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Rapid and simple preparation of N-linked oligosaccharides by cellulose-column chromatography

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Abstract

As a means of preparing N-linked oligosaccharides from hydrazinolysates of glycoproteins in a rapid and simple manner, a method has been developed using cellulose-column chromatography. Hydrazinolysates of human IgG, containing a series of biantennary complex type oligosaccharides, were applied to a cellulose column equilibrated with (4:1:1, v/v) 1-butanol–ethanol–water. The N-linked oligosaccharides were eluted with (1:1, v/v) ethanol–water, and analyzed by HPLC in combination with sequential glycosidase digestion. The oligosaccharides, with or without sialic acid, were quantitatively recovered in the fraction eluted with (1:1, v/v) ethanol–water without UV-detectable contamination by impurities derived from protein or the cellulose. Other types of N-linked oligosaccharides of α1-acid glycoprotein (tetraantennary complex-type), ovalbumin (hybrid-type), and ribonuclease B (high mannose-type) were also quantitatively prepared from the hydrazinolysates by elution of the cellulose column with (1:1, v/v) ethanol–water and these had as high a quality as those prepared by conventional paper chromatography.

Keywords: Glycoprotein; Cellulose-column chromatography; *p*-Amino benzoic acid ethyl ester

1. Introduction

N-linked oligosaccharides of glycoproteins have been shown to have biological and pathological significance.[1–4] It is therefore extremely important to determine the nature of the oligosaccharide structures to elucidate roles of carbohydrates and their relationship to disease. For structural and functional analyses, as well as for their application, N-linked oligosaccharides are isolated after liberation from glycoproteins by hydrazinolysis.[5,6] Their separation from other hydrazinolysate products is essential, since the products derived from the protein portion of glycoproteins interfere with subsequent structural and biological analyses. Paper chromatography has conventionally been used for preparation of N-linked oligosaccharides from hydrazinolysates.[5,6] However, paper chromatography involves a lengthy procedure and is not suitable for preparation of oligosaccharides in large quantities or from multiple samples. Therefore, establishment of a simple and rapid method that can overcome these limitations is important.

In this report, we describe a method for the simple and rapid preparation of typical types

Abbreviations: ABEE, *p*-amino benzoic acid ethyl ester; hIgG, human immunoglobulin G; RNase, ribonuclease; HPLC, high performance liquid chromatography.

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of N-linked oligosaccharides from hydrazinolysates of glycoproteins using a cellulose column. The quality of the N-linked oligosaccharides prepared by cellulose-column chromatography was compared with that of the corresponding N-linked oligosaccharides prepared with paper chromatography in a series of structural analyses after their conversion into *p*-amino benzoic acid ethyl ester (ABEE) derivatives.

2. Results

Preparation of biantennary N-linked oligosaccharides by cellulose-column chromatography.—To define a solvent suitable for quantitative elution of oligosaccharides, maltopentaose dissolved in (4:1:1) 1-butanol–ethanol–water was applied to a cellulose column (bed vol, 1 mL) equilibrated with (4:1:1) 1-butanol–ethanol–water and eluted with several solvents. We found that maltopentaose was quantitatively eluted with (1:1) ethanol–water (data not shown). We then attempted to prepare N-linked oligosaccharides from the glycoproteins by cellulose-column chromatography instead of conventional paper chro matography.

Human immunoglobulin G (hIgG, 7 mg), which is known to contain a series of biantennary complex oligosaccharides,[6] was subjected to hydrazinolysis, and half of the hydrazinolysate was separated by paper chromatography according to the conventional method.[5,6] The remaining half was dissolved in (4:1:1) 1-butanol–ethanol–water, and applied to a cellulose column (bed vol, 1 mL) equilibrated with the same solvent. After the column had been washed with the solvent, the column was eluted with 20 mL of (1:1) ethanol–water, followed by elution with 20 mL of water. As shown in Fig. 1, all hydrazinolysates of the protein portions of hIgG, but not oligosaccharides, were eluted in the (4:1:1) 1-butanol–ethanol–water fractions. The oligosaccharides, which were adsorbed to the cellulose column, were eluted in the (1:1) ethanol–water and water fractions.

The oligosaccharides eluted with (1:1) ethanol–water (Peak A in Fig. 1), those eluted with water (Peak B in Fig. 1), and oligosaccharides purified by paper chromatography were converted into ABEE-derivatives and

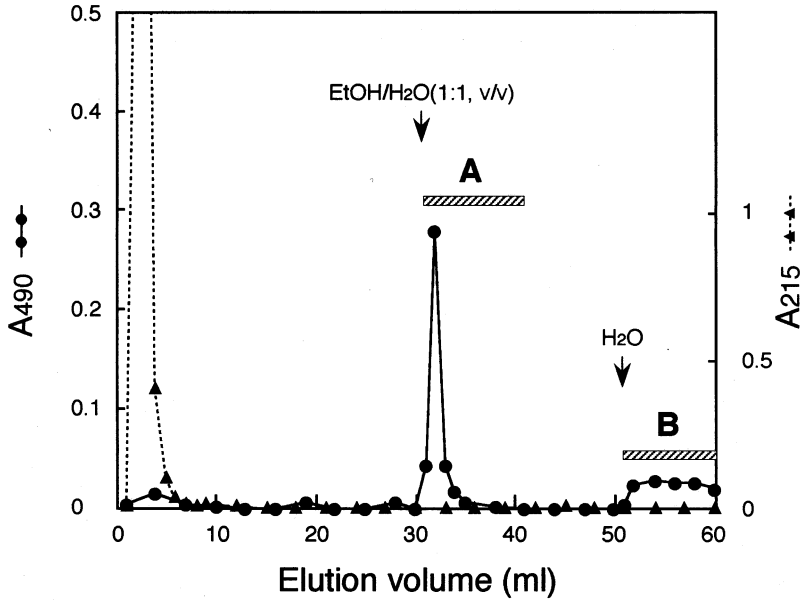

Fig. 1. Preparation of oligosaccharides derived from hIgG by cellulose-column chromatography. hIgG (7 mg) was subjected to hydrazinolysis, and half of the hydrazinolysate was applied to a cellulose column (bed vol, 1 mL) equilibrated with (4:1:1) 1-butanol–EtOH–water. The column was washed with 30 mL of (4:1:1) 1-butanol–EtOH–water, eluted in a stepwise manner with 20 mL each of (1:1) EtOH–water and water, and fractions (1 mL) were collected. The oligosaccharides in these fractions were detected by the phenol–H_2SO_4 method (●) and hydrazinolysate products of the protein portions were detected by measuring absorbance at 215 nm (▲).

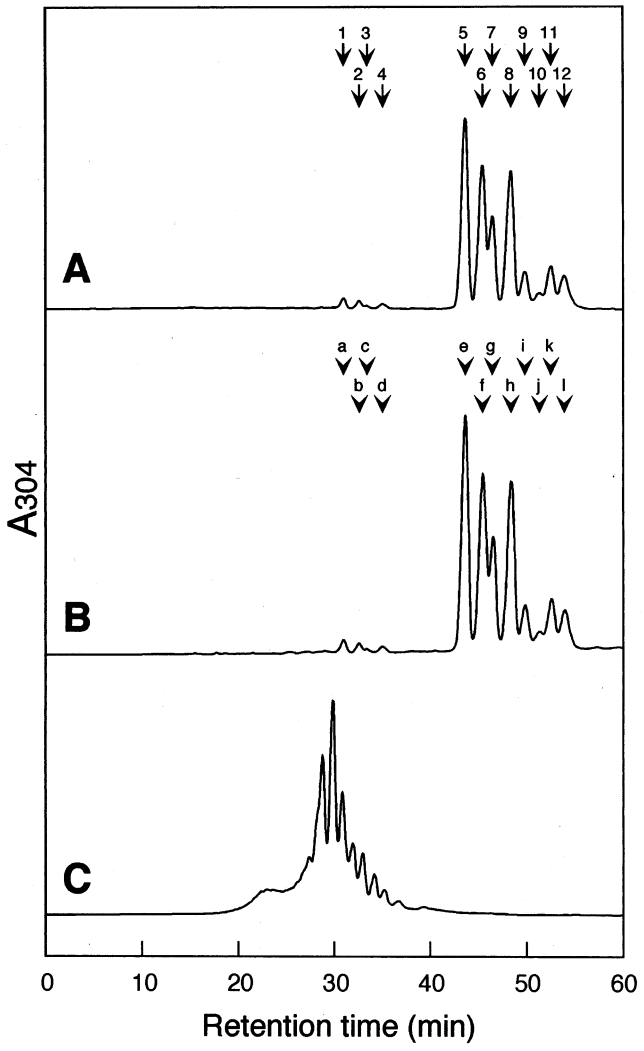

Fig. 2. Reversed-phase HPLC analysis of hIgG oligosaccharides prepared by cellulose-column chromatography. The oligosaccharides of hIgG prepared by cellulose-column chromatography (peaks A and B in Fig. 1) were converted into ABEE-derivatives. In addition, the hIgG oligosaccharides were prepared by paper chromatography and converted into ABEE-derivatives. The ABEE-oligosaccharides were then analyzed with reversed-phase HPLC using a Wakosil 5C18-200 column (0.46 × 25 cm) and eluted as Peaks a–l (indicated by arrowheads). Panels A, B, and C show the elution patterns of ABEE-oligosaccharides prepared by paper chromatography, patterns of those from Peak A (Fig. 1), and those from Peak B (Fig. 1), respectively. Arrows 1–12 indicate the elution positions of standard biantennary oligosaccharides as follows: 1, Gal-β-(1→4)GlcNAc-β-(1→2)Man-α-(1→6)[Gal-β-(1→4)GlcNAc-β-(1→2)Man-α-(1→3)]Man-β-(1→4)GlcNAc-β-(1→4)GlcNAc-ABEE; 2, Gal-β-(1→4)GlcNAc-β-(1→2)-Man - α - (1→6)[GlcNAc - β - (1→2)Man - α - (1→3)]Man - β-(1→4)GlcNAc-β-(1→4)GlcNAc-ABEE; 3, GlcNAc-β-(1→2)Man - α - (1→6)[Gal - β - (1→4)GlcNAc - β - (1→2)Man - α-(1→3)]Man-β-(1→4)GlcNAc-β-(1→4)GlcNAc-ABEE; 4, GlcNAc - β - (1→2)Man - α - (1→6)[GlcNAc - β - (1→2)Man - α-(1→3)]Man-β-(1→4)GlcNAc-β-(1→4)GlcNAc-ABEE; 5, Gal-β-(1→4)GlcNAc-β-(1→2)Man-α-(1→6)[Gal-β-(1→4)-GlcNAc - β - (1→2)Man - α - (1→3)]Man - β - (1→4)GlcNAc - β-(1→4)[Fuc - α - (1→6)]GlcNAc - ABEE; 6, Gal - β - (1→4)GlcNAc-β-(1→2)Man-α-(1→6)[GlcNAc-β-(1→2)Man-α-(1→3)]-Man - β - (1→4)GlcNAc - β - (1→4)[Fuc - α - (1→6)]GlcNAc-ABEE; 7, GlcNAc - β - (1→2)Man - α - (1→6)[Gal - β - (1→4)-GlcNAc - β - (1→2)Man - α - (1→3)]Man - β - (1→4)GlcNAc - β-(1→4)[Fuc-α-(1→6)]GlcNAc-ABEE; 8, GlcNAc-β-(1→2)Man - α - (1→6)[GlcNAc - β - (1→2)Man - α - (1→3)]Man - β-(1→4)GlcNAc-β-(1→4)[Fuc-α-(1→6)]GlcNAc-ABEE; 9, Gal - β - (1→4)GlcNAc - β - (1→2)Man - α - (1→6)[GlcNAc - β-(1→4)][Gal - β - (1→4)GlcNAc - β - (1→2)Man - α - (1→3)]Man-β-(1→4)GlcNAc-β-(1→4)[Fuc-α-(1→6)]GlcNAc-ABEE; 11, Gal - β - (1→4)GlcNAc - β - (1→2)Man - α - (1→6)[GlcNAc - β-(1→4)][GlcNAc - β - (1→2)Man - α - (1→3)]Man - β - (1→4)-GlcNAc-β-(1→4)[Fuc-α-(1→6)]GlcNAc-ABEE; 10, GlcNAc-β - (1→2)Man - β - (1→6)[GlcNAc - β - (1→4)][Gal - β - (1→4)-GlcNAc - β - (1→2)Man - α - (1→3)]Man - β - (1→4)GlcNAc - β-(1→4)[Fuc-α-(1→6)]GlcNAc-ABEE; 12, GlcNAc-β-(1→2)-Man - α - (1→6)[GlcNAc - β - (1→4)][GlcNAc - β - (1→2)Man - α-(1→3)]Man - β - (1→4)GlcNAc - β - (1→4)[Fuc - α - (1→6)]-GlcNAc-ABEE.

treated with sialidase, and the resulting neutral ABEE-oligosaccharides were analyzed by reversed-phase HPLC (Fig. 2). The ABEE-oligosaccharides obtained from the fractions eluted with (1:1) ethanol–water exhibited 12 oligosaccharide peaks (Peaks a–l), whose elution positions were identical to those of the hIgG oligosaccharides obtained by paper chromatography (Fig. 2(A)). Oligosaccharides a–l eluted at the same positions as those of a series of authentic biantennary oligosaccharides (1–12) described in Fig. 2 with structures of ± Gal-β-(1→4)GlcNAc-β-(1→2)Man-α-(1→6)[± GlcNAc-β-(1→4)][± Gal-β-(1→4)-GlcNAc - β - (1→2)Man - α - (1→3)]Man - β-(1→4)GlcNAc - β - (1→4)[± Fuc - α - (1→6)]-GlcNAc-ABEE. The structure of each ABEE-oligosaccharide prepared by cellulose-column chromatography was then analyzed by sequential exoglycosidase treatment with jack bean β-galactosidase and β-*N*-acetylhexosaminidase followed by reversed-phase HPLC. Incubation with jack bean β-galactosidase converted the 12 ABEE-oligosaccharides into three oligosaccharides d, h, and l which eluted at the same positions as the authentic biantennary agalacto-oligosaccharides 4, 8, and 12, respectively. Sequential incubation with β-galactosidase and β-*N*-acetylhexosaminidase of the 12 ABEE-oligosaccharide fractions resulted in their conversion into two oligosaccharide fractions which eluted at the same positions as authentic Man-α-(1→6)[Man - α-

(1→3)]Man - β - (1→4)GlcNAc - β - (1→4)-[Fuc-α-(1→6)]GlcNAc-ABEE and Man-α-(1→6)[Man-α-(1→3)]Man-β-(1→4)GlcNAc-β-(1→4)GlcNAc-ABEE (data not shown). Based on the behavior of oligosaccharides on the reversed-phase HPLC column and the results of sequential exoglycosidase treatment, we confirmed that structures of the ABEE-oligosaccharides in peaks a to l prepared by cellulose-column chromatography formed a series of biantennary N-linked oligosaccharides with the structure of ± Gal-β-(1→4)GlcNAc-β-(1→2)Man-α-(1→6)[± GlcNAc-β-(1→4)][± Gal-β-(1→4)GlcNAc-β-(1→2)-Man - α - (1→3)]Man - β - (1→4)GlcNAc - β-(1→4)[± Fuc-α-(1→6)]GlcNAc-ABEE, as previously shown using oligosaccharides of hIgG prepared by paper chromatography.[3,7] Therefore, oligosaccharides prepared by cellulose-column chromatography can be used for structural analysis with the same confidence as those prepared by paper chromatography after they have been converted into ABEE-derivatives. The molar ratios of ABEE-oligosaccharides prepared by cellulose-column chromatography and paper chromatography were then calculated on the basis of absorbance at 304 nm. As shown in Table 1, the molar ratios of each oligosaccharide in the IgG sample prepared on the cellulose column exhibited the same values as those prepared by conventional paper chromatography with an error of under 5%. On the other hand, ABEE-derivatives of oligosaccharides eluted with water exhibited intense peaks at a different position from that at which the major ABEE-oligosaccharides of hIgG eluted (Peaks e–l in Fig. 2(B)). These oligosaccharide peaks of the water-eluted fractions seemed to be impurities derived from the cellulose, since the oligosaccharides which eluted with water were also observed when chromatography was carried out without applying any sample (data not shown). It should be noted that in the fraction eluted with water, none of Peaks e–l corresponding to the major ABEE-oligosaccharides of hIgG were detected. On MALDI-TOF mass spectrometry analysis, none of these intense peaks gave a mass number corresponding to those calculated from the known structures of ABEE-oligosaccharides of IgG (data not shown), suggesting that IgG oligosaccharides were not present in the fraction eluted with water. In addition, ABEE-oligosaccharide peaks corresponding to the impurities were not detected in the fraction eluted with (1:1) ethanol–water (Fig. 2(B)). In the fractions, which eluted with (4:1:1) 1-butanol–ethanol–water, no evidence of ABEE-derivatives was detected on reversed-phase HPLC after these fractions had been subjected to the ABEE-derivative conversion (data not shown).

Table 1
Molar ratios of oligosaccharides in IgG prepared by cellulose-column chromatography and paper chromatography

Oligosaccharide	Oligosaccharides prepared with		Ratio (%)
	Cellulose-column chromatography (% of molar ratio)	Paper chromatography (% of molar ratio)	
a	1.07	1.08	99.1
b	0.86	0.85	101.2
c	0.22	0.21	104.8
d	0.58	0.56	103.6
e	26.00	25.64	101.4
f	21.17	20.95	101.1
g	10.55	11.10	95.0
h	20.61	20.37	101.2
i	5.26	5.35	98.3
j	2.03	2.09	97.1
k	6.17	6.40	96.4
l	5.48	5.40	101.5

ABEE-oligosaccharides prepared by cellulose-column chromatography or by paper chromatography were subjected to DEAE-HPLC to compare the recovery of sialylated oligosaccharides. Samples of the ABEE-oligosaccharides derived from hIgG separated into a neutral oligosaccharide fraction (N) and two sialylated oligosaccharide fractions (A1 and A2), as described previously.[7] The molar ratio of N, A1, and A2 of the oligosaccharides prepared by cellulose-column chromatography was 85.4:12.9:1.7 and that of the oligosaccharides prepared by paper chromatography was 84.9:13.4:1.7. These results indicated that the biantennary complex-type N-linked oligosaccharides (with or without sialic acid) from human IgG can be completely separated from hydrazinolysates of the protein portion of IgG and impurities derived from cellulose, and quantitatively recovered in fractions eluted with (1:1) ethanol–water without any selection of these oligosaccharides.

When the oligosaccharides were prepared from the hydrazinolysates of 200 μg of hIgG (which contained approximately 6 μg of oligosaccharides) by means of cellulose-column chromatography, elution profiles of the ABEE-oligosaccharides and the ratio of each ABEE-oligosaccharide were the same on reversed-phase HPLC as in the case of oligosaccharides from 3.5 mg hIgG (data not shown). This indicated that the oligosaccharides could be quantitatively prepared by cellulose-column chromatography even from 200 μg of hIgG without contamination of impurities and selection of oligosaccharides.

Preparation of other types of N-linked oligosaccharides by cellulose-column chromatography.—We examined whether the conditions for the above elution from the cellulose column could be applied to the preparation of other types of N-linked oligosaccharides of glycoproteins, i.e., the tri- and tetraantennary complex type, the hybrid type or the high mannose type. α1-Acid glycoprotein, ovalbumin and RNase B (each 5 mg), which are known to contain tri- and tetraantennary complex type oligosaccharides with or without sialic acid, hybrid type oligosaccharides and high mannose type oligosaccharides, respectively,[8–10] were subjected to hydrazinolysis, and the resulting hydrazinolysates were subjected to cellulose-column chromatography as described above. The fractions which eluted with (1:1) ethanol–water were collected and analyzed by reversed-phase HPLC after being treated with sialidase and subjected to the procedure for conversion into ABEE-derivatives. ABEE-oligosaccharides of α1-acid glycoprotein (Fig. 3(A)), ovalbumin (Fig. 3(B)) or RNase B (Fig. 3(C)) prepared by cellulose-column chromatography exhibited elution profiles on reversed-phase HPLC which were identical to those prepared by paper chromatography. The peak ratios of these ABEE-oligosaccharides prepared on the cellulose column were also identical to those prepared by paper chromatography with an error of under 5% (data not shown). These results indicate that oligosaccharides of the tri- and tetraantennary complex type with or without sialic acid, the hybrid type and the high mannose type can also be quantitatively isolated from hydrazinolysates of glycoproteins without serious contamination or selection of oligosaccharides using cellulose-column chromatography and elution with (1:1) ethanol–water.

Large scale preparation of oligosaccharides.—In order to investigate whether cellulose-column chromatography can be applied to the preparation of a large amount of N-linked oligosaccharides, the hydrazinolysate of 100 mg of RNase B (4.2 mg as hexose) was subjected to chromatography on a cellulose column (bed vol, 1 mL). As shown in Fig. 4, oligosaccharides were not eluted in the (4:1:1) 1-butanol–ethanol–water fraction. Instead, they were eluted with (1:1) ethanol–water, and the total amount of hexoses in these fractions was 4.1 mg, indicating that 97.6% of the N-linked oligosaccharides derived from the 100 mg RNase B were recovered in the (1:1) ethanol–water fractions. Therefore, a 1-mL bed volume of cellulose had a sufficient capacity for the N-linked oligosaccharides.

3. Discussion

In the present study, we established a rapid and simple method for the preparation of large amounts of typical types of N-linked oligosaccharides of glycoproteins, i.e., high

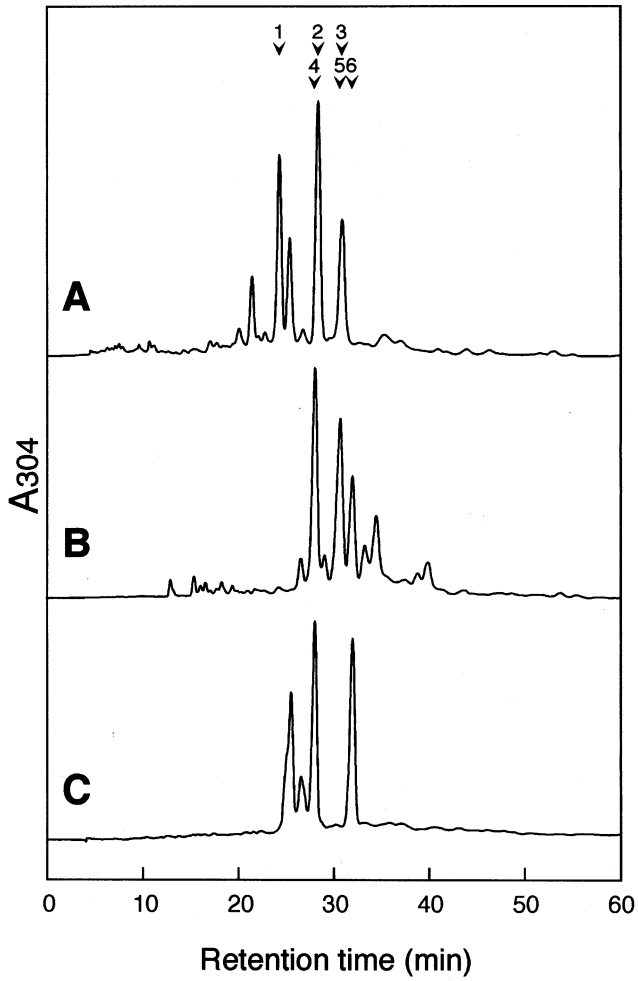

Fig. 3. HPLC analysis of various types of N-linked oligosaccharides prepared by cellulose-column chromatography. α1-Acid glycoprotein (panel A), ovalbumin (panel B), and RNase B (panel C) were subjected to hydrazinolysis and the hydrazinolysates were subjected to cellulose-column chromatography. The oligosaccharides that eluted from the column with (1:1) EtOH–water were converted into ABEE-derivatives and subjected to reversed-phase HPLC using a Wakosil 5C18-200 column as described in the Experimental Section. Arrowheads 1–6 indicate the elution positions of standard oligosaccharides as follows: 1, Gal-β-(1→4)GlcNAc-β-(1→6)[Gal-β-(1→4)GlcNAc-β-(1→2)]Man-α-(1→6)[Gal-β-(1→4)GlcNAc-β-(1→4)[Gal-β-(1→4)GlcNAc-β-(1→2)]Man-α-(1→3)]Man-β-(1→4)GlcNAc-β-(1→4)GlcNAc-ABEE; 2, Gal-β-(1→4)-GlcNAc-β-(1→2)Man-α-(1→6)[Gal-β-(1→4)GlcNAc-β-(1→4)[Gal-β-(1→4)GlcNAc-β-(1→2)]Man-α-(1→3)]Man-β-(1→4)GlcNAc-β-(1→4)GlcNAc-ABEE; 3, Gal-β-(1→4)-GlcNAc-β-(1→2)Man-α-(1→6)[Gal-β-(1→4)GlcNAc-β-(1→2)-Man-α-(1→3)]Man-β-(1→4)GlcNAc-β-(1→4)GlcNAc-ABEE; 4, [Man-α-(1→2)][Man-α-(1→6)][Man-α-(1→3)]Man-α-(1→6)[Man-α-(1→3)]Man-β-(1→4)GlcNAc-β-(1→4)GlcNAc-ABEE; 5, Man-α-(1→6)[Man-α-(1→3)]Man-α-(1→6)[GlcNAc-β-(1→4)][GlcNAc-β-(1→2)Man-α-(1→3)]-Man-β-(1→4)GlcNAc-β-(1→4)GlcNAc-ABEE; 6, [Man-α-(1→6)][Man-α-(1→3)]Man-α-(1→6)[Man-α-(1→3)]Man-β-(1→4)GlcNAc-β-(1→4)GlcNAc-ABEE.

mannose type, hybrid-type, and bi-, tri-, and tetraantennary complex-type oligosaccharides with or without sialic acid, by means of cellulose-column chromatography. These N-linked oligosaccharides were quantitatively eluted with (1:1) ethanol–water from a cellulose column equilibrated with (4:1:1) 1-butanol–ethanol–water without any contamination by hydrazinolysates of the protein portion of glycoproteins or impurities derived from the cellulose. The ABEE-derivatives of the oligosaccharides prepared in this manner exhibited elution patterns identical to those obtained by paper chromatography on subsequent reversed-phase HPLC at the peak positions and in peak ratios. In addition, structures of the N-linked oligosaccharides prepared with the cellulose column could be analyzed by sequential exo-glycosidase digestion as could the oligosaccharides prepared by paper chromatography. These results indicate that oligosaccharides eluted from cellulose columns with (1:1) ethanol–water can be used for structural and biological analyses and have the same quality as those prepared by paper chromatography. Therefore, our method of cellulose-column chromatography can replace conventional paper chromatography for preparation of oligosaccharides. Preparation on a cellulose column and elution with water has already been reported.[11] However, impurities from the cellulose are also eluted with water together with the oligosaccharides and these impurities may interfere with subsequent purification of these oligosaccharides and their structural analyses, since ABEE-derivatives of cellulose-derived impurities gave extensive peaks on reversed-phase HPLC at the same position (retention time 25–35 min) at which ABEE-derivatives of typical types of N-linked oligosaccharides were eluted (Figs. 2 and 3).

Cellulose-column chromatography seems to have an advantage over paper chromatography in preparing N-linked oligosaccharides in view of the following: (i) the former is much simpler and more rapid than the latter; and (ii) is much more suitable for mass preparation of N-linked oligosaccharides from glycoproteins. The 1 mL bed volume of cellulose used in this study adsorbed the oligosaccharides obtained from 100 mg of RNase B (Fig. 4), indicating that 1 mL of cellulose had a sufficient adsorption capacity for the N-linked oligosaccharides. In addition, it is also possi-

ble to prepare N-linked oligosaccharides from multiple samples at the same time by increasing the number of columns.

4. Experimental

Reagents and enzymes.—Human immunoglobulin G, bovine pancreas ribonuclease B, and α1-acid glycoprotein were purchased from Sigma Chemical Co. Maltopentaose and neuraminidase derived from *Arthrobacter ureafaciens* were from Nakalai Tesque Inc. (Kyoto, Japan). Cellulose microcrystalline was purchased from Merck (Germany). *p*-Aminobenzoic acid ethyl ester (ABEE) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Jack bean meal β-galactosidase and β-*N*-acetylhexosaminidase were purified according to a method previously reported.[12] Galactosidase digestion of oligosaccharides was performed as described previously.[5,6,13] Other chemicals used were of reagent grade. The standard ABEE-oligosaccharides were obtained from purified oligosaccharides of hIgG, RNase B, and α1-acid glycoprotein, as described previously.[6,8,9]

Liberation of N-linked oligosaccharides by hydrazinolysis.—Hydrazinolysis was performed according to a method previously reported.[5] Gas-phase hydrazinolysis was performed using Hydraclub S204 (Honen Corporation, Tokyo) at 90 °C for 3 h to liberate N-linked oligosaccharides from glycoprotein samples. The products were then N-acetylated, desalted, and used for separation by cellulose chromatography.

Cellulose-column chromatography.—The cellulose column (0.7 × 2.6 cm, bed vol 1 mL) was washed with 10 mL of water and then with 10 mL of a solvent for elution of N-linked oligosaccharides to prevent contamination by cellulose-derived materials into the oligosaccharide fraction. The column was then equilibrated with 10 mL of (4:1:1, v/v) 1-butanol–EtOH–water. Hydrazinolysates of glycoproteins or oligosaccharides dissolved in (4:1:1, v/v) 1-butanol–EtOH–water were applied to the column. After the column had been washed with 30 mL of (4:1:1, v/v) 1-butanol–EtOH–water, oligosaccharides were

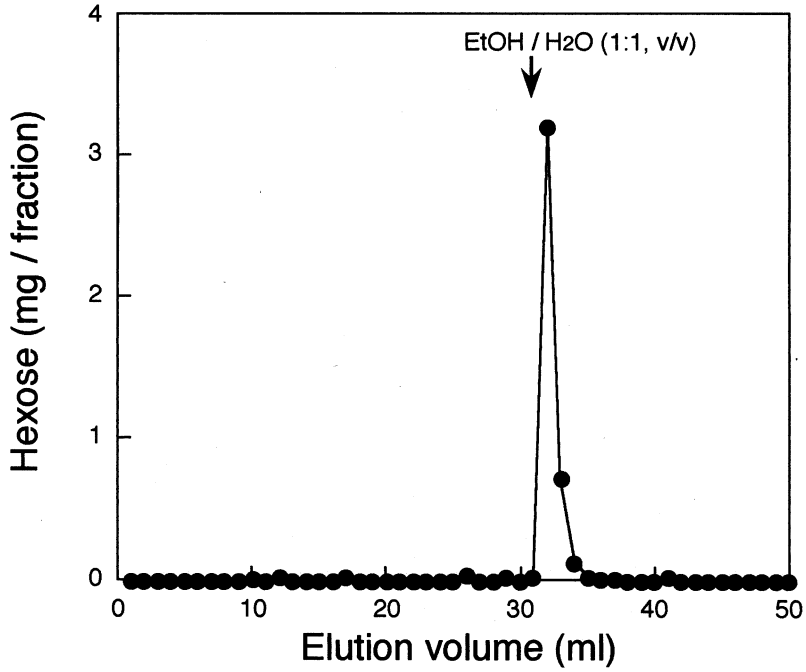

Fig. 4. Cellulose-column chromatography of the hydrazinolysate of 100 mg of ribonuclease B. RNase B (100 mg of protein, 4.2 mg of hexose) was subjected to hydrazinolysis and the hydrazinolysate was applied to a cellulose column (1 mL bed vol) equilibrated with (4:1:1) 1-butanol–EtOH–water. The column was washed with 30 mL of (4:1:1) 1-butanol–EtOH–water and eluted with 20 mL of (1:1) EtOH–water. Fractions (1 mL) were collected, and hexoses in the fractions were determined by the phenol–H_2SO_4 method.

eluted with appropriate volumes of elution solvents (water or (1:1, v/v) EtOH–water). Fractions (1 mL) were collected and dried, and after dissolving each fraction in 1 mL of water, aliquots were treated using the phenol–H_2SO_4 method to determine hexoses contained in the oligosaccharides. Hydrazinolysate products of the protein portion of glycoprotein were detected by measuring absorbance at 215 nm.

Paper chromatography.—Paper chromatography was performed according to a method previously reported.[5,6] Hydrazinolysates of glycoproteins were applied onto a filter paper (Whattman 3MM) and developed with (4:1:1, v/v) 1-butanol–EtOH–water overnight. Then, an area containing oligosaccharides was cut out and the oligosaccharides were extracted from the paper with water.

Structural analysis of oligosaccharides.—The oligosaccharides prepared with cellulose-column chromatography or paper chromatography were converted into ABEE-derivatives by introducing ABEE at their reducing termini by reductive amination, as described previously.[7,14]

For structural analyses of oligosaccharides derived from hIgG and α1-acid glycoprotein, the ABEE-derivatives of the respective oligosaccharides were digested with 100 mU of neuraminidase in 70 μL of 0.1 M acetate buffer (pH 5.0) at 37 °C for 18 h, and the resulting neutral oligosaccharides were collected by ion-exchange HPLC with a COSMOGEL DEAE column (7.5×75 mm, Nacalai Tesque Inc.). Desialylated oligosaccharides derived from the glycoproteins were analyzed with reversed-phase HPLC using a Wakosil 5C18-200 column (0.46×25 cm, Wako Pure Chemical Industries) as described previously.[7] ABEE-derivatives were detected by monitoring absorbance at 304 nm using an ultraviolet spectrophotometer SPD-6AV (Shimadzu). Analysis of sialylation of the ABEE-oligosaccharides derived from hIgG was performed by ion-exchange HPLC with a COSMOGEL DEAE column (7.5×75 mm) as described previously.[7]

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