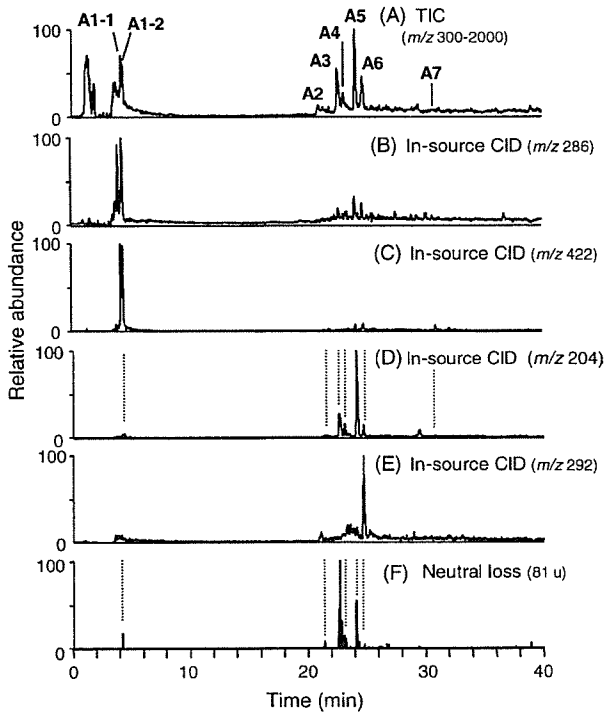

Fig. 6. Total ion chromatogram (TIC) of Asp-N digested protein at 20–25 kDa (*m/z* 300–2000) (A), mass chromatograms from TIC with in-source CID of *m/z* 286 (B), 422 (C), 204 (D), and 292 (E), and neutral loss chromatogram of 81 u by data-dependent CID-MS/MS (F).

ion spectra. Glycopeptides in peak T2 were characterized as Ala73-Lys78 glycosylated at Asn74 with *N*-glycans consisting of $dHex_{0-2}Hex_{3-6}HexNAc_{2-5}$. These *N*-glycans can be identified as high-mannose-type oligosaccharide (M5), and complex-type and hybrid-type oligosaccharides containing Fuc attached to inner trimannosyl core GlcNAc. Their structural assignments are summarized in Table 1. Glycopeptides in peak T3 can be identified as a mixture of peptide His21-His31 and His21-Glu32 glycosylated at Asn23, and Ser96-Asp106 glycosylated at Asn98. Asn23 was attached by high-mannose-type oligosaccharides, M5, 6, and 7, and Asn98 was occupied by *N*-glycan consisting of $dHex_1Hex_4HexNAc_4$ with a Lewis a/x structure as a partial structure. Glycopeptides in peak T5 were characterized as peptide His21-Phe33 glycosylated at Asn23 with high-mannose-type oligosaccharide, M6. Glycopeptides in peak T7 were assigned to be Val69-Lys78 glycosylated at Asn74 with *N*-glycans composed of $dHex_{1-2}Hex_{4-6}HexNAc_{3-6}NeuAc$.

3.4. Analysis of the GPI moiety of rat Thy-1

Since trypsin digestion provided Cys-GPI, which could not be retained on the C_{18} column, Asp-N digestion was also performed to obtain more hydrophobic peptides attached by GPI (GPI-peptides). Fig. 6(A) shows the peptide/glycopeptide map obtained by LC/ITMS of Asp-N digested Thy-1. We localize the GPI-peptides using marker ions, EtN-PO_4-Man$^+$ at *m/z* 286 and GlcN-inositol-PO_4^+ at *m/z* 422, originating from the core structure of the GPI moiety by in-source CID (EtN, ethanolamine; GlcN, glucosamine). Mass chromatograms of *m/z* 286 and 422 suggest the locations of the GPI-peptides to be around 4.2 (peak A1-1) and 4.4 min (peak A1-2) (Fig. 6(B and C)). Using product ions originated from GPI moiety, such as GlcN-inositol-PO_4^+ and PO_4-Man-GlcN$^+$ (*m/z* 422 and 404), as marker ions, four product ion spectra of GPI-peptides were sorted out from all product ion spectra around peaks A1-1 and 1-2. Their precursor ions were doubly charged ions at *m/z* 1132 and 1213 (peak A1-1), 1051 and 1151 (peak A1-2). Based on these product ion spectra, we characterized GPI-peptides as the peptide Asp106-Cys111 with a GPI core structure plus Hex_{0-2}, $HexNAc_{1-2}$ and PO_4-EtN.

Fig. 7(A) shows the product ion spectrum of the doubly charged GPI-peptide ion at *m/z* 1051 in peak A1-2. In addition to product ions at *m/z* 422, those originating from the GPI moiety were detected at *m/z* 404 (PO_4-Man-GlcN$^+$), 447 (EtN-PO_4-Man-GlcN$^+$), 650 (EtN-PO_4-(HexNAc-)Man-GlcN$^+$), 787 (peptide-EtN$^+$), 868 (peptide-EtN-PO_4^+), 1191 (peptide-EtN-PO_4-Man-Man$^+$), 1477 (peptide-EtN-PO_4-Man-Man-(EtN-PO_4-)Man$^+$), 1638 (peptide-EtN-PO_4-Man-Man-(EtN-PO_4-)Man-GlcN$^+$), and 1898 (peptide-EtN-PO_4-Man-Man-(EtN-PO_4-)Man-GlcN-inositol-PO_4^+). From these fragments, it can be deduced that this peptide is Asp106-Cys111 carrying the GPI, as indicated in the inset in Fig. 7(A).

The other GPI-peptide in peak A1-1 was characterized as having side chains; -Hex attached to M1, -PO_4-EtN and -HexNAc attached to M3, based on the product ion spectrum of the doubly charged precursor ion at *m/z* 1132 (data not shown). These two GPI structures are identical to those that have been previously reported [24].

Product ion spectra of doubly charged ion at *m/z* 1151 and 1213 suggested that they contained GPI which bear one HexNAc or two Hex in addition to GPI in Fig. 7(A) respectively. Fig. 7(B) shows the product ion spectra of the doubly charged precursor ions at *m/z* 1151 in peak A1-2. In addition to *m/z* 422, we detected product ions at *m/z* 366 (HexNAc-Man$^+$), 447 (EtN-PO_4-Man-GlcN$^+$), 650 (EtN-PO_4-(HexNAc-)Man-GlcN$^+$), 1229 (peptide-EtN-PO_4-(HexNAc-)Man$^+$), 1391 (peptide-EtN-PO_4-(HexNAc-)Man-Man$^+$), 1676 (peptide-EtN-PO_4-(HexNAc-)Man-Man-(EtN-PO_4-)Man$^+$), 1838 (peptide-EtN-PO_4-(HexNAc-)Man-Man-(EtN-PO_4-)Man-GlcN$^+$), and 1880 (peptide-EtN-PO_4-(HexNAc-)Man-Man-(EtN-PO_4-)(HexNAc-)Man$^+$). These fragment ions suggest the attachment of -HexNAc to Man1, and -PO_4-EtN and -HexNAc to Man3 as indicated in the inset of Fig. 7(B). Similarly, product ion spectra of the doubly charged precursor ion at *m/z* 1213 indicate the attachment of 2Hex and HexNAc to Man1 and Man3-PO_4-EtN (data not shown). To our knowledge, this is the first report of these two GPI structures in Thy-1.

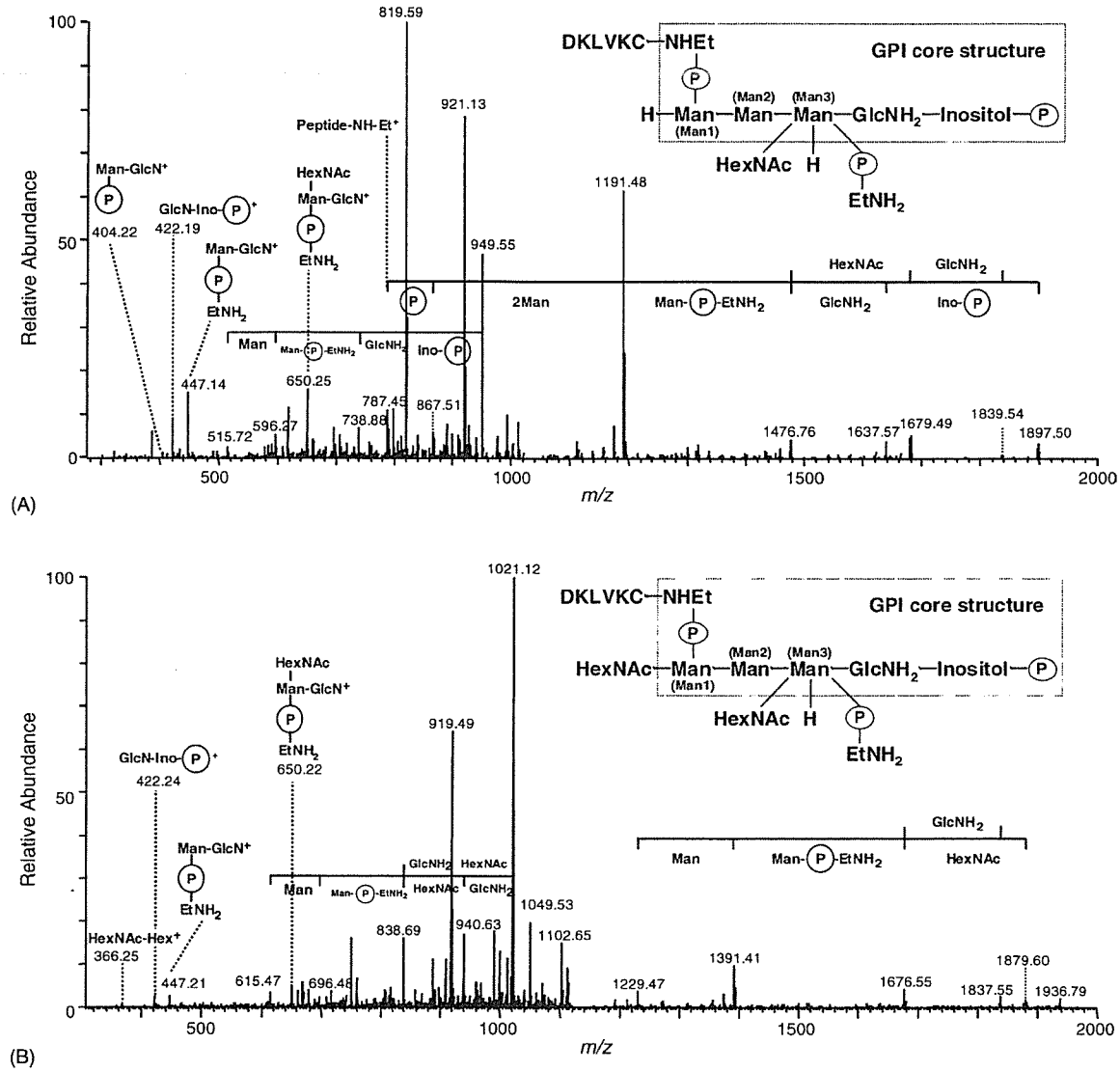

Fig. 7. Product ion spectra of the doubly charged GPI-peptide at *m/z* 1051 (A), and at *m/z* 1151 (B) in peak A1-2. The inset is the deduced structure of the GPI-peptide, and the core structure of GPI is the inside dashed line. Man, mannose; HexNAc, *N*-acetylhexosamine; $GlcNH_2$, glucosamine; $EtNH_2$-P, phosphorylethanolamine; Ino-P, inositol-phosphate.

3.5. *Analysis of Asp-N digested Thy-1*

Glycopeptides obtained by Asp-N digestion were also localized by in-source CID using marker ions at *m/z* 204 and 292 (Fig. 6(D and E)), and neutral loss of 81 u by data-dependent CID-MS/MS (Fig. 6(F)). Product ion spectra of glycopeptides were sorted by using B series ions as marker ions from those acquired around localized elution positions. Consequently, peaks A2-7 were identified as those of glycopeptides (Fig. 6(A)). The oligosaccharide structures in the glycopeptides were then characterized based on their product ion spectra (Table 1). In addition to the high-mannose-type oligosaccharides, M5, 6, and 7 deduced by LC/MS^n of tryptic digests, the oligosaccharide at Asn23 was characterized as $dHex_{0-1}Hex_{3,5,6}HexNAc_{3-5}NeuAc_{0,1}$, complex-type and hybrid-type oligosaccharides containing Lewis a/x or bisecting GlcNAc as a partial structure. Asn74 is attached by *N*-glycans with $dHex_{0-2}Hex_{3-6}HexNAc_{2,4,5}NeuAc_{0,1}$. They were high-mannose-type oligosaccharide, M5, complex-type oligosaccharides containing core Fuc and Lewis a/x as a partial structure, and hybrid-type oligosaccharides with core Fuc. Asn98 is occupied by high-mannose-type oligosaccharides, M5, and *N*-glycans with $dHex_{0-2}Hex_{3,5,6}HexNAc_{2-5}NeuAc_{0,1}$, hybrid-type oligosaccharides containing Lewis a/x or blood group H-determinant as a partial structure, which were found to be of greater diversity than those deduced by analysis of tryptic digests.

4. Discussion

In the present study, we have developed an efficient and convenient strategy for characterization, including protein identification and glycosylation analysis, of a small amount of unknown protein. We used gel electrophoresis, which is a powerful tool for separation of a small amount of protein from complex proteins mixture, especially from insoluble membrane fractions. For the complete glycosylation analysis, we examined the extraction of a whole glycoprotein from the gel, followed by trypsin digestion. Additionally, for the effective glycopeptide analysis, we studied mass spectrometric peptide/glycopeptide mapping by LC/MS^n with in-source CID and data-dependent MS^n. The glycopeptides were localized in the peptide/glycopeptide map by using oxonium ions as marker ions such as $HexNAc^+$ and $NeuAc^+$, which were generated by in-source CID, and neutral loss by data-dependent CID-MS/MS. For simultaneous identification of both peptides and glycopeptides, we conducted the database search analysis using search parameters containing a possible glycosylation at Asn with GlcNAc (203 Da). We successfully determined the sequences of peptides and some of the glycopeptides, which were localized by in-source CID and data-dependent CID-MS^n. The database search analysis using these search parameters was useful for identifying the glycopeptides resulting from predictable proteinase digestion. Glycopeptides caused by irregular digestion could be identified by assignment of peptide b and y series ions, which arose from further MS^n. The oligosaccharide structures of the identified glycopeptides were characterized on the basis of their product ion spectra. In this way, we were able to isolate rat brain Thy-1 and to elucidate *N*-glycosylation at Asn23, 74, and 98 as well as the structure of the GPIs at Cys111.

Post-translationally modified peptides could not be identified by the database search analysis. It has been particularly difficult to identify glycopeptides by database search analysis due to their complicated product ions resulting from the cleavage of glycosidic bonds. It has recently been reported that peptide + GlcNAc ion generated from a glycopeptide by CID-MS/MS yields b and y series ions by further MS^n, and that these ions can be utilized for identification of the peptide backbone and its glycosylation site [15,16,18]. Additionally, search analysis using the database including the possibility of glycosylation at Asn with all possible cleavage products of the known glycopeptides can be utilized for identification of glycopeptides in the peptide/glycopeptide map [19]. This ability would be helpful in the identification of glycoproteins whose glycosylation are already known. In the present study, we carried out a database search analysis using search parameters containing a possible glycosylation at Asn with only GlcNAc (203 Da), and successfully identified an unknown glycoprotein and *N*-glycosylated sites. This search analysis can be used for the identification of *O*-glycosylation, which has no consensus amino acid sequence, by using search parameters containing possible glycosylations at Ser/Thr with Hex, HexNAc, and dHex.

Precursor ion scans have been used for the localization the glycopeptides in peptide/glycopeptide mapping [10,11,13]. Although this method can be used for monitoring the peptides with predictable modification by setting mass of fragment ions prior to scanning, peptides with unpredictable modification cannot be detected. In contrast, in-source CID and CID-MS/MS are capable of localizing of the modified peptides after just one data acquisition using objective oxonium ions and neutral losses. In the present study, we were able to localize GPI-peptides in the peptide/glycopeptide map using EtN-PO_4-Man^+ and GlcN-Inositol-PO_4^+ generated by in-source CID [25] and to elucidate the GPI structures. We also could localize the glycopeptides with dHex, HexNAc, and NeuAc at the non-reducing ends as well as Hex using neutral loss by CID-MS/MS.

Site-specific glycosylation analysis of rat brain Thy-1 was performed after purification with monoclonal antibody affinity chromatography. Released oligosaccharides from fractionated trypsin-digested glycopeptides were analyzed by conventional analytical methods, including exoglycosidase digestion and methylation analysis [26]. In the present study, we separated PIPLC-treated GPI-anchored proteins of rat brain by SDS-PAGE, and conducted site-specific glycosylation analysis by LC/MS^n. Using a simpler step, we could elucidate the glycosylation at each glycosylation site with a greater variety of oligosaccharides than that reported previously and four GPI structures, including two novel attached structures.

Our strategy presented herein can relatively simply facilitate complete site-specific glycosylation analysis that used to require a series of complicated steps and is applicable to characterization of unknown proteins on 2-DE gel in proteomic study. Even in a mixture of multiple unknown glycoproteins, glycosylation of each glycoprotein can be determined based on the product ion spectra. Our method would be helpful for study of the alternation of glycosylation with growth, aging, and disease [27,28].

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References

[1] A. Varki, Glycobiology 3 (1993) 97.
[2] H. Sasaki, B. Bothner, A. Dell, M. Fukuda, J. Biol. Chem. 262 (1987) 12059.
[3] F. Wang, A. Nakouzi, R.H. Angeletti, A. Casadevall, Anal. Biochem. 314 (2003) 266.
[4] K. Hirayama, R. Yuji, N. Yamada, K. Kato, Y. Arata, I. Shimada, Anal. Chem. 70 (1998) 2718.
[5] M. Ohta, N. Kawasaki, S. Itoh, T. Hayakawa, Biologicals 30 (2002) 235.
[6] E. Mortz, T. Sareneva, S. Haebel, I. Julkunen, P. Roepstorff, Electrophoresis 17 (1996) 925.
[7] F.G. Hanisch, M. Jovanovic, J. Peter-Katalinic, Anal. Biochem. 290 (2001) 47.
[8] D. von Witzendorff, M. Ekhlasi-Hundrieser, Z. Dostalova, M. Resch, D. Rath, H.W. Michelmann, E. Topfer-Petersen, Glycobiology 15 (2005) 475.
[9] B. Küster, T.N. Krogh, E. Mortz, D.J. Harvey, Proteomics 1 (2001) 350.
[10] S.A. Carr, M.J. Huddleston, M.F. Bean, Protein Sci. 2 (1993) 183.
[11] M.J. Huddleston, M.F. Bean, S.A. Carr, Anal. Chem. 65 (1993) 877.
[12] R.S. Annan, S.A. Carr, J. Protein Chem. 16 (1997) 391.
[13] K. Sandra, I. Stals, P. Sandra, M. Claeyssens, J. Van Beeumen, B. Devreese, J. Chromatogr. A 1058 (2004) 263.
[14] A. Harazono, N. Kawasaki, T. Kawanishi, T. Hayakawa, Glycobiology 15 (2005) 447.
[15] U.M. Demelbauer, M. Zehl, A. Plematl, G. Allmaier, A. Rizzi, Rapid Commun. Mass Spectrom. 18 (2004) 1575.
[16] Y. Wada, M. Tajiri, S. Yoshida, Anal. Chem. 76 (2004) 6560.
[17] B. Sullivan, T.A. Addona, S.A. Carr, Anal. Chem. 76 (2004) 3112.
[18] S. Zhang, D. Chelius, J. Biomol. Tech. 15 (2004) 120.
[19] S. Wu, P. Bondarenko, T. Shaler, P. Shieh, W. Hancock, Thermo Finnigan LC/MS" Application Report Application Report No. 300.
[20] C. Bordier, J. Biol. Chem. 256 (1981) 1604.
[21] M.P. Lisanti, M. Sargiacomo, L. Graeve, A.R. Saltiel, E. Rodriguez-Boulan, Proc. Natl. Acad. Sci. U.S.A. 85 (1988) 9557.
[22] S. Itoh, N. Kawasaki, M. Ohta, T. Hayakawa, J. Chromatogr. A 978 (2002) 141.
[23] B. Domon, C.E. Costello, J. Glycoconjugate 5 (1988) 397.
[24] S.W. Homans, M.A. Ferguson, R.A. Dwek, T.W. Rademacher, R. Anand, A.F. Williams, Nature 333 (1988) 269.
[25] K. Fukushima, Y. Ikehara, M. Kanai, N. Kochibe, M. Kuroki, K. Yamashita, J. Biol. Chem. 278 (2003) 36296.
[26] R.B. Parekh, A.G. Tse, R.A. Dwek, A.F. Williams, T.W. Rademacher, EMBO J. 6 (1987) 1233.
[27] Y. Sato, M. Kimura, C. Yasuda, Y. Nakano, M. Tomita, A. Kobata, T. Endo, Glycobiology 9 (1999) 655.
[28] G. Durand, N. Seta, Clin. Chem. 46 (2000) 795.

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Regulatory perspectives from Japan – Comparability of biopharmaceuticals☆

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Abstract

In Japan there is no official guideline about comparability assessment of biotechnological products at present. However, there is some notifications which should be referred to, when the manufacturer changes the manufacturing process. Here, regulatory perspectives from Japan on the comparability assessment are presented. When establishing the comparability of biotechnological products derived from different manufacturing processes and the validity of modified manufacturing process, rational step-by-step approaches based on both product and process aspects would be useful. At first, relevant physicochemical and biological properties of products including purity, impurity profiles and stability should be compared before and after the manufacturing change, depending on the type and nature of the desired products. It is also necessary to examine the capacities of the new manufacturing process for ensuring the consistent production of the active protein product as well as the anticipated elimination of potential impurities and contaminants. Further relevant assessment of preclinical and clinical comparability of product may be necessary in some cases.

Keywords: Biotechnological product; Comparability; Japan; ICH-guideline; Regulatory perspectives; Harmonization

1. Introduction

Biotechnological products were developed and produced based on many innovative technologies, which are always advancing by themselves. The products are, therefore, often subject to change in the manufacturing process for improvement of the product quality and production economy, increase in production yield, and so on. It is not reasonable that the manufacturers are required to submit the same full data to obtain the authorization of the manufacturing change as to obtain the new drug authorization. USA-FDA and EU-CPMA have already set each guideline for comparability assessment of biotechnological/biological products. We had also started the discussion about the comparability guideline in Japan. However, we stopped developing it, because comparability assessment of biotechnological products was nominated as a candidate of the new topic in the ICH-Quality. Drafting of the harmonized guideline has just started in the ICH-EWG. Here, I would like to give the regulatory perspectives about the comparability assessment of biopharmaceuticals from Japan.

2. Present official notifications relating with comparability assessment of biotechnological products before and after manufacturing changes in Japan

In Japan, we do not have any official guideline for the comparability assessment of biotechnological/biological products whose manufacturing processes are changed, yet. However, there is a notification, which should be referred to, when the manufacturer changes the manufacturing process of biotechnology-derived drugs which have already been approved. That is the Notification No. 243 from the Pharmaceutical Affairs Bureau, MHW of 1984. However, nearly 20 years have already passed since the Notification was made and some parts

☆ The perspectives are before the discussion in the ICH-EWG.

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of the requirement are assumed to be too strict. At present we usually treat each case as summarized below.

The following recombinant drugs would be treated as "not new drugs", which are categorized as "1-(8) other drugs" in the Pharmaceutical Affairs Bureau Notification No. 698: the first is the product which contains identical active ingredient although the culture method is different from the approved drug; the second is that which contains identical active ingredient although the purification process is different from the approved drug; and the third is the other drug in which difference is not specified. The followings are also usually treated as "not new drugs" but decided on a case-by-case basis: the product which contains identical active ingredient but its structure gene is identified by different process; and the product which contains identical active ingredient but host cell/vector system is different from the approved drug. In the case of the category 1-(8) other drugs as "not new drug", the data on specification and test methods, stability, and bioequivalence are required to be submitted for the registration as the pharmaceuticals, and a list of literature references concerning toxicity, pharmacological action, absorption, distribution, metabolism and excretion, and clinical trials for active ingredients concerned, as well as an outline of the list contents and the results of evaluation test are also required. In addition, in the case of the biotechnology-derived drugs, the following data are also needed on a case-by-case basis:

data on the manufacturing process, physicochemical analysis, specifications and test methods, stability;
data on single dose administration toxicity in one species of animals;
data on bioequivalency study;
data on clinical study for safety, etc.

The present notifications relating with the comparability of the products between before and after the changes in the manufacturing process in Japan are very simple, as summarized above. However, we have discussed much how to assess comparability of biotechnological products to draft the guideline, within Japanese experts. The following is the perspectives obtained from the discussion.

3. Regulatory perspectives from Japan: "how should we assess comparability of biotechnological products before and after the manufacturing change?"

To date, various topics related to the characterization and quality assessment as well as the manufacturing process for biotechnological products have been the subject of ICH harmonized guidelines and have proven very useful, in allowing manufacturers to develop a global approach to these issues. However, there is no specific international guideline on comparability of biotechnological products subject to changes in the manufacturing process. The subject we are facing is how to develop and establish rational concepts and approaches for establishing comparability of protein products derived from different biopharmaceutical manufacturing processes.

3.1. When is comparability assessment needed?

A comparability assessment is needed when a manufacturer wants to claim that the product of new manufacturing process Y is comparable to the already existing product of manufacturing process X with respect to quality, safety and efficacy (Fig. 1). The new process Y would be employed by either the same manufacturer, innovator or by different subsequent-entry manufacturer(s). The existing product from process X may be either an already licensed one or one under development for new drug application for approval. In case where there is an already licensed drug, subsequent-entry product(s) from different manufacturer(s) will be dealt with as a so-called generic product(s). On the other hand, the application from the innovator will be handled as a partial variation from already licensed conditions for the drug with respect to the manufacturing process. In the case of manufacturing variation of the product under development, the issue becomes the verification of such change within a single manufacturer at various stages of product development from early stage research to pre-approval. Here, the followings should be mentioned: it has been already decided that the generic products are excluded from the scope of the ICH-Q5E comparability guideline, but in Japan we still think that the comparability of the generic products could be evaluated following the same scientific approach.

3.2. General principles of comparability assessment

When establishing the comparability of biotechnological products derived from different manufacturing processes and the validity of modified manufacturing process, rational step-by-step approaches based on both product and process aspects would be useful. In this approach, the following parameters should be considered as key points:

(1) physicochemical and biological characterizations;
(2) impurities profile and the presence of potential contaminants;
(3) batch analysis;
(4) product stability;
(5) manufacturing process evaluation/validation studies; and in wider perspective
(6) preclinical and clinical studies.

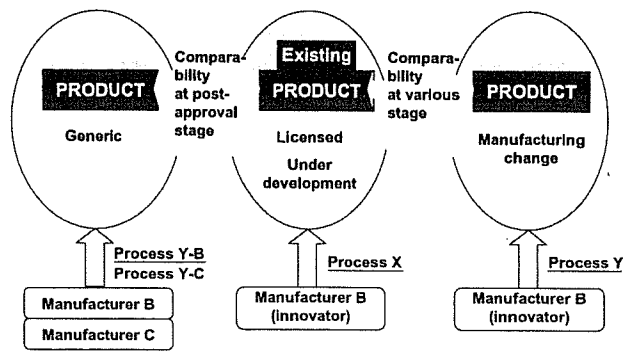

Fig. 1. Various cases of comparability assessment.

3.3. Strategies for comparability assessment

From the viewpoint of product aspects, the essential and critical first step is to establish whether the new candidate product in question is comparable to the existing product in terms of molecular and quality attributes. This is because whatever changes (minor or major) in the manufacturing process are made, if the new candidate product in question is not comparable to the existing product in terms of molecular and quality attributes, the new one will rather be regarded as a novel molecular entity for new drug application, but not as a qualified candidate for further comparability studies. The candidate product should be, therefore, the subject of extensive identification and characterization, as well as quality assessments including tests on impurities profile and the presence of potential contaminants. If these attributes of the candidate product and process are found to be comparable to those of the previous ones, further assessment of preclinical and clinical comparability would be performed, where necessary and appropriate.

3.4. Comparability from product aspects

Before going into some details about the need for further assessment of preclinical and clinical comparability, however, one should ask the following key question: "what is the identity or comparability of the biosynthetic protein product which possesses the inherent degree of structural heterogeneity?" In other words, what kind of criteria should be applied for establishing the identity or comparability of the candidate product(s) compared to the previous product with respect to molecular and quality attributes?

To answer this question, we should remind new concepts in the ICH-Q6B document. In the document we have introduced the concept, which has defined the desired product and variants, so that an inherent degree of structural and quality heterogeneity can be dealt within a relevant way. Desired product is defined as: (1) the protein which has the expected structure, or (2) the protein which is expected from the DNA sequence and anticipated post-translational modification (including glyco-forms), and from the intended downstream modification to produce an active biological molecule. When molecular variants of the desired product are formed during manufacture and/or storage and have properties comparable to the desired product, they are considered to be product-related substances and incorporated into active ingredient. When molecular variants of the desired product do not have properties comparable to those of the desired product, they are considered to be product-related impurities. In the concept, active ingredient may be composed of the desired product and multiple product-related substances; the desired product can be a mixture of several molecular entities derived from anticipated post-translational modification. Impurities may be either process-related or product-related (Fig. 2).

Various cases are considered for minimum qualification for further comparability assessments depending on each following specific type of desired product (A–D):

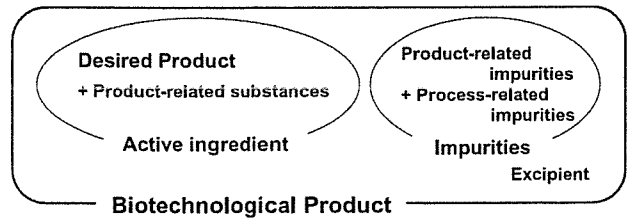

Fig. 2. New concept about biotechnological product in the ICH-Q6B.

(A) the protein which has the expected structure (e.g., monoclonal antibodies);
(B) the protein which is expected from the DNA sequence (simple protein);
(C) the protein which is expected from the DNA sequence and anticipated post-translational modification; and
(D) the protein which is expected from the intended downstream modification to produce an active biological molecule.

In the case of the "Desired product" being defined as the protein which has the expected structure, like monoclonal antibodies, minimum qualification for a candidate product for further comparability assessments should be that the product is derived from the same initial cell clone as a previous one and has comparable molecular and quality attributes compared to a previous one with respect to: (1) structural features, (2) physicochemical, (3) immunological properties, and (4) impurities profile. Variation of carbohydrate heterogeneity due to changes in culture conditions should be considered on a case-by-case basis.

In the case of the "Desired product" being defined as the protein which is expected from the DNA sequence, like recombinant insulin, minimum qualification for a candidate (product) for further comparability assessments should be that the product is the same as an already existing one with respect to protein structure, physicochemical and biological properties, as well as comparable impurities profiles.

In cases where the in vivo biological activity is closely related to the intended clinical effectiveness, further preclinical and clinical assessments with respect to efficacy may be omitted.

In the case of the "Desired product" being defined as the protein which is expected from the DNA sequence structure and anticipated post-translational modification, typically like glycoproteins, minimum qualification for a candidate product for further comparability assessments should be that the product is derived from the same initial cell clone as a previous product and has the same protein structure, comparable physicochemical properties, comparable carbohydrate patterns compared to a previous product with respect to the types of sialic acids and their contents, and antennary profile. Here, comparable biological properties, especially ensuring higher-order structure, in vivo activity and representing the clinical effectiveness, if any, is a critical factor for the qualification.

In the case of the protein which is expected from the intended downstream modification to produce an active

biological molecule, qualification for further comparability assessment of this type of products should be considered as a case-by-case issue, taking into account of types of modification and process change. Where necessary and appropriate, manufacturers should refer to the above cases A–C.

In this way, each specific type of candidate product can be qualified to be comparable to the pre-existing product with respect to molecular and quality attributes including impurity profile. The quality and extent of data obtained from studies on the molecular and quality attributes of the candidate would become one of the crucial elements for determining the necessity and extent of further comparability assessments, as well as for establishing the entire comparability to the pre-existing product.

3.5. Comparability from process aspect

As another aspect of quality comparability assessments, it is necessary to examine the capacities of a new manufacturing process for ensuring the consistent production of the active protein product as well as the anticipated elimination of potential impurities and contaminants. The capacities of the new process should not be less potent than those of the old process.

Changes in the manufacturing process used to make a particular product can be made in a variety of stages or steps of the process. Examples of such changes include: (1) method for generating cell substrate; (2) cell culture methods; (3) isolation and purification procedure; and (4) final product formulation. For changes in a certain stage of manufacturing process including cell substrate matters, relevant and complementary use of the ICH-guidelines (Q5A, Q5B, Q5EC, 5D, Q6B, and S6) would be encouraged.

Whatever changes in the manufacturing process are made, the effects of the changes, both direct and indirect, on the consistent production of the product should be considered and the modified process should be re-evaluated or re-validated as needed. The appropriate process re-evaluation or re-validation programs and criteria will vary depending on the nature and extent of the change. According to the results of process re-evaluation/re-validation studies on the new process, sometimes applicants may need to modify in-process controls including in-process testing and specifications of critical intermediates or final product (Fig. 3). The applicant should provide justification of such modification, if any.

3.6. Suitability of analytical method

As another dimension to comparability study, it is necessary to consider suitability of available analytical methods. Manufacturers should provide assurance that an appropriate set of analytical methods has been selected in order to assess the comparability of the product and to what extent the analytical methods used are suitable for comparability studies. The validation of the analytical methods used should be appropriate.

New analytical technology and modifications to existing technology are continually being developed and should be utilized when appropriate.

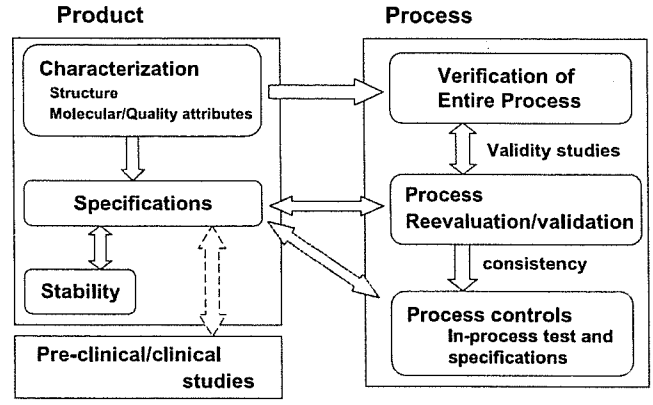

Fig. 3. Elements for ensuring product quality and consistency.

3.7. Preclinical and clinical studies

Further relevant assessment of preclinical and clinical comparability of product may be necessary, when it cannot be determined if the pre-existing product and the candidate product are comparable or not from the quality studies. The extent and nature of preclinical and clinical studies should be determined on a case-by-case basis in consideration of various factors. These include the followings:

- the nature of the product;
- intended clinical use;
- the extent of comparability of the candidate product to the existing counterpart with respect to molecular and quality attributes including impurity profile;
- the nature and extent of changes in manufacturing process;
- the results of the evaluation/validation studies on the new process including the results of relevant in-process tests;
- the capabilities and limitations of tests used for any comparability study;
- availability of existing preclinical and clinical data;
- the extent of existing information and experiences pertaining to the product in question; and
- stage of the product development.

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Capillary affinity electrophoresis using lectins for the analysis of milk oligosaccharide structure and its application to bovine colostrum oligosaccharides

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Abstract

Animal colostrum and milk contain complex mixtures of oligosaccharides, which have species-specific profiles. Milk oligosaccharides have various types of structure related to the core structures of glycolipids and *N*- and *O*-glycans of glycoproteins and provide a good library to examine the binding of oligosaccharides to various lectins. Recently, we reported a capillary affinity electrophoresis (CAE) method for analyzing the interactions between lectins and complex mixtures of *N*-linked oligosaccharides prepared from serum glycoproteins [K. Nakajima, Y. Oda, M. Kinoshita, K. Kakehi, *J. Proteome Res.* 2 (2003) 81–88]. The present paper reports the interactions between 24 milk oligosaccharides and six lectins (PA-I, RCA_{120}, SBA, WGA, UEA-I, and AAL) analyzed using CAE. Based on the resulting data, we constructed a library that enables us to determine nonreducing terminal monosaccharides, such as Gal, GalNAc, GlcNAc, and Fuc, and to differentiate Gal- or Fuc-linked isomers, such as lacto-*N*-tetraose, lacto-*N*-neotetraose, and lacto-*N*-fucopentaose II and III. In addition, using the library, we show that a combination of the lectins can characterize the neutral oligosaccharides derived from bovine colostrum.

Keywords: Capillary affinity electrophoresis; Milk oligosaccharides; Lectins

Mammalian milk and colostrum contain a variety of neutral and acidic oligosaccharides. Messer and Urashima [1] reported that milk oligosaccharides were interesting targets for elucidating the oligosaccharide structures bound by various types of carbohydrate recognition molecules.

Milk oligosaccharides commonly have a lactose unit at their reducing end and are synthesized by various combinations of specific glycosyltransferases. The structures of milk oligosaccharides are diverse and species specific [2–8]. For example, human milk contains more than 100 oligosaccharides and does not contain *N*-glycoylneuraminic acid (NeuGc) [3]. Bear milk contains oligosaccharides having an α-Gal epitope (Galα1-3Galβ1-4GlcNAcβ1-) together with A [GalNAcα1-3(Fucα1-2)Galβ1-] or B [Galα1-3(Fucα1-2)Galβ1-] blood group or $Lewis^x$ [Galβ1-4(Fucα1-3)GlcNAc] antigens alone or in combination [4–6]. Seal milk contains large amounts of oligosaccharides with NeuAcα2-6 or Fucα1-2 linkage and complex oligosaccharides with a high molecular mass [7].

Previously, we developed a capillary affinity electrophoresis (CAE)[1] method for analyzing oligosaccharides based

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[1] *Abbreviations used:* CAE, capillary affinity electrophoresis; MALDI-QIT-TOF, matrix-assisted laser desorption ionization–quadrupole ion trap–time-of-flight; GU, glucose unit; CE, capillary electrophoresis.

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on their affinities for lectins [9–11]. CAE is a powerful tool for analyzing complex mixtures of oligosaccharides in biological samples, because the structural determination is achieved without isolating individual oligosaccharides. CAE analysis involves the following two steps. In the first step, a mixture of oligosaccharides is analyzed in the absence of a lectin. In the second step, the same mixture is analyzed in the presence of a lectin, the specificity of which is well established. Changes in migration in the presence of the lectin suggest partial structures characteristic of the lectin binding. The comparison of migrations obtained with an appropriate set of lectins allows characterization of the structures of the oligosaccharides, even in mixtures. In addition, the accumulation of such data will enable us to suggest structures of unknown oligosaccharides [9,10].

In this study, we applied CAE analysis to 24 milk oligosaccharides using six lectins. The data on the interactions between these oligosaccharides and a set of lectins enabled us to construct a library and we used the library to differentiate the linkage isomers of four oligosaccharides with Gal and Fuc residues at different positions. This technique was also applied to the analysis of neutral oligosaccharides in bovine colostrum and we propose the structures of two oligosaccharides.

Materials and methods

Materials

Twelve human milk oligosaccharides (**1**, **4**, **5**, **6**, **10**, **11**, **12**, **15**, **16**, **17**, **20**, and **22** listed in Table 1) were purchased from Seikagaku Kogyo (Tokyo, Japan). Six oligosaccharides (**7**, **13**, **18**, **19**, **21**, and **24** listed in Table 1) were purified from the milk of Japanese black bears, one oligosaccharide (**23** in Table 1) was purified from polar bear milk, and these structures were confirmed in the previous reports [4,5]. Lyophilized bovine colostrum was obtained from Synertek Colostrum (Wyoming, USA). Seven bovine milk oligosaccharides (**1**, **2**, **3**, **7**, **8**, **9**, and **14** listed in Table 1) were purified from lyophilized bovine colostrum, as reported previously [12]. *Pseudomonas aeruginosa* lectin (PA-I) was obtained from Sigma–Aldrich Japan (Tokyo, Japan). *Ulex europaeus* agglutinin (UEA-I), *Ricinus communis* agglutinin (RCA_{120}), wheat germ agglutinin (WGA), soybean agglutinin (SBA), and *Aleuria aurantia* lectin (AAL) were obtained from Seikagaku Kogyo. α1-2 Fucosidase derived from *Corynebacterium* sp. was purchased from Takara Biomedicals (Kusatsu, Japan). All reagents were analytical or HPLC grade.

Table 1
Library for structure analysis of milk oligosaccharides

	Oligosaccharide	Structure[a]	GU[b]	Added concentration (μM)[c]					
				SBA	PA-I	RCA_{120}	WGA	UEA-I	AAL
1	Lactose	Galβ1-4Glc	1.9	—[d]	—	12.0	—	—	—
2	Lactosamine	Galβ1-4GlcNAc	2.1	—	—	3.0	3.0	—	—
3	*N*-Acetylgalactosaminylglucose	GalNAcβ1-4Glc	2.2	3.0	—	—	—	—	—
4	2-Fucosyllactose	Fucα1-2Galβ1-4Glc	2.7	—	—	—	—	12.0	0.8
5	Lewis[a] trisaccharide	Fucα1-4(Galβ1-3)GlcNAc	2.8	—	—	—	—	—	3.0
6	Lewis[x] trisaccharide	Galβ1-4(Fucα1-3)GlcNAc	2.9	—	—	—	—	—	0.2
7	Isoglobotriose	Galα1-3Galβ1-4Glc	2.9	—	3.0	—	—	—	—
8	β4′-Galactosyllactose	Galβ1-4Galβ1-4Glc	3.0	—	—	0.8	—	—	—
9	α3′-*N*-Acetylgalacotaminyllactose	GalNAcα1-3Galβ1-4Glc	3.1	0.8	3.0	—	—	—	—
10	Di-fucosyllactose	Fucα1-2 Galβ1-4(Fucα1-3)Glc	3.5	—	—	—	—	12.0	0.8
11	Lacto-*N*-neotetraose	Galβ1-4GlcNAcβ1-3Galβ1-4Glc	4.3	—	—	0.8	12.0	—	—
12	Lacto-*N*-tetraose	Galβ1-3GlcNAcβ1-3Galβ1-4Glc	4.3	—	—	—	—	—	—
13	B-pentasaccharide	Fucα1-2Galα1-3Galβ1-4(Fucα1-3)Glc	4.4	—	—	—	—	—	0.8
14	Lacto-*N*-novopentaose I	Galβ1-4GlcNAcβ1-6(Galβ1-3)Galβ1-4Glc	5.1	—	—	0.8	3.0	—	—
15	Lacto-*N*-fucopentaose II	Fucα1-4(Galβ1-3)GlcNAcβ1-3Galβ1-4Glc	5.1	—	—	—	—	—	12.0
16	Lacto-*N*-fucopentaose III	Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc	5.1	—	—	—	—	—	3.0
17	Lacto-*N*-difucopentaose II	Fucα1-4(Galβ1-3)GlcNAcβ1-3Galβ1-4(Fucα1-3)Glc	5.7	—	—	—	—	—	12.0
18	Galactosyl lacto-*N*-fucopentaose III	Galα1-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc	6.1	—	3.0	—	—	—	0.8
19	B-Heptasaccharide-typeII chain	Galα1-3(Fucα1-2)Galβ1-4 (Fucα1-3)GlcNAcβ1-3Galβ1-4Glc	6.6	—	—	—	—	—	—
20	Lacto-*N*-neohexaose	Galβ1-4GlcNAcβ1-6(Galβ1-4GlcNAcβ1-3)Galβ1-4Glc	6.8	—	—	0.8	3.0	—	—
21	Galactosyl-difucosyl lacto-*N*-neotetraose	Galα1-3Galβ1-4(Fucα1-3) GlcNAcβ1-3Galβ1-4(Fucα1-3)Glc	7.2	—	3.0	—	—	—	0.2
22	Difucosyl lacto-*N*-neohexaose	Galβ1-4(Fucα1-3)GlcNAcβ1-6[Galβ1-4 (Fucα1-3) GlcNAcβ1-3]Galβ1-4Glc	8.2	—	—	—	—	—	0.8
23	Digalactosyl lacto-*N*-neohexaose	Galα1-3Galβ1-4GlcNAcβ1-6 (Galα1-3Galβ1-4GlcNAcβ1-3)Galβ1-4Glc	9.0	—	3.0	—	3.0	—	—
24	Digalactosyl-difucosyl lacto- *N*-neohexaose	Galα1-3Galβ1-4(Fucα1-3)GlcNAcβ1-6[Galα1-3 Galβ1-4(Fucα1-3)GlcNAcβ1-3]Galβ1-4Glc	10.2	—	3.0	—	—	—	0.8

[a] Abbreviations used for the structures: GlcNAc, *N*-acetylglucosamine; Glc, glucose; GalNAc, *N*-acetylgalactosamine; Gal, galactose; Fuc, fucose.
[b] GUs are relative electrophoretic mobility to glucose oligomer.
[c] Number shows the concentrations of lectins, which clearly changed the migration time.
[d] No effects.

Preparation of a neutral oligosaccharide mixture from bovine colostrum

A mixture of neutral oligosaccharides from bovine colostrum was prepared according to a reported method [12]. Lyophilized powder (90 g) was suspended in 100 ml water, and the supernatant, collected after centrifugation at 4000*g* for 30 min, was shaken with 300 ml of chloroform–methanol (2:1, v/v). After centrifugation at 4000*g* for 30 min at 4 °C, the upper layer was collected, concentrated using a rotary evaporator, and lyophilized. The lyophilized material (7.0 g) was dissolved in 20 ml of water and 10 ml of the solution was applied to a column packed with Bio-Gel P2 (2.5 × 120 cm) equilibrated with aqueous 50 mM ammonium bicarbonate. The oligosaccharides were eluted with the same solvent at a flow rate of 0.15 ml/min, and 6 ml portions were collected. Fractions reacting to phenol–sulfuric acid reagent eluted in the void volume were pooled and lyophilized. The total weight was 1.02 g. A portion of the lyophilized material (200 mg) was dissolved in 20 ml of water and passed through a DEAE–Sephadex A-25 column (1.5 × 50 cm, acetate form) equilibrated with 50 mM pyridine–acetate buffer, pH 5.0. The fraction that passed through was collected and lyophilized as the neutral oligosaccharide fraction.

Fluorescent labeling of oligosaccharides with 3-aminobenzoic acid (3AA)

Each of the oligosaccharides **1** to **24** (5 nmol) and a mixture of oligosaccharides (1 mg) derived from bovine colostrum were labeled with 3AA as reported previously [13]. Briefly, a dried sample was dissolved in 30 μl of 0.7 M 3AA in dimethylsulfoxide:acetic acid (7:3 by volume). A solution (30 μl) of freshly prepared 2 M sodium cyanoborohydride in the same solvent was added to the mixture and incubated for 1 h at 50 °C. The reaction mixture was diluted with water (200 μl) and applied to a small column (1.0 × 30 cm) of Sephadex LH-20 equilibrated with 50% aqueous methanol. The void fractions containing 3AA-labeled oligosaccharides were collected and evaporated to dryness.

Capillary affinity electrophoresis

A Beckman Coulter P/ACE MDQ Glycoprotein System with a helium–cadmium laser-induced fluorescence detection system was used. Fluorescence-labeled oligosaccharides were detected with a 405-nm filter for emission and a 325-nm filter for excitation. The compounds were separated using an eCAP N-CHO capillary (Beckman–Coulter; 20 cm effective length, 30 cm total length, 50 μm i.d.). The inner surface is chemically modified with polyvinyl alcohol and is ideal for affinity electrophoresis using lectins due to negligible levels of lectin–capillary surface interaction. Tris–acetate buffer (100 mM, pH 7.4) was used as the electrolyte. Sample solutions were injected using a pressure method (0.5 p.s.i, 5 s). The data were collected and analyzed with standard 32 Karat software (Version 4.0, Beckman Coulter) on Microsoft Windows 2000.

Before CAE analysis, a mixture of 3AA-labeled oligosaccharides was analyzed in the absence of a lectin. Their migration times are expressed as glucose units (GUs). GU values were determined with a 3AA-labeled glucose oligomer mixture prepared from a partial hydrolysate of dextran as an external standard.

For CAE analysis, the capillary was filled with the same electrolyte containing a lectin at a specified concentration, and the same mixture of the oligosaccharides was analyzed. Changes in the migration of each oligosaccharide were detected.

Purification of two oligosaccharides (XI and XII) from bovine colostrum

We found two oligosaccharides (**XI** and **XII**) whose structures were not confirmed by CAE analysis with commercially available standard oligosaccharides from bovine colostrum. These oligosaccharides were purified using a combination of normal-phase and reversed-phase HPLC, as reported previously [14,15].

A mixture of 3AA-labeled colostrum oligosaccharides (20 μg) was separated by HPLC with an amine-bonded polymer column (Shodex Asahipak-NH_2 P-50 4E, 4.6 × 250 mm; Showa Denko, Tokyo, Japan) at 50 °C using a linear gradient formed with 2% (v/v) acetic acid in acetonitrile (solvent A) and 5% acetic acid and 3% triethylamine in water (solvent B). The column was initially equilibrated and eluted for 2 min with 70% solvent A, and then solvent B was increased to 95% over 80 min. The flow rate was 1.0 ml/min. The detected oligosaccharides were collected and lyophilized. Each oligosaccharide was then separated at room temperature by HPLC with an ODS column (PALPAK Type R column, 4.6 × 250 mm, Takara Biomedicals) using a linear gradient formed with 50 mM ammonium formate buffer (pH 4.4) (solvent A) and 20% acetonitrile in the same buffer (solvent B). The column was initially equilibrated and eluted with 8% solvent B, and then solvent B was increased to 12% over 70 min. The flow rate was 0.8 ml/min. Each oligosaccharide peak was pooled, lyophilized, and analyzed by capillary electrophoresis and matrix-assisted laser desorption ionization–quadrupole ion trap–time-of-flight mass spectrometry (MALDI-QIT-TOF MS).

MALDI-QIT-TOF MS of XI and XII

MALDI-QIT-TOF mass spectra were obtained using an AXIMA-QIT mass spectrometer (Shimadzu; Nakagyo-ku, Kyoto, Japan) equipped with a nitrogen-pulsed laser (337 nm). Argon was used for collision-induced dissociation. 3AA-labeled oligosaccharides were analyzed in the positive and reflectron modes. 2,5-Dihydroxybenzoic acid was used as the matrix at a concentration of 10 mg/ml in 50% ethanol. A sample solution (2 μl, 10 pmol) was mixed with 2 μl of matrix solution and applied to a polished

stainless steel target. The target was left at room temperature until the mixture had dried.

Results and discussion

Interaction between milk oligosaccharides and lectins

We selected the 24 oligosaccharides shown in Table 1. These oligosaccharides contain various Fuc and Gal linkages. To distinguish these oligosaccharides, we selected six lectins, each of which has a different binding specificity to Gal/GalNAc, Fuc, and GlcNAc. CAE measured (1) the migration time of each oligosaccharide in the absence of lectin and (2) the change in migration times in the presence of a lectin. A change in migration time indicates the binding of the oligosaccharide to a lectin.

Capillary electrophoresis of 24 milk oligosaccharides

Initially, we analyzed a mixture containing the 3AA-labeled oligosaccharides in equimolar concentrations. The smaller oligosaccharides moved faster than the larger oligosaccharides on the basis of their charge/mass ratios, because electroosmotic flow is negligible under the conditions that we used. Oligosaccharides **1**, **2**, **4**, **5**, **8**, **9**, **10**, **11**, and **16** were detected at 1.9, 2.1, 2.7, 2.8, 3.0, 3.1, 3.5, 4.3, and 5.1 GUs, respectively (Fig. 1). The difference in the GUs of **1** and **8** indicates that the addition of a Hex increases GU by 1.1, that between **8** and **11** indicates that the addition of a HexNAc increases GU by 1.3, and that between **11** and **16** indicates that the addition of a Fuc increases GU by 0.8. GUs correlate with the molecular sizes of oligosaccharides. The separations of **4**, **5**, **6**, **7**, **8**, and **9**; **11**, **12**, and **13**; and **14**, **15**, and **16** by CE were incomplete. To perform the analysis within a practical and short time, we selected these CE conditions but the incomplete separation is covered by the analysis in the presence of lectins because all overlapping peaks were resolved in the presence of lectins. Oligosaccharides **5** and **6**, **11** and **12**, and **15** and **16** are linkage isomers. These linkage isomers were separated in the presence of the Gal-binding lectin RCA_{120} and the Fuc-binding lectin AAL, as described below.

CAE for the differentiation of linkage isomers of Gal

We prepared two sets of mixtures: mixture A containing **4**, **6**, **10**, **11**, and **16** and mixture B containing **1**, **5**, **12**, **15**, and **17**. Each mixture contained **23** as the internal standard. Baenziger and Fiete [16] reported that RCA_{120} binds to the Galβ1-4 linkage more strongly than to the Galβ1-3 linkage. Therefore, we selected RCA_{120} to separate Gal linkage isomers, **11** (Galβ1-4GlcNAcβ1-3Galβ1-4Glc) and **12** (Galβ1-3GlcNAcβ1-3Galβ1-4Glc). The oligosaccharides **11** and **12** migrated together without RCA_{120}, but **11** disappeared in the presence of 0.8 μM RCA_{120} (Fig. 2). By contrast, **12** did not change its migration.

CAE for the differentiation of linkage isomers of Fuc

To differentiate **5** [Galβ1-3(Fucα1-4)GlcNAc],**6**[Galβ1-4(Fucα1-3)GlcNAc], **15** [Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc], and **16** [Galβ1-4(Fucα1-3)GlcNAcβ1-3 Galβ1-4Glc],

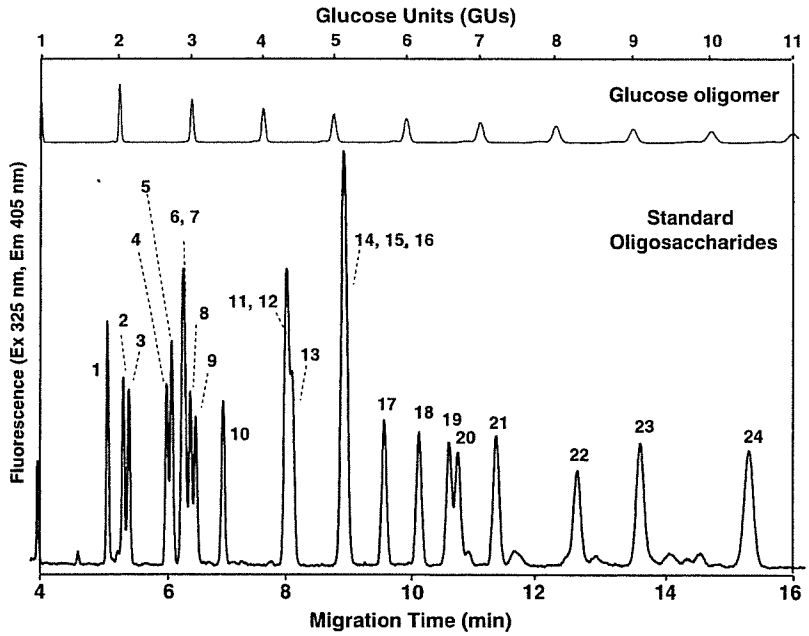

Fig. 1. Analysis of 24 3AA-labeled milk oligosaccharides by capillary electrophoresis. Glucose units (GUs) were determined with glucose oligomers. Analytical conditions: eCAP N-CHO coated capillary; 30 cm length (effective length, 20 cm, 50 μm i.d.); applied potential, 18 kV; injection, pressure method (0.5 psi for 5 s); and running buffer, 100 mM Tris–acetate buffer (pH 7.4) containing 0.5% polyethylene glycol. Fluorescence was detected at 405 nm by the excitation at 325 nm of a helium–cadmium laser. The structures of oligosaccharides **1** to **24** are shown in Table 1.

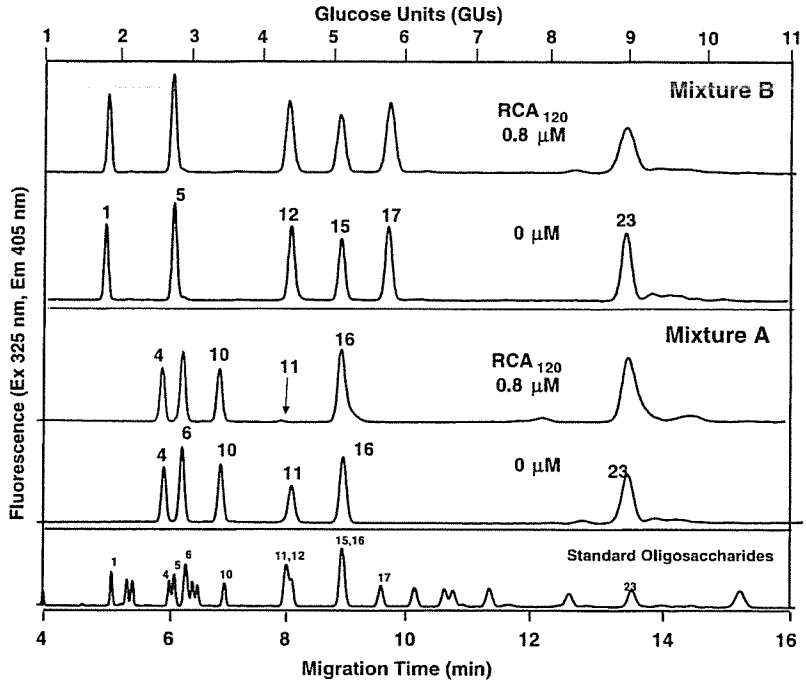

Fig. 2. Capillary affinity electrophoresis for differentiating the linkage isomers of Gal using RCA_{120}. The electropherogram of the standard oligosaccharides is shown at the bottom. CAE of A and B mixtures of human milk oligosaccharides was performed in the presence of RCA_{120} at 0 and 0.8 μM in the electrolyte. The analytical conditions were the same as those described in Fig. 1. Oligosaccharides **11** and **12** migrated together without RCA_{120}, but **11** disappeared in the presence of 0.8 μM.

we used AAL, which has been used to differentiate oligosaccharides having Fuc residues due to its broad specificity toward Lewis^x, Lewis^a, and O antigens [17].

As shown in Fig. 3, only peak **6** (Lewis^x antigen) migrated slower in the presence of 0.2 μM AAL. At 0.8 μM AAL, **6** disappeared, and **4** and **10** containing an α1-2-linked Fuc migrated very slowly. As shown in Fig. 3, **5** (Lewis^a antigen) and **16** [lacto-*N*-fucopentaose III: Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc] migrated slightly slower in 0.8 μM AAL. By contrast, neither **15** nor **17** containing an α1-4-linked Fuc changed their migration at this concentration. Fig. 4 shows the analysis of bear milk oligosaccharides, indicating that **18** (galactosyl-lacto-*N*-fucopentaose III) migrated slow at 0.8 μM AAL, in a manner similar to that of **16**.

CAE for the analysis of difucosylated oligosaccharides

AAL also has the possibility to differentiate the oligosaccharides carrying Fuc residues at multiple sites. Difucosylated oligosaccharides with Fucα1-2 or α1-3 linkage have been found in milk of the Japanese black bear, polar bear, and Ezo brown bear [4–6].

Oligosaccharide **21** has two Fuc residues. One forms the Lewis^x antigen [Galβ1-4(Fucα1-3)GlcNAc] and the other attaches to the Glc residue through α1-3 linkage. Due to these two α1-3-linked Fuc residues, the oligosaccharide was retarded in 0.2 μM AAL (Fig. 4). By contrast, **24**, which has two Lewis^x antigens, was slightly retarded at 0.2 μM AAL and further retarded at 0.8 μM. In addition, **19**, with one Lewis^x antigen and one α1-2-linked Fuc, was not retarded at 0.8 μM. These results for oligosaccharides **19**, **21**, and **24** in the presence of AAL indicate that their binding to AAL is strongly influenced by the number, linkage combination, and binding sites of their Fuc residues.

As discussed above, milk oligosaccharide structures can now be characterized by comparing the migration time and change in migration in the presence of various lectins. Therefore, construction of a library, collecting comprehensive data on a large number of oligosaccharides, should be very effective for future structure identification.

Library for the structure analysis of milk oligosaccharides

To construct a library, we measured the interactions between 24 milk oligosaccharides and six lectins: three Gal/GalNAc-binding lectins (SBA, PA-I, and RCA_{120}), one GlcNAc-binding lectin (WGA), and two Fuc-binding lectins (UEA-I and AAL) [16–21]. The results of these interactions are summarized in Table 1. The library lists the GU of each oligosaccharide and the lectin concentrations that change the migration time. We previously reported that the obvious changes in the migration time allowed the characterization of oligosaccharides and the concentrations of lectins that produced these changes are good indicators for the characterization of oligosaccharide structures.

In the library, SBA identifies the presence of GalNAc and differentiates GalNAcα or β linkage at two concentrations.

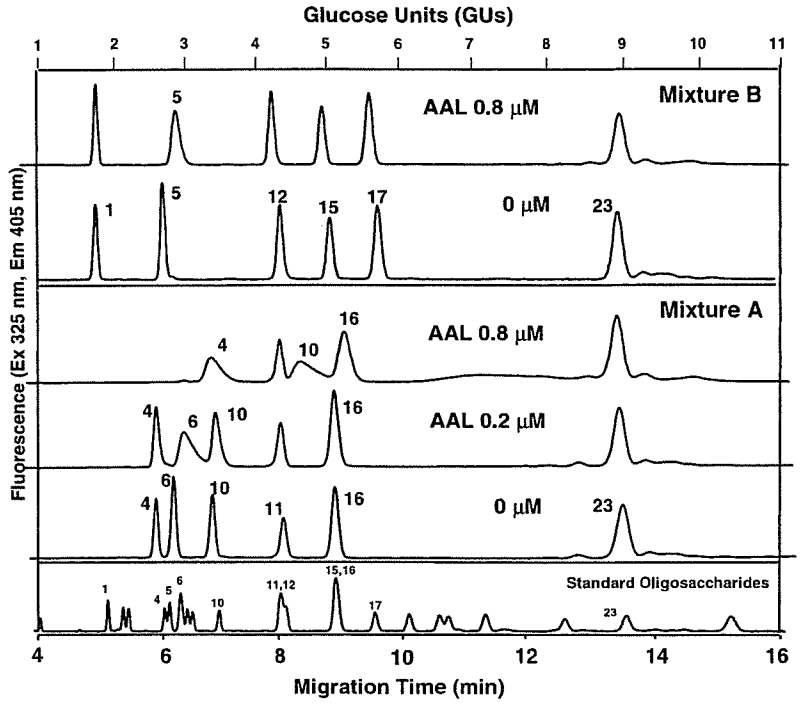

Fig. 3. Capillary affinity electrophoresis for the differentiation of linkage isomers of Fuc using AAL. CAE of A and B mixtures of 3AA-labeled human milk oligosaccharides was performed in the presence of AAL (0, 0.2, and 0.8 μM) in the electrolyte. The analytical conditions were the same as those described in Fig. 1. Peaks **5** and **6** and peaks **15** and **16** separate from each other in the presence of AAL.

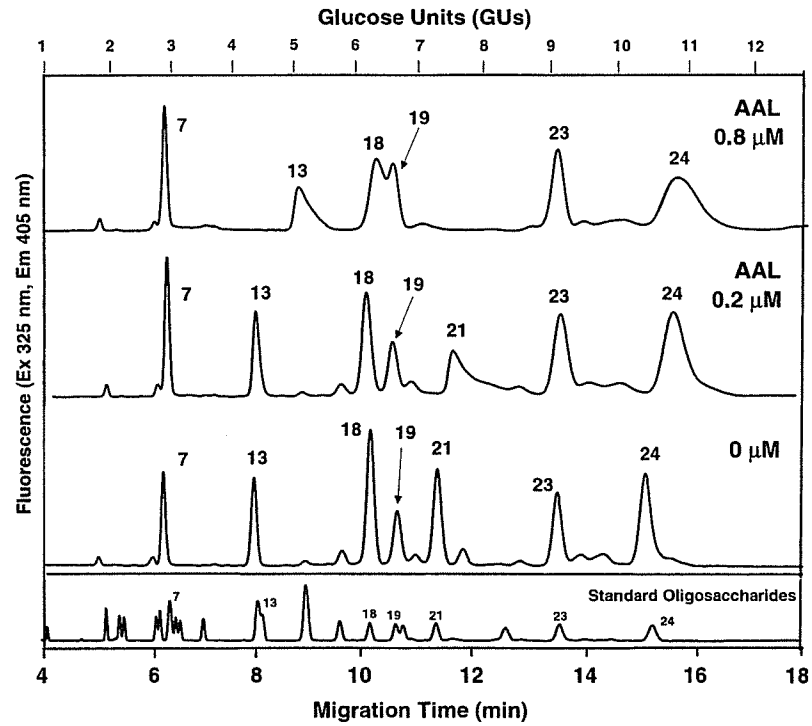

Fig. 4. Capillary affinity electrophoresis for the analysis of 3AA-labeled difucosylated oligosaccharides derived from bear milk using AAL. CAE of bear milk oligosaccharides **7**, **13**, **18**, **19**, **21**, **23**, and **24** was performed in the presence of AAL (0, 0.2, and 0.8 μM) in the electrolyte. The analytical conditions were the same as those described in Fig. 1. Peaks **19**, **21**, and **24** are differentiated in the presence of different concentrations of AAL.

Oligosaccharide **9** which has a GalNAcα1-3 linkage changed its migration in the presence of 0.8 μM SBA, and **3** which has a GalNAcβ1-4 linkage changed its migration in the presence of 3.0 μM SBA. PA-I indicates the presence of Gal/GalNAc with an α1-3 linkage, as in the cases of **7**, **9**, **18**, **21**, **23**, and **24**, while PA-I did not change the migrations of **13** and **19**, indicating that the Fucα1-2 modification on the βGal of Galα1-3Galβ1- inhibits PA-I binding. RCA_{120} affected the migration of oligosaccharides **1**, **2**, **8**, **11**, **14**, and **20**, which have a Galβ1-4 linkage at their nonreducing terminal, as already discussed, but did not affect those of oligosaccharides **6**, **7**, **16**, **18**, **22**, **23**, and **24**, which have Galβ1-4GlcNAc modified with Fucα1-3 or Galα1-3 or both. WGA affected the migration of oligosaccharides **2**, **11**, **14**, **20**, and **23** having Galβ1-4GlcNAc but did not affect that of **12** having Galβ1-3GlcNAc and **6**, **16**, **18**, **19**, **21**, **22**, and **24**, which contain Galβ1-4GlcNAc modified with Fuc (Table 1). The effect of WGA on **11** was smaller than that on **2**, **14**, **20**, and **23**, indicating that WGA binds to the GlcNAcβ1-3 linkage more weakly than to the GlcNAcβ1-6 linkage. These results indicate that RCA_{120} is a useful lectin for detecting the presence of the Galβ1-4 linkage and WGA is useful for detecting the presence of Galβ1-4GlcNAc and differentiating between GlcNAc β1-3 and -6 linkages, but neither lectin binds the structures if the oligosaccharides are fucosylated.

Fucosylated oligosaccharides are efficiently characterized by a combination of UEA-I and AAL. UEA-I detects the presence of O antigen, Fucα1-2Gal, except for those of oligosaccharides **13** and **19**, which are modified with an α-Gal at the Galβ1- of Fucα1-2Galβ1-. By contrast, AAL characterizes most fucosylated oligosaccharides, except for **19**, if we use four concentrations, 0.2, 0.8, 3.0, and 12.0 μM. The presence of 0.2, 0.8, and 3.0 μM AAL differentiates Fucα1-3, -2, and -4 linkages, respectively, as shown in the cases of **6**, **4**, and **5**. Thus, AAL is a useful lectin and the use of AAL in combination with UEA-I can resolve a wide range of fucosylated oligosaccharides.

As discussed above, each lectin enables us to determine the nonreducing terminal monosaccharide and linkage pattern and to differentiate Gal- and Fuc-linked isomers. Isomers **15** and **16** were not clearly separated by normal- and reverse-phase HPLC [22,23] and can be separated if HPLC conditions are optimized. However, CAE can clearly differentiate these isomers in the presence of 3.0 μM AAL (Fig. 3). Moreover, the library can characterize oligosaccharides in biological samples by matching the result of the affinity to the lectins. We demonstrate this with an example of colostrum oligosaccharide analysis.

Analysis of neutral oligosaccharides derived from bovine colostrum

We analyzed neutral oligosaccharides derived from bovine colostrum as an application of CAE using the library. Bovine colostrum contains isoglobotriose (**7**) and α3-*N*-acetylgalactosaminyllactose (**9**). In addition, bovine colostrum contains unique oligosaccharides, such as *N*-acetylgalactosaminylglucose (**3**), lacto-*N*-novopentaose (**14**), and other minor unidentified oligosaccharides [8]. Figs. 5 and 6 show electropherograms of a mixture of 3AA-labeled neutral oligosaccharides in the absence and presence of six lectins. More than 10 peaks were detected from 1.9 to 7.0 GU and seven peaks (**1**, **2**, **3**, **7**, **8**, **9**, and **14**) were

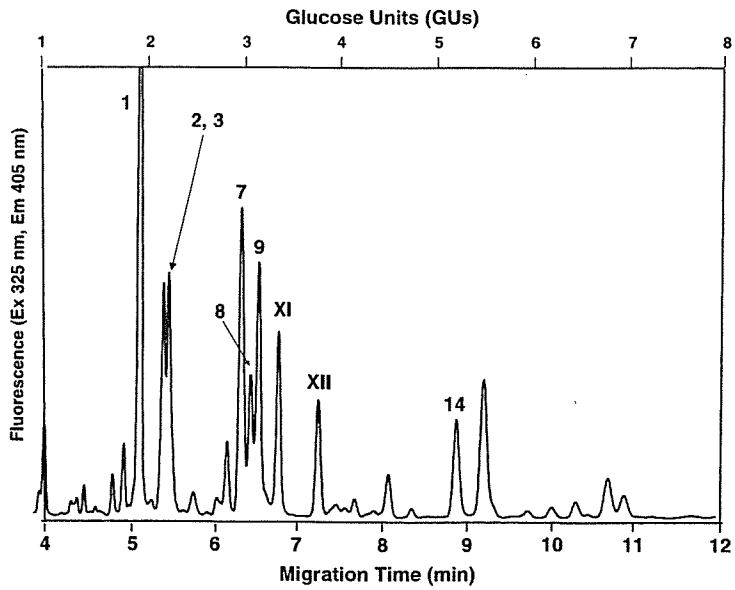

Fig. 5. Analysis of 3AA-labeled neutral oligosaccharides derived from bovine colostrum in the absence of lectin. The analytical conditions were the same as those described in Fig. 1. Peaks **1**, **2**, **3**, **7**, **8**, **9**, and **14** were identified using the library. Peaks **XI** and **XII** are subjected to further analysis and not included in the library shown in Table 1.

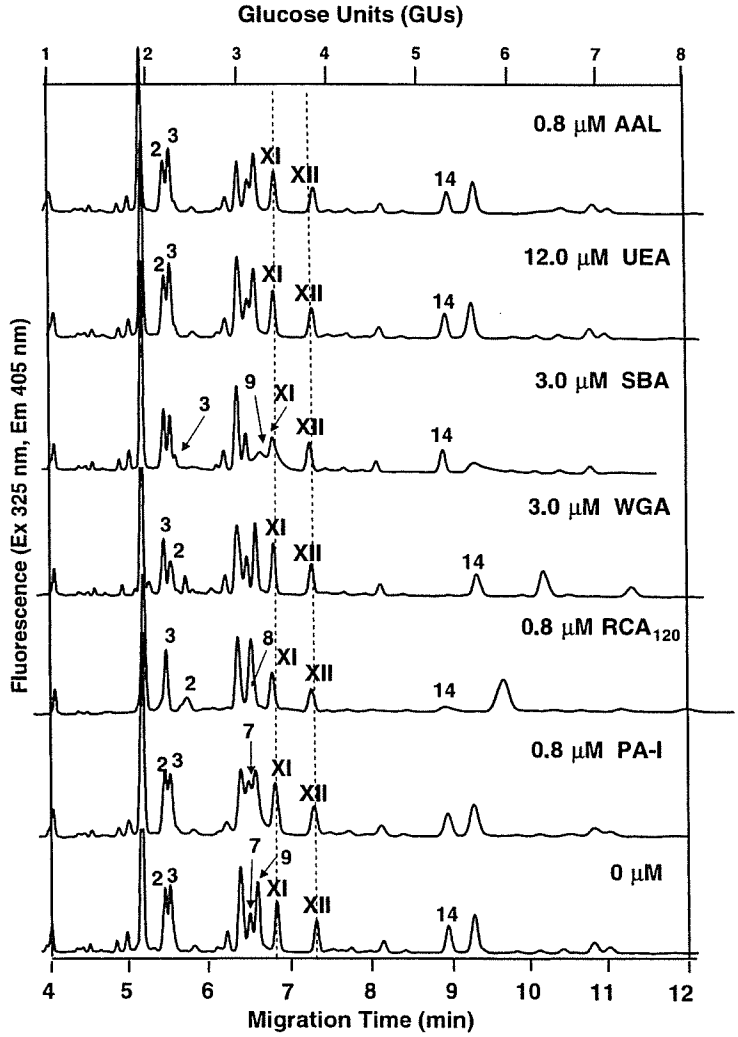

Fig. 6. Capillary affinity electrophoresis for the analysis of 3AA-labeled neutral oligosaccharides using RCA_{120}, PA-I, WGA, SBA, UEA-I, and AAL. The electropherogram (0 μM) was the same as that in Fig. 5. Neutral oligosaccharides were characterized in the presence of 3.0 μM SBA, 0.8 μM PA-I, 0.8 μM RCA_{120}, 3.0 μM WGA, 12.0 μM UEA-I, and 0.8 μM AAL. Peaks **1**, **2**, **3**, **7**, **8**, **9**, and **14** were identified using the library. Peak **XI** changed in the presence of SBA. The analytical conditions were the same as those described in Fig. 1.

identified using the library by comparing the results with those of standard oligosaccharides, with regard to migration time and change of migration in the presence of various lectins (shown in Table 1). However, **XI** and **XII**, detected at 3.3 and 3.8 GU, respectively, could not be identified. We characterized these peaks by CAE and proposed structures based on the CAE results. We were then able to confirm these structures using MALDI-QIT-TOF MS after purifying the individual oligosaccharides.

Structural analysis of XI and XII

Initially, we deduced that **XI** was a trisaccharide based on its migration, 3.3 GU (Table 2). **XI** showed a decrease in peak height and migration time in the presence of SBA at 3.0 μM (Fig. 6). However, WGA, RCA_{120}, and PA-I did not affect peak **XI**, indicating that **XI** has no Gal or GlcNAc but has GalNAc at the nonreducing terminal. The characteristic binding specificities, no affinity to PA-I, and an affinity to SBA that is similar to that of GalNAcβ1-4Glc (Table 1) indicate that **XI** has a terminal GalNAcβ1 structure. We purified **XI** by HPLC and analyzed it using MALDI-QIT-TOF MS according to a reported method [14,15,24]. Fig. 7 shows the MS/MS spectrum at *m/z* 687 of the $[M+Na]^+$ for the purified **XI**, detecting sodiated ions in a series of Y and B fragments. The Y ion at *m/z* 484, corresponding to a loss of 203 (HexNAc-18) from $[M+Na]^+$, indicates that the nonreducing terminal is a HexNAc. The Y ion at *m/z* 322, which lost 162 (Hex-18) from *m/z* 484, indicates the presence of a Hex. These results indicate that the sequence of **XI** is HexNAc-Hex-Hex. The analytical results for **XI** are summarized in Table 2. Based on these results, we propose that the structure of **XI** is GalNAcβ1-Galβ1-4Glc. The linkage of GalNAcβ1- was not determined.

Table 2
Sequence of oligosaccharides XI and XII estimated by CAE and MALDI-QIT-TOF MS

	CE		Effective concentration (μM) and no interactions (–)						Partial structure	MALDI-QIT-TOF MS	
	GU	Molecular size	SBA	PA-I	RCA_{120}	WGA	UEA-I	AAL		*m/z*	Sequence
XI	3.3	Trisaccharide	3.0	—	—	—	—	—	GalNAcβ-	687	HexNAc-Hex-Hex
XII	3.8	Tetrasaccharide	—	—	—	—	—	—	—	833	HexNAc-(Fuc)Hex-Hex

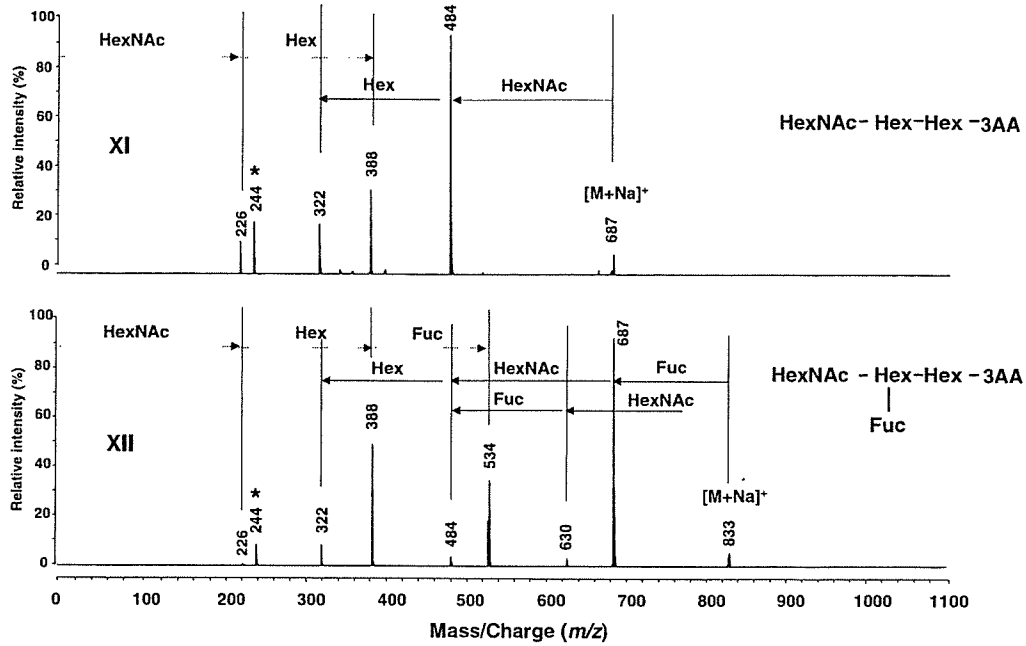

Fig. 7. MS^2 spectra of $[M+Na]^+$ of oligosaccharides **XI** and **XII** obtained from bovine colostrum as precursor ions. The normal and dotted arrows indicate Y and B type cleavage, respectively, according to Domon and Costello's [25] nomenclature. The asterisk indicates the C-type ion. The proposed sequences are shown in the inset.

We deduced that **XII** was a tetrasaccharide based on its migration, 3.8 GU. In the CAE analysis, **XII** did not show any affinity toward the six lectins. MALDI-QIT-TOF MS clearly detected a molecular ion $[M+Na]^+$ at *m/z* 833, indicating that the molecular composition is $Hex_2HexNAc_1Fuc_1$. In the MS/MS spectrum (Fig. 7), a defucosylated Y ion at *m/z* 687, corresponding to a loss of 146, and Y ions at *m/z* 484 and 322, which correspond to losses of 203 (HexNAc-18) and 365 (HexNAc+Hex-36) from *m/z* 687, respectively, were also detected. Y ions at *m/z* 687, 630, and 484 indicate that **XII** is a branched oligosaccharide. B ions at *m/z* 534 (HexNAc+Hex+Fuc+Na-54) and 388 (HexNAc+Hex+Na-36) indicate that the oligosaccharide has a branching Fuc attaching to Hex of HexNAc-Hex. These results indicate that the sequence is HexNAc-[Fuc]-Hex-Hex (Table 2). The product of enzyme degradation of **XII** with α1-2 fucosidase derived from *Corynebacterium* sp. migrated at 3.1 GU, suggesting that the product is the oligosaccharide **9**. The defucosylated product changed its migration in the presence of PA-I and SBA, confirming that the product is GalNAcα1-3Galβ1-4Glc (data not shown). Combined, these results indicate that **XII** is the group A-tetrasaccharide [GalNAcα1-3(Fucα1-2)Galβ1-4Glc].

In summary, we propose the structures GalNAcβ1-Galβ1-4Glc for **XI** and GalNAcα1-3(Fucα1-2)Galβ1-4Glc for **XII**. **XII** is a group A-tetrasaccharide that has not been found in bovine colostrum.

In conclusion, we confirmed that CAE can determine oligosaccharide structures if the library contains the same oligosaccharides. In addition, it is a unique method which can provide information on oligosaccharide structures without isolating individual oligosaccharides. Therefore, CAE is a valuable technique as a screening method. If we discover interesting oligosaccharides by CAE analysis, the oligosaccharides should be isolated and analyzed by a conventional strategy including MS and NMR. However, CAE has several limitations. First, the use of multiple lectin concentrations is required to eliminate false negative results. Therefore, we selected a set of six lectins, the specificities of which are well characterized, and used optimized concentrations. Second, false negative results are also caused by the masking of the epitope with the various types of substations. This drawback is improved by the use of the library, which enables us to clarify the masking effect, i.e., that the binding of RCA_{120}, WGA, PA-I, and UEA-1 is inhibited by Galα1-3 or Fucα1-3, Fucα1-3, Fucα1-2, and Galα1-3 modifications, respectively. Third, this method cannot be used as

a stand-alone method to identify unknown oligosaccharide structures. This is the case, but accumulation and compilation of data in the library are able to minimize the described limitations, increase the number of identifiable oligosaccharides, and establish the comprehensive analysis of oligosaccharides. Therefore, we are compiling data and constructing a Web-based database to utilize CAE more effectively.

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References

[1] M. Messer, T. Urashima, Evolution of milk oligosaccharides and lactose, Trends. Glycosci. Glycotechnol. 14 (2002) 153–176.
[2] T. Urashima, T. Saito, T. Nakamura, M. Messer, Oligosaccharides of milk and colostrums in non-human mammals, Glycoconj. J. 18 (2001) 357–371.
[3] A. Kobata, K. Yamashita, Y. Tachibana, Oligosaccharides from human milk, Methods Enzymol. 50 (1978) 216–220.
[4] T. Urashima, W. Sumiyoshi, T. Nakamura, I. Arai, T. Saito, T. Komatsu, T. Tsubota, Chemical characterization of milk oligosaccharides of the Japanese black bear, *Ursus thibetanus japonicus*, Biochim. Biophys. Acta 1472 (1999) 290–306.
[5] T. Urashima, T. Yamashita, T. Nakamura, I. Arai, T. Saito, A.E. Derocher, O. Wiig, Chemical characterization of milk oligosaccharides of the polar bear, *Ursus maritimus*, Biochim. Biophys. Acta 1475 (2000) 395–408.
[6] T. Urashima, Y. Kusaka, T. Nakamura, T. Saito, N. Maeda, M. Messer, Chemical characterization of milk oligosaccharides of the brown bear, *Ursus arctos yesoensis*, Biochim. Biophys. Acta 1334 (1997) 247–255.
[7] T. Urashima, M. Arita, M. Yoshida, T. Nakamura, I. Arai, T. Saito, J.P. Amould, K.M. Kovacs, C. Lydersen, Chemical characterisation of the oligosaccharides in hooded seal (*Cystophora cristata*) and Australian fur seal (*Arctocephalus pusillus doriferus*) milk, Comp. Biochem. Physiol. B. Biochem. Mol. Biol. 128 (2001) 307–323.
[8] P.K. Gopal, H.S. Gill, Oligosaccharides and glycoconjugates in bovine milk and colostrum, Br. J. Nutr. 84 (2000) S69–S74.
[9] K. Nakajima, Y. Oda, M. Kinoshita, K. Kakehi, Capillary affinity electrophoresis for the screening of post-translational modification of proteins with carbohydrates, J. Proteome Res. 2 (2003) 81–88.
[10] K. Nakajima, M. Kinoshita, Y. Oda, T. Masuko, H. Kaku, N. Shibuya, K. Kakehi, Screening method of carbohydrate-binding proteins in biological sources by capillary affinity electrophoresis and its application to determination of *Tulipa gesneriana* agglutinin in tulip bulbs, Glycobiology 14 (2004) 793–804.
[11] M. Kinoshita, K. Kakehi, Analysis of the interaction between hyaluronan and hyaluronan-binding proteins by capillary affinity electrophoresis: significance of hyaluronan molecular size on binding reaction, J. Chromatogr. B. Anal. Technol. Biomed. Life Sci. 816 (2004) 289–295.
[12] R.W. Veh, J.C. Michalski, A.P. Corfield, M. Sander-Wewer, D. Gies, R. Schauer, New chromatographic system for the rapid analysis and preparation of colostrum sialyloligosaccharides, J. Chromatogr. 212 (1981) 313–322.
[13] S. Kamoda, C. Nomura, M. Kinoshita, S. Nishiura, R. Ishikawa, K. Kakehi, N. Kawasaki, T. Hayakawa, Profiling analysis of oligosaccharides in antibody pharmaceuticals by capillary electrophoresis, J. Chromatogr. A. 1050 (2004) 211–216.
[14] K.R. Anumula, S.T. Dhume, High resolution and high sensitivity methods for oligosaccharide mapping and characterization by normal phase high performance liquid chromatography following derivatization with highly fluorescent anthranilic acid, Glycobiology 8 (1998) 685–694.
[15] K. Kakehi, M. Kinoshita, D. Kawakami, J. Tanaka, K. Sei, K. Endo, Y. Oda, M. Iwaki, T. Masuko, Capillary electrophoresis of sialic acid-containing glycoprotein. Effect of the heterogeneity of carbohydrate chains on glycoform separation using an α1-acid glycoprotein as a model, Anal. Chem. 73 (2001) 2640–2647.
[16] J.U. Baenziger, D. Fiete, Structural determinants of *Ricinus communis* agglutinin and toxin specificity for oligosaccharides, J. Biol. Chem. 19 (1979) 9795–9799.
[17] M. Wimmerova, E. Mitchell, J.F. Sanchez, C. Gautier, A. Imberty, Crystal structure of fungal lectin, J. Biol. Chem. 278 (2003) 27059–27067.
[18] M.E. Pereira, E.A. Kabat, Immunochemical studies on the combining site of the blood group H-specific lectin I from *Ulex europaeus* seeds, Arch. Biochem. Biophys. 185 (1978) 108–115.
[19] B. Lanne, J. Ciopraga, J. Bergstrom, C. Motas, K.A. Karlsson, Binding of the galactose-specific *Pseudomonas aeruginosa* lectin, PA-I, to glycosphingolipids and other glycoconjugates, Glycoconj. J. 4 (1994) 292–298.
[20] K. Yamamoto, T. Tsuji, I. Matsumoto, T. Osawa, Structural requirements for the binding of oligosaccharides and glycopeptides to immobilized wheat germ agglutinin, Biochemistry 20 (1981) 5894–5899.
[21] M.E.A. Pereira, E.A. Kabat, Immunochemical studies on the specificity of soybean agglutinin, Carbohydr. Res. 37 (1974) 89–102.
[22] S. Hase, High-performance liquid chromatography of pyridyl-aminated saccharides, Methods Enzymol. 230 (1994) 225–237.
[23] M. Suzuki, A. Suzuki, Structural characterization of fucose-containing oligosaccharides by high-performance liquid chromatography and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, Biol. Chem. 382 (2001) 251–257.
[24] N. Ojima, K. Masuda, K. Tanaka, O. Nishimura, Analysis of neutral oligosaccharides for structural characterization by matrix-assisted laser desorption/ionization quadrupole ion trap time-of-flight mass spectrometry, J. Mass Spectrom. 40 (2005) 380–388.
[25] B. Domon, C.E. Costello, A systematic nomenclature for carbohydrate fragmentations in FAB-MS/MS spectra of glycoconjugates, Glycoconj. J. 5 (1988) 397–409.

research articles Journal of proteome research

Analysis of Total *N*-Glycans in Cell Membrane Fractions of Cancer Cells Using a Combination of Serotonin Affinity Chromatography and Normal Phase Chromatography

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Cell surface glycans and recognition molecules of these glycans play important roles in cellular recognition and trafficking, such as in the inflammation response by sialyl LewisX oligosaccharides. Malignant cells also utilize a similar mechanism during colonization and establishment of tumor tissues in the host. These considerations prompt us to develop a screening method for comprehensive analysis of *N*-glycans derived from membrane fractions of cancer cells. The method involves two step separations. Initially, *N*-glycans released from cell membrane fractions with *N*-glycoamidase F were labeled with 2-aminobenzoic acid and separated based on the number of sialic acid residues attached to the oligosaccharides using affinity chromatography on a serotonin-immobilized stationary phase. Each of the nonretarded fractions containing asialo- and high-mannose type oligosaccharides and mono-, di-, tri-, and tetra-sialooligosaccharide fractions which were desialylated with neuraminidase was analyzed by a combination of HPLC using an Amide-80 column as the stationary phase and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). We analyzed total *N*-glycan pools of membrane fractions obtained from some cancer cells, and found that U937 cells (Histocytic lymphoma cells) expressed a large amount of oligosaccharides having polylactosamine residues and MKN45 cells (Gastric adenocarcinoma cells) contained hyper-fucosylated oligosaccharides which contained multiple fucose residues. The method described here will be a powerful technique for glycomics studies in cell surface glycoproteins, and will enable one to search marker oligosaccharides characteristically observed in various diseases such as cancer, inflammation, and congenital disorder.

Keywords: cancer cell • *N*-glycan • serotonin-affinity chromatography • polylactosamine-type oligosaccharide • hyperfucosylated oligosaccharides • MALDI-TOF MS

Introduction

Genomic and proteomic approaches are essential for the elucidation of biological functions of proteins. Glycomics is also an emerging field and has been attracting the interest in the research area of glycobiology as the post-genome project.

N-Glycosylation of proteins has been recognized as one of the most common but complex post-translational modification of proteins in eukaryotes.[1] Variation even in the expression of a single glycosyltransferase can alter the glycosylation profile of multiple proteins,[2] and *N*-glycosylation process plays critical roles in protein folding, intracellular transport, and degradation of proteins.[3–5] It has also been revealed that glycans on the cell surface play various key roles in cell–cell interactions and cell proliferations. Because *N*-glycans are synthesized by the actions of many related enzymes, it is difficult to focus the research target on single gene of the enzyme without prior analysis of total *N*-glycans.

Due to the similarities and complex structures of *N*-glycans, separation of oligosaccharides has been a challenging work. Dwek's group achieved excellent works for glycan analysis by HPLC using 2-aminobenzamide as the fluorescent labeling reagent after releasing glycans.[6] Takahashi et al. also analyzed more than 500 *N*-glycans labeled with 2-aminopyridine (PA) by reductive amination method.[7] PA-glycans, which had been previously separated into several groups based on the number of sialic acid residues by anion-exchange chromatography, were analyzed by a combination of normal phase and reversed-phase high performance liquid chromatography (HPLC). Combination with specific exo-glycosidases allowed structure confirmation of unknown oligosaccharides.

We recently reported a strategy for ultrahighly sensitive analysis of carbohydrate chains by capillary affinity electrophoresis using a set of lectins which can discriminate various carbohydrate chains.[8,9] Lectins having broad specificity toward oligosaccharides have also been used to isolate glycopeptides from the digestion mixtures of whole proteins derived from biological sources.[10] Two separate groups reported similar works on the comparison of the relative degree of sialylation

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among human serum glycoproteins carrying N-linked complex-type diantennary, hybrid, and high-mannose oligosaccharides. Comparisons were made by coupling lectin affinity selection with stable isotope coding of peptides from tryptic digests of serum samples.[2,11]

Many research groups attempted to find marker glycans for various diseases such as cancer and inflammation using various techniques as described above. For example, alterations of the carbohydrate chains of glycoproeins, which have been found in a variety of tumors, can be expected to be the basis of various abnormal social behaviors of tumor cells. Comparative studies of the specific carbohydrate chains of glycoproteins, as produced by malignant cells and by their corresponding normal cells, can provide useful information for the diagnosis, prognosis, and immunotherapy of tumors.[12] Callewaert et al. developed a DNA sequence/fragment analyzer-based *N*-glycan profiling method to clinical glycomics, and found that *N*-glycans of the total serum protein yielded an excellent biomarker for the detection of liver cirrhosis.[13]

Biosynthesis of carbohydrate chains is tissue specific and developmentally regulated by glycosyltransferases such as fucosyltransferases.[14] Aberrant glycosylation of membrane components also may be of great relevance in cellular recognition processes and leads to the development of cancer cell subpopulations with different adhesion properties. Enhanced enzyme activity, for example, such as sialyltransferase may cause decrease of cell−cell adhesion. For the studies of such changes on cell surface, a number of histochemical and gene expression studies were reported.[15,16]

On the basis of the considerations described above, we developed a method for comprehensive analysis of *N*-glycans, and applied the method to the analysis of *N*-glycans derived from cancer cell membrane fractions. *N*-Glycans of the cell membrane fractions were released with *N*-glycoamidase F, and fluorescently labeled with 2-aminobenzoic acid (2AA). The 2AA labeled oligosaccharides were separated based on the number of sialic acid residues using serotonin affinity chromatography, and each of the oligosaccharide fractions obtained by serotonin affinity chromatography was analyzed by an Amide-80 column after desialylation with neuraminidase and also by matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF MS). We found that a few cancer cells contained characteristic oligosaccharides which will be a specific marker for malignancy.

Methods and Materials

Materials. Peptide *N*-glycoamidase F was from Roche Diagnostics (Mannheim, Germany). Sephadex LH-20 was from Amerscham Bioscience (Upsala, Sweden). A serotonin-immobilized column was obtained from Seikagaku Kogyo (Chuo-ku, Tokyo, Japan). 2-Aminobenzoic acid (2AA) and sodium cyanoborohydride for fluorescent labeling of oligosaccharides were from Tokyo Kasai (Chuo-ku, Tokyo, Japan). α-Mannosidase, β-galactosidase, and β-*N*-acetylhexosaminidase were from Jack bean, and obtained from Seikagaku Kogyo. Neuraminidase (*Arthrobacter ureafaciens*) was kindly donated from Dr. Ohta (Marukin-Bio, Uji, Kyoto, Japan). Protein inhibitor cocktail for animal cells was obtained from Nakarai Tesque (Nakagyo-ku, Kyoto, Japan). All other reagents and solvents were of the highest grade commercially available or of high-performance liquid chromatography grade. All aqueous solutions were prepared using water purified with a Mili-Q purification system (Millipore, Bedford, MA).

Cell Culture. In the present study, we used HeLa cells (human cervix cancer cells), U937 cells (human T-lymphoma cells), ACHN cells (human kidney glandular cancer cells), MKN45 cells (human gastric cancer cells), A549 cells (human lung cancer cells), and AGS cells (human gastric epithelial cells). The cells (HeLa, ACHN, A549, and AGS) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum and puromycin (Sigma-Aldrich Japan, Minato-ku, Tokyo) at a final concentration of 0.375 μg/mL. U937 cells and MKN45 cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal calf serum. Fetal calf serum was previously kept at 50 °C for 30 min. The cells were cultured at 37 °C under 5% CO_2 atmosphere, and harvested at 80% confluent state. Cells ($n = 10^8$) were washed with phosphate buffered saline (PBS), and collected by centrifugation at 3000 rpm for 20 min.

***N*-Glycan Pool from Cell Membrane Fractions.** Collected cells (1×10^8 cells) were homogenized in 10 mM phosphate buffer (pH 7.3, 5 mL) containing a set of protein inhibitors at 1% concentration with a glass homogenizer for 5 min at 0 °C. A 0.5 M sucrose solution in 0.02 M Tris buffer (pH 7.3, 10 mL) was added to the homogenate, and the mixture was centrifuged at 2000 × *g* for 15 min. The supernatant was collected and centrifuged at 4000 × *g* for 30 min, and the pellet was collected and used as cell membrane fractions. The membrane fractions were suspended in water (400 μL), and a portion (1×10^7 cells, 40 μL) was mixed with an aqueous solution (40 μL) containing 10% SDS and 10% 2-mercaptoethanol. The mixture was kept in the boiling water bath for 10 min. After cooling, 10% NP40 solution (2 μL) and 0.5 M phosphate buffer (pH 7.5, 2 μL) were added. After addition of *N*-glycoamidase F (2 units, 4 μL), the mixture was kept at 37 °C overnight. After keeping the enzyme reaction mixture in the boiling water bath for 10 min, the mixture was centrifuged at 5000 × *g* for 10 min. The supernatant was collected and lyophilized to dryness.

N-Glycans in the lyophilized material were labeled with 2-aminobenzoic acid (2AA) according to the method reported previously.[17,18] Briefly, to the lyophilized material, was added a solution (200 μL) of 2AA and sodium cyanoborohydride, freshly prepared by dissolution of both compounds (30 mg each) in methanol (1 mL) containing sodium acetate and 2% boric acid. The mixture was kept at 80 °C for 1 h. After cooling, the solution was applied to a column of Sephadex LH-20 (1 × 30 cm) equilibrated with 50% aqueous methanol. Earlier eluted fractions showing fluorescence at 410 nm with irradiating at a 335 nm-light were collected and evaporated to dryness. The residue was dissolved in water (100 μL), and a portion (10 μL) was analyzed by HPLC, and the peaks were collected for MS measurement. Another portion was also used for confirmation of the oligosaccharides by capillary electrophoresis and capillary affinity electrophoresis.[8,9]

Serotonin-Affinity Chromatography for Group Separation of Oligosaccharides based on the Number of Sialic Acid Residues. HPLC was performed with a Jasco apparatus equipped with two PU-980 pumps and a Jasco FP-920 fluorescence detector. The *N*-glycan pool obtained from cancer cells as described above was separated based on the number of sialic acid residues using a serotonin-immobilized column (4.6 × 150 mm) with a linear gradient from water (solvent A) to 40 mM ammonium acetate (solvent B) at a flow rate of 0.5 mL/min. Initially, solvent B was used at 5% concentration as eluent for 2 min, and then linear gradient elution was performed to