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

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Abstract

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

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

1. Introduction

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

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

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

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

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

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

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

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

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

2. Experimental

2.1. Materials

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

2.2. Releasing of N-linked oligosaccharides

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

2.3. Fluorescent derivatization of oligosaccharides with 3-AA

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

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

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

2.4. Enzymatic desialylation of sialyl oligosaccharides

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

2.5. Capillary electrophoresis of 3-AA labeled oligosaccharides

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

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

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

3. Results and discussion

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

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

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

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

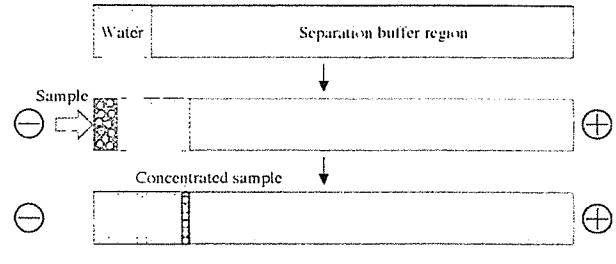

Fig. 1. Principle of on-line concentration using stacking method.

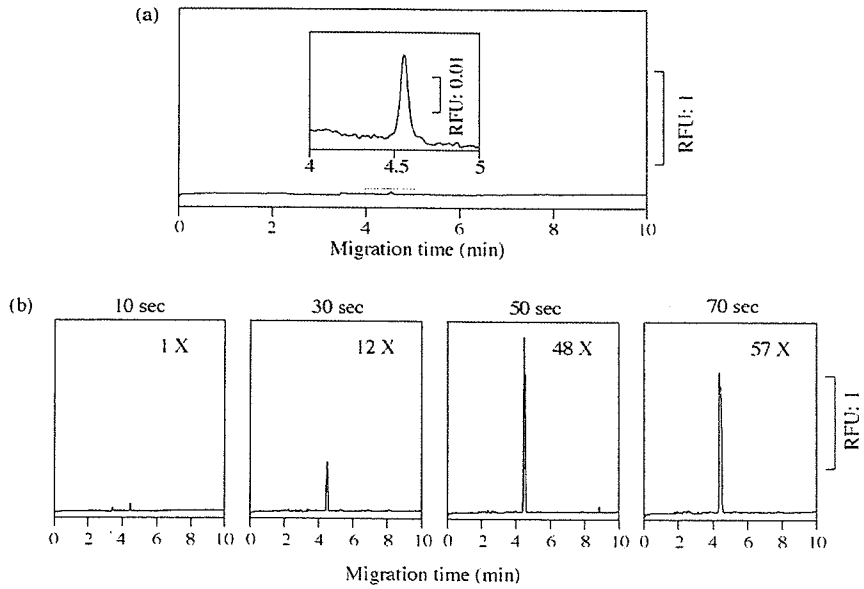

Fig. 2. Analysis of 3AA-Glc_5 using pressure injection method and electrokinetic injection method. 100 mM Tris–borate buffer (pH 8.3) containing 10% PEG70000 was used as the separation buffer with a DB-1 capillary (100 μm I.D., 30 cm, 20 cm effective length). Applied potential, 25 kV at 25 °C. Fluorescent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

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

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

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

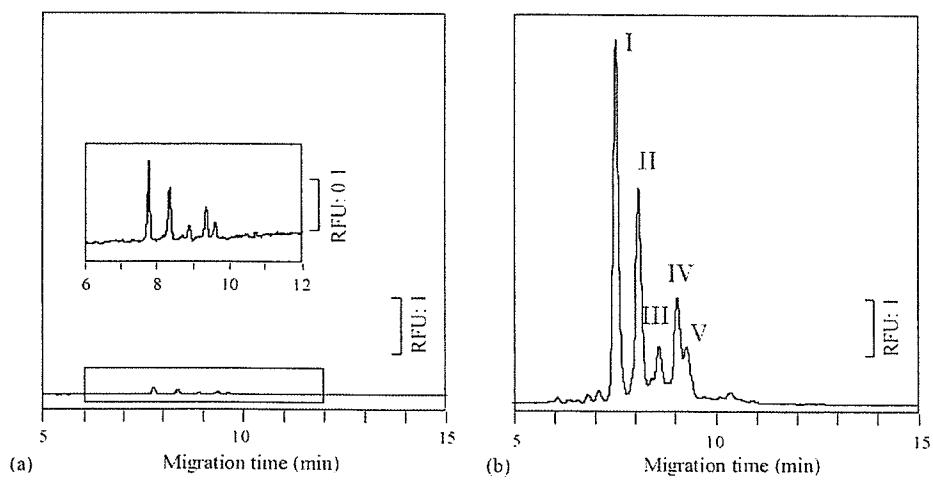

Fig. 3. Analysis of neutral-carbohydrate chains from ribonuclease B using pressure injection method and electrokinetic injection. (a) Pressure injection at 1 psi for 10 s, (b) Electrokinetic injection at 10 kV for 50 s after pre-injection of water at 1 psi for 10 s. Structures of I–V are $Man_5GlcNAc_2$,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

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

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

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

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

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

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

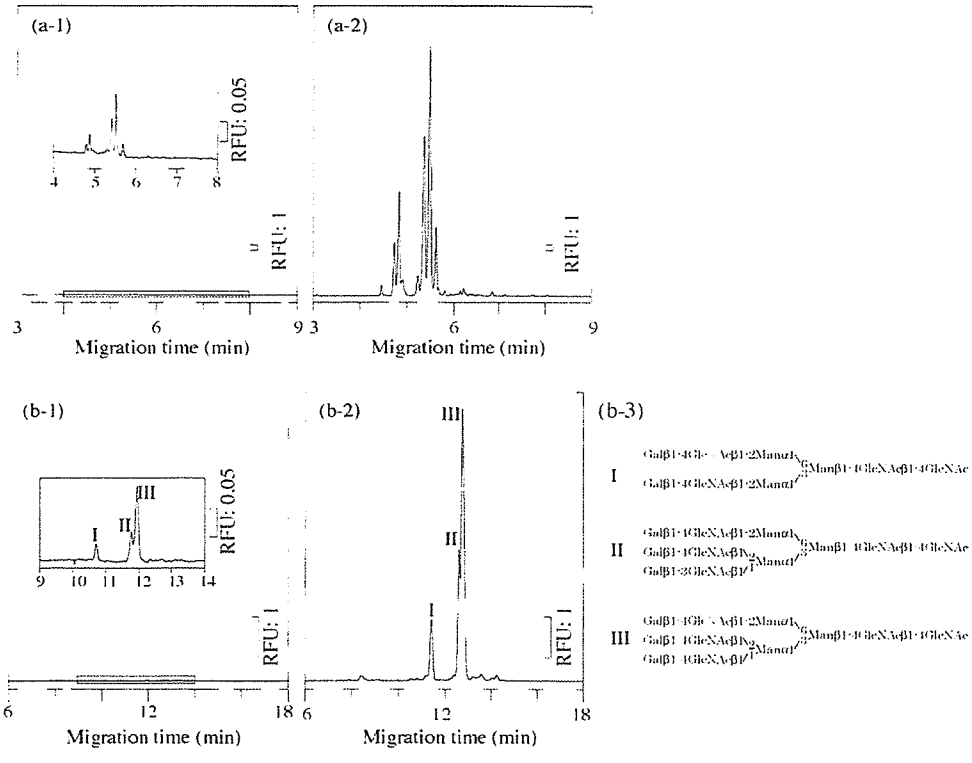

Fig. 4. Analysis of sialo- and asialo-carbohydrate chains from fetuin using pressure method and electrokinetic method. (a-1) Sialo-oligosaccharides by pressure injection at 1 psi for 10 s, (a-2) sialo-oligosaccharides by electrokinetic injection at 10 kV for 50 s after pre-injection of 50 mM phosphate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

Table 1
On-line concentration using some glycoprotein samples

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

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

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

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

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

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

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

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

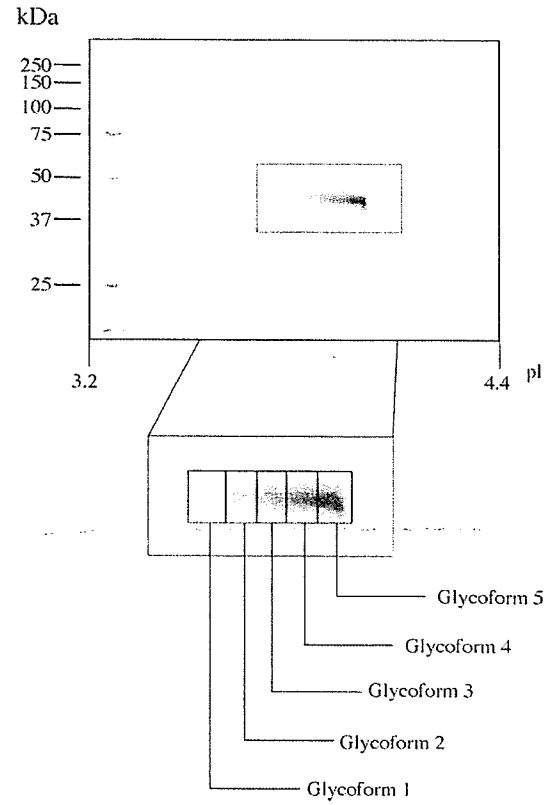

Fig. 5. Separation of AGP glycoforms by 2D-PAGE. Detection was performed with Coomassie blue staining method.

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

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

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

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

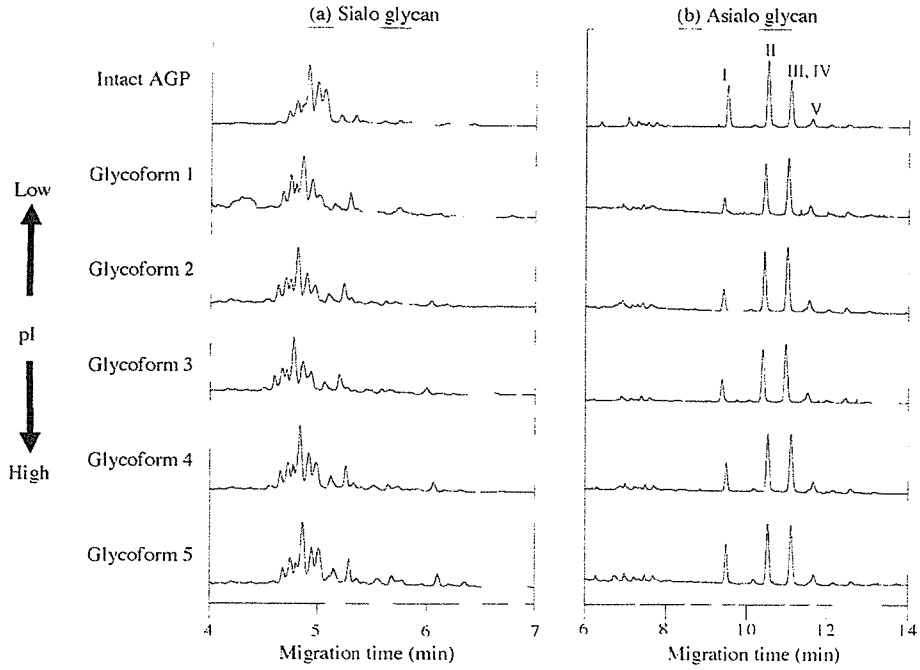

Fig. 6. Analysis of carbohydrate chains from glycoforms of AGP in gel spots after in-gel digestion. (a) Sialo-oligosaccharides by electrokinetic injection at 10 kV for 50 s after pre-injection of 50 mM phosphate buffer, (b) asialo-oligosaccharides by electrokinetic injection at 10 kV for 50 s after pre-injection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

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

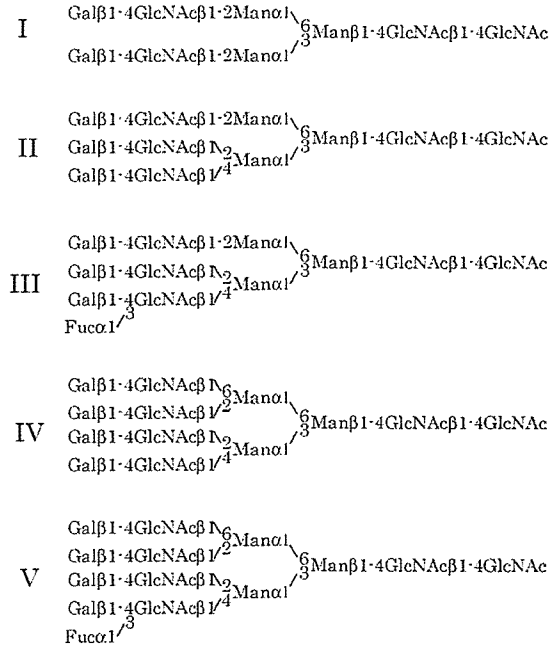

Fig. 7. List of asialo oligosaccharide structures of AGP.

Table 2
Oligosaccharide distributions of AGP glycoforms

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

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

4. Conclusion

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

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

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

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Review

Capillary electrophoresis for the analysis of glycoprotein pharmaceuticals

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

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

1 Introduction

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

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

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

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

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

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

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

2 Glycoform analysis

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

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

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

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

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

3 Structure analysis

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

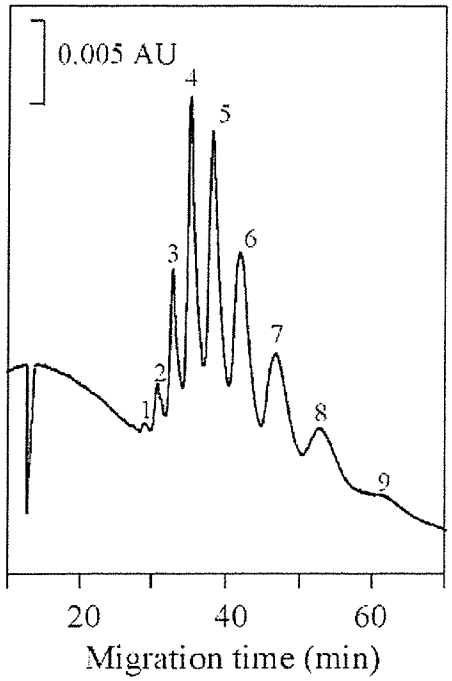

Figure 1. Analysis of rhuEPO glycoforms by CE. Analytical conditions: capillary, DB-1 capillary (57 cm (effective length, 50 cm), 100 μm id); running buffer, 10 mM acetate buffer (pH 5.7) containing 0.5% w/v of HPMC; applied potential, 12.5 kV; detection, UV absorption at 200 nm. Peaks 3–7 correspond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

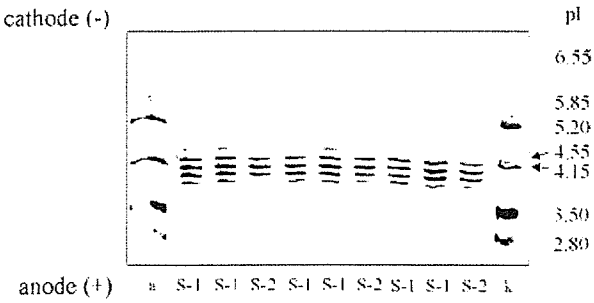

Figure 2. IEF gel electrophoresis of rhuEPO. Two pharmaceutical preparations of rhuEPO (S-1 and S-2) were analyzed. (a) and (k), p*I* marker. Bands 3–7 observed in the lanes of S-1 and S-2 correspond to the peaks 3–7 in Fig. 1, respectively. For details, see [26]. Reproduced with permission from the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

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

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

3.1 Sequencing of oligosaccharides by exoglycosidase digestion

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

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

3.2 Capillary affinity electrophoresis (CAE)

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

In the initial step, a mixture of fluorescent-labeled carbohydrates is analyzed by CE in the absence of lectin in the electrolyte (Fig. 3a). Then the same sample is analyzed in the presence of a lectin whose specificity is well established. When the lectin recognizes carbohydrate A, the peak A is observed later due to the equilibrium formation between the conjugate form with the lectin and the free form. On the contrary, carbohydrate C, which does not show affinity to the lectin, is observed at the same migration time as that in the absence of the lectin. Carbohydrate B shows weak affinity to the lectin and is observed slightly later. Thus, the migration order of the carbohydrate

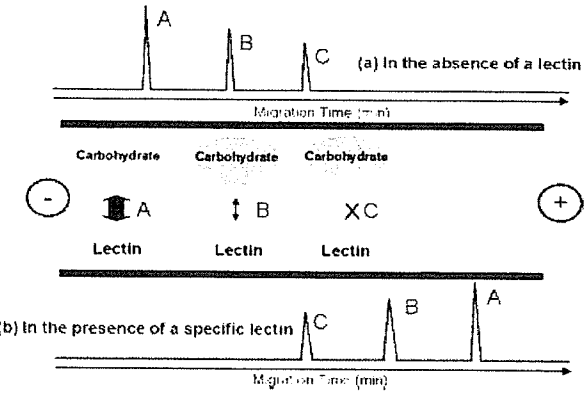

Figure 3. Principle for categorization of carbohydrate chains by CAE. For details, see [41]. Reproduced with permission from the publisher.

chains A–C, changes as shown in Fig. 3b. By repeating the procedures using an appropriate set of lectins, we can categorize all carbohydrate chains. An example of CAE analysis of asialo-oligosaccharides derived from AGP is shown in Fig. 4. AGP contains di-, tri-, and tetra-antennary oligosaccharides as shown in Table 1. Some of tri- and

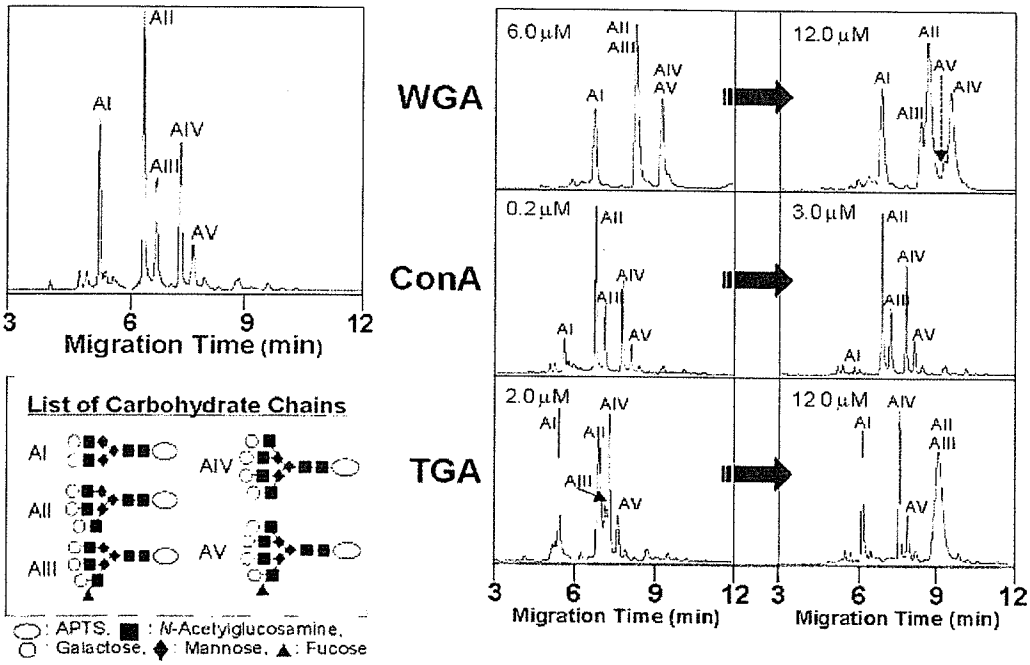

Figure 4. CAE of carbohydrate chains derived from AGP. Analytical conditions: Capillary, eCAP N-CHO capillary (30 cm (effective length, 10 cm), 50 μm id); running buffer, 100 mM Tris-acetate buffer (pH 7.4) containing 0.5% w/v PEG (PEG70000) and lectins; applied potential, 10 kV; fluorescent detection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

Table 1. List of the asialocarbohydrate chains derived from α1-acid glycoprotein

Peak number	Structure
AI	Galβ1-4GlcNAcβ1-2Manα1-6 \ Manβ1-4GlcNAcβ1-4GlcNAc-APTS / Galβ1-4GlcNAcβ1-2Manα1-3
AII	Galβ1-4GlcNAcβ1-2Manα1-6 \ Manβ1-4GlcNAcβ1-4GlcNAc-APTS / Galβ1-4GlcNAcβ1-2Manα1-3 / Galβ-4GlcNAcβ1-4
AIII	Galβ1-4GlcNAcβ1-2Manα1-6 \ Manβ1-4GlcNAcβ1-4GlcNAc-APTS / Galβ1-4GlcNAcβ1-2Manα1-3 / Galβ1-4GlcNAcβ1-4 / Fucα1-3
AIV	Galβ1-4GlcNAcβ1-6 \ Galβ1-4GlcNAcβ1-2Manα1-6 \ Manβ1-4GlcNAcβ1-4GlcNAc-APTS / Galβ1-4GlcNAcβ1-2Manα1-3 / Galβ1-4GlcNAcβ1-4
AV	Galβ1-4GlcNAcβ1-6 \ Galβ1-4GlcNAcβ1-2Manα1-6 \ Manβ1-4GlcNAcβ1-4GlcNAc-APTS / Galβ1-4GlcNAcβ1-2Manα1-3 / Galβ1-4GlcNAcβ1-4 / Fucα1-3

tetra-antennary oligosaccharides are substituted with fucose at the nonreducing terminal lactosamine residue to form Lewis X structure [43].

CAE using wheat germ agglutinin (WGA) showed the reversal of migration orders of AII and AIII, and AIV and AV, indicating that the fucose residue attached to the branches of tri- and tetra-antennary oligosaccharides decreased the binding with WGA. Con A showed specific interaction with di-antennary oligosaccharide, and the peak intensity (AI) was decreased in the presence of Con A. Addition of Tulipa gesneriana agglutinin (TGA) resulted in retardation of the migration times of triantennary structures (AII and AIII), indicating that this lectin is quite sensitive toward tri-antennary structures. Thus, this technique can be useful for the classification of a

mixture of oligosaccharides using a set of lectins of which binding specificities are well known. It should be noted that kinetic analysis also can be performed in CAE by observing the change of migrations at different concentrations of lectins.

CAE is not only a powerful tool for structure analysis of oligosaccharides but also a base technology of glycomics for oligosaccharide–protein interactions, and we can find carbohydrate-binding proteins in biological samples by employing a set of standard oligosaccharides as ligand [41, 42].

3.3 CE-MS

MS is a powerful technology for direct assignment of oligosaccharide structure by combination with HPLC, and MS/MS technique is often used for sequential analysis. For HPLC analysis, both on-line and off-line MS techniques are available because the peaks of interest are easily collected after detection. On the other hand, it is generally difficult to collect the peaks on CE run, although several works have been reported for collection of glycoprotein peaks using CE for measurement by MALDI-TOF MS [32]. Several studies for oligosaccharide analysis in biomedical research using CE-MS have also been reported (see [44] for a review). To date, there has been little work on CE-MS for glycan structure analysis. Che *et al.* [45] performed CE-MS using CE-quadrupole IT (QIT)-MS for the analysis of 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS)-derivatized dextran oligosaccharide standards. Gennaro *et al.* [46] demonstrated CE-QIT-MS analysis of ANTS-labeled oligosaccharides including *N*-linked glycans derived from ribonuclease B and fetuin. CE separation was performed in 20 mM 6-aminocaproic acid buffer (pH 4.12) with a polyvinyl alcohol (PVA)-coated capillary. This system was also available for multiple stage MS sequencing of sialylated oligosaccharides.

4 Glycan profiling

In the initial studies of glycoprotein pharmaceuticals, glycoform analysis is useful for the evaluation of glycoproteins as a whole molecule based on the carbohydrate heterogeneity. However, glycoform analysis described above (see Section 2) is mainly based on the difference in p*I*s of molecular species which have different number of sialic acid residues. Therefore, information about oligosaccharide structures obtained by glycoform analysis is limited.

Oligosaccharides of glycoprotein pharmaceuticals have been reported to influence its biological activity, pharmacokinetics, and stability [9, 10, 47, 48]. Newly emerging glycoprotein pharmaceuticals have new glycosylation sites in molecules to improve their pharmacokinetic characteristics. Furthermore, oligosaccharides attached to the core protein are intentionally changed to strengthen the biological activity by controlling glycosyltransferases in host cells by knock-in and knock-out techniques. To properly evaluate such glycoprotein pharmaceuticals, more detailed information on oligosaccharides is required for pharmaceutical development. For approved study and quality control, pharmaceutical companies have to elucidate "glycan profile" of their product, including heterogeneity of oligosaccharide structures and their abundances. CE is also a powerful tool for glycan profiling because of its high resolution to separate complex mixture of oligosaccharides.

4.1 Release of *N*-linked glycans from core protein

We have to release oligosaccharides from the protein core prior to analysis. There are several methods for releasing oligosaccharides from the core protein. Hydrazinolysis has been widely used for releasing all types of glycans. Peptide N^4-(acetyl-β-D-glucosaminyl)asparagine amidase (PNGase F; EC 3.2.2.18) is currently most frequently used for releasing complex-type, hybrid-type, and high-mannose *N*-linked oligosaccharides in case of the analysis of glycoprotein pharmaceuticals [49–51]. The method is quite convenient although the enzyme cannot release some specific type of *N*-linked oligosaccharide having core α1-3 fucose found in glycoproteins derived from plants and insects [52–55]. For the release of such oligosaccharides, glycoamidase A (almond) is available [55, 56].

4.2 Analysis of released glycans by CE

The released oligosaccharides using chemical or enzymatic methods as described above are usually labeled with fluorogenic or chromophoric compounds (see [57] for a review). A large number of labeling methods have been hitherto reported and most of the methods employ reductive amination [58]. The labeling reaction starts with the attack of the lone pair of amino groups of labeling reagent to the carbon of carbonyl groups of reducing end of carbohydrates, yielding a Schiff base under mild acidic conditions. The Schiff base is reduced to stable secondary amine in the presence of reducing reagent such as sodium cyanoborohydride.

In selection of labeling method for CE analysis, labeling reagents having negative or sometimes positive charges are often employed because they have to be resolved in the electric field. The labeling reagents for helium–cad-

mium or argon LIF detection system are preferred in CE analysis. This is important for highly sensitive detection of oligosaccharides at femtomole or lower levels.

Reductive amination of carbohydrates with 2-aminopyridine (PA) was reported by Honda *et al.* [59, 60] for high-resolution CE separation of mono- and oligosaccharides using borate buffer. Chiesa and Horvath [61] employed ANTS for derivatization of oligosaccharides and performed CE separation using triethylammonium phosphate buffer. Successful separation of ANTS-labeled carbohydrates was also achieved using various buffer systems including borate buffers [62, 63], acetate buffers [64, 65], and buffers containing sieving matrix material [66, 67].

8-Aminopyrene-1,3,6-trisulfonate (APTS) is currently one of the most popular derivatization reagents for CE analysis of oligosaccharides derived from various biological sources [32, 39, 68–74]. The APTS-labeled oligosaccharides have excitation at 455 nm, and show intense fluorescence at 520 nm. APTS derivatives are migrated at fast velocity and well resolved based on their negative charges as reported by many groups [32, 39, 68–74].

2- or 3-Aminobenzoic acid (AA) has also been reported for derivatization of oligosaccharides derived from glycoprotein samples, and used for CE analysis [75]. Labeling with 2-AA has been widely used for HPLC analysis of monosaccharides and oligosaccharides [76–78]. Among AA derivatives, the 3-substituted one shows the highest reaction efficiency. Because 3-AA derivatization can be achieved at mild conditions, sialic acids are not released during derivatization reaction. These aminobenzene derivatives can be detected at high sensitivity with a helium–cadmium LIF detection in CE analysis.

4.3 Application to glycan profiling of therapeutic antibody pharmaceuticals

Recombinant Igs (MAbs) are emerging as major therapeutic glycoprotein pharmaceuticals [8]. Relationship between biological functions such as antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) and oligosaccharides attached to the Fc region is important for pharmaceutical development. Terminal galactose (Gal), bisecting *N*-acetylglucosamine (GlcNAc), and core fucose (Fuc) residues have been revealed to be important for expression of biological activities [79–84].

Figure 5 shows the results of CE-LIF analysis of *N*-glycans derived from trastuzumab (a humanized mAb for treating metastatic breast cancer) after derivatization with APTS (Fig. 5a) and 3-AA (Fig. 5b). Separations were performed using a DB-1 capillary in buffers containing polyethylenglycol as sieving polymer.

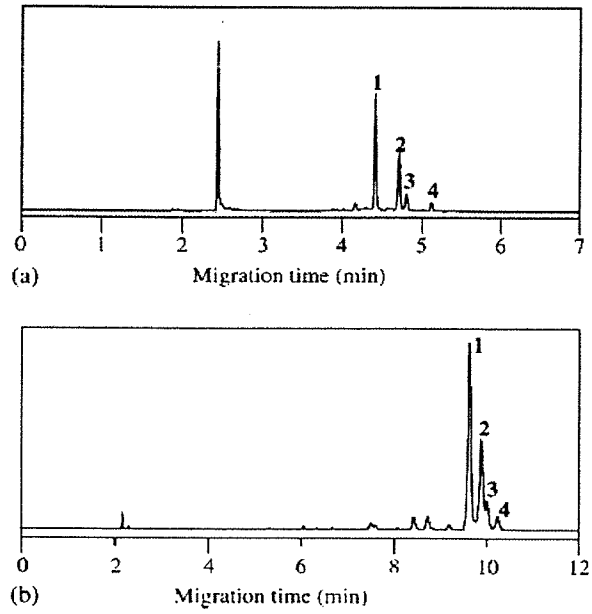

Figure 5. Oligosaccharide maps of trastuzumab by CE. (a) Oligosaccharides derivatized with APTS, (b) oligosaccharides derivatized with 3-AA. Analytical conditions: (a) Capillary, DB-1 capillary (30 cm (effective length 20 cm), 50 μm id); running buffer, 50 mM Tris-acetate buffer (pH 7.0) containing 0.5% w/v PEG70000; applied potential, 18 kV; fluorescent detection at 520 nm excited with argon-laser with 488 nm filter. (b) Capillary, DB-1 capillary (30 cm (effective length 20 cm), 100 μm id); running buffer, 100 mM Tris-borate buffer (pH 8.3) containing 10% w/v PEG70000; applied potential, 25 kV; fluorescent detection at 405 nm excited with helium–cadmium-laser with 325 nm filter. Structures of peaks 1–4 are shown in Table 2. For details, see [85]. Reproduced with permission from the publisher.

The list of the four major oligosaccharides in trastuzumab is shown in Table 2. In both electropherograms, two isomers of diantennary oligosaccharide to which one galactose residue attaches to one of the two nonreducing ends (**2** and **3**) were successfully separated within 6 and 11 min, respectively. It should be noted that the resolution of these oligosaccharides is difficult in HPLC analysis [85]. Although these oligosaccharides are fully fucosylated at the reducing GlcNAc residue, nonfucosylated glycans experimentally prepared by fucosidase treatment can be completely separated from fucosylated glycans at faster migration times (Fig. 6).

4.4 Validation for glycan profiling of therapeutic antibody pharmaceuticals

Determination of oligosaccharide distributions in glycoprotein therapeutics is a significant requirement in product assurance. Glycan-profiling method by CE can be

Table 2. List of the major oligosaccharides in IgG_1

Peak number	Structure
1	GlcNAcβ1-2Manα1\6 Fucα1\6 Manβ1-4GlcNAcβ1-4GlcNAc GlcNAcβ1-2Manα1/3
2	Galβ1-4GlcNAcβ1-2Manα1\6 Fucα1\6 Manβ1-4GlcNAcβ1-4GlcNAc GlcNAcβ1-2Manα1/3
3	GlcNAcβ1-2Manα1\6 Fucα1\6 Manβ1-4GlcNAcβ1-4GlcNAc Galβ1-4GlcNAcβ1-2Manα1/3
4	Galβ1-4GlcNAcβ1-2Manα1\6 Fucα1\6 Manβ1-4GlcNAcβ1-4GlcNAc Galβ1-4GlcNAcβ1-2Manα1/3

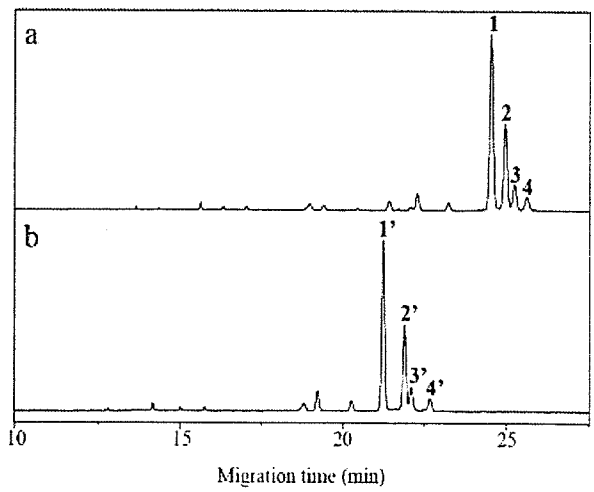

Figure 6. 3-AA-Labeled oligosaccharide maps of trastuzumab before (a) and after (b) fucosidase digestion. Analytical conditions: capillary, DB-1 capillary (50 cm (effective length 40 cm), 50 μm id); running buffer, 100 mM Tris-borate buffer (pH 8.3) containing 7.5% w/v PEG70000; applied potential, 30 kV; detection, fluorescent detection at 405 nm excited with helium–cadmium-laser with 325 nm filter. Structures of peaks 1–4 are shown in Table 2 and peaks 1'–4' correspond to defucosylated structures. Preparation of oligosaccharide mixture derivatized with 3-AA was performed according to the procedure reported in [85]. Fucosidase digestion was carried out using α(1-2,3,4,6) fucosidase (from bovine kidney, Glyko, Novato, CA). 3-AA-Labeled oligosaccharides mixture was dissolved in 20 mM sodium citrate/phosphate buffer (pH 6.0, 25 μL) followed by addition of fucosidase (25 mU, 5 μL) and incubated at 37°C overnight. Kamoda *et al.*, unpublished data.

applied to the lot release test if the method is validated. United States Food and Drug Administration (FDA) requires all assays for the release of commercial products to be validated [86], and a set of experiments must be conducted according to the International Conference on Harmonization (ICH) guidelines to ensure that the assay is appropriate for the intended use and to determine that the assay is suitable for routine analysis [86]. In method development, key parameters such as enzymatic digestion, derivatization, and CE conditions have to be optimized. In addition, the method should be confirmed based on its robustness and has to be validated according to ICH guideline in accuracy, linearity, precision, and specificity. Precision includes repeatability, intermediate precision, and reproducibility.

There have been some reports on the performance of CE-LIF for the analysis of oligosaccharides derived from recombinant monoclonal IgGs. We evaluated the repeatability of CE analysis of oligosaccharide mixture from single preparation of therapeutic IgG (trastuzumab), in quantitative determination of the relative distribution of *N*-linked glycans using APTS and 3-AA-labeling method [85]. The results showed that the precisions of migration times and relative corrected peak areas for four major peaks (see Fig. 5, peak 1–4) were sufficiently high in both two derivatization methods. Ma and Nashabeh [39] evaluated reproducibility, which means interlaboratory precision, in the similar assay for rituximab using APTS-labeling method, and demonstrated good precision with the RSDs below 1% for the corrected area percent of nongalactosylated (G0), mono-galactosylated (G1), and di-galactosylated (G2)

glycans. They also evaluated the accuracy by G0 recovery in three different ways, *i.e.*, addition of known amount of G0 standard, mixing two rituximab lots have different G0 distribution, and dilution of the initial sample. All three studies showed excellent performance with the recovery around 100%. From these results, CE-LIF method for quantitative determination of relative distribution of *N*-linked glycans has been proved to be a potential for routine lot release testing of therapeutic antibody in pharmaceutical industry.

5 Conclusions

In the field of analytical science, CE has been an important choice for analyzing oligosaccharides derived from glycoprotein due to its high resolution and rapid separation. Recent development in analytical method and analytical device allow general use of CE technologies for regulatory science in the development of glycoprotein pharmaceuticals in pharmaceutical industry. In this review, glycoform analysis and profiling of released *N*-linked glycans using CE are reviewed including various structure analysis techniques. Glycoform assay of glycoprotein using CE has a potential to be substituted with conventional slab gel electrophoresis method due to its convenient manipulation, high resolution, and automation. Glycan profiling using CE can be a complementary or substitutive technique for HPLC due to rapid separation, high resolution, and unique separation mode.

CE has some disadvantages in structure analysis due to difficulty in collecting sample after separation. Development of CE-MS^n is promising under diverse derivatization techniques, but various separation buffers should be developed for general and practical use.

Introduction of CE to the pharmaceutical analytical field potentially provides additional and detailed information on the oligosaccharide moieties in glycoprotein pharmaceuticals, and will contribute to the development of more effective and safe pharmaceuticals.

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Research Article

Comparative studies on the analysis of urinary trypsin inhibitor (ulinastatin) preparations

Urinary trypsin inhibitor (ulinastatin) is a characteristic protein pharmaceutical which contains both glycosaminoglycans and *N*-linked glycans in its molecule and has been used for treatment of acute pancreatitis. The comparability of ulinastatin preparations of different lots or from different companies was studied by using conventional analytical approaches such as SDS-PAGE, cellulose acetate membrane electrophoresis, and HP size-exclusion chromatography (SEC) and also by using newly developed techniques such as CE and MALDI-TOF MS. The methods using SEC and SDS-PAGE according to *The Japanese Pharmacopoeia* showed similar molecular masses for two different preparations, and the estimated molecular masses were significantly different from those observed with MALDI-TOF MS. We also showed that the electrophoretic methods using cellulose acetate membrane electrophoresis and CE can be used for comparability assessments of ulinastatin preparations. In addition, we analyzed the unsaturated disaccharides derived from glycosaminoglycan (chondroitin 4-sulfate chain) and *N*-linked oligosaccharides attached to ulinastatin by CE after releasing them by enzymatic digestion followed by fluorescent labeling with 2-aminoacridone and 2-aminobenzoic acid, respectively. The results indicated that carbohydrate chains are important as markers for comparability assessments of ulinastatin pharmaceutical preparations.

Keywords: Capillary electrophoresis / Carbohydrate chains / Glycoprotein pharmaceuticals / Ulinastatin
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1 Introduction

Carbohydrate moieties in glycoprotein pharmaceuticals show various effects on the expression of their biological activities such as metabolic rate, stability, and solubility [1, 2]. Several studies that focus on comparability of carbohydrate chains have been reported for the assessment of glycoprotein pharmaceuticals such as erythropoietin [3, 4], granulocyte-macrophage colony-stimulating factor [5], thrombopoietin [6], and interferon-γ [7]. These studies revealed that carbohydrate chains of these products were highly heterogeneous and their analysis was important for quality assessment.

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Abbreviations: 2AA, 2-aminobenzoic acid; **AMAC**, 2-aminoacridone; **ChS**, chondroitin 4-sulfate; **ChSrUTI**, ChS-removed UTI; **PNGaseF**, peptide *N*-glycoamidase F; **ppUTI**, peptide portion of UTI; **SEC**, size-exclusion chromatography; **UTI**, urinary trypsin inhibitor

Urinary trypsin inhibitor (ulinastatin, UTI) is an intrinsic serine-protease inhibitor which is extracted and purified from human urine [8, 9]. UTI is approved as a therapeutic agent for acute pancreatitis by the Ministry of Health, Labor, and Welfare of Japan, and has been used for the management of acute pancreatitis or rheumatoid arthritis mainly in Japan and China [10, 11]. UTI is a characteristic glycoprotein pharmaceutical and is considered to be a metabolite of inter-α-trypsin inhibitor (ITI) [12, 13]. The amino acid sequence of UTI is shown in Fig. 1.

The peptide portion composed of 143 amino acid residues has a molecular mass of 15 340 Da [14], and has *N*-glycan at Asn-45 [15, 16] as well as a glycosaminoglycan chain of low-sulfated chondroitin 4-sulfate (ChS) which is attached to Ser-10 [15, 17]. The ChS chain of UTI does not seem to be involved in protease-inhibitor activity [18], but is considered to play an important role in maintenance of physiological, physicochemical, and biological properties.

UTI belongs to a class of biopharmaceuticals, and its physicochemical properties are defined in *The Japanese Pharmacopoeia* (JP) [19]. In JP, several items are de-

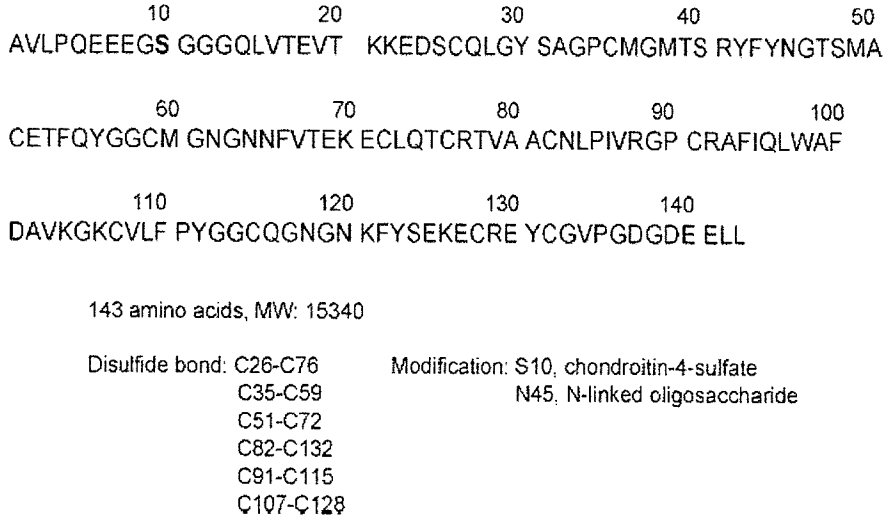

Figure 1. Structure of UTI.

scribed for quality assessment of UTI. These are an identification test, pH of the solution, specific activity, purity, molecular mass, antigenicity, and toxicity [19]. A colorimetric assay by phenol-sulfuric acid method, spectrophotometry, an inhibitory assay on the action of trypsin to *N*-α-benzoyl-L-arginine-4-nitroanilide, and a double immuno-diffusion test (Ouchterlony method) are employed. SDS-PAGE is used to determine purity so as to confirm the absence of other urinary proteins. For molecular mass determination, size-exclusion chromatography (SEC) is employed, and the molecular mass of UTI ranged from 62 kDa to 72 kDa.

Although the methods described above are appropriate for routine testing of protein pharmaceuticals, molecular masses of protein pharmaceuticals containing carbohydrate chains are often overestimated due to the bulkiness of carbohydrates. Moreover, it should be noted that the defined molecular mass range is often higher than the theoretical molecular masses.

In a previous report, we developed a method for the profiling analysis of oligosaccharides in antibody pharmaceuticals by CE [20], and we demonstrated that analysis of carbohydrate chains in glycoprotein pharmaceuticals by CE with LIF detection is useful for quality assurance of glycoprotein pharmaceuticals. In follow-up studies, we developed various methods for the assessment of UTI preparations and the methods were used for comparative studies on the analysis of different UTI preparations. In addition, we compared conventional analytical techniques that are certified by JP with some newly developed analytical techniques such as CE and MALDI-TOF MS.

2 Materials and methods

2.1 Reagents

Pharmaceutical preparations of UTI (preparation A and B) for injection, which are commercially available from two different companies in Japan, were purchased and used after dialysis against water for 2 days, changing the water several times at 4°C. Chondroitinase ABC (Chase ABC) and standard samples of unsaturated disaccharides derived from glycosaminoglycans were obtained from Seikagaku Kogyo (Chuo-ku, Tokyo, Japan). Peptide *N*-glycoamidase F (PNGase F, recombinant) was obtained from Roche Diagnostics (Mannheim, Germany). Neuraminidase (*Arthrobacter ureafaciens*) was kindly supplied by Dr. Ohta of Marukin-Bio (Uji, Kyoto, Japan). 2-Aminobenzoic acid (2AA) and 2-aminoacridone (AMAC) were obtained from Tokyo Kasei (Chuo-ku, Tokyo, Japan) and Molecular Probes (Eugene, OR, USA), respectively, and used without further purification. DB-1 capillary, of which the inner surface is chemically modified with PDMS, was obtained from J&W scientific (Folsom, CA, USA). PEG (PEG70000, average molecular weight 70 000) was obtained from Wako Pure Chemicals (Dosho-machi, Osaka, Japan). Sephadex LH-20 was purchased from Amersham Bioscience (Piscataway, NJ, USA). Other reagents and solvents were of the highest grade commercially available.

2.2 Digestion of UTI preparations with Chase ABC

UTI preparations were analyzed after stepwise glycosidase digestion (Fig. 2).