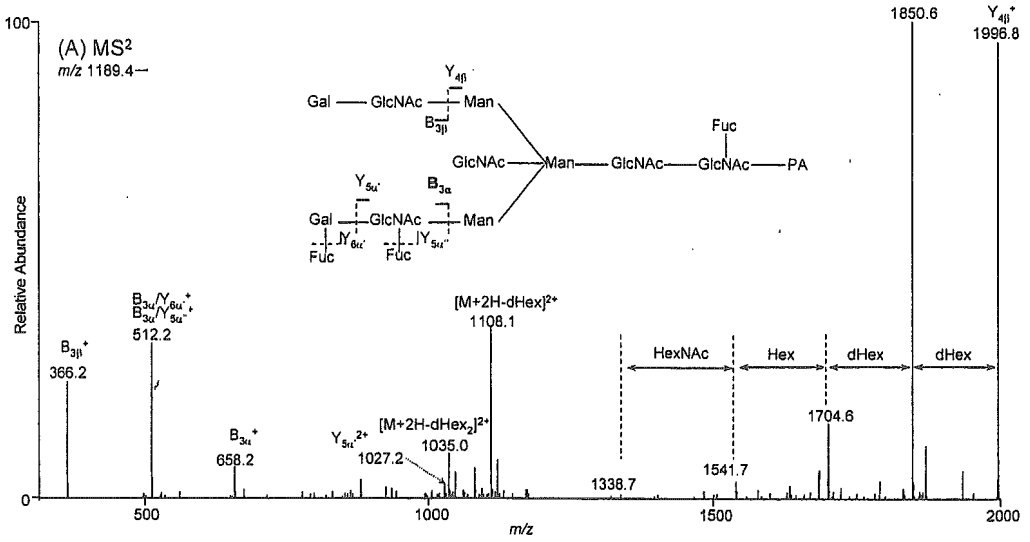

Figure 9. MS^2 spectrum of oligosaccharide f by LC/ESI-MS^n: precursor ion, $[M+H+NH_4]^{2+}$ at *m/z* 1189.9.

Le^x-oligosaccharides in biological samples. Our method, based on a sequential scan for the structure-characteristic ions, may be applicable to the analyses of oligosaccharides carrying other partial motifs, such as sialyl Le^x and sulfated sugar.

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REGULAR ARTICLE

Glycomic/glycoproteomic analysis by liquid chromatography/mass spectrometry: Analysis of glycan structural alteration in cells

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The alteration of glycosyltransferase expression and the subsequent changes in oligosaccharide structures are reported in several diseases. The analysis of glycan structural alteration in glycoproteins is becoming increasingly important in the discovery of therapies and diagnostic markers. In this study, we propose a strategy for glycomic/glycoproteomic analysis based on oligosaccharide profiling by LC/MS followed by proteomic approaches, including 2-DE and 2-D lectin blot. As a model of aberrant cells, we used Chinese hamster ovary cells transfected with *N*-acetylglucosaminyltransferase III (GnT-III), which catalyzes the addition of a bisecting *N*-acetylglucosamine (GlcNAc) to β-mannose of the mannosyl core of *N*-linked oligosaccharides. LC/MS equipped with a graphitized carbon column (GCC) enabled us to elucidate the structural alteration induced by the GnT-III expression. Using 2-D lectin blot followed by LC/MS/MS, the protein carrying an extra *N*-acetylhexosamine in cells transfected with GnT-III was successfully identified as integrin α3. Thus, oligosaccharide profiling by GCC-LC/MS followed by proteomic methods can be a powerful tool for glycomic/glycoproteomic analysis.

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1 Introduction

It is common knowledge that approximately 50% of proteins in mammalian cells are glycosylated and that glycans play crucial roles in various biological events including cell recognition [1], adhesion [2] and cell-cell interaction [3]. The alteration of glycosyltransferase expression and subsequent changes in oligosaccharide structures are reported in several diseases, including inherited diseases [4], the progression of cancer [5] and autoimmune diseases [6–8]. The analysis of glycan structural alteration in glycoproteins is becoming increasingly important in the discovery of therapies and diagnostic markers.

Comprehensive analysis of proteins in a given cellular sample is the most effective means of elucidating the disease mechanism. Simultaneous separation and characterization of proteins by 2-DE and 2-D LC followed by MS have been utilized as the fundamental approaches to proteomic analysis; however, these approaches alone are ineffectual for the elucidation of the glycan structural alteration in glycoproteins. A strategy based on qualitative and quantitative glycomic analysis is necessary for the study of glycosylation-associated diseases.

LC/MS is widely used for glycosylation analysis in glycoproteins. Previously, we demonstrated that LC/MS equipped with a graphitized carbon column (GCC-LC/MS) is a useful means of oligosaccharide profiling and for the structural analysis of carbohydrates [9–12]. Using this method, oligosaccharides, including high mannose, hybrid and complex

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Abbreviations: CHO, Chinese hamster ovary; **CHO-III cells**, CHO cells transfected with *N*-acetylglucosaminyltransferase III; **dHex**, deoxyhexose; **GCC**, graphitized carbon column; **GlcNAc**, *N*-acetylglucosamine; **GnT-III**, *N*-acetylglucosaminyltransferase III; **Hex**, hexose; **HexNAc**, *N*-acetylhexosamine; **NeuAc**, *N*-acetylneuraminic acid; **PNGase F**, peptide *N*-glycosidase F

types with or without sialic acids, can be separated, and structural information can be obtained from their mass spectra and chromatographic behavior.

Here we propose a strategy for performing glycomic/glycoproteomic analysis based on a combination of GCC-LC/MS and proteomic approaches.

First, GCC-LC/MS is applied to the analysis of oligosaccharide structural alteration in aberrant cells. Chinese hamster ovary (CHO) cells, used as a model of aberrant cells, were transfected with *N*-acetylglucosaminyltransferase III (GnT-III), which catalyzes the addition of bisecting *N*-acetylglucosamine (GlcNAc) to the trimannosyl core of *N*-linked oligosaccharides [13] and is associated with cell adhesion [14] and the suppression of tumor cell metastasis [15–17]. Then, 2-D lectin blotting followed by LC/MS/MS was used to identify the protein in which glycosylation was altered by the expression of GnT-III.

2 Materials and methods

2.1 Cell lines and culture

The CHO cells were obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan). The human GnT-III cDNA was cloned into the pCI-neo vector. The expression vector was transfected into CHO cells with LipofectAMINE plus reagent, according to the manufacturer's instructions. To screen the transformants, the transfectants were cultured with Ham's F12 medium supplemented with 10% fetal calf serum (FCS) and 1 mg/mL G418. After 2 weeks, the colonies were lifted with a micropipette. A high GnT-III-expressing clone was used in succeeding experiments.

The CHO cells and GnT-III-transfected CHO cells (CHO-III cells) were cultured in Ham's F12 medium supplemented with 10% FCS, 100 U/mL of penicillin and 100 μg/mL of streptomycin under a humidified atmosphere of 95% air and 5% CO_2. After harvesting CHO and CHO-III cells, they were rinsed with PBS containing protease inhibitors and 2 mM EDTA.

2.2 Preparation of insoluble and soluble fractions

The insoluble and soluble fractions were prepared from CHO and CHO-III cells using a Mem-PER Eukaryotic Membrane Protein Extraction Reagent Kit (Pierce Biotechnology, P.O., USA). The detergent in these fractions was removed with Detergent-OUT (Geno Technology, M.O., USA) three times. For desalting and degreasing, seven volumes of acetone were added to the sample solution, and the mixture was stirred and sonicated. The mixture was then incubated at −20°C for 1 h and centrifuged at 4°C for 15 min, 15 000 × g. The supernatants were discarded, and the pellets dried. The protein concentrations were determined using a BCA protein assay kit (Pierce).

2.3 Preparation of *N*-linked oligosaccharide alditols

The protein (500 μg) from each fraction was dissolved in 810 μL of 0.5 M Tris-HCl containing 8 M guanidine-HCl and 5 mM EDTA (pH 8.6), and then 6.0 μL of 2-mercaptoethanol were added in the solution. After incubation at room temperature for 2 h, freshly prepared 0.6 M sodium monoiodoacetamide (135 μL) was added to the solution. After incubation at room temperature for 2 h in the dark, the solution was desalted with PD10 column (Amersham Biosciences, NJ, USA), and the elute was lyophilized. The carboxylmethylated proteins were dissolved in 500 μL of 100 mM PBS (pH 7.2), and 20 U of peptide *N*-glycosidase F (PNGase F) (Roche Diagnostics, Mannheim, Germany) were added to the solution. After incubation at 37°C for 4 days, 1.74 mL of cold ethanol was added to the solution, the mixture was incubated at −20°C for 3 h, and proteins were removed by centrifugation at 4°C for 10 min (15 000 × g). The supernatants containing oligosaccharides were evaporated, and then lyophilized. The oligosaccharides were incubated with 500 μL of 0.5 M $NaBH_4$ at room temperature for 16 h, and then neutralized with 10% (v/v) acetic acid to pH 6.5 and desalted with Envi-Carb (Supelco, Bellefonte, USA).

2.4 GCC-LC/MS

LC was carried out using a MAGIC 2002 system (Michrom BioResources, Auburn, CA, USA). The GCC used was a Hypercarb column (150 × 0.2 mm, ThermoFinnigan, San Jose, CA, USA). The eluents were 5 mM ammonium acetate, pH 8.5, containing 2% ACN (pump A) and 5 mM ammonium acetate, pH 8.5, containing 80% ACN (pump B). The borohydride-reduced oligosaccharides were eluted at a flow rate of 2 μL/min with a gradient of 10–45% of pump B in 90 min. Mass spectra were recorded on a TSQ 7000 triple-stage quadruple mass spectrometer (ThermoFinnigan) equipped with a nanoelectorospray ion source (AMR, Inc., Tokyo, Japan). The mass spectrometer was operated in positive ion mode. Ions in the range of m/z 900–2400 were acquired with a scan duration of 3 s. The ESI voltage was set at 2.0 kV, and the capillary temperature was 175°C. The electron multiplier was set at 1.0 kV. Collisions for MS/MS were carried out with collision energy of 25%, scan duration of 4 s., and mass range of m/z 100–2000.

2.5 1-D SDS-PAGE and lectin blotting

Proteins were separated by 1-D SDS-PAGE (12.5% T, 3% C) as described by Laemmli [18] and stained with SYPRO Orange (Bio-Rad, Richmond, CA, USA) at room temperature for 30 min in transfer buffer (25 mM Tris-HCl, 20 mM glycine and 20% methanol). The gel images were scanned on a Tyhoon 9400 (Amersham Biosciences) at an excitation wavelength of 540/25 nm and an emission wavelength of 590/30 nm. After saving the gel image, the proteins were blotted to a PVDF membrane (Immun-Blot PVDF membrane,

0.2 μm, Bio-Rad) at 3.0 mA/cm^2, 20 V for 30 min in transfer buffer containing 0.1% SDS using a semi-dry blotter (Trans-blot SD sel, Bio-Rad). The efficiency and position of the transfer were confirmed using SYPRO Orange transferred together with proteins. Nonspecific sites on the membrane were blocked at 4°C for 16 h in 0.5% casein-PBS. After the membranes were washed with 0.05% Tween-PBS (T-PBS) three times, they were treated with 0.1 U/mL of sialidase (Nacalai Tesque, Kyoto, Japan) at 37°C for 16 h in 0.5 M acetate buffer (pH 5.0). The membranes were then re-blocked with 0.5% casein-PBS at 37°C for 15 min, washed with T-PBS three times, and incubated with biotinylated phytohemagglutinin-E4 (PHA-E4, 2 μg/mL) at 4°C for 2 h in PBS (pH 7.4). The membranes were then washed with T-PBS and incubated with 1:1000 diluted avidin-alkaline phosphatase (AP) complex solution at 4°C for 1 h in PBS.

2.6 Concentration of target proteins in the gel

The band detected by lectin blotting on 1-D gel was excised and then mashed in 20 mM Tris-HCl (pH 8.0) containing 2% SDS. The proteins in the gel particles were extracted by intermittent sonication at 4°C for 30 min, followed by shaking at room temperature for 16 h. After extraction, the gel particles were removed by centrifugation (15 000 × g). The proteins in the supernatant were precipitated with sevenfold acetone at −20°C for 3 h, and then the precipitates were washed with acetone three times to remove salts and detergent.

2.7 2-DE

For first dimension IEF of the sample, Immobiline DryStrip gel (13 cm, pH4–7 NL, Amersham Biosciences) was used. The samples were dissolved in IEF solution containing 7 M urea, 2 M thiourea, 18 mM DTT, 0.5% IPG buffer, 2% CHAPS, and bromophenol blue. Dried IPG strips were rehydrated overnight in the sample solution. IEF was then performed using the following steps: 500 V for 1 h, 100 V for 1 h, and 8000 V for 2 h, *i.e.* a total of 17.5 kVh.

IPG strips were treated with 10 mL of 50 mM Tris-HCl (pH 8.8) containing 2% SDS, 6 M urea, 30% glycerol and 65 mM DTT for 15 min, and then treated with 10 mL of 50 mM Tris-Cl (pH 8.8) containing 2% SDS, 6 M urea, 30% glycerol and 135 mM iodoacetamide for 15 min in order to reduce the disulfide bonds of cysteinyl residues. SDS-polyacrylamide gels (7.5%T, 3%C, size 140 × 140 × 1 mm) and running buffer containing 25 mM Tris-HCl, 192 mM glycine and 0.1% SDS were used for the 2-DE. The gels were run at 25 mA/gel after setting the IPG strip on the gel. Fluorescent staining and scanning of gel, followed by lectin blotting, were performed as mentioned above. In 2-D lectin blotting, the proteins were blotted to a PVDF membrane at 3.0 mA/cm^2, 20 V for 90 min.

2.8 In-gel digestion and protein identification by LC/MS/MS

Interesting spots were excised from the 2-DE gel for in-gel trypsin digestion. The gel particles were destained with 20 mM ammonium bicarbonate containing 50% methanol in microcentrifuge tubes, and dehydrated in 100% ACN. Enzymatic digestion was performed overnight at 37°C with 5 μL of 20 μg/mL trypsin (Promega, Madison, WI, USA) in 20 mM ammonium bicarbonate (pH 8.5). Digested peptides were extracted with 1% TFA in 50% ACN, and samples were dried with a Speed-Vac and redissolved in 0.1% TFA for LC/MS.

LC was carried out using a Paradigm MS4 (Michrom BioResources) equipped with Magic C18 column (50 × 0.2 mm, Michrom BioResources). The eluents were 0.1% formic acid containing 2% ACN (pump A), and 0.1% formic acid containing 90% ACN (pump B). The peptides were eluted at a flow rate of 2 μL/min with a gradient of 5–70% of pump B in 30 min. Mass spectra were recorded on an API QSTAR Pulsar i (Applied Biosystems, Foster City, CA, USA) in the positive ion mode. The proteins were identified by searching the Swiss-Prot database using MASCOT (Matrix Science, UK). The mass range and MS/MS range were *m/z* 400–2000 and *m/z* 100–2000, respectively, and the ESI voltage was set at 2.5 kV.

3 Results

3.1 Analysis of glycans in the insoluble fractions

N-linked oligosaccharides were released from soluble and insoluble fractions by PNGase F and reduced with $NaBH_4$ to prevent the separation of anomers by GCC. Figure 1A shows the *N*-linked oligosaccharide profile of the insoluble fraction from CHO cells (5 × 10^7). Diverse oligosaccharide ions were detected by full scan in the positive ion mode of MS. Oligosaccharides were numbered with the labels on peaks where they were detected, and the multiple oligosaccharides in single peak were classified by the digits behind alphabets, such as peaks A1 and A2. Their monosaccharide compositions were deduced from the *m/z* values as shown in Table 1. *N*-linked oligosaccharides from CHO cells have a high proportion of high mannose-type and bi-, tri- and tetra-antennary complex type oligosaccharides [19, 20]. High mannose-type oligosaccharides, $[Man]_{5-9}[GlcNAc]_2$ were detected at 9–23 min (peaks A–E and K). Major components (peaks N2, Q2, R1, S1, T1, U1 and V2) were deduced as fucosylated and non-fucosylated biantennary forms with mono- and di-sialic acids from previous articles and their monosaccharide compositions. Various oligosaccharides, including mono- (peak N1 and Q1), tri- (peak P1, U2, V1), tetra-antennary (peak V3), and hybrid-type (peak F1 and I1) oligosaccharides were detected as minor components together with low molecular weight oligosaccharides such as the trimannosyl core (peaks G1 and O1).

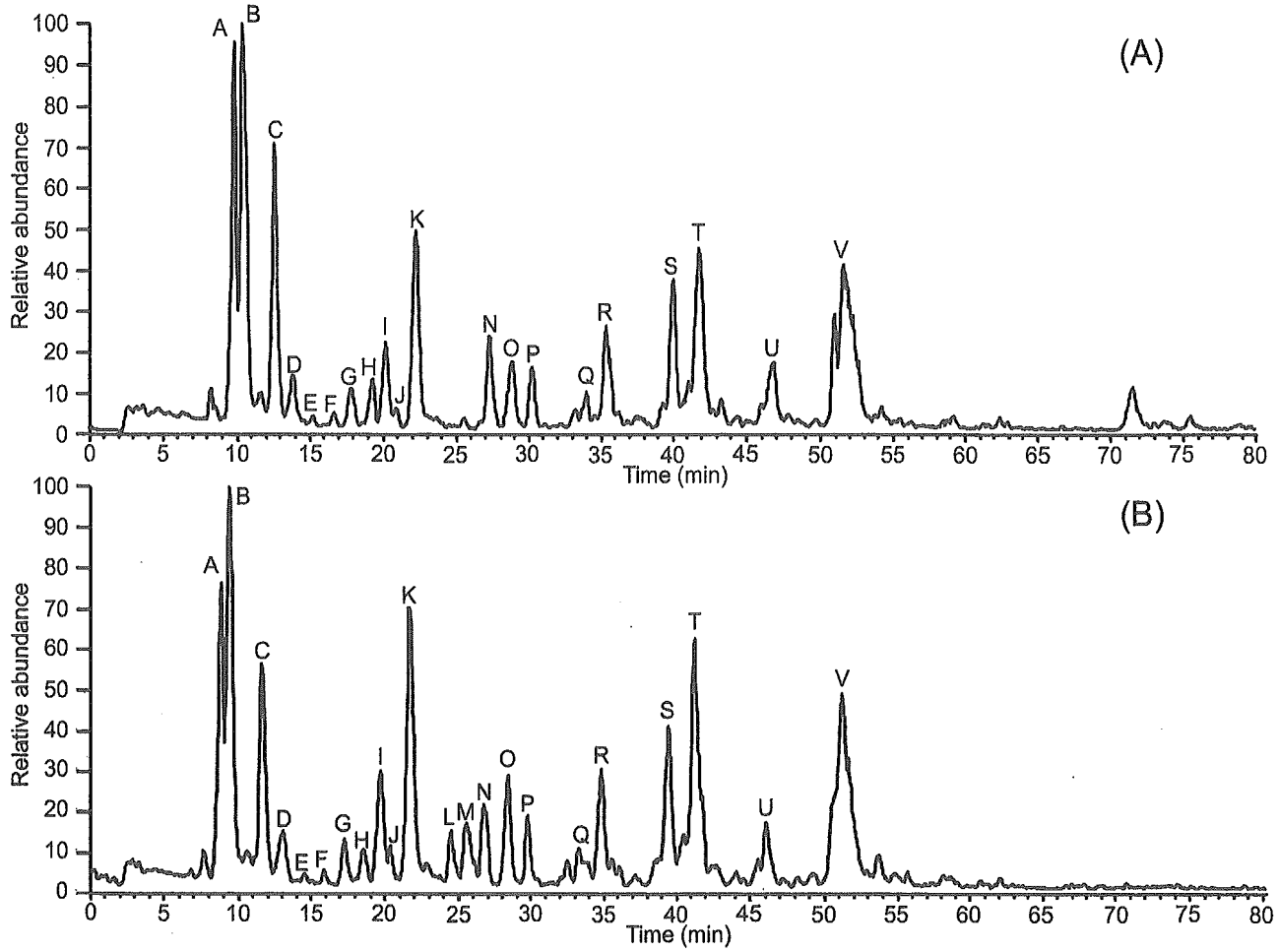

Figure 1. Total ion chromatograms of GCC-LC/MS of borohydride-reduced *N*-linked oligosaccharides released from insoluble fraction of CHO (A) and CHO-III (B) in positive ion mode. Pump A: 5 mM ammonium acetate, pH 8.5, containing 2% ACN. Pump B: 5 mM ammonium acetate, pH 8.5, containing 80% ACN. The borohydride-reduced oligosaccharides were eluted at a flow rate of 2 μL/min with a gradient of 10–45% of pump B in 90 min.

Figure 1B shows the oligosaccharide profile of the CHO-III-insoluble fraction. The distribution of oligosaccharides in CHO-III was different from that in the CHO cell insoluble fraction. Some additional peaks (peaks L1 and M1) were detected in the CHO-III-insoluble fraction, and their doubly charged ions at *m/z* 1143.2 and 1143.0 were consistent with the theoretical *m/z* values of fucosylated biantennary-bearing NeuAc with one additional HexNAc. Figure 2 shows the MS/MS spectrum of peak M1. Detection of $B_{1\gamma}/Y_6^{2+}$ at *m/z* 894.1 and an intense ion of $[HexNAc]^+$, at *m/z* 204 suggest that the oligosaccharide (peak M1) carries one GlcNAc at either of the non-reducing ends. Peak M1 is possibly assigned to bisected biantennary form. In addition, peaks D1, I2 and S2, which were not found in the profile of CHO, were detected in that of CHO-III (Fig. 1B). They can also be deduced as bisected biantennary forms from their MS/MS spectra. Other than these oligosaccharides bearing GlcNAc at either of the non-reducing ends in CHO-III cells, there was no significant difference in glycosylation between CHO and CHO-III cells. These results suggest that only limited oligosaccharides are altered by the expression of GnT-III.

3.2 Analysis of glycans in the soluble fractions

Figure 3A and B shows the *N*-linked oligosaccharide profiles of the soluble fractions of CHO and CHO-III, respectively. The oligosaccharide components of soluble fractions are very different from those of insoluble fractions (Table 1). High mannose-type oligosaccharides, $[Man]_{5-9}[GlcNAc]_2$, were detected as major components (peaks A–C and K), and complex-type and hybrid-type oligosaccharides were detected as minor oligosaccharides in the soluble fraction. Oligosaccharides bearing extra GlcNAc (D1, L1 and M1) were also detected in the soluble fraction of CHO-III.

Table 1. Observed *m/z* values and carbohydrate compositions of peaks A-V in total ion chromatogram 3 of CHO-insoluble (Fig. 1A), CHO-III-insoluble (Fig. 1B), CHO-soluble (Fig. 3A) and CHOIII-soluble (Fig. 3B) fractions

Carbohydrate composition[a]	Theoretical mass[b]	Peak No.		Insoluble fraction				Soluble fraction			
				CHO		CHO-III		CHO		CHO-III	
				Charge state	Observed *m/z*	Charge state	Observed *m/z*	Charge state	Observed *m/z*	Charge state	Observed *m/z*
$[Hex]_7[HexNAc]_2$	1561.4	A	A1	H^+	1562.2	H^+	1562.0	Na^+	1584.4	Na^+	1584.2
$[Hex]_8[HexNAc]_2$	1723.5		A2	Na^+	1746.3	Na^+	1746.3	Na^+	1746.5	Na^+	1746.1
$[Hex]_9[HexNAc]_2$	1885.7	B	B1	Na^+	1908.4	Na^+	1908.5	Na^+	1908.4	Na^+	1908.9
$[Hex]_6[HexNAc]_2$	1399.3	C	C1	H^+	1400.1	H^+	1400.0	H^+	1399.7	H^+	1399.9
$[Hex]_7[HexNAc]_2$	1561.4		C2	Na^+	1584.2	Na^+	1584.0	Na^+	1584.8	Na^+	1584.0
$[dHex]_1[Hex]_5[HexNAc]_5$	1992.9	D	D1		N.D.[c]	$2H^+$	**997.4**		N.D.	$2H^+$	**997.5**
$[Hex]_6[HexNAc]_2$	1399.3		D2	Na^+	1422.0	Na^+	1421.9		N.D.		N.D.
$[Hex]_7[HexNAc]_2$	1561.4		D3	Na^+	1584.2	Na^+	1584.1		N.D.		N.D.
$[Hex]_8[HexNAc]_2$	1723.5		D4	Na^+	1746.2	Na^+	1746.4		N.D.		N.D.
$[Hex]_4[HexNAc]_2$	1075.0	E	E1	Na^+	1097.9	Na^+	1097.6		N.D.		N.D.
$[Hex]_6[HexNAc]_2$	1399.3		E2	H^+	1400.1	H^+	1400.0		N.D.		N.D.
$[Hex]_6[HexNAc]_3$	1602.5	F	F1	H^+	1604.0	H^+	1603.1		N.D.		N.D.
$[Hex]_3[HexNAc]_2$	912.8	G	G1	H^+	913.7	H^+	913.7	Na^+	935.7	Na^+	935.6
$[Hex]_5[HexNAc]_4$	1643.5	H	H1	H^+	1644.5	H^+	1644.2	Na^+	1666.3	Na^+	1666.4
$[Hex]_6[HexNAc]_4$	1827.6	I	I1	$2Na^+$	914.7	$2Na^+$	914.7		N.D.		N.D.
$[Hex]_5[HexNAc]_5[NeuAc]_1$	2137.9		I2		N.D.	$2H^+$	**1069.8**		N.D.		N.D.
$[dHex]_1[Hex]_3[HexNAc]_4$	1465.4	J	J1	H^+	1466.1	H^+	1466.1	Na^+	1488.2	Na^+	1487.9
$[Hex]_5[HexNAc]_2$	1237.1	K	K1	H^+	1238.0	H^+	1238.0	H^+	1237.9	H^+	1237.9
$[dHex]_1[Hex]_5[HexNAc]_5[NeuAc]_1$	2284.1	L	L1		N.D.	$2H^+$	**1143.2**		N.D.	$2H^+$	**1142.9**
$[dHex]_1[Hex]_5[HexNAc]_6[NeuAc]_1$	2284.1	M	M1		N.D.	$2H^+$	**1143.0**		N.D.	$2H^+$	**1143.3**
$[dHex]_1[Hex]_4[HexNAc]_3$	1424.3	N	N1	H^+	1425.4	H^+	1425.3	Na^+	1447.1	Na^+	1447.1
$[dHex]_1[Hex]_5[HexNAc]_4$	1789.7		N2	H^+	1790.1	H^+	1790.3	Na^+	1812.3	Na^+	1812.1
$[dHex]_1[Hex]_3[HexNAc]_2$	1059.0	O	O1	H^+	1059.7	H^+	1059.7	H^+	1059.8	H^+	1059.7
$[dHex]_1[Hex]_6[HexNAc]_5$	2155.0	P	P1	$2H^+$	1078.5	$2H^+$	1078.5		N.D.		N.D.
$[dHex]_1[Hex]_3[HexNAc]_3$	1262.2	Q	Q1	H^+	1263.0	H^+	1263.0		N.D.		N.D.
$[Hex]_5[HexNAc]_4[NeuAc]_1$	1934.7		Q2	$2H^+$	968.4	$2H^+$	968.4		N.D.		N.D.
$[Hex]_5[HexNAc]_4[NeuAc]_1$	1934.7	R	R1	$2H^+$	968.4	$2H^+$	968.4	$2H^+$	968.7	$2H^+$	968.2
$[dHex]_1[Hex]_5[HexNAc]_4[NeuAc]_1$	2080.9	S	S1	$2H^+$	1041.4	$2H^+$	1041.4	$2H^+$	1041.4	$2H^+$	1041.3
$[dHex]_1[Hex]_5[HexNAc]_5[NeuAc]_2$	2574.0		S2		N.D.	$2H^+$	**1288.5**		N.D.		N.D.
$[dHex]_1[Hex]_5[HexNAc]_4[NeuAc]_1$	2080.9	T	T1	$2H^+$	1041.4	$2H^+$	1041.5	$2H^+$	1041.4	$2H^+$	1041.3
$[Hex]_5[HexNAc]_4[NeuAc]_2$	2226.0	U	U1	$2H^+$	1114.0	$2H^+$	1113.9	$2H^+$	1113.9	$2H^+$	1113.9
$[dHex]_1[Hex]_6[HexNAc]_5[NeuAc]_1$	2446.2		U2	$2H^+$	1224.2	$2H^+$	1224.3	$2Na^+$	1124.9		N.D.
$[dHex]_1[Hex]_6[HexNAc]_5[NeuAc]_2$	2737.5	V	V1	$2H^+$	1370.0	$2H^+$	1370.0		N.D.		N.D.
$[dHex]_1[Hex]_5[HexNAc]_4[NeuAc]_2$	2372.1		V2	$2H^+$	1187.1	$2H^+$	1187.1	$2H^+$	1187.2	$2H^+$	1187.2
$[dHex]_1[Hex]_7[HexNAc]_6[NeuAc]_1$	2811.6		V3	$2H^+$	1406.8	$2H^+$	1406.6		N.D.		N.D.

The characteristic *m/z* values observed in total ion chromatograms of CHO-III are depicted in bold type.

a) [dHex], deoxyhexose; [Hex], hexose; [HexNAc], *N*-acetylhexosamine; [NeuAc], *N*-acetylneuraminic acid.

b) Monoisotopic mass values.

c) Not detected.

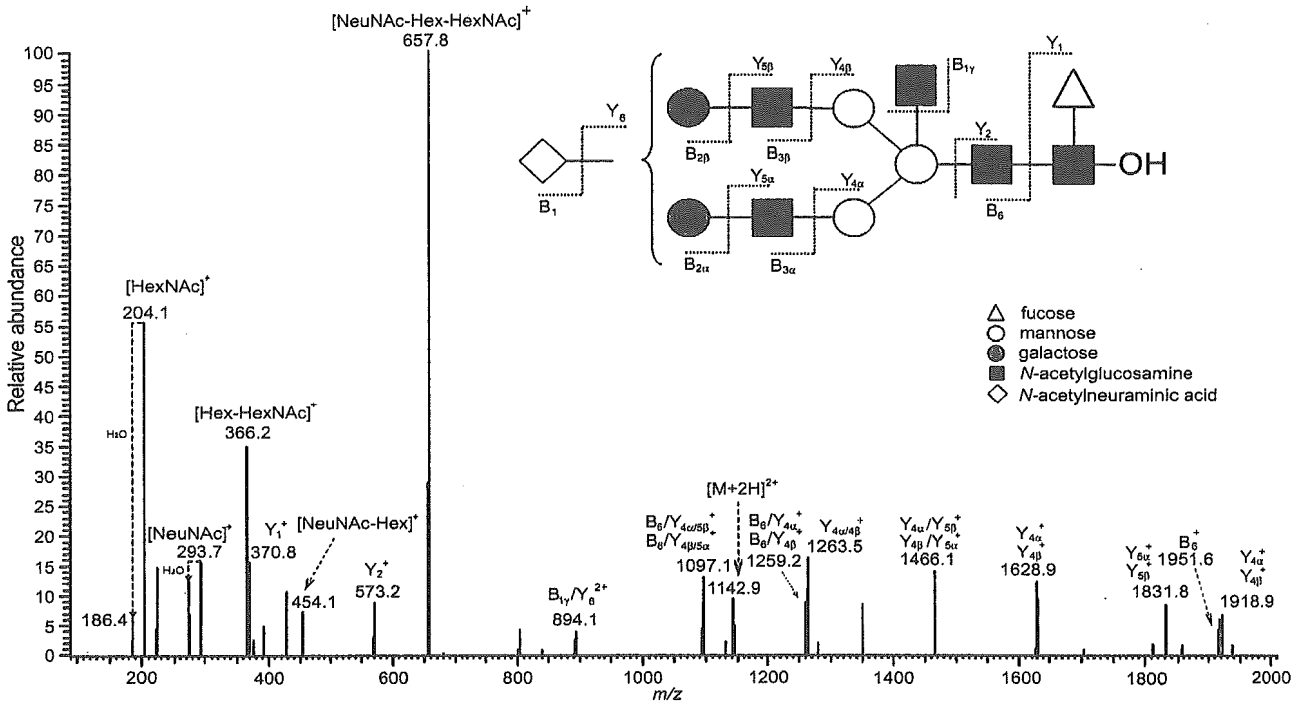

Figure 2. MS/MS spectrum of fucosylated biantennary *N*-linked oligosaccharide (peak M1) detected in the insoluble fraction from CHO-III.

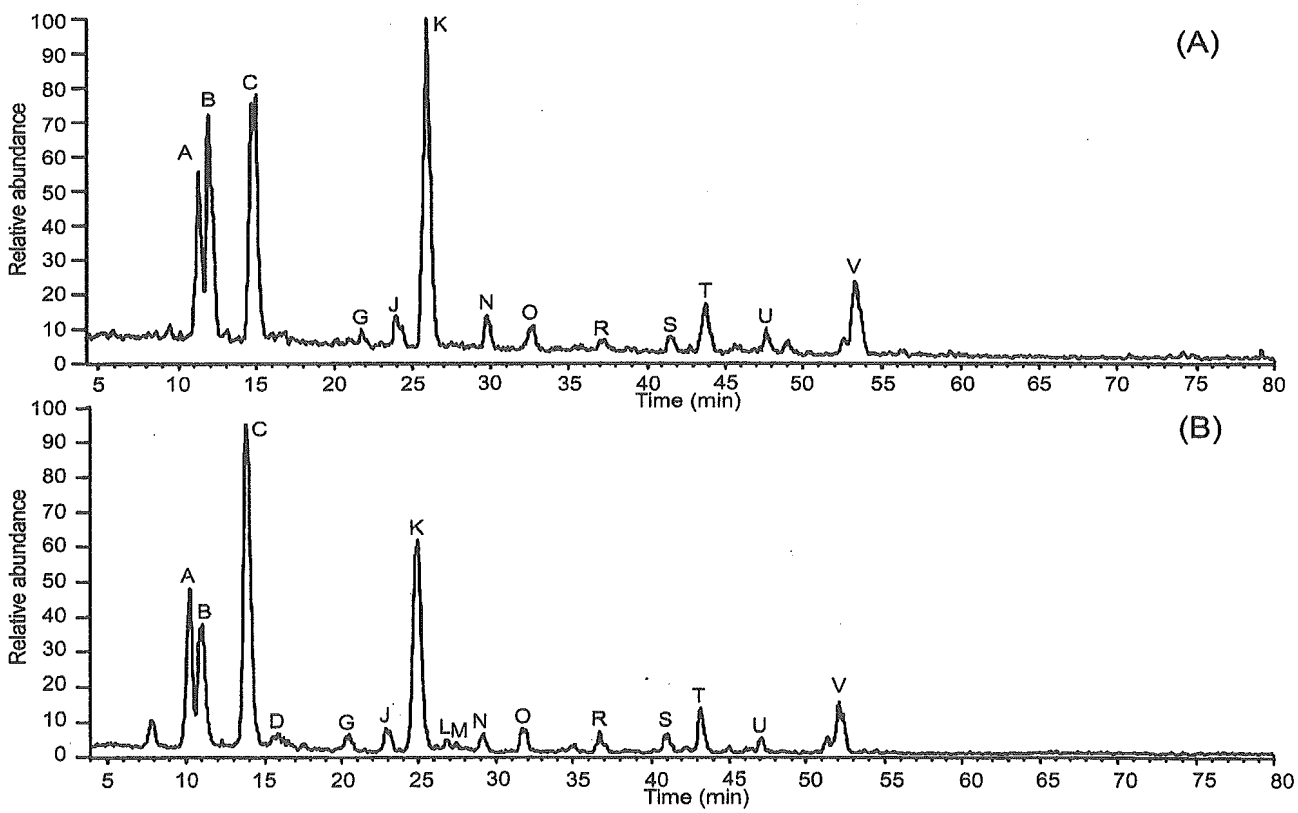

Figure 3. Total ion chromatograms of GCC-LC/MS of borohydride-reduced *N*-linked oligosaccharides released from the soluble fractions of CHO (A) and CHO-III (B) in positive ion mode. Pump A: 5 mM ammonium acetate, pH 8.5, containing 2% ACN. Pump B: 5 mM ammonium acetate, pH 8.5, containing 80% ACN. The borohydride-reduced oligosaccharides were eluted at a flow rate of 2 μL/min with a gradient of 10–45% of pump B in 90 min.

3.3 Identification of protein bearing bisected oligosaccharides

To identify proteins with altered glycans by the expression of GnT-III, we performed 2-DE followed by lectin blotting using PHA-E4 lectin, which recognizes bisecting GlcNAc in complex-type oligosaccharides. Although some bisected glycoproteins (70–120 kDa) could be visualized by 2-D lectin blotting, their expressions were too low to be detected on 2-DE gel. Lectin affinity chromatography, which is generally used for the concentration of glycoproteins, cannot be used for the insoluble fraction due to the presence of detergent in the solvent medium. Therefore, we first performed 1-D SDS-PAGE followed by lectin blotting, and then proteins in the range of 70–120 kDa were extracted from the gel (Fig. 4). 2-DE followed by lectin blotting was then performed, and interesting spots were successfully detected on 2-DE gel. Figure 5A and B shows the 2-DE gel images and the 2-D lectin blot of extracted proteins, respectively. The remarkable train spots (120 Da) of glycoprotein were picked up and in-gel digested with trypsin. The digest was subjected to LC/MS/MS, and the integrin α3 precursor was identified as the GnT-III target protein.

4 Discussion

The development of a simple and rapid method to explore glycan structural alteration in a complex mixture is required to elucidate the mechanisms of diseases involving glycan alteration. In this study, we demonstrated that GCC-LC/MS, which is used for glycosylation analysis in glycoproteins, is applicable for the exploration of changes in glycosylation between samples. Using GCC-LC/MS, high mannose, hybrid, and complex types as well as neutral and acidic oligosaccharides could be separated and characterized by a single analysis. GCC-LC/MS clearly shows differences in glycosylation between soluble and insoluble fractions. High mannose-type oligosaccharides were detected as major components in the soluble fraction. The soluble fraction contains endoplasmic reticulum and Golgi apparatus, where *N*-linked oligosaccharides are constructed. The predominance of high-mannose-type oligosaccharides in the soluble fraction may be the cause of immature oligosaccharides in the process of biosynthesis. In contrast, complex and hybrid types with or without sialic acids were detected in the insoluble fraction, suggesting that membrane proteins carry mature oligosaccharides.

In addition, GCC-LC/MS revealed differences in glycosylation between control cells and aberrant model cells. Biantennary forms bearing extra GlcNAc were obviously increased in cells transfected with the GnT-III gene, indicating that our methodology allows us to explore changes in the glycosyltransferase expression followed by glycan alteration. Although MS is frequently used for the analysis of glycosylation, identification of oligosaccharide isomers by MS alone

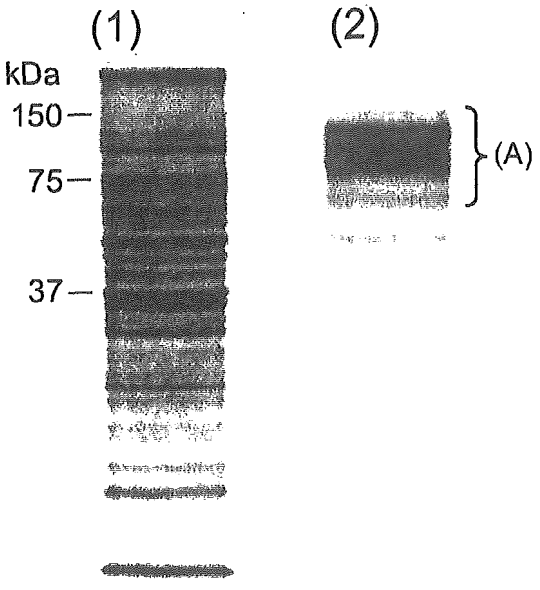

Figure 4. (1) 1-D SDS-PAGE and (2) lectin blot images of the CHO-III insoluble fraction. Proteins were separated on a 12.5% SDS-PAGE gel and stained with SYPRO Orange.

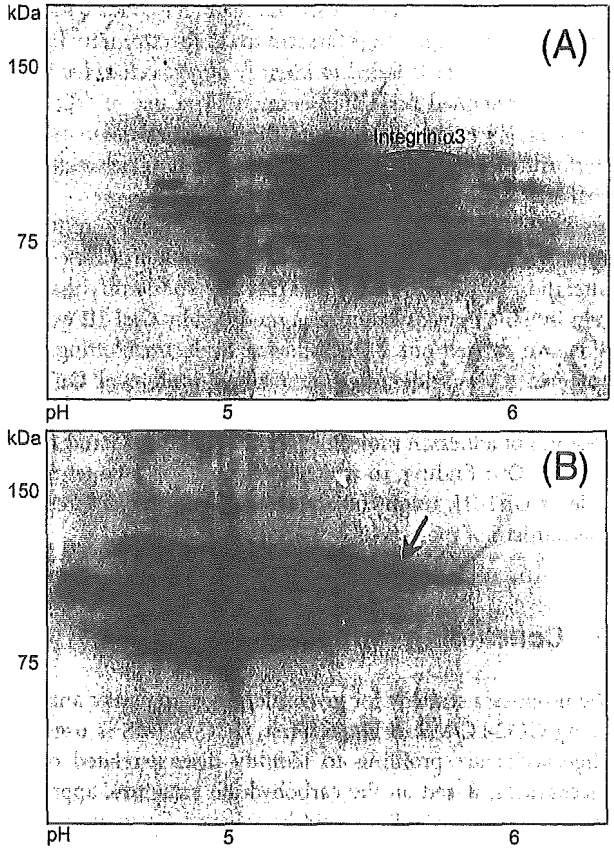

Figure 5. Enlarged partial (A) 2-DE and (B) lectin blot images from band A in Fig. 4.

still remains challenges. Positional isomers could be differentiated by multiple-stage tandem mass spectrometry (MS^n); however, MS^n itself failed to identify oligosaccharides if the sample contained positional isomers. The use of GCC-LC/MS enables us to differentiate the structural isomers and perform differential analysis in glycosylation between normal and aberrant cells.

GnT-III is reported to involve the suppression of tumor cell metastasis and is assumed to be a marker of cancerous alteration in hepatic carcinoma [21, 22]. To identify the protein in which glycosylation was modified by GnT-III expression, we carried out 2-DE followed by lectin blotting, and Integrin α3 was identified as a target protein of GnT-III. Integrin α3, a type I membrane protein, is known to be a receptor of adhesion molecules, such as laminin 5 and 10/11 [23–25]. Our finding, in which integrin α3 is a target molecule of GnT-III, might be a clue to clarify the suppression mechanism of metastasis by GnT-III.

5 Concluding remarks

We propose a strategy for glycomic/glycoproteomic analysis using GCC-LC/MS in Fig. 6. First, GCC-LC/MS is used for oligosaccharide profiling to identify disease-related oligosaccharides. Based on the carbohydrate structure, appropriate lectins or antibodies could be selected for western blotting. Proteins carrying disease-related oligosaccharides could then be identified by 2-D lectin blotting followed by MS/MS analysis. Using several groups, 2-D lectin blotting has been proposed for the characterization of glycoproteins on gel [26, 27]. The use of mass spectrometric oligosaccharide profiling, which can directly characterize glycan structures, is worthwhile to obtain structural information about disease-related carbohydrate and is helpful in the subsequent choice of appropriate lectins and antibodies. Our method is expected to be a powerful tool for glycomic/glycoproteomic analysis.

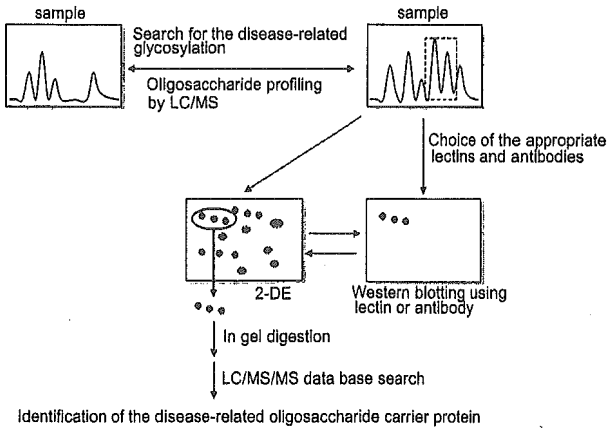

Figure 6. Strategy for glycomics/glycoproteomics using GCC-LC/MS, 2-DE and 2-D Western blotting using lectin or antibody.

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バイオロジクスのトランスレーショナルリサーチ（その1）

最近医薬品を含めた医療技術開発におけるトランスレーショナルリサーチ（TR）の重要性が叫ばれ，様々な学会等でトピックとして取り上げられている．我が国の医薬品開発環境の最も大きな問題点の一つは，臨床研究環境の未整備であることは衆目の一致するところであるが，取り上げられる機会が多い割には，TRの要点についての理解は必ずしも深まってはいないように思われる．そこで本稿ではバイオロジクス，特に筆者の専門とするバイオテクノロジー応用医薬品（バイオ医薬品）の開発を目指してTRを実施する上で考慮すべき点を，規制ガイドラインを参考に，2回に分けて考えてみる．

TRの本来の意味は，「探索的臨床研究＝基礎的研究成果を臨床へ導入するための臨床開発の初期段階のプロセス」であるが，今現在本来の意味のTRを行う上での条件等を定めた公的なガイドラインはない．しかしTRの対象を治験レベルまで広げると，その実施の条件，および注意点は既存の各種ガイドライン等から浮かび上がる．

バイオ医薬品の中で既に最も実用化が実現している医薬品は，遺伝子組換え技術や細胞培養技術を用いて製造されたタンパク質性医薬品（エリスロポエチン，インスリン類，成長ホルモン，インターフェロン，ヒトモノクローナル抗体等）であるが，我が国においてこれら医薬品開発にあたってまず参照すべきガイドラインは，(1)薬審第243号通知（昭和59年3月30日）「組換えDNA技術応用医薬品ガイドライン」；(2)薬審1第10号通知（昭和63年6月6日）「細胞培養技術応用医薬品ガイドライン」；(3)都道府県衛生主管部(局)薬務主管課宛事務連絡（平成元年5月）「薬審1第10号通知に関する質疑応答」である．しかしその後，医薬品の開発段階で考慮することが必要な技術的要件に関するICH国際調和ガイドラインが作成され，国内ガイドラインとして公表されている．これらは企業による新薬開発を対象としたものであるが，生体内タンパク質の医薬品への応用をめざしたトランスジェニックリサーチの実施の条件を考える上での参考となる．以下がこれらのガイドラインである：(1)「遺伝子発現構成体ガイドライン（厚生省医薬安全局審査管理課長通知 医薬審第3号 平成10年1月6日）」：主として遺伝子組換え技術を用いて医薬品製造用細胞を作製する場合の遺伝子発現構成体の設計，作製，細胞への導入，導入後の安定性チェックに関する注意点；(2)「細胞基材ガイドライン（厚生省医薬安全局審査管理課長通知 医薬審第873号 平成12年7月14日）」：バイオ医薬品の製造に使用する細胞基材の由来，調整，特性解析，管理に関する注意点；(3)「安定性ガイドライン（厚生省医薬安全局審査管理課長通知 医薬審第6号 平成10年1月6日）」：タンパク質性医薬品の安定性試験に関する注意点；(4)「ウィルス安全性ガイドライン（厚生省医薬安全局審査管理課長通知 医薬審第329号 平成12年2月22日）」：製品のウィルス汚染に関する配慮，試験に関する注意点；(5)「タンパク質性医薬品の特性解析・品質規格ガイドライン（厚生省医薬安全局審査管理課長通知 医薬審第571号 平成13年5月1日）」：医薬品の特性解析および品質規格設定にあたっての注意点；(6)「同等性・同質性ガイドライン（厚生労働省医薬食品局審査管理課長通知 薬食審発第0426001号 平成17年4月26日）」：製造工程の変更にともなう医薬品の同等性／同質性評価にあたっての注意点；(7)「非臨床安全性評価ガイドライン（厚生省医薬安全局審査管理課長通知 医薬審第326号 平成12年2月22日）」：臨床試験に先立つ非臨床安全性試験に関する注意点．

これらタンパク性医薬品を低分子化学合成医薬品と比較すると，(1)タンパク質の高次構造解析に限界があるため，構造の完全な同定，確認がしばしば困難であり，生物活性に基づく評価が重要；(2)製造の一定性を確保することが困難な細胞を利用して製造し，また翻訳後修飾等により分子多様性がある物質が多いので，物質の一定性の確保が重要；(3)常温で不安定な物質が多く，実時間での安定性の確認および保存条件の確保が重要；(4)製造に生体由来原料を使用するので，感染症に対する配慮が重要；(5)品質確保のために遺伝子発現構成体，細胞基材，宿主由来不純物など製造工程管理が重要；(6)物質としては天然のタンパク質に近いので，薬理作用および作用メカニズムの予測は容易であるが，種差により動物を用いた非臨床試験が不適切な場合もある；(7)体内動態試験は方法論に限界がある場合がある；(8)ヒトタンパク質の場合，種差，抗原性等により，げっ歯類動物を用いた非臨床安全性試験の予測性に限界がある，といった特徴がある．とりわけバイオロジクスの場合は被験物質の一定性の確保は，いかなるTRにおいてもデータの信頼性をはかる上で極めて重要であり，大学等での研究では見逃されがちな点である．（次号に続く）

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Mass Spectrometry of Glycoproteins

糖タンパク質の質量分析

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Key Words: *MS, MS/MS, MS^n, glycoprotein, oligosaccharide, glycopeptide*

Abstract

This review presents mass spectrometric methods for glycoprotein identification; determination of glycosylation sites, structural elucidation of carbohydrates, and their applications to glycomics and proteomics.

要　約

本レビューでは、質量分析を用いた糖タンパク質同定、糖鎖結合位置の決定、糖鎖構造解析、及びグライコミクス・プロテオミクスへの応用について紹介する。

A. Introduction

Mass spectrometry (MS) has become a powerful tool for glycoprotein identification, and the determination of glycosylation sites, and structural features of carbohydrates, such as sequence, linkage and branching at each glycosylation site. Generally, the mass spectrometric characterization of glycoprotein involves the following steps: 1) fractionation of enzymatically or chemically liberated glycans followed by MS, and 2) fractionation of glycopeptides from proteolytic digests followed by MS. Here we present the MS of glycan and glycopeptides using the latest applications.

B. MS of Liberated Glycans

Matrix-assisted laser desorption/ ionization (MALDI) (1), and electrospray ionization (ESI) (2), which are soft ionization techniques, are often used for glycan molecular mass determination. MALDI has been used by preference for rapid microanalyses, however, it generates metastable ions and the consequent various fragmentations, including the depletion of terminal sialic acids (known as post-source decay, PSD) (3). Although ESI used to have a problem with sensitivity, the introduction of nanospray technology allows us to use ESI to analyze femtomole levels of glycans (4). To measure all types of glycans, including neutral glycans and sulfated or sialylated acidic glycans, we suggest mass spectrometric glycan analysis in both positive and negative ion modes.

MALDI and ESI are combined with several types of

A. 緒　言

質量分析 (MS) は、糖タンパク質の同定、糖鎖結合位置の決定、並びに各結合位置における糖鎖の配列、結合位置、及び分岐等を含む構造特性解析の有用な手段として利用されている。現在、MS を用いた糖鎖解析のアプローチとしては、1) 酵素的または化学的に切り出された糖鎖の分画と MS、及び 2) 糖タンパク質酵素消化物からの糖ペプチドの分画と MS、が一般的である。そこで、ここでは、遊離糖鎖と糖ペプチドの MS について、最近の分析例を取り上げながら解説する。

B. 遊離糖鎖の MS

糖鎖分子の分析に適したソフトイオン化法として、マトリクス支援レーザー脱離イオン化法 (MALDI) (1)、及びエレクトロスプレーイオン化法 (ESI) (2) がよく利用されている. MALDI は迅速分析及び微量分析に適しているが、準安定イオンが生成し、MS^1 スペクトル上にはシアル酸が脱離したイオンをはじめとする様々なフラグメントが検出される (ポストソース分解、PSD) (3)。ESI は感度上の問題が指摘されてきたが、現在ではナノスプレーの開発によって、フェムトモルレベルの糖鎖分析が可能となっている (4)。糖鎖には中性糖鎖だけでなく、シアル酸や硫酸基などが結合した酸性糖鎖が存在するので、未知試料を分析する際には、ポジティブ及びネガティブ両イオンモードで測定するのが望ましい。

MALDI や ESI は様々なアナライザー (分析計) と組み合

analyzer, such as quadrupole (Q) (5), quadrupole ion trap (IT) (6), time-of-flight (TOF) (7), and Fourier transform ion cyclotron resonance (FTICR) (8). Tandem mass spectrometers with various combinations of these analyzers have recently become available. Tandem mass spectrometry (MS/MS) is widely recognized as an effective means of structural elucidation, including molecular mass measurement by MS^1 and oligosaccharide sequencing by collision-induced dissociation (CID)-MS/MS (9–11). In particular, ITMS instruments are becoming popular for multistage tandem mass spectrometry (MS^n), which offers multiple precursor selections and CID experiments (12, 13).

Fig. 1A shows types of carbohydrate fragmentation by CID-MS/MS (14). In the positive ion mode the most common fragmentation involves cleavage of the glycosidic bond with retention of the glycosidic oxygen atom by species formed from the reducing end (Fig. 1B). Fragment ions generated by this cleavage are represented as B-ion (non-reducing end) and Y-ion (reducing end). The cleavage of carbon-carbon bonds of the sugar ring yields A-ion and X-ion. These cross ring fragments are often used as decisive ions in linkage determination (15, 16).

Fig. 2 shows the positive ion ESI-MS/MS and MS^3 spectra of pyridylaminated agalacto-triantennary oligosaccharide (I) (Figs. 2A, A') and bisected agalacto-biantennary oligosaccharide (II) (Figs. 2B, B'). These are positional isomers whose one GlcNAc is attached to either α1-3/6 Man or β1-4Man in the trimannosyl core and cannot

わせて利用されている。アナライザーには四重極型 (Q) (5)、イオントラップ型 (IT) (6)、飛行時間型 (TOF) (7)、及びフーリエ変換イオンサイクロトロン共鳴型 (FTICR) (8) MS 装置などが用いられている．現在では、これらの分析装置を様々に組み合わせたタンデム質量分析装置の利用が可能である．タンデム質量分析 (MS/MS) は、MS^1 による糖鎖の質量測定と、衝突誘起解離 (CID)-MS/MS による糖鎖の配列解析を同時に行うことができる (9–11)。特に ITMS 装置は、前駆イオンの選択と CID-MS/MS を繰り返す多段階 MS/MS (MS^n) が可能であることから、近年、糖鎖解析用装置としての人気が高い (12、13)。

図 1A は、CID-MS/MS における糖鎖の開裂を示したものである (14)。ポジティブイオンモードで測定した糖鎖の CID-MS/MS では主に、グリコシド結合の酸素原子を還元末端側糖鎖に残した開裂が生じる (図 1B)．そのとき生じた非還元末端側イオンは B イオン、還元末端側イオンは Y イオンと呼ばれ、糖鎖の配列解析に利用される。ピラノース環の炭素 - 炭素間の結合が開裂して生じたイオンは A、及び X イオンと呼ばれ、糖鎖結合位置の決め手となることがある (15、16)。

図 2 は、ピリジルアミノ化されたアガラクト 3 本鎖糖鎖 (I) 及びアガラクトバイセクト 2 本鎖糖鎖 (II) のポジティブイオン MS/MS (図 2A、B)、及び MS^3 (図 2A'、B') スペクトルである．これらの糖鎖は GlcNAc 1 分子がトリマンノシルコアの α1-3/6Man または β1-4Man に結合した位置異性体で、MS^1 による分子量測定では区別することはできない。しかし、MS^n によっ

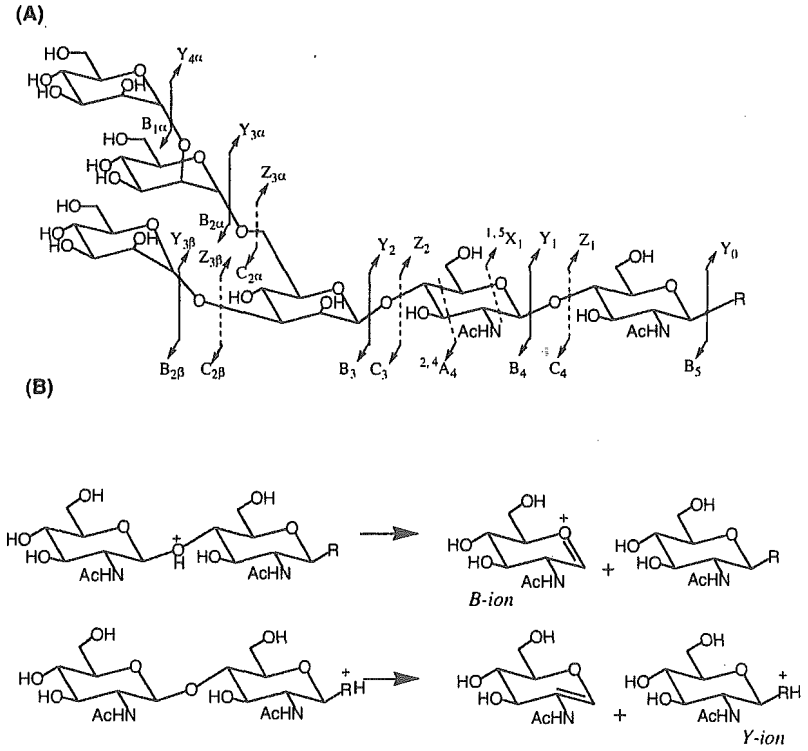

Fig. 1. (A) Types of carbohydrate fragmentation. (B) Production of B- and Y-ions in the positive ion mode.

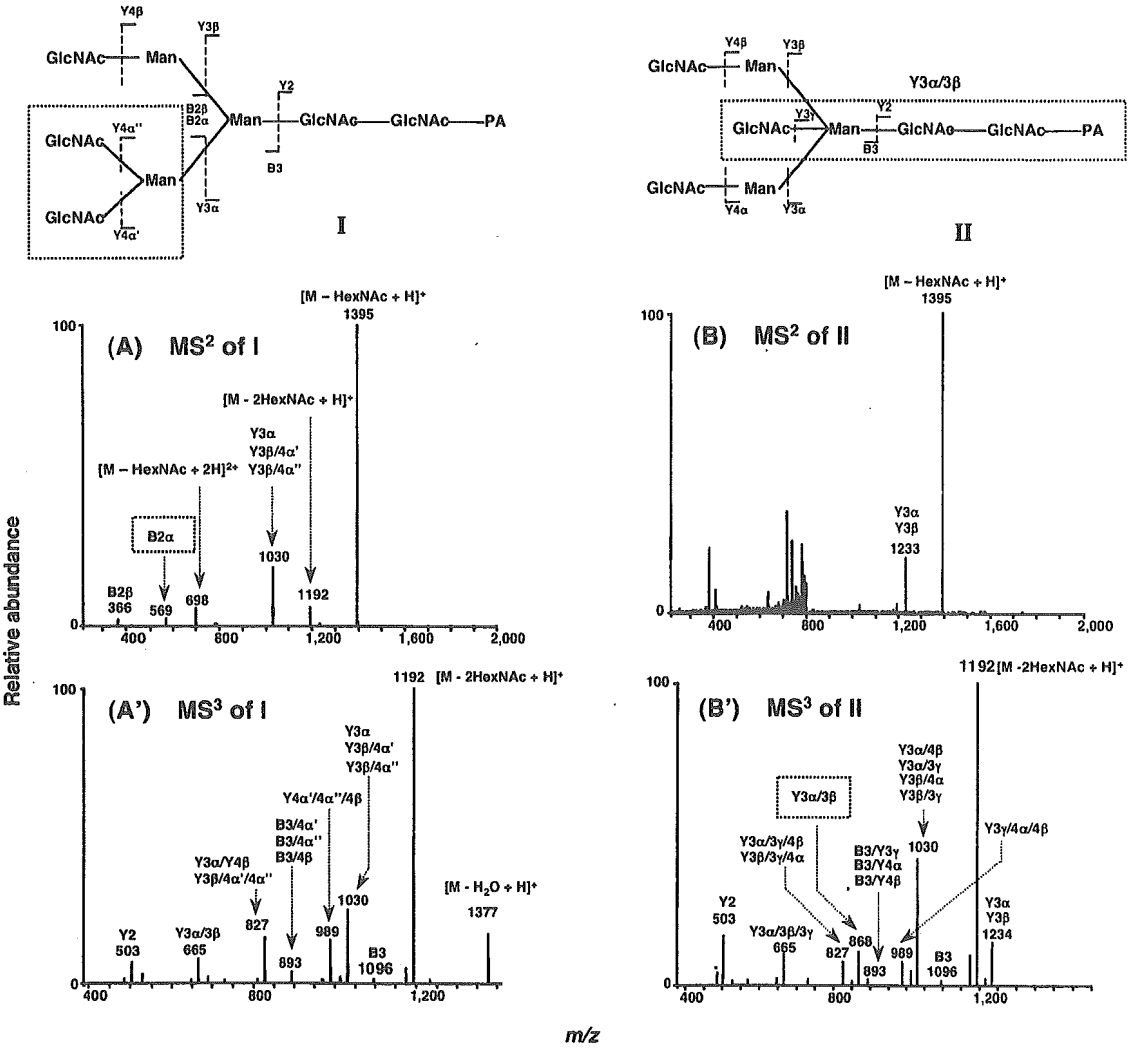

Fig. 2. MS^n spectra of oligosaccharides. (A) MS/MS spectrum of oligosaccharide I (precursor ion: *m/z* 800.8). (A') MS^3 spectrum of oligosaccharide I (precursor ion: *m/z* 1,395.4). (B) MS/MS spectrum of oligosaccharide II (precursor ion: *m/z* 800.5). (B') MS^3 spectrum of oligosaccharide II (precursor ion: *m/z* 1,395.4). MS: LTQ (Thermo Electron).

be discriminated by MS^1 (molecular mass determination). In contrast, MS^n spectra clearly show structural differences between two glycans. B-ion corresponding to $GlcNAc_2Man^+$ is observed at *m/z* 569 in the MS/MS spectrum of glycan I (Fig. 2A), while Y-ion at *m/z* 868 corresponding to [GlcNAc-Man-GlcNAc-GlcNAc-PA + H]$^+$ is detected in MS^3 spectrum of glycan II (Fig. 2B'). In particular, glycan II can be determined as bisected oligosaccharides on the basis of a bisected *N*-glycan diagnostic ion at *m/z* 868.

The MS^n spectra of some glycans exhibit characteristic fragment patterns. Hence, even if no diagnostic ion, such as a bisected glycan-specific fragment, is detected, the glycan structure can be sometimes deduced from ion intensity ratios obtained by MS^n (17,18). For instance, structures of branched arms were deduced from mass spectrometric patterns obtained by MS/MS which causes a cleavage of α1-3 linkage more than α1-6 linkage (19–21). However, complete structural

て両者の構造の違いを明確にすることができる。例えば、糖鎖 I の MS/MS スペクトルには *m/z* 569 に $GlcNAc_2Man^+$ に相当する B イオンが検出され（図 2A）、糖鎖 II の MS^3 スペクトル（前駆イオン：*m/z* 1395 [M-HexNAc + H]$^+$）には [GlcNAc-Man-GlcNAc-GlcNAc-PA + H]$^+$ に相当するイオンが *m/z* 868 に検出されている（図 2B'）。特に、[GlcNAc-Man-GlcNAc-GlcNAc-PA + H]$^+$ (*m/z* 868) はバイセクト糖鎖に特異的なフラグメントであり、糖鎖 II がバイセクト糖鎖であることを決定づけている。

糖鎖の MS^n は特徴的なスペクトルパターンを示すことが多いので、構造特異的イオンが検出されない場合でも、MS^n スペクトルのパターンより糖鎖構造を推定できる場合がある (17,18)。例えば、トリマンノシルコアの α1-3 結合が α1-6 結合よりも開裂しやすいことを利用して、分岐構造を解析した例が報告されている (19–21)。しかし、MS 単独で糖鎖構造を完

elucidation by MS alone is still a great challenge, and additional experiments are required, such as exoglycosidase digestion (22), lectin affinity chromatography (23), and sugar mapping (17,24,25).

C. Glycan Profiling by LC/MS

Many different oligosaccharides are attached to a glycoprotein. The development of derivatization and separation techniques for glycans has been an important part of structural glycobiology (26–34). The derivatization of glycan with a hydrophobic molecule improves the ionization efficiency of hydrophilic glycans and offers higher sensitivity (31,35). A combination of various HPLC techniques with off-line MALDI-MS or on-line ESI-MS has been successful in oligosaccharide profiling as well as the elucidation of structural details (36–39). As an example, we present the mass spectrometric *N*-glycan profiling of CHO cells and cells transfected with *N*-acetylglucosaminyltransferase III, which catalyzes the addition of GlcNAc to β1-4Man in the trimannosyl core (Fig. 3) (40). Mass spectrometric analysis reveals the difference in glycosylation between two samples and the appearance of peaks with additional HexNAc (203 Da) in the transfected cells.

We recently demonstrated quantitative oligosaccharide

全に同定することが困難である場合が多く、MSはエキソグリコシダーゼによる段階的消化法(22)、レクチンアフィニティークロマトグラフィー(23)、及び糖鎖マッピング(17,24,25)などと組み合わせて用いられることが多い。

C. LC/MSを用いた糖鎖プロファイリング

糖タンパク質には様々な糖鎖が結合しているので、糖鎖の標識法と分離技術の開発は、構造糖鎖生物学において重要な位置を占めている(26–34)。糖鎖を分離・検出するために開発された疎水性物質による誘導体化は、親水性の高い糖鎖のイオン化効率を向上させるので、MSにおける高感度化においても有用である(31,35)。さらに、様々なHPLCによる分離法とオフラインMALDI-MS、あるいはオンラインESI-MSを組み合わせた分析方法は、糖鎖プロファイリングと糖鎖構造解析を兼ね備えた方法として利用され、多くの成果を上げている(36–39)。一例として図3にCHO細胞、及びトリマンノシルコアのβ1-4ManにGlcNAcを付加させる*N*-アセチルグルコサミン転移酵素III(GnT-III)遺伝子を導入したCHO細胞のMSを用いた糖鎖プロファイリングの結果を示す(40)。MSを利用することによって、GnT-III導入細胞で新たに出現した糖鎖は、HexNAc1分子(203Da)増加した糖鎖であることが確認できる。

筆者らは最近、安定同位体標識化2-アミノピリジン(AP)を用いた標識法とLC/MSを組み合わせた定量的糖鎖プロファ

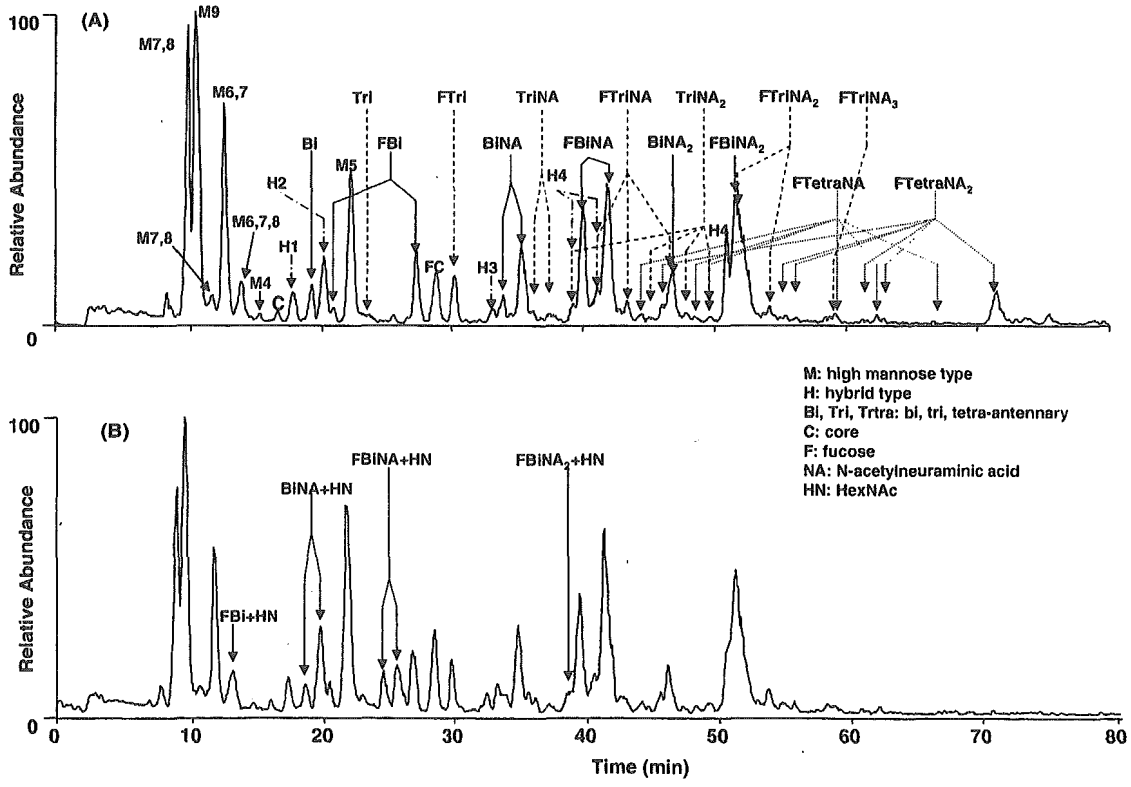

Fig. 3. TICs of *N*-linked oligosaccharides released from the insoluble fractions. (A) CHO cells. (B) *N*-acetyl glucosaminyltransferase III-transfected CHO cells. Column: Hypercarb (0.2 × 150 mm, Thermo Electron), LC: Magic 2002 (Michrome BioResources), MS: TSQ-7000 (Thermo Electron), Eluent: 5 mM ammonium acetate containing acetonitrile.

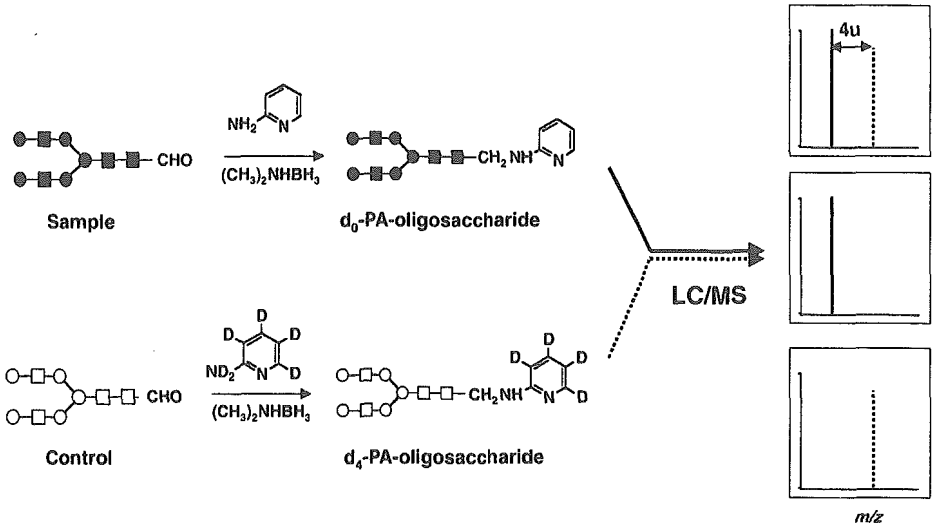

Fig. 4. Quantitative oligosaccharide profiling using LC/MS and an isotope tagging method.

profiling using isotope-labeled 2-aminopyridine (AP) and LC/MS (Fig. 4) (41). In this procedure, oligosaccharides released from an analyte and standard glycoprotein are tagged with d_0-AP and d_6-AP, respectively, and an equal amount of d_0-PA and d_4-PA oligosaccharides is analyzed by LC/MS. Oligosaccharides existing in either the analyte or standard glycoprotein appear as single ions, and oligosaccharides that exist in both analyte and standard glycoprotein are detected as paired ions with a difference of 4 u. The relative amount of analyte oligosaccharides can be determined on the basis of the analyte/ internal standard ion pair intensity ratio. This method can improve the precision of the mass spectrometric quantification and be used for glycan differential analysis among multiple samples.

D. MS of Glycopeptides

When glycans are released from protein, all information about their attachment to the protein is lost. Protein identification, determination of glycosylation sites and site-specific glycosylation analyses are generally achieved by the mass spectrometric analysis of glycopeptides. ESI and MALDI coupled with several analyzers are employed for glycopeptide analysis. ESI allows the accurate mass measurement of relatively large glycopeptide/protein because of the generation of multiple charged ions, whereas ions of large glycopeptide molecule ions are sometimes missed by MALDI-MS due to PSD and their poor ionization efficiency (42).

Fig. 5 illustrates three possible cleavages of the peptide backbone. The most common C-terminal and N-terminal fragment ions are b-ion and y-ion, respectively (37). In many cases, CID induces glycosidic bond cleavages rather than peptide backbones. Electron capture dissociation with FT-ICRMS is reported as a means for preferential cleavage of the

イリングを開発した(図4)(41)。この方法は、標準糖タンパク質及び検体糖タンパク質から切り出した糖鎖をそれぞれ6重水素置換AP(d_6-AP)、及び未置換AP (d_0-AP)で標識し、得られたd_4-PA糖鎖及びd_0-PA糖鎖を1対1の混合物としてLC/MSで分析するものである。標準糖タンパク質または検体糖タンパク質のどちらか一方にしか結合していない糖鎖は、d_4-PA糖鎖またはd_0-PA糖鎖どちらかの単独イオンとして検出される。標準糖タンパク質及び検体糖タンパク質に共通して存在する糖鎖は4u異なる1対のイオンとして検出され、d_4-PA糖鎖及びd_0-PA糖鎖のイオン強度比から相対糖鎖結合量を求めることができる。同位体標識糖鎖を内部標準物質として用いることによって、MSを用いた定量解析における再現性が改良されるので、定量的糖鎖プロファイリングは、複数のサンプル間の糖鎖の差異を質的量的に比較する場合に有用である。

D. 糖ペプチドのMS

タンパク質から糖鎖を切り離すと、糖鎖とタンパク質間の結合に関する情報が失われてしまうので、糖鎖含有タンパク質の同定、糖鎖結合位置の決定、及び部位特異的糖鎖不均一性の解析などには糖ペプチドのMSが適している。糖ペプチドの分析においても、ESIあるいはMALDIに様々なアナライザーを組み合わせた装置が利用されている。MALDI-MSでは、ほぼ一価イオンが生成するが、ESI-MSでは多価イオンが生成するため比較的高分子量の糖ペプチドの質量を正確に測定することが可能である。また、MALDI-MSを用いて糖鎖の割合が高い糖ペプチドを分析する場合、イオン化の抑制やPSDによって、分子関連イオンが測定されにくいとする報告がある(42)。

図5はペプチド骨格の開裂を示している。ペプチド結合の開裂によって生じたN末端側はbイオン、C末端側はyイオンと呼ばれる(43)。通常、糖ペプチドのCID-MS/MSでは、ペプチドよりも糖鎖の開裂が優先される。ペプチド部分が優先的に開裂する方法としてFT-ICRMS装置を用いた電子捕獲解

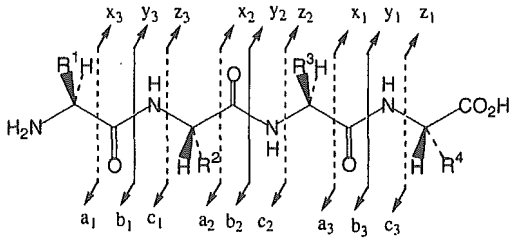

Fig. 5. Types of peptide fragmentation.

peptide backbone (44,45).

For the cleavages of both glycosidic and peptide bonds CID-MS/MS is carried out with relatively high energy (approx. 50V/1000 u) (46–51). Hence, it allows both peptide sequencing and estimation of the carbohydrate structure on the basis of b/y-ions and B/Y-ions. Fig. 6A presents the CID-MS/MS spectrum of a glycopeptide derived from human α-fetoprotein using the ESI-QqTOFMS instrument. Carbohydrate-specific B-ions, such as $HexNAc^+$ and $NeuAc^+$ are observed at *m/z* 204, 292 as well as y-series ions. Based on the y-series ions and peptide ion, this peptide is identified

糖鎖とペプチド部分を同時に開裂させるためには比較的高いエネルギー(約50V/1000 u)を与えてCID-MS/MSを行う(46–51)。生成したb、yイオン、及びB、Yイオンから、ペプチド部分のアミノ酸配列や、糖鎖構造を解析することができる。図6Aは、ESI-四重極飛行時間型MSを用いたCID-MS/MSによって得られたヒトアルファフェトプロテイン由来糖ペプチドのMS/MSスペクトルである。糖鎖Bイオンである*m/z* 204 ($HexNAc^+$)、及び292 ($NeuAc^+$)等と一緒に、ペプチドに由来する一連のyイオンが検出されていることがわかる。これら一

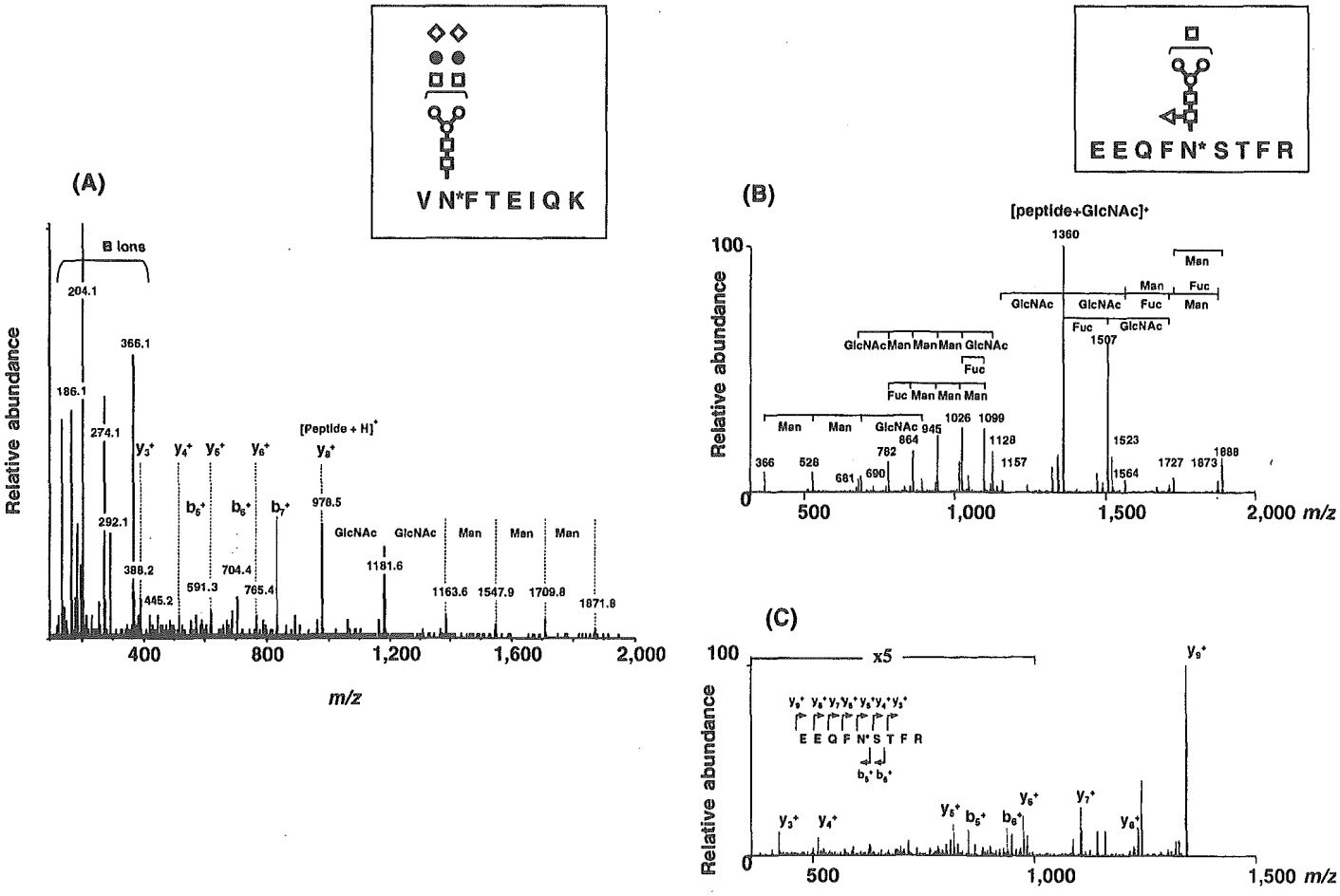

Fig. 6. CID-MS^n spectra of glycopeptides. (A) CID-MS/MS with relatively high energy (precursor ion, *m/z* 1,061.8), Sample: glycopeptide from human alpha-fetoprotein, MS: QSTAR (Applied Biosystems). (B) Low-energy CID-MS/MS (precursor ion, *m/z* 1,200). (C) Low-energy CID-MS^3 (precursor ion, *m/z* 1,360), Sample: glycopeptide from mouse IgG1, MS: LTQ (Thermo Electron).

as VNFTEIQK. The oligosaccharide structure can be deduced as disialylated biantennary from the molecular mass of the carbohydrate moiety together with B-ions in the MS/MS spectrum.

B- and Y-series ions are produced by the low-energy CID-MS/MS of glycopeptides (12, 52–55). Fig. 6B shows the low energy (approx. 5–20V/1000 u) CID-MS/MS spectrum of glycopeptide derived from mouse IgG1. Based on the carbohydrate related-ions, the carbohydrate structure could be determined as fucosylated biantennary. When using an ITMS instrument, MS^3 is automatically carried out for intense ions. In this experiment, $[peptide + HexNAc + H]^+$, which is generally detected as intense ion, was subjected to further product ion scan, and b- and y-series ions appeared in the MS^3 spectrum (Fig. 6C). Peptides can be identified by comparing experimental fragment ions with predictable fragment ions derived from proteins in a database. Moreover, the database analysis with the possibility of glycosylation at Asn and Ser/Thr with HexNAc, Hex, and dHex allows the identification of glycopeptides and glycosylation sites (56). For instance, this peptide was identified as EEQFN*STFR glycosylated with HexNAc at N*. This method would enable the glycosylation analysis of unknown glycoproteins and a mixture of glycoproteins.

E. Site-Specific Glycosylation Analysis of Glycoproteins

Fig. 7 illustrates the strategy for the site-specific glycosylation analysis of glycoproteins. First, a glycoprotein is digested with an appropriate proteinase, which provides

連のｙイオン、及びペプチドイオンの質量から、このペプチド部分はVNFTEIQKと同定することができる。また、糖鎖構造は、糖鎖部分の分子量とＢイオンからジシアロ２本鎖糖鎖と推定される。

低エネルギーCID-MS/MSでは、グリコシド結合が優先して開裂し、B、及びＹイオンが検出される(12,52–55)。図6Bはマウス IgG1から得られた糖ペプチドの低エネルギーCID-MS/MS(約5-20V/1000 u)スペクトルで、糖鎖関連イオンから、糖鎖構造を推定することができる。糖ペプチドのMS/MSでは一般に、[ペプチド+HexNAc+H]$^+$が比較的強く検出される。そこで、ITMS装置を用いて[ペプチド+HexNAc+H]$^+$を前駆イオンとして選択し、さらにプロダクトイオンスキャンを行うと、MS^3スペクトル上にｂ及びｙイオンが検出される(図6C)。これらのｂ及びｙイオンの実測値をタンパク質データベースに登録されているタンパク質の予測プロダクトイオンの理論質量と比較することによって、ペプチドを同定することができる．さらに、AsnやSer/ThrにHexNAc、Hex、あるいはdHex等による糖鎖修飾の可能性を追加してデータベース検索をすることによって、糖鎖結合位置を決定できる場合がある(56)。例えば、ここで分析された糖ペプチドはデータベース検索エンジンを用いて、EEQFN*STFR(N*はHexNAc修飾Asn)と同定された。この方法を利用することによって、混合物中の糖タンパク質や、未知の糖タンパク質の部位特異的な糖鎖解析が可能となるものと期待される。

E. 糖タンパク質の部位特異的糖鎖解析

糖タンパク質の部位特異的糖鎖解析の流れを図7にまと

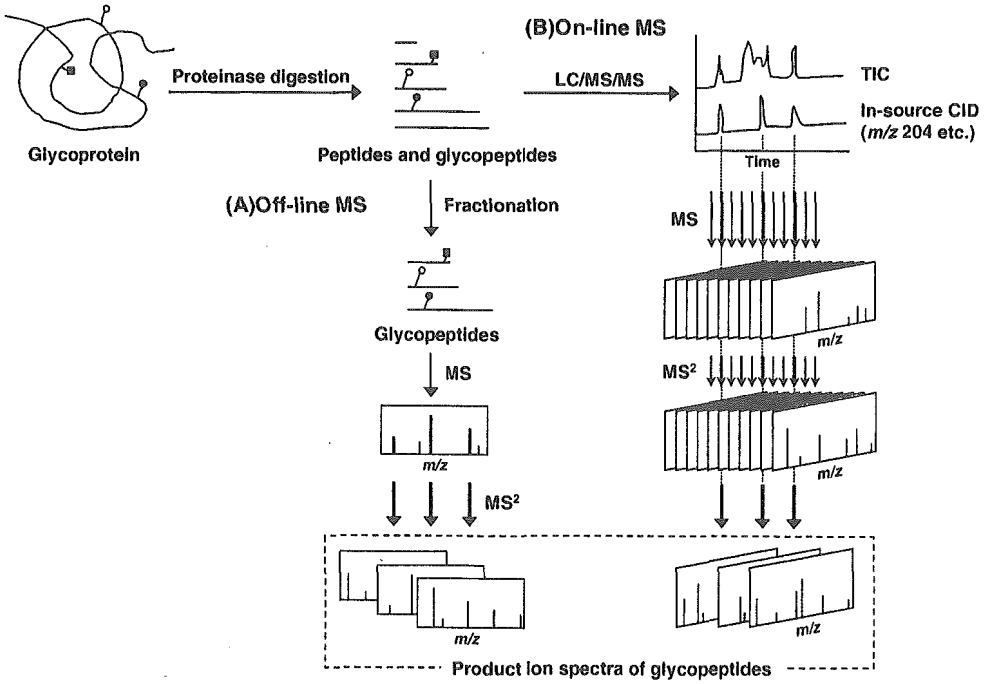

Fig. 7. Strategy for site-specific glycosylation analysis by LC off-line MS (A) and on-line LC/MS (B).

glycopeptides, including a single glycosylation site. Trypsin, Lys-C, Glu-C and Asp-N are commonly used for glycosylation analysis. Since glycopeptide ions are sometimes missed by MS in the presence of excess peptides due to their lower ionization efficiency, several methods have been proposed for the fractionation of glycopeptides, such as HPLC and affinity chromatography (57,58), followed by off-line MALDI-MS. LC on-line ESI/MS is an efficient method for the direct elucidation of glycopeptides in a complex mixture. Although LC/MS provide a complicated chromatogram, glycopeptides in a choromatographic position can be localized by the appearance of marker ions, such as $HexNAc^+$ and $HexHexNAc^+$, resulting from precursor ion scanning and in-source CID (59–66). However, for peptide identification these two means require additional CID-MS/MS scan for some intense ions (data-dependent CID-MS/MS scan). Recently we presented an alternative method, with which product ion spectra of glycopeptides can be selected directly using marker ions arising from glycopeptides by data-dependent CID-MS/MS scan with relatively high energy (49).

める。まず、糖タンパク質を適切な酵素で糖ペプチドに断片化する。この際、同一ペプチドに複数の糖鎖結合部位が含まれないように酵素を選択する。基質特異性の高いトリプシン、Lys-C、Glu-C、及びAsp-N等がよく用いられている。糖ペプチドはペプチドに比べてイオン化効率が悪く、ペプチドが混在するとマススペクトルが得られにくい。そのため、アフィニティークロマトグラフィーや、HPLC等で予め糖ペプチドを分画してから(57,58)、マススペクトルを測定するオフライン法(図7A)や、C18カラム等を用いたLC/MSでペプチド・糖ペプチドを分離しながら直接マススペクトルを測定するオンライン法がよく用いられている(図7B)。オンラインLC/MSでは複雑なクロマトグラムが得られるが、プリカーサーイオンスキャンやインソースCIDによって生じた糖鎖に特徴的なBイオン(例えば、$HexNAc^+$、*m/z* 204や$HexHexNAc^+$、*m/z* 366など)を利用することによって、糖ペプチドの溶出位置を推定することができる(59–66)。ただしこれらの方法は、ペプチドを同定するために、別途、強度の高いイオンに対する自動的CID-MS/MSスキャン(データ依存的CID-MS/MSスキャン)を行う必要がある。そこで、我々は別法として、データ依存的なCID-MS/MSによって生成したBイオンを利用して糖ペ

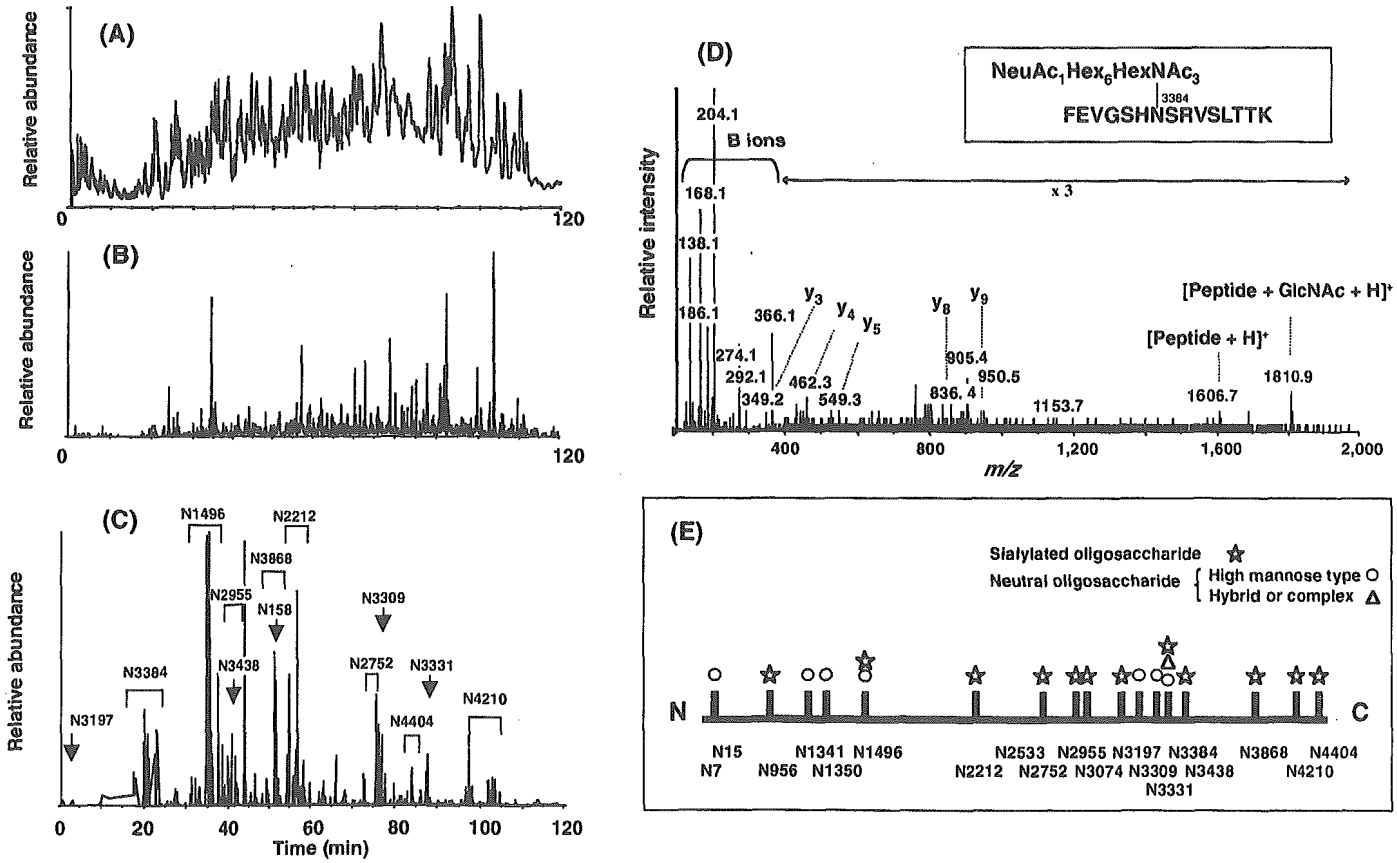

Fig. 8. Site-specific glycosylation analysis of apoB100 by LC/ESI-MS/MS. (A) TIC of full MS scan (*m/z* 400-2,000). (B) TIC of data-dependent CID-MS/MS scan (*m/z* 100-2,000). (C) Mass chromatogram at *m/z* 204 in data-dependent CID-MS/MS scan. (D) MS/MS spectrum of glycopeptide (precursor ion: *m/z* 1,160.4). (E) *N*-glycosylation of apoB100. Sample: tryptic digest of apoB100 (4 μg), LC: Paradigm (Michrome BioResources), Column: Magic C18 (0.2 × 50 mm, Michrome BioResources), MS: QSTAR (Applied Biosystems).

Here we demonstrate the site-specific glycosylation analysis of human apolipoprotein B100 (apoB100). Tryptic digest of apoB (4 μg) was injected into an LC/ESI-QqTOFMS instrument equipped with a C18 column. Figs. 8A and B are total ion chromatograms (TIC) obtained by full MS^1 scan and data-dependent CID-MS/MS scan, respectively (49). These chromatograms appear complicated due to a number of peptide-related ions derived from a large glycoprotein molecule of 500,000 Da. In order to localize glycopeptides in the peptide map, carbohydrate marker ion, $HexNAc^+$ at *m/z* 204, was extracted from the TIC of data-dependent CID-MS/MS scan (Fig. 8C). The MS/MS spectra of glycopeptides were then sorted from the peaks appearing in the mass chromatogram (Fig. 8D). We successfully identified 17 *N*-glycosylation sites among 19 potential *N*-glycosylation sites and deduced glycosylation at each glycosylation site from the mass of carbohydrate moieties (Fig. 8E).

F. Application in Proteomics and Glycomics

MS enables us to elucidate a small number of glycoproteins isolated by electrophoresis (67, 68) and micro HPLC (69). This method could be applied in comprehensive or carbohydrate structure-specific glycoprotein analysis by a combination with proteomic approaches such as 2-dimensional (2D) gel electrophoresis (GE) and 2D-LC followed by MS. Some applications have been already demonstrated, for instance, glycoprotein expression analysis using 2D-GE coupled with carbohydrate-specific dyeing or immunoblotting (70), and LC/MS combined with lectin affinity chromatography (71,72). These glycomic/proteomic technologies are expected to be a powerful tool for the functional study of glycoproteins, finding disease-related glycoproteins and identifying proteins attached to some glyco-epitopes.

プチドの MS/MS スペクトルを選び出す方法を見出した (49)。

図 8 に、我々が最近行ったオンライン法によるヒトアポリポプロテイン B100 (apoB100) の部位特異的な糖鎖解析例を示す (49)。apoB100 のトリプシン消化物 (4 μg) を C18 カラムを用いた LC/ESI-QqTOFMS 装置で分析した。図 8A 及び B はフル MS^1 スキャン (*m/z* 1,000-2,000) 及びデータ依存的 CID-MS/MS スキャンによって得られたトータルイオンクロマトグラム (TIC) である。apoB100 は分子量約 500,000 Da の大きな糖タンパク質なので、非常に多くのペプチドイオンが検出されている。そこで、糖ペプチドの溶出位置を推定するために、データ依存的 CID-MS/MS スキャンによって生じた *m/z* 204 イオンのみを抜き出した (図 8C)。出現したピーク周辺から B イオンを指標に糖ペプチドの MS/MS スペクトルを探し出し、それらのスペクトル上のプロダクトイオンを帰属した (図 8D)。その結果、19 カ所の推定 *N* 結合型糖鎖結合部位のうち 17 カ所に糖鎖が結合していることを明らかにするとともに、それぞれの部位に結合している糖鎖を推定することができた (図 8E)。

F. グライコミクス・プロテオミクスへの応用

現在では、電気泳動 (67、68) やミクロ液体クロマトグラフィー (69) で分離された僅かな糖タンパク質からでも、MS によって、多くの糖鎖構造情報を得ることができるようになった。これらの糖鎖解析技術とプロテオミクス的アプローチ、すなわち、2 次元電気泳動や 2 次元クロマトグラフィーによるタンパク質発現解析と MS を組み合わせることによって、糖タンパク質の網羅的解析や、任意の糖鎖構造を持つタンパク質の解析が可能になるものと期待されている。すでに、2 次元電気泳動と糖タンパク質特異的染色法や免疫ブロットを組み合わせた糖タンパク質発現解析や (70)、レクチンアフィニティークロマトグラフィーと各種 LC/MS を組み合わせた糖タンパク質の網羅的解析の例が報告されている (71、72)。今後、これらのグライコミクス・プロテオミクス解析技術が、糖鎖の機能研究や、疾患等に関与する糖鎖・糖タンパク質の探索、並びに様々な糖鎖エピトープ結合タンパク質の特定に貢献できるものと期待される。

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