Available online at www.sciencedirect.com
SCIENCE DIRECT
ELSEVIER
Analytical Biochemistry 348 (2006) 105–114
ANALYTICAL BIOCHEMISTRY
www.elsevier.com/locate/yabio

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

Kazuki Nakajima [a,b], Mitsuhiro Kinoshita [a], Namiko Matsushita [a], Tadasu Urashima [c], Minoru Suzuki [b], Akemi Suzuki [b,*], Kazuaki Kakehi [a]

[a] *Faculty of Pharmaceutical Sciences, Kinki University, Kowakae 3-4-1, Higashiosaka-shi, Osaka 577-8502, Japan*
[b] *Sphingolipid Expression Laboratory, Supra-Biomolecular System Research Group, RIKEN Frontier Research System, Hirosawa 2-1, Wako-shi, Saitama 351-0198, Japan*
[c] *Department of Animal Science, Obihiro University of Agriculture and Veterinary Medicine, Inadacho, Obihiro-shi, Hokkaido 080-8555, Japan*

Received 4 August 2005
Available online 26 October 2005

**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

* Corresponding author. Fax: +81 48 462 4692.
*E-mail address:* aksuzuki@riken.jp (A. Suzuki).

[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.

0003-2697/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2005.10.010

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],

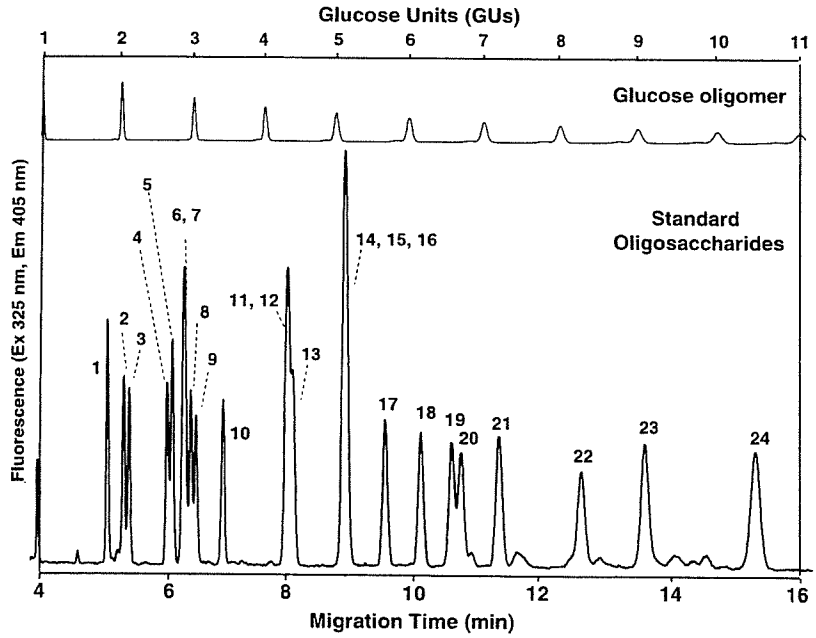


Fig. 1. Analysis of 24 3AA-labeled milk oligosaccharides by capillary electrophoresis. Glucose units (GUs) were determined with glucose oligomers. Analytical conditions: eCAP N-CHO coated capillary; 30 cm length (effective length, 20 cm, 50 μm i.d.); applied potential, 18 kV; injection, pressure method (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.

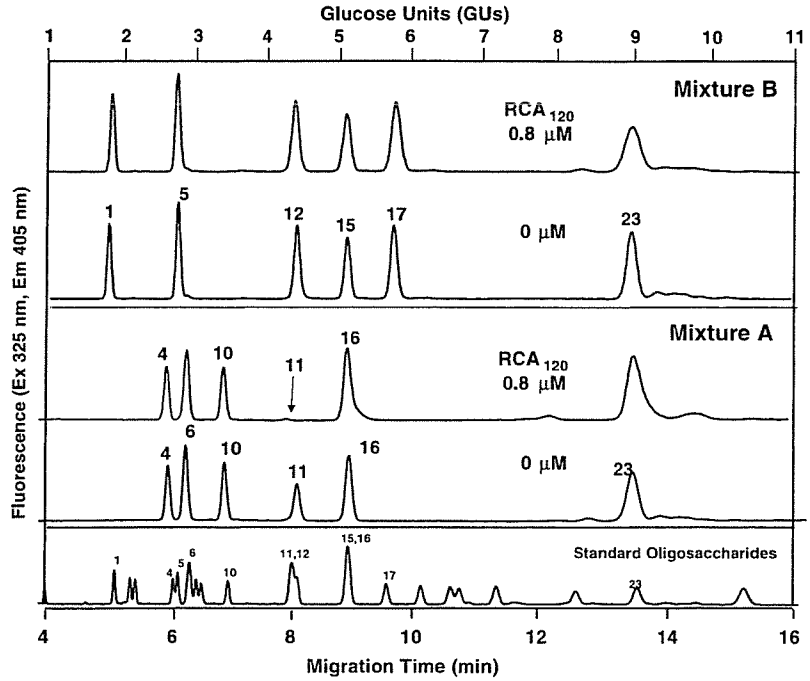


Fig. 2. Capillary affinity electrophoresis for differentiating the linkage isomers of Gal using $RCA_{120}$. The electropherogram of the standard oligosaccharides is shown at the bottom. CAE of A and B mixtures of human milk oligosaccharides was performed in the presence of $RCA_{120}$ at 0 and 0.8 μM 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.

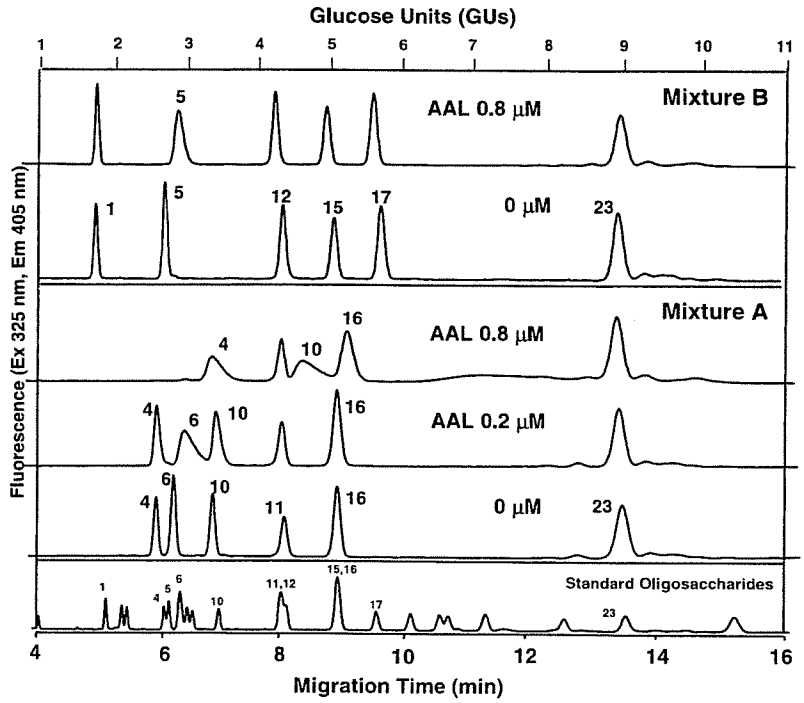


Fig. 3. Capillary affinity electrophoresis for the differentiation of linkage isomers of Fuc using AAL. CAE of A and B mixtures of 3AA-labeled human milk oligosaccharides was performed in the presence of AAL (0, 0.2, and 0.8 μM) in the electrolyte. The analytical conditions were the same as those described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

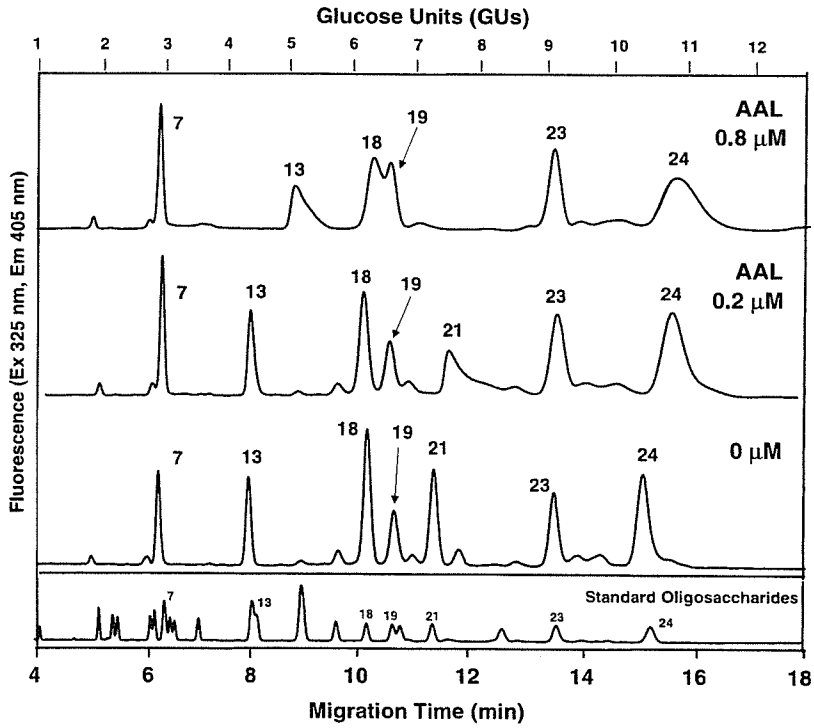


Fig. 4. Capillary affinity electrophoresis for the analysis of 3AA-labeled difucosylated oligosaccharides derived from bear milk using AAL. CAE of bear milk oligosaccharides **7**, **13**, **18**, **19**, **21**, **23**, and **24** was performed in the presence of AAL (0, 0.2, and 0.8 μM) in the electrolyte. 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

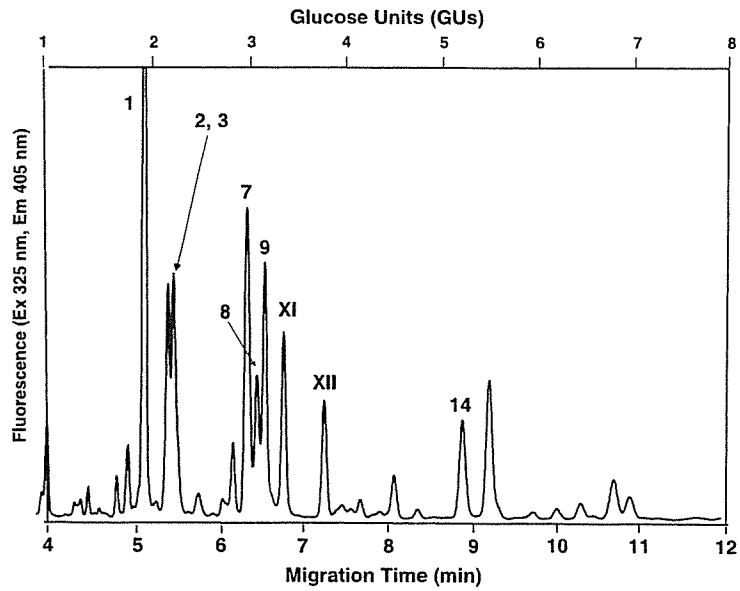


Fig. 5. Analysis of 3AA-labeled neutral oligosaccharides derived from bovine colostrum in the absence of lectin. The analytical conditions were the same as those described in Fig. 1. Peaks **1**, **2**, **3**, **7**, **8**, **9**, and **14** were identified using the library. Peaks **XI** and **XII**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

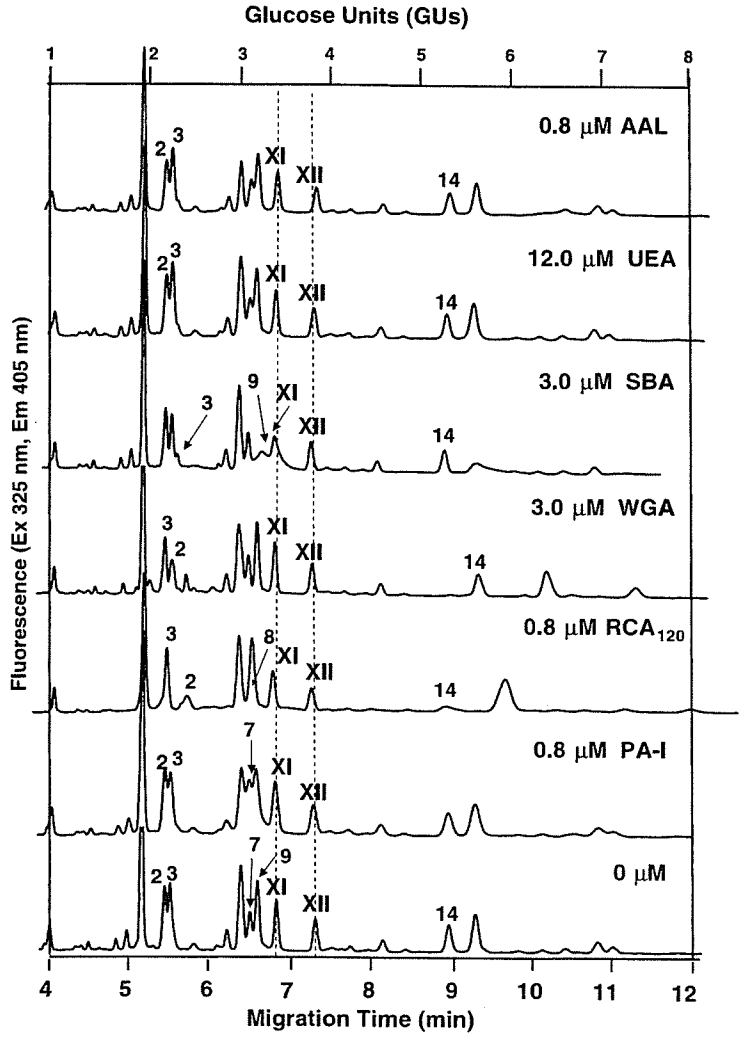


Fig. 6. Capillary affinity electrophoresis for the analysis of 3AA-labeled neutral oligosaccharides using $RCA_{120}$, PA-I, WGA, SBA, UEA-I, and AAL. The electropherogram (0 μM) was the same as that in Fig. 5. Neutral oligosaccharides were characterized in the presence of 3.0 μM SBA, 0.8 μM PA-I, 0.8 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 ($\mu$M) 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 |

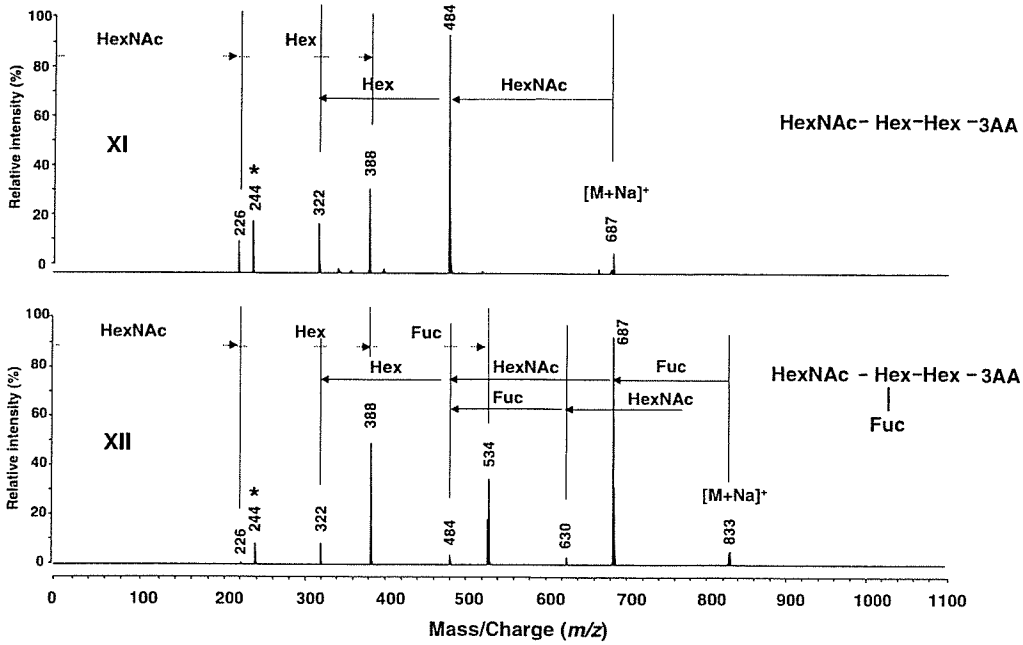


Fig. 7. $MS^2$ spectra of $[M+Na]^+$ of oligosaccharides **XI** and **XII** obtained from bovine colostrum as precursor ions. The normal and dotted arrows indicate Y and B type cleavage, respectively, according to Domon and Costello's [25] nomenclature. The asterisk indicates the C-type ion. The proposed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

## Acknowledgments

We thank Professor T. Tsubota, University of Gifu, Dr. T. Komatsu, Ani Institute of the Japanese Black Bear, Dr. A.T. Derocher, University of Alberta, and Dr. O. Wiig, University of Oslo, for providing bear milk. This work was partly supported by the New Energy and Industrial Technology Organization.

## 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

**Ryosuke Naka,[†] Satoru Kamoda,[†,‡] Aya Ishizuka,[†] Mitsuhiro Kinoshita,[†] and Kazuaki Kakehi*[,†]**

*Faculty of Pharmaceutical Sciences, Kinki University, Kowakae 3–4–1, Higashi-Osaka 577-8502, Japan, and Kirin Brewery Co., Ltd., Hagiwara-machi 100-1, Takasaki 370-0013, Japan*

**Received September 4, 2005**

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

* To whom correspondence should be addressed. E-mail: k_kakehi@phar.kindai.ac.jp.
[†] Kinki University.
[‡] Kirin Brewery Co., Ltd.

10.1021/pr0502976 CCC: $33.50 

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 ($1 \times 10^8$ 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 ($1 \times 10^7$ 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

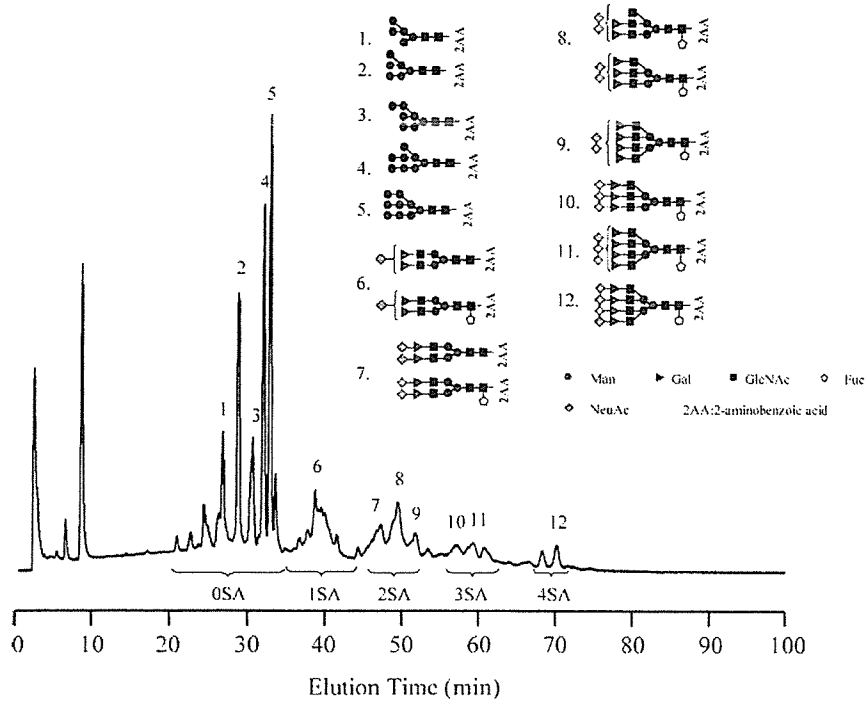


**Figure 1.** Analysis of *N*-glycans released from cell membrane fractions of U937 cells. 0SA, 1SA, 2SA, 3SA, and 4SA indicate that asialo-, monosialo-, disialo-, trisialo-, and tetrasialo-glycans are observed in these regions. Analytical conditions: column, Asahi Shodex NH2P-50 4E(4.6 × 250 mm); eluent, solvent A, 2% $CH_3COOH$ in acetonitrile; solvent B, 5% $CH_3COOH$-3% triethylamine in water; gradient condition: a linear gradient (30–95% solvent B) from 2 to 82 min, maintained for 20 min.

30 mM ammonium acetate for 35 min, and finally the eluent was changed to solvent B (40 mM ammonium acetate) during the following 10 min. Peaks were collected and lyophilized to dryness.

**Preparation of Asialo-Oligosaccharides.** After separation of *N*-glycan pool based on the number of sialic acid residues as described above, oligosaccharide fractions containing sialic acids were digested with neuraminidase.[18] Neuraminidase (2 munits, 4 μL) was added to the mixture of sialooligosaccharides in 20 mM acetate buffer (pH 5.0, 20 μL), and the mixture was incubated at 37 °C overnight. After keeping the mixture in the boiling water bath for 3 min followed by centrifugation, a portion of the supernatant was used for HPLC analysis. It is important to fractionate *N*-glycan pool based on the number of sialic acid residues prior to the separation by normal phase HPLC (see below). It should be noticed that direct digestion of total *N*-glycan pool with neuraminidase makes it difficult to resolve all oligosaccharides.

**HPLC of the 2AA-Labeled Asialo-Oligosaccharides.** The apparatus was the same as described in the analysis of sialic acid-containing oligosaccharides. Separation was done with a polymer-based Asahi Shodex NH2P-50 4E column (Showa Denko, Tokyo, 4.6 × 250 mm) or an Amide-80 column (Tosoh, 4.6 × 250 mm) using a linear gradient formed by 2% acetic acid in acetonitrile (solvent A) and 5% acetic acid in water containing 3% triethylamine (solvent B). The column was initially equilibrated and eluted with 70% solvent A for 2 min, at which point solvent B was increased to 95% over 80 min and kept at this composition for further 100 min.

**MALDI-TOF MS.** MALDI-TOF mass spectra of the labeled oligosaccharides were observed on a Voyager DE-PRO apparatus (PE Biosystems, Framingham, MA). A nitrogen laser was used to irradiate samples at 337 nm, and an average of 50 shots was taken. The instrument was operated in linear mode using negative polarity at an accelerating voltage of 20 kV. An aqueous sample solution (ca. 10 pmol, 0.5 μL) was applied to a polished stainless steel target, to which was added a solution (0.5 μL) of 2,5-dihydroxybenzoic acid in a mixture of methanol–water (1:1). The mixture was dried in atmosphere by keeping it at room temperature for several minutes. The mass numbers of the molecular ion peaks were corrected using a mixture of 2AA labeled dextran oligomers as mass markers.

**Specific Enzyme Reactions for Confirmation of Polylactosamine-Type *N*-Glycans.** Digestion of polylactosamine-type oligosaccharides with β-galactosidase (25 mU, 1 μL) was performed in 20 mM citrate buffer (pH 3.5, 20 μl) at 37 °C for 24 h. After keeping the mixture in the boiling water bath for 5 min followed by centrifugation, a portion (1 μL) of the supernatant was used for MALDI-TOF MS analysis. Digestion with β-*N*-acetylhexosaminidase was also performed in the similar manner in 20 mM citrate buffer (pH 5.0, 20 μL), and analyzed by MALDI-TOF MS method.

## Results and Discussion

**Release of *N*-Glycans from Cell Membrane Fractions and Fluorescent Derivatization with 2AA.** In the present report, glycoproteins derived from membrane fractions were digested with *N*-glycoamidase F according to the manufacturer's protocol. We did not examine if the enzyme released all glycans in glycoproteins because easy operation and fast analysis are the most important for total *N*-glycan analysis. For the same

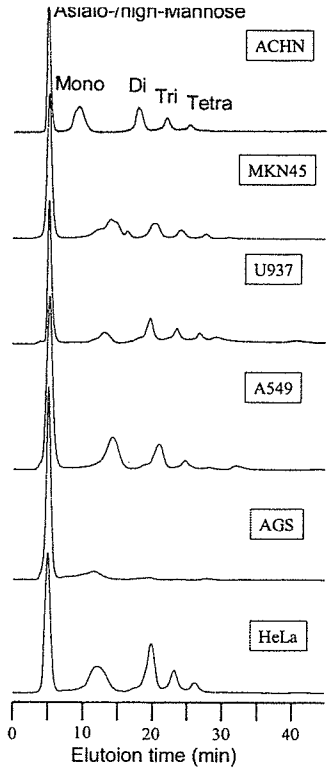


**Figure 2.** Serotonin-affinity chromatography of *N*-glycans from six different cancer cells. Asialo-/high-mannose, mono, di, tri, and tetra indicate that asialo- and high-mannose, monosialo-, disialo-, trisialo-, and tetrasialo-glycans are observed in these regions. Analytical conditions: eluent, solvent A, water; solvent B, 40 mM ammonium acetate; gradient condition, linear gradient (5–75% solvent B) from 2 to 37 min and 75–100% solvent B from 37 to 45 min.

**Table 1.** Relative Ratios of Five Categories of *N*-Glycans in Cancer Cells

| | relative abundance (%) of oligosaccharides | | | | |
|---|---|---|---|---|---|
| cancer cell | high-mannose /asialo | mono-sialo | di-sialo | tri-sialo | tetra-sialo |
| ACHN | 45 | 25 | 18 | 9 | 3 |
| A549 | 56 | 25 | 14 | 4 | 1 |
| MKN45 | 50 | 28 | 13 | 6 | 3 |
| U937 | 62 | 14 | 16 | 5 | 3 |
| AGS | 85 | 12 | 2 | 1 | 0 |
| HeLa | 41 | 25 | 21 | 10 | 3 |

reason, we did not choose hydrazinolysis for glycan release either. In addition, we did not use proteolysis of the membrane fractions with protease prior to digestion with *N*-glycoamidase F to reduce total time required for the analysis.

Although a number of methods have been reported for fluorescent labeling of oligosaccharides,[19–21] we selected 2AA as the labeling reagent due to its high sensitivity and excellent resolution in HPLC of *N*-glycans.[17] In addition, negative charge of the carboxylic acid group of 2AA is useful as a driving force in capillary electrophoresis of the labeled oligosaccharides, and we can employ capillary affinity electrophoresis to obtain structure information by analyzing the interaction between carbohydrates and lectins.[8,9,18]

**Analysis of Total *N*-Glycan Pool Derived from Membrane Fractions of U937 Cells Using a Polymer-Based Amino Column.** In the previous paper on the detailed analysis of sialic acid-containing oligosaccharides derived from α1-acid glycoprotein samples of several different animals, we proposed the use of a polymer-based amino-bonded stationary phase for separation of sialo-oligosaccharides.[18]

In the initial attempt, we analyzed total *N*-glycans released from membrane fractions according to the method described above after digestion with *N*-glycoamidase F followed by labeling with 2AA. Figure 1 shows a typical example observed in the analysis of total *N*-glycans derived from membrane fractions of U937 cells. We observed five peak groups based on the number of sialic acid residues.

The first group observed between 22 and 35 min were due to asialo- and high-mannose type oligosaccharides. Five abundant peaks (**1**–**5**) observed at this region were due to high-mannose type oligosaccharides which were confirmed by comparison of the elution time with standard oligosaccharide samples derived from ribonuclease B and MALDI-TOF MS and α-mannosidase digestion (data not shown). Furthermore, we also used capillary electrophoresis for confirmation of the peaks by comigration with the standard oligosaccharides.[8]

Oligosaccharides having mono-, di-, tri- and tetra-sialic acid residue(s) were observed at 35–43 min, 45–55 min, 56–63 min and 67–73 min, respectively. Because resolution among sialic acid-containing oligosaccharides in each group was incomplete, we confirmed only 12 peaks due to complexity of the peaks even by combination of MALDI TOF-MS and capillary electrophoresis. This is because some sialo-oligosaccharides of di-, tri-, and tetra-antennary types were not fully substituted with sialic acids and various types of multiantennary oligosaccharides were present as complex mixture in each group (see below).

**Group Separation of *N*-Glycan Pool Based on the Number of Sialic Acid Residues.** Takahashi et al. reported a three-dimensional (3-D) sugar-mapping technique for the analysis of pyridylaminated neutral and sialyl oligosaccharides.[7] At the initial step, anion-exchange chromatography was employed for group separation of sialic acid-containing oligosaccharides.

In the present report, we examined the use of serotonin-affinity chromatography for group separation of sialic acid-containing oligosaccharides, because the serotonin-immobilized column for HPLC is commercially available and shows excellent resolution with volatile ammonium acetate buffer. The molecular basis of the interaction between serotonin and *N*-acetylneuraminic acid was examined by proton NMR,[22] and the principle was applied to purification of some glycoproteins.[23] Sturgeon and Sturgeon also reported affinity chromatography of sialoglycoproteins.[24] El Rassi et al. developed the technique to the analysis of sialo-oligosaccharides and gangliosides by high-performance liquid chromatography.[25]

Results obtained from the analysis of *N*-glycans from six cancer cells (ACHN, MKN45, U937, A549, AGS, and HeLa) are shown in Figure 2.

Asialo- and high-mannose oligosaccharides were not retarded by the column and observed early at ca. 5 min. Mono-, di-, tri-, and tetra-sialo-oligosaccharides were observed at ca. 10–15 min, 20, 23, and 27 min, respectively. Relative ratios of these groups of oligosaccharides are summarized in Table 1.

Fractions containing high-mannose and asialo-oligosaccharides (the earliest appearing peaks) were especially abundant

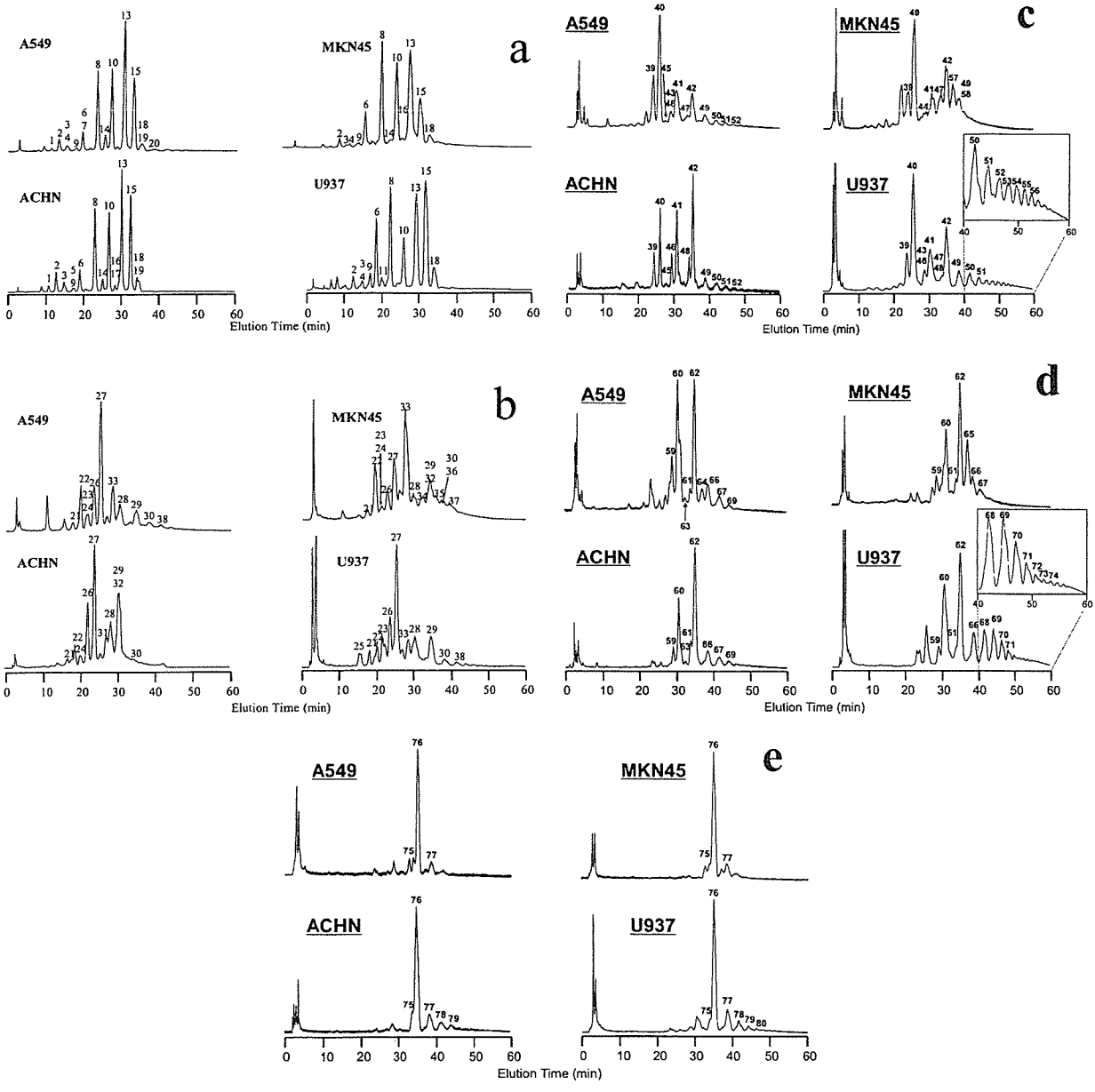


**Figure 3.** Analysis of carbohydrate chains of asialo/high-mannose (a), monosialo- (b), disialo- (c), trisialo-, (d) and tetrasialo-, (e) oligosaccharide fractions obtained by serotonin affinity chromatography. Analytical conditions were the same as described in Figure 1 except for using an Amide80 column as the stationary phase..

(85%) in AGS cells. U937 cells also contained large amount of high-mannose and asialo-oligosaccharides (62%). Other cancer cells contained 40–60% of high-mannose and asialo-oligosaccharides. One-fourth of the total glycans was occupied with mono-sialooligosaccharides in ACHN, A549, MKN45 and HeLa cells, but U937 and AGS cells contained smaller amount (14% and 12%, respectively) of monosialo-oligosaccharides. We have to confirm if differences in relative abundance of these oligosaccharide groups are due to cancer or tissue specific changes, but these data will be useful for classification of cell lines.

**Analysis of *N*-Glycans Resolved by Serotonin Affinity Chromatography.** In the present paper, we focused on the analysis of oligosaccharides derived from ACHN, MKN45, U937 and A549 cells, because these cell lines contained relatively large amount of sialic acid-containing oligosaccharides. The fractions containing sialo-oligosaccharides were analyzed after removing sialic acids with neuraminidase to improve resolutions by HPLC using an Amide80 column. The results are shown in Figure 3.

In nonretarded fractions containing asialo- and high-mannnose type oligosaccharides (Figure 3a), we found large amount of high-mannose oligosaccharides as major oligosaccharides in all cells. Peaks **6**, **8**, **10**, **13**, and **15** are derived from M5, M6, M7, M8, and M9, respectively. Structures of these high-mannose oligosaccharides were easily confirmed by a combination of MALDI-TOF MS and α-mannosidase digestion (data

**Table 2.** List of Oligosaccharides Observed in Cancer Cells

| peak no. | observed molecular mass (calculated molecular mass) | monosaccharide composition | cancer cell |
|---|---|---|---|
| | | Asialo- and High-Mannose Oligosaccharides | |
| 1 | 1029.8 (1031.4) | $Man_3GlcNAc_2$−2AA | A549, ACHN |
| 2 | 1176.9 (1177.4) | $Man_3FucGlcNAc_2$−2AA | A549, ACHN, MKN45, U937 |
| 3 | 1192.8 (1193.4) | $Man_4GlcNAc_2$−2AA | A549, ACHN, MKN45, U937 |
| 4 | 1380.9 (1380.5) | $Man_3FucGlcNAc_3$−2AA | A549, MKN45, U937 |
| 5 | 1395.7 (1396.5) | $Man_3GalGlcNAc_3$−2AA | ACHN |
| 6 | 1356.1 (1355.5) | $Man_5GlcNAc_2$−2AA | A549, MKN45, ACHN, U937 |
| 7 | 1542.0 (1542.6) | $Man_3GalFucGlcNAc_3$−2AA | A549 |
| 8 | 1517.3 (1517.5) | $Man_6GlcNAc_2$−2AA | A549, ACHN, MKN45, U937 |
| 9 | 1583.5 (1583.6) | $Man_3FucGlcNAc_4$−2AA | A549, ACHN, MKN45, U937 |
| 10 | 1679.1 (1679.6) | $Man_7GlcNAc_2$−2AA | A549, ACHN, MKN45, U937 |
| 11 | 1783.5 (1786.7) | $Man_3FucGlcNAc_5$−2AA | U937 |
| 12 | 1761.2 (1761.7) | $Man_3Gal_2GlcNAc_4$−2AA | ACHN |
| 13 | 1830.6 (1841.7) | $Man_8GlcNAc_2$−2AA | A549, ACHN, MKN45, U937 |
| 14 | 1908.0 (1907.7) | $Man_3Gal_2FucGlcNAc_4$−2AA | A549, ACHN, MKN45 |
| 15 | 2003.8 (2003.7) | $Man_9GlcNAc_2$−2AA | A549, ACHN, MKN45, U937 |
| 16 | 2053.8 (2053.8) | $Man_3Gal_2Fuc_2GlcNAc_4$−2AA | ACHN, MKN45 |
| 17 | 2127.1 (2126.8) | $Man_3Gal_3GlcNAc_5$−2AA | ACHN |
| 18 | 2166.3 (2167.8) | $Man_3Gal_2GlcNAc_6$−2AA | A549, ACHN, MKN45, U937 |
| 19 | 2639.6 (2638.0) | $Man_3Gal_4FucGlcNAc_6$−2AA | A549, ACHN |
| 20 | 3004.7 (3003.1) | $Man_3Gal_5FucGlcNAc_7$−2AA | A549 |
| | | Mono-sialooligosaccharides (as Asialoglycans) | |
| 21 | 1394.8 (1396.5) | $NeuAcMan_3GalGlcNAc_3$−2AA | A549, ACHN, MKN45, U937 |
| 22 | 1543.2 (1542.6) | $NeuAcMan_3GalFucGlcNAc_3$−2AA | A549, ACHN, MKN45, U937 |
| 23 | 1556.9 (1558.6) | $NeuAcMan_4GalGlcNAc_3$−2AA | A549, MKN45, U937 |
| 24 | 1746.8 (1745.7) | $NeuAcMan_3GalFucGlcNAc_4$−2AA | A549, ACHN, MKN45 |
| 25 | 1597.6 (1599.6) | $NeuAcMan_3GalGlcNAc_4$−2AA | U937 |
| 26 | 1759.8 (1761.7) | $NeuAcMan_3Gal_2GlcNAc_4$−2AA | A549, ACHN, MKN45, U937 |
| 27 | 1906.3 (1907.7) | $NeuAcMan_3Gal_2FucGlcNAc_4$−2AA | A549, ACHN, MKN45, U937 |
| 28 | 2271.5 (2272.8) | $NeuAcMan_3Gal_3FucGlcNAc_5$−2AA | A549, ACHN, MKN45, U937 |
| 29 | 2636.4 (2638.0) | $NeuAcMan_3Gal_4FucGlcNAc_6$−2AA | A549, ACHN, MKN45, U937 |
| 30 | 3000.9 (3003.1) | $NeuAcMan_3Gal_5FucGlcNAc_7$−2AA | A549, ACHN, MKN45, U937 |
| 31 | 2128.4 (2126.8) | $NeuAcMan_3Gal_3$-$GlcNAc_5$−2AA | ACHN |
| 32 | 2489.8 (2491.6) | $NeuAcMan_3Gal_4$-$GlcNAc_6$−2AA | ACHN, MKN45 |
| 33 | 2052.7 (2053.8) | $NeuAcMan_3Gal_2Fuc_2GlcNAc_4$−2AA | A549, MKN45, U937 |
| 34 | 2417.9 (2418.9) | $NeuAcMan_3Gal_3Fuc_2GlcNAc_5$−2AA | MKN45 |
| 35 | 2563.4 (2565.0) | $NeuAcMan_3Gal_3Fuc_3GlcNAc_5$−2AA | MKN45 |
| 36 | 2929.2 (2930.1) | $NeuAcMan_3Gal_4Fuc_3GlcNAc_6$−2AA | MKN45 |
| 37 | 3149.4 (3149.2) | $NeuAcMan_3Gal_5Fuc_2GlcNAc_7$−2AA | MKN45 |
| 38 | 3368.4 (3368.2) | $NeuAcMan_3Gal_6FucGlcNAc_8$−2AA | A549, U937 |
| | | Di-sialooligosaccharides (as Asialoglycans) | |
| 39 | 1759.9 (1761.7) | $NeuAc_2Man_3Gal_2GlcNAc_4$−2AA | A549, ACHN, MKN45, U937 |
| 40 | 1906.2 (1907.7) | $NeuAc_2Man_3Gal_2FucGlcNAc_4$−2AA | A549, ACHN, MKN45, U937 |
| 41 | 2272.5 (2272.8) | $NeuAc_2Man_3Gal_3FucGlcNAc_5$−2AA | A549, ACHN, MKN45, U937 |
| 42 | 2637.6 (2638.0) | $NeuAc_2Man_3Gal_4FucGlcNAc_6$−2AA | A549, ACHN, MKN45, U937 |
| 43 | 2051.6 (2053.8) | $NeuAc_2Man_3Gal_2Fuc_2GlcNAc_4$−2AA | A549, ACHN |
| 44 | 2067.3 (2069.8) | $NeuAc_2Man_4Gal_2FucGlcNAc_4$−2AA | MKN45 |
| 45 | 2109.1 (2110.8) | $NeuAc_2Man_3Gal_2FucGlcNAc_5$−2AA | A549, ACHN |
| 46 | 2127.1 (2126.8) | $NeuAc_2Man_3Gal_3GlcNAc_5$−2AA | A549, ACHN, U937 |
| 47 | 2418.9 (2418.9) | $NeuAc_2Man_3Gal_3Fuc_2GlcNAc_5$−2AA | A549, MKN45, U937 ] |
| 48 | 2492.0 (2491.9) | $NeuAc_2Man_3Gal_4GlcNAc_6$−2AA | ACHN, U937 |
| 49 | 3004.8 (3003.1) | $NeuAc_2Man_3Gal_5FucGlcNAc_7$−2AA | A549, ACHN, MKN45, U937 |
| 50 | 3368.05 (3368.2) | $NeuAc_2Man_3Gal_6FucGlcNAc_8$−2AA | A549, ACHN, U937 |
| 51 | 3734.5 (3733.4) | $NeuAc_2Man_3Gal_7FucGlcNAc_9$−2AA | A549, ACHN, U937 |
| 52 | 4066.7 (4068.5) | $NeuAc_2Man_3Gal_8FucGlcNAc_{10}$−2AA | A549, ACHN, U937 |
| 53 | 4462.5 (4463.6) | $NeuAc_2Man_3Gal_9FucGlcNAc_{11}$−2AA | U937 |
| 54 | 4826.7 (4828.8) | $NeuAc_2Man_3Gal_{10}FucGlcNAc_{12}$−2AA | U937 |
| 55 | 5195.7 (5193.9) | $NeuAc_2Man_3Gal_{11}FucGlcNAc_{13}$−2AA | U937 |
| 56 | 5557.8 (5559.0) | $NeuAc_2Man_3Gal_{12}FucGlcNAc_{14}$−2AA | U937 |
| 57 | 2783.0 (2784.0) | $NeuAc_2Man_3Gal_4Fuc_2GlcNAc_6$−2AA | MKN45 |
| 58 | 2929.2 (2930.1) | $NeuAc_2Man_3Gal_4Fuc_3GlcNAc_6$−2AA | MKN45 |
| | | Tri-sialooligosaccharides (as Asialoglycans) | |
| 59 | 2128.5 (2126.8) | $NeuAc_3Man_3Gal_3GlcNAc_5$−2AA | A549, ACHN, MKN45, U937 |
| 60 | 2271.7 (2272.8) | $NeuAc_3Man_3Gal_3FucGlcNAc_5$−2AA | A549, ACHN, MKN45, U937 |
| 61 | 2490.3 (2491.9) | $NeuAc_3Man_3Gal_4$-$GlcNAc_6$−2AA | A549, ACHN, MKN45, U937 |
| 62 | 2636.9 (2638.0) | $NeuAc_3Man_3Gal_4FucGlcNAc_6$−2AA | A549, ACHN, MKN45, U937 |
| 63 | 2476.2 (2475.9) | $NeuAc_3Man_3Gal_3FucGlcNAc_6$−2AA | A549, ACHN |
| 64 | 2563.6 (2565.0) | $NeuAc_3Man_3Gal_3Fuc_3GlcNAc_5$−2AA | A549 |
| 65 | 2783.2 (2784.0) | $NeuAc_3Man_3Gal_4Fuc_2GlcNAc_6$−2AA | MKN45 |
| 66 | 3003.6 (3003.1) | $NeuAc_3Man_3Gal_5FucGlcNAc_7$−2AA | A549, ACHN, MKN45, U937 |

**Table 2.** (Continued)

| peak no. | observed molecular mass (calculated molecular mass) | monosaccharide composition | cancer cell |
|---|---|---|---|
| 67 | 3150.7 (3149.1) | $NeuAc_3Man_3Gal_5Fuc_2GlcNAc_7$−2AA | A549, ACHN, MKN45 |
| 68 | 3366.5 (3368.2) | $NeuAc_3Man_3Gal_6Fuc$-$GlcNAc_8$−2AA | U937 |
| 69 | 3727.9 (3733.4) | $NeuAc_3Man_3Gal_7FucGlcNAc_9$−2AA | A549, ACHN, U937 |
| 70 | 4097.3 (4098.5) | $NeuAc_3Man_3Gal_8FucGlcNAc_{10}$−2AA | U937 |
| 71 | 4462.2 (4463.6) | $NeuAc_3Man_3Gal_9FucGlcNAc_{11}$−2AA | U937 |
| 72 | 4826.9 (4828.8) | $NeuAc_3Man_3Gal_{10}FucGlcNAc_{12}$−2AA | U937 |
| 73 | 5192.6 (5193.9) | $NeuAc_3Man_3Gal_{11}FucGlcNAc_{13}$−2AA | U937 |
| 74 | 5557.6 (5559.0) | $NeuAc_3Man_3Gal_{12}FucGlcNAc_{14}$−2AA | U937 |
| | | Tetra-sialooligosaccharides (as Asialoglycans) | |
| 75 | 2492.8 (2491.9) | $NeuAc_4Man_3Gal_4GlcNAc_6$−2AA | A549, ACHN, MKN45, U937 |
| 76 | 2636.9 (2638.0) | $NeuAc_4Man_3Gal_4FucGlcNAc_6$−2AA | A549, ACHN, MKN45, U937 |
| 77 | 3000.8 (3003.1) | $NeuAc_4Man_3Gal_5FucGlcNAc_7$−2AA | A549, ACHN, MKN45, U937 |
| 78 | 3366.5 (3368.2) | $NeuAc_4Man_3Gal_6FucGlcNAc_8$−2AA | ACHN, U937 |
| 79 | 3731.6 (3733.4) | $NeuAc_4Man_3Gal_7FucGlcNAc_9$−2AA | ACHN, U937 |
| 80 | 4096.7 (4098.5) | $NeuAc_4Man_3Gal_8FucGlcNAc_{10}$−2AA | U937 |

not shown). Proposed list of the structures derived from oligosaccharide peaks are shown in Table 2. Although their linkage positions and configurations were arbitrarily assigned as indicated in Figure 4, the structures of the oligosaccharides were also confirmed by comparison of the migration times with those of standard samples using capillary electrophoresis as reported in the previous paper.[8,9,26−28]

The relative abundances of these high-mannose oligosaccharides were different among cancer cells but the difference among cells was not significant. We also found some small oligosaccharides (**1**−**5**) that are partial structures of asialoglycans of di, tri- and tetra-antennary oligosaccharides. Further studies will be necessary if these small oligosaccharides are the characteristic products of cancer cells.

After neuraminidase digestion of mono-, di-, tri-, and tetrasialooligosaccharides, each group of oligosaccharides was analyzed by HPLC using an Amide80 column. In monosialooligosaccharide fractions (Figure 3b), peak **27** derived from diantennary oligosaccharide having a fucose residue was abundant in A549, U937, and ACHN cells, and the structure was confirmed by comparison with the oligosaccharide obtained from IgG.[9] Interestingly, MKN45 cells contained a characteristic peak (**33**) derived from the monosialo-diantennary oligosaccharide containing two fucose residues as major oligosaccharide. Structure characteristics of the oligosaccharide was confirmed by capillary affinity electrophoresis using two fucose-specific lectins (RSL for the fucose residue linked to the reducing terminal through α1−6 linkage[29] and AAL for the fucose residue of LewisX structure[30] (data not shown). Furthermore, we found tri- and tetra-antennary oligosaccharides containing three fucose residues (peak **35** and peak **36**) as minor oligosacchrides in MKN45 and ACHN cells. ACHN cells contained a large amount of peak **32**, which was derived from a complex-type monosialo tetra-antennary oligosaccharide. At the same elution time, we also detected small amount of

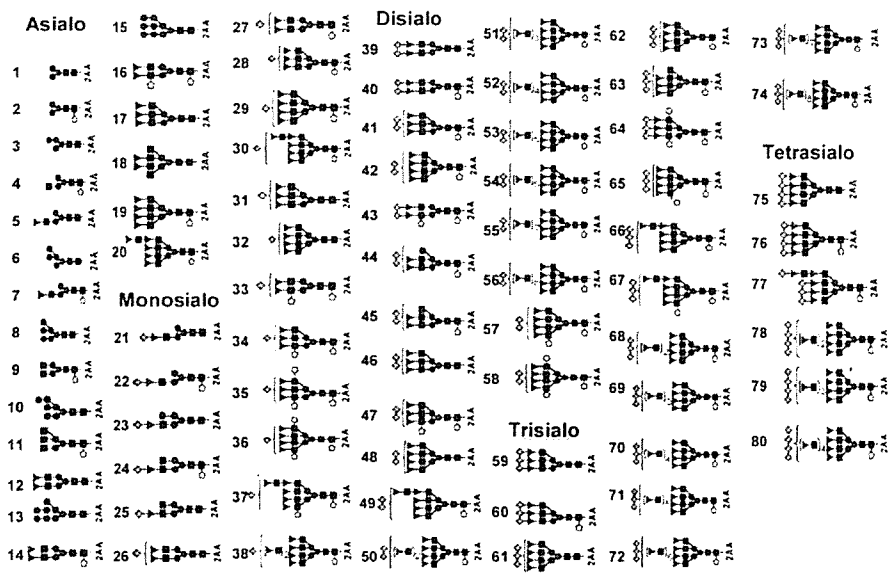


**Figure 4.** List of the structures of oligosaccharides found in membrane fractions of four cancer cells. Asialo/high-mannose, monosialo, disialo, trisialo, and tetrasialo-oligosaccharides are marked in yellow, green, blue, orange, and purple colors. Linkage positons are tentatively assigned.

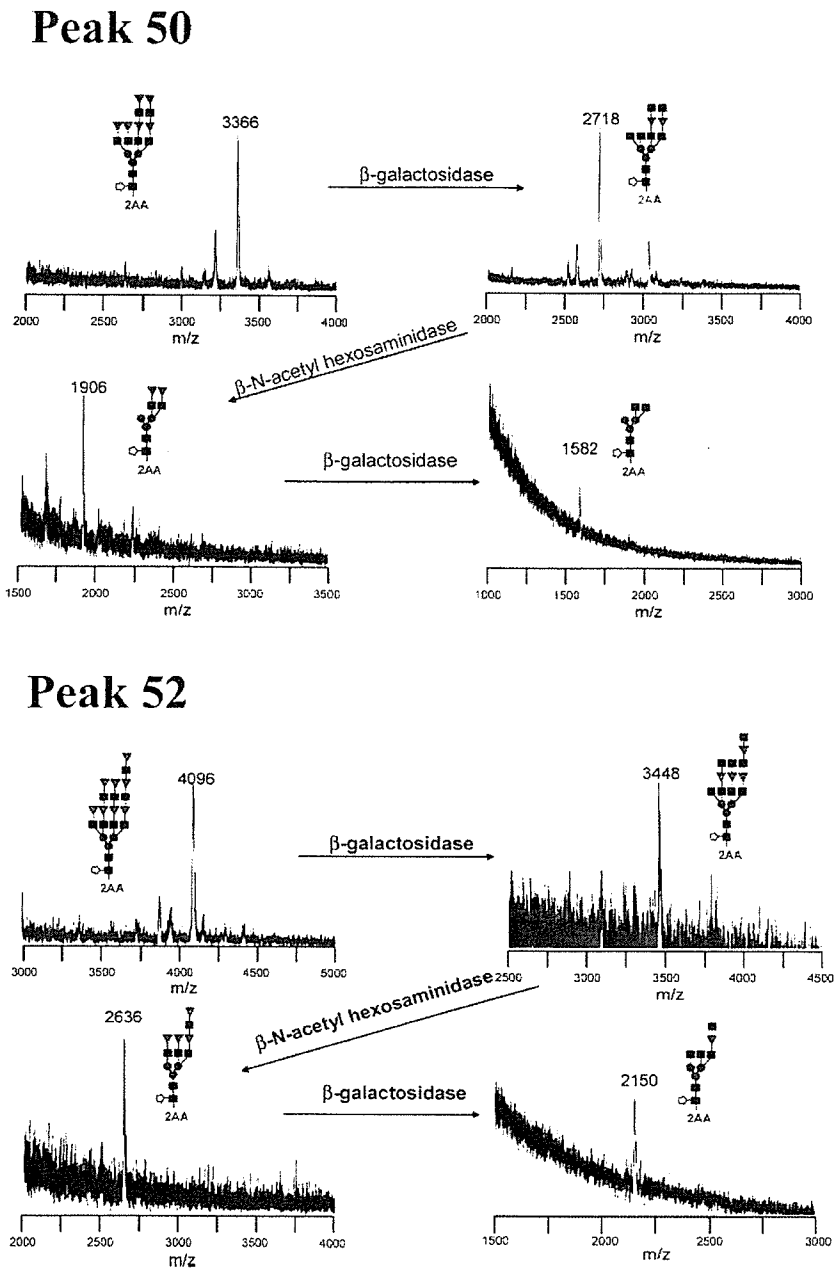


**Figure 5.** Stepwise exoglycosidase digestion of polylactosamine-type oligosaccharides derived from peak 50 and peak 52 observed in Figure 3. Conditions for enzymatic digestion of *N*-glycans with *β*-galactosidase and *β*-*N*-acetylhexosaminidase are shown in the Experimental Section.

monosialo tetra-antennary oligosaccharide containing a fucose residue (**29**).

In disialo-oligosaccharide fractions (Figure 3c), A549, MKN45, and U937 cells contained a diantennary oligosaccharide containing a fucose residue (peak **40**) as the major oligosaccharide. In contrast, ACHN showed characteristic oligosaccharide profile, and contained tri- and tetra-antennary oligosaccharides containing a fucose residue (peak **41** and peak **42**) as well as peak **40**. Oligosaccharides derived from U937 cells showed interesting and characteristic ladder peaks between 40 and 60 min. These ladder peaks were due to tetra-antennary oligosaccharides having different numbers of lactosamine residues (see below).

In trisialo-oligosaccharide fractions (Figure 3d), all cell lines commonly contained tri- and tetra-antennay oligosaccharides having a fucose residue as major oligosaccharides (**60** and **62**). We found an oligosaccharide (**74**) having large molecular mass (*m*/*z* 5556 by MALDI-TOF−MS) in U937 cells which contained characteristic ladder peaks as also observed in disialo-oligosaccharide fractions. These ladder peaks from peak **68** to peak **74** were due to tetraantennary oligosaccharides having multiple poly-lactosamine residues (for confirmation of the structure, see below).

In tetrasialo-oligosaccharide fractions (Figure 3e), we found that tetraantennary oligosaccharide having a fucose residue (**76**) was a major oligosaccharide in all cancer cells.

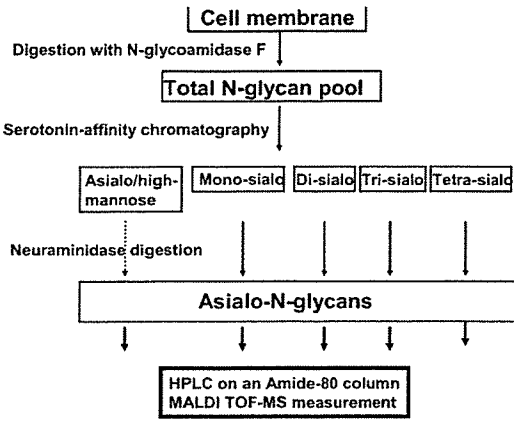


**Figure 6.** Outline of the analysis of total *N*-glycans of cancer cell membrane fractions.

By combination of serotonin affinity chromatography of sialo-oligosaccharides and normal phase chromatography using an Amide80 column as the stationary phase, we identified 80 kinds of oligosaccharides as shown in Table 2. Modification of *N*-glycans with various numbers of sialic acid and fucose residues was significant in complex-type oligosaccharides. Tetra-antennary oligosaccharides containing multiple lactosamine residues (see Figure 3c,d) have di- or tri-sialic acid residues, and one or two branches of these oligosaccharides were not substituted with sialic acids. Further studies are required to understand the reasons why sialic acids do not fully occupy the branches.

**Structure Analysis of the Oligosaccharides which Showed Ladder Peaks in U937 Cells.** As described above, we found that polylactosamine-type oligosaccharides were abundantly present in U937 cells, and peak **50** and peak **52** were collected as nearly pure state (see Figure 5).

The oligosaccharides **50** and **52** showed molecular ions at *m/z* 3366 and *m/z* 4096, respectively, by MALDI-TOF mass spectrometry as shown in Figure 5. We analyzed the structures of these oligosaccharides by combination of digestion with specific glycosidases and MALDI-TOF MS. Digestion of **50** with $\beta$-galactosidase caused loss of four galactose residues (648 mass units), and the product showed a molecular ion at *m/z* 2718. This result indicates that **50** is a tetra-antennary oligosaccharide and galactose residues occupy all branches. The oligosaccharide showing molecular ion at *m/z* 2718 was then digested with $\beta$-*N*-acetylhexosaminidase, and a new peak was observed at *m/z* 1906 by loss of four *N*-acetylglucosamine residues. The oligosaccharide at *m/z* 1906 was further digested with $\beta$-galactosidase to produce a peak at *m/z* 1582. This result indicated that two galactose residues were released. From these results, we concluded that the oligosaccharide derived from peak **50** was a tetraantennary oligosaccharide to which a fucose residue was linked to the GlcNAc in the reducing end, and two of the branches were substituted with lactosamine residues as shown in Figure 4. In the similar manner, we analyzed the structure of **52**. The oligosaccharide **52** showed a molecular ion peak at *m/z* 4096, which was larger than the oligosaccharide **52** by 770 mass units which were obviously due to two lactosamine residues. Digestion of **52** with $\beta$-galactosidase caused loss of four galactose reidues, and a new ion peak was observed at *m/z* 3448. The oligosaccharide at *m/z* 3448 was then digested with $\beta$-*N*-acetylhexosaminidase to afford a peak at *m/z* 2636. The peak was again digested with $\beta$-galactosidase to afford a peak at *m/z* 2150. The loss of 486 mass units indicates that three galactose residues were released. These course of digestion by a combination of exoglycosidases revealed that the oligosaccharide **52** was a tetraantennary oligosaccharide. One of the branches was substituted with one lactosamine residue and two of the branches were modified with tetrasaccharide

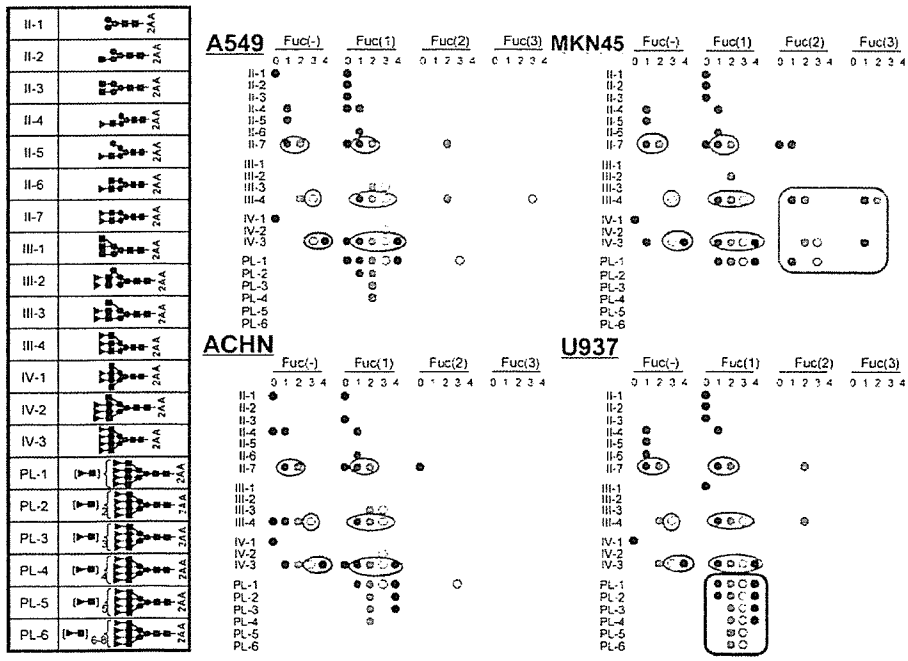


**Figure 7.** Oligosaccharide profiles in cell membrane fractions of A549, MKN45, ACHN, and U937 cells. The oligosaccharides marked in blue circle were commonly found in all cancer cells, those marked in green and red squares were characteristically found in MKN45 cells and U937 cells, respectively.

having two lactosamine residues. The last branch was substituted with a hexasaccharide composed from three lactosamine residues.

## Conclusion

In the present paper, we developed a method for comprehensive analysis of *N*-glycans present in cell membrane fractions, and applied the technique to the analysis of *N*-glycans derived from cancer cell membrane fractions. The overall procedures are summarized in Figure 6.

The total *N*-glycan pool was obtained by digestion with *N*-glycoamidase F, and separated based on the number of sialic acid residues by serotonin-affinity chromatography. Relative abundances of neutral (i.e., high-mannose and asialo-complex oligosaccharides), mono-, di-, tri- and tetra-sialooligosaccharides were characteristic for cancer cell lines.

After digestion of sialic acid-containing oligosaccharide fractions with neuraminidase, each oligosaccharide fraction was analyzed using an Amide80 column. We observed characteristic *N*-glycan profiles in each cell line as shown in Figure 3, and arbitrarily assigned their structures as shown in Table 2 and Figure 4. High-mannose oligosaccharides were commonly observed in all cells. In addition, most of the complex-type oligosaccharides were also commonly present in all tumor cell lines examined in the present study. In contrast, we found characteristic oligosaccharides which contained multi-fucose residues in MKN45 cells. In U937 cells, polylactosamine-type oligosaccharides were abundantly observed. The results are summarized in Figure 7.

The column in the left side of Figure 7 shows the list of complex-type asialooligosaccharides found in A549, MKN45, ACHN, and U937 cells. The oligosaccharides are further categorized whether they contain fucose residue(s). Oligosaccharides marked in blue circle were commonly observed in all cancer cells. Oligosaccharides which contain multiple fucose residues were observed in MKN45 cells (see green square). U937 cells contain tetraantennary oligosaccharides to which polylactosamine residues are attached (see red square).

In conclusion, the method developed in the present paper will enable comprehensive analysis of oligosaccharides in cancer cells, and will be a powerful tool for finding specific oligosaccharides to cancer cells. Presence of fucose-rich oligosaccharides in MKN45 cells and polylactosamine-rich oligosaccharides in U937 cells strongly suggests the (over)-expression of the related enzymes which may be marker proteins for these malignancies. At present, we do not have examined total *N*-glycans from normal (control) cells, and cannot conclude that these oligosaccharides are due to malignancy or tissue specific. However, Taniguchi et al. reported that GnT-V ($\beta$-*N*-acetylglucosaminyltransferase) responsible for biosynthesis of tetraantennary oligosaccharides overexpressed in some cancer tissues.[31,32] Expression of a large amount of tetra-antennay polylactosamine-type oligosaccharides is interesting in relation to prognosis and histology to malignancy. LewisX and sialyl-LewisX structure [Gal$\beta$1−4(Fuc$\alpha$1−3)GlcNAc core sequence] were also reported to be often overexpressed in human carcinomas.[33] At present, we do not have any evidence on their biological roles, but further studies on carbohydrate chains of various cancer cells and normal tissues may reveal their significance in diagnostic studies. Furthermore, regulation studies of these enzymes responsible for biosynthesis of these *N*-glycans may lead to a method for treatment of cancers.

**Acknowledgment.** This work was supported by Grant-in-Aid of the New Energy and Industrial Technology Development Organization (NEDO) and Research on Health Science focusing on Drug Innovation from The Japan Health Science Foundation.

## References

(1) Varki, A. *Glycobiology* **1992**, *2*, 25−40.
(2) Qui, R.; Regnier, F. E. *Anal. Chem.* **2005**, *77*, 2802−2809.
(3) Frigerio, L.; Lord, J. M. *Curr. Biol.* **2000**, *21*, R674 − R677.
(4) Parodi, A. J. *Annu. Rev. Biochem.* **2000**, *69*, 69−93.
(5) Helenius, A.; Aebi, M. *Science* **2001**, *291*, 2364−2369.
(6) Burker, M.; Quelhas, D.; Critchley, A. J.; Carchon, H.; Heberstreit, H. F.; Hibbert, R. G.; Vilarinho, L.; Teles, E.; Malthijs, G.; Scholken, E.; Argibay, P.; Harvey, D. J.; Dwek, R. A.; Jacken, J.; Rudd, P. M. *Glycobiology* **2003**, *13* (9), 601−622.
(7) Takahashi, N.; Nakagawa, H.; Fujikawa, K.; Kawamura, Y.; Tomiya, N. *Anal. Biochem.* **1995**, *226*, 139−146.
(8) Nakajima, K.; Oda, Y.; Kinoshita, M.; Kakehi, K. *J. Proteome Res.* **2003**, *2*, 81−88.
(9) Nakajima, K.; kinoshita, M.; Oda, Y.; Masuko, T.; Kaku, H.; Shibuya, N.; Kakehi, K. *Glycobiology* **2004**, *14*, 793−804.
(10) Hirabayashi, J.; Kasai, K. *J. Chromatogr. B.* **2002**, *771*, 67−87.
(11) Madera, M.; Mechref, Y.; Novotny, M. V. *Anal. Chem.* **2005**, in press (Web released).
(12) Kobata, A.; Amano, J. *Immunol. Cell Biol.* **2005**, *83*, 429−439.
(13) Calleaert, N.; Van Vlierberghe, H.; Van Hecke, A.; Laroy, W.; Delanghe, J.; Contreras, R. *Nat. Med.* **2004**, *10* (4), 429−434.
(14) Petretti, T.; Kemmner, W.; Schlze, B.; Schlag, P. M. *Gut* **2000**, *46*, 359−366.
(15) Ishida, H.; Togayachi, A.; Sakai, T.; Iwai, T.; Hiruma, T.; Sato, T.; Okubo, R.; Inaba, N.; Kudo, T.; Gotoh, M.; Shoda, J.; Tanaka, N.; Narimatsu, H. *FEBS Lett.* **2005**, *579*, 71−78.
(16) Kanoh, A.; Ota, M.; Narimatsu, H.; Irimura, T. *Biochem. Biophys. Res. Commun.* **2003**, *303*, 896−901.
(17) Anumula, K. R.; Dhume, S. T. *Glycobiology* **1998**, *8* (7), 685−694.
(18) Nakano, M.; Kakehi, K.; Tsai, M. H.; Lee, Y. C. *Glycobiology* **2004**, *14* (3), 431−441.
(19) Anumula, K. R. *Anal. Biochem.* **2000**, *283*, 17−26.
(20) Rudd, P. M.; Dwek, R. A. *Curr. Opin. Biotechnol.* **1997**, *8*, 488−497.
(21) Hase, S. *J. Chromatogr. A* **1996**, *720*, 173−182.
(22) Berry, L. R.; Puzzuoli, F. V.; Hatton, M. W. *Can. J. Biochem. Cell Biol.* **1985**, *63*, 757−763.
(23) Pask-Hughes, R. A. *J. Chromatogr.* **1987**, *393*, 273−284.
(24) Sturgeon, R. J.; Sturgeon, C. M. *Carbohydr. Res.* **1982**, *103*, 213−219.
(25) El Rassi, Z.; Horvath, C.; Yu, R. K.; Ariga, T. *J. Chromatogr.* **1989**, *488*, 229−236.
(26) Sei, K.; Nakano, M.; Kinoshita, M.; Masuko, T.; Kakehi, K. *J. Chromatogr. A.* **2002**, *958*, 273−281.
(27) Kinoshita, M.; Murakami, E.; Oda, Y.; Funakubo, T.; Kawakami, D.; Kakehi, K.; Kawasaki, N.; Morimoto, K.; Hayakawa, T. *J. Chromatogr. A* **2000**, *866*, 261−271.
(28) Kakehi, K.; Funakubo, T.; Suzuki, S.; Oda, Y.; Kitada, Y. *J. Chromatogr.* **1999**, *863*, 205−218.
(29) Yasuo Oda; Tsutomu Senaha; Yuuki Matsuno; Kazuki Nakajima; Ryousuke Naka; Mitsuhiro Kinoshita; Eiko Honda; Itaru Furuta; Kakehi, K. *J. Biol. Chem.* **2003**, *278*, 32439−32447.
(30) Nakajima, K.; Kinoshita, M.; Matsuhita, N.; Urashima, T.; Suzuki, M.; Suzuki, A.; Kakehi, K. *Anal. Biochem* **2005**, in press.
(31) Dosaka-Akita, H.; Miyoshi, E.; Suzuki, O.; Itoh, T.; Katoh, H.; Taniguchi, N. *Clin. Cancer Res.* **2004**, *10*, 1773−1779.
(32) Dennis, J. W.; Granovsky, M.; Warren, C. E. *Biochim. Biophys. Acta* **1999**, *1473*, 21−34.
(33) Itzkowitz, S. H.; Yuan, M.; Fukushi, Y.; Palekar, A.; Phelphs, P. C.; Shamuddin, A. M.; Trumps, B. F.; Hakomori, S.; Kim, Y. S. *Cancer Res.* **1986**, *46*, 2627−2632.

PR0502976