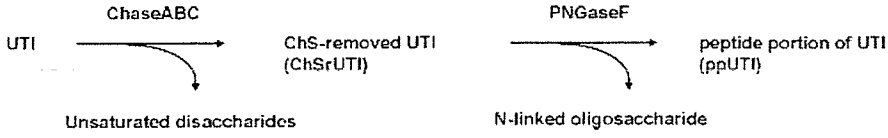

Figure 2. Stepwise glycosidase digestion of UTI.

Initially, UTI was digested with Chase ABC to remove the ChS chain. A sample of lyophilized UTI preparations (1 mg) was dissolved in 20 mM Tris-HCl buffer (pH 8.0, 50 μL), Chase ABC (100 mU, 2 μL) was added to the solution, and the mixture was incubated at 37°C for 12 h. After drying the mixture with a vacuum evaporator (SpeedVac, Savant, Farmingdale, NY, USA), the lyophilized material was used as ChS-removed UTI (ChSrUTI).

2.3 Digestion of ChSrUTI with PNGase F

A portion of ChSrUTI (0.5 mg) was dissolved in 20 mM phosphate buffer (pH 7.0, 50 μL), PNGase F (5 U, 5 μL) was added to the solution, and the mixture was incubated at 37°C for 12 h. The reaction mixture was then analyzed as a peptide portion of UTI (ppUTI).

2.4 SEC

UTI preparations, ChSrUTI, and ppUTI were prepared as described in the previous sections and analyzed by SEC. Analysis was performed on an SC-8020 apparatus equipped with an 8020-type UV absorption detector (TOSOH, Minato-ku, Tokyo, Japan). A portion (50 μL) of aqueous solutions of prepared UTI samples (2.5 mg/mL as concentration) was injected into a TSKgel G2000SW column (TOSOH, 30 cm × 7.5 mm id) using 0.1 M phosphate buffer (pH 6.8) containing 0.1 M NaCl as eluent at a flow rate of 1.0 mL/min. Detection was performed by UV absorption at 280 nm. Phosphorylase b (rabbit muscle, 97 200 Da), BSA (66 200 Da) and lysozyme (chicken egg white, 14 400 Da), each 0.5 mg/mL, were used as molecular mass standards.

2.5 SDS-PAGE

SDS-PAGE of UTI preparations was performed using a 12% acrylamide gel in a vertical slab gel apparatus. Samples were analyzed as non-reduced and reduced forms. For the preparation of non-reduced samples, UTI and ChSrUTI (5 and 10 μg, respectively) were dissolved in 10 μL of sample buffer (125 mM Tris-HCl buffer, pH 6.8, 2% SDS, 10% glycerol) and then boiled at 100°C for 5 min. For the preparation of reduced samples, sample buffer containing 5% 2-mercaptoethanol was used. After BPB solution (0.05%, 1 μL) was mixed with the sample solution, the mixture was loaded on the gel. Electrophoresis was performed at 5 mA while samples were in the stacking gel. When the dye reached the resolving gel, the current was increased to 10 mA. CBB R250 was used for the staining.

2.6 MALDI-TOF MS

MALDI-TOF MS spectra of UTI preparations were acquired on a Voyager DE-Pro mass spectrometer (PE Biosystems, Framingham, MA, USA) in negative-ion linear mode. Nitrogen laser (337 nm) was used for the ionization. The accelerating voltage was 22 kV, and delayed extraction was performed after 1500 ns. 2,5-Dihydroxybenzoic acid (DHB) was used as matrix. Subsequently, 1 μL matrix solution (10 mg DHB in 1 mL 50% aqueous methanol) was loaded on the sample plate, dried at room temperature, and 1 μL UTI or ChSrUTI solution (1 mg/mL) was loaded. After drying, 1 μL matrix solution was loaded again and used for MS analysis.

2.7 Cellulose acetate membrane electrophoresis

Cellulose acetate membrane electrophoresis was performed using an SE-33 apparatus (Toyo kagaku) using a SELECA-V membrane (Advantec Toyo). As a running buffer, 0.1 M pyridine-0.47 M formic acid (pH 3.0) was used. A portion (1 μL) of each UTI sample solution (2 mg/mL) was applied to the membrane. Electrophoresis was performed using constant current mode at 0.5 mA/cm for 1 h. After electrophoresis, the membrane was kept for 10 min in a solution of 0.1% w/v Alcian blue in 0.1% v/v acetic acid, and was then washed with 0.1% v/v acetic acid for a few minutes to remove the background color.

2.8 CE

CE of UTI preparations was performed on a Beckman P/ACE 2200 apparatus. Separation was carried out at 25°C using a fused silica capillary (50 μm id, 40 cm length) in 50 mM borate buffer (pH 9.3) containing 100 mM SDS at the applied potential of 20 kV. The sample solution was injected by pressure method (0.5 psi) for 10 s. Detection was performed by UV absorption at 214 nm. AMAC-labeled unsaturated disaccharides derived from ChS

were analyzed using a Beckman MDQ Glycoprotein System with a DB-1 capillary (50 μm id, 40 cm length) in 100 mM Tris-borate buffer (pH 8.0) containing 1% PEG70000, and detection was performed by an argon LIF detector (Ex 488 nm, Em 520 nm). CE of 2AA-labeled *N*-linked oligosaccharides was performed in the similar manner using a helium-cadmium LIF detector (Ex 320 nm, Em 405 nm) with a DB-1 capillary (50 μm id, 40 cm length) in 100 mM Tris-borate buffer (pH 8.3) containing 10% PEG70000.

2.9 Fluorescent derivatization of unsaturated disaccharides derived from ChS with AMAC

Fluorescent labeling of unsaturated disaccharides derived from ChS chains was performed according to a previously reported procedure [21]. UTI (1 mg) was dissolved in 20 mM Tris-HCl buffer (pH 8.0, 50 μL) and an aqueous solution of Chase ABC (100 mU, 2 μL) was added. After incubating the mixture at 37°C for 12 h, the reaction mixture was filtered through an ultrafiltration membrane (Millipore, 10 000 Da cut-off) and the filtrate was lyophilized. The lyophilized material was dissolved in 100 mM AMAC in a mixture (10 μL) of DMSO-acetic acid (17:3 v/v) and 1 M sodium cyanoborohydride (10 μL) in the same solvent. After keeping the mixture at 90°C for 30 min, water (500 μL) and chloroform (500 μL) were added to the reaction mixture and mixed vigorously by a vortex mixer. After removing the chloroform layer, the aqueous phase was washed again with chloroform (500 μL), and the procedure was repeated five times. A portion of the aqueous phase was used for CE analysis.

2.10 Fluorescent derivatization of *N*-linked oligosaccharides with 2AA

Because PNGase F does not efficiently release *N*-linked oligosaccharides from intact UTI preparations due to the presence of ChS chains, we used ChSrUTI for the release of *N*-linked oligosaccharides with PNGase F. The released *N*-glycans were labeled with 2AA according to a previously reported procedure [22]. The ChSrUTI product (1 mg as UTI) was dissolved in 20 mM phosphate buffer (pH 7.0, 50 μL), and PNGase F (5 U, 5 μL) in the same buffer was added to the mixture. The mixture was incubated at 37°C for 12 h. After keeping the mixture in a boiling water bath for 5 min, it was lyophilized to dryness. The lyophilized material was dissolved in 2AA solution (500 μL) which was freshly prepared by dissolution of 2AA and sodium cyanoborohydride (15 mg each) in methanol (1 mL) containing 4% sodium acetate and 2% boric acid, and the mixture was kept at 80°C for 1 h. After cooling, water (200 μL) was added to the mixture, which was then applied on a Sephadex LH-20 (1 × 30 cm) column equilibrated with 50% aqueous methanol. The earlier eluted fluorescent fractions were pooled and evaporated to dryness. The residue was dissolved in water (100 μL) and a portion (10 μL) of the solution was used for analysis by CE and MALDI-TOF MS.

3 Results and discussion

Chemical and physicochemical methods have been developed for the evaluation of glycoprotein pharmaceuticals. UTI is evaluated by SEC and SDS-PAGE in JP. As initial studies, we analyzed UTI preparations according to the methods described in JP.

3.1 Molecular masses of UTI preparations

3.1.1 SEC

Figure 3 shows the results of the analysis of UTI preparations and their digestion products with glycosidases by SEC using a TSKgel G2000SW column.

Both preparations (A and B) of native UTI were observed at ~8.1 min (Fig. 3a), and their molecular weights were estimated as ~70 kDa from the calibration curves. Digested products with Chase ABC (ChSrUTI) were observed at later elution times (~9.8 min) than those of native UTI (Fig. 3b), and their molecular weights were estimated as ~53 kDa. ChSrUTI preparations were further treated with PNGase F. The products (ppUTI) obtained from both preparations (A and B) showed similar elutions, and a major peak was observed at ~9.9 min (Fig. 3c). The peaks observed at around 13 min were derived from *N*-linked oligosaccharides released from the protein core of UTI. Molecular weights of ppUTI were estimated as 52 kDa. These results indicate that overestimated molecular masses of UTI are obviously due to the presence of the carbohydrate chains. However, SEC is useful for equivalency tests for different pharmaceutical products, but it does not seem appropriate for evaluation of quality.

3.1.2 SDS-PAGE

Figure 4 shows the results of SDS-PAGE analysis of native UTI and ChSrUTI preparations under non-reduced conditions.

Native UTI preparations A (lanes 1, 5) and B (lanes 2, 6) showed a broad and smear band at ~37 kDa. In contrast, ChSrUTI (A, lanes 3 and 7; B, lanes 4 and 8; indicated as

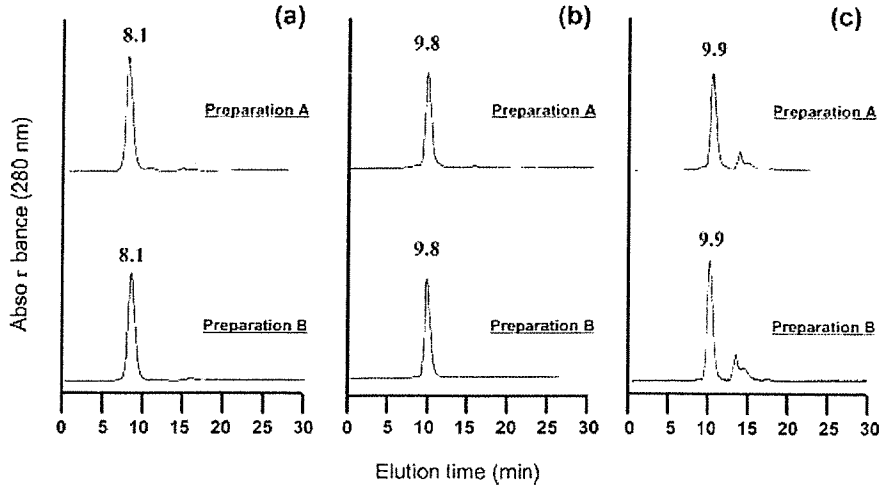

Figure 3. SEC of native UTI and glycosidase-treated UTI samples. Preparations A and B are shown in the upper and lower panel, respectively. UTI was analyzed as native form (a), ChSrUTI (b), and ppUTI (c). The numbers at each peak indicate elution times (min).

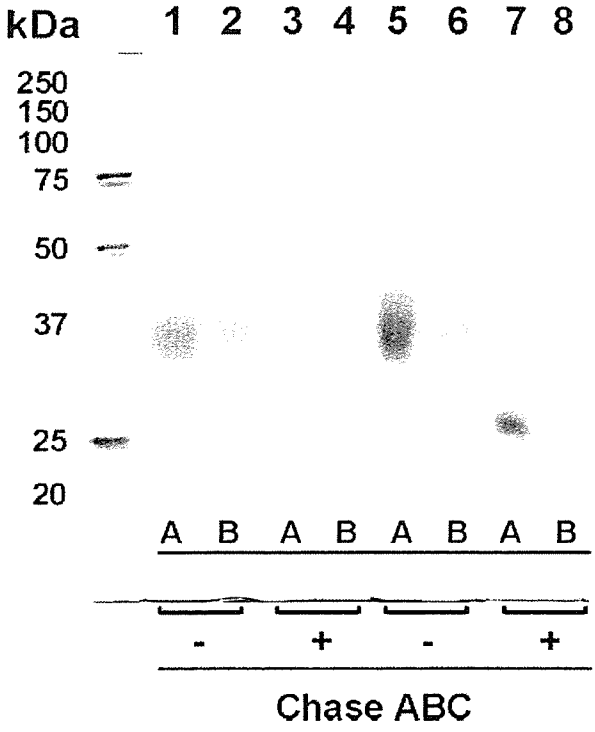

Figure 4. SDS-PAGE analysis of native UTI and ChSrUTI. UTI samples were analyzed with a 12% acrylamide gel; 5 μg (lanes 1–4) and 10 μg (lanes 5–8) of UTI samples were applied.

Chase ABC +) showed a single band at ~25 kDa. Minor bands observed at 70 kDa for both preparations were probably due to native UTI dimers, because the band disappeared after digestion with Chase ABC (see lanes 3, 4 and 7, 8). The broad and smear band of native UTI is due to the large contents and heterogeneity of carbohydrate chains, especially ChS chains. Reduced UTI preparations with 2-mercaptoethanol showed similar results (data not shown). In SDS-PAGE analysis, UTI preparations showed smaller molecular masses than those estimated by SEC. This observation indicates that determination of precise molecular masses of UTI preparations is not possible with SEC and SDS-PAGE methods, although they have been widely used as the standard methods for evaluation of glycoprotein pharmaceuticals.

3.1.3 MALDI-TOF MS

We analyzed native UTI and ChSrUTI by MALDI-TOF MS. Figure 5 shows the mass spectra of both preparations obtained in negative-ion mode.

Native UTI samples showed a broad molecular ion peak at *m/z* 24 300 for both preparations (Fig. 5a). Small peaks at *m/z* 48 000 were due to dimer forms. In contrast, ChSrUTI samples of both preparations were detected at *m/z* 18 600 and the peaks became narrower than those obtained from native UTI preparations (Fig. 5b). Ions due to the dimeric and trimeric forms were also observed at *m/z* 38 000 and 56 000, respectively.

Molecular weights of UTI estimated in the present study are summarized in Table 1.

Molecular weights of UTI determined by MALDI-TOF MS showed similar values to the calculated data as reported previously [16, 17]. These results indicate that a combination of MALDI-TOF MS and SDS-PAGE or SEC was useful for both the evaluation of molecular weights and the assessment of purity. Furthermore, these methods

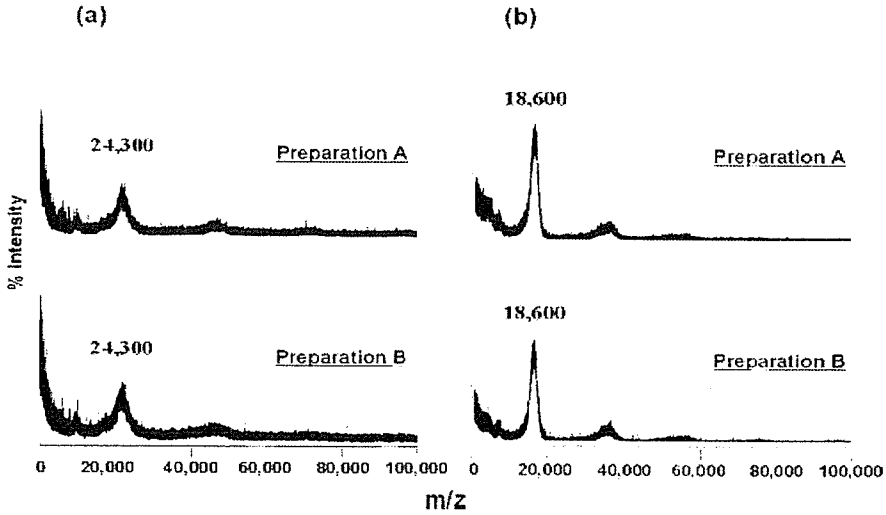

Figure 5. MALDI-TOF MS spectra of UTI samples. Native UTI (a) and ChSrUTI (b) were analyzed in negative-ion mode. Preparations A and B are shown in the upper and lower panel, respectively.

Table 1. Estimated molecular weights of UTI preparations with some of the methods employed in the present study

Method	Preparation A	Preparation B
SEC	70 kDa	70 kDa
SDS-PAGE	37 kDa	37 kDa
MALDI-TOF MS	24 kDa	24 kDa

were also useful for confirmation of the equivalence of UTI preparations as exemplified by the analysis of preparation A and B.

3.2 Analysis of UTI by electrophoretic methods

3.2.1 Cellulose acetate membrane electrophoresis

Cellulose acetate membrane electrophoresis has been widely used for the analysis of glycosaminoglycans, and is still useful for the diagnosis of congenital disorders of glycosaminoglycans [23, 24]. UTI is a pharmaceutical that contains glycosaminoglycans.

We used cellulose acetate membrane electrophoresis for the analysis of UTI and ChSrUTI samples. Native UTI preparations (A and B) were observed at the same position as a single spot with no significant difference (data note shown). In the analysis with cellulose acetate membrane electrophoresis, UTI preparations were migrated by negative charges due to sulfate groups from the ChS chains. These results indicate that both preparations A and B were substituted with the same numbers of sulfate groups in the ChS chains. In contrast, both UTI preparations (ChSrUTI) were not detected after digestion with Chase ABC, because Alcian blue is a specific staining reagent for detection of polyanions such as glycosaminoglycans.

3.2.2 CE

CE is useful for the analysis of acidic proteins such as sialic acid containing glycoproteins [25–27]. In this study, we used MEKC for the analysis of UTI preparations, and compared migrations of both preparations (Fig. 6).

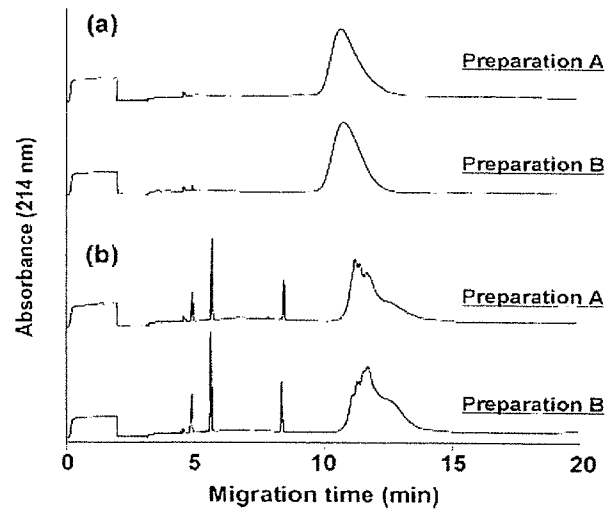

Figure 6. CE of UTI samples. Analytical conditions: fused-silica capillary (40 cm, 50 μm id); buffer, 50 mM borate buffer (pH 9.3) containing 100 mM SDS; applied voltage, 20 kV; detection, UV absorption at 214 nm. (a) Native UTI, (b) ChSrUTI.

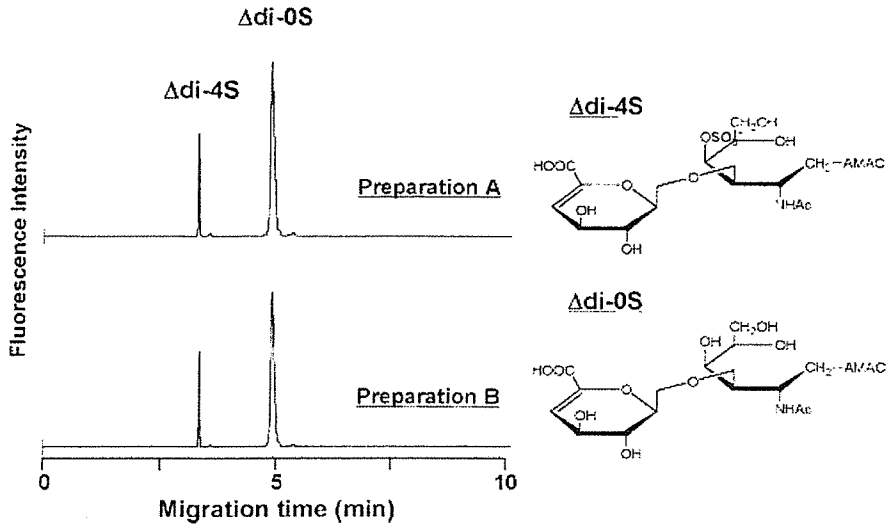

Figure 7. CE of AMAC-labeled unsaturated disaccharides derived from a ChS chain. Preparations A and B were digested with Chase ABC, and the produced unsaturated disaccharides were derivatized with AMAC and detected by an Ar LIF detector. Analytical conditions: capillary, DB-1 capillary (40 cm, 50 μm id); buffer, 100 mM Tris-borate buffer (pH 8.0) containing 1% PEG70000; applied voltage, 15 kV.

Both preparations A and B were observed at ~11 min as a single broad peak (Fig. 6a). After digestion of both preparations with Chase ABC, ChSrUTI gave a group of several peaks due to the microheterogeneity of *N*-glycans between 11 and 14 min, with a few peaks between 5 and 9 min due to unsaturated disaccharides derived from ChS chains (Fig. 6b).

3.3 Analysis of carbohydrate chains

3.3.1 Analysis of ChS chains

UTI contains a single ChS-like glycosaminoglycan chain (low-sulfated chondroitin 4-sulfate) attached to Ser-10 (see Fig. 1). We analyzed unsaturated disaccharides derived from low-sulfated ChS of UTI after digestion with Chase ABC followed by labeling with AMAC. Figure 7 shows the results of the disaccharide analysis by CE.

Both preparations gave two major peaks upon digestion with Chase ABC, which were confirmed as Δdi-4S and Δdi-0S, respectively, by comparison with commercially available standard unsaturated disaccharides. Relative ratios of Δdi-4S and Δdi-0S were 38 ± 0.8% and 62 ± 0.8% (mean ± SD, n = 4), respectively, for both preparations. The ratios of Δdi-4S and Δdi-0S will be a good parameter for quality assurance of UTI preparations as well as comparative studies.

3.3.2 Analysis of *N*-linked oligosaccharides

UTI also has a single *N*-linked oligosaccharide chain attached to Asn-45. In the evaluation of glycoprotein pharmaceuticals, determination of *N*-linked oligosaccharides is essential. We analyzed *N*-linked oligosaccharides using ChSrUTI products for both preparations by digestion with PNGase F, because PNGase F could act efficiently on ChSrUTI, and an approximately tenfold larger amount of *N*-linked oligosaccharides was released than that from native UTI (data not shown). The analysis of the *N*-glycans was made with CE after labeling the released oligosaccharides with 2-AA. The results are shown in Fig. 8.

Two major peaks (peaks I and II) with a minor peak (peak III) derived from N-linked oligosaccharides were observed at 7.0, 8.8, and 13.2 min, respectively (Fig. 8a). After digestion with neuraminidase, these peaks were observed later (13.2 min) as a single peak (peak III, Fig. 8b). From these results, peaks I, II, and III were confirmed as disialo-, monosialo-, and asialo-diantennary complex-type oligosaccharides, respectively, by comparing the migration times with those from standard diantennary oligosaccharides derived from human transferrin, and by MALDI-TOF MS analysis (data not shown). It should be noted that there was no significant difference between preparations A and B.

3.3.3 Lot-to-lot analysis

UTI preparations of different lot products were evaluated by analyzing *N*-linked oligosaccharides. We used four lots from each preparation, and compared relative corrected peak areas of *N*-linked oligosaccharides (Fig. 9).

Electropherograms obtained from four lots (a–d) of preparation A (left panel) and B (right panel) showed a similar appearance, but their oligosaccharide compositions exhibited significant variation. In the analysis of prep-

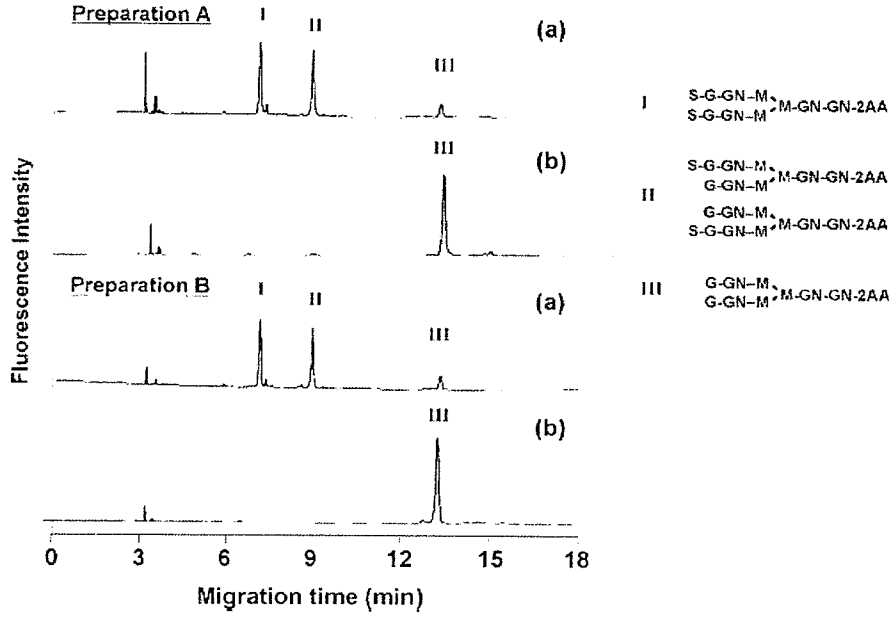

Figure 8. CE of 2AA-labeled *N*-linked oligosaccharides in UTI. *N*-Linked oligosaccharides were analyzed as sialo (a) and asialo (b) forms. Analytical conditions: capillary, DB-1 capillary (40 cm, 50 μm id); buffer, 100 mM Tris-borate (pH 8.3) containing 10% PEG70000; applied voltage, 20 kV; He-Cd LIF detection.

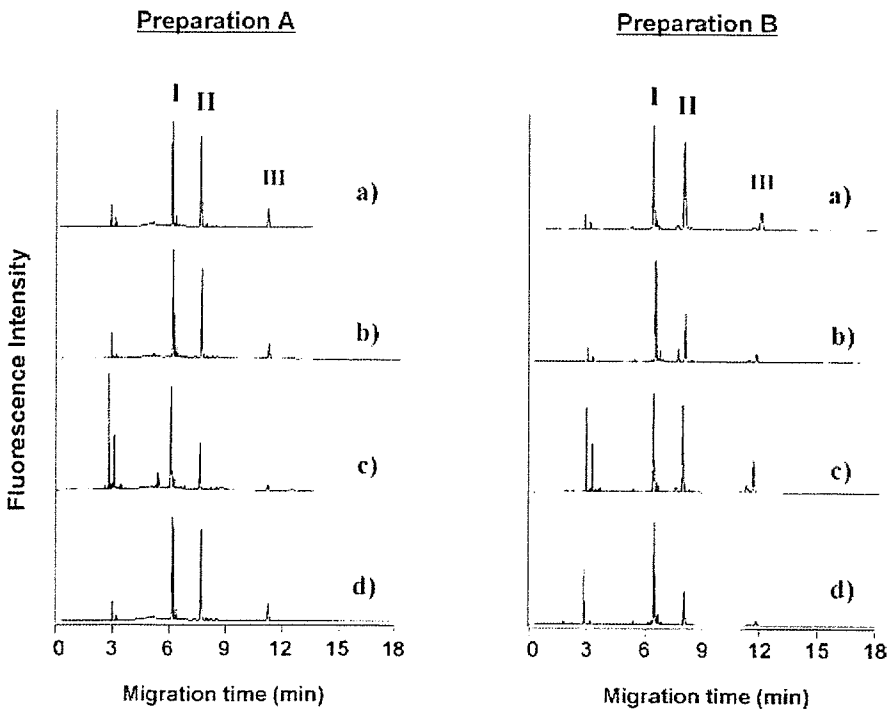

Figure 9. Analysis of 2AA-labeled *N*-linked oligosaccharides of different lot preparations. Analytical conditions were the same as in Fig. 8. Structures of peaks I–III are also shown in Fig. 8.

aration A, the relative corrected peak areas of each peak (I, II, and III) were 58.9 ± 8.5%, 34.6 ± 6.7%, and 6.4 ± 1.9%, respectively. The RSD values of peaks I, II, and III were 14.4%, 19.4%, and 29.3%, respectively. In contrast, in the analysis of preparation B, the relative corrected peak areas of each peak (I, II, and III) were 60.6 ± 14.4%, 31.0 ± 9.7%, and 8.4 ± 5.9%, respectively. The RSD values of peaks I, II, and III were 23.8%, 31.2%, and 70.6%, respectively. In a previous study, Kamoda *et al.* [20] reported variation of oligosaccharide compositions in different lots of antibody pharmaceuticals, and showed that the RSD values of peak areas

were below 28.7%. As shown in this report, oligosaccharide compositions in different lots of glycoprotein pharmaceuticals were significantly different. In particular, preparation B showed critical RSD values. Because UTIs contain highly acidic ChS chains in their molecules, the acidity of the preparations may cause hydrolysis of acid-labile sialic acid residues in *N*-glycans. Accordingly, it should be noted that lot-to-lot variation of oligosaccharide compositions in UTI preparations is an important issue for quality assurance.

4 Concluding remarks

We compared two commercial UTI preparations supplied from different companies by using various analytical methods, and demonstrated that the molecular masses of both preparations showed similar values in analyses using SEC and SDS-PAGE methods which are adopted in JP. However, the observed molecular masses showed significantly higher values than those observed by MALDI-TOF MS.

We examined two electrophoretic methods for the analysis of glycan portions of UTI preparations. Cellulose acetate membrane electrophoresis and MEKC were useful for comparative studies of UTI preparations. In addition, analysis of AMAC-labeled unsaturated disaccharides and 2AA-labeled *N*-linked oligosaccharides by CE was quite useful for comparative studies of UTI preparations.

We demonstrated that the equivalence assessment of glycan portions was important for comparative analysis of UTI preparations, and evaluated various methods including CE. For the assessment of glycoprotein pharmaceuticals such as UTI, a detailed evaluation that focuses on carbohydrate chains should be performed.

5 References

[1] Fukuda, M. N., Sasaki, H., Lopez, L., Fukuda, M., *Blood* 1989, *73*, 84–89.

[2] Donahue, R. E., Wang, E. A., Kaufman, R. J., Foutch, L. *et al.*, *Cold Spring Harbor Symp. Quant. Biol.* 1986, *51*, 685–692.

[3] Kawasaki, N., Haishima, Y., Ohta, M., Itoh, S. *et al.*, *Glycobiology* 2001, *11*, 1043–1049.

[4] Kawasaki, N., Ohta, M., Itoh, S., Hyuga, M. *et al.*, *Biologicals* 2002, *30*, 113–123.

[5] Forno, G., Fogolin, B. M., Oggero, M., Kratje, R. *et al.*, *Eur. J. Biochem.* 2004, *271*, 907–919.

[6] Inoue, N., Watanabe, T., Kutsukake, T., Saitoh, H. *et al.*, *Glycoconj. J.* 1999, *16*, 707–718.

[7] James, D. C., Freedman, R. B., Hoare, M., Ogonah, O. W. *et al.*, *Biotechnology (NY).* 1995, *13*, 592–596.

[8] Tanaka, Y., Maehara, S., Sumi, H., Toki, N. *et al.*, *Biochim. Biophys. Acta* 1982, *705*, 192–199.

[9] Bromke, B. J., Kueppers, F., *Biochem. Med.* 1982, *27*, 56–57.

[10] Tujino, T., Komatsu, Y., Isayama, H., Hirano, K. *et al.*, *Clin. Gastroenterol. Hepatol.* 2005, *3*, 376–383.

[11] Chen, S. Y., Wang, J. Y., *Chin. J. Dig.* 2002, *3*, 70–74.

[12] Hochstrasser, K., Bretzel, G., Feuth, H., Hilla, W. *et al.*, *Hoppe-Seyler's Z. Physiol. Chem.* 1976, *357*, 153–162.

[13] Balduyck, M., Laroui, S., Mizon, C., Mizon, J., *Biol. Chem. Hoppe-Seyler* 1989, *370*, 329–336.

[14] Wachter, E., Hochstrasser, K., *Hoppe-Seyler's Z. Physiol. Chem.* 1981, *362*, 1351–1355.

[15] Hochstrasser, K., Schonberger, O. L., Rossmanith, I., Wachter, E., *Hoppe-Seyler's Z. Physiol. Chem.* 1981, *362*, 1357–1362.

[16] Toyoda, H., Ikei, T., Demachi, Y., Toida, T. *et al.*, *Chem. Pharm. Bull.* 1992, *40*, 2882–2884.

[17] Toyoda, H., Kobayashi, S., Sakamoto, S., Toida, T. *et al.*, *Biol. Pharm. Bull.* 1993, *16*, 945–947.

[18] Selloum, L., Davril, M., Mizon, C., Balduyck, M. *et al.*, *Biol. Chem. Hoppe-Seyler* 1987, *368*, 47–55.

[19] *The Japanese Pharmacopoeia*, 14th Edition, Hirokawa Publishing, Tokyo 2001, pp. 833–835.

[20] Kamoda, S., Nomura, C., Kinoshita, M., Nishiura, S. *et al.*, *J. Chromatogr. A* 2004, *1050*, 211–216.

[21] Matsuno, Y., Kinoshita, M., Kakehi, K., *J. Pharm. Biomed. Anal.* 2004, *36*, 9–15.

[22] Nakano, M., Kakehi, K., Tsai, M. H., Lee, Y. C., *Glycobiology* 2004, *14*, 431–441.

[23] Kodama, C., Kodama, T., Yosizawa, Z., *J. Chromatogr.* 1988, *429*, 293–313.

[24] Piraud, M., Boyer, S., Mathieu, M., Maire, I., *Clin. Chim. Acta.* 1993, *221*, 171–181.

[25] Sei, K., Nakano, M., Kinoshita, M., Masuko, T. *et al.*, *J. Chromatogr. A* 2002, *958*, 273–281.

[26] Kakehi, K., Kinoshita, M., Kawakami, D., Tanaka, J. *et al.*, *Anal. Chem.* 2001, *73*, 2640–2647.

[27] Kinoshita, M., Murakami, E., Oda, Y., Funakubo, T. *et al.*, *J. Chromatogr. A* 2000, *866*, 261–271.

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JOURNAL OF CHROMATOGRAPHY A

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Capillary electrophoresis with laser-induced fluorescence detection for detailed studies on *N*-linked oligosaccharide profile of therapeutic recombinant monoclonal antibodies

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Received 28 May 2006; received in revised form 5 August 2006; accepted 11 August 2006
Available online 1 September 2006

Abstract

Total *N*-linked oligosaccharide profiling method for recombinant monoclonal antibody (rmAb) using capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) and an approach for detailed structural analysis of *N*-linked oligosaccharide were developed. A CE-LIF method using 2-aminobenzoic acid (2-AA) as a fluorogenic reagent allowed sensitive detection of several minor peaks besides typical asialo-biantennary complex type oligosaccharides in the analysis of *N*-linked oligosaccharide from a commercial rmAb pharmaceutical, rituximab. These minor peaks were successfully assigned as sialo-biantennary complex type and high-mannose type oligosaccharides by comparison with the migration times of 2-AA derivatized oligosaccharides which were separately fractionated and determined by high-performance liquid chromatography (HPLC) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). In development of biopharmaceuticals, it is important to evaluate these minor oligosaccharides, because some of these minor glycans are likely to influence immunogenicity and clearance rate *in vivo*. The repetitive analysis using CE-LIF showed excellent precision in relative corrected peak areas. These results demonstrate that the present CE-LIF method is applicable for both structural characterization and quantitative profiling of *N*-linked oligosaccharides derived from rmAb pharmaceuticals. The present method will be a powerful tool for rapid, quantitative and exhaustive evaluation of *N*-linked oligosaccharides in various stages of rmAb pharmaceutical development such as clone selection, bioprocess control, and routine lot release testing to ensure product efficacy and consistency.

Keywords: *N*-linked oligosaccharide; Monoclonal antibody; Capillary electrophoresis; 2-Aminobenzoic acid; High-mannose type; MALDI-TOF-MS

1. Introduction

Glycosylation is one of the most common modifications of proteins, and more than 50% of proteins are glycosylated [1]. Carbohydrate moieties of such proteins are involved in expression of cellular functions including recognition, cell-to-cell signaling [2], protein folding, canceration [3], immune response, fertilization [4] and differentiation.

For recombinant monoclonal antibody (rmAb) pharmaceuticals, which contain a conserved *N*-glycosylation site in the CH_2 region of heavy chain [5], glycosylation is known to influence the biological, pharmacological and physicochemical properties of IgGs [6,7]. The biological functions affected by the oligosaccharides of mAb include resistance to proteases, binding to monocyte Fc receptors, interaction with complement component C1q, and circulatory half-life *in vivo* [8–11]. Furthermore, specific changes in oligosaccharide structure affect biological function such as antibody-dependent cellular cytotoxity (ADCC). Presence of bisecting *N*-acetylglucosamine (GlcNAc) has been reported to improve ADCC [12,13]. Recent reports showed that the absence of core α1–6 linked fucose (Fuc) residue caused more obvious enhancement of ADCC activity [14,15]. The oligosaccharides of mAb have microheterogeneity, and their profile is often altered even under a defined set of culture and purification protocol. Therefore, it is quite important to assess detailed oligosaccharide profile for the development and manufacturing of rmAb pharmaceuticals.

Various methods for the analysis of protein modification with carbohydrates have been developed such as using high-pH anion-exchange chromatography with pulsed amperometric

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doi:10.1016/j.chroma.2006.08.028

detection (HPAEC-PAD) [16,17] and high-performance liquid chromatography (HPLC) after derivatization with fluorogenic reagents [18–23]. These chromatographic techniques enable 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 [24–29], and some groups investigated the quantitative analysis or structural studies on the oligosaccharides of mAb by CE-LIF using 8-aminopyrene-1,3,6-trisulfonate (APTS) as a fluorogenic reagent [25,27]. In these reports, major oligosaccharides of rmAb such as asialo-*N*-linked complex biantennary structure with 0, 1 and 2 galactose (Gal) residue(s) were studied.

Recently, we also developed an analytical method for *N*-linked oligosaccharide profiling of rmAb pharmaceuticals using a derivatization reagent, 3-aminobenzoic acid [30]. During this study, we observed many minor peaks in the earlier migration times than those for typical asialo biantennary oligosaccharides when *N*-linked oligosaccharide of rituximab and trastuzumab were analyzed, although the structures of these minor peaks had not been assigned. In the biopharmaceutical development, it is important to evaluate the minor oligosaccharides because some of them affect such as immunogenicity and clearance rate *in vivo* [31–33].

In the present study, we developed a CE-LIF method for profiling *N*-linked oligosaccharides from rmAb pharmaceuticals using 2-aminobenzoic acid (2-AA), which has been widely used for the analysis of *N*-linked oligosaccharides including mass spectrometry, HPLC and CE. By combination with HPLC and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), we could assign all minor peaks observed in the analysis of *N*-linked oligosaccharides derived from rituximab, a chimeric IgG1-type mAb pharmaceutical which is widely used for the treatment for non-Hodgkin's lymphoma.

We found that rituximab and other two commercial IgG-type rmAb pharmaceuticals commonly contain sialo-oligosaccharides and high-mannose type oligosaccharides as minor component. We demonstrate that the present CE-LIF method is a powerful tool for exhaustive characterization of *N*-linked oligosaccharides derived from rmAb pharmaceuticals.

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 obtained from Nacalai tesque (Nakagyo-ku, Kyoto, Japan). α-Mannosidase (Jack bean) was obtained from Oxford Glycosystems (Bedford, MA, USA). A kit for capillary electrophoresis of sodium dodecyl sulfate (SDS)-protein complexes using a nongel sieving matrix (CE-SDS), SDS-Gel MW Analysis Kit, was purchased from Beckman Coulter (Fullerton, CA, USA), which includes SDS sample buffer and SDS-gel buffer. 2-Aminobenzoic acid (2-AA) was obtained from Wako (Dosho-machi, Osaka, Japan). Sodium cyanoborohydride was obtained from Aldrich (Milwaukee, WI, USA). Polyethylene glycol (MW; 35000, PEG35000) was purchased from Fluka (Buchs, Switzerland). A pharmaceutical preparation of rmAb, rituximab, trastuzumab, and palivizumab, was kindly donated from Ms. Nishiura of Kinki University Nara Hospital. The solution of rmAb 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 rmAb (0.5 mg) was dissolved in 49 μl of 20 mM phosphate buffer (pH 7.0) in a sample tube (1.5 ml). 2-Mercaptoethanol (1 μl) and PNGase F (10 units, 10 μl) were added to the mixture, and incubated at 37 °C for 20–24 h. After addition of ethanol (150 μl), the mixture was centrifuged at 15,000 × *g* for 15 min. The supernatant containing the released oligosaccharides was transferred to a new sample tube and evaporated to dryness.

2.3. CE-SDS analysis of reduced mAb

The efficiency of *N*-linked oligosaccharide digestion with PNGase F was monitored by CE-SDS under reducing condition using SDS-Gel MW Analysis Kit (Beckman Coulter). A portion of digested mAb sample with PNGase F (80 μg in 10 μl) was mixed with SDS sample buffer (85 μl) and 2-mercaptoetanol (5 μl) and the mixture was incubated at 65 °C for 10 min. CE analysis was performed using a fused-silica capillary (50 μm I.D., 20 cm effective length, 30 cm total length, Beckman Coulter) in SDS-gel buffer. Prior to sample injection, the capillary was rinsed with 0.1 M NaOH, 0.1 M HCl, water, and SDS-gel buffer for 3 min, 1 min, 1 min and 10 min, respectively. Then samples were injected electrophoretically for 20 s at −5 kV. Separation was conducted in the negative polarity mode (−15 kV) at 25 °C for 30 min. UV detection was conducted at 220 nm.

2.4. Fluorescent derivatization of oligosaccharides with 2-AA

N-linked oligosaccharides in the mixture were labeled with 2-aminobenzoic acid (2-AA) according to the method reported previously [20,21,23]. Briefly, water (20 μl) was added to a dried oligosaccharide sample. A derivatization reagent was freshly prepared by dissolution of 2-AA and sodium cyanoborohydride (30 mg and 20 mg, respectively) in methanol (1 ml) containing 4% sodium acetate and 2% boric acid, and the reagent (100 μl) was added to the oligosaccharide solution. The mixture was kept at 80 °C for 1 h. After cooling followed by addi-

tion of water (30 μl), the oligosaccharide mixture was purified using a solid-phase extraction column (Oasis HLB cartridges, 1 ml, Waters, Milford, MA) [20]. The reaction 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), the fluorescent labeled oligosaccharides were eluted with acetonitrile–water (20:80, 1 ml) and the eluate was evaporated to dryness by a centrifugal evaporator. The residue was dissolved in water (100 μl), and a portion (typically 5 μl) was used for the analysis by CE-LIF. For collecting peaks by HPLC analysis, 50 μl of a sample solution was used.

2.5. Enzymatic digestion of 2-AA labeled oligosaccharides

For sialidase digestion, neuraminidase (1 munit, 1 μl) was added to an aqueous solution of 2-AA labeled oligosaccharides (2 μl) prepared as described above, and the mixture was incubated at 37 °C for 16 h. The reaction mixture was boiled for 5 min, and centrifuged at 15,000 × *g* for 15 min, and supernatant was used for CE analysis. For α-mannosidase digestion, α-mannosidase (1 munit, 1 μl) was added to an aqueous solution of 2-AA labeled oligosaccharides (2 μl), and incubated at 37 °C for 16 h. The reaction mixture was boiled for 5 min and used for CE analysis in the same manner.

2.6. Capillary electrophoresis of 2-AA labeled oligosaccharides

Capillary electrophoresis was performed on a ProteomeLab PA800 system (Beckman Coulter) equipped with a helium-cadmium laser induced fluorescence detector (ex. 325 nm, em. 405 nm) using a DB-1 capillary (100 μm I.D., 30 cm effective length, 40 cm total length, Agilent/J&D scientific, Palo Alto, CA) in 100 mM Tris-borate buffer (pH 8.3) containing 10% PEG35000 as the running buffer. PEG is added to diminish electroendoosmotic flow and improve the resolution. For pressure injection, sample solutions were introduced to the capillary at 1 psi for 10 s. Separation was performed by applying 25 kV at 25 °C at reverse polarity.

2.7. Fractionation of N-linked oligosaccharide by HPLC

HPLC was performed with a HITACHI apparatus equipped with two L-7100 pumps and a HITACHI F-7100 fluorescence detector (ex. 350 nm, em. 425 nm). Separation was done with a polymer-based Asahi Shodex NH2P-50 4E column (Showa Denko, Tokyo, 250 mm × 4.6 mm) using a linear gradient formed by 2% acetic acid in acetonitrile (solvent A) and 5% acetic acid in water containing 3% triethylamine (solvent B) at 1 ml/min. The column was initially equilibrated and eluted with 70% solvent A for 2 min, at which point solvent B was increased to 95% over 80 min and kept at this composition for additional 15 min. The peaks observed were collected into 1.5 ml sample tubes and eluate was evaporated to dryness.

2.8. Structural determination by MALDI-TOF-MS

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) of the labeled oligosaccharides was performed on an AutoflexII (Bruker daltonics, Bremen, Germany). A nitrogen laser was used to irradiate samples at 337 nm, and an average of 50 shots was taken. The instrument was operated in reflector mode using negative polarity. The parameters were: ion source 1, 19.0 kV; ion source 2, 16.95 kV; lens voltage, 8.75 kV; pulsed ion extraction, 130 ns; matrix suppression, 400 Da. An aqueous solution of oligosaccharide (ca. 10 pmol, 0.5 μl) was applied to a standard steel target (Bruker daltonics), to which was added a solution (0.5 μl) of 2,5-dihydroxybenzoic acid (DHB, 10 mg/ml) in a mixture of methanol–water (1:1). The mixture was dried in atmosphere by keeping it at room temperature for several minutes. Observed ion peaks were calibrated using a mixture of 2-AA labeled dextran oligomers as mass markers.

3. Results and discussion

3.1. N-linked oligosaccharide profiling of rituximab by CE-LIF

N-linked oligosaccharides of rituximab were released from core protein by digestion with PNGase F. We monitored the digestion efficiency by CE-SDS method under reducing condition (Fig. 1).

The peak of glycosylated form of heavy chain (HC) was fully shifted to earlier migration time (non-glycosylated form of heavy chain, NGHC) after PNGase F digestion. The result indicates that complete digestion was achieved in the present enzyme reaction.

Although a number of methods have been reported for fluorescent labeling of oligosaccharides, we selected 2-aminobenzoic acid as the labeling reagent due to its high sensi-

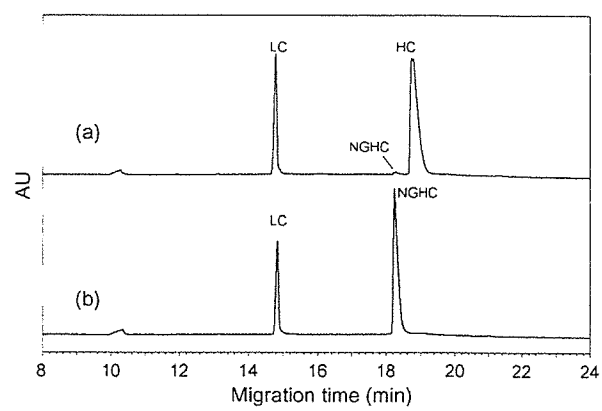

Fig. 1. CE-SDS analysis of reduced preparation of rituximab. LC, light chain; HC, glycosylated form of heavy chain; NGHC, non-glycosylated form of heavy chain. Analytical conditions: buffer, SDS-gel buffer (Beckman Coulter); capillary, fused-silica capillary (50 μm i.d., 30 cm, 20 cm effective length); injection, 20 s at −5 kV; applied potential, −15 kV; temperature, 25 °C; detection, UV at 220 nm. (a) Rituximab before treatment with PNGase F, (b) rituximab after treatment with PNGase F.

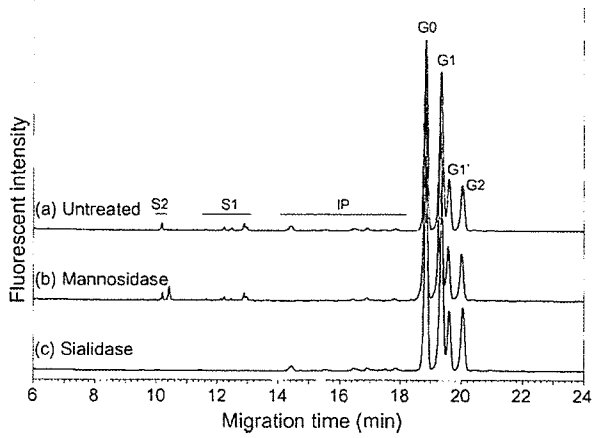

Fig. 2. Analysis of 2-AA labeled *N*-linked oligosaccharides derived from rituximab. 100 mM Tris-borate buffer (pH 8.3) containing 10% polyethylene glycol (MW: 35,000) was used as the separation buffer with a DB-1 capillary (100 μm i.d., 40 cm, 30 cm effective length). Applied potential, −25 kV at 25 °C, fluorescent detection at 325 nm excitation with He-Cd laser with a 405 nm emission filter, pressure injection method at 1 psi for 10 s. (a) The oligosaccharides were released with PNGase F. (b) The oligosaccharides (a) was digested with sialidase. (c) The oligosaccharides (a) was digested with α-mannosidase.

tivity and excellent resolution in both HPLC and CE, and also high sensitivity in MALDI-TOF-MS analysis.

Typical electropherogram of *N*-linked oligosaccharides labeled with 2-AA derived from rituximab is shown in Fig. 2a.

Four major peaks (G0, G1, G1′ and G2) are due to typical oligosaccharides of mAb. These oligosaccharides have asialo-, biantennary and core-fucosylated complex type structures (see Table 1). G0, G1/G1′ and G2 refer to agalacto-, mono- and di-galactosylated structures, respectively. The isomeric oligosaccharides, G1 and G1′, having one galactose (Gal) residue attached to either of the branches, were well resolved. Our previous study showed these two peaks could be resolved completely when using longer capillary (70 cm effective length) although longer analysis time was required (about 90 min) [30]. However, the present conditions achieved sufficient resolution for profiling and characterization of oligosaccharides from mAb within 30 min. The characterization of major four peaks was well studied previously [25,30]. We also observed many minor peaks in faster migration regions (S2, S1 and IP). Most peaks at IP region decreased or disappeared after α-mannosidase treatment (Fig. 2b). In contrast, peaks in S2 and S1 regions completely disappeared after sialidase treatment (Fig. 2c). These results clearly indicate that small amount of high-mannose type and sialylated oligosaccharides are present in rituximab and the present method conveniently allows monitoring these oligosaccharides.

3.2. HPLC fractionation and MALDI-TOF-MS analysis of 2-AA oligosaccharides derived from rituximab

The sample of 2-AA labeled oligosaccharide mixture from rituximab was separated by normal phase-anion exchange HPLC using polymer-based amino-stationary phase (Fig. 3).

The observed peaks (**a–m**) were collected and used for MALDI-TOF-MS analysis (Fig. 4).

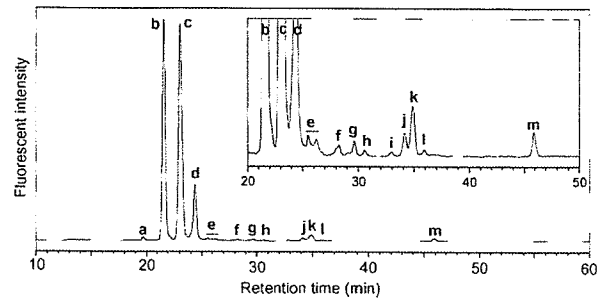

Fig. 3. Fractionation of 2-AA labeled oligosaccharides derived from rituximab by HPLC. Analytical conditions: column, Asahi Shodex NH2P-50 4E (250 mm × 4.6 mm); eluent, solvent A, 2% CH_3COOH in acetonitrile; solvent B, 5% CH_3COOH-3% triethylamine in water; gradient condition: a linear gradient (30–95% solvent B) from 2 to 82 min, maintained for 15 min, detection, ex. 350 nm; em. 425 nm. Peaks **a–m** were collected and applied to MALDI-TOF-MS analysis and CE analysis.

The list of the oligosaccharides is shown in Table 1.

Peak **a** showed a molecular ion (*m/z* 1379.5) of complex type, agalactosylated biantennary oligosaccharide with one GlcNAc residue at the non-reducing terminal (GN1G0). Peak **b** (*m/z* 1582.5) is due to G0, and peak **c** (*m/z* 1744.6) is due to G1 and G1′ (data not shown). These are major oligosaccharides reported in mAb. Peak **d** showed a molecular ion at *m/z* 1354.3 which corresponds to that of the high-mannose type oligosaccharide (M5 in Table 1) as well as the molecular ion of typical G2 structure (*m/z* 1906.1). Peaks **e–h** (*m/z* 1516.2, 1678.3, 1840.3 and 2002.1, respectively) are also due to high-mannose type oligosaccharides (M6–M9). Peaks **i–m** were eluted in the region of sialylated oligosaccharides [20,21,23]. Peaks **i–k** showed molecular ions at *m/z* 1832.2, 2035.3 and 2197.2, respectively, and are monosialo-type biantennary oligosaccharides as shown in GN1G1S1, G1S1/G1′S1 and G2S1 (Table 1). Peak **l** is speculated to be a sialo-hybrid type (Table 1, HybridS1) based on its molecular mass (*m/z* 2156.1) although further structural analysis will be needed for confirmation. Several sialo-hybrid type oligosaccharides were also observed in a recombinant glycoprotein produced by Chinese hamster ovary (CHO) cell line [34]. Finally, peak **m** showed a molecular ion (*m/z* 2488.21) of disialylated form of G2S2 (Table 1).

3.3. Peak assignment in CE for profiling oligosaccharides of rituximab

In order to assign the peaks observed in Fig. 2, the oligosaccharides confirmed by HPLC and MALDI-TOF-MS as described above were analyzed by CE and compared with the total oligosaccharide profile of rituximab (Figs. 5 and 6). The results suggest that the fastest migrated peak was disialylated-biantennary structure (G2S2), and then monosialylated oligosaccharides were observed (Fig. 5).

In monosialylated groups, the oligosaccharides having lower molecular masses (i.e. higher charge to mass ratio) were observed earlier. It should be noticed that fraction **j** which is eluted as a single peak in HPLC, was resolved into two peaks by CE. These two peaks in CE were confirmed by sialidase diges-

Table 1
List of the 2-AA labeled oligosaccharides from rituximab

Peak	Observed mass (theoretical mass[a])	Structure[b]	Abbreviation
a	1379.5 (1379.5)	GlcNAc β1→2 {Man α1 ↘6, Man α1 ↗3} Man β1→4GlcNAc β1→4GlcNAc; Fuc α1↘6	GN1G0
b	1582.5 (1582.6)	GlcNAc β1→2Man α1 ↘6, GlcNAc β1→2Man α1 ↗3 Man β1→4GlcNAc β1→4GlcNAc; Fuc α1↘6	G0
c	1744.6 (1744.6)	Gal β1→4GlcNAc β1→2Man α1 ↘6, GlcNAc β1→2Man α1 ↗3 Man β1→4GlcNAc β1→4GlcNAc; Fuc α1↘6	G1
		GlcNAc β1→2Man α1 ↘6, Gal β1→4GlcNAc β1→2Man α1 ↗3 Man β1→4GlcNAc β1→4GlcNAc; Fuc α1↘6	G1′
d	1906.1 (1906.7)	Gal β1→4GlcNAc β1→2Man α1 ↘6, Gal β1→4GlcNAc β1→2Man α1 ↗3 Man β1→4GlcNAc β1→4GlcNAc; Fuc α1↘6	G2
	1354.3 (1354.5)	Man α1 ↘6, Man α1 ↗3 Man α1 ↘6, Man α1 ↗3 Man β1→4GlcNAc β1→4GlcNAc	Man5
e	1516.2 (1516.5)	Man α1 ↘6, Man α1 ↗3 Man α1 ↘6, Man α1 →3Man α1 ↗3 Man β1→4GlcNAc β1→4GlcNAc	Man6
		Man α1 ↘6, Man α1 →2Man α1 ↗3 Man α1 ↘6, Man α1 ↗3 Man β1→4GlcN Acβ1→4GlcNAc	
f	1678.3 (1678.6)	Man α1 →2 {Man α1 ↘6, Man α1 ↗3 Man α1 ↘6, Man α1 →3Man α1 ↗3} Man β1→4GlcNAc β1→4GlcNAc	Man7
g	1840.3 (1840.6)	Man α1 →2, Man α1 →2 {Man α1 ↘6, Man α1 ↗3 Man α1 ↘6, Man α1 →3Man α1 ↗3} Man β1→4GlcNAc β1→4GlcNAc	Man8
h	2002.1 (2002.7)	Man α1 →2Man α1 ↘6, Man α1 →2Man α1 ↗3 Man α1 ↘6, Man α1 →2Man α1→3Man α1 ↗3 Man β1→4GlcNAc β1→4GlcNAc	Man9
i	1832.2 (1832.7)	NeuAc α2→3Gal β1→4GlcNAc β1→2 {Man α1 ↘6, Man α1 ↗3} Man β1→4GlcNAc β1→4GlcNAc; Fuc α1↘6	GN1G1S1
j	2035.3 (2035.7)	NeuAc α2→3Gal β1→4GlcNAc β1→2Man α1↘6, GlcNAc β1→2Man α1↗3 Man β1→4GlcNAc β1→4GlcNAc; Fuc α1↘6	G1S1
		GlcNAc β1→2Man α1↘6, NeuAc α2→3Gal β1→4GlcNAc β1→2Man α1↗3 Man β1→4GlcNAc β1→4GlcNAc; Fuc α1↘6	G1′S1

Table 1 (*Continued*)

Peak	Observed mass (theoretical mass[a])	Structure[b]	Abbreviation
k	2197.2 (2197.8)	NeuAc α2→3Gal β1→4GlcNAc β1→2Man α1↘6 / Gal β1→4GlcNAc β1→2Man α1↗3 Man β1→4GlcNAc β1→4GlcNAc (Fuc α1↘6) / Gal β1→4GlcNAc β1→2Man α1↘6 / NeuAc α2→3Gal β1→4GlcNAc β1→2Man α1↗3 Man β1→4GlcNAc β1→4GlcNAc (Fuc α1↘6)	G2S1
l	2156.1 (2156.8)	Man α1↘6 / Man α1↗3 Man α1↘6 / NeuAc α2→3Gal β1→4GlcNAc β1→2Man α1↗3 Man β1→4GlcNAc β1→4GlcNAc (Fuc α1↘6)	HybridS1
m	2488.2 (2488.9)	NeuAc α2→3Gal β1→4GlcNAc β1→2Man α1↘6 / NeuAc α2→3Gal β1→4GlcNAc β1→2Man α1↗3 Man β1→4GlcNAc β1→4GlcNAc (Fuc α1↘6)	G2S2

[a] Theoretical masses are of 2-AA labeled oligosaccharides, calculated as monoisotopic mass of $[M-H]^-$.
[b] Linkage positions are tentatively assigned. All structures are derivatized with 2-AA at the reducing ends.

tion. After sialidase digestion of fraction **j**, the bigger left peak moved to G1, smaller right peak moved to G1′ (data not shown). From these data, we could assign the peaks due to G1S1 and G1′S1 (Table 1) by CE.

After sialylated species were observed, high-mannose type oligosaccharides (M5 to M9) were migrated in this order (Fig. 6).

GN1G0 was also included in this region. The IgG molecules having these high-mannose type oligosaccharides are assumed to be secreted from cultured cells after incomplete processing of biosynthesis as reported previously [35]. In the biosynthetic pathway of *N*-linked oligosaccharides in mammalian cells, initial form of glycosylation in protein is $Glc_3Man_9GlcNAc_2$-Asn. The oligosaccharide is trimmed to $Man_9GlcNAc_2$ (M9) by glucosidase I and II, and then further trimmed by mannosidase from $Man_8GlcNAc_2$ (M8) to yield $Man_5GlcNAc_2$ (M5). Then, GlcNAc residue is transferred to mannose of trimannosyl core by GlcNAc-transferase I, resulting in hybrid type oligosaccharide. After continuous digestion of two mannose residues, a Fuc residue is transferred to core GlcNAc, yielding a complex

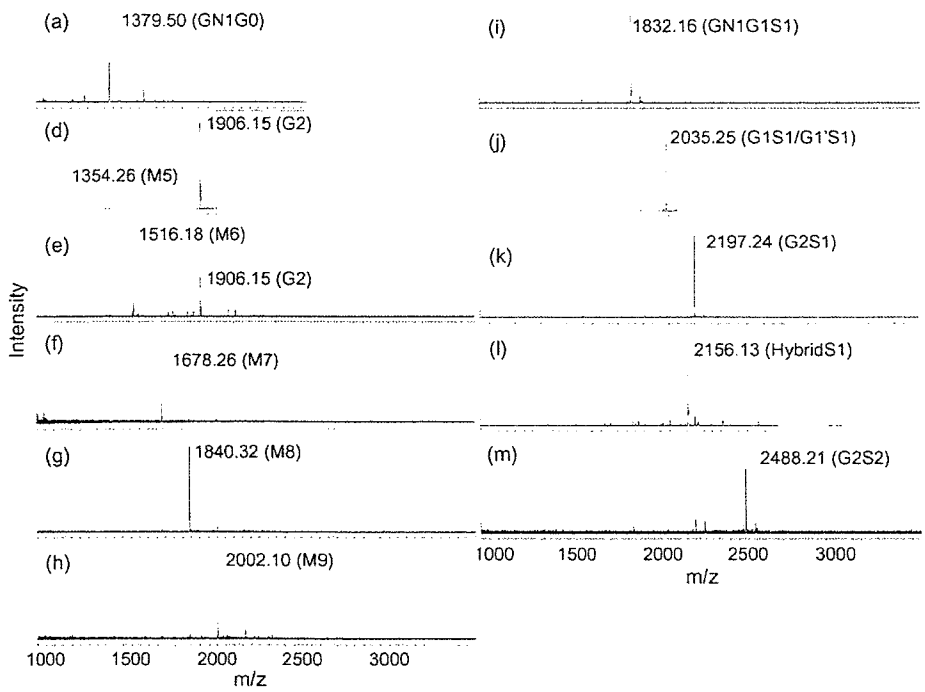

Fig. 4. MALDI-TOF mass spectra of 2-AA labeled oligosaccharides from rituximab. Peaks **a**, **d**–**m** are indicated in Fig. 3. Mass spectra were observed in negative/reflector mode using DHB as matrix as described in Section 2. Abbreviation of oligosaccharide structures and observed masses (monoisotopic mass) are also shown. The list of oligosaccharides is shown in Table 1.

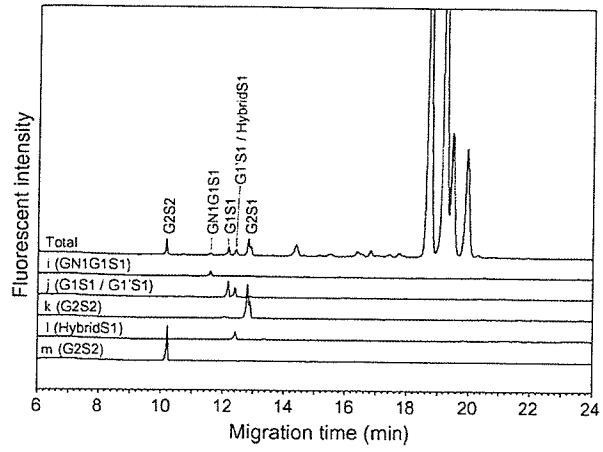

Fig. 5. Peak assignment of sialic acid-containing oligosaccharides of rituximab. Total mixture of oligosaccharides from rituximab was analyzed by CE-LIF (total). The same sample was separated by HPLC (Fig. 2, **a**, **d–i**) and the collected peaks determined by MALDI-TOF-MS (Fig. 4) were analyzed by CE. Assigned structures are shown on the peaks as abbreviations (Table 1). The analytical conditions of CE were described in Fig. 2.

type GN1G0. Further processing by a series of glycosyltransferases occurs in the Golgi compartments, and finally completely processed complex type oligosaccharides are synthesized [36]. However, it is accepted that individual glycosylation reaction does not always proceed to completion, leading to the secretion of incompletely processed high-mannose type, hybrid type oligosaccharides as minor oligosaccharides [34,35,37]. These observations support that high-mannose type and hybrid type oligosaccharides were detected as minor oligosaccharide species in the case of rmAb produced by CHO cell line.

After these incompletely processed-species (IP), major oligosaccharides (G0, G1, G1′ and G2) which are previously reported as typical oligosaccharides in rmAb, were observed from 18.2 to 20 min.

The results indicate that the *N*-linked oligosaccharides of mAb show high heterogeneity in minor oligosaccharides and the

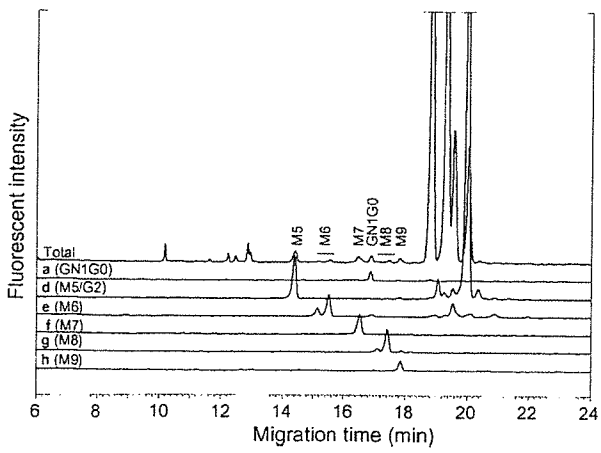

Fig. 6. Peak assignment of asialo- and high-mannose type oligosaccharides of rituximab. Sialic acid-containing oligosaccharides were assigned in the same manner as described in Fig. 5.

Table 2
Precision in oligosaccharide profiling of rituximab

Preparation	Corrected area (%)							
	S2	S1	M5-M9	GN1G0	G0	G1	G1′	G2
1	0.9	2.7	4.0	0.6	38.0	34.2	10.1	9.6
2	1.0	2.6	4.1	0.6	38.2	34.2	10.0	9.4
3	0.9	3.0	3.9	0.6	37.9	34.2	10.1	9.4
Average	0.9	2.8	4.0	0.6	38.0	34.2	10.1	9.5
SD	0.1	0.2	0.1	0.0	0.2	0.0	0.1	0.1
RSD (%)	6.2	7.5	2.5	0.0	0.4	0.0	0.6	1.2

Three preparations of 2-AA labeled *N*-linked oligosaccharides from rituximab were analyzed by CE-LIF.

present CE-LIF method for profiling of *N*-linked oligosaccharide using 2-AA labeling has high resolving ability to separate these oligosaccharides. In contrast, HPLC method using amino-column requires much longer time, and cannot separate of M5 and G2.

3.4. Quantitative evaluation and analysis of other rmAb pharmaceuticals

We evaluated the precision reproducibility of relative distribution of oligosaccharides including S2, S1, high-mannose type oligosaccharides using the present technique by repeated analysis of rituximab. Three preparations of 2-AA labeled oligosaccharides from a sample of rituximab were analyzed on CE-LIF. The result is summarized in Table 2.

Good reproducibility with the RSD of relative corrected peak areas of less than 7.5% (SD were 0.2% or below) was shown even in the analysis of minor oligosaccharides such as S2 and S1. As for major oligosaccharides (G0, G1, G1′ and G2), the RSD showed excellent values, and were 0.4, 0.0, 0.6 and 1.2%, respectively.

Based on the results on precision analysis, we applied the present method to the *N*-linked oligosaccharide profiling of some rmAb pharmaceuticals, rituximab, trastuzumab and palivizumab (Fig. 7).

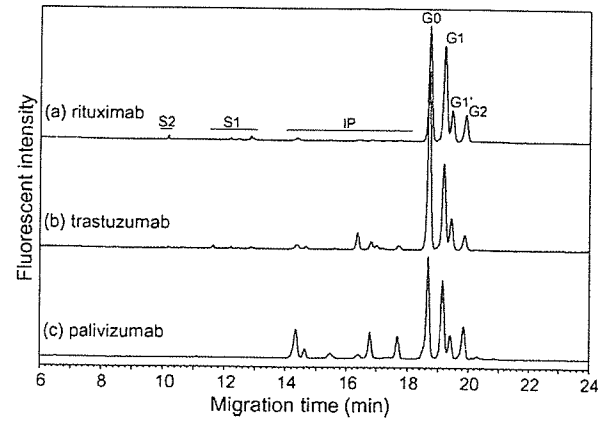

Fig. 7. Comparison of *N*-linked oligosaccharide profile from some rmAb pharmaceuticals by CE-LIF after 2-AA derivatization. The conditions of CE-LIF analysis were as in Fig. 2.

All three preparations included G0, G1, G1′ and G2 as major oligosaccharides. However, the profiles of minor oligosaccharides such as sialyl oligosaccharides (S2 and S1) and IP are specific to each preparation. In trastuzumab (Fig. 7b), S2 and S1 peaks are quite small but IP peaks are more abundant than those of rituximab. The data observed in these two rmAb preparations are well comparable with lot-analysis data reported previously [30]. Palivizumab showed characteristic profiles, and large amount of IP was observed. In contrast, there were no peaks in the region of S2 and S1.

The present method can monitor the processing of oligosaccharides in rmAb samples. For example, rituximab contains minute amount of high-mannose type oligosaccharides, indicating processing is nearly complete but palivizumab contains a large amount of IP. Difference of the amount of sialyl- and high-mannose type oligosaccharides in rmAb possibly affects clearance or biological activity of the pharmaceutical preparations [31–33]. Moreover, high-mannose structures are likely to influence the effector functions of the antibody and may alter the structure of the Fc region [38]. It has been demonstrated that the proportion of high-mannose type oligosaccharides increase during cell culture [39]. Cell culture under low glutamine or glucose concentration leads to increase hybrid type and high-mannose type oligosaccharides [34]. Therefore, it is important to evaluate these minor oligosaccharides for pharmaceutical development of rmAb. The present method will be a powerful tool to evaluate total *N*-linked oligosaccharides of rmAb pharmaceuticals for such purpose.

4. Conclusion

In this study, we developed CE-LIF method for high-resolution analysis of *N*-linked oligosaccharides from mAb pharmaceuticals, and assigned all peaks in the electropherogram using a combination of HPLC and MALDI-TOF-MS. In the model study using rituximab, we demonstrated that the present CE-LIF method could separate total *N*-linked oligosaccharides not only typical asialo-biantennary complex type but also minor sialo-complex type, high-mannose type and hybrid type oligosaccharides with high resolution and high reproducibility. Our results show that the present method is quite useful for rapid, quantitative and exhaustive evaluation of *N*-linked oligosaccharide profile of mAb pharmaceuticals in various development stages such as, for example, clone selection and bioprocess control, detailed characterization for approval application and lot release testing.

References

[1] A. Varki, R. Cummings, J. Esko, H. Freeze, G. Hart, J. Marth, Essentials of Glycobiology, Cold Spring Harbor Laboratory Press, New York, 1999.
[2] T. Muramatsu, Glycobiology 3 (1993) 291.
[3] M. Fukuda, Cancer Res. 56 (1996) 2237.
[4] J. Zara, R.K. Naz, Front. Biosci. 3 (1998) 1028.
[5] B.J. Sutton, D.C. Phillips, Biochem. Soc. Trans. 11 (1983) 130.
[6] T. Mizouchi, T. Taniguchi, A. Shimizu, A. Kobata, J. Immunol. 129 (1982) 2016.
[7] S. Fujii, T. Nishiura, A. Nishikawa, R. Miura, N. Taniguchi, J. Biol. Chem. 265 (1990) 6009.
[8] R.J. Ratherbarrow, N. Richardson, A. Feinstein, Mol. Immunol. 22 (1985) 407.
[9] M.H. Tao, S.L. Morrison, J. Immunol. 143 (1989) 2595.
[10] M.R. Walker, J. Lund, K.M. Thomsom, R. Jefferis, Biochem. J. 259 (1989) 347.
[11] K.A. Leader, B.M. Kumpel, A.G. Hadley, B.A. Bradley, Immunology 72 (1991) 481.
[12] P. Umana, J. Jean-Mairet, R. Moudry, H. Amstutz, J.E. Bailey, Nat. Biotechnol. 17 (1999) 176.
[13] J. Davies, L. Jiang, L.Z. Pan, M.J. LaBarre, D. Anderson, M. Reff, Biotechnol. Bioeng. 4 (2001) 288.
[14] R.L. Shields, J. Lai, R. Keck, L.Y. O'Connell, K. Hong, Y.G. Meng, S.H. Weikert, L.G. Presta, J. Biol. Chem. 277 (2002) 26733.
[15] T. Shinkawa, K. Nakamura, N. Yamane, E. Shoji-Hosaka, Y. Kanda, M. Sakurada, K. Uchida, H. Anazawa, M. Satoh, M. Yamasaki, N. Hanai, K. Shitara, J. Biol. Chem. 278 (2003) 3466.
[16] M.R. Hardy, R.R. Townsend, Y.C. Lee, Methods Enzymol. 179 (1989) 65.
[17] M. Spellman, Anal. Chem. 62 (1990) 1714.
[18] K.R. Anumula, Anal. Biochem. 220 (1994) 275.
[19] J.C. Bigge, T.P. Patel, J.A. Bruce, P.N. Goulding, S.M. Charles, B. Parekh, Anal. Biochem. 230 (1995) 229.
[20] K.R. Anumula, S.T. Dhume, Glycobiology 8 (1998) 685.
[21] M. Nakano, K. Kakehi, M.H. Tsai, Y.C. Lee, Glycobiology 14 (2004) 431.
[22] S. Kamoda, M. Nakano, R. Ishikawa, S. Suzuki, K. Kakehi, J. Proteome Res. 4 (2005) 146.
[23] R. Naka, S. Kamoda, A. Ishizuka, M. Kinoshita, K. Kakehi, J. Proteome Res. 5 (2006) 88.
[24] F.T. Chen, R.A. Evangelista, Electrophoresis 19 (1998) 2639.
[25] S. Ma, W. Nashabeh, Anal. Chem. 71 (1999) 5185.
[26] K. Kakehi, T. Funakubo, S. Suzuki, Y. Oda, Y. Kitada, J. Chromatogr. A 863 (1999) 205.
[27] T.S. Raju, Anal. Biochem. 283 (2000) 125.
[28] K. Nakajima, Y. Oda, M. Kinoshita, K. Kakehi, J. Proteome Res. 2 (2003) 81.
[29] S. Ma, W. Lau, R.G. Keck, J.B. Briggs, A.J. Jones, K. Moorhouse, W. Nashabeh, Methods Mol. Biol. 308 (2005) 397.
[30] S. Kamoda, C. Nomura, M. Kinoshita, S. Nishiura, R. Ishikawa, K. Kakehi, N. Kawasaki, T. Hayakawa, J. Chromatogr. A 1050 (2004) 211.
[31] V. Gross, K. Steube, T.-A. Tran-Thi, D. Haussinger, G. Legler, K. Decker, P.C. Heinrich, W. Gerok, Eur. J. Biochem. 162 (1987) 83.
[32] N. Jenkins, E.M. Curling, Enzyme Microb. Technol. 16 (1994) 354.
[33] A. Wright, S.L. Morrison, J. Exp. Med. 180 (1994) 1087.
[34] D.C.F. Wong, K.T.K. Wong, L.T. Goh, C.K. Heng, M.G.S. Yap, Biotechnol. Bioeng. 89 (2005) 164.
[35] M.J. Bailey, A.D. Hooker, C.S. Adams, S. Zhang, D.C. James, J. Chromatogr. B 826 (2005) 177.
[36] R. Kornfeld, S. Kornfeld, Annu. Rev. Biochem. 54 (1985) 631.
[37] A.D. Hooker, N.H. Green, A.J. Baines, A.T. Bull, N. Jenkins, P.G. Strange, D.C. James, Biotechnol. Bioeng. 63 (1999) 560.
[38] R. Jefferis, J. Lund, M. Goodall, Immunol. Lett. 44 (1995) 111.
[39] A.D. Hooker, M.H. Goldman, N.H. Markham, D.C. James, A.P. Ison, A.T. Bull, N. Jenkins, Biotechnol. Bioeng. 48 (1995) 639.

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Analytical Biochemistry 362 (2007) 245–257

ANALYTICAL BIOCHEMISTRY

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Development of an apparatus for rapid release of oligosaccharides at the glycosaminoglycan–protein linkage region in chondroitin sulfate-type proteoglycans

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Received 14 November 2006

Available online 20 December 2006

Abstract

An apparatus, AutoGlycoCutter (AGC), was developed as a tool for rapid release of *O*-linked-type glycans under alkaline conditions. This system allowed rapid release of oligosaccharides at the glycosaminoglycan–protein linkage region in proteoglycans (PGs). After digestion of PGs with chondroitinase ABC, the oligosaccharides at the linkage region were successfully released from the protein core by AGC within 3 min. The reducing ends of the released oligosaccharides were labeled with 2-aminobenzoic acid and analyzed by a combination of capillary electrophoresis (CE) and matrix-assisted laser desorption time-of-flight mass spectrometry. In addition, the unsaturated disaccharides produced by chondroitinase ABC derived from the outer parts of the glycans were labeled with 2-aminoacridone and analyzed by CE to determine the disaccharide compositions. We evaluated AGC as a method for structural analysis of glycosaminoglycans in some chondroitin-sulfate-type PGs (urinary trypsin inhibitor, bovine nasal cartilage PG, bovine aggrecan, bovine decorin, and bovine biglycan). Recoveries of the released oligosaccharides were 57–73% for all PGs tested in the present study. In particular, we emphasize that the use of AGC achieved ca. 1000-fold rapid release of *O*-glycans compared with the conventional method.

Keywords: Proteoglycan; In-line flow system; Capillary electrophoresis; Linkage region

Proteoglycans (PGs)[1] consist of a protein core substituted with glycosaminoglycan (GAG) chain(s) and are ubiquitously found in the extracellular matrix. PGs are associated with the cell surface of eucaryotic cells [1,2] and have been recognized as important molecules in development and connective tissue assembly [3,4]. PGs are also directly implicated in a large variety of human diseases. For instance, the significant change of PG content or structural alterations of GAGs in tumor states have been reported [5,6].

GAGs attached to PGs also perform a variety of biological functions based on the interactions with various proteins such as collagen and growth factors [7]. GAG chains are linear polymers composed of repeating disaccharides of hexosamine and hexuronic acid and are generally sulfated except for hyaluronic acid (HA), while keratan sulfate (KS) consists of a poly-*N*-acetyllactosamine backbone.

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[1] *Abbreviations used:* PGs, proteoglycans; GAG, glycosaminoglycan; HA, hyaluronic acid; KS, keratan sulfate; CS/DS, chondroitin/dermatan sulfate; HP/HS heparin/heparan sulfate; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; UTI urinary trypsin inhibitor; BNC, bovine nasal cartilage; 2AA, 2-aminobenzoic acid; AMAC, 2-aminoacridone; PEG, polyethyleneglycol; AGC, Autoglycocutter; DHB,2,5-dihydroxybenzoic acid; CE, capillary electrophoresis.

0003-2697/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2006.12.027

GAGs except HA are covalently attached to serine residues of core protein in an *O*-linked manner through the core tetrasaccharide structure of GlcAβ1-3Galβ1-3Galβ1-4Xylβ1-*O*-Ser [8,9]. The linkage-region glycan is synthesized through stepwise sequential addition of each monosaccharide from the corresponding sugar nucleotide to a specific serine residue in a core protein. Chondroitin/dermatan sulfate (CS/DS) is synthesized once GalNAc is transferred to the common linkage region, while heparin/heparan sulfate (HP/HS) is formed if GlcNAc is first added. So far, a variety of linkage-region structures modified with various sulfation patterns have been identified in chondroitin sulfate PGs derived from many cartilages such as whale cartilage [10], bovine nasal septum cartilage [11,12], shark cartilage [13,14], and bovine articular cartilage [15,16]. Because the sulfation of Gal residues in the glycans at the linkage region has not been found in HP/HS–PGs [17,18], sulfated Gal structures are considered to play an important role in GAG biosynthesis, especially in the sorting mechanisms leading to CS/DS or to HP/HS. Phosphorylation at Xyl residue in the linkage region has also been found in some PGs [14,19,20]. Previous studies suggested that dephosphorylation took place soon after the transfer reaction of GlcA to the trisaccharide, Galβ1-3Galβ1-4Xyl [21,22], and *in vitro* enzyme assays using human glucuronyltransferase I demonstrated that this enzyme showed higher activity toward the phosphorylated acceptor substrate, Gal-Gal-Xyl(2-*O*-phosphate)-Ser [23].

From these observations, structural analysis of linkage-region oligosaccharides is important for elucidating the mechanism of GAG biosynthesis. To analyze the oligosaccharides at the linkage region, they are usually released from the core protein by β-elimination reaction in alkaline solution. The releasing reaction is usually performed under mild conditions for a long time (typically, 0.5 M LiOH at 4 °C for 15 h) to prevent degradation by peeling reaction or random cleavage [24]. Endo-β-xylosidase and cellulases that exhibit endo-β-xylosidase activity have been reported for releasing GAGs from the core protein [25,26], but specificity of the enzyme is a big problem for routine use.

So far, structural analysis of the oligosaccharides at the linkage region has been performed by various analytical methods such as high-performance liquid chromatography (HPLC), ^{1}H-NMR spectroscopy, and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). ^{1}H-NMR spectroscopy is a powerful technique for determination of oligosaccharide structure but usually requires large amounts of the sample (~mg). For sensitive analysis, nonreductive alkaline β-elimination followed by fluorescent labeling with 2-aminobenzamide was reported [24].

In the present study, we fabricated an apparatus for rapid release of GAGs from the core protein by using an in-line flow system and evaluated the method for the analysis of linkage oligosaccharides derived from five CS/DS–PGs.

Materials and methods

Urinary trypsin inhibitor (UTI, ulinastatin) was obtained from Mochida Pharmaceutical Co. (Shinjuku-ku, Tokyo, Japan) and used after dialysis against water. Bovine nasal cartilage proteoglycan (BNC–PG), chondroitinase ABC, chondroitinase AC-II, chondro-4-sulfatase, and standard samples of unsaturated disaccharides were obtained from Seikagaku Corp. (Chuo-ku, Tokyo, Japan). Calf intestine alkaline phosphatase was obtained from Roche Applied Science (Minato-ku, Tokyo, Japan). Aggrecan, decorin, and biglycan (all from bovine articular cartilage) were obtained from Sigma–Aldrich, Japan (Chuo-ku, Tokyo, Japan). 2-Aminobenzoic acid (2AA) and 2-aminoacridone (AMAC) were purchased from Tokyo Kasei Kogyo (Chuo-ku, Tokyo, Japan) and Molecular Probes (Eugene, OR, USA), respectively. DB-1 capillary, of which the inner surface is chemically modified with dimethylpolysiloxane, was obtained from J&W Scientific (Folsom, CA, USA). Polyethyleneglycol (PEG70000, average molecular mass 70,000) was from Wako Pure Chemicals (Dosho-machi, Osaka, Japan). Other reagents and solvents were of the highest grade commercially available.

Digestion of PGs with chondroitinase ABC

PG samples (100 μg) were dissolved in 50 mM Tris–HCl buffer (pH 8.0, 100 μl), and chondroitinase ABC (200 mU, 4 μl) dissolved in the same buffer was added to the solution. The enzyme reaction was carried out at 37 °C overnight, and the reaction mixture was passed through a filter device (M.W. 10,000 cutoff, Ultrafree-MC, Millipore) at 10,000*g* to separate the unsaturated disaccharides and core protein containing linkage oligosaccharides. After washing the core protein fraction with water (200 μl) by centrifugation three times, filtrate and washing (unsaturated disaccharides fraction) were combined and lyophilized to dryness by a centrifugal evaporator (SpeedVac, Servant). Lyophilized materials were used for fluorescent labeling with AMAC. The core protein containing linkage oligosaccharide in the filter cup was collected with water (100 μl) and used for releasing of oligosaccharides by AGC.

Release of oligosaccharides at the linkage region by AGC

To release oligosaccharides at the linkage region, the solution (100 μl) of core protein containing linkage oligosaccharides obtained by the method as described above was injected into AGC. An aqueous solution of 0.5 M LiOH was used as alkaline solution for releasing oligosaccharides at a flow rate of 0.5 ml/min, and temperature of the reaction coil in the reactor was set at 70 °C. After passing through the reactor, the reaction mixture was desalted by a cation-exchange cartridge in in-line mode. The eluate from the cation-exchange cartridge was collected into the sample reservoir by monitoring the absorbance at 230 nm. The process from sample injection to collection

of the released oligosaccharides was completed within 3 min. After the run, the cation-exchange cartridge was regenerated with 0.25 M H_2SO_4. The collected solution (ca. 900 μl) containing released oligosaccharides was lyophilized to dryness by a centrifugal evaporator, and the lyophilized material was used for fluorescent labeling with 2AA.

Fluorescent labeling of unsaturated disaccharides with AMAC

A mixture of unsaturated disaccharides obtained by the digestion with chondroitinase ABC was labeled with AMAC using the method reported previously [27]. Briefly, the mixture was dissolved in 100 mM AMAC in a mixture (10 μl) of dimethyl sulfoxide–acetic acid (17:3, v/v), and 1 M sodium cyanoborohydride (10 μl) in the same solvent was added. After maintaining the mixture at 90 °C for 30 min, water (500 μl) and chloroform (500 μl) were added to the reaction mixture and mixed vigorously by a vortex mixer. After removing the chloroform layer, the aqueous phase was washed again with chloroform (500 μl), and the procedure was repeated five times. A portion of the aqueous phase was used for the analysis by capillary electrophoresis (CE).

Fluorescent labeling of linkage oligosaccharides with 2AA

Released linkage oligosaccharides were derivatized with 2AA according to the method reported previously [28]. The linkage oligosaccharides released by AGC were dissolved in 2AA solution (100 μl) which was freshly prepared by dissolution of 2AA and sodium cyanoborohydride (15 mg each) in methanol (1 ml) containing 4% sodium acetate and 2% boric acid, and the mixture was kept at 80 °C for 1 h. After cooling, water (100 μl) was added to the mixture, and the mixture was applied on a column of Sephadex LH-20 (1 × 30 cm) previously equilibrated with 50% aqueous methanol. The earlier eluted fluorescent fractions were pooled and evaporated to dryness. The residue was dissolved in water (100 μl) and a portion of the solution was used for the analysis by capillary electrophoresis and MALDI-TOF MS.

Capillary electrophoresis

Capillary electrophoresis was performed on a Beckman MDQ Glycoprotein System. For the analysis of AMAC-labeled unsaturated disaccharides, electrophoresis was performed with a DB-1 capillary (50 μm i.d., 30 cm length) in 100 mM Tris–borate buffer (pH 8.0) containing 1% PEG70000, and detection was performed by an argon-laser-induced fluorescence detector (Ex 488 nm, Em 520 nm), whereas electrophoresis of 2AA-labeled linkage oligosaccharides was performed with a DB-1 capillary (100 μm i.d., 30 cm length) in 100 mM Tris–borate buffer (pH 8.0) containing 10% PEG, and detection was performed by a helium–cadmium–laser induced fluorescence detector (Ex 325 nm, Em 405 nm).

Chondro-4-sulfatase digestion

Digestion was performed according to the procedure recommended by the manufacturer. A portion of 2AA-labeled linkage oligosaccharides (7.6 nmol) was incubated with 20 mU of chondro-4-sulfatase in 50 mM Tris–HCl buffer (pH 7.5, 20 μl) containing 50 mM sodium acetate and 0.01% bovine serum albumin at 37 °C for 30 min. The reaction was terminated by maintaining the mixture on the boiling water bath for 5 min. A portion of the mixture (2 μl) was diluted with water (10 μl) and analyzed by capillary electrophoresis.

Chondroitinase AC-II digestion

Chondroitinase AC-II digestion was performed in a manner similar to that for chondro-4-sulfatase digestion. A portion of 2AA-labeled linkage oligosaccharides (7.6 nmol) was incubated with 25 mU of chondroitinase AC-II in 50 mM acetate buffer (pH 6.0) containing 50 mM sodium acetate and 0.01% bovine serum albumin at 37 °C for 30 min. After maintaining the mixture on the boiling water bath for 5 min, a portion of the mixture (2 μl) was diluted with water (10 μl) and analyzed by capillary electrophoresis.

MALDI-TOF MS

MALDI-TOF MS spectra of 2AA-labeled linkage oligosaccharides were acquired on a Voyager DE-Pro mass spectrometer (PE Biosystems, Framingham, MA, USA) in negative-ion linear mode. Nitrogen laser (337 nm) was used for the ionization. Accelerating voltage was 19 kV, and delayed extraction was performed after 500 ns. 2,5-Dihydroxybenzoic acid (DHB) was used as a matrix throughout the work.

Results

The outline of the method for the analysis of glycan portions of PGs is shown in Fig. 1. PGs afford a mixture of unsaturated disaccharides and the core protein bearing hexa- or higher oligosaccharides as linkage oligosaccharides by digestion with lyases [15]. In the present study, a sample of PG is extensively digested with chondroitinase ABC, and the reaction mixture is fractionated using a centrifugal filter device to two fractions of a mixture of unsaturated disaccharides and the protein portion to which linkage glycans are attached. The mixture of produced unsaturated disaccharides was labeled with AMAC and analyzed by CE. The protein core to which linkage oligosaccharides are attached is subjected to the automated glycan releasing apparatus, AutoGlycoCutter (AGC), to release oligosaccharides. The released oligosaccharides

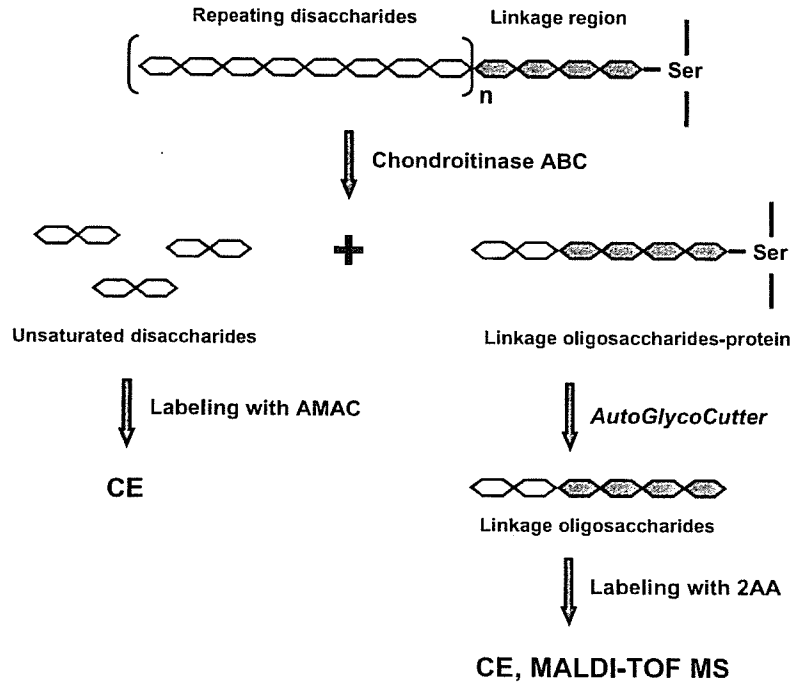

Fig. 1. Outline for the analysis of GAGs in PGs.

are then labeled with 2AA, and analyzed by CE and MALDI-TOF MS.

AGC is an apparatus for releasing *O*-linked-type oligosaccharides using an in-line flow system. AGC and the diagram of the system are shown in Fig. 2. AGC is an automated bench top apparatus composed of several units as shown in Fig. 2a. A portion (1–100 μl) of the sample solution of PGs after digestion with lyases as described above is introduced by an automatic injector into the flow of alkali solution supplied by a plunger pump at a flow rate of 0.1–1.5 ml/min. Glycans attached to the core protein through *O*-glycosidic linkages are released in the reaction coil (0.25 mm i.d., 10 m length) thermostated at constant temperature (40–150 °C) in the reactor. The alkaline solution containing released oligosaccharides is immediately cooled and neutralized by passing through a cartridge packed with cation-exchange resin (ca. 1 ml volume) to avoid further degradation of the released glycans. The effluent from the cartridge is detected by a UV absorption detector at 230 nm and the fractions containing released glycans are collected by a fraction collector. After the glycan-releasing reaction, the cartridge is regenerated with 0.25 M H_2SO_4 at a flow rate of 1.2 ml/min followed by washing with water by an automatic valve changing device.

Analysis of linkage oligosaccharides of UTI and efficiency in releasing reaction using AGC

Urinary trypsin inhibitor is a characteristic PG extracted from human urine [29,30] and used as a protease inhibitor for treatment of acute pancreatitis. UTI contains a low-sulfated chondroitin-4-sulfate chain at Ser10 [31,32] and has a disulfated hexasaccharide at the linkage region [33]. In the glycan-releasing reaction of UTI, we employed a reaction coil of 10 m length (0.25 mm i.d.) and 0.5 M LiOH as the alkali solution at a flow rate of 0.5 ml/min. Temperature of the reactor was set at 70 °C. Total time required for the releasing reaction of a PG sample was ca. 3 min. After the releasing reaction using AGC, we analyzed the linkage oligosaccharides as 2AA derivatives. The results were compared with those obtained by the conventional method reported previously (Fig. 3) [24]. In CE analysis, we employed a DB-1 capillary with the inner surface chemically modified with dimethylpolysiloxane. Therefore, oligosaccharides having higher negative charges move faster and are observed at earlier migration times. The linkage oligosaccharides released by AGC were observed nearly as a single peak at ca. 3 min (Fig. 3a). This result was clearly comparable with that obtained from the analysis of linkage oligosaccharides released by the conventional method (Fig. 3b). In MALDI-TOF MS analysis of 2AA-labeled linkage oligosaccharides obtained by the present method, a molecular ion at *m/z* 1311.9 corresponding to $[M+Na-2H]^-$ was observed (Fig. 3c) and, accordingly, the composition $\Delta HexAHexAHexNAcHex_2Pen(SO_3H)_2$ 2AA was predicted. A fragment ion at *m/z* 1209.9 due to loss of $NaSO_3^-$ ion was also observed. Similar results were also observed for the MALDI-TOF MS analysis of the oligosaccharides released by the conventional method (data not shown). MS analysis of polysulfated carbohydrates often gives fragment ions due to loss of sulfate groups [34]. After digestion with chondro-4-sulfatase, the major

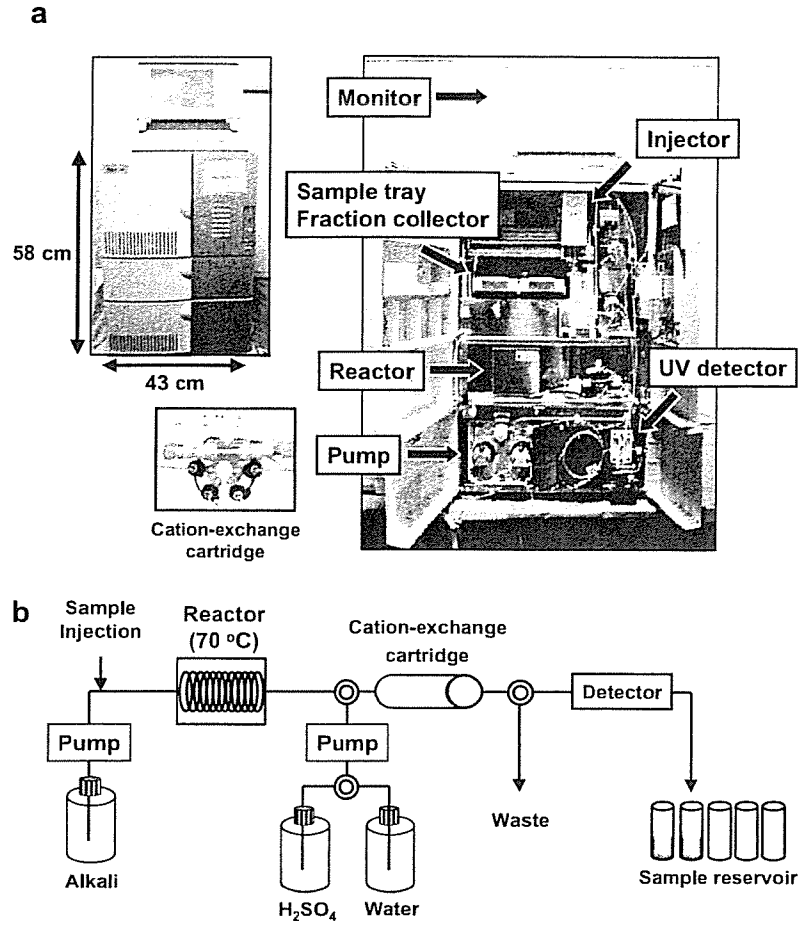

Fig. 2. AutoGlycoCutter (AGC) for releasing glycans from the core protein. (a) Appratus. (b) Diagram of AGC for oligosaccharide releasing.

peak at 3 min in CE was observed later at ca. 4.4 min as a single peak (data not shown). Chondro-4-sulfatase has been demonstrated to release the sulfate groups at C4 position of Gal and that of GalNAc in the disulfated hexasaccharide [10]. From these results, we concluded that AGC gave results similar to those obtained by the conventional method, and the oligosaccharide released by AGC has the following structure as reported previously [33]: ΔHexAα1-3GalNAc(4-sulfate)β1-4GlcAβ1-3Gal(4-sulfate)-β1-3Galβ1-4Xyl-2AA. Efficiency in recovery of oligosaccharides at the linkage region was $64 \pm 3\%$ ($n = 7$) as determined by CE.

Evaluation of AGC using model PGs

PG derived from bovine nasal cartilage is a high-molecular-weight PG that contains up to 150 chondroitin sulfate chains and 30–50 keratan sulfate chains [11,35]. The GAG chains are attached to the core protein through *O*-glycosidic linkages. The hexasaccharides derived from the linkage region have both C4 and C6 sulfate groups [11]. In a manner similar to that described above, we analyzed the oligosaccharides derived from the linkage region of BNC–PG by CE after the releasing reaction followed by derivatization with 2AA (Fig. 4). Peaks **2** and **3** were assigned as monosulfated hexasaccharides, and peak **4** was assigned as nonsulfated hexasaccharides from their migration times. Upon digestion of the mixture of linkage oligosaccharides of BNC–PG with chondro-4-sulfatase, the peaks observed at 2.9 and 3.4 min (peaks **1** and **2**) disappeared and the relative intensity of peak **4** increased. We also found that peaks **2** and **3** were shifted to later migration times after digestion with chondroitinase AC-II (data not shown). These results indicate that a sulfate group in the hexasaccharides observed as peaks **2** and **3** is on the GalNAc residue. The results are also confirmed by MS data as shown in Fig. 4c. The linkage oligosaccharides derived from BNC–PG showed two ions at *m/z* 1210.9 and *m/z* 1130.8 which correspond to monosulfated hexasaccharides (ΔHexA-HexAHexNAcHex$_2$Pen(SO$_3$H)2AA) and nonsulfated hexasaccharides (ΔHexAHexAHexNAcHex$_2$Pen2AA), respectively. From these observations, it was revealed that peak **1** is a disulfated hexasaccharide as a minor component, and peak **2** is a hexasaccharide that has a sulfate group at the C4 position of the GalNAc residue. In a previous study, it was demonstrated that the monosulfated