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High-performance capillary electrophoresis of O-glycosidically linked sialic acid-containing oligosaccharides in glycoproteins as their alditol derivatives with low-wavelength UV monitoring

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Abstract

Several O-glycosidically linked monosialooligosaccharides from glycoproteins were separated as their alditol derivatives in ca. 10 min in borate buffer (pH 9.6) containing sodium dodecyl sulfate (SDS), and sensitively detected at the 10^{-4} *M* level by measuring absorption at 185 nm. Oligosaccharides having higher degree of polymerizations migrated faster, and N-acetyl- and N-glycolylneuraminic acid-containing oligosaccharide analogues could be resolved from each other under the conditions employed. Good linearity was demonstrated between 0.9 and 20 m*M* concentrations for relative response of N-acetylneuraminyllactose as a model compound to lactobionic acid as an internal standard. The detection limit was 0.2 m*M*, which corresponded to 0.80 pmol as the injected amount. The relative standard deviation of relative response at 9 m*M* was 1.97% ($n = 7$). The established system was successfully applied to microanalysis of sialooligosaccharides in bovine submaxillary mucin and swallow nest material.

1. Introduction

The analysis of oligosaccharides and polysaccharides is important for understanding their biological roles in bioactive glycoproteins, and particular attention has been paid for the analysis of sialooligosaccharides to elucidate the physiological role of the sialic acid residues.

There are a few established methods, such as hydrazinolysis and digestion with glycopeptidase, for the release of N-glycosidically linked sialooligosaccharides from glycoproteins [1]. The released oligosaccharides may be analyzed directly by high-performance anion-exchange chromatography [2] or high-performance capillary electrophoresis (HPCE) [3]. They may be also analyzed by high-performance liquid chromatography (HPLC) or HPCE after derivatization with some chromogenic or fluorogenic reagents [4,5].

On the other hand, there are no good methods for the release of O-glycosidically linked oligosaccharides to give free oligosaccharides. Treatment with alkali in the presence of borohydride can liberate O-glycosidically linked oligosaccharides [6], but the released oligosaccharides are converted concurrently to the corresponding alditols. Such alditols may be analyzed by high-performance-anion exchange chromatography with pulsed amperometric detection [7,8], but they cannot be sensitively detected by UV or visible light absorption in intact state and they cannot be detected by fluorescence at all. Conversion to absorbing or fluorescent derivatives is

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also hampered, because they lack the aldehyde group as the key functional group.

Under these circumstances, HPCE is expected to become an alternative to high-performance-anion exchange chromatography, since sensitive low-wavelength monitors have been recently developed. Some simple mono- and oligosaccharides have been separated as their borate complexes in basic conditions and detected at 195 nm [9]. Sialooligosaccharides from glycoproteins are considered to absorb such low-wavelength UV light more strongly, because of the presence of N-acyl groups in both sialic acid and hexosamine residues.

This paper presents a study of the analysis of O-glycosically linked sialooligosaccharides as their alditols, based on HPCE with low-wavelength UV detection.

2. Materials and methods

Bovine submaxillary mucin was prepared from bovine submaxillary glands according to the literature [10]. The nest material of Chinese swiftlet was commercially available from a Chinese foodstuff shop (Kobe, Japan). N-Acetylneuraminyllactose (NeuAc-Lac) was obtained from Sigma (St. Louis, MO, USA). Lactobionic acid used as the internal standard for calibration was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). ^{1}H NMR spectra were recorded in deuterium oxide at room temperature with a JEOL JNM GSX-500 spectrometer (JEOL, Tokyo, Japan) at 500 MHz. The proton signals were referenced to the methyl proton signal of the internal acetone (2.225 ppm) in δ (ppm). Negative ion fast-atom bombardment mass spectra were obtained in glycerol matrix using a JEOL SX102 mass spectrometer. The energy of the primary xenon beam was 8 kV. Calibration of mass number was carried out by using Ultramark (available from JEOL) as the mass reference. Evaporation of small volume of solutions (smaller than 1 ml) was carried out by a centrifugal concentrator CC-101 (Tomy, Tokyo, Japan) at room temperature. The reagents and solvents for chromatography were of the highest grade commercially available. Deionized water double distilled in a glass-made apparatus was used throughout the work.

2.1. Isolation of sialooligosaccharide alditol standards from bovine submaxillary mucin

Bovine submaxillary mucin (100 mg) was treated with 50 m*M* sodium hydroxide (10 ml) containing sodium borohydride to a concentration of 1 *M* for 72 h at 37°C. Acetic acid was carefully added to the mixture to destroy the excess amount of sodium borohydride, and the mixture was centrifuged. The supernatant solution was collected and lyophilized to dryness. Methanol (50 ml) was added to the residue, and the mixture was evaporated to dryness. The procedures were repeated five times to remove boric acid completely. The residue was then dissolved in a small volume of water (3 ml), and applied to a column (100 cm × 3.0 cm I.D.) of Sephadex G-50 (Pharmacia, Uppsala, Sweden) equilibrated with 30 m*M* ammonium hydrogen carbonate. The column was eluted with the same solution, and 10-ml fractions were collected. The fractions containing sialic acids, as monitored by the resorcinol–hydrochloric acid assay [11], were collected and lyophilized.

Reduced oligosaccharides (**1**, **2**, **3** and **4** in Fig. 1) were isolated by preparative HPLC in the following manner [12].

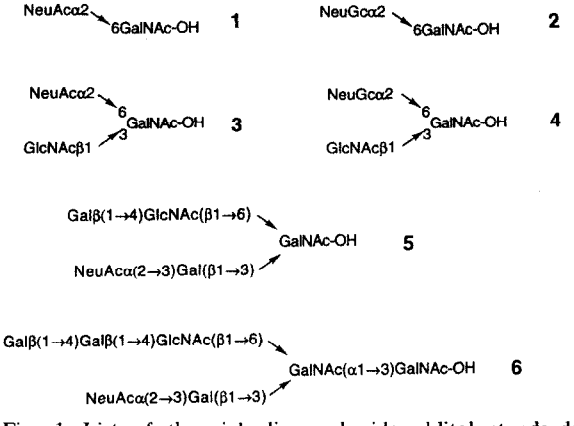

Fig. 1. List of the sialooligosaccharide alditol standards employed in the present work.

The residue obtained from the resorcinol-positive Sephadex G-50 fractions was applied to a column (250 mm × 4.6 mm I.D., 5 μm) of Takara PalPak-N (Takara Shuzo, Kyoto, Japan) and the column eluted isocratically with a mixture of acetonitrile–15 m*M* potassium dihydrogenphosphate solution (pH 5.2) (80:20, v/v) for 15 min, followed by gradient elution by changing the volume ratio of acetonitrile and the phosphate buffer linearly from 80:20 to 50:50 during 45 min at a flow-rate of 0.8 ml/min. The effluent was monitored at 210 nm. The effluents giving peaks at 25, 28, 33 and 37 min were collected and evaporated to dryness at room temperature. Each of the residues was dissolved in a small amount of water, and desalted on a small column (30 cm × 1 cm I.D.) of Sephadex G-25. The fractions which showed positive reaction to resorcinol were collected and lyophilized, to afford the sialooligosaccharide alditol standards.

2.2. Isolation of monosialooligosaccharide alditol standards from Chinese swiftlet

The procedure for isolation of monosialooligosaccharide alditols (**5** and **6** in Fig. 1) was similar to that described by Wieruszeski et al. [12]. Edible bird's nest material (1 g) was added in 50 m*M* sodium hydroxide (50 ml) containing sodium borohydride to a concentration of 1 *M*, and the mixture was incubated for 72 h at 37°C. The reaction was stopped by dropwise addition of acetic acid for neutralization. The resultant solution was centrifuged, the supernatant was lyophilized, a small volume of methanol (30 ml) was added to the residue, and the mixture was evaporated to dryness. The process of the addition and evaporation of methanol was repeated four times more. The residue was dissolved in a small volume of 30 m*M* aqueous ammonium bicarbonate, the solution was applied to a column (110 cm × 2.5 cm I.D.) of Sephadex G-50, and the column was eluted with the same solution. Fractions of 10 ml were collected, and assayed for sialic acid by the resorcinol–hydrochloric acid method. The 320–400-ml fraction was collected and lyophilized to dryness. The residue was dissolved in a small volume of pyridine acetate buffer (1 m*M* pyridine and 2 m*M* acetic acid, pH 5.6), and applied to a column (25 cm × 1.5 cm I.D.) of DEAE-Sephadex A-25 (Pharmacia), equilibrated with the same buffer. The column was eluted by a linear gradient made by the 1 m*M* pyridine–2 m*M* acetic acid buffer and the 150 m*M* pyridine–300 m*M* acetic acid buffer at a flow-rate of 10 ml/h. The volume of the starting and final buffers was 300 ml. Fractions of 5 ml were collected and assayed by the resorcinol–hydrochloric acid method. The fractions eluted between 250 and 325 ml were collected and lyophilized to dryness. The fractions showed the presence of three major peaks at 36, 42 and 45 min in HPLC on the amine-bonded silica column under the conditions described above for the isolation of sialooligosaccharides from bovine submaxillary mucin. The oligosaccharides giving peaks at 36 min and 45 min were isolated as pure state. These oligosaccharides showed molecular masses of 1406 and 1040, respectively, in negative fast atom bombardment-MS, and their ^{1}H NMR spectra were the same as those of oligosaccharides **5** and **6** (Fig. 1), respectively, described by Wierunszeski et al. [12].

2.3. Micro scale analysis of sialooligosaccharides in bovine submaxillary mucin and Chinese swiftlet

A sample of bovine submaxillary mucin (100 μg) was dissolved in 50 m*M* sodium hydroxide (100 μl) containing sodium borohydride to a concentration of 1.0 *M* in a polypropylene tube (1.5 ml), and the mixture was incubated for 72 h at 37°C. The excess amount of sodium borohydride was decomposed by careful addition of acetic acid (10 μl). The mixture was then passed through a small column of Amberlite CG-120 (H^+ form, 2 ml), and the column was washed with water (20 ml). The eluate and the washing fluid were combined and evaporated to dryness at 30°C by a rotary evaporator. The residue was dissolved in a small volume of methanol (5 ml) and the solution was evaporated to dryness. The methanol addition-dry up procedures were repeated four times more to remove boric acid

completely. The residue was dissolved in water (50 μl), and an aliquot was analyzed by HPCE.

A mixture of O-linked sialooligosaccharide alditols from Chinese swiftlet (10 μg) was also obtained similarly as described above. The amounts of the reagents and the volume of the solvent were reduced to one tenth of those used for the analysis of oligosaccharides in bovine submaxillary mucin.

2.4. HPCE

A Waters Quanta 4000 model (Millipore Japan, Tokyo, Japan) was used to carry out electrophoresis. An uncoated open tubular fused-silica capillary tube (50 cm × 360 μm O.D.; 50 μm I.D.), obtained form Polymicro Technologies (Phoenix, AZ, USA), was used in all experiments. The tube was fixed to the detector at the 7 cm position from the cathodic end of the capillary. The polyimide coating (2 mm section) was burned off to make a window for UV detection. For all experiments, absorbance at 185 nm was used for monitoring oligosaccharide alditols. All samples were introduced by hydraulic pressure for 10 s at a 10 cm height, and analysis was performed at 17 kV. The carrier solution was made from 200 m*M* borate buffer (pH 9.6) and contained sodium dodecyl sulfate (SDS) to a concentration of 100 m*M*.

3. Results and Discussion

3.1. Separation of monosialooligosaccharide alditols by HPCE

The electroosmotic flow is toward the cathode, since an uncoated capillary tube of fused silica having a negative charge on its inner wall is used in this work. On the other hand, sialooligosaccharides have the carboxyl groups which are dissociated to give anions under neutral and alkaline conditions. Therefore, they are expected to be held back when introduced from the anodic end. Thus, the combined effects of electroosmosis and electrostatic phenomenon will give peaks in the order of increasing charge to size ratios.

We investigated conditions for separation of the six oligosaccharide alditol standards, **1–6** (Fig. 1). Alkaline borate buffer gave basically a similar migration profile to that in alkaline phosphate buffer, indicating no significant contribution of borate complex formation. At pH 9.6, separation was optimal, but resolution was not satisfactory for the N-acetyl and N-glycolylneuraminic acid containing pairs, **1–2** and **3–4**. Addition of SDS to a concentration of 100 m*M*, however, resulted in baseline resolution of these pairs, as shown in Fig. 2. Table 1 lists the electrophoretic mobilities of **1–6** together with those of N-acetyl- and N-glycolylneuraminic acids.

N-Glycolylneuraminic acid-containing oligosaccharides (peaks **2** and **4**) showed slightly longer migration times than the N-acetylneuraminic acid-containing analogues (peaks **1** and **3**). Under the alkaline conditions employed, the carboxyl groups in these sialooligosaccharides are considered to be almost completely dissociated. Therefore, the operating mechanism of separation is exclusively zone electrophoresis. For these reasons the improvement of resolution by addition of SDS should be attributable to alteration of the electric charge to molecular size

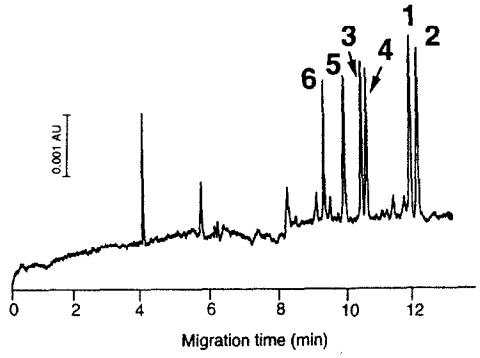

Fig. 2. Separation of an equimolar mixture of sialooligosaccharide alditol standards. Peak numbers refer to compounds in Fig. 1. Analytical conditions: capillary, fused silica (50 cm × 50 μm I.D.); carrier, 200 m*M* borate buffer (pH 9.6) containing SDS (0.1 *M*); applied voltage, 17 kV; detection, UV absorbance at 185 nm.

Table 1
Electrophoretic mobilities of some sialooligosaccharides

Sialooligosaccharide	μ_{ep} (cm^2 min^{-1} V^{-1}, $\times 10^3$)
NeuAc	14.44
NeuGc	14.66
NeuAcα(2→6)GalNAcOH	13.04
NeuGcα(3→6)GalNAcOH	13.26
GalNAcβ(1→3) [NeuAcα(2→6)]GalNAcOH	11.39
GalNAcβ(1→3) [NeuGcα(2→6)]GalNAcOH	11.57
Galβ(1→4)GlcNAcβ(1→6)GalNAcOH	10.67
NeuAc(α2→6)Galβ(1→3)/ Galβ(1→4)GlcNAcβ(1→6)GalNAc(α1→3)GalNAcOH	9.73
NeuAc(α2→6)Galβ(1→3)/	

Ac = Acetyl; Gal = galactose; Gc = glycol; Neu = neuraminic acid.

ratio. If the electric charge is significantly different between the pairs, they should have been resolved even without addition of SDS. Consequently the most probable mechanism will be alteration of molecular size. It is anticipated that the addition of SDS might change the conformation of these oligosaccharides to different magnitudes resulting in variation of molecular size.

3.2. *Quantitative analysis of sialooligosaccharide alditols by HPCE at 185 nm*

The calibration curve of N-acetylneuraminyllactose was prepared by using a commercially available authentic specimen. Fig. 3 shows

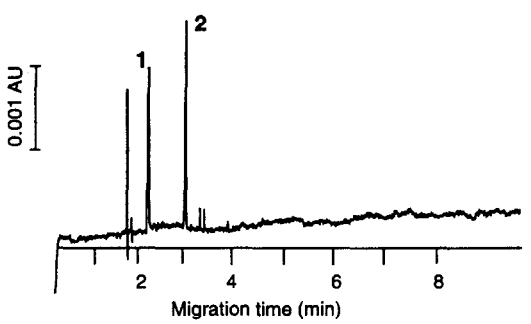

Fig. 3. Analysis of N-acetylneuraminyllactose by using lactobionic acid as an internal standard. Analytical conditions as in Fig. 2, except that the applied voltage was 25 kV. Peaks: 1 = N-acetylneuraminyllactose; 2 = lactobionic acid (internal standard).

the separation of this model sialooligosaccharide and lactobionic acid used as an internal standard.

In this case, a higher voltage (25 kV) was applied, hence the separation was rapid (in ca. 3 min). The plot of the relative peak response vs. the concentration of N-acetylneuraminyllactose showed good linearity between 0.9 and 20 m*M*. The lower limit of concentration (0.9 m*M*) in quantitative analysis corresponded to 3.6 pmol as the absolute injected amount, as the injected volume was 4.0 nl. This volume was obtained as $\pi r^2 l$, where r and l are the radius and the length of the plug of diluted rede ink introduced hydrodynamically in the same manner as sample solutions of reduced oligosaccharides. The minimum detectable concentration was 0.2 m*M* (signal-to-noise ratio 3). This is corresponding to 0.80 pmol as injected amount. We employed a 5-μl volume for the sample solutions, and the minimum sample amount in this volume was 1.0 nmol, since the lowest detectable concentration was 0.2 m*M*. This limit is higher than that obtained by high-performance anion-exchange chromatography (20 pmol, [13]), but the minimum amount injected to the capillary tube (0.8 pmol) was by far smaller than this amount. In addition, it was an advantage of the present method that greater portion of the sample could be recovered after analysis. The relative standard deviation of the relative peak response at 10 m*M* was 1.97% ($n = 7$).

3.3. Micro scale analysis of sialooligosaccharides in mucin samples

On the basis of the foregoing results the O-glycosidically linked sialooligosaccharides were released as alditol derivatives from a minute amount (100 μg) of bovine submaxillary mucin, and the derivatives were analyzed under the optimized conditions. The result is shown in Fig. 4.

Two predominant peaks due to NeuAcα-(2→6)GalNAc-OH (peak 1) and GalNAcβ(1→3)[NeuAcα(2→6)]GalNAc-OH (peak 3) were observed, accompanied by the peaks of the corresponding N-glycolyl analogues, NeuGcα(2→6)GalNAc-OH (peak 2) and GalNAcβ(1→3)[NeuGcα(2→6)]GalNAc-OH (peak 4). The total amounts of these major oligosaccharides were 91% of the overall peak areas. The broad peaks observed between 7 and 9 min were not identified in the present study, but these are presumably due to larger sialooligosaccharides having smaller charge to size ratio under these conditions.

Another example for analysis of the oligosaccharides in Chinese swiftlet is shown in Fig. 5.

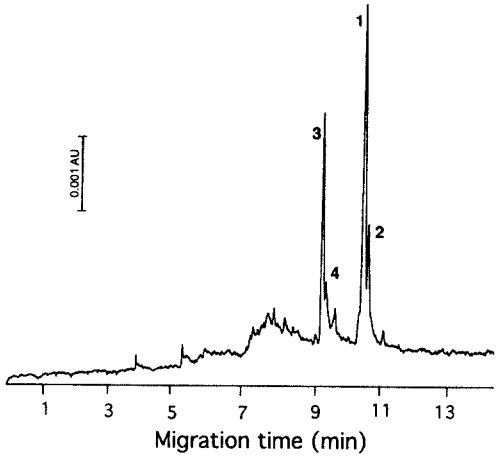

Fig. 4. Micro scale analysis of O-glycosidically linked sialooligosaccharides in bovine submaxillary mucin. Analytical conditions as in Fig. 2; peak numbers refer to compounds in Fig. 1. For the procedure for the release of oligosaccharides, see Materials and methods section.

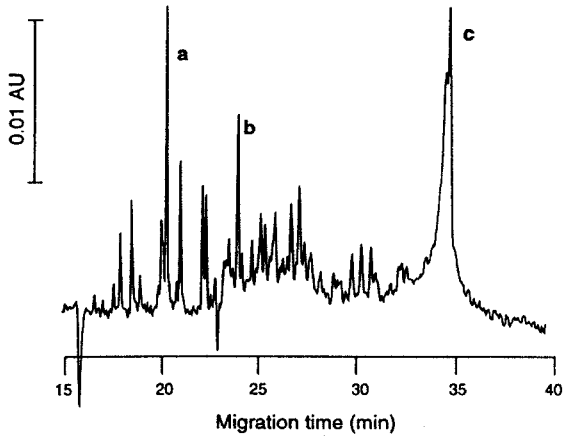

Fig. 5. Micro scale analysis of O-glycosidically linked sialooligosaccharides in Chinese swiftlet. Analytical conditions as in Fig. 2 except that the tube length was 100 cm. Peaks a, b and c were assigned to oligosaccharide alditols **5** and **6**, and N-acetylneuraminic acid, respectively. Concerning the procedure for the release of oligosaccharides, see the Materials and methods section.

Complex patterns were observed. Peaks a and b were identified as oligosaccharide alditols **6** and **5**, respectively, by co-migration with the standards. The large peak (peak c) observed at 35 min is due to that of N-acetylneuraminic acid introduced as the internal marker. In this case, a longer capillary (1 m) than that used for the analysis of oligosaccharides in bovine submaxillary mucin was used to obtain better separation, hence longer analysis time (35 min) was required.

The series of operations in these analyses are simple, because the processes of the release of oligosaccharides and reduction to alditols are conducted in one-pot fashion. Although considerable interference should be taken into consideration in cases of impure glycoprotein samples, the overall procedure is quite practical and convenient.

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