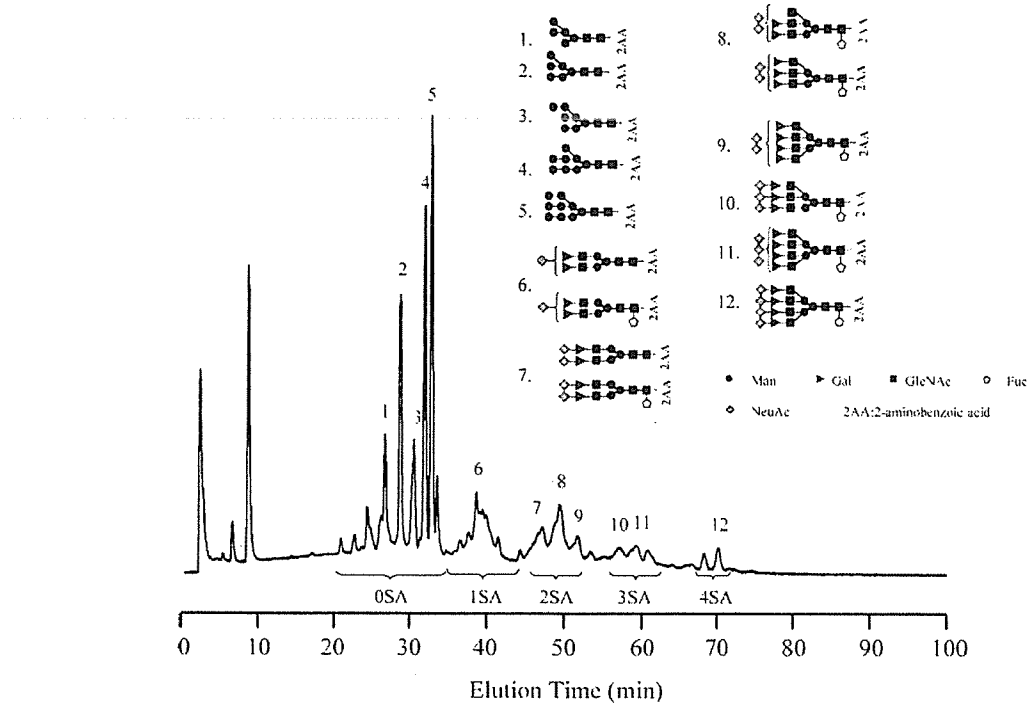

Figure 1. Analysis of *N*-glycans released from cell membrane fractions of U937 cells. 0SA, 1SA, 2SA, 3SA, and 4SA indicate that asialo-, monosialo-, disialo-, trisialo-, and tetrasialo-glycans are observed in these regions. Analytical conditions: column, Asahi Shodex NH2P-50 4E(4.6 × 250 mm); eluent, 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

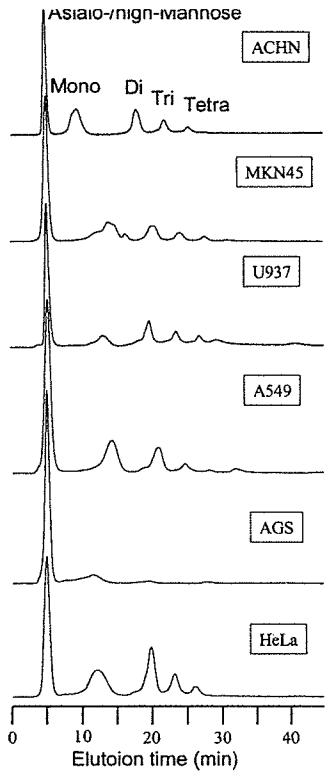

Figure 2. Serotonin-affinity chromatography of *N*-glycans from six different cancer cells. Asialo-/high-mannose, mono, di, tri, and tetra indicate that asialo- and high-mannose, monosialo-, disialo-, trisialo-, and tetrasialo-glycans are observed in these regions. Analytical conditions: eluent, solvent 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

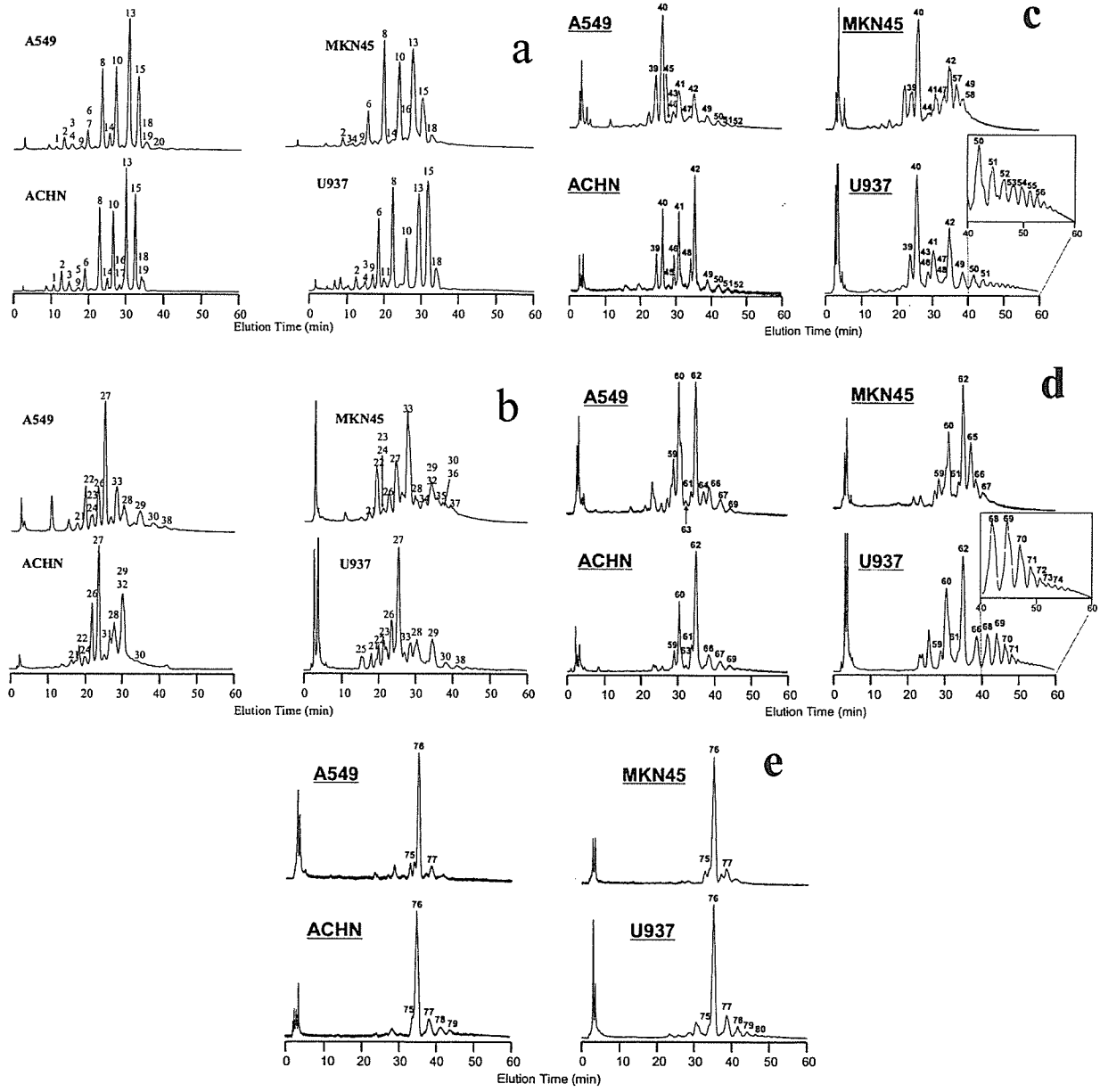

Figure 3. Analysis of carbohydrate chains of asialo/high-mannose (a), monosialo- (b), disialo- (c), trisialo-, (d) and tetrasialo-, (e) oligosaccharide fractions obtained by serotonin affinity chromatography. Analytical conditions were the same as described in Figure 1 except for using an Amide80 column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

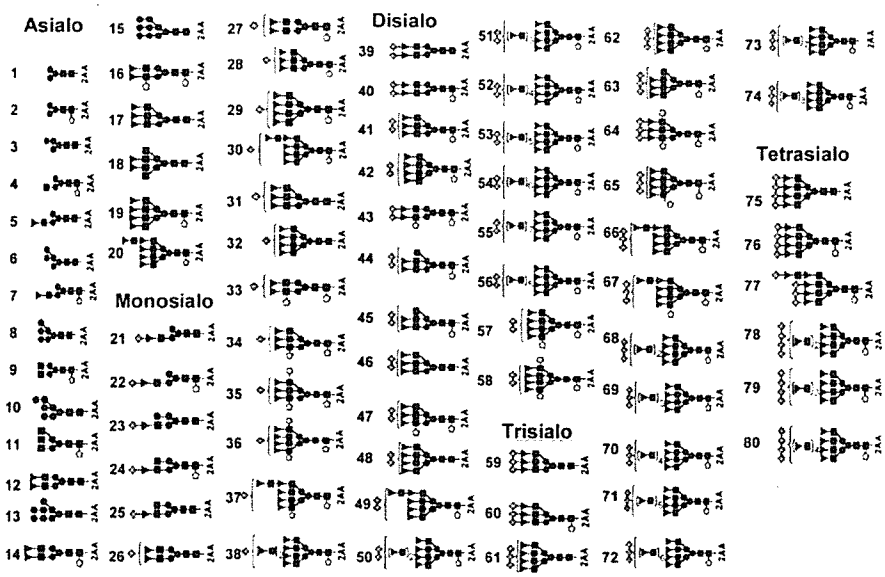

Figure 4. List of the structures of oligosaccharides found in membrane fractions of four cancer cells. Asialo/high-mannose, monosialo, disialo, trisialo, and tetrasialo-oligosaccharides are marked in yellow, green, blue, orange, and purple colors. Linkage positons are tentatively assigned.

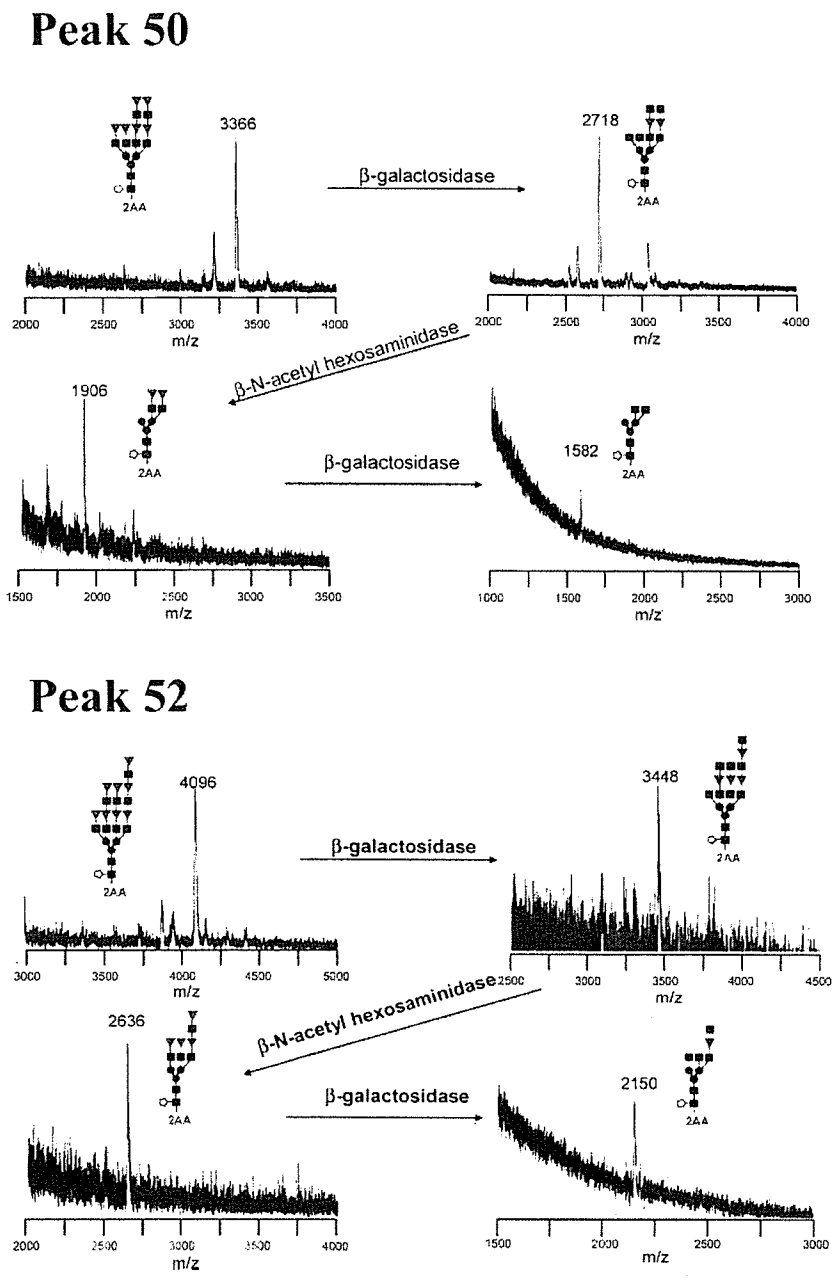

Figure 5. Stepwise exoglycosidase digestion of polylactosamine-type oligosaccharides derived from peak 50 and peak 52 observed in Figure 3. Conditions for enzymatic digestion of *N*-glycans with *β*-galactosidase and *β*-*N*-acetylhexosaminidase are shown in the Experimental Section.

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.

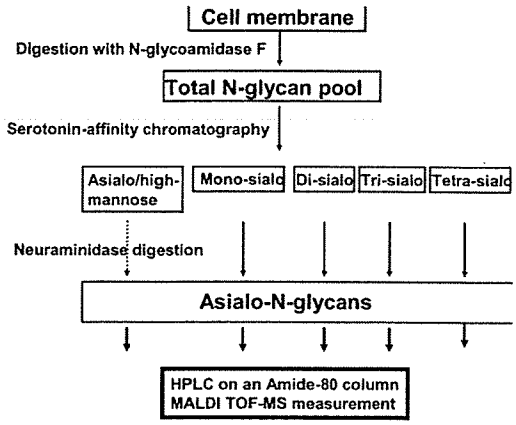

Figure 6. Outline of the analysis of total *N*-glycans of cancer cell membrane fractions.

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 β-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 β-*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 β-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 β-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 β-*N*-acetylhexosaminidase to afford a peak at *m/z* 2636. The peak was again digested with β-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

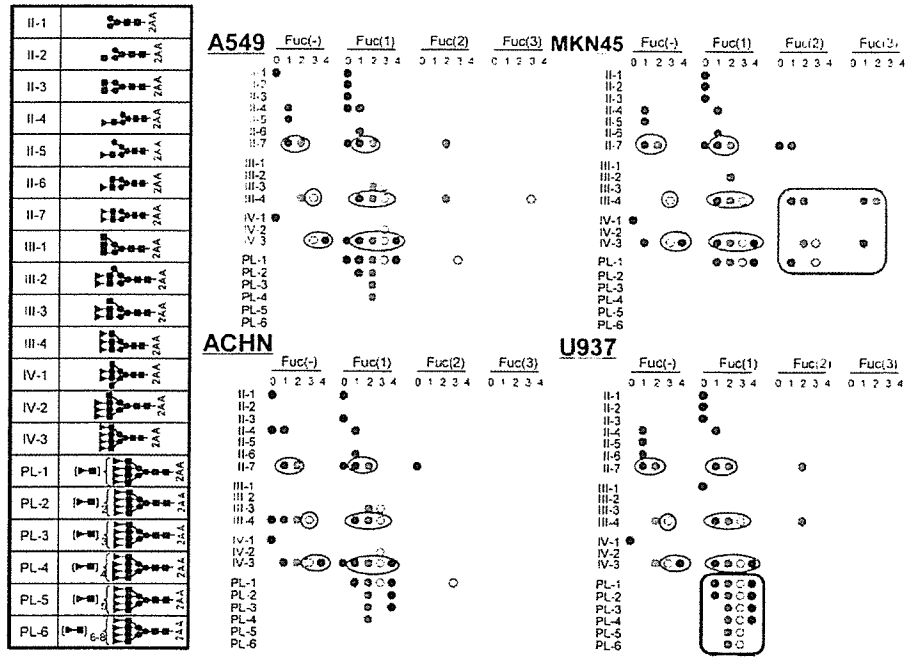

Figure 7. Oligosaccharide profiles in cell membrane fractions of A549, MKN45, ACHN, and U937 cells. The oligosaccharides marked in blue circle were commonly found in all cancer cells, those marked in green and red squares were characteristically found in MKN45 cells and U937 cells, respectively.

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 (β-*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 [Galβ1−4(Fucα1−3)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.

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Analysis of glycoprotein-derived oligosaccharides in glycoproteins detected on two-dimensional gel by capillary electrophoresis using on-line concentration method

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Available online 31 August 2005

Abstract

Capillary electrophoresis (CE) is an effective tool to analyze carbohydrate mixture derived from glycoproteins with high resolution. However, CE has a disadvantage that a few nanoliters of a sample solution are injected to a narrow capillary. Therefore, we have to prepare a sample solution of high concentration for CE analysis. In the present study, we applied head column field-amplified sample stacking method to the analysis of *N*-linked oligosaccharides derived from glycoprotein separated by two-dimensional gel electrophoresis. Model studies demonstrated that we achieved 60–360 times concentration effect on the analysis of carbohydrate chains labeled with 3-aminobenzoic acid (3-AA). The method was applied to the analysis of *N*-linked oligosaccharides from glycoproteins separated and detected on PAGE gel. Heterogeneity of α1-acid glycoprotein (AGP), i.e. glycoforms, was examined by 2D-PAGE and *N*-linked oligosaccharides were released by in-gel digestion with PNGase F. The released oligosaccharides were derivatized with 3-AA and analyzed by CE. The results showed that glycoforms having lower p*I* values contained a larger amount of tetra- and tri-antennary oligosaccharides. In contrast, glycoforms having higher p*I* values contained bi-antennary oligosaccharides abundantly. The result clearly indicated that the spot of a glycoprotein glycoform detected by Coomassie brilliant blue staining on 2D-PAGE gel is sufficient for quantitative profiling of oligosaccharides.

Keywords: Oligosaccharide; Capillary electrophoresis; On-line concentration; Glycoprotein; Two-dimensional gel electrophoresis

1. Introduction

It is important to investigate post translational modification of proteins for the study of protein functions in the era of proteome after genome. Glycosylation is one of the most common modifications of proteins, and more than 50% of proteins are glycosylated [1], and involved in expression of cellular functions including recognition, cell-to-cell signaling [2], protein folding, canceration [3], immune response, fertilization [4] and differentiation.

Protein modification with carbohydrates has been examined by analyzing carbohydrates after releasing them by chemical or enzymatic method. Various separation techniques for the analysis of carbohydrates were employed such as high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and high-performance liquid chromatography (HPLC) after derivatization with fluorogenic reagents [5–7]. These chromatographic techniques enabled the analysis of carbohydrates with good resolution and high sensitivity.

Capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) is also a powerful tool which provides a rapid, high resolution analysis of complex mixture of fluorescent-labeled oligosaccharides with excellent sensitivity [8–12]. The CE-LIF method using 8-aminopylene-1,3,6-trisulfonate (APTS) as the labeling reagent for carbohydrates is currently one of the most useful method due to the ability for rapid separation and high sensitivity [11,13]. However, when analyzing unknown oligosaccharides such as a novel glycoprotein, we have to obtain other information such as molecular masses, for example, by mass spectrometry. Unfortunately, it is often difficult to observe clear molecular

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doi:10.1016/j.chroma.2005.08.052

ions of APTS derivatives by matrix-assisted laser desorption/ionization time-of-flight-mass spectrometry (MALDI-TOF MS) technique, which affords valuable information on the structure. We previously developed a CE-LIF method using 3-aminobenzoic acid (3-AA) as labeling reagent [12,14] and this method also has high sensitivity as well as APTS, and has some advantages including good compatibility with MALDI-TOF/MS.

Although CE-LIF methods have high sensitivity, CE method is not appropriate for the separation of low-concentration sample solution due to limited sample volume to introduce into a narrow capillary and short path length for detection, and we have to introduce a concentrated sample solution.

In the proteome study, two-dimensional polyacrylamide gel electrophoresis technique (2D-PAGE) is one of the most popular techniques for separation of total proteins derived from biological samples [15,16]. However, the amount of protein mixture applicable to 2D-PAGE is usually less than 100 μg. And it is usually difficult to analyze carbohydrate chains of a glycoprotein in the single spot observed on the gel.

Several on-line sample concentration techniques for enhancement of sensitivity in CE analysis have been reported including sweeping, isotachophoretic sample stacking (ITPSS), hydrodynamic injection and field-amplified sample stacking (FASS). Sweeping method is usually employed in MEKC and essentially requires some detergents such as sodium dodecyl sulfate for micelle formation in separation buffer for concentration of sample zone [17–20]. ITPSS is a popular stacking technique typically employed in capillary zone electrophoresis (CZE) mode by introducing sample solution between the leading and terminating buffer providing a 10–100-fold sensitivity enhancement [21]. Head-column FASS (HC-FASS) provides the highest sensitivity enhancement of all stacking techniques and can be easily carried out by introducing low conductivity solution at the end of the capillary inlet before sample introduction [22,23]. For FASS, samples introduced have to be desalted prior to the analysis to keep the conductivity of sample zone low. Zhang and Thormann have developed a robust stacking method providing more than 1000-fold sensitivity enhancement using HC-FASS for the analysis of positively charged hydrophobic compounds [24,25].

On-line concentration method has been utilized to analyze a sample solution at low concentration especially in case of the analysis of environmental pollutants in wastewater and river water [26], and also employed for the analysis of catecholamines [27], aromatic carboxylic acids, steroids [17] and various pharmaceuticals [26] to achieve ultra highly sensitive detection. For example, Quirino and Terabe achieved almost million-fold increase in detection sensitivity for cationic analytes by the combination of stacking and sweeping method [28].

In the present study, we have developed an on-line concentration method using HC-FASS technique for CE-LIF analysis of *N*-linked oligosaccharides labeled with 3-aminobenzoic acid, and achieved highly sensitive detection of carbohydrate chains from a glycoprotein spot on the gel after 2D-PAGE.

2. Experimental

2.1. Materials

Peptide-N^4-(acetyl-β-D-glucosaminyl)asparagine amidase (PNGase F; EC 3.2.2.18, recombinant) was obtained from Roche Diagnostics (Mannheim, Germany). Neuraminidase (*Arthrobacter ureafaciens*) was a gift from Dr. Ohta (Marukin-Chuyu, Uji, Kyoto, Japan). 3-Aminobenzoic acid (3-AA) was obtained from Tokyo Kasei (Chuo-ku, Tokyo, Japan). Maltopentaose (Glc_5) was purchased from Hayashibara Biochemical Laboratories, Inc. (Okayama-shi, Okayama, Japan). Sodium cyanoborohydride was obtained from Aldrich (Milwaukee, WI). Fetuin (bovine) was obtained from Gibco (Invitrogen, Chuo-ku, Tokyo, Japan). α1-Acid glycoprotein (AGP, human), transferrin (human), fibrinogen (human), ribonuclease B (bovine pancreas) and thyroglobulin (porcine) were obtained from Sigma (St. Louis, MO). A pharmaceutical preparation of recombinant immunoglobulin (rIgG), rituximab, was kindly donated from Ms. Nishiura of Kinki University Nara Hospital. The solution of rIgG was dialyzed against distilled water for 3 days with changing water several times at 4 °C using cellulose membrane tubing (Sanko Junyaku, Chiyoda-ku, Tokyo, Japan), and then freeze-dried. Other reagents and solvents were the reagent grade or HPLC grade and purchased from Wako (Dosho-machi, Osaka, Japan).

2.2. Releasing of N-linked oligosaccharides

A sample of glycoprotein (1 mg) was dissolved in 50 μl of 20 mM phosphate buffer (pH 7.0) in a sample tube (1.5 ml). PNGase F (1 unit, 2 μl) was added to the mixture, and the mixture was incubated at 37 °C for 24 h. After boiled for 5 min, the mixture was evaporated to dryness.

2.3. Fluorescent derivatization of oligosaccharides with 3-AA

To Glc_5 (1 mg) or the released oligosaccharides from glycoproteins as described above, a solution (30 μl) of 0.7 M 3-AA in DMSO-acetic acid (7:3, v/v) and a freshly prepared solution (30 μl) of 2 M $NaBH_3CN$ in the same solvent were added. Derivatization reaction was carried out at 50 °C for 60 min. The reaction mixture was applied on a column of Sephadex LH-20 (1 cm I.D., 30 cm length) equilibrated with aqueous 50% (v/v) methanol. The earlier eluted fluorescent fractions (Ex. 305 nm, Em. 405 nm) were pooled and evaporated to dryness. The residue was dissolved in water (100 μl) and a portion was used for the analysis by capillary electrophoresis. Mixture of labeled oligosaccharides at small scale was conveniently purified using a solid-phase extraction column (Oasis HLB cartridge, 1 ml, Waters, Milford, MA) [7] instead of purification by Sephadex LH-20 column due to difficulty in monitoring the fluorescence signal. Briefly, the solution was diluted with 1.0 ml of acetonitrile-water (95:5) and mixed vigorously, and was applied to a cartridge previously equilibrated with the same solvent (1 ml × 2). After washing the cartridge with

acetonitrile-water (95:5, 1 ml × 2), oligosaccharides were eluted with acetonitrile-water (20:80, 1 ml) and the eluate was evaporated to dryness by a centrifugal evaporator.

Both methods are available for removing salts and excess labeling reagents from the reaction mixture for the analysis by HC-FASS technique.

2.4. Enzymatic desialylation of sialyl oligosaccharides

3-AA labeled oligosaccharides were dissolved in 20 mM acetate buffer (pH 5.0, 10 μl). Neuraminidase was added (1 munit, 1 μl) to the buffer and the mixture was incubated at 37 °C for 24 h. The reaction mixture was boiled for 5 min for inactivation of enzyme.

2.5. Capillary electrophoresis of 3-AA labeled oligosaccharides

Separation conditions for capillary electrophoresis were similar to that described in the previous report [12,14]. Capillary electrophoresis was performed on a P/ACE MDQ glycoprotein system (Beckman-Coulter, Fullerton, CA) equipped with a helium–cadmium laser induced fluorescence detector (Ex. 325 nm, Em. 405 nm) using a DB-1 capillary (100 μm I.D., 20 cm effective length, 30 cm total length) in 100 mM Tris–borate buffer (pH 8.3) containing 10% PEG70000 as the running buffer. DB-1 capillary of which surface is chemically modified with dimethylpolysiloxane does not generate electroosmotic flow during electrophoresis. Therefore, negative charges due to sialic acids and the carboxylic acid group of 3-AA are major driving forces [12,14]. For pressure injection, sample solutions were introduced to the capillary at 1 psi for 10 s. For electrokinetic injection, sample solutions were introduced at 10 kV for 50 s after pre-injection of water at 1 psi for 10 s to concentrate the sample ions. Separation was performed by applying 25 kV at 25 °C. For the analysis of sialyl oligosaccharides, 50 mM phosphate buffer (pH 4.0) was used as a boundary zone prior to sample injection to improve concentration efficiency.

2.6. Oligosaccharide analysis of the spots observed on the gel after two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

A sample of AGP (5 μg) was dissolved in 125 μl of 8 M urea solution containing 2 M thiourea, 4% CHAPS and 100 mM DTT. The solution was applied to an immobilized p*I* gel (IPG strip gel, p*I* 3–6, 12 cm, Bio-Rad, Hercules, CA). The gel was rehydrated for 12 h and then subjected to electrophoresis in a Protean IEF Cell (Bio-Rad). Isoelectric focusing was performed at 3500 V for 17 h. The IPG gel was then equilibrated in the first equilibration buffer (50 mM Tris–HCl, 8 M urea, 30% glycerol, 10% SDS, 100 mM DTT) for 5 min at room temperature followed by keeping the gel in the second equilibration buffer (50 mM Tris–HCl, 8 M urea, 30% glycerol, 10% SDS, 250 mM iodoacetoamide, 0.001% BPB) for 5 min at room temperature. Second dimension analysis was performed as described by Laemmli [29] using a slab gel (7 cm × 10 cm) of 9% polyacrylamide with a Mini Protean 3 cell (Bio-Rad) and a power supply (Power Pac 3000, Bio-Rad) at 10 mA for 2.5 h. Protein spots were visualized with Coomassie brilliant blue (R-250). After destaining the background color with 40% methanol–10% acetic acid, the gel was washed with water several times to remove destaining reagent. The protein spots were excised and collected in a 1.5 ml centrifuge tube. The gel pieces were dehydrated with acetonitrile (100 μl) for 30 min at room temperature. Acetonitrile was removed and the gel was dried by a centrifugal evaporator. PNGase F (2 units, 4 μl) and 10 mM phosphate buffer (pH 7.0, 100 μl) were added to the dried gel pieces, and the mixture was incubated at 37 °C overnight [30,31]. The aqueous layer was collected and evaporated to dryness. The residue was derivatized with 3-AA and analyzed by CE-LIF as described above.

3. Results and discussion

3.1. Injection of a sample solution using on-line concentration method

APTS derivatization method is the most poplar derivatization method for oligosaccharide analysis by CE-LIF due to its rapidity, and is often used for the analysis of oligosaccharides in stability test and lot release test of pharmaceutical glycoproteins. Derivatization with 3-AA is available for structure analysis of unknown samples, because 3-AA labeled oligosaccharides can be easily analyzed by MALDI-TOF/MS with high sensitivity and by HPLC by a combination with specific glycosidase digestion. The scope of the present study is to develop the method which can be applied for the oligosaccharide analysis in the spot on 2D PAGE gel. From these reasons, we chose 3-aminobenzaoic acid (3-AA) as a derivatization reagent for oligosaccharides.

We examined head-column field-amplified sample stacking (HC-FASS) method (Fig. 1) to increase the sensitivity in the analysis of glycoprotein glycans.

At the initial step, separation buffer is filled in the capillary. A small volume of water is introduced to the capillary from one end of the capillary by pressure method (typically 1 psi, 10 s) which is required to establish a short zone of low conductivity at the capillary inlet. Then, a sample solution is introduced by the electrokinetic method. Because all oligosaccharide molecules in the sample solution is negatively charged due to the presence of the carboxyl group of 3-aminobenozoic acid (3-AA) attached to the reducing end, the oligosaccharide molecules are

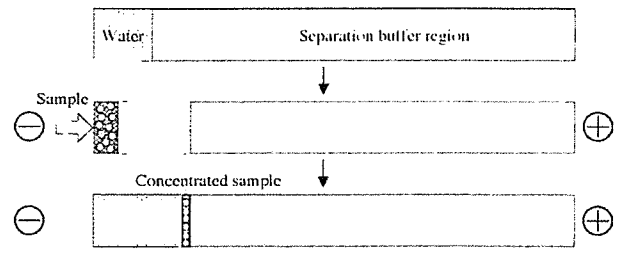

Fig. 1. Principle of on-line concentration using stacking method.

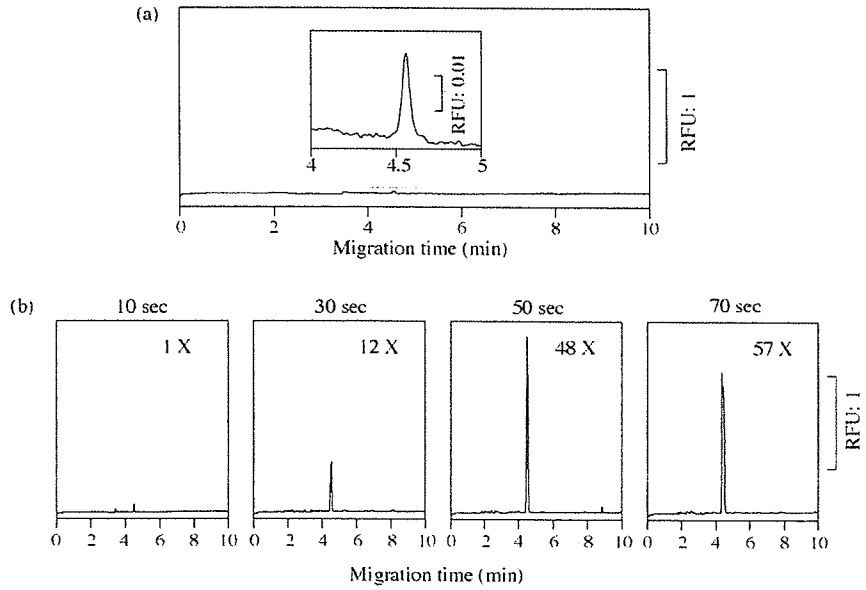

Fig. 2. Analysis of 3AA-Glc_5 using pressure injection method and electrokinetic injection method. 100 mM Tris–borate buffer (pH 8.3) containing 10% PEG70000 was used as the separation buffer with a DB-1 capillary (100 μm I.D., 30 cm, 20 cm effective length). Applied potential, 25 kV at 25 °C. Fluorescent detection at 325 nm excitation with He–Cd laser with a 405 nm emission filter. (a) Pressure injection method at 1 psi for 10 s. (b) Electrokinetic injection method at 10 kV for 10–70 s after pre-injection of water at 1 psi for 10 s.

migrated toward the anode. The electric field in water region is much bigger than that in the separation buffer, and the migration velocity of sample becomes much faster in the pre-injected water region than in the separation buffer. Therefore, oligosaccharide molecules in the sample solution are concentrated at the boundary position between water and the separation buffer. It should be noted that desalting procedure is required to achieve stacking in good efficiency. Although this stacking technique provides the sensitivity enhancement with one to three order magnitudes, there are some disadvantages such as poor reproducibility by repetitive injections from the same sample solution [24].

We investigated the effect of on-line concentration using 3-AA labeled Glc_5 as a model. A few examples are shown in Fig. 2.

When a solution (0.1 μg/ml) of 3-AA Glc_5 was injected by pressure injection method (1 psi, 10 s), a small peak was observed at ca. 4.5 min with the signal to noise (S/N) ratio of 4.5 (Fig. 2(a)). Stacking effect of the electrokinetic method is shown in Fig. 2(b) for various intervals of injection times from 10 to 70 s. Peak areas were increased with increasing the injection periods, and best concentration effect was observed for injection times of 50 s. Longer injection times caused lowering the theoretical numbers of plate as indicated in the analysis for 70 s injection times. Based on the optimization studies, we achieved, ca. 48 times higher sensitive detection than that observed for pressure injection method with keeping the similar theoretical number of plates (more than 10,000). These data indicated that

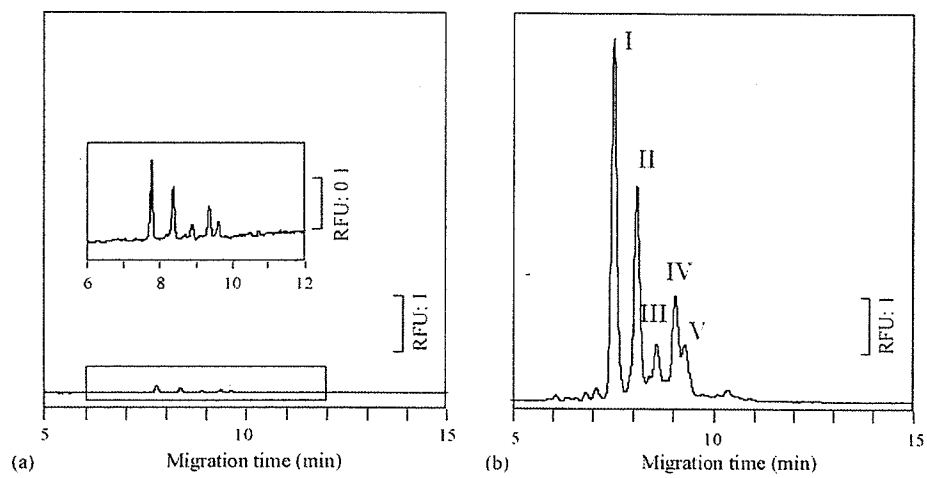

Fig. 3. Analysis of neutral-carbohydrate chains from ribonuclease B using pressure injection method and electrokinetic injection. (a) Pressure injection at 1 psi for 10 s, (b) Electrokinetic injection at 10 kV for 50 s after pre-injection of water at 1 psi for 10 s. Structures of I–V are $Man_5GlcNAc_2$, $Man_6GlcNAc_2$, $Man_7GlcNAc_2$, $Man_8GlcNAc_2$ and $Man_9GlcNAc_2$, respectively. Other analytical conditions were the same as in Fig. 2.

we could detect 2 ng/ml of 3-AA Glc_5 (2.4 nM). When 3 μl of the sample solution are used for injection, we can analyze oligosaccharides at fmol level.

3.2. Application of on-line concentration method to the analysis of N-linked oligosaccharides derived from some glycoprotein samples

We applied the present method to the analysis of *N*-linked oligosaccharides derived from a few glycoprotein samples. Ribonuclease B (bovine pancreas) which contains neutral *N*-linked oligosaccharides was used as model sample. These oligosaccharide structures are reported as high-mannose type of $Man_{5-9}GlcNAc_2$ [12,14,32]. We prepared 3-AA labeled oligosaccharides as described in Section 2, and analyzed by CE-LIF. When a highly diluted solution of oligosaccharide sample (100 μg/ml as original glycoprotein) was injected by pressure method, the oligosaccharide peaks were very small (Fig. 3(a)).

In contrast, when the same solution was analyzed by on-line concentration using HC-FASS technique, we successfully achieved ca. 93 times higher sensitivity (Fig. 3(b)). The enhancement efficiency in this assay is higher than that in the model study using 3-AA Glc_5 (Fig. 2). This difference is possibly due to difference of charge-molecular mass ratios, suggesting that the present technique might be more efficient for high molecular weight oligosaccharides such as those from glycoprotein.

We also applied the present method to the analysis of sialyl oligosaccharides derived from fetuin (bovine) as a model glycoprotein containing sialyl oligosaccharides. Structures of fetuin oligosaccharides have been characterized in detail previously [12,33–35]. Modification of injection conditions was required to achieve higher effective on-line concentration for the analysis of sialyl oligosaccharides because the negative charges due to sialic acid residues caused faster migration rate in the boundary zone between pre-injected water and separation buffer. In order to diminish the charge effect based on sialic acid residues, we used 50 mM phosphate buffer (pH 4.0) as a pre-injected buffer in place of water. We prepared a sample solution of 3-AA labeled oligosaccharides derived from fetuin (100 μg/ml as original glycoprotein concentration) and compared both injection methods. The results are shown in Fig. 4.

When 50 mM phosphate buffer (pH 4.0) was used as boundary zone, sialo-oligosaccharides were well resolved as shown in Fig. 4(a-2) and the sensitivity became ca. 264-fold higher than that of the pressure method (Fig. 4(a-1)). We could also achieve good resolution and high concentration effect (362-fold) for the analysis of asialo-oligosaccharides (Fig. 4(b-2)). The analysis of a mixture of highly sialylated oligosaccharides, for example, such as from erythropoietin and AGP, are generally difficult due to their heterogeneity in glycosidic linkages of sialic acids to galactose residues, and sialo-oligosaccharide samples are often analyzed as asialo form after neuraminidase treatment [13]. Peak splitting at 12.6 min (Fig. 4(b-2)) indicates that triantennary

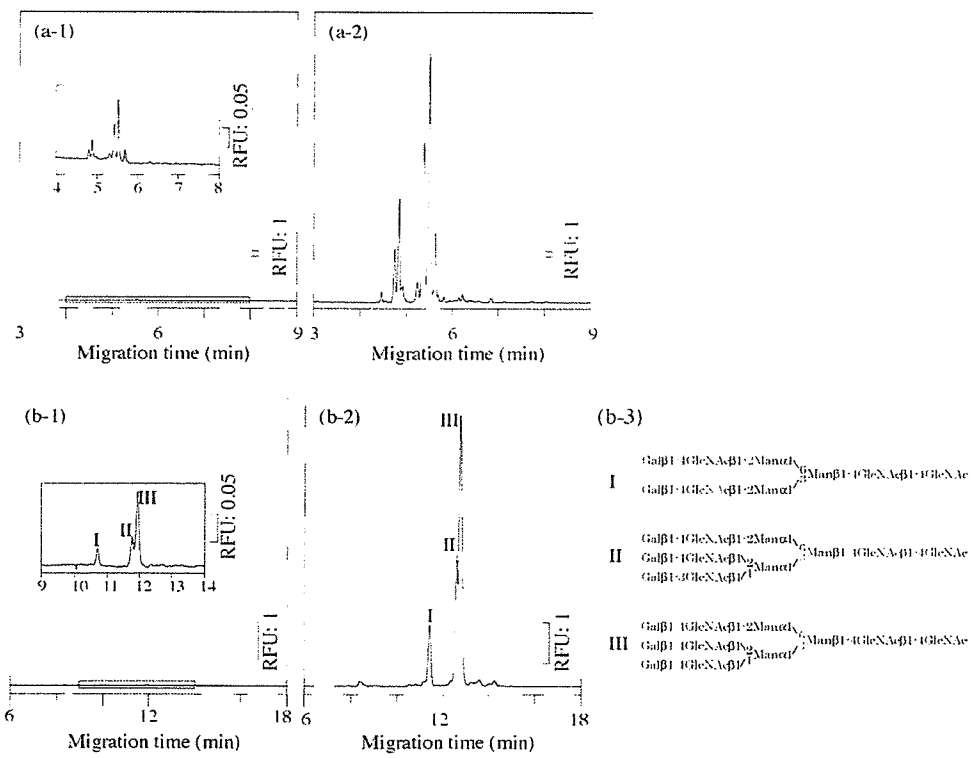

Fig. 4. Analysis of sialo- and asialo-carbohydrate chains from fetuin using pressure method and electrokinetic method. (a-1) Sialo-oligosaccharides by pressure injection at 1 psi for 10 s, (a-2) sialo-oligosaccharides by electrokinetic injection at 10 kV for 50 s after pre-injection of 50 mM phosphate buffer, (b-1) asialo-oligosaccharides by pressure injection at 1 psi for 10 s, (b-2) asialo-oligosaccharides by electrokinetic injection at 10 kV for 50 s after pre-injection of water at 1 psi for 10 s. Other analytical conditions were the same as in Fig. 2. (b-3) List of asialo-oligosaccharide structures of fetuin.

Table 1
On-line concentration using some glycoprotein samples

Glycoprotein	Concentration effect
Ribonuclease B	93-fold
Rituximab	90-fold
AGP	
Sialo	69-fold
Asialo	65-fold
Transferrin	
Sialo	60-fold
Asialo	250-fold
Fetuin	
Sialo	264-fold
Asialo	362-fold
Fibrinogen	
Sialo	116-fold
Asialo	67-fold

oligosaccharide having a Galβ1-3 branch can be resolved from the major oligosaccharide having only Galβ1-4 branches (Fig. 4(b-3)). These data clearly showed that on-line concentration worked well for the analysis of asialo-oligosaccharides.

Based on the optimization studies on on-line concentration method described above, we applied the present method to some glycoprotein samples (rituximab, AGP, fibrinogen and transferrin). The results are summarized in Table 1.

We successfully observed on-line concentration effect in all samples although the efficiencies were different among samples. Difference of efficiency is probably due to differences of charge-molecular mass ratios of oligosaccharides, but three-dimensional structures of oligosaccharides also should be considered. Apparent molecular sizes of oligosaccharides, which greatly affect their mobilities in CE, are possibly varied with rigidity or flexibility of their inner-glycosidic linkages [36,37].

3.3. Analysis of oligosaccharides in a glycoprotein spot observed on 2D-PAGE gel

The ultimate purpose of the present study is to establish the method which enables analyzing *N*-linked oligosaccharides in a glycoprotein spot observed on a gel after two-dimensional gel electrophoresis. A glycoprotein often shows multiple spots on 2D gel, which are due to heterogeneity of carbohydrate chains in the molecule. These multispots are called "glycoform".

We separated glycoforms of AGP [38] by 2D-PAGE and analyzed oligosaccharides in each spot. The AGP sample was resolved into five glycoforms (glycoforms 1–5 from low p*I* values) along p*I* dimension (p*I* 3.8–4.0) on 2D-PAGE gel as shown in Fig. 5, although all spots showed smear shape and were not clearly identified. The glycoform 1 was almost lower detection limit of 2D-PAGE by Coomassie blue staining.

After collecting each spot by cutting the gel, staining reagent and electrophoresis buffer were removed from the gel and the gel was dehydrated. After in-gel digestion with PNGase F to release the oligosaccharides, the released oligosaccharides

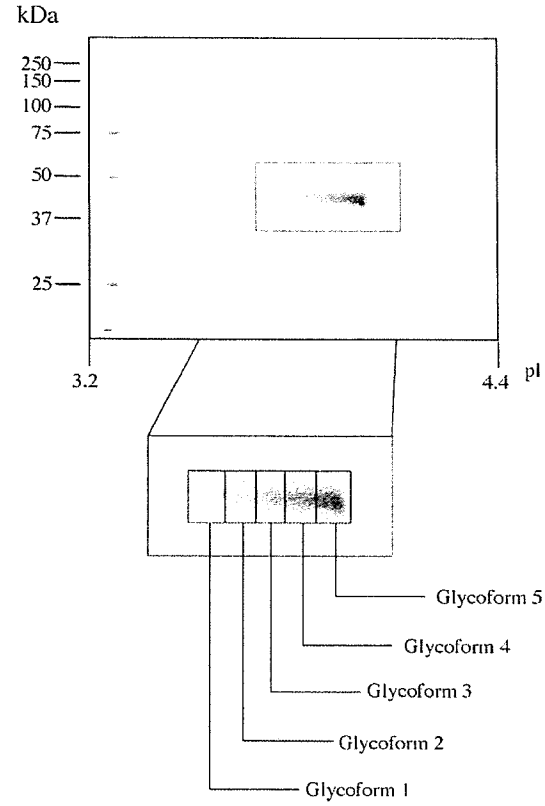

Fig. 5. Separation of AGP glycoforms by 2D-PAGE. Detection was performed with Coomassie blue staining method.

were analyzed by the present on-line concentration method. The results are shown in Fig. 6.

Although we could not confirm each peak in the analysis of sialo-oligosaccharides (Fig. 6(a)), peaks observed at earlier migration times contains larger number of sialic acid residues and slower migrated peaks contain smaller number of sialic acid residues, because molecules having less negative charges moves slower than those having higher negative charges in DB-1 capillary which does not generate electroosmotic flow. Fig. 6(a) clearly indicates that the glycoform having higher p*I* values contain slow-moving peaks, namely contain smaller numbers of sialic acids. The peaks observed in asialo-oligosaccharide analysis (Fig. 6(b)) were confirmed as shown in Fig. 7 according to the previous works [35,38–41].

AGP contains di-, tri-, and tetra-antennary carbohydrate chains, and some of tri- and tetra-antennary carbohydrate chains have Lewis-X structure (α-fucose attached to GlcNAc of Gal-GlcNAc arm). Although III and IV were not resolved in the present analytical conditions, asialo-oligosaccharides in the spots observed on 2D-gel showed characteristic ratios among the peaks. The relative abundances of oligosaccharides in each glycoform are summarized in Table 2.

The glycoforms having lower p*I* values contained a larger amount of tri- and tetra-antennary oligosaccharides (III–V). On the contrary, glycoforms having higher p*I* values contained bi-antennary oligosaccharides (I) abundantly (Table 2). These results suggest that the oligosaccharide profile in each

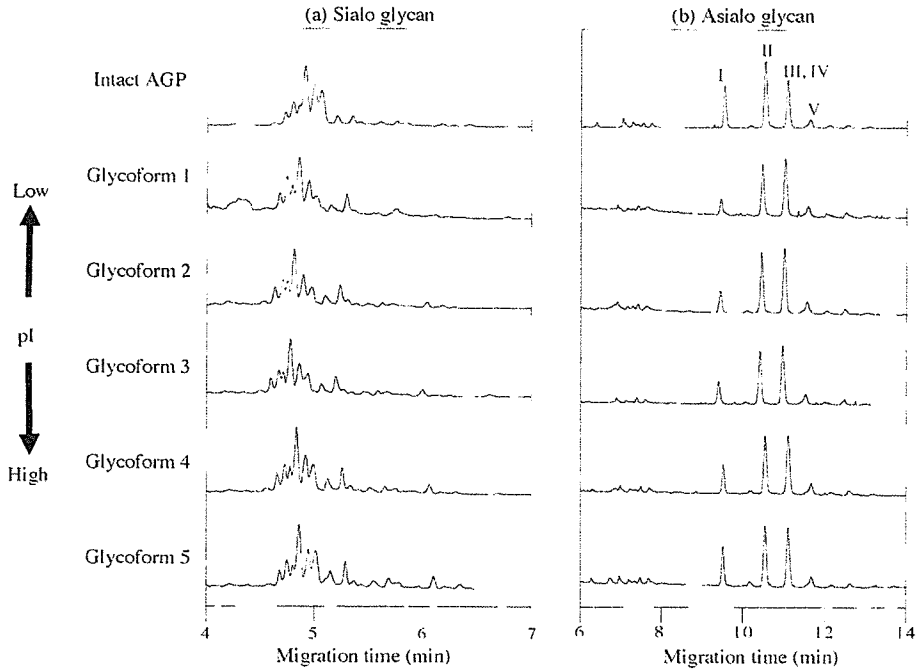

Fig. 6. Analysis of carbohydrate chains from glycoforms of AGP in gel spots after in-gel digestion. (a) Sialo-oligosaccharides by electrokinetic injection at 10 kV for 50 s after pre-injection of 50 mM phosphate buffer, (b) asialo-oligosaccharides by electrokinetic injection at 10 kV for 50 s after pre-injection of water at 1 psi for 10 s. Other analytical conditions were the same as in Fig. 2. Structures of peaks I–V are shown in Fig. 7.

glycoform is different in not only sialic acid content but also antennary structure. It should be noted that the present method enables to analyze carbohydrate chains quantitatively even in the spots of almost lower detection limit on 2D-gel detected by Coomassie blue staining method.

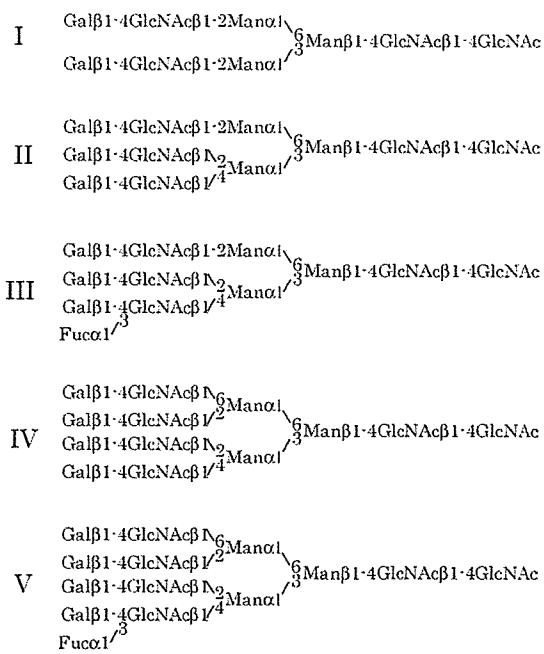

Fig. 7. List of asialo oligosaccharide structures of AGP.

Table 2
Oligosaccharide distributions of AGP glycoforms

Structure	Oligosaccharide distribution (%)		
	I	II	III–V
Intact AGP	14.8	37.2	47.0
Glycoform 1 (3.78)	11.3	36.9	52.9
Glycoform 2 (3.84)	11.9	37.8	51.3
Glycoform 3 (3.89)	14.3	37.7	48.9
Glycoform 4 (3.94)	16.0	37.1	47.9
Glycoform 5 (3.99)	20.3	35.6	45.1

Structures I–V as in Fig. 7. The numbers in parentheses indicate the p*I* values.

4. Conclusion

In the present study, we developed an on-line concentration method using HC-FASS technique for CE-LIF analysis of *N*-linked oligosaccharides released from glycoproteins. We achieved 60–362-fold on-line concentration effect in the analysis of both neutral and sialyl oligosaccharides in comparison with the conventional pressure injection method. We applied the present method to the oligosaccharide analysis in the spots observed on 2D-gel stained with Coomassie blue, and successfully analyzed oligosaccharides even from the spots almost at the lower detection limit by Coomassie brilliant blue staining.

In the present study, we used 3-AA as derivatization reagent for oligosaccharides, but the essential method will be applicable to other frequently used derivatization reagent having negative charges such as 2-aminoanthranilic acid (2-AA) and 8-aminopyrene-1,3,6-trisulfonate (APTS). The methodology will overcome the disadvantage of low concentration sensitivity in

CE, and will be a powerful tool for characterizing oligosaccharide modifications for proteomics and glycomics studies.

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Received November 19, 2005
Revised January 6, 2006
Accepted January 10, 2006

Review

Capillary electrophoresis for the analysis of glycoprotein pharmaceuticals

Carbohydrate chains in glycoprotein pharmaceuticals play important roles for the expression of their biological activities, but the structure and compositions of carbohydrate chains are dependent on the conditions for their production. Therefore, evaluation of the carbohydrate chains is quite important for productive process development, characterization of product for approval application, and routine quality control. The oligosaccharides themselves have complex structure including blanching and various glycosidic linkages, and oligosaccharides in one glycoprotein pharmaceutical generally have high heterogeneity, and characterization of oligosaccharide moiety in glycoprotein has been a challenging target. In these situations, CE has been realized as a powerful tool for oligosaccharide analysis due to its high resolution and automatic operating system. This review focuses on the application of CE to the glycoform analysis of glycoproteins and profiling of the *N*-linked glycans released from glycoprotein pharmaceuticals. Current applications for structure analysis using CE-MS^n technique and glycan profiling method for therapeutic antibody are also described.

Keywords: Capillary electrophoresis / Glycoforms / Glycoprotein pharmaceuticals / *N*-Linked oligosaccharides DOI 10.1002/elps.200500853

1 Introduction

For approval studies of glycoprotein pharmaceuticals, determination of post-translational modification on core protein is required. Glycosylation is one of the most common modifications of proteins, and is involved in expression of cellular functions including recognition, cell-to-cell signaling, protein folding, canceration, immune response, fertilization, and differentiation [1–3].

Among many biopharmaceutical drugs currently approved [4], erythropoietin and various therapeutic antibody pharmaceuticals are the most successful products in recombinant technologies [4–8]. Oligosaccharide profiles of these glycoprotein pharmaceuticals are influenced by host cells, cell culture conditions, and purification step through the productive process. Glycosylation in glycoprotein pharmaceuticals has been reported to affect pharmacokinetics and biological activity [9, 10], and pharmaceutical companies are required to keep consistency in quality of the products with respect to monosaccharide compositions, sialic acid contents, oligosaccharide profiles, and oligosaccharide sequences for regulatory requirements.

CE is currently one of the most powerful methods with high sensitivity and high resolution, which enables to detect even 10^{-15}–10^{-18} mol of oligosaccharide samples when using a suitable fluorescent labeling method and LIF detection. Such a high sensitivity is quite useful for monitoring biosynthesis of oligosaccharides even in a single cell [11]. A multicapillary device enables simultaneous multisample analysis and achieves high-throughput analysis. Recently, high-throughput clinical analysis of *N*-linked oligosaccharides from serum protein of liver disease patients was performed using multicapillary CE-based DNA analyzer combined with 96-well-based sample preparation method and a thermal cycler [12].

One of the most distinct points of CE is its high-resolving power compared with that of HPLC. In CE, separation is achieved based on charge to mass ratios of the analyte ions in the sample solution. CGE is also available by using a gel matrix of high molecular weight in the separation

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Abbreviations: AA, aminobenzoic acid; **AGP**, α1-acid glycoprotein; **ANTS**, 8-aminonaphthalene-1,3,6-trisulfonic acid; **APTS**, 8-aminopyrene-1,3,6-trisulfonate; **CAE**, capillary affinity electrophoresis; **rhuEPO**, recombinant human erythropoietin

buffer. CGE has been widely used for sequencing of DNA [13, 14]. CGE is also useful for high-resolution separation of enzyme digestion mixture of glycosaminoglycans, such as hyaluronic acid and polysialic acids [15, 16]. We successfully separated molecular species of hyaluronic acid having molecular masses up to 32 000 Da in a buffer containing PEG as gel matrix [16]. Polysaccharide peaks having more than 150 monosaccharide units were clearly discriminated within 70 min. High-performance anion-exchange chromatography using pulsed amperometric detection is also useful for such purpose [17], but conventional HPLC is not generally available for high-resolution separation of highly hydrophilic and acidic polysaccharides.

More than 50% of proteins present in human sera are glycoproteins except for albumin and α-amylase. Carbohydrate chains attached to these glycoproteins are highly heterogeneous in their abundance, structure, and linkage position, and are varied with changes in physiological conditions such as cancer, inflammation, and aging [18, 19]. Glycans are not directly controlled by genes, and we cannot obtain information on carbohydrate chains only by the analysis of expression of related enzymes. From these reasons, it is mandatory to analyze carbohydrate chains attached to the protein.

2 Glycoform analysis

Recombinant technologies have made it possible to produce protein pharmaceuticals using living cells. However, we observe inherent structural heterogeneity in these proteins during production by living cells. In glycoprotein pharmaceuticals, products are mixtures of heterogeneous oligosaccharides (*i.e.*, glycoforms). Each glycoform may have different biological activity and safety, and manufactures should define the heterogeneity of the product and demonstrate consistency of the products. Furthermore, profiles of heterogeneity should be characterized to assure lot-to-lot consistency.

Oligosaccharides in glycoprotein pharmaceuticals are generally heterogeneous mixtures because the oligosaccharides are synthesized by multienzyme actions. In addition, most of the glycoproteins have multiple sites to which oligosaccharides are linked. Consequently, heterogeneity in structures and linkage sites results in multiforms of glycoprotein (*i.e.*, glycoform). If a glycoprotein has five oligosaccharides in its molecule and five possible linkage sites, the glycoprotein has at least $5^5 = 3125$ possible glycoforms. Although it is not possible to resolve such complex mixtures by the present technology, CE is one of the best tools due to its high resolving power.

Recombinant human erythropoietin (rhuEPO) has three *N*-linked and one *O*-linked glycosylation sites and the glycans have variable numbers of sialic acid residues [20]. IEF on a slab gel has been widely used for glycoform analysis of rhuEPO based mainly on the differences in sialic acid contents [21]. While the resolution among glycoform bands obtained by gel IEF method is excellent, the technique is time-consuming in gel preparation, staining–destaining procedures, and scanning for quantitative determination of each band. Several separation methods using capillary format have been developed to overcome the problems in slab gel IEF [22–27] and also described in European Pharmacopoeia [28]. In the methodology using CE described in the 2002 European Pharmacopoeia, the separation was performed using a bare fused silica and separation buffer containing putrescine and urea. Although excellent resolution among seven glycoforms is observed in the first analysis, we often encounter difficulty in obtaining reproducible separation [26]. The glycoform resolution becomes worse irreversibly after repeating several analyses [25, 27–30]. Kinoshita *et al.* [26] successfully separated rhuEPO glycoforms using commercially available surface-modified capillaries for capillary GC in the running buffer containing hydroxypropylmethylcellulose (HPMC) with good reproducibility [26]. The separation of rhuEPO using this technique is shown in Fig. 1. An example for separation by slab gel IEF is also shown in Fig. 2. CE method showed larger number of peaks indicating higher resolving ability for glycoform separation.

The same group also reported that glycoforms of α1-acid glycoprotein (AGP), which is a major acidic glycoprotein in sera, were also analyzed in the similar conditions [31, 32]. The proposed method employing the running buffer which had slightly higher pH values (*ca.* 1.0) than p*I* values of the target glycoproteins allowed good resolution of glycoforms.

Glycoform separation by CE is an effective tool to trace the change of glycoprotein heterogeneity due to changes in attached oligosaccharides. Currently, the method has started to be used for lot release and stability testing in quality control in biopharmaceutical companies [33].

3 Structure analysis

In the approval application of biopharmaceuticals, it is required to determine the structures of oligosaccharides (*i.e.*, branching structure and monosaccharide sequences) attached to the core protein in detail. For such purpose, standard samples of *N*-linked oligosaccharide are employed for identification by comparing the migration times or coinjection with the sample solution. Due to dif-

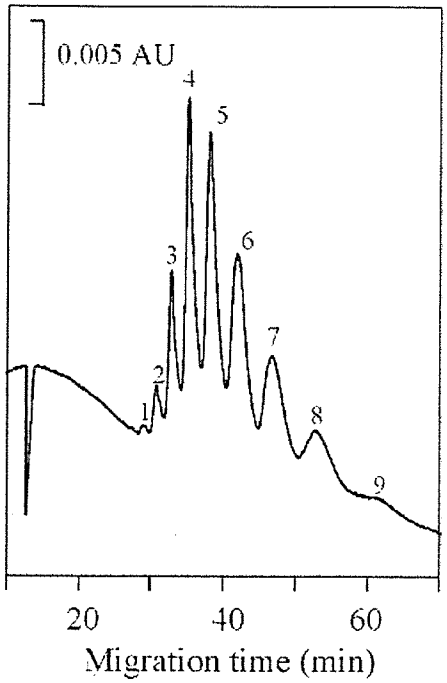

Figure 1. Analysis of rhuEPO glycoforms by CE. Analytical conditions: capillary, DB-1 capillary (57 cm (effective length, 50 cm), 100 μm id); running buffer, 10 mM acetate buffer (pH 5.7) containing 0.5% w/v of HPMC; applied potential, 12.5 kV; detection, UV absorption at 200 nm. Peaks 3–7 correspond to the bands 3–7 in Fig. 2, respectively. For details, see [26]. Reproduced with permission from the publisher.

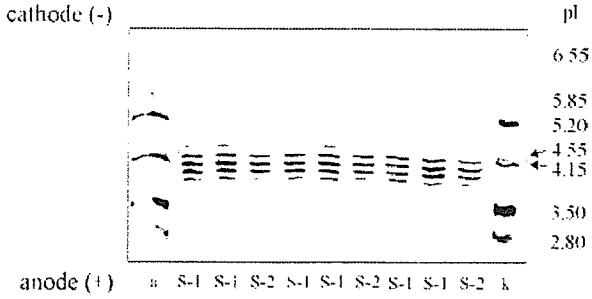

Figure 2. IEF gel electrophoresis of rhuEPO. Two pharmaceutical preparations of rhuEPO (S-1 and S-2) were analyzed. (a) and (k), *pI* marker. Bands 3–7 observed in the lanes of S-1 and S-2 correspond to the peaks 3–7 in Fig. 1, respectively. For details, see [26]. Reproduced with permission from the publisher.

ficulty in preparation of standard samples of oligosaccharides, we developed a new method for preparing *N*-glycans as free oligosaccharides after releasing *N*-glycans from core protein followed by derivatization with 9-fluorenylmethyl chloroformate (Fmoc) [34].

Identification of oligosaccharides by simple comparison of migration times in CE or elution times in HPLC cannot fully explain the structures of unknown oligosaccharide peaks. As for HPLC analysis, MS and MS/MS technology are used to identify the unknown peak by LC-ESI-MS or MALDI-TOF-MS after collecting the peak [35–38]. In contrast, it is often difficult to determine the oligosaccharide structure of the peak observed on the electropherogram in CE, because absolute amount of oligosaccharide is quite small due to limited sample volume introduced into a narrow capillary and manipulation for collection of the peak is difficult.

3.1 Sequencing of oligosaccharides by exoglycosidase digestion

Digestion of an oligosaccharide sample with specific glycosidases is an easy and simple technique for the determination of oligosaccharide structure by CE. After digestion with specific exoglycosidases, the peaks are moved to the new positions by loss of monosaccharide residues. Careful observation of peak shift after digestion with combinations of various glycosidases enables to identify the oligosaccharide sequence even if the sample has high heterogeneity. Callewaert *et al.* [12] confirmed the partial structures of some glycans in complex glycan mixture from total serum proteins by sequential exoglycosidase digestions using a combination of neuraminidase, β1-4 galactosidase, fucosidase, and *N*-acetylhexosaminidase. Glycosidase digestion assay is also useful to distinguish the positional isomers or glycosidic linkages. For example, Galβ1-4GlcNAc and Galβ1-3GlcNAc at the nonreducing end can be discriminated by using specific β1-4galactosidase or β1-3galactosidase. In glycan profiling of a therapeutic antibody, rituximab, two positional isomers of bi-antennary *N*-linked oligosaccharide with one galactose at the nonreducing terminal of different arms, which can be separated by CE (see Fig. 5, peaks 2 and 3), could be identified using α1-2 and α1-3 mannosidase digestion after *N*-acetylhexosaminidase treatment [39]. These subtle differences cannot be distinguished by the present MS or MS/MS technique.

Commercially available glycosidases for oligosaccharide sequence are limited and optimization studies for digestion are required for each enzyme. However, this technique is simple and useful for identification of the oligosaccharide sequence including glycosidic linkages and isomers of glycans. Moreover, the injection volume of glycan sample to the capillary is quite small (generally of nanoliter order). Therefore, a few microliters of the sample is sufficient for each digestion reaction and almost all the rest of sample solution after injection can be available for further studies using other enzymes. This is one of the advantages for the sequential exoglycosidase assay using CE.

3.2 Capillary affinity electrophoresis (CAE)

Analysis of the interactions between carbohydrates and carbohydrate-binding proteins (*i.e.*, lectins) is a useful tool for acquiring structure information of carbohydrates [40]. Nakajima *et al.* have developed CAE technique for high-throughput structure analysis of oligosaccharides [41, 42]. This technique is based on the analysis of the specific-binding reactions between oligosaccharides and carbohydrate-binding proteins (*i.e.*, lectin). The principle of CAE is shown in Fig. 3.

In the initial step, a mixture of fluorescent-labeled carbohydrates is analyzed by CE in the absence of lectin in the electrolyte (Fig. 3a). Then the same sample is analyzed in the presence of a lectin whose specificity is well established. When the lectin recognizes carbohydrate A, the peak A is observed later due to the equilibrium formation between the conjugate form with the lectin and the free form. On the contrary, carbohydrate C, which does not show affinity to the lectin, is observed at the same migration time as that in the absence of the lectin. Carbohydrate B shows weak affinity to the lectin and is observed slightly later. Thus, the migration order of the carbohydrate chains A–C, changes as shown in Fig. 3b. By repeating the procedures using an appropriate set of lectins, we can categorize all carbohydrate chains. An example of CAE analysis of asialo-oligosaccharides derived from AGP is shown in Fig. 4. AGP contains di-, tri-, and tetra-antennary oligosaccharides as shown in Table 1. Some of tri- and

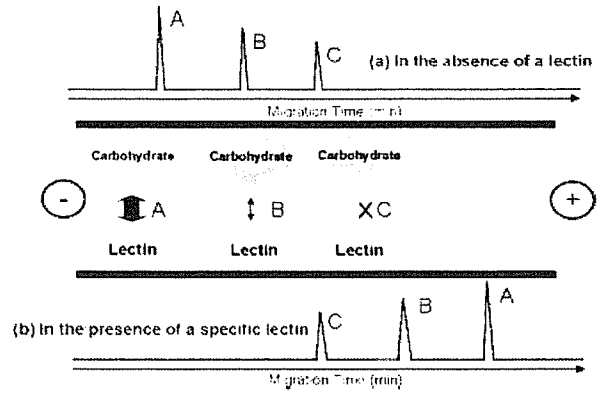

Figure 3. Principle for categorization of carbohydrate chains by CAE. For details, see [41]. Reproduced with permission from the publisher.

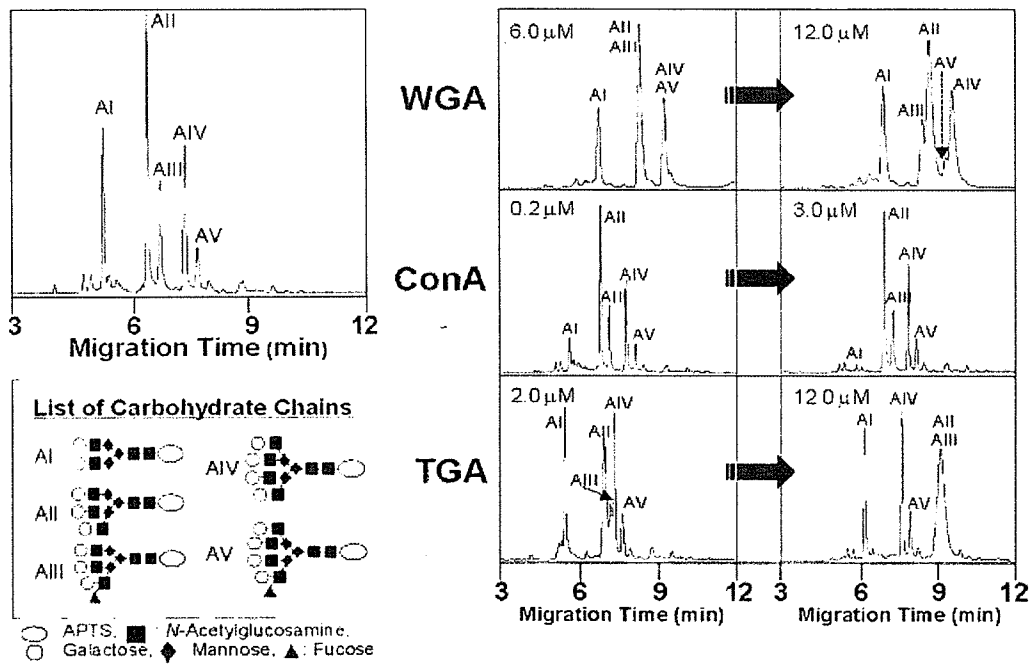

Figure 4. CAE of carbohydrate chains derived from AGP. Analytical conditions: Capillary, eCAP N-CHO capillary (30 cm (effective length, 10 cm), 50 μm id); running buffer, 100 mM Tris-acetate buffer (pH 7.4) containing 0.5% w/v PEG (PEG70000) and lectins; applied potential, 10 kV; fluorescent detection at 520 nm excited with argon-laser with 488 nm filter. Accurate structures of the oligosaccharides are shown in Table 1. Left electropherogram is the profile of carbohydrate chains from AGP in the analysis without lectin, and the right electropherograms are those with lectins. For details, see [41]. Reproduced with permission from the publisher.

