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Profiling analysis of oligosaccharides in antibody pharmaceuticals by capillary electrophoresis

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Abstract

Carbohydrate chains in glycoprotein pharmaceuticals have important roles for the expression of their biological activities. Therefore, development of an assessment method for the carbohydrate chains is an important parameter for quality control of glycoprotein pharmaceuticals such as newly developed therapeutic antibodies. In this report, we applied capillary electrophoresis with laser-induced fluorescence detection to the analysis of carbohydrate chains after releasing with glycoamidase followed by derivatization with 3-aminobenzoic acid. We found that four major oligosaccharides present in antibody pharmaceuticals were successfully separated with good resolution. The present method showed good precision in both migration times and relative peak areas, and gave comparable accuracy with that using a derivatization method with 8-aminopyrene-1,3,6-trisulfonate.

Keywords: Oligosaccharide; Capillary electrophoresis; 3-Aminobenzoic acid; 8-Aminopyrene-1,3,6-trisulfonate; Antibody pharmaceutical

1. Introduction

Carbohydrate chains in glycoprotein pharmaceuticals have important roles for the expression of their biological functions such as bioactivity and pharmacokinetic characteristics. For example, recombinant human erythropoietin (rhEPO) produced in Chinese hamster ovary (CHO) cells contains a variable number of sialic acid residues on the non-reducing terminals of the oligosaccharide chains, and its biological activity obviously depends on the sialic acid contents [1,2]. Furthermore, increase in the number of carbohydrate-attaching sites causes the increase of half-life in blood.

Recombinant immunoglobulins are emerging as powerful pharmaceuticals for therapeutic use to treat cancer and other life-threatening diseases. Some therapeutic monoclonal antibodies have been approved, and over one hundred monoclonal antibodies has been under on-going clinical trials, and the total income generated from these therapeutic antibodies is predicted to rise to US$ 10–20 $\times$ 10^9 by 2010 [3].

Relationship between biological functions and oligosaccharides of antibody pharmaceuticals has been extensively studied. Kumpel et al. [4,5] reported that lactosamine structure (i.e. presence of galactose (Gal) residues in the non-reducing terminals) affect antibody-dependent cellular cytotoxicity (ADCC), which is a major function of some therapeutic antibodies. Presence of bisecting *N*-acetylglucosamine (GlcNAc) also has been reported to improve ADCC [6,7]. Furthermore, recent reports indicated that the absence of fucose (Fuc) residue at the innermost GlcNAc of reducing ends showed more obvious ADCC than that caused by the presence of bisecting GlcNAc [8,9].

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Conditions for industrial production of glycoprotein pharmaceuticals are quite important to maintain the consistency of carbohydrate chains [10,11]. Therefore, assessment studies of oligosaccharides in glycoprotein pharmaceuticals are crucial for their quality assurance. Regulatory agencies have increasingly required determining the oligosaccharide distributions in quantitative base.

Many methods have been developed for characterizing oligosaccharides with good resolution and high sensitivity using various separation techniques such as high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD) [12] and high-performance liquid chromatography (HPLC) after derivatization with fluorogenic reagents [13–15]. Recent advances in capillary electrophoresis with laser-induced fluorescence detection (CE–LIF) have provided a rapid, high resolution, and high sensitivity analysis of the mixture of fluorescent-labeled oligosaccharides. The CE–LIF method for oligosaccharide analysis using 8-aminopyrene-1,3,6-trisulfonate (APTS) has been widely employed due to rapidity of analysis and high sensitivity [16–21]. Recently, we developed a CE–LIF method for oligosaccharide analysis using 3-aminobenzoic acid (3-AA) as labeling reagent, and revealed the usefulness when detected by a helium–cadmium (He–Cd) laser-induced fluorescent detector [22].

In this report, we applied a combination of 3-AA labeling method and CE–LIF to the oligosaccharide mapping of commercial therapeutic antibody pharmaceuticals: a chimeric monoclonal antibody for treating non-Hodgkin's lymphoma (rituximab) and a humanized monoclonal antibody for treating metastatic breast cancer (trastuzumab), and compared 3-AA derivatization method with APTS derivatization method.

2. Experimental

2.1. Materials

Peptide *N*-glycoamidase (PNGase F; EC 3.2.2.18, recombinant) was obtained from Roche Diagnostics (Mannheim, Germany). 8-Aminopyrene-1,3,6-trisulfonate was obtained from Beckman-Coulter (Tokyo, Japan). It should be noted that APTS often contains isomers to which sulfonic acid residues are attached in different positions. 3-Aminobenzoic acid was obtained from Tokyo Kasei (Tokyo, Japan) and used without further purification. Sodium cyanoborohydride was obtained from Aldrich (Milwaukee, WI, USA). DB-1 capillary was obtained from J&W Scientific (Folsom, CA, USA). Dimethyl sulfoxide (DMSO) and polyethylene glycol (PEG70000, average molecular mass 70 000) were from Wako (Tokyo, Japan). Sephadex G-25 and Sephadex LH-20 were purchased from Amersham Bioscience (Piscataway, NJ, USA). Therapeutic antibody products, rituximab and trastuzumab, were collected from the vials immediately after clinical use in Kinki University Nara Hospital. The solutions of antibody pharmaceuticals were dialyzed against distilled water for 3 days with changing water several times at 4 °C using cellulose membrane tubing (Sanko-junyaku, Tokyo, Japan), and then freeze-dried.

2.2. Releasing of N-linked oligosaccharides from antibody pharmaceuticals

A freeze-dried antibody pharmaceutical sample (1 mg) was dissolved in 20 mM phosphate buffer (pH 7.0, 50 μl) in a sample tube (1.5 ml) followed by addition of PNGase F (1 unit, 2 μl). The digestion was carried out at 37 °C overnight, and the reaction mixture was boiled for 5 min and centrifuged (10 000 × *g* for 10 min). The supernatant was dried by centrifugal vacuum evaporator (SpeedVac, Savant, Farmingdale, NY, USA).

2.3. Fluorescent derivatization of oligosaccharides with APTS

Fluorescent labeling of the oligosaccharides was performed according to the previously reported procedure [21]. Briefly, the enzyme reaction mixture obtained as described above was dissolved in 100 mM APTS solution in 15% acetic acid (2 μl). Then freshly prepared solution of 1 M $NaBH_3CN$ in tetrahydrofuran (2 μl) was added, and the mixture was overlaid with mineral oil (100 μl) to prevent evaporation of the reaction solvent. The derivatization was carried out at 55 °C for 90 min. The reaction mixture was diluted with water (100 μl), and the aqueous layer was applied on a column of Sephadex G-25 (30 cm × 1 cm i.d.) equilibrated with water. The earlier eluted yellowish fractions including fluorescent-labeled oligosaccharides [excitation (Ex.) 488 nm, emission (Em.) 520 nm] were pooled and evaporated to dryness. The residue was dissolved in 100 μl of water and a portion was used for the analysis by capillary electrophoresis.

2.4. Fluorescent derivatization of oligosaccharides with 3-AA

Procedures for derivatization of oligosaccharides with 3-AA were similar to those described in the previous report [22]. To the enzyme reaction mixture, a solution (30 μl) of 0.7 M 3-AA in DMSO-acetic acid (7:3, v/v) and freshly prepared solution (30 μl) of 2 M $NaBH_3CN$ in the same solvent were added. The derivatization was carried out at 50 °C for 60 min, and water (140 μl) was added to the mixture. The reaction mixture was applied on a column of Sephadex LH-20 (30 cm × 1 cm i.d.) 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.

2.5. Capillary electrophoresis of APTS labeled oligosaccharides

Capillary electrophoresis was performed on a P/ACE MDQ glycoprotein system (Beckman-Coulter) equipped with an argon-laser induced fluorescence detector (Ex. 488 nm, Em. 520 nm). Separations were performed using a DB-1 capillary (50 μm i.d., 20 cm effective length, 30 cm total length) in 50 mM Tris–acetate buffer (pH 7.0) containing 0.5% PEG70000 as the running buffer. Sample solutions were introduced to the capillary by pressure method (0.5 p.s.i. for 5 s; 1 p.s.i. = 6894.76 Pa). Analysis was performed by applying 18 kV at 25 °C.

2.6. Capillary electrophoresis of 3-AA labeled oligosaccharides

Capillary electrophoresis was performed on the same apparatus as described above, but a helium–cadmium laser induced fluorescence detector (Ex. 325 nm, Em. 405 nm) was installed to the apparatus. Separations were performed 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. Sample solutions were introduced to the capillary by pressure method (1 p.s.i. for 10 s). Analysis was performed by applying 25 kV at 25 °C.

We also used a longer capillary (100 μm i.d., 70 cm effective length, 80 cm total length) to achieve better resolution at 30 kV. The sample solution was introduced at 1 p.s.i. for 40 s. Other conditions were the same as described when a 30 cm capillary was used.

3. Results and discussion

3.1. Oligosaccharides in antibody pharmaceuticals, trastuzumab and rituximab

Oligosaccharides derived from some immunoglobulins have been investigated by capillary electrophoresis after releasing carbohydrate chains with PNGase F followed by labeling with APTS [18,19]. List of the major oligosaccharides in rituximab (an antibody drug for treatment of non-Hodgkin's lymphoma) is shown in Fig. 1 [18].

As reported previously, rituximab contains four major oligosaccharides. All oligosaccharides have a Fuc residue in the innermost GlcNAc residue. The most abundant oligosaccharide (**1** in Fig. 1) lacks both Gal residues in the non-reducing ends of the diantennary chain. The oligosaccharides **2** and **3** are the positional isomers. The oligosaccharide **2** has a Gal residue attached to the Manα1-6Man branch and **3** has a Gal residue at the Manα1-3Man branch. A small amount of the oligosaccharide **4** having lactosamine residues at both branches is also present.

We could easily confirm the oligosaccharide structures of trastuzumab by comparing electropherograms after

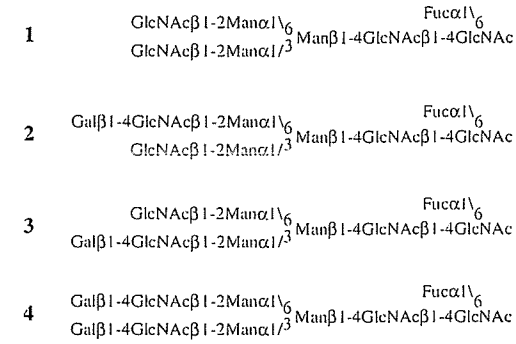

Fig. 1. List of the major oligosaccharides in rituximab.

analysis of oligosaccharides derived from rituximab and trastuzumab. A small amount of oligosaccharides (less than 3%) were observed in trastuzumab at earlier migration times (5.2 min–9.3 min). Matrix-assisted laser desorption/deionization time-of-flight mass (MALDI-TOF/MS) analysis clearly showed that these minor oligosaccharides were the oligosaccharide **1** lacking a GlcNAc residue, oligosaccharides (**2**, **3** and **4**) that have *N*-acetylneuraminic acid residues (data not shown). The oligosaccharide map of trastuzumab after derivatization with APTS is shown in Fig. 2a.

Because electroosmotic flow was negligible in the present analytical conditions using a capillary (DB-1) of which

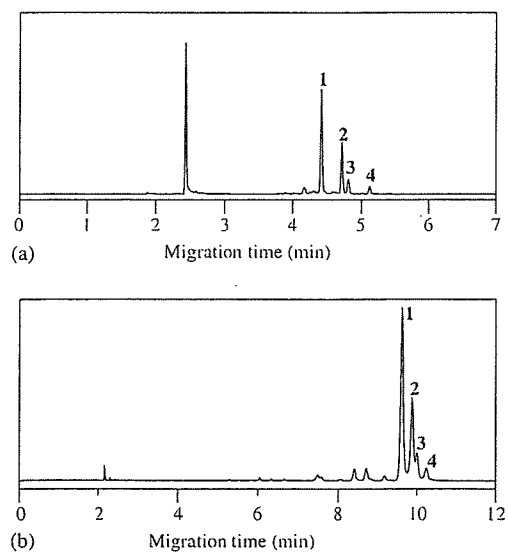

Fig. 2. Oligosaccharide maps of trastuzumab by capillary electrophoresis. (a) Oligosaccharides derivatized with APTS, (b) oligosaccharides derivatized with 3-AA. Analytical conditions: (a) capillary, DB-1 (50 μm i.d., 20 cm effective length, 30 cm total length); running buffer, 50 mM Tris–acetate buffer (pH 7.0) containing 0.5% PEG70000; injection, pressure method (0.5 p.s.i. for 5 s); applied voltage, 18 kV at 25 °C. (b) Capillary, DB-1 (100 μm i.d., 20 cm effective length, 30 cm total length); running buffer, 100 mM Tris–borate buffer (pH 8.3) containing 10% PEG70000; injection, pressure method (1.0 p.s.i. for 10 s); applied voltage, 25 kV at 25 °C. Structures of peak **1–4** are shown in Fig. 1.

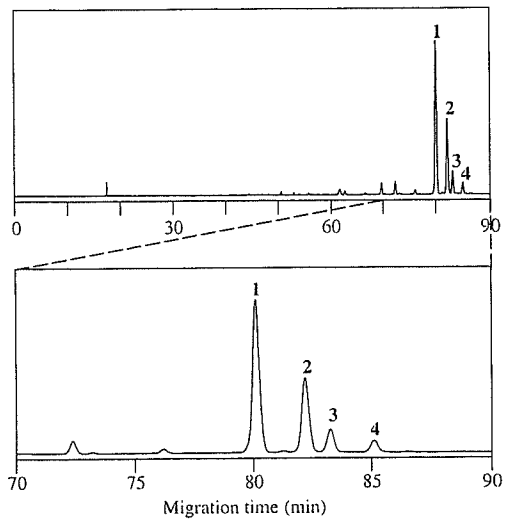

Fig. 3. Oligosaccharide map of 3-AA labeled oligosaccharides using a longer capillary (70 cm effective length). Analytical conditions: capillary, DB-1 (100 μm i.d., 70 cm effective length, 80 cm total length); running buffer, 100 mM Tris–borate buffer (pH 8.3) containing 10% PEG70000; injection, pressure method (1.0 p.s.i. for 40 s); applied voltage, 30 kV at 25 °C. Structures **1–4** as in Fig. 1.

surface is chemically modified with dimethylpolysiloxane, smaller oligosaccharides were observed earlier based on their charge/mass ratios. The oligosaccharide **3** having a Gal residue at Manα1-3Man branch has a more rigid conformation than that of the oligosaccharide **2**, and possibly has an apparent large molecular size [23]. Thus, oligosaccharide **3** having a Gal residue at Manα1-3Man branch was observed later than the oligosaccharide **2**.

We also analyzed oligosaccharides labeled with 3-AA. Because the 3-AA labeled oligosaccharides have negative charges due to a carboxylic acid residue, these oligosaccharides were also separated by capillary electrophoresis as shown in Fig. 2b. The resolution between **2** and **3** was not complete, but the analysis was completed within 12 min. The results showed basically similar electropherograms to that obtained for the analysis of APTS-labeled oligosaccharides (Fig. 2a). 3-AA labeling method has following advantages. (1) Highest grade reagent is easily commercially available or purified by recrystallization with methanol-water. (2) 3-AA reacts with reducing oligosaccharides in the mildest conditions without release of sialic acid residues [22]. This is important for the analysis of oligosaccharides in glycoproteins. (3) 3-AA labeled oligosaccharides can be analyzed by normal- or reversed-phase HPLC [14], which leads the applications for structural analysis of oligosaccharides. Furthermore, labeled oligosaccharides can be stable at −20 °C for several months.

In order to obtain sufficient resolution equivalent to APTS derivatization method, we used a longer capillary (70 cm effective length, 80 cm total length) for the analysis of 3-AA labeled oligosaccharides, and successfully obtained good resolution for all oligosaccharides, although longer analysis time (about 90 min) was required (Fig. 3).

Table 1
Precision of electrophoresis in migration times and relative corrected peak areas in APTS derivatization method

Injection	Migration time (min)				Relative corrected peak area (%)			
	1	2	3	4	1	2	3	4
1	4.41	4.71	4.80	5.12	61.8	26.9	7.9	3.6
2	4.43	4.73	4.83	5.14	60.7	27.6	8.1	3.5
3	4.43	4.74	4.83	5.15	59.4	28.5	8.5	3.6
4	4.43	4.74	4.84	5.16	58.6	28.8	8.7	3.9
5	4.44	4.75	4.85	5.16	58.2	29.0	8.8	4.0
6	4.44	4.75	4.85	5.17	58.4	28.8	8.7	4.1
Mean	4.43	4.74	4.83	5.15	59.5	28.3	8.5	3.8
SD	0.01	0.02	0.02	0.02	1.4	0.8	0.3	0.2
RSD (%)	0.2	0.3	0.4	0.3	2.4	2.9	4.3	6.6

Single preparation from trastuzumab was injected six times. **1–4** as in Fig. 1. Relative corrected peak areas were calculated as follows: corrected peak area = measured peak area/migration time, relative corrected peak area = corrected peak area/total corrected peak area × 100.

3.2. Repetitive analysis of APTS labeled oligosaccharides

We evaluated the APTS derivatization method. The continuous injections ($n = 6$) were performed from single preparation sample. The results suggested that the precision of migration time was sufficiently high with relative standard deviations (RSDs) below 0.4% (Table 1).

We also evaluated the precision in relative corrected peak areas (%). This is an important parameter because these values represent composition of oligosaccharides. The results indicated that precision was sufficiently high with RSD values below 6.6% (Table 1).

3.3. Repetitive analysis of 3-AA labeled oligosaccharides

We also evaluated 3-AA derivatization method. In the same manner as described in Table 1, continuous six times injections from single preparation sample were performed. The results show that the precision of migration time was sufficiently high with RSD below 1.9% (Table 2), and the precision of relative peak areas was also sufficiently high with the RSD below 6.1% (Table 2).

In the analysis, we employed a short capillary used in Fig. 2b. Although resolution between peak **2** and peak **3** was incomplete, the ratio showed almost the same values with that observed in APTS method (Table 1). These results indicate that 3-AA method has almost equivalent accuracy in relative peak areas with those observed in APTS method. Thus, we found that APTS and 3-AA derivatization methods showed similar accuracy and precisions both in migration times and relative peak areas. Easy derivatization and purity of the derivatization reagent are strong point for 3-AA, and rapidness in analysis time is a merit for APTS.

Table 2
Precision of electrophoresis in migration times and relative corrected peak areas in 3-AA derivatization method

Injection	Migration time (min)				Relative corrected peak area (%)			
	1	2	3	4	1	2	3	4
1	9.70	9.87	9.99	10.15	57.2	29.8	8.7	4.4
2	9.61	9.88	10.00	10.23	57.3	29.1	9.2	4.5
3	9.85	10.12	10.25	10.49	57.7	29.7	8.2	4.5
4	9.85	10.12	10.25	10.49	57.5	29.7	8.3	4.6
5	9.93	10.20	10.33	10.58	56.6	29.6	9.6	4.2
6	9.98	10.25	10.38	10.63	57.2	29.5	8.7	4.6
Mean	9.82	10.07	10.20	10.43	57.2	29.6	8.8	4.5
SD	0.14	0.16	0.17	0.19	0.4	0.2	0.5	0.2
RSD (%)	1.4	1.5	1.6	1.9	0.7	0.8	6.1	3.4

Single preparation from trastuzumab was injected six times. **1–4** as in Fig. 1. Relative corrected peak areas were calculated by the equation in Table 1.

Table 3
Analysis of oligosaccharides from different lot preparations of therapeutic antibodies by 3-AA derivatization method

Lot	Relative corrected peak areas (%)							
	Rituximab				Trastuzumab			
	1	2	3	4	1	2	3	4
a	37.5	30.5	15.5	16.5	55.8	26.3	11.4	6.5
b	43.4	37.2	9.9	9.5	56.5	27.9	10.5	5.0
c	43.1	36.4	10.4	10.1	53.7	28.6	12.6	5.1
d	47.1	36.0	9.4	7.5	57.6	26.5	10.8	5.0
e	46.4	35.8	9.6	8.1	55.1	30.3	9.2	5.4
f	42.1	33.3	12.8	11.8	55.9	29.1	10.3	4.8
g	42.3	37.5	10.4	9.8				
Mean	43.1	35.2	11.1	10.5	55.8	28.1	10.8	5.3
SD	3.2	2.5	2.2	3.0	1.3	1.5	1.1	0.6
RSD (%)	7.3	7.1	20.0	28.7	2.4	5.5	10.6	11.7

Structures **1–4** as in Fig. 1. Relative corrected peak areas were calculated by the equation in Table 1.

3.4. Lot-to-lot analysis

We applied the 3-AA derivatization method to the lot-to-lot analysis of therapeutic antibody pharmaceuticals. We used 7 and 6 lots from rituximab and trastuzumab, respectively. The electropherograms showed almost the same electropherograms for all lot preparations as shown in Fig. 4. Compositions of oligosaccharides were shown in Table 3.

The RSD values of corrected relative peak areas were below 28.7% in rituximab, and 11.7% in trastuzumab. It should be noticed that these values include not only precision of lot production, namely actual variation of oligosaccharide composition from lot-to-lot, but also the precision of experimental procedure. Furthermore, experimental procedure includes sample preparation (release of oligosaccharide, derivatization and purification) and electrophoresis (injection, migration and integration). Latter electrophoresis precision is shown above (Section 3.3). As for the sample preparation precision, we observed good precision with the RSD of relative peak areas below 3.2% in the experiment of simultaneous three preparations from the same lot of antibody and each single injection to CE (data not shown). The RSDs observed in the lot-to-lot analysis (Table 3) were bigger than that of experimental precision, suggesting that the variations of oligosaccharide composition observed in Table 3 were derived mainly from the difference of actual oligosaccharide composition and that the present method could successfully detected it.

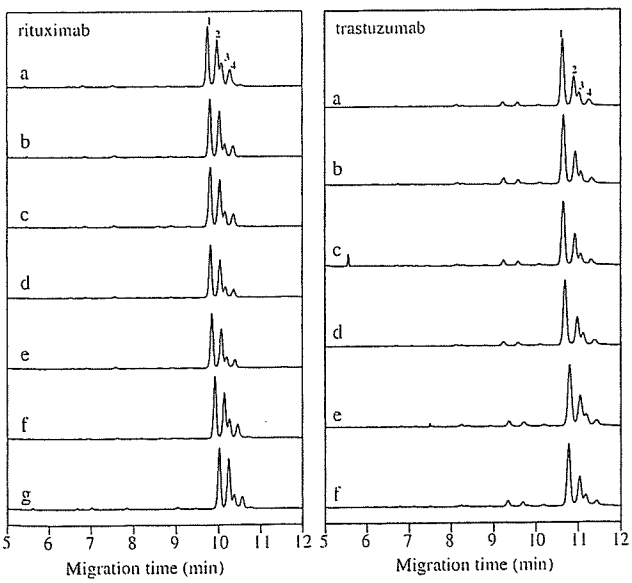

Fig. 4. Oligosaccharide maps from different lot preparations of therapeutic antibodies, rituximab (left) and trastuzumab (right) by 3-AA derivatization method. Analytical conditions are the same as in Fig. 2b. Structures **1–4** as in Fig. 1.

4. Conclusion

In capillary electrophoresis of carbohydrates in therapeutic glycoprotein pharmaceuticals, APTS derivatization method is widely used due to its rapid analysis time and high sensitivity. In the present paper, we applied 3-AA derivatization method to the analysis of carbohydrates in therapeutic antibody pharmaceuticals and found that this method had sufficient resolution to characterize the oligosaccharide composition in antibody pharmaceuticals and almost equivalent repeatability and accuracy with APTS derivatization method. Although 3-AA method needs longer analysis time than APTS method, it is noted that CE apparatus is generally equipped with an auto-sampler.

Furthermore, the 3-AA derivatization method has some advantages; high purity reagent is easily available from any vendors, 3-AA labeled oligosaccharides can be analyzed by HPLC and be stable at −20 °C for several months.

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Electrophoretic analysis of di- and oligosaccharides derived from glycosaminoglycans on microchip format

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Abstract

Microchip electrophoresis is a powerful tool for fast analysis of nucleic acids and has expanded its applicability to the analysis of various biological materials including proteins and carbohydrates. Glycosaminoglycans have intrinsic negative charges, and are good targets for electrophoretic analysis. In the present paper, we developed a method to analyze oligosaccharides and unsaturated disaccharides derived from some glycosaminoglycans after digestion with specific enzymes followed by derivatization with 2-aminoacrydone (AMAC) by reductive amination. The method described here allowed rapid analysis of oligosaccharides derived from glycosaminoglycans within 150 s with high sensitivity. We show an application of the present technique to the glycosaminoglycan analysis in cultured HeLa cells.

Keywords: Microchip electrophoresis; Capillary electrophoresis; Glycosaminoglycan; Oligosaccharide

1. Introduction

Capillary electrophoresis on microchip format is a new technology that promises fast and sensitive analysis in genome areas [1]. It has a potential to simultaneously analyze hundreds of samples in a matter of minutes or less using multi channels. Electrophoresis on microchip is now applied to the routine analysis of nucleic acids, proteins. Some instruments for microchip electrophoresis are commercially available.

Microchip electrophoresis has been applied to the analysis of some proteins labeled with fluorescein [2], fluorescein isothiocyanate-labeled amino acid enantiomers [3], carbohydrates [4,5] and DNA fragments [6]. Separation performance of microchip electrophoresis is basically similar to that of capillary electrophoresis, but microchip electrophoresis can be performed on a time scale of seconds.

A variety of modern chromatographic techniques such as high-performance liquid chromatography, polyacrylamide gel electrophoresis and capillary electrophoresis have been reported for the analysis of carbohydrates [7]. Capillary electrophoresis has revealed the ability in separation of carbohydrates, although most carbohydrates must be converted to ion forms prior to the analysis. Furthermore, carbohydrates should be labeled with chromophores or fluorophores for sensitive detection [8].

Glycosaminoglycans such as heparin, chondroitin/dermatan sulfate, or keratan sulfate are the constituents of proteoglycans. Hyaluronic acid (hyaluronan) is an important constituent of tissue matrices. Chondroitin sulfate (ChS) and dermatan sulfate (DS), usually categorized as galactosaminoglycans composed of repeating disaccharide units of an uronic acid and an aminosugar are highly charged polymers due to the presence of sulfate groups [9]. Sulfation of hydroxyl and amino groups plays a key role in determination of physiological characteristics of proteoglycans. Altered sulfation patterns are often encountered in pathological state [10]. Hyaluronic acid is a macromolecular anionic polysaccharide, and widely used for medical use. Oligosaccharides derived from hyaluronic acid show some interesting biological activities such as angiogenesis and inhibition of cell apoptosis [11,12].

In the analysis of glycosaminoglycans in biological materials, glycosaminoglycans are collected by a combination of precipitation methods using quaternary ammonium salts such as cetylpyridinium chloride and ethanol [13], and exam-

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ined mainly by two methods. The one is an electrophoretic method using cellulose acetate membrane, and can examine glycosaminoglycans as native state after staining with specific reagents. The other uses specific enzymes such as lyases and hydrolases. Identification and quantitation of the oligosaccharides thus obtained are performed using gel electrophoresis [14], high-performance liquid chromatography [15,16] or capillary electrophoresis [9,17].

In the present report, we propose a rapid method to analyze di- and oligosaccharides derived from glycosaminoglycans using microchip electrophoresis after derivatization with 2-aminoacrydone (AMAC).

2. Materials and methods

2.1. Chemicals

Hyaluronic acid (*Streptococcus zooepidemicus*, HA) was obtained from Nakalai Tesque (Nakagyo-ku, Kyoto, Jpana). Hyaluronidase (sheep testis) was obtained from Roche Diagnostics (Minato-ku, Tokyo, Japan). AMAC was obtained from molecular probes (Eugen, OR). Chondroitin 4- and 6-sulfate and chondroitinase ABC and standard samples of unsaturated disaccharides were from Seikagaku Kogyo (Nihonbashi, Chuo-ku, Tokyo, Japan). Pronase was obtained from Calbiochem (San Diego, CA, USA). Polyethyleneglycol (MW 70,000) was obtained from Wako Pure Chemicals (Dosho-machi, Osaka, Japan). A chemically-modified capillary (DB-1) was purchased from J&W Scientific (Folsom, CA, USA). Other reagents and solvents were of the highest grade commercially available.

2.2. Preparation of oligosaccharides derived from hyaluronic acid (HA) and chondroitin sulfate (ChS)

Oligosaccharides derived from HA (HA oligomers) were prepared by digestion of HA with hyaluronidase. Briefly, HA (*S. zooepidemics*, 100 mg) was dissolved in 150 mM citrate buffer (pH 5.3, 10 ml). Hyaluronidase (sheep testis, 10 mg) was added to the solution, and kept at 37 °C for 24 h. After keeping the mixture in a boiling water bath for 10 min, the mixture was centrifuged and the supernatant was collected. The supernatant (ca. 10 ml) was mixed with 95% ethanol (30 ml) containing 1.3% potassium acetate and 0.27 mM EDTA and kept on an ice bath for 2 h. The precipitate was collected, dried under reduced pressure and used as the mixture of HA oligomers. The mixture of oligosaccharides contained from tetrasaccharide to polymers containing ca. 50 monosaccharides. Oligomers having a defined size were purified by HPLC on an amine-bonded silica column (YMC-PAC PA5) using NaH_2PO_4 as eluent according to the method reported previously [17].

Oligosaccharides derived from chondroitin sulfates (1 mg) were also obtained after digestion with hyaluronidase (sheep testis, 0.5 mg) in the similar manner as above.

2.3. Fluorescent labeling of oligosaccharides with AMAC

Labeling reaction of oligosaccharides with AMAC was performed using the method reported previously [9]. A mixture of oligosaccharides or the standard sample of an oligosaccharide (100 μg) was dissolved in 100 mM AMAC in a mixture (10 μl) of dimethylsulfoxide-acetic acid (17:3, v/v). Into the solution, was added 1 M sodium cyanoborohydride (10 μl) in the same solvent. After keeping the mixture at 90 °C for 30 min, water (500 μl) and chloroform (500 μl) were to the reaction mixture and mixed vigorously by a vortex mixer. After removing the chloroform layer, the aqueous phase was washed with chloroform (500 μl). After evaporation of the aqueous phase, the residue was dissolved in water (100 μl) and a portion was used for microchip electrophoresis.

2.4. Digestion of AMAC-labeled HA 20 mer (HA_{20})

We examined time course of digestion of HA_{20} with hyaluronidase. Preparation of HA_{20} and fluorescent labeling reaction with AMAC were performed as described above. An aqueous solution (10 μl) of AMAC-labeled HA_{20} (20 μg/ml) was mixed with 200 munits of hyaluronidase (10 μl) in 10 mM acetate buffer (pH 5.3), and kept at 37 °C. At specified intervals, the mixture was analyzed by microchip electrophoresis.

2.5. Digestion of chondroitin sulfates with chondroitinase ABC

Chondroitin 4-sulfate, chondroitin 6-sulfate or the equal amount of the mixture (100 μg each) was dissolved in 20 mM Tris–hydrochloric acid buffer (pH 8.0, 100 μl), and an aqueous solution of chondroitinase ABC (100 munits, 4 μl) was added. After incubating the mixture overnight at 37 °C, the mixture was kept in a boiling water bath for 10 min. After cooling, the mixture was evaporated to dryness by a centrifugal evaporator (SpeedVac, Servant). The residue was dissolved in a mixture (10 μl) of dimethylsulfoxide-acetic acid (17:3, v/v), and labeling reactions with AMAC was performed as described above.

2.6. Microchip electrophoresis (ME) of AMAC-labeled oligosaccharides

ME was performed on a Hitachi Microchip electrophoresis apparatus (Type SV1100) with an LED detector. The excitation wavelength of the LED detector is preset at 470 nm by the manufacturer, and a detection filter for 580 nm or longer wavelengths is installed. The chip made of polymethylmetacrylate (PMMA) has a simple cross channel of 100 μm width and 30 μm depth. The distances from the channel intersection to the sample, sample waste, buffer and buffer waste wells are 5.25, 5.25, 5.75, and 37.5 mm, respectively. The effective length for separation is 30 mm.

Buffer solutions were introduced into the microchannels with a syringe. All reservoirs on the microchips were filled with either running buffer or a sample solution prior to analysis. In the sample loading step, 300 V was applied to the sample well, and separation was performed by applying the potential of 750 V (130 V at the sample introduction side).

2.7. Capillary electrophoresis (CE) of AMAC-labeled oligosaccharides

CE was performed on a Beckman P/ACE MDQ Glycoprotein System fitted with an LIF detector, a laser module (3 mW, air-cooled argon laser with $\lambda_{ex} = 488$ nm). Detection was performed using a 520 nm filter. Separation was carried out at 25 °C using a DB-1 capillary (50 μm i.d., 60 cm length) in 100 mM Tris–acetate buffer (pH 7.5) containing 1% polyethyleneglycol. The sample solution was injected by electrokinetic method (15 kV, 10 s). The applied voltage for separation was 25 kV.

2.8. Glycosaminoglycans from HeLa cells

HeLa cells were cultured in DMEM containing 10% newborn calf sera (NCS) under 5% CO_2 atmosphere at 37 °C. The cells were collected with a cell scraper, and washed with phosphate buffered saline (PBS, 1 ml) several times. Cells (10^7 cells) were suspended in 20 mM phosphate buffer (pH 7.0, 1 ml) and incubated for 20 min at room temperature to remove culture medium. The cells were homogenized in the same buffer (1 ml) with a Teflon-glass homogenizer, and centrifuged at 8000 × *g* for 10 min at 4 °C. The supernatant was collected and diluted with 0.5 M Tris–hydrochloric acid buffer (pH 8.0, 1 ml), and the mixture was digested with pronase (2 mg) at 37 °C overnight. After keeping the mixture in a boiling water bath for 10 min, the mixture was centrifuged at 8000 × *g*, and the supernatant was collected. Ninety-five percent ethanol containing 1.3% potassium acetate and 0.27 mM EDTA (6 ml) was added to the mixture, and kept at 0 °C for 2 h. The precipitate (500 μg) was dissolved in 20 mM Tris–HCl buffer (pH 8.0, 100 μl), and incubated with chondroitinase ABC (4 μl) at 37 °C overnight. After keeping the mixture in a boiling water bath for 5 min followed by centrifugation, the supernatant was lyophilized and labeled with AMAC as described above.

3. Results and discussion

3.1. Selection of fluorescent-labeling reagent

Most commercially available equipments for microchip electrophoresis are designed for the analysis of nucleic acids, and fluorescence due to intercalation with ethidium bromide is monitored using an LED light source (Ex = 518 nm; Em = 605 nm). In the present study, we aimed at rapid analysis of oligosaccharides derived from glycosaminoglycans obtained after digestion with enzymes. Therefore, we selected AMAC as the derivatization reagent [17], because AMAC can be easily removed by extraction with chloroform after derivatization by reductive amination in the presence of $NaBH_3CN$ as described in Section 2. Other reagents such as 8-aminopyrene-1,3,6-trisulfonate (APTS) and 3-aminobenzoic acid requires purification of the labeled oligosaccharides by gel chromatography [18,19], and did not show good sensitivity at this wavelength.

3.2. Analysis of oligosaccharides derived from hyaluronic acid, chondroitin sulfate A and chondroitin sulfate C

Fig. 1 shows the separation of oligosaccharides derived from hyaluronic acid, chondroitin sulfate A and C using ME and CE after digestion of each glycosaminoglycan with hyaluronidase (sheep testis).

In ME, larger oligomers of hyaluronic acids were observed earlier, and all oligomer peaks were observed between 100 and 150 s (Fig. 1A(a)). Hexa- and tetra-saccharides derived from chondroitin sulfate A were observed within 100 s (Fig. 1A(b)). Oligosaccharide mixture derived from chondroitin sulfate C was also observed within 100 s. However, CE showed better resolutions for these oligosaccharides, although longer analysis times were required. In the analysis of oligosaccharides derived hyaluronic acid by CE, we easily confirmed larger oligosaccharides composed of 14 monosaccharide units (Fig. 1B(a)). In ME, we employed a chip made of polymethylmetacrylate. Hydrophobic interaction between AMAC and surface of the chip was one of the reasons for worse resolution among each oligosaccharide peaks than that observed in CE. Polyethyleneglycol was also added at 1.0% concentration to prevent non-specific interactions. At this concentration, molecular sieving effect is negligible, and larger oligomers were observed earlier based on charge/molecular sizes.

3.3. Digestion of hyaluronic acid 20 mer (HA_{20}) with hyaluronidase

Hyaluronidase (sheep testis) is an *endo*-type enzyme, and cleaves the glycosidic bonds to form a mixture of oligosaccharides. In detailed examination of an *endo*-type enzyme reaction, we have to analyze the reaction products during the course of enzyme reaction. We digested AMAC-labeled HA_{20} with hyaluronidase, and monitored the course of enzyme reaction. The results are shown in Fig. 2.

After 0.5 h, HA_8, HA_{10} and larger oligomers were clearly observed (Fig. 2(b)). After 1 h, HA_8, HA_{10} and HA_{12} were observed as the major products. Finally, HA_4 was observed abundantly, and HA_6 was also observed as the minor product after 2 h (Fig. 2(e)). These data indicate that hyaluronidase did not cleave HA_8 to HA_6 and HA_2 but produced two molecules of HA_4. These characteristic patterns of enzyme action were in good agreement with those as reported previously [20].

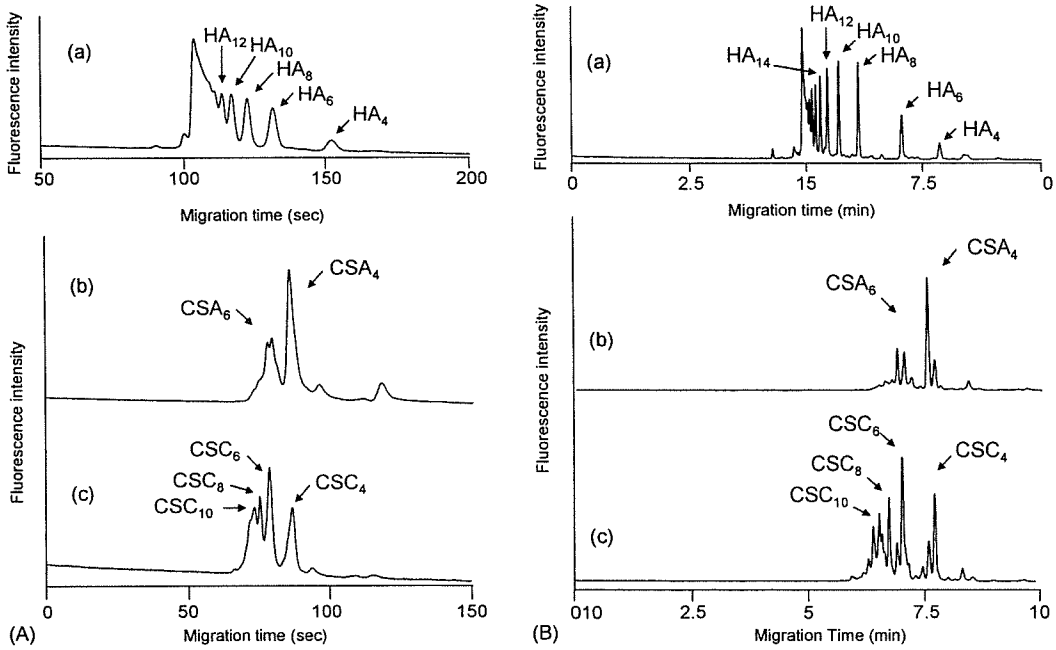

Fig. 1. Microchip electrophoresis (A) and capillary electrophoresis (B) of oligosaccharides derived from hyaluronic acid (a), chondroitin sulfate A (b) and chondroitin sulfate C (c). The samples were obtained by digestion with hyaluronidase followed by labeling with AMAC. The numbers in the figure indicate the degree of polymerization. Analytical conditions for microchip electrophoresis: buffer, 0.1 M Tris–acetate (pH 7.5) containing 1% polyethyleneglycol 70,000; applied voltage for sample injection: 300 V; for analysis: 750 V. Analytical conditions for capillary electrophoresis: capillary, fused silica capillary coated with dimethylpolysiloxane (60 cm, 50 μm i.d., effective length of 50 cm); buffer, 0.1 M Tris–acetate (pH 7.5) containing 1% polyethyleneglycol 70,000; applied voltage, 20 kV; temperature, 25 °C. Fluorescence detection was performed with a 520 nm light filter for emission by irradiating with an argon laser (488 nm). The sample solution was introduced by pressure method (1 psi) for 10 s. The numbers indicate the degree of polymerization.

It should be noticed that the time required for each run was only 3 min, and ME made it possible to monitor the reaction course using a portion of the reaction mixture. This is quite important for monitoring the enzyme reaction course, because other chromatographic and electrophoretic methods require longer operation times.

3.4. Analysis of unsaturated disaccharides derived from chondroitin sulfates

Glycosaminoglycans such as chondroitin/dermatan sulfate and hyaluronic acid are usually converted to unsaturated disaccharides through the action of chondroitin sulfate lyases, and these disaccharides are analyzed by HPLC or CE [21,22]. In the analysis of these unsaturated disaccharides by ME, it is important to find appropriate conditions for resolving ΔDi-4*S* and ΔDi-6*S* (positional isomers of sulfate groups). Fig. 3 shows pH effect on the resolution of ΔDi-4*S* and ΔDi-6*S* derived from chondroitin 4-sulfate and 6-sulfate, respectively.

We examined Tris–acetate buffer and Tris–borate buffer at some pHs that showed good resolution in the CE analysis of sialic acid-containing oligosaccharides [18]. When 0.1 M Tris–acetate buffer was used as the running buffer, resolution was poor as shown in Fig. 3A(a–d). However, both unsaturated disaccharides were successfully resolved in 0.1 M Tris–borate buffer at pH 8.0 (Fig. 3A(f)), although baseline resolution was not achieved. In the carbohydrate analyses, Tris–borate buffer often shows better separation efficiency than Tris–acetate buffer probably due to competitive complex formation between carbohydrates/Tris and borate ions. Low-operation voltage in Tris–borate buffer is also preferable for electrophoresis [8].

Under the conditions described above, we analyzed several commercially available standard samples of unsaturated disaccharides (Fig. 3B). ΔDi-4*S* and ΔDi-6*S* were observed at 95 s and 90 s, respectively, and ΔDi-UA2*S* was observed at 95 s (Fig. 3B(a–c)). ΔDi-diS_B containing two sulfate groups was observed earliest at 70 s. From these results, we could determine all the sulfate-containing unsaturated disaccharides within 100 s.

We digested chondroitin sulfate A, C and D and dermatan sulfate with chondroitinase ABC, and analyzed the unsaturated disaccharides after labeling with AMAC (Fig. 4).

In digestion of chondroitin sulfate A (i.e. chondroitin 4-sulfate), ΔDi-4*S* was observed abundantly, and a small peak of ΔDi-6*S* was also observed (Fig. 4(a)). Chondroitin sulfate C (i.e. chondroitin 6-sulfate) gave ΔDi-6*S* with a small peak of ΔDi-4*S* as shown in Fig. 4(b). In digestion of dermatan sulfate, ΔDi-4*S* was observed at the same migration time with that of ΔDi-4*S* derived from

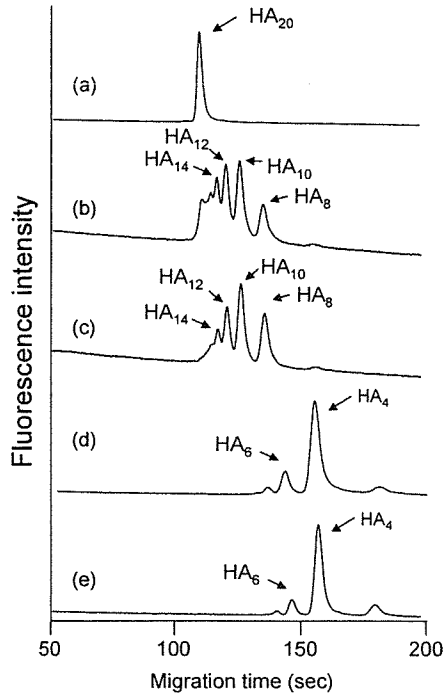

Fig. 2. Digestion of 20 mer of hyaluronic acid (HA_{20}) with hyaluronidase. HA_{20} was incubated with hyaluronidase at 37 °C. After a specified intervals from 0 h to 3 h, a portion was analyzed by ME. Reaction time: (a) 0 h, (b) 0.5 h, (c) 1.0 h, (d) 2.0 h and (e) 3.0 h. Analytical conditions were the same as in Fig. 1. The numbers indicate the degree of polymerization.

chondroitin sulfate A. On the contrary, chondroitin sulfate D gave almost equal amount of ΔDi-6*S* and ΔDi-4*S* at around 100 s. All electropherograms also showed a peak due to unsaturated disaccharide containing disulfate groups (ΔDi-diS_D and ΔDi-diS_B) around 70 s. Especially, chondroitin sulfate D gave a large amount of ΔDi-2*S*6*S* (ΔDi-diS_D) on digestion with chondroitinase ABC (Fig. 4(d)).

3.5. Detection limit and calibration curves

Fig. 5 shows the electropherograms of unsaturated disaccharides derived from chondroitin sulfate A at three different concentration levels.

At 10 ng/ml as the amount of chondroitin sulfate A, we could confirm ΔDi-4*S* at the signal to noise ratio of ca. 3 (Fig. 5(c)).

The apparatus used in the present study is customized for the analysis of nucleic acids, and the peak responses are normalized using specific software. Therefore, we observed linearity for quite narrow range from 10 μg/ml to 100 μg/ml. Thus, we could not show linearity data in the present work. However, it is often necessary to identify which glycosaminoglycans are present in routine clinical analysis, and the present technique will be useful for such purpose.

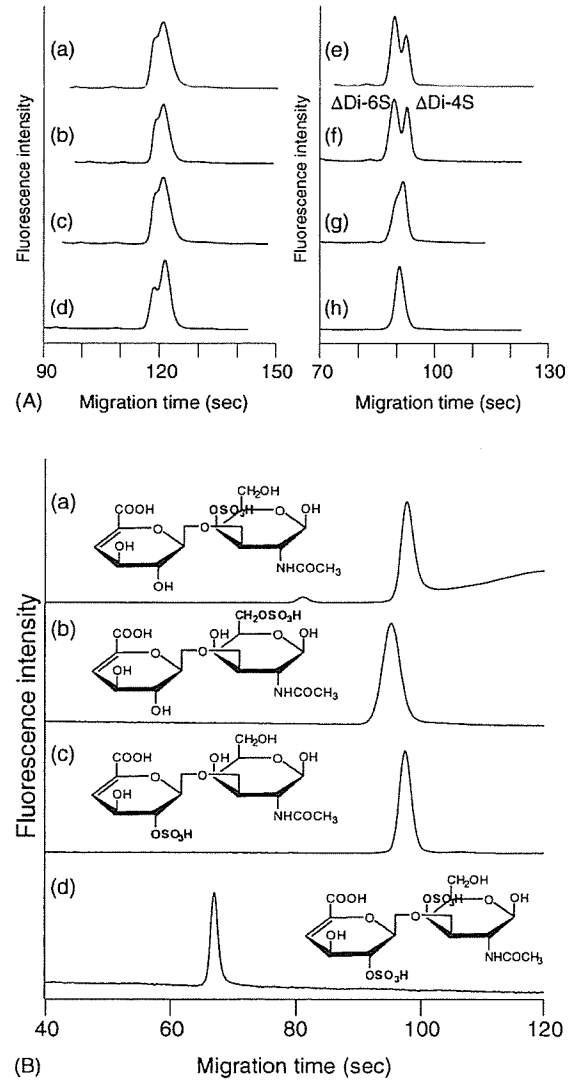

Fig. 3. Optimization for the analysis of unsaturated disaccharides (ΔDi-4*S* and ΔDi-6*S*) derived from chondroitin sulfates and analysis of some unsaturated sulfated disaccharides. (A) Mixture of ΔDi-4*S* and ΔDi-6*S* (unsaturated disaccharides derived from chondroitin sulfate A and C, respectively) was analyzed in 0.1 M Tris–acetate buffer containing 1% polyethyleneglycol 70,000 at pH 6.0 (a), pH 7.0 (b), pH 8.0 (c) and pH 9.0 (d), and also analyzed in 0.1 M Tris–borate buffer containing 1% polyethyleneglycol 70,000 at pH 7.5 (e), pH 8.0 (f), pH 8.5 (g) and pH 9.0 (h). (B) Analysis of ΔDi-4*S* (a), ΔDi-6*S* (b), ΔDi-UA2*S* (c) and ΔDi-diS_B (ΔDi-di2*S*4*S*) (d). Analytical conditions for Fig. 3B: running buffer, 0.1 M Tris–borate buffer containing 1% polyethyleneglycol 70,000 at pH 8.0.

3.6. Application

We applied the present method to the analysis of glycosaminoglycans derived from HeLa cells. The results are shown in Fig. 6.

Proteoglycan fractions derived from HeLa cells gave six peaks after digestion with chondroitinase ABC followed by labeling with AMAC. By comparison with standard samples

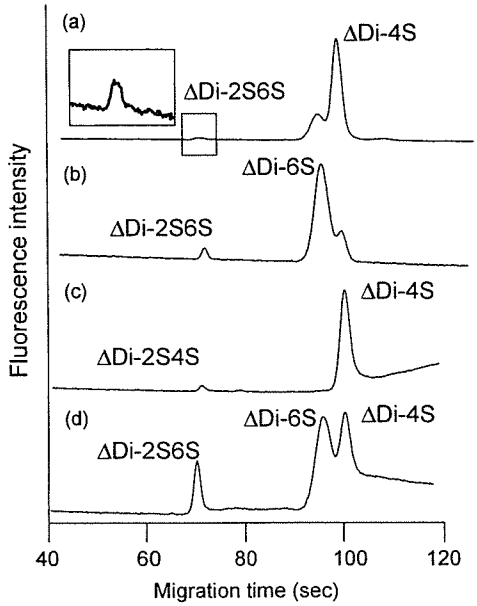

Fig. 4. Analysis of unsaturated disaccharides after digestion of chondroitin sulfates followed by labeling with AMAC. (a) Chondroitin sulfate A, (b) chondroitin sulfate C, (c) dermatan sulfate and (d) chondroitin sulfate D. Analytical conditions were the same as described in Fig. 3B.

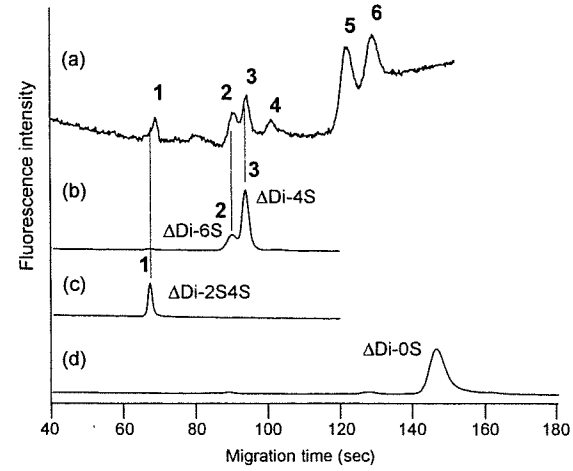

Fig. 6. Analysis of unsaturated disaccharides derived from glycosaminoglycan fractions obtained from HeLa cells. Analytical conditions: buffer; 0.1 M Tris–borate/1% polyethyleneglycol 70,000 (pH 8.0), applied voltage; for sample injection: 300 V; for analysis: 750 V. (a) Unsaturated disaccharides derived from HeLa cells digested with chondroitinase ABC. (b) Unsaturated disaccharides derived from chondroitin sulfate A (whale cartilage). (c) A commercial sample of ΔDi-diS_B (ΔDi-di2*S*4*S*). (d) A commercial sample of ΔDi-0*S*. The peak of ΔDi-HA was observed at the later migration time than ΔDi-0*S* (date not shown).

of unsaturated disaccharides, we identified the earlier observed three peaks (1, 2 and 3). Peak 1 was due to ΔDi-di*S*, and peaks 2 and 3 were due to ΔDi-6*S* and ΔDi-4*S*, respectively. These data indicated the presence of chondroitin sulfate A, and C and dermatan sulfate in HeLa cells. We compared three peaks (4, 5 and 6) with those of ΔDi-0*S* and ΔDi-HA. ΔDi-HA was observed at later migration times and not included in the figure. We found that these peaks were not due to ΔDi-0*S* and ΔDi-HA, and further studies are required.

From these results, microchip electrophoresis is a powerful tool for fast examination of glycosaminoglycans in cultured cells (within 150 s), and will be useful for the studies on cell cultures and tissue matrices.

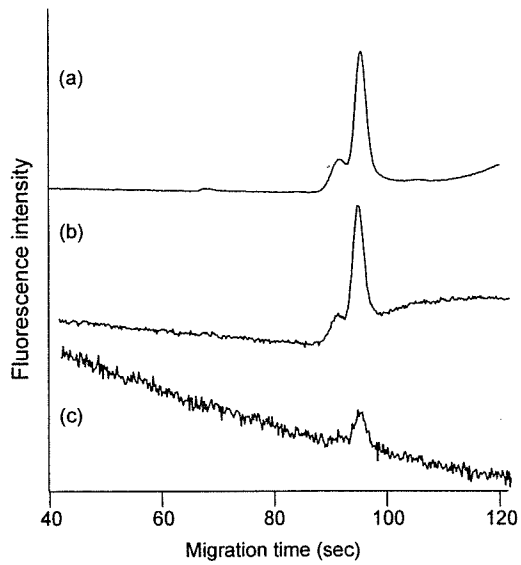

Fig. 5. Analysis of ΔDi-4*S* derived from chondroitin sulfate A at (a) 1.0 μg/ml, (b) 100 ng/ml and (c) 10 ng/ml. Analytical conditions were the same as in Fig. 3B.

4. Conclusion

Microchip electrophoresis has been used for the analysis of nucleic acids, and the rapidness in the analysis will be useful to clinical analysis. Furthermore, fabrication of the apparatus with multichannel mode will allow the analysis of many samples at a time. In the present paper, we digested some glycosaminoglycan samples with hyaluronidase (sheep testis) and chondroitinase ABC, and the di- and oligosaccharides were labeled with AMAC in the presence of sodium cyanoborohydride. The labeling method using AMAC is quite easy, because the excess amount of the reagent can be removed by extraction with chloroform. Unfortunately, the wavelength of AMAC derivative has the excitation maximum at 425 nm, and is different from that of LED installed in the apparatus.

All unsaturated disaccharides labeled with AMAC were observed within 150 s. ΔDi-4*S* was detected at 10 ng/ml with signal to noise ratio of 3. If more appropriate light source is available, we will be able to detect the enzyme reaction products at higher sensitivity.

We applied the present method to the analysis of the digestion course of HA_{20} with hyaluronidase, and showed that

the rapidness is especially useful for the assay of *endo*-type enzyme such as hyaluronidase.

In conclusion, microchip electrophoresis will be a promising alternative to HPLC or CE in the analysis of unsaturated disaccharides derived from glycosaminoglycans.

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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

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We developed capillary affinity electrophoresis (CAE) to analyze the molecular interaction between carbohydrate chains and proteins in solution state (Nakajima *et al.* [2003] *J. Proteome Res.*, 2, 81–88). A mixture of oligosaccharides derived from a glycoprotein was labeled with 8-aminopyrene-1,3,6-trisulfonate (APTS), and used as glycan library without isolation. Interaction of a carbohydrate-binding protein with each oligosaccharide in the mixture could be simultaneously observed, and relative affinities of oligosaccharides toward the protein were accurately determined. In this study, we applied CAE to detect the presence of lectins in some plants (Japanese elderberry bark and tulip bulb). In the crude extract of the elderberry bark, binding activity toward sialocarbohydrate chains could be easily detected. We also examined the presence of lectins in the crude extract of tulip bulbs and determined the detailed carbohydrate-binding specificity of *Tulipa gesneriana* agglutinin (TGA), one of the lectins from tulip bulbs. Kinetic studies demonstrated that TGA showed novel carbohydrate-binding specificity and preferentially recognized triantennary oligosaccharides with Gal residues at nonreducing termini and a Fuc residue linked through α(1-6) linkage at chitobiose portion of the reducing termini but not tetraantennary carbohydrates. The results described here indicate that CAE will be a valuable method for both screening of lectins in natural sources and determination of their detailed carbohydrate-binding specificities.

Key words: capillary affinity electrophoresis/carbohydrate-binding specificity/8-aminopyrene-1,3,6-trisulfonate/lectin

Introduction

Glycosylation is one of the most important events of posttranslational modification of proteins, and it is involved in various processes, such as protein folding, molecular recognition, and protein stabilization (Hakomori, 2000; Lasky, 1995). To understand the function of carbohydrates in glycoproteins, it is important to elucidate their structures and distributions. Lectins are a versatile tool for categorizing carbohydrates because they specifically recognize carbohydrate structures. When a lectin is used for recognition of carbohydrates, carbohydrate-binding specificity of the lectin should be strictly defined. Although a large number of lectins have been reported, only a limited number of reports are available for carbohydrate-binding studies. This is because most of the binding specificities have been determined based on semiquantitative methods, such as an agglutination assay, which is still one of the most popular methods.

Most of the assay methods for carbohydrate-binding proteins, such as surface plasmon resonance (Shinohara *et al.*, 1997), fluorescence polarization (Oda *et al.*, 1998), and time-resolved fluorometry (Lee *et al.*, 1998; Nakajima *et al.*, 2002), essentially require carbohydrates in a pure state. However, it is often too laborious to obtain pure carbohydrates in amounts necessary for kinetic studies by either chemical synthesis or isolation from natural sources.

We have developed capillary affinity electrophoresis (CAE) to analyze the molecular interaction between carbohydrates and proteins in solution state (Nakajima *et al.*, 2003). A mixture of oligosaccharides derived from a glycoprotein was labeled with 8-aminopyrene-1,3,6-trisulfonate (APTS) and used without isolation of each oligosaccharide. Interaction between a lectin and each carbohydrate chain in a mixture was determined simultaneously based on their change of electrophoretic mobilities or peak intensities. Furthermore, we found that CAE allowed calculation of affinity constants without determination of the accurate concentrations of carbohydrates (i.e., ligands) that are often difficult to measure. We showed that CAE was applied to classify carbohydrate chains in biological samples using a few lectins (Nakajima *et al.*, 2003).

In the present study, we show that CAE can examine the presence of a lectin in a crude biological extract using an appropriate set of oligosaccharides. At the initial step, a mixture of oligosaccharides of known compositions is analyzed in the absence of lectin. Then the same mixture is analyzed in the same buffer containing the extract. If we observe changes of migration of oligosaccharides, such changes indicate that a substance (typically lectins) that interacts with the carbohydrates is present in the extract. Another set of a mixture of different oligosaccharides is analyzed in the same manner as described. By repeating these procedures, we can confirm the presence of a lectin and determine the detailed binding specificity of the lectin.

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It is important to select an appropriate set of carbohydrates from various glycoproteins. In the previous study, we showed that human α1-acid glycoprotein (AGP), bovine IgGs, porcine thyroglobulin, bovine ribonuclease B, and bovine fetuin were appropriate as sources of N-linked oligosaccharides, because oligosaccharide compositions of these glycoproteins have been well characterized by a number of studies. The N-linked oligosaccharides or their desialylated oligosaccharides derived from these glycoproteins is shown in Table I. These oligosaccharides were previously labeled with APTS before use.

N-linked oligosaccharides of AGP, porcine thyroglobulin, and fetuin are available for detection of both sialic acid–binding lectins and lectins recognizing complex-type oligosaccharides (Kakehi *et al.*, 1999; Nakajima *et al.*, 2003; Yamamoto *et al.*, 1981). Thyroglobulin is also available for finding mannose-specific lectins because it contains high-mannose type oligosaccharides. AGP and thyroglobulin also contain oligosaccharides with α(1-3) and α(1-6) linked fucose residues, respectively, and they are used for detection of fucose-binding lectins. Asialofetuin contains one di- and two triantennary complex-type oligosaccharides. One of the asialo-triantennary oligosaccharides has three Galβ(1-4)GlcNAc residues, and another oligosaccharide has one Galβ(1-3)GlcNAc branch and two Galβ(1-4)GlcNAc residues (Green *et al.*, 1988). Bovine IgG has diantennary complex-type oligosaccharides with α(1-6) linked fucose residues (Raju *et al.*, 2000). Oligosaccharides of ribonuclease B are high-mannose type (Man5 ~ Man9) (Fu *et al.*, 1994). By combination of these sets as glycan libraries, we can easily obtain information on the carbohydrate-binding specificity of a novel lectin.

Results

Detection of lectin activity in a crude extract of the bark of Sambucus sieboldiana

Two lectins with different carbohydrate-binding specificity, sialic acid–specific *Sambucus sieboldiana* agglutinin (SSA) and galactose-specific ribosome-inactivating protein (RIP) were reported in Japanese elderberry (*S. sieboldiana*) bark (Kaku *et al.*, 1996; Rojo *et al.*, 1997). We examined the presence of these lectins by observing the change of migrations using a mixture of oligosaccharides derived from AGP in the electrolyte containing the crude extract from *S. sieboldiana* bark. As shown in Figure 1A, addition of crude protein fraction (250 μg/ml) in the electrolyte obviously caused change of migrations of sialo-oligosaccharide peaks.

At a concentration of 500 μg/ml crude protein fraction, the peaks almost disappeared. In contrast, migrations of asialo-oligosaccharides were not obviously affected (Figure 1B), although nonspecific retardation of migration times was observed due to the presence of proteins in the crude extract. These data clearly indicated that the crude extract contained a sialic acid–binding protein but did not show clear affinity toward asialo-oligosaccharides derived from AGP.

Migrations of the oligosaccharides in the presence of purified SSA and RIP are shown in Figure 2. The purified SSA obviously lowered the peak height of sialo-oligosaccharides and the peaks disappeared at the concentration of 0.8 μM (Figure 2A) as observed for the crude extract, but SSA did not affect the migration of asialo-oligosaccharides (Figure 2B). In contrast, RIP did not cause change of migrations of sialo-oligosaccharides (Figure 2A). However, asialo-oligosaccharides (**2** and **4**) showed higher affinities to RIP than those having a fucose residue (**3** and **5**) and were observed gradually later with increase of the concentrations of the lectin. The peaks of these carbohydrates were overlapped at 8 μM RIP (Figure 2B). It should be noted that SSA showed clear binding at 0.8 μM, but RIP required 8 μM concentration for specific binding to asialo-oligosaccharides.

Detection of lectin activity in crude extract of tulip bulbs

Two carbohydrate-binding proteins have been reported in tulip bulbs. One is a mannose-specific lectin, *Tulipa gesneriana* lectin (TGL), which preferentially binds α(1-6) linked manno-oligosaccharides derived from mannan (yeast cells) (Oda and Minami, 1986). The other is *T. gesneriana* agglutinin (TGA), which agglutinates animal blood cells. Asialothyroglobulin showed the most potent inhibition toward binding of ^{125}I-labeled TGA to mouse erythrocytes among the examined glycoproteins, and asialo-AGP also showed inhibitory effect (Oda *et al.*, 1987). However, precise binding specificity of TGA has not been determined.

Interactions between crude protein fraction in tulip bulbs and the carbohydrates derived from a few glycoproteins are shown in Figure 3. In the electrolyte-containing crude extract (5 mg/ml), migrations of asialo-oligosaccharides including (**10**) derived from porcine thyroglobulin showed dramatic changes, and the major peaks became broad (Figure 3AII). Asialo-oligosaccharides derived from AGP showed interesting binding to the crude extract, and peaks derived from triantennary oligosaccharides (**2** and **3**) obviously became broader and appeared later on addition of the crude extract in the buffer (Figure 3BII). In contrast, presence of the crude extract of tulip bulbs did not cause obvious changes of migrations of any oligosaccharides derived from ribonuclease B (Figure 3CII). In the previous article, Oda and Minami (1986) reported that TGL showed specific affinity to mannan derived from yeast. Because the structures of mannan from yeast and high-mannose oligosaccharides are different, TGL did not show affinity toward high-mannose oligosaccharides derived from ribonuclease B.

Detailed studies on carbohydrate-binding specificity of TGA

We used four sets of oligosaccharide mixture derived from AGP, fetuin, porcine thyroglobulin, and bovine IgG as glycan libraries to determine the detailed carbohydrate-binding specificity of TGA using CAE.

AGP contains di- (**1**), tri- (**2** and **3**), and tetra- (**4** and **5**) antennary oligosaccharides. Some of the tri- and tetraantennary oligosaccharides are substituted with a fucose residue at one of the lactosamine branch to form sialyl Lewis x structure (**3** and **5**).

Figure 4 shows the interactions between asialo-oligosaccharides of AGP and TGA at various concentrations. Addition of TGA in the electrolyte caused specific retardation

Table I. List of oligosaccharides

Peak number	Structure[*1,*2]
1	Galβ1-4GlcNAcβ1-2Manα1-6 \ Manβ1-4GlcNAcβ1-4GlcNAc-APTS / Galβ1-4GlcNAcβ1-2Manα1-3
2	Galβ1-4GlcNAcβ1-2Manα1-6 \ Manβ1-4GlcNAcβ1-4GlcNAc-APTS / Galβ1-4GlcNAcβ1-2Manα1-3 / Galβ1-4GlcNAcβ1-4
3	Galβ1-4GlcNAcβ1-2Manα1-6 \ Manβ1-4GlcNAcβ1-4GlcNAc-APTS / Galβ1-4GlcNAcβ1-2Manα1-3 / Galβ1-4GlcNAcβ1-4 / Fucα1-3
4	Galβ1-4GlcNAcβ1-6 \ Galβ1-4GlcNAcβ1-2Manα1-6 \ Manβ1-4GlcNAcβ1-4GlcNAc-APTS / Galβ1-4GlcNAcβ1-2Manα1-3 / Galβ1-4GlcNAcβ1-4
5	Galβ1-4GlcNAcβ1-6 \ Galβ1-4GlcNAcβ1-2Manα1-6 \ Manβ1-4GlcNAcβ1-4GlcNAc-APTS / Galβ1-4GlcNAcβ1-2Manα1-3 / Galβ1-4GlcNAcβ1-4 / Fucα1-3
6	Galβ1-4GlcNAcβ1-2Manα1-6 \ Manβ1-4GlcNAcβ1-4GlcNAc-APTS / Galβ1-3GlcNAcβ1-2Manα1-3 / Galβ1-4GlcNAcβ1-4
7	GlcNAcβ1-2Manα1-6 \ Manβ1-4GlcNAcβ1-4GlcNAc-APTS / / GlcNAcβ1-2Manα1-3 Fucα(1-6)
8	Galβ1-4GlcNAcβ1-2Manα1-6 \ Manβ1-4GlcNAcβ1-4GlcNAc-APTS / / GlcNAcβ1-2Manα1-3 Fucα(1-6)
9	GlcNAcβ1-2Manα1-6 \ Manβ1-4GlcNAcβ1-4GlcNAc-APTS / / Galβ1-4GlcNAcβ1-2Manα1-3 Fucα(1-6)

Table I. continued

Peak number	Structure[*1*2]
10	Galβ1-4GlcNAcβ1-2Manα1-6 Manβ1-4GlcNAcβ1-4GlcNAc-APTS Galβ1-4GlcNAcβ1-2Manα1-3 Fucα(1-6)
11	GlcNAcβ1-2Manα1-6 Manβ1-4GlcNAcβ1-4GlcNAc-APTS GlcNAcβ1-2Manα1-3
12	Galβ1-4GlcNAcβ1-2Manα1-6 Manβ1-4GlcNAcβ1-4GlcNAc-APTS GlcNAcβ1-2Manα1-3
13	GlcNAcβ1-2Manα1-6 Manβ1-4GlcNAcβ1-4GlcNAc-APTS Galβ1-4GlcNAcβ1-2Manα1-3
14	Galβ1-4GlcNAcβ1-2Manα1-6 Manβ1-4GlcNAcβ1-4GlcNAc-APTS Galβ1-4GlcNAcβ1-2Manα1-3 Fucα(1-6) Galβ1-4GlcNAcβ1-4
15	NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-6 Manβ1-4GlcNAcβ1-4GlcNAc-APTS NeuAcα2-3Galβ1-4GlcNAcβ1-2Manα1-3
16	NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-6 Manβ1-4GlcNAcβ1-4GlcNAc-APTS NeuAcα2-3Galβ1-4GlcNAcβ1-2Manα1-3 NeuAcα2-6Galβ1-4GlcNAcβ1-4
17	NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-6 Manβ1-4GlcNAcβ1-4GlcNAc-APTS NeuAcα2-3Galβ1-4GlcNAcβ1-2Manα1-3 NeuAcα2-3Galβ1-4GlcNAcβ1-4
18	Galβ1-4GlcNAcβ1-2Manα1-6 Manβ1-4GlcNAcβ1-4GlcNAc-APTS NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-3 Fucα(1-6)
19	Manα1-6 Manα1-3Manα1-6 Manβ1-4GlcNAcβ1-4GlcNAc-APTS Manα1-3
20	Manα1-6 Manα1-3Manα1-6 Manβ1-4GlcNAcβ1-4GlcNAc-APTS Manα1-2Manα1-3

Table I. continued

Peak number	Structure[*1*2]
21	Manα1-6 \ Manα1-3Manα1-6 \ Manβ1-4GlcNAcβ1-4GlcNAc-APTS / Manα1-2Manα1-2Manα1-3
22	Manα1-2Manα1-6 \ Manα1-3Manα1-6 \ Manβ1-4GlcNAcβ1-4GlcNAc-APTS / Manα1-2Manα1-3
23	Manα1-6 \ Manα1-2Manα1-3Manα1-6 \ Manβ1-4GlcNAcβ1-4GlcNAc-APTS / Manα1-2Manα1-3
24	Manα1-6 \ Manα1-2Manα1-3Manα1-6 \ Manβ1-4GlcNAcβ1-4GlcNAc-APTS / Manα1-2Manα1-2Manα1-3
25	Manα1-2Manα1-6 \ Manα1-2Manα1-3Manα1-6 \ Manβ1-4GlcNAcβ1-4GlcNAc-APTS / Manα1-2Manα1-2Manα1-3

[*1] The abbreviations used for the structures are: Gal, galactose; GlcNAc, *N*-acetylglucosamine; Man, mannose; Fuc, fucose; NeuAc, *N*-acetylneuraminic acid.
[*2] All oligosaccharides were used after labeling with 8-aminopyrene-1,3,6-trisufonate APTS by reductive amination.

of the migration times of triantennary carbohydrates (**2** and **3**), but did not affect the migrations of tetraantennary carbohydrates (**4** and **5**). Peaks of tri- (**2** and **3**) and tetra- (**4** and **5**) antennary carbohydrate chains were overlapped at 4.5 μM TGA, and finally, the migration order of them were reversed and the peaks of **2** and **3** were fused to a broad peak at 12.0 μM TGA and observed at 7.7 min.

TGA showed a small effect on migration of diantennary oligosaccharide (**1**) compared to those of triantennary carbohydrates. At 12.0 μM TGA, the migration time of (**1**) was observed slightly later (5.8 min) than in the absence of the lectin (5.2 min).

Fetuin contains two triantennary oligosaccharides (**2** and **6**) and a diantennary oligosaccharide (**1**). One (**2**) of the triantennary oligosaccharides has three Galβ(1-4)GlcNAc branches, and the other (**6**) contains one Galβ(1-3)GlcNAc as well as two Galβ(1-4)GlcNAc branches (Table I). TGA clearly distinguished these triantennary oligosaccharides, as shown in Figure 5.

The mobility of triantennary oligosaccharide (**6**), which has a Galβ(1-3)GlcNAc branch, became obviously smaller than that of (**2**) in the presence of TGA and was observed later at higher concentrations of TGA. The migration order of triantennary oligosaccharides (**2** and **6**) was reversed at 6.0 μM TGA. These data indicate that oligosaccharide (**6**) has higher affinity toward TGA.

Bovine IgG contains four major diantennary oligosaccharides (**7–10**) that have α(1-6) linked fucose residues at chitobiose portion of the reducing end. Interestingly, TGA showed obvious retardation effect on the migration times of these oligosaccharides (**7–10**), as shown in Figure 6. The complete form (**10**) of diantennary oligosaccharide was observed latest, and the one lacking both Gal residues (**7**) showed the weakest interaction with TGA (Figure 6A).

Although GlcNAc residue in the reducing end of these oligosaccharides is an open-chain form (i.e., N-acetylglucosaminitol), these oligosaccharides show distinct affinities toward TGA. In contrast, diantennary oligosaccharides that do not have a fucose residue in the reducing end did not show affinity toward TGA as mentioned in the binding to the oligosaccharides derived from fetuin and AGP. To

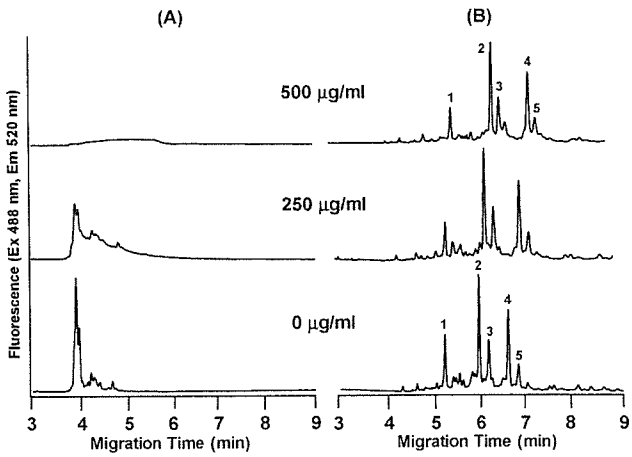

Fig. 1. Capillary affinity electrophoresis of oligosaccharides derived from AGP in the presence of crude protein fractions from *S. sieboldiana* bark. An aqueous solution (10 μl) of the mixture of (**A**) sialo- and (**B**) asialo-oligosaccharides derived from AGP was assayed in the presence of crude protein fraction from *S. sieboldiana* bark at the concentrations of 0, 250, and 500 μg/ml as protein, respectively. Running buffer: 100 mM Tris-acetate buffer (pH 7.4) containing 0.5% polyethylene glycol (PEG 70000) and crude protein extract. Capillary: eCAP N-CHO coated capillary, 30 cm length (effective length, 20 cm, 50 μm ID). Applied potential: 18 kV. Injection: pressure method (0.5 psi for 5 s). Fluorescent detection at 520 nm excited with an argon laser with a 488 nm filter. The structures of the oligosaccharides are shown in Table I.

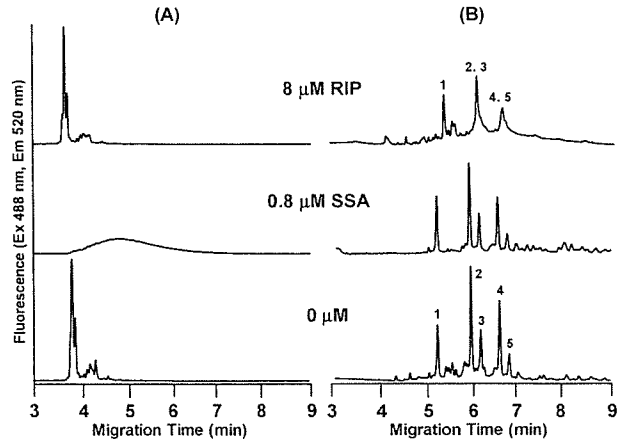

Fig. 2. Capillary affinity electrophoresis of sialo- and asialo-oliogsaccharides derived from AGP in the presence of purified lectins from *S. sieboldiana*. (**A**) Sialo- and (**B**) asialo-oligosaccharides. The analytical conditions were the same as those described in Figure 1.

confirm these observations, we digested the oligosaccharides with fucosidase and examined the binding of defucosylated oligosaccharides (**11–13**, and **1**). We found that these defucosylated oligosaccharides did not show obvious interaction with TGA (Figure 6B).

Porcine thyroglobulin contains complex type oligosaccharides (**10** and **14**) and high-mannose type oligosaccharides (HM in Figure 7) as minor oligosaccharides (Kakehi *et al.*, 1999). Addition of TGA decreased the peak intensity of triantennary oligosaccharide (**14**) even at 0.2 μM TGA in the electrolyte and also retarded diantennary oligosaccharide (**10**) at higher concentrations than 0.8 μM TGA, as observed for the interactions with oligosaccharides derived from IgG. Migration times of high-mannose oligosaccharides were not changed even in the presence of 12.0 μM TGA.

Finally, we examined the interactions between TGA and sialic acid–containing oligosaccharides derived from fetuin (Figure 8A) and porcine thyroglobulin (Figure 8B). A group of sialo triantennary oligosaccharides observed at 3.9 min were resolved into three groups around 4.5 min and 5.2 min at 12.0 μM TGA (Figure 8A). In the analogy of the analysis of asialo-oligosaccharides, we speculated that sialo-triantennary oligosaccharides having Galβ(1-3)GlcNAc branch showed different affinity to TGA, although further studies are required. In Figure 8B, results on the interactions betweens TGA and sialo-oligosaccharides from thyroglobulin are shown. High-mannose type oligosaccharides showed no interactions (see small peaks at 4.5~5.5 min). In contrast, **18** showed obvious retardation of migration times in the presence of TGA.

Determination of binding affinity constants

Binding constants for interactions between TGA and various carbohydrates were determined according to the method reported previously (Nakajima *et al.*, 2003). The values of association constant (K_a) to oligosaccharides are summarized in Table II.

The method using CAE does not require the data concerning the concentrations of ligand (i.e., oligosaccharide), therefore is quite useful for the binding studies using oligosaccharide mixture as ligands. The oligosaccharide (**14**) that has a triantennary structure with an α(1-6) linked fucose branch showed the highest affinity ($Ka = 1.7 \times 10^6$ M^{-1}), followed by **6** ($K_a = 6.4 \times 10^5$ M^{-1}) and **10** ($K_a = 5.8 \times 10^5$ M^{-1}). Oligosaccharides (**2** and **3**) showed almost the same affinities, indicating that fucose residue with α(1-3) linkage in the outer chain was not involved in the binding. Comparison of **10** with **7** and **1** indicates that Gal residues at nonreducing termini and α(1-6) linked fucose residue at the reducing terminal are important for the binding. The K_a values for sialo-carbohydrates are smaller than those of corresponding asialo-carbohydrates: 1.5 and 0.6×10^5 M^{-1} for **1** and **15**, 4.4 and 2.0×10^5 M^{-1} for **2** and **16**, **17** and 5.8 and 3.4×10^5 M^{-1} for **10** and **18**, respectively. These indicate that masking of Gal residues at nonreducing termini with sialic acids considerably lowers the affinity.

Discussion

We observed the interactions between the crude extract from Japanese elderberry bark or tulip bulbs and some sets of oligosaccharides derived from a few glycoproteins using CAE. Addition of crude extract from Japanese elderberry bark to the electrolyte obviously changed the mobility of sialo-oligosaccharides of AGP. These results were clearly due to the presence of SSA in the extract. However, another lectin, RIP, in the crude extract was not detected. The