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Analysis of Glycopeptides Using Lectin Affinity Chromatography with MALDI-TOF Mass Spectrometry

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Glycopeptides prepared from 1 nmol of a mixture of glycoproteins, transferrin, and ribonuclease B by lysylendopeptidase digestion were isolated by lectin and cellulose column chromatographies, and then they were analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry and MALDI-quadrupole ion trap (QIT)-TOF mass spectrometry which enables the performance of MS^n analysis. The lectin affinity preparation of glycopeptides with *Sambucus nigra* agglutinin and concanavalin A provides the glycan structure outlines for the sialyl linkage and the core structure of *N*-glycans. Such structural estimation was confirmed by MALDI-TOF MS and MALDI-QIT-TOF MS/MS. Amino acid sequences and location of glycosylation sites were determined by MALDI-QIT-TOF MS/MS/MS. Taken together, the combination of lectin column chromatography, MALDI-TOF MS, and MALDI-QIT-TOF MS^n provides an easy way for the structural estimation of glycans and the rapid analysis of glycoproteomics.

Glycosylation is one of the most common post-translational modifications of proteins. It is estimated that over half of mammalian proteins are glycosylated. The biosynthesis of glycans is not directly controlled by genetic template and depends on the concerted action of glycosyltransferases, and the structures of glycans are much more variable than those of proteins and nucleic acids. In addition, the structures of glycans can be easily altered by changes of the physiological condition of the cells.[1,2] Recent studies suggest that glycans modify the functions of glycoproteins and have roles in molecular recognition processes that occur in bacterial and viral infections, cell adhesion in inflammation and metastasis, differentiation, development, and many other events characterized by intercellular communication.[1,3,4] The functional significance of glycans, particularly those attached to a protein at specific glycosylation sites, has been elucidated in various facets of biology.[5–9] To understand the structure−function relationship in glycoproteins in detail, glycoproteomics analysis is required, e.g., the determination of protein, glycosylation sites, and structures of glycans attached to each glycosylated site.

Sialylation and *N*-glycan core structures are changed in various circumstances. For example, α2-6 sialylation is involved in promoting B lymphocyte activation, anti-inflammatory activity of immunoglobulin G, and amyloid-β production.[10–12] Change of the *N*-glycan core is observed in the course of tumorigenesis, brain aging, and differentiation.[13–15] Lectin column chromatography is a useful technique to fractionate glycans and glycopeptides,[16] and it is feasible to isolate glycopeptides based on lectin affinity. To isolate Siaα2-6 glycopeptides, *Sambucus nigra* agglutinin (SNA) is useful, because SNA specifically binds to the Siaα2-6 moiety.[17] Concanavalin A (Con A) is useful to isolate glycopeptides having high mannose-type glycans, because it binds high mannose-type

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glycans but does not bind multiantennary complex-type glycans.[18,19] Thus, SNA- and Con A-affinity chromatographies can provide substantial information in terms of the presence of Siaα2-6 linkage and high mannose-type glycans.

Mass spectrometry (MS) is currently the most efficient and promising analytical tool for elucidating both the peptide and glycan structures of glycopeptides. However, structural information obtained by MS is not complete because the typical mass difference for monosaccharides (162 and 203 Da for hexose and *N*-acetylhexosamine, respectively) cannot discriminate each monosaccharide, e.g., galactose, mannose, and glucose as hexose and *N*-acetylgalactosamine and *N*-acetylglucosamine as *N*-acetylhexosamine. Also, it is difficult to obtain anomeric and linkage information of glycans by MS, and it is necessary to perform a sequential glycosidase digestion to obtain such information.[20–22] However, the partial glycan structure can be estimated on the basis of its binding specificity to various lectins,[16] and their structures can be confirmed by MS analysis. The combination of lectin chromatography and MS analysis provides useful information of glycan structures as a highly sensitive detection and makes further biological approaches possible.

Several peptides and glycopeptides will be generated from a given glycoprotein by protease digestion. However, the ionization efficiency of glycopeptides in MS analysis is very low, leading to decreased sensitivity for glycopeptides compared with that of peptides. Therefore, the MS analysis of glycopeptides is not easy. Recently, cellulose column chromatography was reported as an effective tool for the separation of glycopeptides from peptides, rendering MS analysis of glycopeptides feasible.[23,24] Matrix-assisted laser desorption/ionization-quadrupole ion trap time-of-flight mass spectrometry (MALDI-QIT-TOF MS) is an ion trap type of MS which enables the performance of MS^n analysis.[25] In this study, we intended to develop a rapid and sensitive method for analyzing glycopeptides by introducing lectin and cellulose column chromatographies in combination with MALDI-TOF MS and MALDI-QIT-TOF MS^n.

EXPERIMENTAL SECTION

Materials. Human serum transferrin, bovine pancreatic ribonuclease B (RNase B), and guanidine thiocyanate (GT) were purchased from Sigma-Aldrich (St. Louis, MO). Lysylendopeptidase (Lys-C) and recrystalized 2,5-dihydroxybenzoic acid (DHB) were purchased from Wako Pure Chemical, Inc. (Osaka, Japan). Top Tips were purchased from Glygen Corp. (Columbia, MD). SNA-agarose was purchased from Vector Laboratories (Burlingame, CA). All solvents used in this study were HPLC grade.

Lys-C Digestion of Glycoproteins. A mixture of 1 nmol each of transferrin and RNase B was treated in 12.5 μL of 4 M urea containing 4 mM 2-mercaptoethanol by heating at 37 °C for 60 min. After adding 12.5 μL of 0.1 M $NaHCO_3$ (pH 8.5) and 1.5 μL of Lys-C (36 pmol) dissolved in 20 mM Tris−HCl (pH 9.0), the mixture was incubated at 37 °C for 20 h, and then 100 μL of 1-butanol and 25 μL of ethanol were added.

Cellulose Column Affinity Chromatography. A Top Tip filled with 1.4 mg of cellulose (TT1CEL) was sequentially washed with 20 μL of water, 20 μL of ethanol/water (1/1, v/v) (solvent A), and 20 μL of 1-butanol/ethanol/water (4/1/1, v/v) (solvent B). The Lys-C-digested sample solution obtained as described above was applied to the column. Then, the cellulose column was washed five times with 20 μL of solvent B. The bound materials were eluted twice with 20 μL of solvent A, and then they were completely dried.

SNA-Agarose Column Affinity Chromatography. Forty microliters of SNA-agarose filled in a tip was washed three times with 100 μL of phosphate-buffered saline (PBS). The bound fraction to the cellulose column was dissolved in 100 μL of PBS and applied to the SNA-agarose column. Then, the SNA column was washed four times with 100 μL of PBS. The bound materials were eluted with 100 μL of 6 M GT, and 1-butanol (400 μL) and ethanol (100 μL) were added to the fraction. The solution was applied to a cellulose column as described. After washing four times with 20 μL of solvent B, the bound materials were recovered from the column by 20 μL of solvent A.

Con A-Agarose Column Affinity Chromatography. A Top Tip filled with 40 μg of Con A-agarose (TT1CONA) was washed three times with 20 μL of PBS. The cellulose-bound fraction, dried and dissolved in 20 μL of PBS, was applied to the Con A column. Then, the Con A column was washed four times with 20 μL of PBS. The bound fraction was recovered with 20 μL of 6 M GT. 1-Butanol (80 μL) and ethanol (20 μL) were added to the Con A-bound fraction, and then the fraction was applied to the cellulose column in the same way as described. After the washing of the cellulose column five times with 20 μL of solvent B, the bound fraction was recovered with 20 μL of solvent A.

Mass Spectrometry (MS). Positive ion MALDI-TOF mass spectra and MS^n spectra of the glycopeptides were obtained by AXIMA-CFR and AXIMA-QIT (Shimadzu Corp., Kyoto, Japan) with a nitrogen laser (337 nm). For collision-induced dissociation, argon was used as the collision gas. For both MS and MS^n measurements, dried samples were dissolved in 10 μL of water. One-tenth (1 μL) of the solution was desalted by a C18+Carbon NuTip and mixed on a stainless-steel target with 1 μL of the matrix solution (25 mg/mL DHB in 40% methanol in 0.1% aqueous trifluoroacetic acid), and the mixture was dried under a gentle steam of air. In order to calibrate the mass spectra of glycopeptides, angiotensin II (m/z 1046.54), adrenocorticotropic hormone (ACTH) fragment 18−39 (m/z 2465.20), bovine insulin oxidized B chain (m/z 3494.65), and bovine insulin peptide (m/z 5730.61) were used. Mascot (Matrix Science, Ltd., London, U.K.) was used for protein identification based on MALDI-QIT-TOF MS/MS/MS spectra.[26]

RESULTS AND DISCUSSION

Purification of Glycopeptides from a Mixture of Human Transferrin and Bovine Pancreatic RNase B. The schematic

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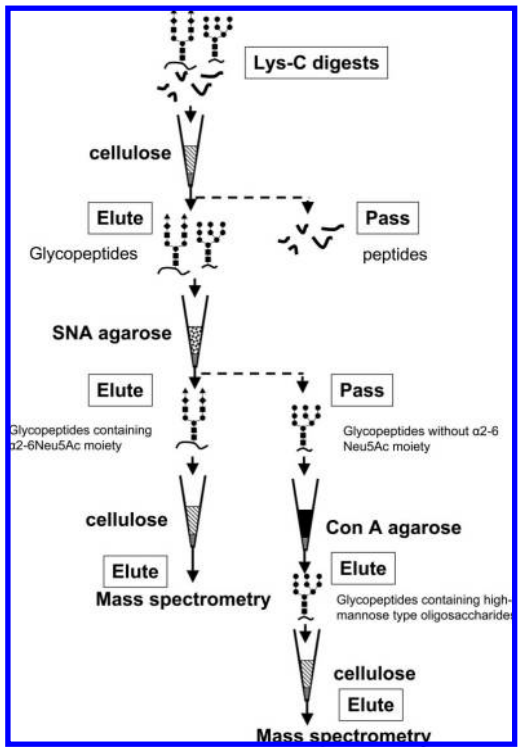

Figure 1. Schematic illustration of purification of glycopeptides by the cellulose and lectin columns. A mixture of glycosylated and nonglycosylated peptides was subjected to cellulose column chromatography. The bound fraction eluted with solvent A (ethanol/water (1:1, v/v)) was applied to the SNA-agarose column, and the bound fraction was eluted with GT. The bound fraction was applied to the second cellulose column. The fraction bound to the second cellulose column was recovered with solvent A. The passed-through fraction to the SNA-agarose column was applied to the Con A-column, and the bound fraction was eluted with GT. The bound fraction was applied to the second column as described above. Purified glycopeptides thus obtained were subjected to MALDI-(QIT)-TOF MS.

illustration of glycopeptide purification is shown in Figure 1. A mixture of 1 nmol each of transferrin and RNase B was digested by Lys-C, and the generated glycosylated and nonglycosylated peptides were subjected to cellulose column chromatography. The bound fraction obtained with solvent A was applied to a SNA-agarose column, and the bound fraction was recovered with guanidine thiocyanate (GT). The bound fraction was applied to the second cellulose column for removing GT because high concentration of GT inhibited ionization. The bound fraction to the second cellulose column was recovered with solvent A. The passed-through fraction to the SNA-agarose column was applied to a Con A-column and the bound fraction was eluted with GT. The bound fraction was applied to the second column for removing GT as described above. Predicted glycopeptides of transferrin and RNase B with glycan structures and amino acid sequences are shown in Figure 2.

MALDI-TOF MS of Glycopeptides Obtained by SNA-Agarose Column Chromatography and Con A-Agarose Column Chromatography. Fractionated glycopeptides were subjected to MALDI-TOF MS. Several ions were detected with strong intensity in the mass spectrum of the SNA-bound fraction (Figure 3A). Among them, *m*/*z* values of two ions (*m*/*z* 3624.4 and 5431.8) were comparable to the theoretical molecular weights of glycopeptides carrying a sialylated-biantennary glycan, C421-K433 and

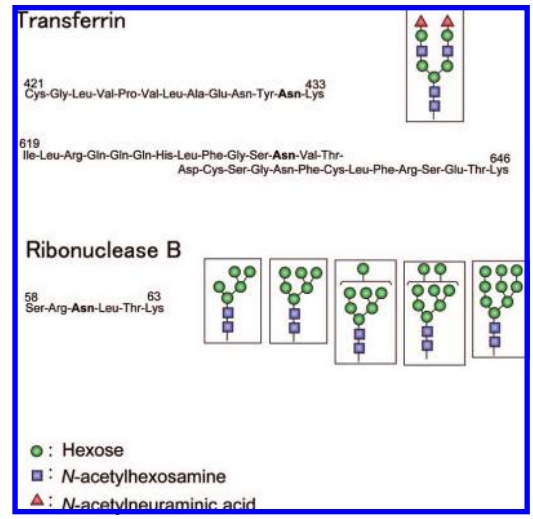

Figure 2. Predicted glycopeptides of transferrin and ribonuclease B with *N*-glycan structures and amino acid sequences. Numbers indicate amino acid residues of each peptide, and boldfaced Asns are *N*-glycosylated.

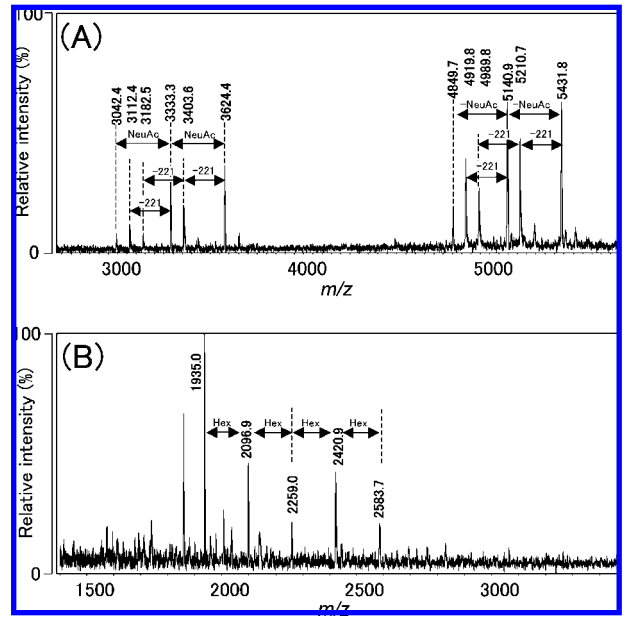

Figure 3. MALDI-TOF mass spectra of SNA-bound glycopeptides (A) and Con A-bound glycopeptides (B) prepared as shown in Figure 1. (A) *m*/*z* 3624.4 and 5431.8 were identical to the theoretical molecular weights of glycopeptides having sialylbiantennary structure. *m*/*z* 3042.4 and 3333.3 and 4849.7 and 5140.9 were deleted two and one Neu5Ac residues from ions at *m*/*z* 3624.4 and 5431.8, respectively. Six other ions at *m*/*z* 3112.4, 3182.5, 3403.6, 4919.8, 4989.8, and 5210.7 were the cross-ring cleavage products of the glycopeptides having two Neu5Acα2-6 linkages. (B) MS spectrum of Con A-bound glycopeptides. Ions at *m*/*z* 1935.0, 2096.9, 2259.0, 2420.9, and 2583.7 were identical to the theoretical molecular weights of glycopeptides having different numbers of mannose residues.

I619-K646, respectively, shown in Figure 2 (DDBJ/GenBank/EBI Data Bank with accession number NP 001054 for human transferrin). Ions at *m*/*z* 3042.4 and 3333.3 were observed with less intensity than that of the ion at *m*/*z* 3624.4. The mass differences between 3624.4 and 3042.4 and 3624.4 and 3333.3 were 582 and 291 Da, respectively, suggesting that they were produced by the elimination of 2 mol and 1 mol of Neu5Ac. Ions at *m*/*z* 4849.7

and 5140.9 would be derived from the ion at *m/z* 5431.8 due to the loss of 2 mol and 1 mol of Neu5Ac, respectively.

Ions at *m/z* 3182.5 and 3403.6 were observed with less intensity than the ions at *m/z* 3624.4 in the mass spectrum. The mass difference of each ion was 442 and 221 Da, respectively. Since the mass difference of 221 is derived from the ring cleavage of Neu5Ac that binds to the galactose residue by the α2-6 linkage,[27] we concluded that the ion at *m/z* 3624.4 has two α2-6-linked sialic acids in the molecule. The ion at *m/z* 3112.4 was observed with less intensity than the ion at *m/z* 3333.3. The mass difference of these ions was 221, leading to the conclusion that the ion at *m/z* 3333.3 has one α2-6-linked sialic acid. The detection of these ions was consistent with the binding specificity of SNA lectin, which can bind to glycopeptides containing the Siaα2-6 moiety.[17] The ring cleavage ions of Neu5Ac were observed at *m/z* 4989.8 and 5210.7, and 4919.8 could be generated from the ion at *m/z* 5431.8 and 5140.9, respectively. The mass differences of ions at *m/z* 4989.8, 5210.7, and 5431.8 were 221 Da, indicating that the ion at *m/z* 5431.8 has two α2-6-linked sialic acids. Since the mass difference between *m/z* 5140.9 and 4919.8 was 221 Da, it is also concluded that one α2-6-linked sialic acid moiety is in the ion observed at *m/z* 5140.9. These results indicate that the glycopeptides containing Neu5Acα2-6Gal moieties were fractionated by sequential SNA and cellulose column chromatographies, and their partial structures were characterized by MALDI-TOF MS.

The Con A-bound fraction was analyzed by MALDI-TOF MS (Figure 3B). Several intense ions were detected. They were expected to include the high mannose-type *N*-glycans because they bound to the Con A column.[16] Among these ion peaks, the *m/z* values of five ions (*m/z* 1935.0, 2096.9, 2259.0, 2420.9, and 2583.7) were comparable to the theoretical molecular weights of glycopeptides that are composed of a series of high mannose-type *N*-glycans (Man5, Man6, Man7, Man8, and Man9, respectively) and peptide (Figure 2, S58-K63, DDBJ/GenBank/EBI Data Bank with accession number X07283 for bovine pancreatic RNase B). The mass difference of each ion, 162 Da, is responsible for the elimination of hexose.

From the results described above, glycopeptides containing Neu5Acα2-6 moieties were recovered in the SNA-bound fraction, and glycopeptides containing high-mannose-type glycans were recovered in the Con A-bound fraction. These results indicate that lectin affinity column chromatography is very effective for the preparation of glycopeptides for MS analysis.

MALDI-QIT-TOF MS of Glycopeptides Obtained by SNA-Agarose Column Chromatography and Con A-Agarose Column Chromatography. The fraction bound to the SNA-agarose column was analyzed by MALDI-QIT-TOF MS (Figure 4A). The mass difference of 584 (*m/z* 3624.4 vs *m/z* 3042.4) was identical to the molecular weight of two Neu5Ac residues. The mass difference of 291 (*m/z* 3624.4 vs *m/z* 3333.4; *m/z* 3333.4 vs *m/z* 3042.4; *m/z* 5140.9 vs *m/z* 4849.7) was identical to the molecular weight of one Neu5Ac residue. These results suggested that both ions at *m/z* 3042.4 and 4849.7 were asialoglycopeptides. Ions with mass difference of 221, indicating the ring cleavage of Neu5Ac, were not detected by MALDI-QIT-TOF MS in contrast to MALDI-TOF MS. Further, the ion at *m/z* 3624.4 corresponding to the

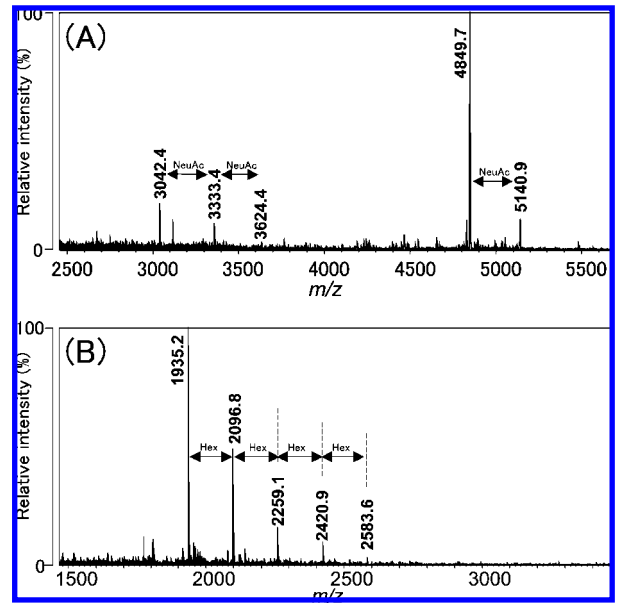

Figure 4. MALDI-QIT-TOF mass spectra of SNA-bound glycopeptides (A) and Con A-bound glycopeptides (B) prepared as shown in Figure 1. (A) MS spectrum of SNA-bound glycopeptides. The mass difference (*m/z* 582, *m/z* 3624.4 vs *m/z* 3042.4) was identical to the molecular weight of two dehydroxylatd Neu5Ac residues. The mass difference (*m/z* 291, *m/z* 3624.4 vs *m/z* 3333.4; *m/z* 3333.4 vs *m/z* 3042.4; *m/z* 5140.9 vs *m/z* 4849.7) was identical to the molecular weight of one Neu5Ac residue. (B) MS spectrum of Con A-bound glycopeptides. The *m/z* values of 1935.2, 2096.8, 2259.1, 2420.9, and 2583.6 were identical to the theoretical molecular weights of glycopeptides having different numbers of mannose residues.

molecular weight of theoretical glycopeptide having sialylated-biantennary glycan (C421−K433) was very weak. In addition, the ion at *m/z* 5431.5 corresponding to the theoretical molecular weight of glycopeptide having sialylated-biantennary glycan (I619−K646) was not detected by MALDI-QIT-TOF MS. These results indicate that the sialyl linkages of these glycopeptides were degraded under our experimental conditions of the MALDI-QIT-TOF instrument.

The fraction bound to the Con A-agarose column was also analyzed by MALDI-QIT-TOF MS (Figure 4B). Several intensive ions similar to those detected by MALDI-TOF MS (Figure 3B) were found. The results indicate that glycopeptides containing high mannose-type glycans gave similar fragment patterns by the MALDI-TOF MS and MALDI-QIT-TOF MS analyses.

MALDI-QIT-TOF MS/MS and MS/MS/MS of Glycopeptides Obtained by SNA-Agarose Column Chromatography. To obtain the structural information in detail, multistage analyses (MS/MS and MS/MS/MS) of ions, *m/z* 3624.4 and 5140.9, were performed by MALDI-QIT-TOF MS. However, a sufficient amount of these ions corresponding to the sialylated glycopeptides could not be detected, probably due to the limitations of the attainable resolution.

The MS/MS analysis of the ion at *m/z* 3042.4 is shown in Figure 5A. Several intensive ions (*m/z* 2879.9, 2677.0, 2514.8, 2311.7, 2149.6, 1987.8, 1824.9, 1622.1, and 1419.0) were observed. The mass differences of each ion were 162 or 203, suggesting that they were responsible for the elimination of hexose or *N*-acetylhexosamine. MS/MS analysis of the ion at *m/z* 3042.4 indicates that it contains five hexoses and four *N*-acetylhex-

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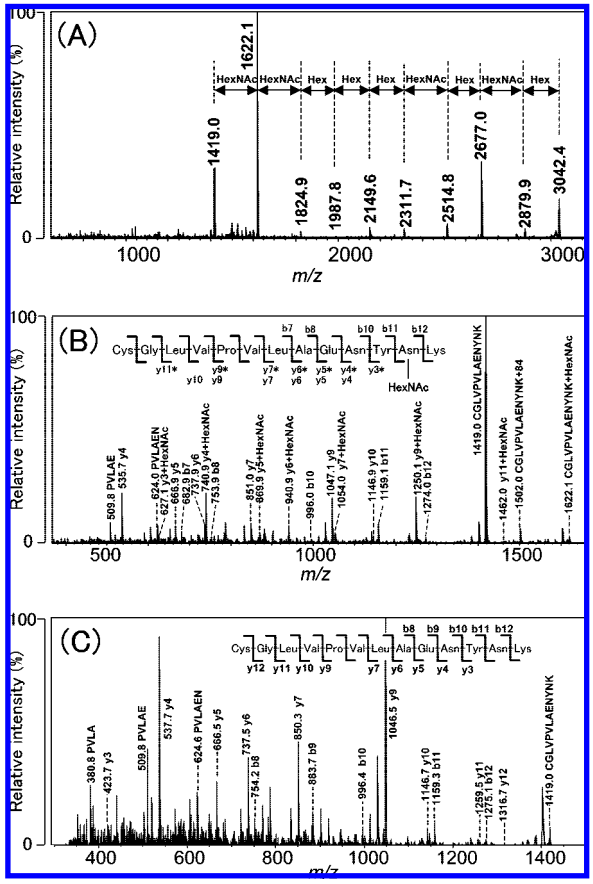

Figure 5. MALDI-QIT-TOF MS/MS and MS/MS/MS spectra of SNA-bound glycopeptides. (A) MS/MS spectrum of the ion at *m/z* 3042.4 (Figure 4A). The difference between detected ions indicated that dehydroxy hexose and *N*-acetylhexosamine were deleted from a precursor ion. (B) MS/MS/MS spectrum of the ion at *m/z* 1622.1. Amino acid sequence of precursor ion is shown in the upper part of the figure. Asterisks indicate the ions derived from each peptide having one GlcNAc residue. (C) MS/MS/MS spectrum of the ion at *m/z* 1419.0. The amino acid sequence and glycan structure of precursor ion are shown in the upper part of the figure. The fragmentation scheme is in accordance with the nomenclature of Domon and Costello.[36]

osamines (Figure 5A). The fragment pattern suggests that at first two lactosamine residues (Galβ1-4GlcNAc) were eliminated, then three mannoses and chitobiose (GlcNAcβ1-4GlcNAc) were sequentially eliminated. After the summing up of these data, the ion at *m/z* 3624.4 is characterized to be an *N*-glycan having two Neu5Ac residues, two Galβ1-4GlcNAc structures, and trimannosyl core structure [Manα1-3(Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc]. It is consistent with the results in a previous report stating that the major *N*-glycan of transferrin was a sialylbiantennary structure.[28]

Among the detected ions in Figure 5A, the ions at *m/z* 1622.1 and 1419.0 were intensive. They were suggested to be a peptide (C421-K433, CGLVPVLAENYNK) modified and not modified by one *N*-acetylglucosamine (GlcNAc), respectively. To obtain further structural information, these ions were subjected to MS/MS/MS analysis.

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The result of MS/MS/MS analysis of the ion at *m/z* 1622.1 is shown in Figure 5B. One intensive ion was detected at *m/z* 1419.0. Because the mass difference from the parent ion was 203, this ion was characterized to be a peptide formed by the loss of GlcNAc (CGLVPVLAENYNK). The fragment ions derived from *m/z* 1622.1 are shown at the upper part of Figure 5B. Five b-series and six y-series ions were assigned. The ion generated by the cleavage of two peptide bonds (PVLAE and PVLAEN) and seven glycopeptide ions having one GlcNAc residue were also assigned (y3+HexNAc, y4+HexNAc, y5+HexNAc, y6+HexNAc, y7+HexNAc, y9+HexNAc, and y11+HexNAc, indicated by asterisks in Figure 5B). It was predicted that the ion at *m/z* 1502.0 contained a fragment that was derived from the ring cleavage of GlcNAc residue on the basis of its *m/z* value (CGLVPVLAENYNK+84).[25]

A glycan-linked amino acid could not be identified by multistage analysis, because two fragment ions were not detected (y2-HexNAc and b12-HexNAc). However, in the predicted amino acid sequence (C421-K433), there was only one potential *N*-glycosylated site (N432, Figure 2). Taken together, these data indicate that the multistage analysis of glycopeptide is very powerful for the determination of the location of glycosylation sites.

MS/MS/MS analysis of the ion at *m/z* 1419.1 is shown in Figure 5C. Many ions derived from ion at *m/z* 1419.0 were detected. By Mascot database search, the mass spectrum generated from the ion at *m/z* 1419.0 was identified to be human transferrin. In the mass spectrum shown in Figure 5C, five b-series ions and 9 y-series ions were assigned. The three ions that were generated by the cleavage of three peptide bonds (PVLA, PVLAE, and PVLAEN) were also assigned. The predicted amino acid sequence is consistent with this estimation.

MALDI-QIT-TOF MS/MS and MS/MS/MS of Glycopeptides Obtained by Con A-Agarose Column Chromatography. MS/MS analysis of the ion at *m/z* 1935.2 is shown in Figure 6A. In this case, it was difficult to identify the fragment ions of each monosaccharide deletion because the intensities of these ions were weak. However, Con A-binding specificity helped to assign the fragment ions. It was suspected that these glycopeptides had high mannose-type *N*-glycans. On the basis of this assumption, ions at *m/z* 1773.1, 1610.9, 1448.9, 1286.8, 1124.5, 921.6, and 718.4 were assigned to be sequential fragment ions of glycan due to the elimination of hexose and *N*-acetylhexosamine. On the basis of these results, it was estimated that the glycan moiety was composed of five hexoses and two *N*-acetylhexosamines. Taken together, the ion at *m/z* 1935.2 was assigned to be high mannose-type *N*-glycan having five mannose residues. The ions at *m/z* 921.6 and 801.5 (Figure 6A) were thought to be peptide ions modified by a single GlcNAc and the fragment that derived from the ring cleavage of GlcNAc, respectively.[25] The ion at *m/z* 718.4 was suggested to be a peptide S58-T63 (SRNLTK). To confirm this assumption, these ions (*m/z* 718.4 and 921.6) were subjected to MS/MS/MS analysis.

The result of MS/MS/MS analysis of the ion at *m/z* 921.6 is shown in Figure 6B. On the basis of the mass difference as shown in Figure 6A, it was predicted that the ion at *m/z* 921.6 had one GlcNAc residue. Eight b-series related ions and two y-series ions were detected (Figure 6B). The b3+H_2O+HexNAc (*m/z* 561.1) and y4+HexNAc (*m/z* 678.4) ions were observed. The b3+H_2O+HexNAc ion had an asparagine residue (N60) at its carboxyl

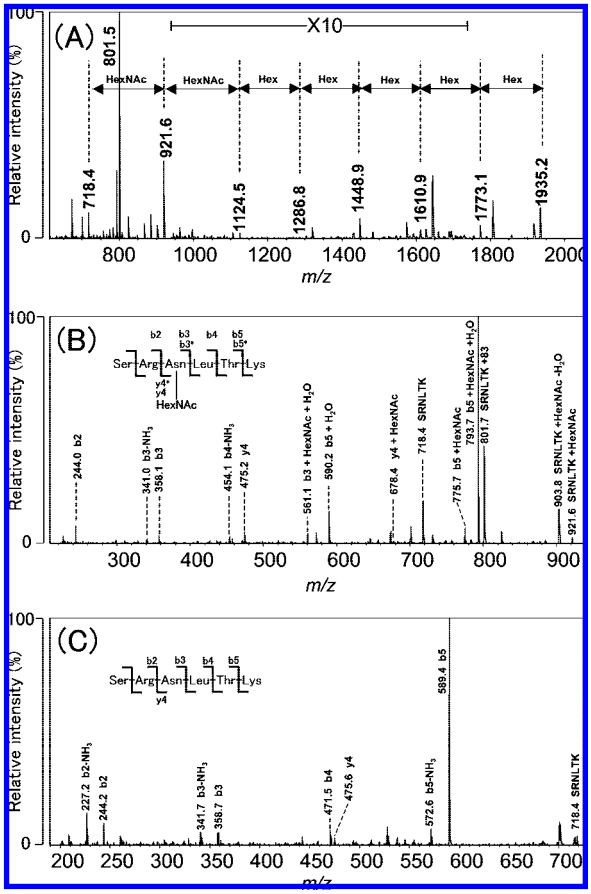

Figure 6. MALDI-QIT-TOF MS/MS and MS/MS/MS spectra of Con A-bound glycopeptides. (A) MS/MS spectrum of the ion at *m/z* 1935.2. The difference between detected ions indicated that dehydroxy hexose and *N*-acetylhexosamine were deleted from a precursor ion. A cross-ring cleavage product of the HexNAc-bearing peptide was an identified ion at *m/z* 801.5. (B) MS/MS/MS spectrum of the ion at *m/z* 921.6. The amino acid sequence and glycan structure of precursor ion are shown in the upper part of figure. Asterisks indicate the ions derived from each peptide having one GlcNAc residue. (C) MS/MS/MS spectrum of the ion at *m/z* 718.4. The amino acid sequence and glycan structure of the precursor ion are shown in the upper part of the figure. The fragmentation scheme is in accordance with the nomenclature as described in Figure 5.

terminus while the y4+HexNAc ion had it at its amino terminus. These results indicate that a GlcNAc residue was attached to N60. It was predicted on the basis of its mass number (SRNLTK+83) that the ion at *m/z* 801.7 contained a fragment that was derived from the ring cleavage of GlcNAc residue.[25]

MS/MS/MS analysis of the ion at *m/z* 718.4 is shown in Figure 6C. Several ions derived from ion at *m/z* 718.4 were detected, and the amino acid sequence agreed with this estimation. The fragment ions derived from *m/z* 718.4 are shown at the upper part of Figure 6C. Four b-series ions and one y-series ion were assigned. By Mascot database, the ion at *m/z* 718.4 was identified to be derived from bovine pancreatic RNase B. This is consistent with a previous report that N60 of RNase B was actually *N*-glycosylated.[25]

In the present study, we have shown that lectin affinity column chromatography was very effective to prepare glycopeptides for MALDI-QIT-TOF MS analysis. By the MALDI multistage MS analysis of several glycopeptides, the identification of the glycoprotein, the location of the glycosylated sites, and the structures of glycans of each glycosylated site were obtained. Binding specificity to the lectin columns and fragment ion analysis of MS/MS made it easy to predict the glycan structures. It should noted that the cellulose column is effective not only to prepare glycopeptides after digestion of glycoprotein by protease[24] but also to remove salts after lectin column chromatography.

Several reports about multistage MS analysis of glycopeptides have been published.[8,29–32] However, in these studies the structural information of glycans was limited because the glycan structures were heterogeneous. To overcome this problem, we combined MS analysis with the lectin columns because the lectin-binding specificity provided the partial structural information. As shown here, the SNA-binding suggested the presence of Neu5Acα2-6Gal in glycopeptides and the Con A-binding suggested the presence of high mannose-type glycans. Such information facilitated the assignment of ions. The introduction of other lectins may provide additional information on glycans and may support the analysis of glycopeptides by MS. We have also succeeded in purifying the glycopeptides comprising multiantennary complex-type *N*-glycans by *Datura stramonium* agglutinin[33,34] and the fucosylated *N*-glycans by *Aleuria aurantia* lectin[35] and in detecting the corresponding glycopeptide ions by MS (data not shown). In our methods, the amount of glycoprotein necessary for multistage MS analysis was about 1 nmol and it took only 2 days from protease digestion to multistage MS analysis. Lectin column chromatography is a useful technique to fractionate not only *N*-glycosylated peptides but also *O*-glycosylated peptides,[16] indicating that lectins have good potential for glycopeptide analysis. Taken together, the combination of lectin affinity chromatography and MALDI-TOF MS might be a powerful tool to analyze complex mixtures of glycopeptides in a relatively rapid fashion.

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