtained from the MS^3 spectra that follow. In the negative-ion mode, however, sialic acid (s) is relatively stable.[37, 50] Therefore, only negative-ion MS^n spectra are discussed below.

Figure 3 shows MS^n (n=2,3) spectra of egg yolk glycopeptide with a silylated N-glycan (Egg Yolk (2A1-200.4) in Scheme 1). The MS^2 spectra in Figure 4A and 4B were derived from the $[M-3H]^{3-}$ precursor ions (m/z 953.8 and 765.9) of the sialylated egg yolk glycopeptide and the standard PA N-glycan 2A1-200.4 in their MS^1 spectra (not shown), respectively. Figure 3A indicates the deprotonated peptide ion with the cross-ring cleavage $^{0,2}X_0$ of GlcNAc residue (m/z 741.5) and the deprotonated cross-ring cleavage $^{0,2}A_7$-type fragment ion (m/z 1060.5 (z2)) and B_6-type fragment ion (m/z 1000.4(z2)) of the N-glycan moiety. Here, z2 means a doubly charged ion. In contrast, the MS^2 spectrum of the standard N-glycan PA 2A1-200.4 (Figure 3B) shows the deprotonated B_6-type fragment ion (m/z 1000.4(z2)) of the N-glycan moiety and the deprotonated Y_1-type fragment ion (PA-GlcNAc, m/z 298.1) in very low abundance. Thus, the MS^3 spectra in Figure 3C and 3D were

derived from the common B_6-type fragment precursor ions (m/z 1000.4(z2)), respectively. Note that these MS^3 spectra are very similar, and the spectral matching (i.e., correlation coefficient) value between them is 0.975. Thus, the MS^3 spectra of the deprotonated B_6-type fragment ions (m/z 1000.4(z2)) are likely to coincide well regardless of origin, as well as the case of the neutral N-glycan discussed above.

Human serum IgG glycopeptides with isomeric neutral N-glycans.

The next test sample consists of the two human serum IgG-2 glycopeptides attaching the neutral fucosylated N-glycans 210.2 and 210.3 isomers (IgG-2 (210.2) and IgG-2(210.3) in Scheme 1). An important point is whether these isomeric N-glycans can be differentiated and assigned without releasing them from the peptides. Figure 4A and 4B show the MS^2 spectra derived from the $[M+H+Na]^{2+}$ precursor ions (m/z 1394.1) of the isomeric N-glycopeptides IgG-2 (210.2) and IgG-2 (210.3), respectively. Sodiated B_5-type fragment ions (m/z 1280.5(Na^+)) of the N-glycan moieties are commonly observed together with other small B_n-type fragment ions. The protonated peptide fragment ion (m/z 1507.7(H^+)) can easily be found from the precursor ion (m/z 1394.1) and the sodiated B_5-type fragment ion (m/z 1442.5(Na^+)) in the MS^2 spectrum (i.e., 1507.7 = 2x 1394.1 – 1280.5). Strictly speaking, the (GlcNAc-Fuc) residue remaining on the peptide moiety is unclear at this stage in the case of an unknown peptide. It can be determined by the MS^3 spectrum derived from

the protonated peptide fragment ion (m/z 1507.7(H^+)), as discussed in the following subsection. Figure 4C and 4D are the MS^3 spectra derived from the sodiated B_5-type fragment ions (m/z 1280.5(Na^+)) in Figure 4A and 4B, respectively. Only the fragment ions corresponding to B_n-type cleavages are denoted with their m/z values (see Table 1A). Here, note that these MS^3 spectra are slightly different from one another.

Figure 5A and 5B are the MS^2 spectra derived from the $[M+H+Na]^{2+}$ precursor ions (m/z 863.3) of the isomeric PA N-glycan standards (PA-210.2 and PA-210.3), respectively. Similar to the cases mentioned above, the sodiated B_5-type fragment ion (m/z 1280.5(Na^+)) and the protonated PA-(GlcNAc-Fuc) ion (m/z 446.2(H^+)) and PA-(GlcNAc) ion (m/z 300.1(H^+)) are observed. The dominant ion (m/z 790.3) corresponds to the fragmentation with neutral loss of a fucose. The protonated B_2-type fragment ion (Hex (Gal or Man)-GlcNAc: m/z 366.1(H^+)) is also relatively abundant. The MS^3 spectra (Figure 5C and 5D) were derived from the sodiated B_5-type fragment ions (m/z 1280.5(Na^+)) of the PA 210.2 and PA 210.3 standards, respectively. The six major fragment ions corresponding to B_n-type cleavages are indicated with their m/z values. Figure 6 summarizes the MS^3 spectral matching values in a table and the ion intensities overlaid in a graph. The correlation coefficients were calculated by considering the six fragment ion intensities observed in Figure 4C, 4D, 5C, and 5D. From the higher correlation coefficients of 0.998 (IgG-2 210.2 vs. PA 210.2) and 0.989

(IgG-2 210.3 vs. PA 210.3) than the other values (0.913 and 0.851), the isomeric N-glycans of the

IgG-2 glycopeptides can probably be distinguished from one another and correctly assigned.

Amino acid sequence analysis of human serum IgG-1 and IgG-2 glycopeptides.

Using the glycopeptides IgG-1 and IgG-2 attaching the same N-glycan 210.4 (IgG-1 (210.4) and

IgG-2(210.4) in Scheme 1), we can easily discuss the determination of the amino acid sequence of the

glycopeptides. Figure 7A and 7B show the MS^2 spectra derived from the $[M+2H]^{2+}$ precursor ions at

m/z 1464.2 of IgG-2 (210.4) and m/z 1480.6 of IgG-1 (210.4) in the MS^1 spectrum (not shown),

respectively. In these MS^2 spectra, no protonated B_5-type fragment ions were observed. The reason

for this might be that the protonated B_5-type fragment ions are unstable rather than the sodiated one

and readily cause further fragmentation. However, the relatively abundant pair of protonated peptide

fragment ions (m/z 1360.6(H^+) and m/z 1507.7(H^+)) and (m/z 1392.6(H^+) and m/z 1538.7(H^+)) are

observed in Figure 7A and 7B, respectively. The mass difference of these ion pairs corresponds to a

neutral loss of Fuc (146.1Da). These results imply that a glycoside-bond cleavage efficiently

occurred between the two GlcNAc in the chitobiose (GlcNAc β1-4 GlcNAc) retaining (GlcNAc-Fuc)

residue on asparagines (N). Figure 8C and 8D show the MS^3 spectra derived from the protonated

peptide fragment ions retaining a GlcNAc residue (m/z 1360.6(H^+) (A) and m/z 1392.6(H^+) (B)) in the

MS^2 spectra, respectively. The peptide sequences (EEQYNSTYR) for IgG-1 and (EEQFNSTFR) for

IgG-2 including the binding site (N) of the GlcNAc residue could easily be confirmed by MASCOT [51]

search for the b/y-type [52] fragment ions observed in the MS^3 spectra (Figure 7C and 7D).

There is a limitation on the approach proposed in this study. As this test sample shows, the linkage

type and position of a fucose cannot be assigned; only fucosylation of the reducing terminal GlcNAc

can be confirmed. Further study is required to solve this problem.

DISCUSSION

The first two samples tested in this study imply that the MS^3 spectra derived from the sodiated

B_5-type fragment ion of the N-glycan moiety probably show a similar fragment pattern regardless of

origin (i.e., N-glycopeptide or PA N-glycan). In other words, if the MS^3 spectra derived from the

B_5-type fragment ion of the PA N-glycan standards are stored in an MS^n spectral library, it might be

possible to determine the structural assignment of the N-glycans of the glycopeptides (although the

core fucosylation problem mentioned above is still open). If a corresponding N-glycan moiety is not

in an MS^n spectral library (i.e. a new N-glycan), structural analysis combining several specific

exoglycosidase digestions and MS^n spectral matching might be possible for the glycopeptides

without releasing the N-glycan, as discussed previously for a novel human serum PA N-glycan.[41]

Very recently, several approaches using positive-ion CID MS^n (n=1-4) spectra acquired by ESI-IT

MS,[22] MALDI-TOF/TOF MS, [24, 25] MALDI-IT TOF MS, [26-28] and ESI-IT TOF MS [21, 28] have been

reported. In these articles, the peptide amino acid sequences were determined by analyzing MS^n

(n=2-4) spectra derived from fragmented peptide ions with a GlcNAc residue or cross-ring cleavage

fragment, which is necessary for determining the glycosylation site (asparagines (N)). Although the

composition of oligosaccharides was also analyzed in detail, the structural assignment (including

differentiation of the isomeric compositions) of the oligosaccharides has received little discussion.

Although the number of samples tested is still limited, the approach based on CID MS^n spectral

matching probably has the potential to provide a useful tool for the direct and simple structural

assignment of neutral and sialylated N-glycans of glycopeptides without releasing them from the

peptides.

CONCLUSION

An approach based on CID MS^n spectra acquired by ESI-Linear IT TOF MS in the positive- and

negative-ion modes has been proposed as a means of structural analysis of N-glycan, peptide amino

acid sequence, and glycosylation site in glycopeptides. For N-glycopeptides with neutral N-glycans,

the sodiated and deprotonated B_5-type fragment ions of N-glycan moiety could be observed. The

positive- and negative-ion MS^3 spectra derived from them were compared with the corresponding

MS^3 spectra of the PA N-glycan standards, and then the structures of the B_5-type fragment ions of the

N-glycopeptides could be assigned. For an N-glycopeptide with a sialylated N-glycan, only the

negative-ion MS^2 spectrum produced the deprotonated B$_6$-type fragment ions of the N-glycan moiety and, similarly, it could be assigned by comparing the MS^3 spectra derived from the sialylated N-glycopeptide with the sialylated PA N-glycan standard. In addition, it was shown that the peptide amino acid sequence including the glycosylation site was determined by the MS^3 spectrum derived from the protonated peptide ions retaining GlcNAc (or GlcNAc-Fuc) residue on the asparagines (N).

Acknowledgments

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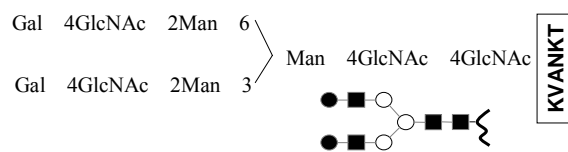35. Hogan JM, Pitteri SJ, Chrisman PA, McLuckey SA. *J. Proteome Res.* 2005**; 4**: 628.

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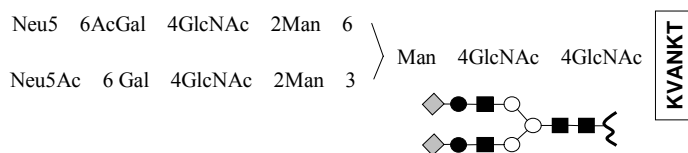
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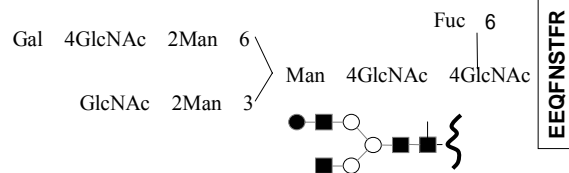52. Roepstorff P, *Biomed. Mass Spectrom.* 1984; **11**: 601.
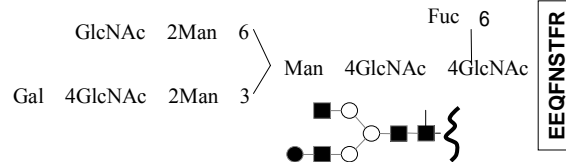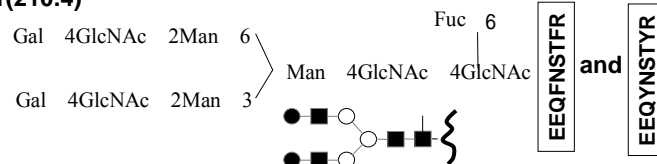
FIGURES and CAPTIONS:

Egg Yolk (200.4)

Gal 4GlcNAc 2Man 6⟩
                              Man 4GlcNAc 4GlcNAc — KVANKT
Gal 4GlcNAc 2Man 3⟩

Egg Yolk (2A1-200.4)

Neu5 6AcGal 4GlcNAc 2Man 6⟩
                              Man 4GlcNAc 4GlcNAc — KVANKT
Neu5Ac 6 Gal 4GlcNAc 2Man 3⟩

IgG-2 (210.2)

                                      Fuc 6
Gal 4GlcNAc 2Man 6⟩
                              Man 4GlcNAc 4GlcNAc — EEQFNSTFR
GlcNAc 2Man 3⟩

IgG-2 (210.3)

                                      Fuc 6
GlcNAc 2Man 6⟩
                              Man 4GlcNAc 4GlcNAc — EEQFNSTFR
Gal 4GlcNAc 2Man 3⟩

IgG-2 and IgG-1(210.4)

                                      Fuc 6
Gal 4GlcNAc 2Man 6⟩
                              Man 4GlcNAc 4GlcNAc — EEQFNSTFR and EEQYNSTYR
Gal 4GlcNAc 2Man 3⟩

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

Scheme 1: Structures of glycopeptides with the complex type N-glycans discussed in this study

Table 1: Assignment of fragment ions of neutral and sialylated N-glycans

(A)

m/z	Positive-ion MS^2 Fragment (type)	
366.1	1Hex1HexNAc (B)	(H$^+$)
388.1	1Hex1HexNAc (B)	(Na$^+$)
550.2	2Hex1HexNAc (B)	(Na$^+$)
712.3	3Hex1HexNAc (B)	(Na$^+$)
874.3	4Hex1HexNAc (B)	(Na$^+$)
915.4	3Hex2HexNAc (B)	(Na$^+$)
1077.4	4Hex2HexNAc (B)	(Na$^+$)
1280.4	4Hex3HexNAc (B)	(Na$^+$)
1442.4	5Hex3HexNAc (B)	(Na$^+$)

Hex:Hexose; HexNAc: N-acetylhexose;

NeuAc:N-acetylneuramic acid

(B)

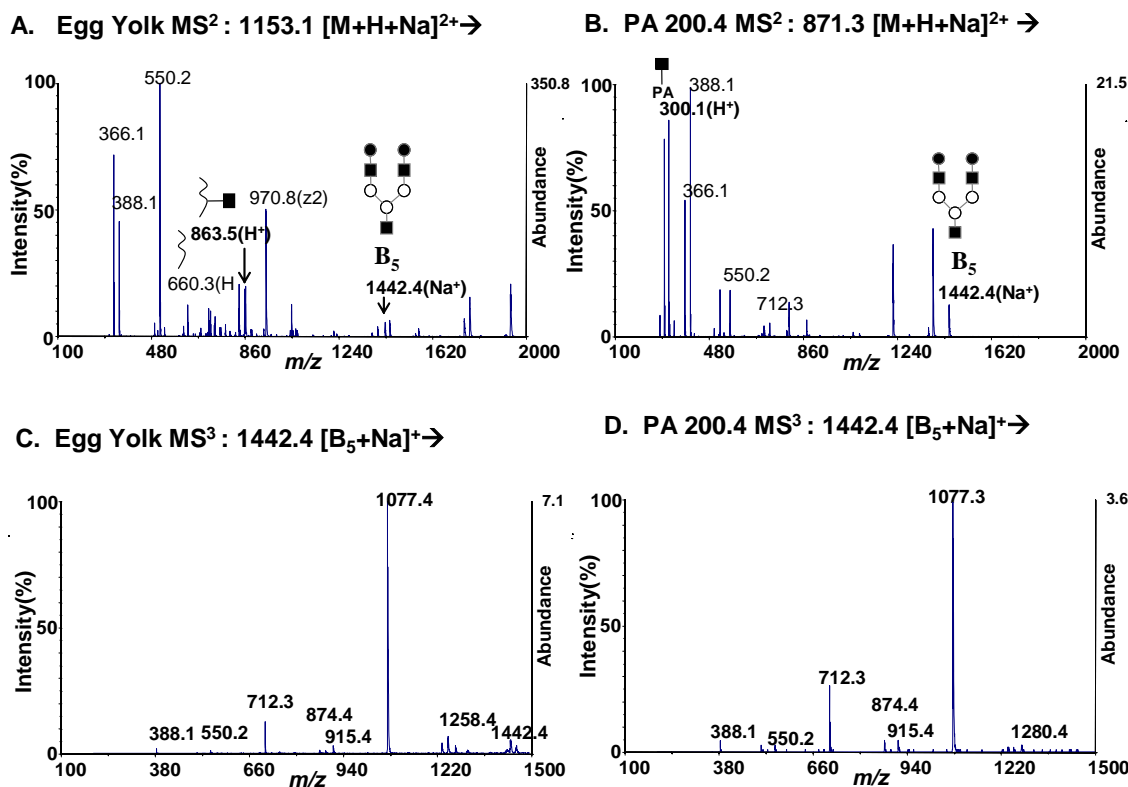| m/z	Negative-ion MS^2 Fragment (type)	
382.2	1Hex1HexNAc (C)	(-H$^+$)
424.2	1Hex1HexNAcHex (^{1,3}A)	(-H$^+$)
586.2	2Hex1HexNAcHex (^{1,3}A)	(-H$^+$)
688.3	3Hex1HexNAc (D)	(-H$^+$)
891.4	3Hex2HexNAc (B)	(-H$^+$)
1053.4	4Hex2HexNAc (B)	(-H$^+$)
1256.6	4Hex3HexNAc (B)	(-H$^+$)
1418.6	5Hex3HexNAc (B)	(-H$^+$)
290.1	1NeuAc (B)	(-H$^+$)
655.3	1NeuAc1Hex1HexNAc(B)	(-H$^+$)
817.4	1NeuAc2Hex1HexNAc (B)	(-H$^+$)
835.4	1NeuAc2Hex1HexNAc (C)	(-H$^+$)
907.4	2NeuAc5Hex2HexNAc (C)	(-2H$^+$)
979.4	1NeuAc3Hex1HexNAc (D)	(-H$^+$)
1000.4	2NeuAc5Hex3HexNAc (B)	(-2H$^+$)
1060.5	2NeuAc5Hex4HexNAc (^{0,2}A)	(-2H$^+$)
1709.5	NeuAc5Hex3HexNAc (B)	(-H$^+$)

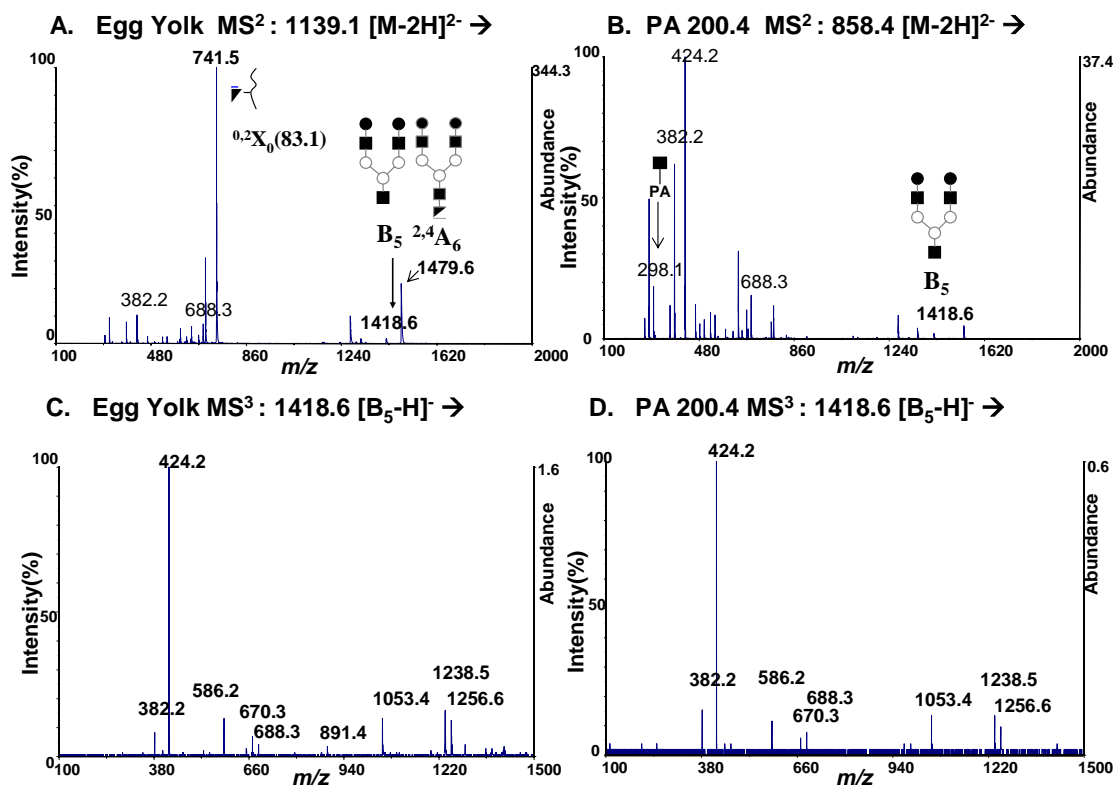**Figure 1.** Positive-ion MS^n (n=2,3) spectra of chicken egg yolk glycopeptides (200.4) and PA

N-glycan (200.4) standard.

(A): MS^2 spectrum derived from the precursor ion ([M+H+Na]$^{2+}$, *m/z* 1153.1) in the MS^1 spectrum of

Egg Yolk (200.4). (B): MS^2 spectrum derived from the precursor ion ([M+H+Na]$^{2+}$, *m/z* 871.3) in the

MS^1 spectrum of PA 200.4 standard. (C) and (D): MS^3 spectra derived from the sodiated B$_5$-type

fragmented ions ([B$_5$+Na]$^+$, *m/z* 1442.4) in (A) and (B), respectively. The correlation coefficient

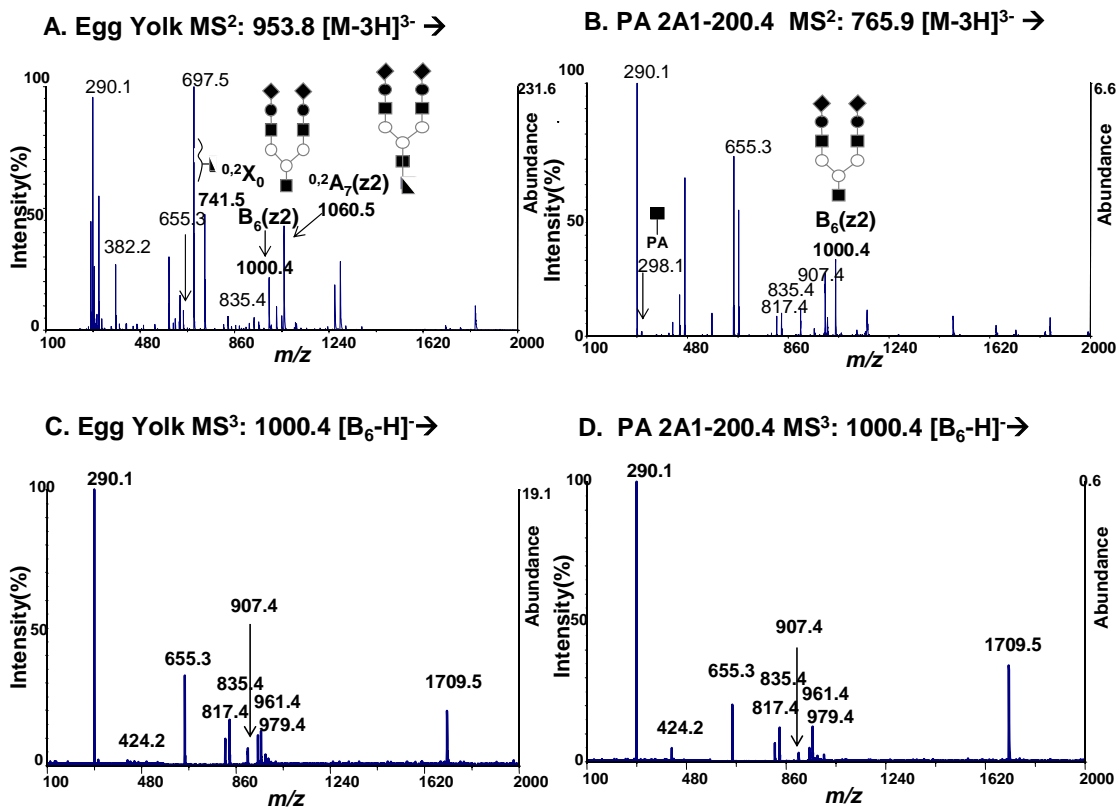between MS^3 spectra (C) and (D) is 0.985.

Figure 2. Negative-ion MS^n (n=2,3) spectra of chicken egg yolk glycopeptides (200.4) and PA

N-glycan (200.4) standard.

(A): MS^2 spectrum derived from the precursor ion ($[M-2H]^{2-}$, *m/z* 1139.1) in the MS^1 spectrum of Egg

Yolk (200.4). (B): MS^2 spectrum derived from the precursor ion ($[M-2H]^{2-}$, *m/z* 858.4) in the MS^1

spectrum of PA 200.4 standard. (C) and (D): MS^3 spectra derived from the deprotonated B_5-type

fragmented ions ($[B_5-H]^-$, *m/z* 1418.6) in (A) and (B), respectively. The correlation coefficient

between MS^3 spectra (C) and (D) is 0.993.

Figure 3. Negative-ion MS^n (n=2,3) spectra of chicken egg yolk glycopeptides (2A1-200.4) and PA

N-glycan (2A1-200.4) standard.

(A): MS^2 spectrum derived from the precursor ion ($[M-3H]^{3-}$, m/z 953.8) in the MS^1 spectrum of Egg

Yolk (2A1-200.4). (B): MS^2 spectrum derived from the precursor ion ($[M-3H]^{3-}$, m/z 765.9) in the

MS^1 spectrum of PA 2A1-200.4 standard. (C) and (D): MS^3 spectra derived from the deprotonated

B_6-type fragmented ions ($[B_6-H]^{2-}$, m/z 1000.4) in (A) and (B), respectively. The correlation

coefficient between MS^3 spectra (C) and (D) is 0.975.
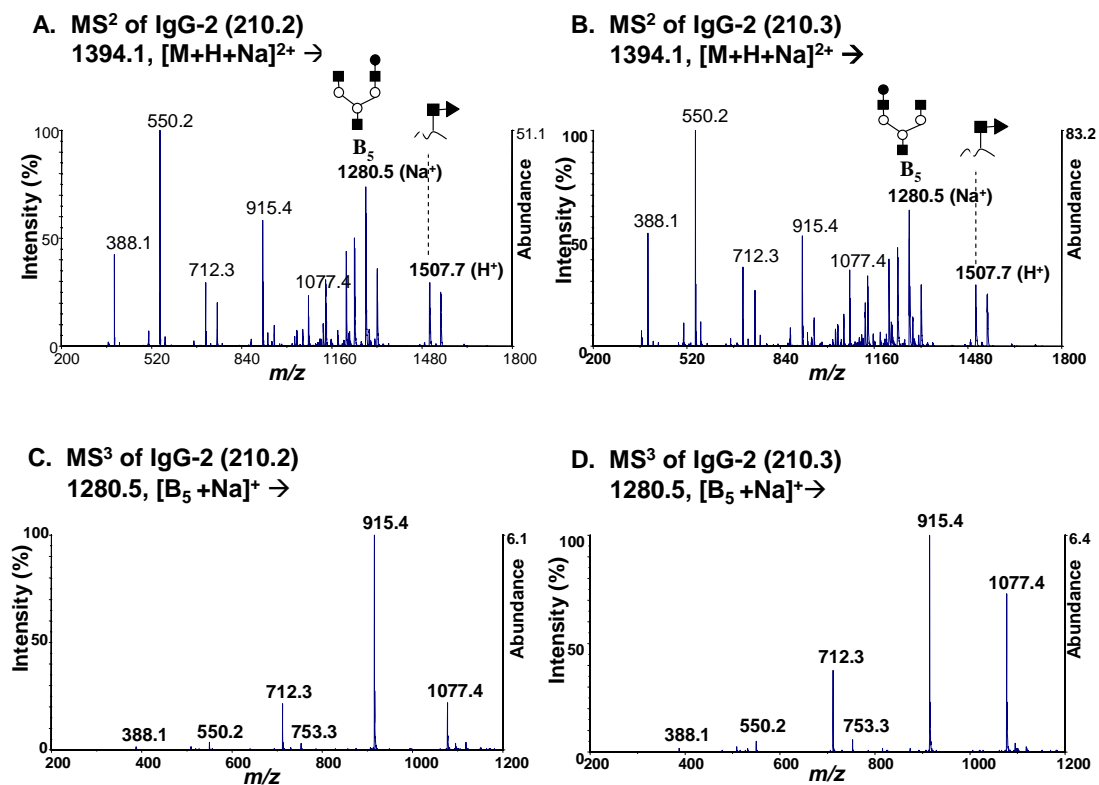
Figure 4. MS^n (n=2-3) spectra derived from $[M+H+Na]^{2+}$ precursor ions of isomeric glycopeptides

IgG-2 (210.2) and IgG-2 (210.3).

(A) and (B): MS^2 spectra derived from $[M+H+Na]^{2+}$ (*m/z* 1394.1) in MS^1 spectra of IgG-2 (210.2)

and IgG-2 (210.3), respectively. (C) and (D): MS^3 spectra derived from the sodiated B_5-type fragment

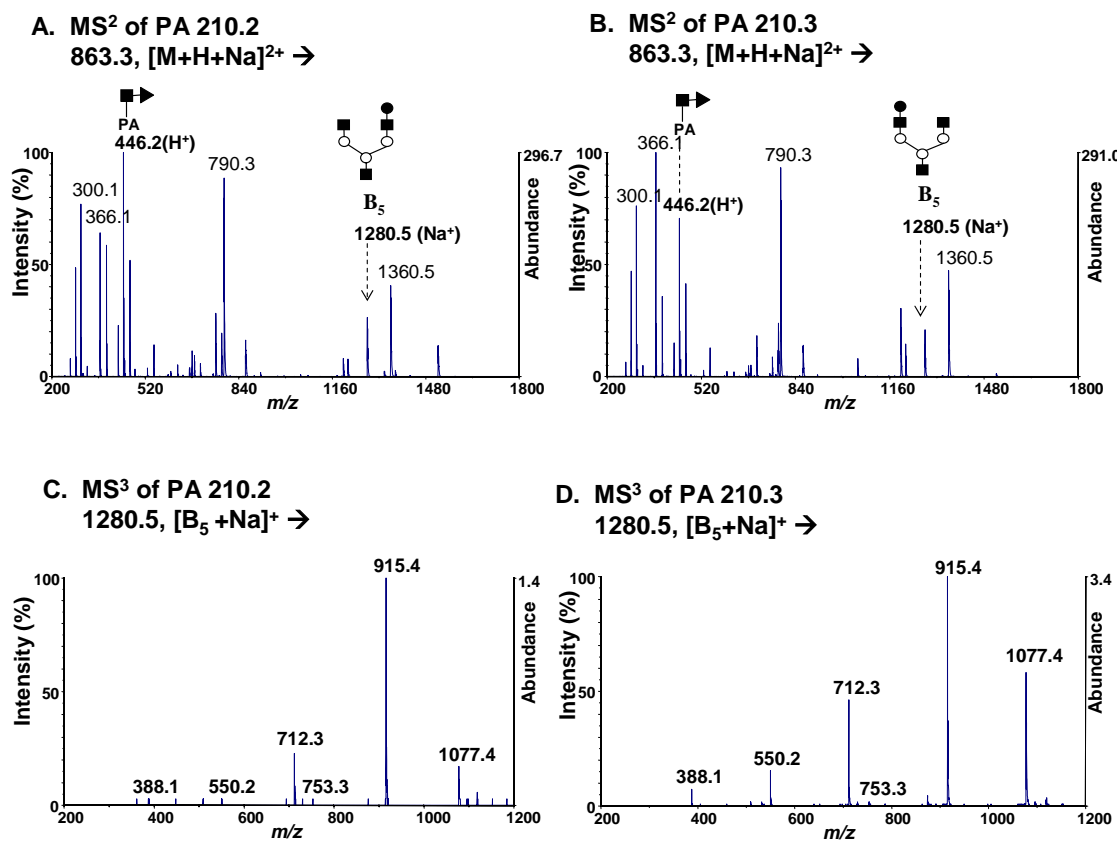ions (*m/z* 1280.5) of N-glycan moieties (210.2 and 210.3) in (A) and (B), respectively.

Figure 5. MS^n (n=2-3) spectra derived from [M+H+Na]$^{2+}$ precursor ions of isomeric PA N-glycan

(210.2 and 210.3) standards .

(A) and (B): MS^2 spectra derived from [M+H+Na]$^{2+}$ (*m/z* 863.3) in MS^1 spectra of PA 210.2 and PA

210.3 standards, respectively. (C) and (D): MS^3 spectra derived from the sodiated B$_5$-type fragment

ions (*m/z* 1280.5) in (A) and (B), respectively.
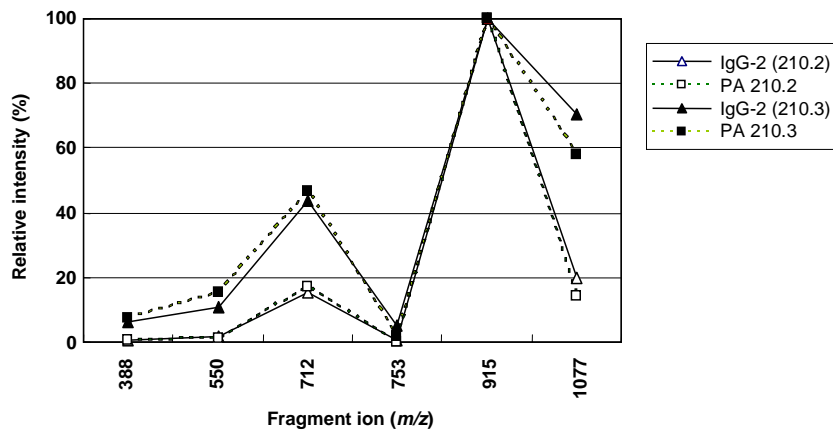
Correlation coefficients	PA 210.2	PA 210.3
IgG-2 (210.2)	**0.998**	0.913
IgG-2 (210.3)	0.851	**0.989**

Figure 6. MS^3 spectral matching results of IgG-2 (210.2), IgG-2 (210.3), PA 210.2, and PA 210.3.

Correlation coefficients were calculated by considering the six fragment ion intensities observed in

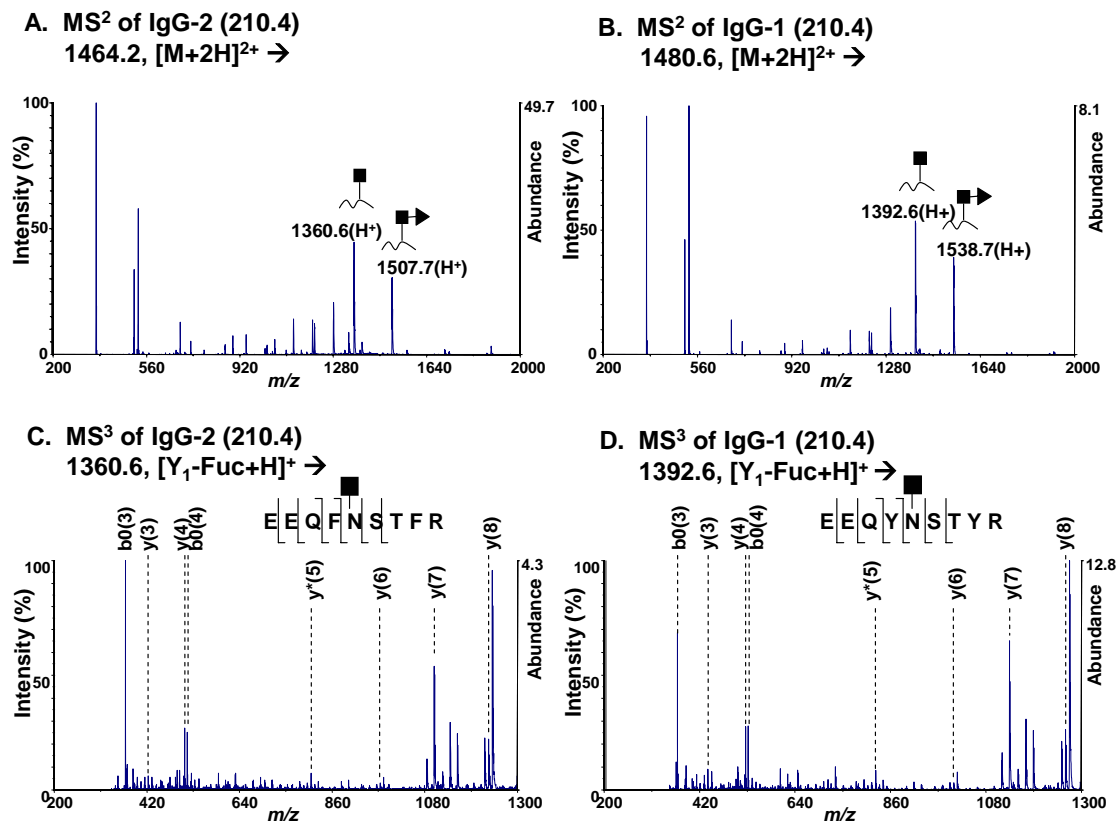Figure 4C, 4D, 5C, and 5D and overlaid in a graph.

Figure 7. MS^n (n=2-3) spectra derived from the protonated ions [M+2H]$^{2+}$ of glycopeptides IgG-2

(210.4) and IgG-1 (210.4).

 (A) and (B): MS^2 spectra derived from [M+2H]$^{2+}$ of IgG-2 (210.4) and IgG-1 (210.4) in MS^1

spectra, respectively. (C) and (D): MS^3 spectra derived from the peptide fragment ions retaining

GlcNAc ([Y$_1$-Fuc+H]$^+$: *m/z* 1360.6 and 1392.6) in (A) and (B), respectively.