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/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.

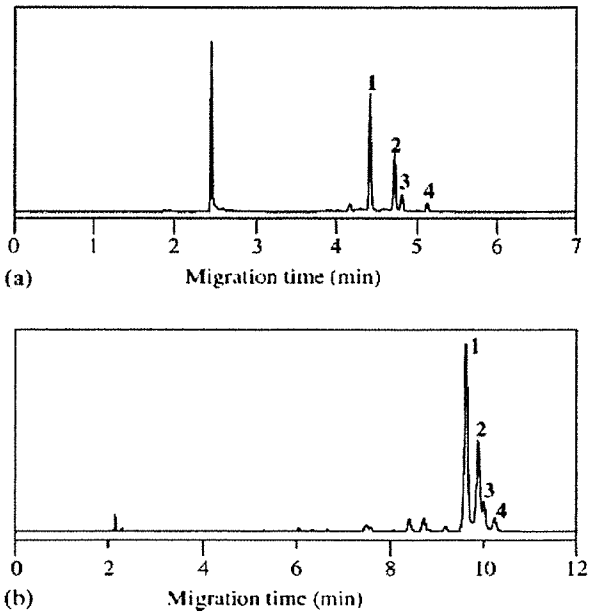

Figure 5. Oligosaccharide maps of trastuzumab by CE. (a) Oligosaccharides derivatized with APTS, (b) oligosaccharides derivatized with 3-AA. Analytical conditions: (a) Capillary, DB-1 capillary (30 cm (effective length 20 cm), 50 μm id); running buffer, 50 mM Tris-acetate buffer (pH 7.0) containing 0.5% 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

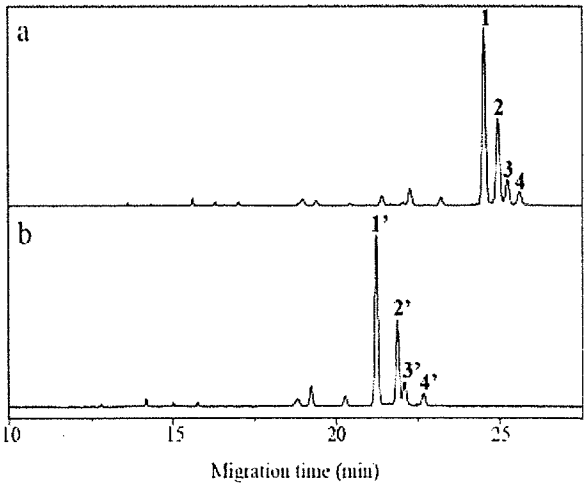

Figure 6. 3-AA-Labeled oligosaccharide maps of trastuzumab before (a) and after (b) fucosidase digestion. Analytical conditions: capillary, DB-1 capillary (50 cm (effective length 40 cm), 50 μm id); running buffer, 100 mM Tris-borate buffer (pH 8.3) containing 7.5% w/v PEG70000; applied potential, 30 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-

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          10         20         30         40         50
AVLPQEEEGS GGGQLVTEVT  KKEDSCQLGY SAGPCMGMTS RYFYNGTSMA

          60         70         80         90        100
CETFQYGGCM GNGNNFVTEK ECLQTCRTVA ACNLPIVRGP CRAFIQLWAF

         110        120        130        140
DAVKGKCVLF PYGGCQGNGN KFYSEKECRE YCGVPGDGDE ELL
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143 amino acids, MW: 15340

Disulfide bond: C26-C76
C35-C59
C51-C72
C82-C132
C91-C115
C107-C128

Modification: S10, chondroitin-4-sulfate
N45, N-linked oligosaccharide

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).

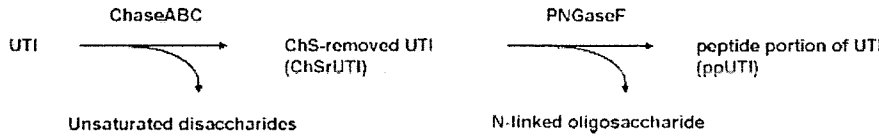

Figure 2. Stepwise glycosidase digestion of UTI.

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

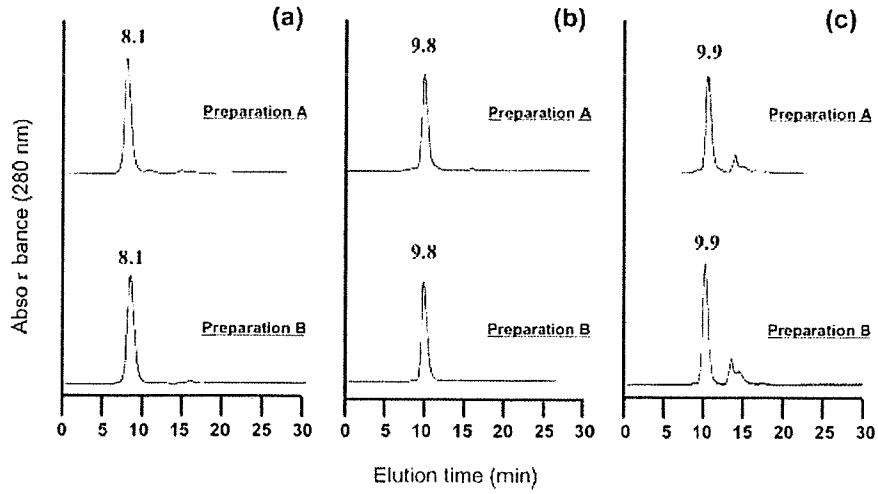

Figure 3. SEC of native UTI and glycosidase-treated UTI samples. Preparations A and B are shown in the upper and lower panel, respectively. UTI was analyzed as native form (a), ChSrUTI (b), and ppUTI (c). The numbers at each peak indicate elution times (min).

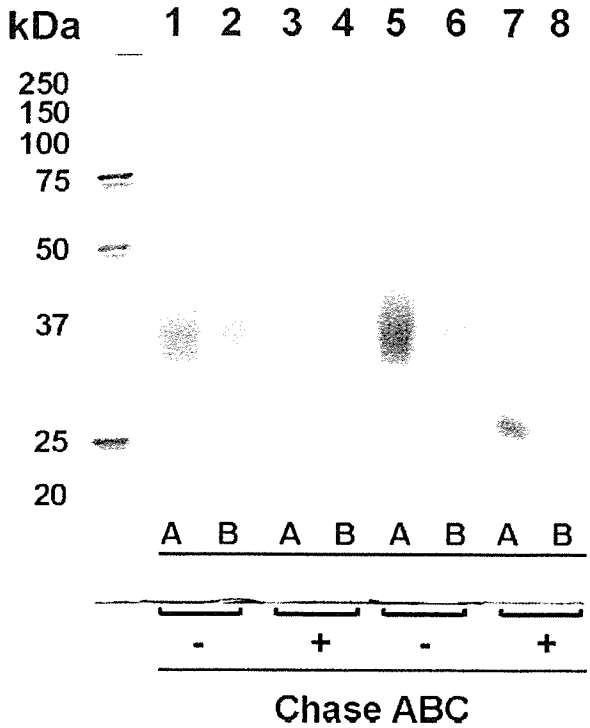

Figure 4. SDS-PAGE analysis of native UTI and ChSrUTI. UTI samples were analyzed with a 12% acrylamide gel; 5 μg (lanes 1–4) and 10 μg (lanes 5–8) of UTI samples were applied.

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

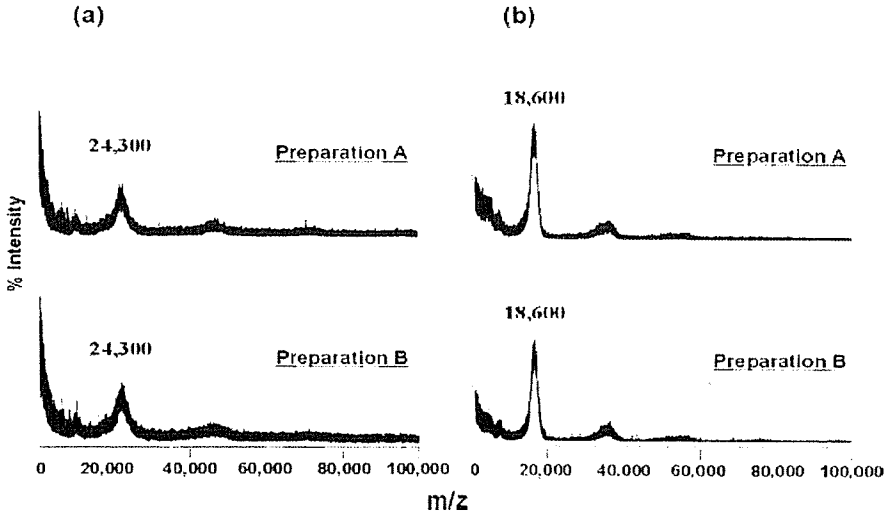

Figure 5. MALDI-TOF MS spectra of UTI samples. Native UTI (a) and ChSrUTI (b) were analyzed in negative-ion mode. Preparations A and B are shown in the upper and lower panel, respectively.

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).

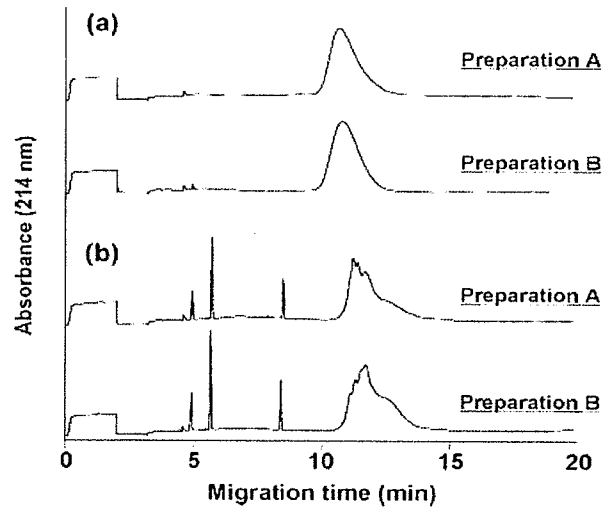

Figure 6. CE of UTI samples. Analytical conditions: fused-silica capillary (40 cm, 50 μm id); buffer, 50 mM borate buffer (pH 9.3) containing 100 mM SDS; applied voltage, 20 kV; detection, UV absorption at 214 nm. (a) Native UTI, (b) ChSrUTI.

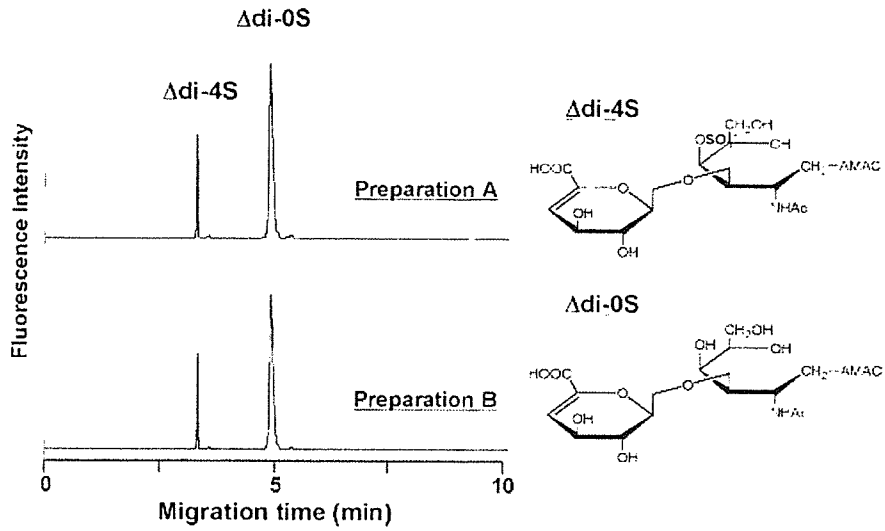

Figure 7. CE of AMAC-labeled unsaturated disaccharides derived from a ChS chain. Preparations A and B were digested with Chase ABC, and the produced unsaturated disaccharides were derivatized with AMAC and detected by an Ar LIF detector. Analytical conditions: capillary, DB-1 capillary (40 cm, 50 μm 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-

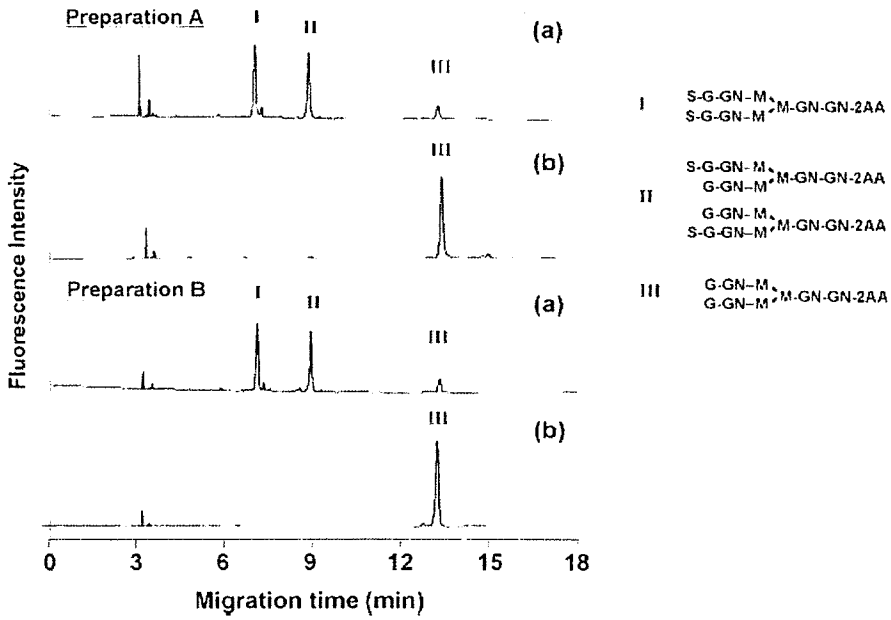

Figure 8. CE of 2AA-labeled *N*-linked oligosaccharides in UTI. *N*-Linked oligosaccharides were analyzed as sialo (a) and asialo (b) forms. Analytical conditions: capillary, DB-1 capillary (40 cm, 50 μm id); buffer, 100 mM Tris-borate (pH 8.3) containing 10% PEG70000; applied voltage, 20 kV; He-Cd LIF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

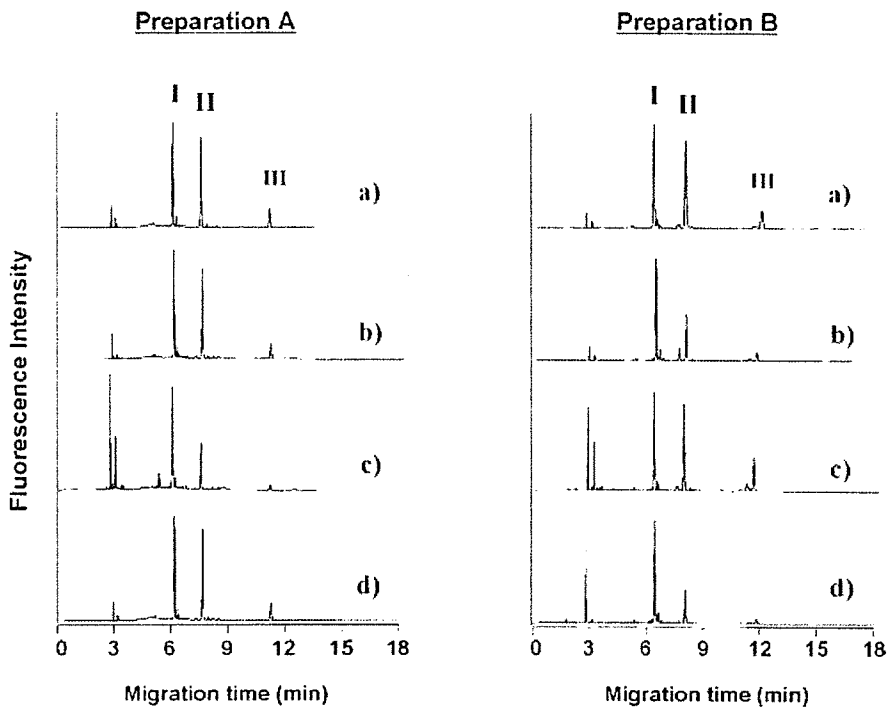

Figure 9. Analysis of 2AA-labeled *N*-linked oligosaccharides of different lot preparations. Analytical conditions were the same as in Fig. 8. Structures of peaks I–III are also shown in Fig. 8.

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.

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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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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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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-

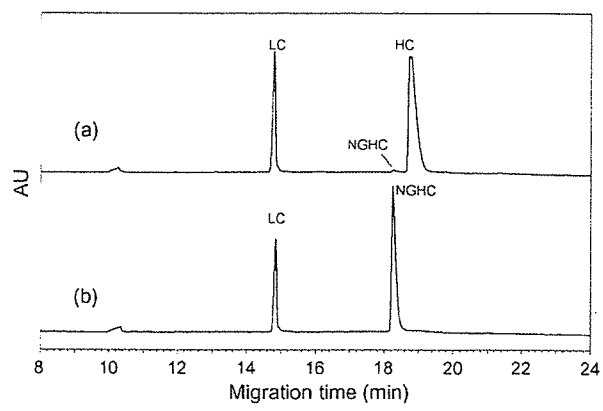

Fig. 1. CE-SDS analysis of reduced preparation of rituximab. LC, light chain; HC, glycosylated form of heavy chain; NGHC, non-glycosylated form of heavy chain. Analytical conditions: buffer, SDS-gel buffer (Beckman Coulter); capillary, fused-silica capillary (50 μm i.d., 30 cm, 20 cm effective length); 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.

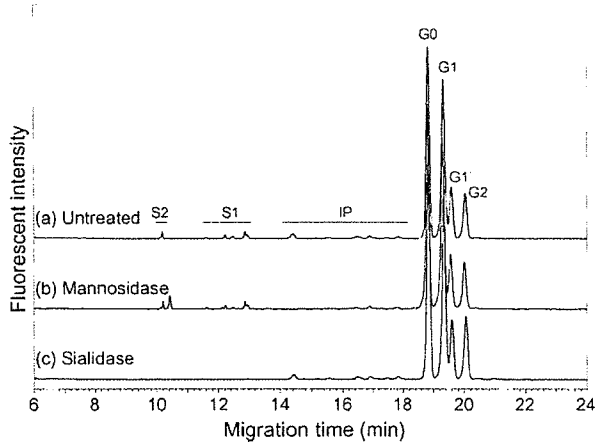

Fig. 2. Analysis of 2-AA labeled *N*-linked oligosaccharides derived from rituximab. 100 mM Tris-borate buffer (pH 8.3) containing 10% polyethylene glycol (MW: 35,000) was used as the separation buffer with a DB-1 capillary (100 μm i.d., 40 cm, 30 cm effective length). Applied potential, −25 kV at 25 °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.

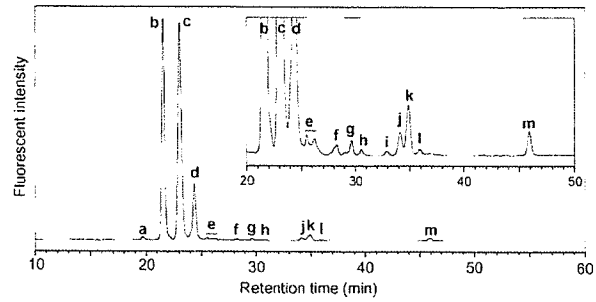

Fig. 3. Fractionation of 2-AA labeled oligosaccharides derived from rituximab by HPLC. Analytical conditions: column, Asahi Shodex NH2P-50 4E (250 mm × 4.6 mm); eluent, solvent A, 2% CH_3COOH in acetonitrile; solvent B, 5% CH_3COOH-3% triethylamine in water; gradient condition: a linear gradient (30–95% 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.

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).

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 Man α1 ↘6 / Man α1 →2Man α1 ↗3 Man α1 ↘6 / Man α1 ↗3 Man β1→4GlcN Acβ1→4GlcNAc	Man6
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