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Collection of α_1-acid glycoprotein molecular species by capillary electrophoresis and the analysis of their molecular masses and carbohydrate chains
Basic studies on the analysis of glycoprotein glycoforms

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Abstract

A highly heterogeneous glycoprotein, α_1-acid glycoprotein, was resolved into their glycoforms by capillary electrophoresis using a surface-modified capillary in 20 m*M* acetate buffer (pH 4.2) containing 0.5% (w/v) hydroxypropylmethylcellulose. We collected the fractions containing each glycoform as nearly pure state by capillary electrophoresis, and examined the molecular masses of these glycoforms by matrix assisted laser desorption time-of-flight mass spectrometry. We also analyzed carbohydrate chains after releasing them with *N*-glycosidase F followed by fluorescent labeling with 8-aminopyrene-1,3,6-trisulfonate. We found that the separation of glycoforms was mostly due to the presence of multiantennary carbohydrate chains. We propose that the present technique is useful for the analysis of post translational modification of proteins with carbohydrate chains.

Keywords: Alpha1-acid glycoprotein; Glycoproteins; Proteins; Carbohydrates; Orosomucoid

1. Introduction

Glycosylation of proteins represents a critically important post-translational modification reaction. Carbohydrate chains of glycoproteins often show variations during biosynthesis in the different type of cell line [1] and the culture conditions [2], and play vital functional roles in biological systems [3,4]. Variations in the oligosaccharides in glycoprotein pharmaceuticals often produce profound effects in vivo. For example, human recombinant erythropoietin (rhEPO) produced in Chinese hamster ovary (CHO) cells shows a number of glycoforms [5], and more prolonged life in blood than the preparation having no carbohydrate chains [6,7]. Each of the glycoforms of rhEPO contains a variable number of sialic acid residues at the non-reducing terminals of the oligosaccharide chains. Variations in sialic acid content along with the differences in the arrangement of complex carbohydrate structures give rise to several rhEPO species with isoelectric points between 4.2 and 4.6 [8].

Capillary electrophoresis is one of the important techniques for the analysis of multiple forms of glycoprotein for the examination of variations in carbohydrate chains [9,10]. In 1989, Kilar and

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Hjertén attempted to resolve human transferrin isoforms (i.e., glycoforms) by high-performance capillary electrophoresis [11]. Since then, a number of separation modes have been developed for the direct resolution of glycoforms. The rhEPO was resolved with high resolution into its glycoforms using a combination of 1,4-diaminobutane and a high concentration of urea (7 *M*) [12]. We compared several methods for resolution of glycoforms of sialic acid-containing glycoprotein samples [8]. We also reported that α_1-acid glycoprotein (AGP) in sera was selectively introduced by electrokinetic injection and successfully resolved into its glycoforms using an electrolyte having slightly higher pH (about 4.5) than its isoelectric point in a surface-modified capillary developed for capillary gas chromatography [13].

Analysis of oligosaccharides of glycoproteins is also essential for the assessment of glycoprotein pharmaceuticals. The development of new technologies, including capillary electrophoresis and two- and three-dimensional chromatographic techniques and fluorophore-assisted carbohydrate electrophoresis in slab gel format, have increased the ease of oligosaccharide analysis [14,15]. Capillary electrophoresis is also a powerful strategy for the analysis of fluorescent-labeled carbohydrate chains. We evaluated some aminobenzene analogues as the fluorescent-labeling reagents for the carbohydrate chains released from protein core [16].

As described above, heterogeneity of glycoproteins based on the variation of carbohydrate chains has been evaluated mostly by two strategies using glycoform analysis of glycoprotein or oligosaccharide analysis after releasing the carbohydrate chains. However, it is important to evaluate the relationship between glycoforms and oligosaccharide heterogeneity for accurate understanding of the biological role of carbohydrate chains. In the previous paper, we examined the effect of carbohydrate chains on glycoform separation of sialic acid-containing glycoproteins by capillary electrophoresis using human AGP [13]. Three conventional chromatography techniques [anion-exchange chromatography, Con A affinity chromatography, Cu(II)–metal affinity chromatography] were used to separate molecular species of AGP. We analyzed the glycoforms and carbohydrate chains by capillary electrophoresis and high-performance liquid chromatography (HPLC), respectively, and showed that it was difficult to predict the changes of the ratios of respective carbohydrate chains during biological and clinical events only by glycoform analysis.

In the present study, we collected each glycoform peak of AGP after separation by capillary electrophoresis. The fractions containing each glycoform were examined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Further, the separated peak fraction was digested with *N*-glycosidase F, and the released oligosaccharides were derivatized with 8-aminopyrene-1,3,6-trisulfonate (APTS) and analyzed by capillary electrophoresis with argon-laser-induced fluorescence detection.

2. Experimental

2.1. Materials

Samples of human AGP were obtained from Sigma (Tokyo, Japan). Highly purified APTS for labeling of carbohydrates was obtained from Beckman Instruments (Fullerton, CA, USA). Peptide-N^4-(acetyl-β-D-glucosaminyl)asparagine amidase (*N*-glycosidase F, EC3.2.2.18) was from Roche Molecular Biochemicals (Tokyo, Japan). Neuraminidase (*Arthrobacter ureafaciens*) was a gift from Drs. Tsukada and Ohta (Marukin-Chuyu, Uji, Kyoto, Japan). α1,3/4-L-Fucosidase (*Streptomyces* sp. 142) was obtained from Takara Biomedicals (Shiga, Japan). 2,5-Dihydroxybenzoic acid (DHB), 2,4,6-trihydroxybenzoic acid, 4-hydroxy-3-methoxycinnamic acid (ferulic acid), 3,5-dimethoxy-4-hydroxycinnamic acid, α-cyano-4-hydroxycinnamic acid, 3,4-dihydroxy-cinnamic acid and 2,5-dihydroxyacetophenone as the matrix materials for MALDI-TOF-MS were from Sigma–Aldrich Japan (Tokyo, Japan). Sodium cyanoborohydride was from Nacalai Tesque (Kyoto, Japan). All other samples and reagents were of the highest grade commercially available or of HPLC grade and used without further purification. A DB-1 capillary column for capillary gas chromatography was obtained from J&W Scientific (Folsom, CA, USA). All aqueous solutions were prepared using water purified with a Milli-Q purification system (Millipore, Bedford, MA, USA).

2.2. Capillary electrophoresis for glycoform analysis and collection of glycoforms

Glycoform analysis of AGP was performed with a P/ACE 5010 system (Beckman). A DB-1 capillary (57 cm or 27 cm total length×100 μm I.D.) was used. The detection window was made at 7 cm from the outlet of the capillary by carefully removing the polyimide coating by burning, and the transparent portion was fixed on the detector block. On-line detection was performed with monitoring of the UV absorption at 200 nm. Acetate buffer (20 m*M*, pH 4.2) containing 0.5% 60SH50 hydroxypropylmethylcellulose (HPMC; Wako, Osaka, Japan) was employed as the electrolyte. The electrophoresis was performed at the applied voltage of 15 kV at 30 °C. The sample solution (1.0 mg/ml) was introduced by pressure mode for 10 s.

For the collection of peak component, we used a solution at 10 mg/ml with a DB-1 capillary of 57 cm (50 cm effective length)×100 μm I.D. In the present system, reproducibility of migration times was excellent as indicated in the previous paper [8]. Therefore, we could collect all the peaks as almost pure state according to the time program as shown in Table 1.

Because the apparatus can stop applying voltage when the outlet vial has to be changed, we can automatically collect each peak in separate vials by editing time-program. Collection procedures were repeated five times. Under the present injection conditions, about 2 μg of AGP (approximately 60 pmol, calculated from the molecular mass of AGP as 35 000) were injected in each injection. After collection of each peak, the electrolyte solution containing the peak component in the outlet vial was passed through an ultra filtration tube (molecular mass cut-off, 10 000; Millipore Ultrafree MC tube). The concentrate on the membrane filter was diluted with water (200 μl), and filtered again by centrifugation. The concentrate was collected and lyophilized by a centrifugal evaporator (SpeedVac, Savant, Farmingdale, NY, USA). The residue was dissolved in water (10 μl), and analyzed by capillary electrophoresis (CE) to confirm the purity. A portion (0.5 μl) was employed for MALDI-TOF-MS analysis and a half portion (ca. 150 pmol, 5 μl) was used for oligosaccharide analysis.

2.3. MALDI-TOF-MS analysis

MALDI-TOF-MS experiments were carried out on a Voyager DE PRO (PE Biosystems, Framingham, MA, USA). A nitrogen laser was used to irradiate samples with an ultraviolet light (337 nm), and an average of 100 shots was taken. The instrument was operated in linear operation using negative polarity. An accelerating voltage of 18 kV was used. The sample solution (0.5 μl) was applied to a polished stainless steel target, to which added a solution (0.3 μl) of 2,5-dihydroxybenzoic acid (DHB) or other matrix materials in a mixture of acetonitrile–water (1:1). The mixture was dried in atmosphere by keeping it for several hours at room temperature before MS measurement.

2.4. Analysis of carbohydrate chains

N-Glycosidase F (5 units) in 20 m*M* phosphate

Table 1
Timetable for fractional collection of glycoform peaks and recovery of the peak component

Peak No.	Mobility (10^3 cm^2 V^{-1} s^{-1})	Migration time (min)	Arrival at the outlet end time (min)	Collection period (min)	Amount obtained by five repeated collections* (μg)
1	9.56	29.1	33.1	28.5–35.2	3.1
2	9.12	30.5	34.7	28.5–35.2	
3	8.66	32.1	36.6	35.2–37.9	3.5
4	7.98	34.8	39.7	37.9–41.4	4.4
5	7.32	38.0	43.3	41.4–45.7	4.1
6	6.64	41.8	47.6	45.7–50.8	2.7
7	5.94	46.7	53.3	50.8–58.0	1.4

*The amount of the collected peak component was calculated from the peak area. The injected amount was calculated according to Ref. [13].

buffer (pH 7.0, 50 μl) was added to the aqueous solution (50 μl) of the fractions containing AGP glycoforms collected by CE as described above. The mixture was incubated for 24 h at 37 °C. After the mixture was kept in the boiling water bath for 5 min, ethanol (200 μl) was added to the mixture, and the mixture was centrifuged. The supernatant was collected and evaporated to dryness. Neuraminidase (5 μU, 5 μl) and 20 m*M* acetate buffer (pH 5.0, 50 μl) were added to the residue, and the mixture was further incubated overnight at 37 °C. Ethanol (200 μl) was added to the mixture and the supernatant was collected after centrifugation and evaporated to dryness as described above. An APTS solution (250 μg/5 μl of 15% aqueous acetic acid) and 1 *M* sodium cyanoborohydride in tetrahydrofuran (THF) (5 μl) were added to the residue. Mineral oil (100 μl; Aldrich, catalog No. 16,140-3) was added to the mixture to avoid evaporation of the reaction solvent. The mixture was kept for 90 min at 55 °C. Water (200 μl) was added to the yellowish solution under the oil layer and the aqueous layer was collected. The same procedure was repeated twice to collect the labeled carbohydrates. The solution thus collected (600 μl) was applied on a column of Sephadex G-25 (32 cm×0.8 cm I.D.) pre-equilibrated with water, and eluted with the same solvent. The fluorescent fractions appeared earlier were pooled and concentrated to dryness under reduced pressure. The residue was dissolved in 20 μl of water, and a portion was analyzed by CE. A portion of the residue was also analyzed by MALDI-TOF-MS to determine the reaction efficiency in derivatization. The analytical conditions for MS measurement was described above.

2.5. Capillary electrophoresis of the APTS-labeled oligosaccharides

We employed a Beckman Coulter P/ACE MDQ Glycoprotein System with argon-laser-induced fluorescence detection system. The detection was performed by installing a 520-nm filter for emission with a 488-nm light for excitation. Separation was performed using a DB-1 capillary at 25 °C throughout the work. The detection window of the capillary was made at 10 cm from the outlet of the capillary by carefully removing the polyimide coating by burning, and the transparent portion was fixed on the detector block. The detailed analytical conditions are given in the figure legends. Injections were performed automatically in the electrokinetic method (10 kV, 15 s). Data were collected and analyzed with a standard 32 Karat software (Version 4.0) on Windows 2000.

2.6. Digestion of a mixture of the labeled carbohydrate chains with α-L-fucosidase

A mixture of the APTS-labeled oligosaccharides was dissolved in 50 m*M* phosphate buffer (pH 6.0, 50 μl). A 10 μU amount of α1,3/4-L-fucosidase (10 μl) was added to the solution, and the mixture was kept for 24 h at 37 °C, then lyophilized to dryness. The residue was dissolved in water (50 μl), and an aliquot of the mixture was analyzed by capillary electrophoresis.

3. Results

3.1. Fractional collection of AGP glycoforms by capillary electrophoresis

The present mode of separation showed excellent reproducibility in migration times as indicated in the previous paper [8]. Therefore, we could easily collect the peak component according to the time program described in the Experimental section (Table 1) by changing the outlet vials at the anodic end. We repeated fraction collection five times using a protein solution (10 mg/ml). The pooled fractions were passed through an ultra-filtration membrane filter (molecular mass cut-off, 10 000), and a portion was analyzed by capillary electrophoresis. The results are shown in Fig. 1.

AGP showed the presence of seven glycoforms in the present analytical conditions. We could isolate six glycoform peaks (from peaks 1 and 2 to peak 7) as almost pure state except for peaks 1 and 2. As shown in Fig. 1, the fractions obtained from peaks 1 and 2 showed a peak around 31 min with a shoulder (29 min) due to peak 1. Although we attempted to isolate peaks 1 and 2, it was quite difficult to isolate them due to the small amount of peak 1. We could not perform further examination on peak 7 either. In

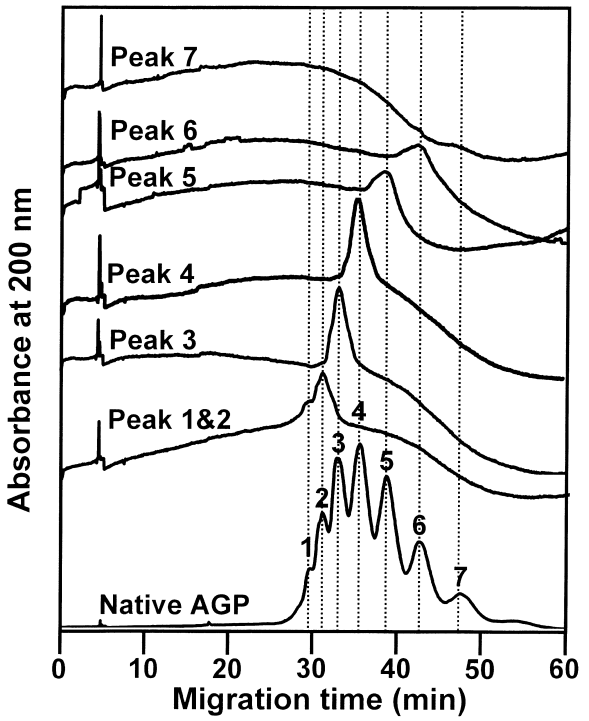

Fig. 1. Fractional collection of AGP glycoforms by capillary electrophoresis. (a) Separation profile of AGP. (b) Analysis of the collected peaks. Analytical conditions: capillary, DB-1 capillary (50 cm×100 μm I.D.); buffer, 20 m*M* sodium acetate buffer (pH 4.2) containing 0.5% Tween 20; applied voltage, 15 kV; operation temperature, 30 °C; injection, 5 s by pressure method; detection, UV absorption at 200 nm. Peaks are numbered according to their migration order.

the electropherogram of each collected peak, the baseline was considerably drifted. This was probably due to the effect of HPMC in the concentrated fractions.

3.2. MALDI-TOF-MS of the collected fractions

Sottani et al. reported MALDI-TOF-MS of AGP using sinapic acid and 4-hydroxy-3-methoxycinnamic acid (ferulic acid) as matrix material. Although the resolution among ions was not complete, they observed six ions from m/z 32 256 to m/z 34 084 [17]. We examined five compounds hitherto reported as the matrix materials for MALDI-TOF-MS of proteins. Although we attempted various procedures to improve the resolution, we could not observe good resolved molecular ions but a quite broad peak.

The original sample showed a peak around m/z 35 670 using DHB as the matrix as shown in Fig. 2.

Peaks 1 and 2 showed a broad ion centered at m/z 36 125. Peak 7 also showed a broad ion at m/z 34 735. These data indicate that slower migrating peaks gradually showed smaller m/z values. Because the electroosmotic flow is negligible in the present separation mode, the slower migrating analyte ions include less negative charges (i.e., less sialic acid residues).

3.3. Analysis of carbohydrate chains released from AGP after labeling with APTS

We analyzed the carbohydrate chains of AGP after labeling with APTS according to the analogous manner described previously [13]. In the present work, we chose APTS as the derivatization reagent for carbohydrates, because of the easy clean-up procedure. The labeled carbohydrates have high

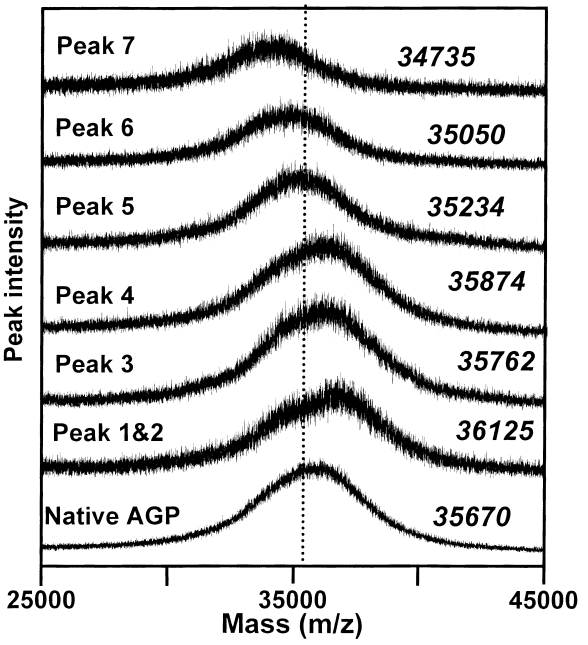

Fig. 2. MALDI-TOF-MS spectra of each glycoform collected by capillary electrophoresis. Peak numbers as in Fig. 1. DHB was used as the matrix material as described in the Experimental section.

negative charges and are analyzed with high sensitivity by argon-laser-induced fluorescence detection within 20 min. The results are shown in Fig. 3.

AGP contains di-, tri- and tetraantennary carbohydrate chains. Some of the tri- and tetraantennary carbohydrate chains are substituted with fucose or *N*-acetyllactosamnie (Galβ1-4GlcNAc) residues. The list of the carbohydrate chains is shown in Fig. 4.

Fig. 3a shows the analysis of sialo-oligosaccharides. Due to strong acidity of sialic acid and the APTS residue in the carbohydrate chains, all oligosaccharides migrated toward the anode even at pH 2.5. The peak observed at the earliest migration time

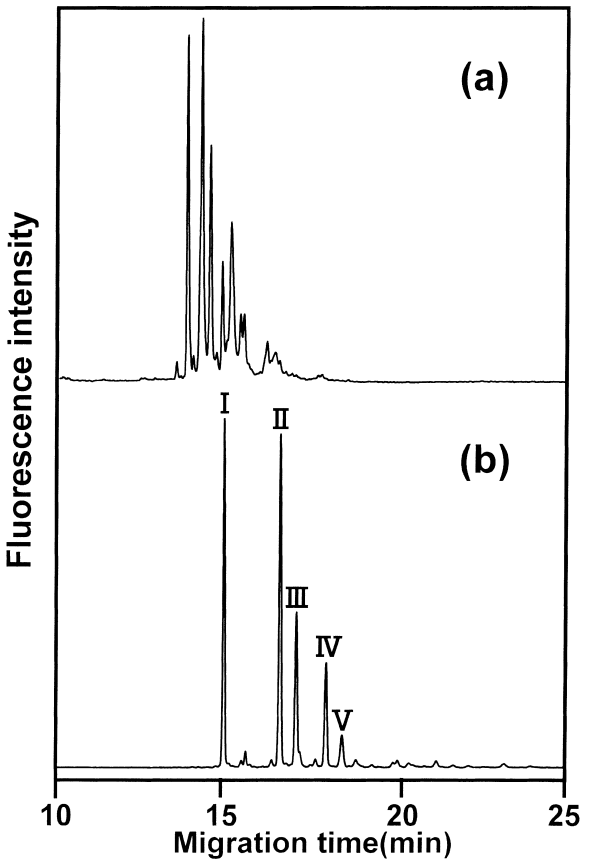

Fig. 3. Capillary electrophoresis of APTS-labeled sialo- (a) and asialo- (b) oligosaccharides derived from native AGP. Numbers in (b) as in Fig. 4. Analytical conditions: capillary, fused-silica (40 cm effective length×50 μm I.D.); electrolyte, 50 m*M* phosphate buffer (pH 2.5); temperature, 25 °C; detection, Ar laser-induced fluorescence detection (excitation, 488 nm; emission, 520 nm); injection, pressure method (3900 Pa, 10 s).

(14.0 min) was due to disialo-diantennary oligosaccharide (I). The large peak following the disialo-diantennary peak was due to trisialo-triantennary oligosaccharide (II). These peaks were easily confirmed by comparison with the oligosaccharides obtained from transferrin and fetuin. Other carbohydrate chains could not be identified, because the standard sialooligosaccharides were not available. Fig. 3b shows the results for the analysis of asialooligosaccharides. Peaks I and II are due to asialo-diantennary and asialo-triantennary oligosacchrides, respectively. Peak IV is due to the tetraantennary oligosaccharide. These peaks were identified by comparison with the results reported previously [13]. Peaks III and V were moved to peaks II and IV, respectively, after digestion with α-fucosidase (data not shown). Thus, peak III is due to the triantennary oligosaccharide to which α-fucose residue attaches, and peak V is due to the tetraantennary oligosaccharide to which α-fucose residue attaches. We could not identify the peak due to the polylactosamine-type oligosaccharide (VI), probably due to the minute amount in the AGP preparation used in the present study.

3.4. Analysis of the carbohydrate chains from each glycoform of AGP

In the estimation of the effect of carbohydrate chains on glycoform separation by capillary electrophoresis, it is necessary to determine the relative abundance of carbohydrate chains [13]. As mentioned in the Experimental section, a sample of AGP (10 μg, about 300 pmol) was separated and glycoforms were collected. Releasing reaction of carbohydrate chains from the protein core of AGP was performed with *N*-glycosidase F according to the recommended manufacturers' sheet as also indicated in the experimental section. The residue obtained from the enzyme reaction was mixed with a solution of APTS in 15% aqueous acetic acid (5 μl) and 1 *M* sodium cyanoborohydride in THF (5 μl), and the total reaction volume was only 10 μl. We observed poor reproducibility in reaction efficiency probably due to evaporation of the reaction solvent. To improve the efficiency in derivatization and recovery, we added mineral oil (100 μl) to avoid evaporation of the solvent. The reaction mixture was

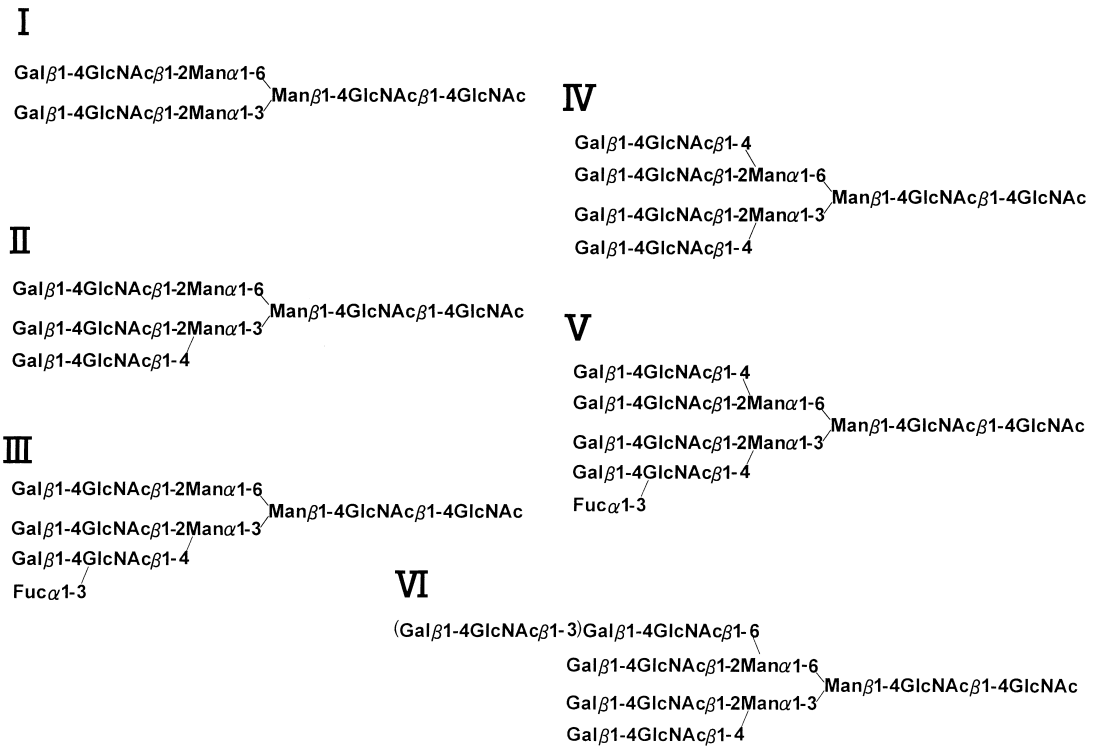

Fig. 4. Carbohydrate chains in α_1-acid glycoprotein.

passed through a small column of Sephadex G-25 with water as the eluent. The fluorescent fractions appeared earlier were analyzed by CE according to the procedure in the Experimental section. The fractions thus collected were examined by MALDI-TOF-MS to confirm that the derivatization proceeded quantitatively. We did not observe any underivatized oligosaccharide ions, and found that the derivatization reaction proceeded quantitatively (data not shown).

The results are shown in Fig. 5 for the analysis of carbohydrate chains in each glycoform (compare the results observed for the analysis of the carbohydrate chains from native AGP, see Fig. 3). The relative abundances of oligosaccharides in each collected peak are summarized in Table 2.

The earlier observed glycoforms (see Fig. 1) contained larger amount of tri- and tetraantennary carbohydrate chains (II and IV). The slower moving fractions (see peak 6 in Fig. 1) contained the diantennary oligosaccharide (I) most abundantly. We could not observe the peaks due to carbohydrate chains from peaks 1 and 2 and peak 7, because of the minute amount of the fractions.

4. Discussion

Molecular species (i.e., glycoforms) of AGP were collected by capillary electrophoresis. Using a sample solution at 10 mg/ml, five repeated procedures for fractional collection could afford enough quantity of the samples to obtain the information about molecular masses by MALDI-TOF-MS and carbohydrate chains. The total amount of AGP sample collected was approximately 10 μg (about 300 pmol). Although we attempted to collect peaks 1 and 2 (see Fig. 1), it was quite difficult to isolate peaks 1 and 2. Other strategies such as conventional chromatography technique may be required prior to the fractional collection by capillary electrophoresis. The baseline drift observed in the analysis of each collected peak (Fig. 1) was probably due to the presence of HPMC in the concentrated fractions. The

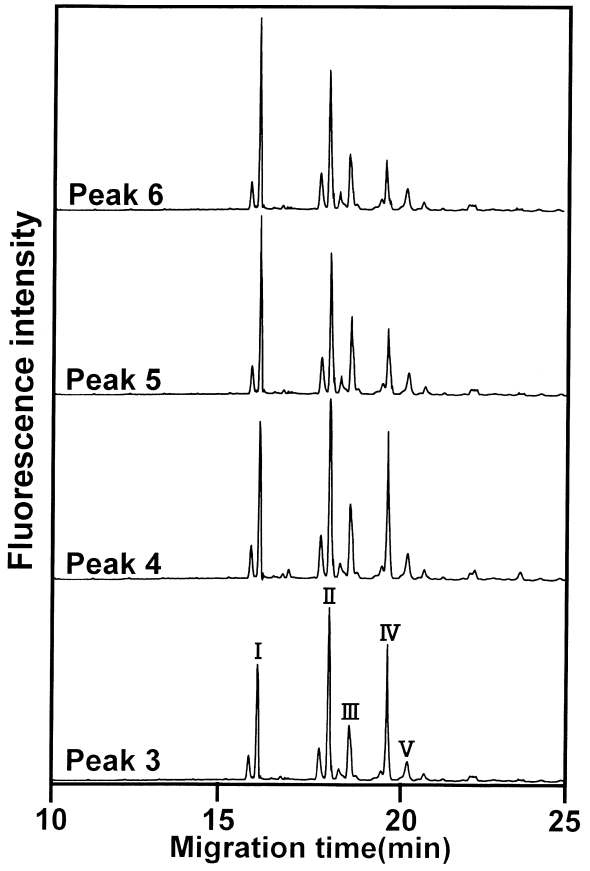

Fig. 5. Analysis of asialo-carbohydrate chains released from the glycoforms. Analytical conditions as in Fig. 3.

presence of HPMC in the electrolyte is important to keep the applied potential constant during analysis. HPMC did not show any effect on the following MALDI-TOF-MS measurement and carbohydrate analysis.

In the MS measurement, Sottani et al. reported that AGP gave incompletely resolved six ions centered at 33 170 using ferulic acid as matrix [17]. We examined various matrix materials including ferulic acid in order to obtain good resolved molecular ions. However, the sample of native human AGP showed a broad ion peak at m/z 35 670. The peak had almost the width of m/z 8000. Fragmentations of sialic acid residues of sialic acid-containing oligosaccharides were often observed during MALDI mass measurement. Highly sialylated carbohydrate chains (i.e., multi-branched carbohydrate chains) did not gave obvious molecular ions [13]. Instability of the sialic acid residues was probably one of the reasons why the molecular species of AGP showed such broad ions. However, fractions collected by CE showed characteristic mass patterns as shown in Fig. 2. The earlier migrated fractions showed large molecular masses (for example, m/z 36 125 for peaks 1 and 2), and later migrated peaks showed smaller molecular masses. These results indicate that the diantennary carbohydrate chains are abundant or the molecular species contain the smaller amount of carbohydrate chains in the later migrated fractions.

Table 2
Relative abundances of the oligosaccharides in the collected peaks

Collected peak	Oligosaccharide (%)				
	I	II	III	IV	V
Peak 3	21	36	11	27	5
Peak 4	26	31	13	26	4
Peak 5	37	28	16	14	4
Peak 6	42	28	12	13	5
Native AGP	36	34	17	11	3

Analysis of asialo-carbohydrate chains of these glycoforms clearly showed that relative ratios of di-, tri- and tetraantennary carbohydrate chains are different among glycoforms (Fig. 5, Table 2). Faster migrating peaks, i.e., highly negatively charged analyte ions, contain tri- and tetraantennary carbohydrate chains more abundantly. Slower moving peaks contain larger amount of the diantennary carbohydrate chain.

In the present study, a few hundred picomoles of the sample were employed for MS measurement and the analysis of carbohydrate chains. Further improvement in sensitivity will be required for the application of the present technique to the analysis of post-translational modifications of proteins in biological studies. We are developing a method for analyzing carbohydrate modifications in a protein separated by two-dimensional gel electrophoresis and will report elsewhere.

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