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Analysis of sialo-*N*-glycans in glycoproteins as 1-phenyl-3-methyl-5-pyrazolone derivatives by capillary electrophoresis

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Abstract

A method for the analysis of the sialo-*N*-glycans in glycoproteins was established by the electrokinetic chromatography mode of capillary electrophoresis (CE) in sodium dodecyl sulfate (SDS) micelles as 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatives, using sialo-*N*-glycans in fetuin as a model. Six major and some minor peaks were observed for the *N*-glycans in fetuin, which were well separated from each other using 50 m*M* phosphate buffer, pH 6.0, containing SDS to a concentration of 30 m*M* in an uncoated fused-silica capillary, and these peaks were assigned to sialo-*N*-glycans having either of the biantennary or β1-3/β1-4 linked galactose-containing complex type triantennary *N*-glycans as the basic structures, by an indirect method based on the assignment of the peaks in high-performance liquid chromatography separated in parallel with CE and peak collation between these two separation methods. The attaching position of the sialic acid residue was determined using the linkage preference of neuraminidase isozymes. The established system is considered to be useful for routine analysis of microheterogeneity of the carbohydrate moiety of this model glycoprotein from the following reasons: (1) the derivatization with PMP proceeds quantitatively under mild conditions without causing release of the sialic acid residue, (2) the derivatives can be sensitively detected by UV absorption, (3) the procedure is simple, rapid and reproducible. Preliminary results of *N*-glycan analysis for several other glycoproteins under these conditions are also presented.

Keywords: Derivatization, electrophoresis; Sialoglycans; Glycans; Glycoproteins; Proteins; Phenylmethylpyrazolone; Fetuin; Carbohydrates

1. Introduction

Recent advancement of glycobiology has revealed the important role of carbohydrates in many biological phenomena, and the significance of carbohydrate analysis has become more and more recognized. Since the carbohydrate comprises a number of homologues having very similar structures and many of them exist concurrently in biological samples, carbohydrate analysis inevitably requires high-resolution separation.

Various methods for analytical separation based on chromatography and electrophoresis have been utilized for carbohydrate analysis. Numerous papers

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have been published for gas chromatography (reviewed in, e.g., Refs. [1,2]) and liquid chromatography (reviewed in, e.g., Ref. [3]) of carbohydrates, but currently capillary electrophoresis (CE) is considered to be the best method for this purpose. It allows high-resolution separation and reproducible quantification by on-column detection. However, CE is basically a method for separation of ions, but most carbohydrates have no electric charge under normal conditions. There is another problem in applying CE to carbohydrates that the majority of carbohydrates cannot be directly detected with high sensitivity by conventional methods based on absorption and fluorescence, because they have neither chromophoric nor fluorophoric groups.

The best strategy to solve these problems at the same time is chemical derivatization. It may endow carbohydrates with not only ionic properties but also sensibility to conventional detection methods. A number of methods for pre-capillary derivatization have been developed, including the methods utilizing 2-aminopyridine (AP) [4], 6-aminoquinoline [5], 2-aminoacridone* [6], 4-aminobenzoic acid [7] as well as its ester [8] and nitrile [9], disulfonated [10] and trisulfonated [11] aminonaphthalenes, trisulfonated aminopyrene* [12], etc. Further methods are described in our recent review [13]. All the above methods are based on reductive amination, and the derivatives have either positive or negative charge of various magnitudes depending on the analytical conditions, and can be sensitively detected by UV absorption and/or fluorescence intensity. The methods using asterisked reagents are especially sensitive to fluorescence detection. Two-step methods, such as the carboxybenzoylquinolinecarboxaldehyde method [14] and the rhodamine succinyl ester method [15] are also available, both of which allow quite sensitive detection with laser-induced fluorescence detection. The first step of these methods is also based on reductive amination. Since reductive amination is basically an acid-catalyzed reaction performed in the presence of an acid, the acid-labile groups such as the sialic acid residue and the sulfate group may be partially decomposed during derivatization. Even though acid decomposition is minimized by careful operation using non-aqueous media, the possibility of such side reactions cannot theoretically be neglected.

We have developed a different type of derivatization method using 1-phenyl-3-methyl-5-pyrazolone (PMP), which is based on a novel type of condensation between the active carbon (C-4) in the PMP reagent and the reducing end of a reducing carbohydrate [16]. This was first developed for high-performance liquid chromatography (HPLC) of carbohydrates [16], and later successfully applied also to CE. Two groups of PMP are introduced to each reducing carbohydrate, to give a bis-PMP derivative, which shows strong absorption in the UV region (λ_{max}, 245 nm; ϵ, ca. 30 000). The condensation is rapid and quantitative under mild conditions (normally 30 min at 70°C) requiring no acid catalyst. Besides these advantages the bis-PMP derivatives can be separated by various modes of CE, including plain zone electrophoresis [17], zone electrophoresis as borate complexes [18], zone electrophoresis as alkaline earth metal complexes [19], micellar electrokinetic chromatography [20], ion-exchange electrokinetic chromatography [21], size-exclusion electrophoresis [22], hydrogen bonding electrophoresis with a certain kind of chiral selector [23], etc. Thus, the PMP method is quite suitable for routine analysis of various kinds of carbohydrates. So far we have successfully applied this method to CE analysis of simple mono- and oligosaccharides, and recently have extended the application studies of this method also to oligosaccharides in glycoconjugates.

In the analytical studies of *N*-glycans in glycoproteins by CE an approach to systematic mapping as AP derivatives was already reported by our group [24] and later by Hermentin et al. [25]. Such an approach should be evaluated, because any kinds of *N*-glycans will be able to be identified, if a complete database is prepared by collecting authentic specimens of all naturally occurring *N*-glycans, but it is quite elaborate and will require tremendous energy and time for its accomplishment. Our second strategy, which will acquire general assessment in practical analysis, is to establish the best analytical system for routine analysis of oligosaccharides in each glycoprotein. Such system will be useful for checking microheterogeneity of glycoprotein sample and the PMP method will be the best suited for this purpose. The present paper constitutes the first one of this series of our work based on this concept. It reports the preliminary result for *N*-glycan analysis,

focusing on the analysis of sialo-*N*-glycans in fetuin as a model.

So far both chemical and enzymatic methods have been well studied for the release of *N*-glycans. The chemical method based on heating in anhydrous hydrazine, a strong base [26] is a non-specific but selective method applicable to all kinds of *N*-glycans but may cause partial release of sialic acid residue in the oligosaccharide chains. In addition it may also cause partial cleavage of *O*-glycans [27]. On the other hand various kinds of enzymes releasing oligosaccharides having the *N*,*N*′-diacetylchitobiose or *N*-acetylglucosamine residue at the reducing termini from glycoproteins have been reported (reviewed in Ref. [28]), but most of them are not non-specific to carbohydrate structure. Since selective release is desirable but specific release is formidable as a general procedure for *N*-glycan release in the studies of carbohydrate microheterogeneity, the glycoamidase from *Flavobacterium meningosepticum* (PNGase F) having the lowest specificity was selected in the present work.

2. Experimental

2.1. Materials

A sample of fetal calf fetuin was obtained from Gibco BRL (Life Technologies, Tokyo, Japan), which had been prepared by ammonium sulfate fractionation of fetal calf serum, followed by gel filtration and lyophilization. The content of free sialic acid was less than 0.2%. The samples of other glycoproteins (α_1-acid glycoprotein, α_1-antitrypsin, α_2-macroglobulin and haptoglobin), all from human blood, were obtained from Sigma (St. Louis, MO, USA). The authentic specimens of the biantennary and triantennary asialo-*N*-glycans, I, II and III, were also obtained from the same source.

PNGase F, cloned from *Flavobacterium meningosepticum* and expressed in *Escherichia coli*, was purchased from New England Biolabs (Beverley, MA, USA). Neuraminidase preparations from *Arthrobacter ureafaciens* and *Salmonella typhimurium* were obtained from Nacalai Tesque (Kyoto, Japan) and Takara (Shiga, Japan), respectively. Neuraminidase from *S. typhimurium* was known to release the α2-3 linked *N*-acetylneuraminic acid (NANA) residue ca. 260 times easier than the α2-6 linked NANA residue, whereas neuraminidase from *A. ureafaciens* was not site-specific, cleaving the α2-3 linked and the α2-6 linked NANA with equal ease.

PMP was obtained from Kishida (Osaka, Japan) and recrystallized from methanol. All other chemicals were of the highest grade commercially available. Deionized and glassware-distilled water was used to prepare the running buffer for CE and the mobile phase for HPLC.

2.2. CE

The analysis of the PMP derivatives of sialo-*N*-glycans from fetuin was performed using a Beckman P/ACE 2000 system equipped with a high-voltage power supply, a pressure injection system for sample introduction, and a changeable lamp/interference filter type UV detector (deuterium lamp, wavelength at 254 nm). A roll of 50-μm I.D. capillary was obtained from Polymicro Technologies (Phoenix, AZ, USA) and a 54-cm piece was cut out from the roll. A detection window was created at a position of 7 cm from the cathodic end by removing polyimide coating by burning, and the capillary was installed in a cassette. The absorbance of the 254-nm light emitted by a mercury lamp was monitored and five data points were collected for every second.

The sialo-*N*-glycans from other glycoproteins were analyzed using a Model 270A capillary electrophoresis system of Applied Biosystems (the company name has been changed to Applera Corp., Foster City, CA, USA) equipped with a spectroscopic UV detector. The absorbance at 245 nm was monitored with data points of 20/s. A capillary of the same size from the same source was installed without using a cassette.

2.3. HPLC

HPLC was carried out using an 880-PU solvent delivery system and a UV-970 detector from Jasco (Tokyo, Japan). Separation by the hydrophobic interaction mode was carried out on a column (15 cm×6 mm I.D.) of Hikarisil ODS (Showa Denko, Tokyo, Japan; particle diameter, 5 μm) with a mixture of 100 m*M* phosphate buffer (pH 7.0)–acetonitrile

(85:15, v/v) as eluent at a flow-rate of 1.0 ml/min at ambient temperature. The detection of the separated asialo-*N*-glycans was performed by measuring UV absorbance at 245 nm.

Separation by the combined hydrophilic interaction and anion-exchange modes was performed on a column (25 cm×4.6 mm I.D.) packed with LiChroCART NH_2 (Merck, Darmstadt, Germany; particle diameter, 5 μm) with a mixture of [200 m*M* acetate–triethylamine buffer (pH 6.5)]–acetonitrile (47:53, v/v) at a flow-rate of 1.0 ml/min. Column temperature was maintained at 40°C and the separated sialo-*N*-glycans and their desialylation products were detected by measuring UV absorbance at 245 nm.

2.4. Release of sialo-N-glycans

It is generally known that preliminary depolymerization to the glycopeptide level by a kind of peptidase such as pronase and trypsin facilitates the following glycan release, but a longer procedure should be avoided in routine analysis. In addition it may cause partial release of the sialic acid residue. From these viewpoints the glycoprotein sample was directly subjected to digestion with PNGase F. In order to facilitate *N*-glycan release the protein sample was denatured by the addition of detergents, SDS and NP-40, and was unfolded by reducing the disulfide bond with 2-mercaptoethanol. The procedure for *N*-glycan release with this enzyme described below was well established by Applied Biosystems (presently belonging to the Perkin-Elmer group).

To a glycoprotein sample (5–50 μg) was added a 10-μl portion of a denaturing reagent (5% SDS, in 10% aqueous solution of 2-mercaptoethanol) and the mixture was heated on a boiling water bath for 5 min. After cooling the solution to room temperature 20 m*M* potassium monohydrogenphosphate, pH 7.2 (buffer for deglycosylation) containing ethylenediaminetetraacetic acid to a concentration of 50 m*M* (5 μl), and a 10% aqueous solution of NP-40 (5 μl) were added, followed by a PNGase F solution (50 units, 3 μl). The whole was gently mixed, incubated for 24 h at 37°C, and heated for 1 min on a boiling water bath to inactivate the enzyme. The mixture was lyophilized and the residue was subjected to derivatization with PMP.

2.5. Derivatization of the released sialo-N-glycans with PMP

This was performed by reducing sample scale in the procedure described in our previous paper [16]. To the residue containing the released sialo-*N*-glycans obtained above were added a 0.5 *M* methanolic solution of PMP (50 μl) and 0.3 *M* (normalized) sodium hydroxide (50 μl), and the mixture was incubated for 30 min for 70°C. After cooling the mixture to room temperature 0.3 *M* (normalized) hydrochloric acid (50 μl) was added for neutralization, and the neutralized solution was evaporated to dryness. To the residue were added water (200 μl) and ethyl acetate (200 μl), and the mixture was shaken vigorously. The ethyl acetate layer was discarded to remove the excess amount of PMP, and the aqueous solution was further extracted by adding another batch of ethyl acetate. This operation was repeated one more time, and the final aqueous layer obtained was evaporated to dryness. The residue was dissolved in a desired volume of water, and the solution was analyzed by CE, directly or after desalting by a Model G0 Asahi Kasei microacylizer using an Aciplex AC-110-02 membrane.

2.6. Desialylation of PMP-derivatized sialo-N-glycans

2.6.1. Digestion with neuraminidase from A. ureafaciens

A half portion of the residue obtained by the evaporation of each HPLC fraction in Fig. 2 was dissolved in 60 m*M* citrate–phosphate buffer, pH 5.0 (30 μl) containing a solution (5 μl) of neuraminidase from *A. ureafaciens* (10 mU), and the mixture was incubated for 16 h at 37°C. The solution was heated for 1 min on a boiling water bath to terminate the enzyme reaction, and subjected to analysis by HPLC in the hydrophobic interaction mode.

2.6.2. Digestion with neuraminidase from S. typhimurium

The other half portion of the residue was dissolved in 100 m*M* citrate buffer, pH 6.0 (10 μl) containing neuraminidase from *S. typhimurium* (10 mU), and the mixture was incubated for 10 min at 37°C. The solution was heated for 1 min on a boiling water bath

to terminate the enzyme reaction, and subjected to analysis by HPLC in the combined modes of hydrophilic interaction and anion-exchange.

3. Results and discussion

3.1. Derivatization of released N-glycans with PMP

A mixture of the *N*-glycans released from each glycoprotein as mentioned above was subjected to derivatization with PMP by the established procedure without intermediate purification from the accompanying proteinaceous substances and small molecular compounds added. The detection limit of the PMP derivatives of reducing carbohydrates by UV absorption is at ca. 1 micromolar concentration. This is corresponding to ca. 10 fmol injected amount, if the introduced sample volume is assumed to be 10 nl. However, more than 1000-fold amounts (10 pmol) were needed, because the minimum volume that we can handle with high reliability is about 10 μl. Therefore, the most appropriate sample amount for practical analysis will be 100 pmol–1 nmol for each *N*-glycan. Although the required amount of glycoprotein sample is dependent on the contents of individual *N*-glycans, we found that the appropriate amount was in a range of 10 (high carbohydrate content)–100 (low carbohydrate content) μg. Use of narrower capillaries and in-capillary pre-concentration can reduce sample amount, though such sample reduction will be reported elsewhere.

3.2. CE analysis of the PMP derivatives of N-glycans

PMP derivatives of reducing carbohydrates can be analyzed by various separation modes of CE as mentioned above [17–23]. Selection of separation mode depends on the combination of sample components. Our preliminary examination for various modes indicated that the micellar electrokinetic chromatography mode in SDS micelles [20] is generally the most suitable for the separation of the PMP derivatives of sialylated oligosaccharides [29]. Therefore, the salt concentration, pH and the SDS concentration were optimized. It was found that the final values of 50 m*M*, 6.0 and 30 m*M*, respectively, gave the greatest number of peaks indicating the best separation.

Fig. 1 shows the electropherogram obtained for 10-μg sample of fetuin under these optimized conditions.

Fluctuation of migration time can be minimized by rinsing the capillary with 100 m*M* sodium hydroxide followed by water, before each run. Relative migration time to PMP gave small RSD values of migration time less than 3%. Six major peaks (a, c, f, g, j and l) were observed, which were well separated from each other in 25 min. Six minor peaks (b, d, e, h, i and k) were also seen, though they were far smaller than the major peaks.

3.3. Peak assignment

Peak assignment of non-commercialized compounds in CE is generally quite a difficult work. Isolation of individual peak substances by CE and determination of their structures by an appropriate means will be the regular tactics. Proton magnetic resonance spectroscopy will give the most useful information for structure elucidation, but the

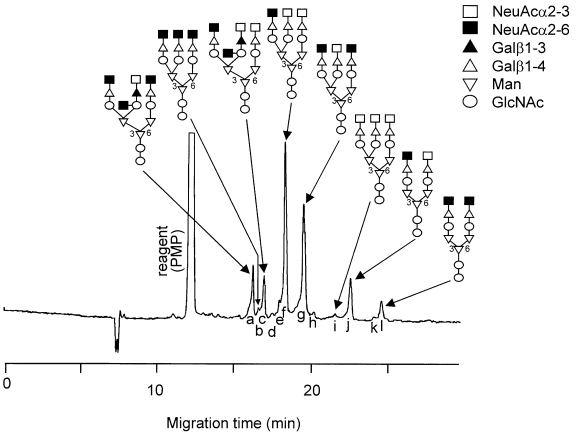

Fig. 1. Separation of the PMP derivatives of sialo-*N*-glycans released from fetuin (10 μg) by digestion with PNGase F, by CE in the micellar electrokinetic chromatography mode in SDS micelles. Capillary, uncoated fused-silica (47 cm×50 μm I.D.); running buffer, 50 m*M* phosphate buffer (pH 6.0) containing SDS to a concentration of 30 m*M*; capillary temperature, 25°C; applied voltage, 15 kV; detection, UV absorption at 254 nm. The numbers, 3 and 6, in the *N*-glycan structures indicate the position of mannosylation. 3: α1-3 linked, 6: α1-6 linked.

amounts of the sample components separated by CE are so small to be analyzed by this method that such structure elucidation is practically impossible. CE–MS, especially on-line coupled CE–MS [30], will be promising, but the apparatus are expensive and information on sialo-*N*-glycans is not enough for the time being. Off-line CE–matrix-assisted laser desorption ionization time-of-flight MS [31] will be an alternative method, but data accumulation seems insufficient to ensure discrimination among positional isomers of sialo-*N*-glycans. Our second best tactic was to separate the PMP derivatives of sialo-*N*-glycans by HPLC and identify the separated derivatives using enzymatic reactions. In this case collation of the HPLC peaks with the CE peaks is essential. HPLC requires much larger amounts of sample, but once peak collation with CE and assignment of HPLC peaks are established, analysis by CE which requires much smaller sample amounts will be allowed, using the established CE pattern.

Our accumulated data on HPLC of oligosaccharides indicated that sialo-*N*-glycans were well separated by the combined modes of hydrophilic interaction and anion-exchange on an aminosilica column with aqueous acetonitrile, whereas asialo-*N*-glycans were resolved much more effectively by the hydrophobic interaction mode on an octadecylsilica column with similar solvents (unpublished results). Based on this information separation of the PMP-derivatized mixture of sialo-*N*-glycans from fetuin was optimized by the combined modes of hydrophilic interaction and anion-exchange. The best separation was obtained using a LiChroCART NH_2 column and a mixture of [200 m*M* acetic acid–triethylamine buffer (pH 6.5)]–acetonitrile as eluent as shown in Fig. 2A. It gave eight peaks of the PMP derivatives of the *N*-glycans (peaks 1–8), although peaks were rather wide compared to the peaks in CE (Fig. 1), presumably due to the slow keto-enol tautomerism in the PMP moiety during separation process, as in capillary electrochromatography [32].

The fraction giving each peak was evaporated to dryness and a small portion of the residue was electrophoresed under the conditions for Fig. 1, in order to collate it with one of the CE peaks. Fig. 2 summarizes the result of the collation of HPLC peaks with CE peaks thus achieved. The result indicated the correspondence of 1 to j, 2 to l, 3 to i, 4 to f, 5 to g, 6 to b, 7 to c/e/h, and 8 to a.

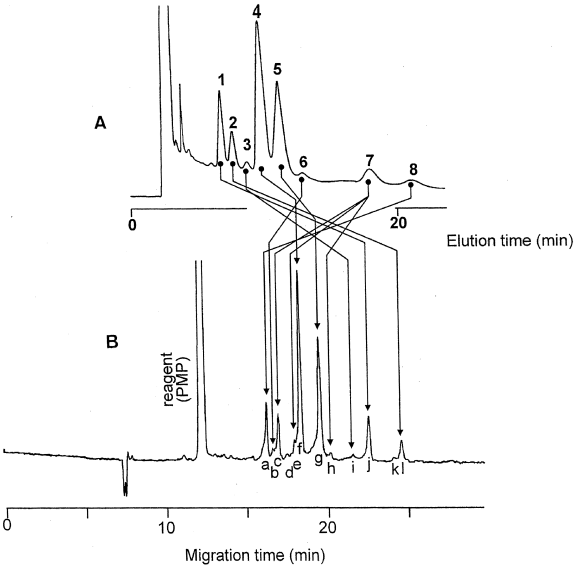

Fig. 2. Collation of the HPLC peaks (A) with CE peaks (B). (A) Column, NH_2 (25 cm×4.6 mm I.D.); eluent, a mixture of [200 m*M* acetic acid–triethylamine buffer (pH 6.5)]–acetonitrile (47:53, v/v); column temperature, 40°C; flow-rate, 1.0 ml/min; detection, UV absorbance at 245 nm. (B) As in Fig. 1.

Fetuin has been known to contain various kinds of *N*-glycans together with *O*-glycans [33]. The *N*-glycans comprise bi- and triantennary complex type oligosaccharides having the basic structures I, II and III, shown in Fig. 3, which was obtained by HPLC in the hydrophobic interaction mode.

The outermost galactose residues are fully or partially substituted by the NANA residue(s). In order to examine the basic structure, about the half portion of the residue from each fraction was subjected to digestion with neuraminidase from *A. ureafaciens* and the product was analyzed by HPLC in the hydrophobic interaction mode using a Hikarisil ODS column. Each fraction gave a peak in a range of 29–36 min, and the peak was identified by comparing the elution time with that of the authentic specimen. The result indicated that fractions giving peaks 1 and 2 (fractions 1 and 2, respectively) gave peak III, fractions 3–6 peak II, and fractions 7 and 8 peak I. Fractions 1–3 also gave minor peaks of II, which can be considered to be a contaminant resulting from incomplete purification by HPLC using the aminocolumn. Since the neuraminidase from *A. ureafaciens* is known to release both of the α2-3 and α2-6 linked NANA residues and each fraction was

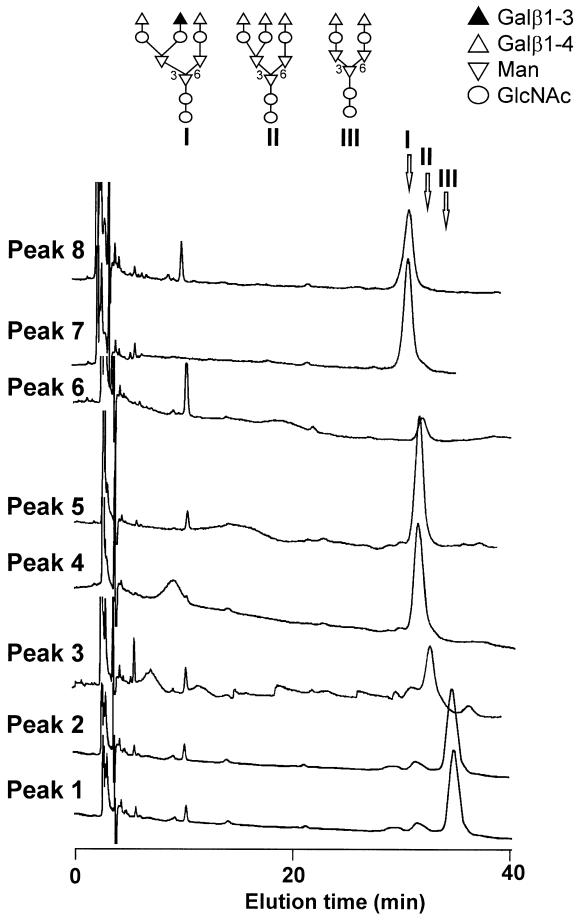

Fig. 3. Analysis of the *C. perfringens* neuraminidase digests of individual fractions giving peaks 1–8 in Fig. 2, by HPLC in the hydrophobic interaction mode. Column, Hikarisil ODS (15 cm×6 mm I.D.); eluent, a mixture of 100 m*M* phosphate buffer (pH 7.0)–acetonitrile (85:15, v/v); column temperature, ambient; flow-rate, 1.0 ml/min; detection, UV absorbance at 245 nm. The numbers, 3 and 6, in the *N*-glycan structures indicate the position of mannosylation. 3: α1-3 linked, 6: α1-6 linked.

digested with a large excess of the enzyme for sufficiently long time (16 h), all NANA residues were considered to have been released from these sialylated *N*-glycans. The basic structures of these *N*-glycans were thus elucidated by the experiment using *A. ureafaciens*, so the remaining problem is the elucidation of the number and the attaching position(s) of the sialic acid residue.

Generally enzyme specificity is an excellent tool to determine the attaching position of glycosidic linkages and if there were sialic acid-releasing enzymes having high positional specificity, they will serve perfectly for the elucidation of attaching positions of the sialic acid residues in sialoglycans. Unfortunately, however, such highly specific sialidases are not commercially available. Nevertheless, the neuraminidase from *S. typhimurium* has been known to release the α2-3 linked NANA 260 times faster than the α2-6 linked NANA [34]. Such extent of preference is not sufficient to release exclusively the α2-3 linked NANA, and prolonged digestion will result in release of both the α2-3 and the α2-6 linked NANAs.

We subjected the other half of the residue from each fraction to limited digestion with neuraminidase from *S. typhimurium* for only 10 min, and the product was analyzed by HPLC by the combined modes of hydrophilic interaction and anion-exchange using the aminosilica column. The result (shown in Fig. 4) was rather complicated due to insufficient specificity and incomplete digestion, but one or few peaks appeared from each fraction due to the preferential release of the α2-3 linked NANA residue.

In the combined modes of hydrophilic interaction and anion-exchange more hydrophilic compounds having higher negativity, i.e., those having larger numbers of the NANA residue are generally more strongly retained, and the triantennary *N*-glycans were eluted slower than the biantennary *N*-glycans. The *N*-glycans having the β1-3 linked galactose residue are much more retarded than the counterparts having the β1-4 linked galactose residue, contrary to the separation by the hydrophobic interaction mode (Fig. 3). In addition the former are known to have the NANA residue attached to the interior *N*-acetylglucosamine residue by the α2-6 linkage in addition to the NANA residues attached to the galactose residues, and therefore are more hydrophilic.

The HPLC analysis of the product of limited digestion of fraction 1 with *S. typhimurium* neuraminidase indicated the appearance of a fast eluting peak at ca. 5 min together with the original peak at ca. 7 min (peak 1). This provides an evidence that this biantennary *N*-glycan has one or two 2,3 linked NANA residue(s). Since the digestion was obviously incomplete considering form the survival of peak 1, the *N*-glycan having two α2-3 linked NANA residues will give two peaks of monosialylated and completely desialylated derivatives under these con-

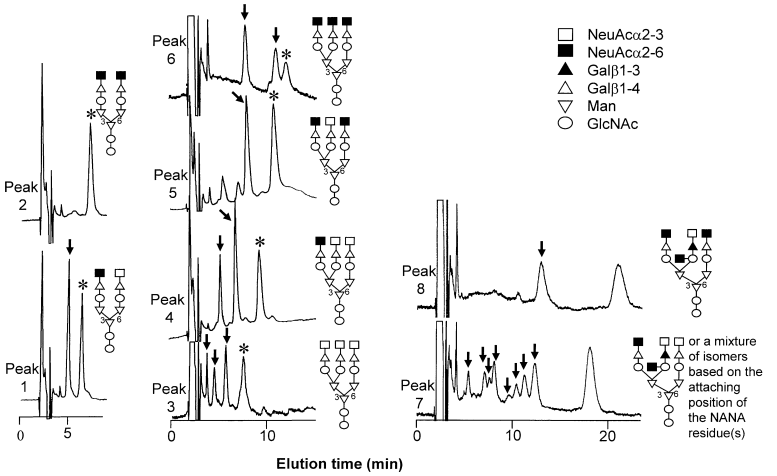

Fig. 4. Analysis of the *S. typhimurium* neuraminidase digests of individual fractions giving peaks 1–8 in Fig. 2, by HPLC in the combined modes of hydrophilic interaction and anion-exchange. Analytical conditions as in Fig. 2. The asterisked peaks are those of the substrates remaining not digested (the original peaks). The arrows indicate the peaks that newly appeared by digestion. The numbers, 3 and 6, in the *N*-glycan structures indicate the position of mannosylation. 3: α1-3 linked, 6: α1-6 linked.

ditions. Therefore, it is more likely that the *N*-glycan giving peak 1 will have one NANA residue attached to the outermost galactose residue by the α2-3 linkage. The other galactose residue is probably substituted by another NANA residue by the α2-6 linkage, because otherwise (if one of the galactose residues is not sialylated) the original and the completely desialylated *N*-glycans thereof would give much faster eluting peaks. Fraction 2 gave no new peaks upon digestion with *S. typhimurium* neuraminidase. Therefore, this biantennary *N*-glycan has no α2-3 linked NANA residue. Since it was eluted near peak 1 (probably α2-3 and α2-6 disialylated biantennary *N*-glycan having the α2-3 and α2-6 linked NANA residues as mentioned above), it can be assigned to the biantennary *N*-glycan having two α2-6 linked NANA residues. Thus, it is most likely that the *N*-glycans giving peaks 1 and 2 have the biantennary structure of III, and one of the galactose residues is substituted by the α2-6 linked NANA residue. The remaining galactose residue is substituted by either the α2-3 linked NANA residue (fraction 1) or the α2-6 linked NANA residue (fraction 2). Comparison of the elution times of these disialylated biantennary *N*-glycans suggested that the isomer having the α2-6 linked NANA residue was more strongly retained than the isomer having the α2-3 linked NANA residue. For the disialylated biantennary *N*-glycan having the α2-3 linked and α2-6 linked NANA residues (fraction 1) there is no evidence from these HPLC experiments to decide which of the two antenna has the α2-3 linked NANA residue, accordingly which has the α2-6 linked NANA residue. The assignment to the structures in Fig. 4 is based on the literature [34], in which the structures of *N*-glycans were determined by ^{1}H-nuclear magnetic resonance (NMR) spectrometry.

Fractions 3–6 commonly possess the sialylated triantennary structure (II) having the β1-4 linked galactose residue, as mentioned above based on the result of digestion with *A. ureafaciens* neuraminidase. If the same rule as in the biantennary sialo-*N*-glycans governs also in these sialylated triantennary *N*-glycans, fraction 3, 4, 5, and 6 will have three, two, one, or zero NANA residue(s) attached to the galactose residue(s) by the α2-3 linkage. The remaining galactose residue(s) in these *N*-glycans is considered to be also substituted by the α2-6 linked NANA residue(s), namely all compounds of peaks 3–6 are considered to have totally three NANA residues linked to the galactose residues, because all these peaks appeared slower than peaks 1 and 2, the biantennary *N*-glycans whose galactose residues are also fully sialylated. Fractions 3 and 4 gave plural peaks together with the original peaks (peaks 3 and 4, respectively), reflecting incomplete digestion. The

positional isomers resulting by the release of one α2-3 linked NANA residue (three species from fraction 3 and two species form fraction 4) will give such chromatograms. Fraction 6 was not completely purified, being contaminated by fraction 5, as evidenced by the presence of both peaks 5 and 6. Since the newly appearing peak (indicated by the arrow) was identical with that from fraction 5, the peak 6 compound is considered to have been unaltered by digestion with *S. typhimurium* neuraminidase. Thus, the structures having three α2-6 linked NANA residues will be reasonable for peak 6. The peak 5 compound is considered to be a trisialylated *N*-glycan having one α2-3 linked and two α2-6 linked NANA residues. Fractions 7 and 8 commonly gave triantennary asialo-*N*-glycan (I) having the β1-3 linked galactose residue in the digestion with *A. ureafaciens* neuraminidase (Fig. 4). Since both fractions gave newly appearing peaks besides the original peaks, they have obviously one or two α2-3 linked NANA residue(s), and fractions 7 and 8 are considered to be trisialo-*N*-glycans having one and two α2-3 linked NANA residues, in analogy with the inference concerning fractions 3–6. Digestion of fraction 7 with *S. typhimurium* neuraminidase, however, showed a complex chromatographic pattern giving several newly appearing peaks. One of these peaks may be due to the disialo-*N*-glycans resulting from the release of one α2-3 linked NANA residue, and one of them may be of the monosialylated *N*-glycan coming from the release of two α2-3 linked NANA residues, but the presence of the other peaks could not be explained. One of the *N*-acetylglucosamine residues was reported to be substituted by the α2-6 linked NANA residue in this sialo-*N*-glycan as in another isomer (peak 8 compound), but the possibility of substitution of the *N*-acetylglucosamine residue by the α2-3 linked NANA residue should not be ruled out. Such substitute might account for the multiplicity of peaks. As already pointed out fraction 7 gave one major peak (c) and two minor peaks (e and h) in the collation experiment with the CE peaks. However, it was difficult to assign all these minor peaks in CE, because the HPLC peak could not be separated any more. They might be such positional isomers. Fractions 8 gave only one major peak on digestion with *S. typhimurium* neuraminidase. Therefore, the release of one (but not two) 2-3 linked NANA residue is plausible considering from the elution time of the digestion product (if two NANA residues are eliminated, the digestion product will give an elution time shorter than 10 min). Based on these results the structures of the trisialylated triantennary *N*-glycans are proposed for peaks 3–8, as shown in Fig. 4. The specification of the antenna having the α2-3 (or α2-6) linked NANA residue was again based on the literature [35].

Peak assignment of sialo-*N*-glycans in fetuin based on this series of neuraminidase digestion studies and the HPLC–CE peak collation study is summarized in Fig. 1. Six major peaks (a, c, f, g, j and l) and two minor peaks (b and i) could thus be assigned to sialylated bi- and triantennary complex type *N*-glycans, but other four minor peaks (d, e, h and k) were unassigned. All the assigned peaks were considered to be of fully sialylated *N*-glycans, but the presence of partially sialylated derivatives should also be taken into account. Green et al. [35] reported the presence of several isomers of disialylated triantennary *N*-glycans having a non-sialylated galactose residue in considerable abundance (ca. 20% in total). In the present work we could not identify such partially sialylated peaks, but careful examination of minor peaks in HPLC analysis by improving separation conditions and increasing sample amount will add further information on minor peaks present in the electropherogram due to incomplete sialylation or partial desialylation.

The assignment of such isomers, as shown in Fig. 1, as well as Figs. 3 and 4, conformed to the literature. Green et al. [35] reported the relative abundance of each sialo-*N*-glycan in fetuin, and these data were useful for the assignment of the major peaks in the present CE analysis. With regards to fetuin *N*-glycans Townsend et al. carried out energetic work [36], and they also confirmed the presence of partially sialylated triantennary *N*-glycans, but their proportions were not high. In addition such partially sialylated *N*-glycans might be artifacts formed during sample preparation, storage, and a series of analytical operations. In the present work the possibility of partial release of the NANA residue can be excluded, because the employed conditions were mild.

Under the analytical conditions employed SDS

micelles are moving from the anode to the cathode by the combined effects of electrophoretic (slow toward the anode) and electroosmotic (fast toward the cathode) movements. In the micellar electrokinetic chromatography mode used in the present system the PMP derivatives of sialo-*N*-glycans are solubilized to these moving micelles with various magnitudes, resulting in divergent migration times. Correlation of the structures to migration order is not easy, but it is clearly shown that the derivatives of biantennary *N*-glycans gave longer migration times than the derivatives of triantennary *N*-glycans. This can be justified, because the latter have smaller carbohydrate moiety than the former, while all derivatives have commonly two PMP groups. Therefore, the contribution of the PMP group in the former to solubilization to SDS micelles will be relatively greater and will give rise to slower migration. The same tendency had been observed for the PMP derivatives of homologous series of oligosaccharides [20].

A similar phenomenon is observed between the derivatives of trisialo- (peaks a and c) and tetrasialo- (peaks f, g and i) *N*-glycans, though there was an exception of peak b. The effect of the substitution site of the sialic acid residue (compare peaks j and l; c. f and I) cannot be correlated to migration order. Comparison of the dissociation constants of the carboxyl groups in the sialic acid residues will be necessary, though it is a future problem.

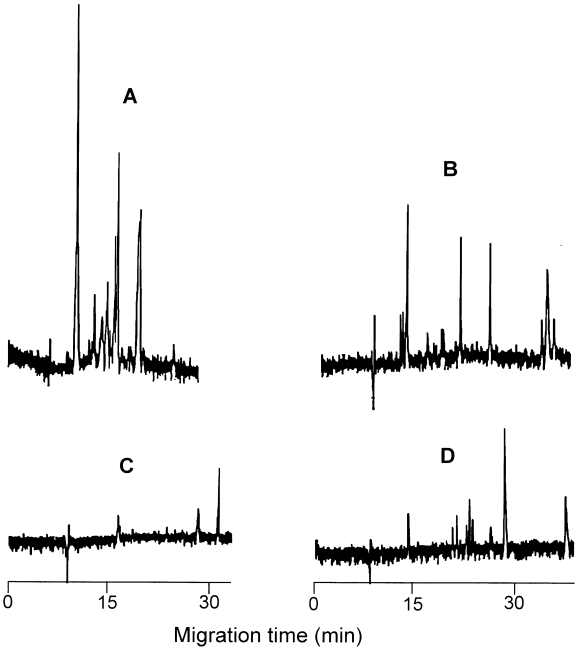

Fig. 5. Analysis of the *N*-glycans from various glycoproteins (10 μg each). Release of the *N*-glycans from the glycoproteins, derivatization of the released *N*-glycans with PMP, and CE analysis were performed under the same conditions as in the analysis of sialo-*N*-glycans in fetuin (Fig. 1). (A) α_1-Acid glycoprotein; (B) α_1-antitrypsin; (C) α_2-macroglobulin; (D) haptoglobin.

3.4. N-Glycan analysis for other glycoproteins

The sialo-*N*-glycans were released from several other glycoproteins, the released sialo-*N*-glycans were derivatized with PMP, and the resultant derivatives were analyzed by the same series of procedure as described above for fetuin. Fig. 5 shows good separation of the derivatives.

The heavier noise than in the CE of the sialo-*N*-glycans from fetuin (Fig. 1) is probably because the deuterium lamp used was not in good conditions. It may also be due to larger data points (20/s). Although peaks were not assigned and the electropherograms may include blank peaks coming from the enzymatic and chemical transformations, the results demonstrate the usefulness of this system for CE analysis as PMP derivatives. Peak assignment will be shown elsewhere in the future.

4. Conclusion

Although peak assignment required tedious operations for fractionation by HPLC and collation of HPLC–CE peaks, the procedure for CE itself was shown to be quite simple and we can point out the priority of the CE method as PMP derivatives to other CE methods in that it gives high yields of derivatives, the reaction proceeds under mild conditions to ensure no release of the sialic acid residue, and the derivatives absorb strongly in the UV region. It is also superior to the high-performance anion-exchange chromatography with pulsed amperometry in selectivity. We cannot neglect the paper by Liu et al. on CE of hydrazine-released glycans from fetuin as isoindole derivatives [14]. This method is sensi-

tive, but is not quantitative and requires long analysis time. In addition it gave no good separation of the derivatives of glycans.

As demonstrated in Fig. 1, the proposed method does not need intermediate purification because of the high selectivity of the derivatization with PMP. We are going to establish further CE systems for *N*-glycan analysis for various glycoproteins based on this principle. However, since no authentic specimens are commercially available as not only free carbohydrates but also PMP derivatives, peak assignment requires much effort and time. The result presented in this paper is only a milestone of this series of work aiming at the establishment of a simple, rapid, reliable method for such difficultly manageable compounds as sialo-*N*-glycans. We have also a wider vision that the proposed method may be extended to oligosaccharide analysis for other glycoconjugates including *O*-glycans in glycoproteins, glycosphingonolipids and plant glycosides, proteoglycan-derived oligosaccharides, etc.

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