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Chromatographic Separation of Asparagine-linked Oligosaccharides Labeled with an Ultraviolet-absorbing Compound, *p*-Aminobenzoic Acid Ethyl Ester

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We have expanded on the suitability of *p*-aminobenzoic acid ethyl ester as an ultraviolet-absorbing reagent [Wang *et al.*, (1984) Anal Biochem 141:366-81] for the analysis of asparagine-linked oligosaccharides derived from glycoproteins. The oligosaccharides released from glycoproteins by hydrazinolysis/*N*-reacetylation were derivatized with *p*-aminobenzoic acid ethyl ester and the derivatives were purified and separated into neutral and acidic oligosaccharides on a PRE-SEP C_{18} cartridge. The acidic oligosaccharides could be further separated into a few species by high-voltage paper electrophoresis.

***p*-Aminobenzoic acid ethyl ester derivatives of neutral oligosaccharides were analyzed by gel permeation chromatography on Bio-Gel P-4 and HPLC on a silica-based amide column. The elution profile and the proportion of the oligosaccharides were in agreement with literature values. The overall yield of oligosaccharides from glycoproteins was approximately 70%. Fifty pmol of oligosaccharide were detectable on Bio-Gel P-4 and 4-5 pmol on HPLC.**

For an understanding of the biological functions of glycoconjugates such as glycolipids and glycoproteins, the complete structures of the carbohydrate moieties of glycoconjugates must be characterized. Numerous methods such as gas-liquid chromatography [1], paper chromatography [2], gel permeation chromatography [3, 4] and high performance liquid chromatography (HPLC) [5] have been developed for the separation of oligosaccharides. Gel filtration requires a relatively long time to develop the column, but is the most useful technique for the separation of oligosaccharides. HPLC has become an especially powerful analytical tool for oligosaccharides [5].

Abbreviations: HPLC, high performance liquid chromatography; NABEE, *p*-aminobenzoic acid ethyl ester; FAB-MS, fast-atom bombardment mass spectrometry; $(GlcNAc)_2$, $(GlcNAc)_3$, $(GlcNAc)_4$, $(GlcNAc)_5$ and $(GlcNAc)_6$, chito-oligosaccharides containing 2, 3, 4, 5 and 6 residues of *N*-acetylglucosamine.

The major drawback has been associated with the detection of oligosaccharides separated by these chromatographic methods. Refractive index monitoring and post-column carbohydrate quantification by colorimetric analysis are not sufficiently sensitive to detect samples at sub-nanomolar levels. Kobata and co-workers [3, 6] have developed a method for the detection of oligosaccharides at a micro-scale level by labeling the oligosaccharide moieties with tritium *via* NaB^3H_4 reduction. This method has been widely used for the structural analysis of many kinds of oligosaccharides. This method, however, requires expensive reagents and instrumentation and special facilities to use radioactive compounds. Fluorescent derivatization methodology of neutral oligosaccharide mixtures has been developed for a rapid and sensitive separation of oligosaccharides on HPLC [7-10]. This method has become one of the most convenient methods for analysis of Asn-linked oligosaccharides.

Wang *et al.* [11] recently reported a method for labeling oligosaccharides with ultraviolet (UV)-absorbing compounds, i.e. aniline, *p*-aminoacetophenone and *p*-aminobenzoic acid ethyl ester (ABEE), by the procedure of reductive amination [12-14]. The UV-absorbing derivatives, especially ABEE derivatives, have been proved to be useful for sensitive separation of oligosaccharides by HPLC. They were also useful as labels for monitoring the sequential glycosidase digestions of oligosaccharides. In addition, the derivatives were suitable for structural analysis of oligosaccharides by fast-atom bombardment-mass spectrometry (FAB-MS).

In this paper, we expand on the suitability of ABEE as the UV-absorbing reagent for separation and detection of Asn-linked oligosaccharides. We describe the derivatization procedure for Asn-linked oligosaccharides released from glycoproteins, purification procedures of the derivatives, and the separation of the derivatives by gel permeation and HPLC techniques.

Materials and Methods

Materials

p-Aminobenzoic acid ethyl ester (ABEE) was purchased from Wako Pure Chemical Industries (Osaka, Japan) and sodium cyanoborohydride from Nakarai Chemicals (Kyoto, Japan). Anhydrous hydrazine was obtained from Aldrich (Milwaukee, WI, USA).

Isomalto-oligosaccharides were prepared by partial acid hydrolysis of dextran [15]. A series of high-mannose oligosaccharides was purified from the urine of patients with α-mannosidosis as described previously [16]. Manβ1-4GlcNAc, Manβ1-4GlcNAcβ1-4GlcNAc, Manβ1-4GlcNAcβ1-4Manβ1-4GlcNAc and Manβ1-4GlcNAcβ1-4Manβ1-4GlcNAcβ1-4GlcNAc were isolated from a kidney of a goat with β-mannosidosis [17]. A series of malto-oligosaccharides was obtained from Hayashibara Biochemical Lab. (Okayama, Japan) and Nakarai Chemicals Ltd. A series of chito-oligosaccharides was purchased from Seikagaku Kogyo Co. (Tokyo, Japan) and purified by Bio-Gel P-4 (200-400 mesh; Bio-Rad Labs, Richmond, CA, USA) column chromatography.

Fetuin (Type IV), asialofetuin (Type II) and hen ovomucoid (type III-O) were obtained from Sigma Chemical Co. (St. Louis, MO, USA), and human chorionic gonadotropin (hCG) from Sankyou Zoki (Tokyo, Japan). Neuraminidase from *Arthrobacter ureafaciens* was obtained from Nakarai Chemicals. All other chemicals were of commercially available analytical grade.

General Methods

Thin-layer chromatography (TLC) was performed on Silica Gel 60 plates (E. Merck, Darmstadt, W. Germany). The plate was developed twice in acetonitrile/water, 4/1 by vol. Following separation, the UV-absorbing oligosaccharides were detected by densitometric TLC-scanning with a Shimadzu CS-930 dual wave length TLC scanner (Shimadzu Co., Kyoto, Japan) at 304 nm or orcinol/H_2SO_4 reagent [18]. Silicic acid column chromatography was performed on a column packed with 20 g of Iatrobeads 6RS 8060 (Iatron Laboratories, Tokyo, Japan) using acetonitrile/water, 4/1 by vol, as eluting solvent. ^{13}C-NMR spectra were recorded for solutions in ^2H_2O using a JEOL FX-100 NMR spectrometer. Chemical shifts are given relative to sodium 2,2-dimethyl-2-silapentane-5-sulphonate. FAB-MS spectra were recorded on a Shimadzu GC-MS 9020-DF mass spectrometer fitted with a FAB ion source. The UV absorption spectra of the ABEE derivatives of oligosaccharides were recorded on a Shimadzu UV-260 spectrophotometer.

High voltage paper electrophoresis was performed using pyridine-acetate buffer, pH 5.4 (pyridine/acetic acid/water, 3/1/387 by vol) at 76 V/cm for 70 min. The UV-absorbing compounds were detected with a densitometric TLC-scanner at 304 nm.

Neuraminidase digestion was performed in 50 mM sodium acetate buffer, pH 5.4 for 20 h at 37°C.

Derivatization of Oligosaccharides with ABEE

Up to 2.8 μmol of the oligosaccharides were dissolved in 10 μl of water. To this solution was added 40 μl of reagent mixture freshly made by mixing 35 mg (210 μmol) of ABEE, 3.5 mg (55 μmol) $NaBH_3CN$, 41 μl of glacial acetic acid and 350 μl of absolute methanol. In a screw-capped reaction vial, the mixture was heated at 80°C for 1 h for neutral oligosaccharides or 30 min for acidic oligosaccharides with continual stirring. To this mixture, 1 ml of water was added. The mixture was extracted five times with 1 ml portions of diethyl ether to remove excess ABEE. The aqueous layer was lyophilized, and the residue was dissolved in an appropriate volume of water for subsequent analysis.

Hydrazinolysis and N-Reacetylation of Glycoprotein

Hydrazinolysis/*N*-reacetylation was performed with the procedures published [19, 20]. Glycoprotein (approx. 20 mg) was thoroughly dried in a vacuum desiccator over P_2O_5 for three days *in vacuo*. The vacuum was released and the sample was suspended in 2.0 ml of anhydrous hydrazine in a glove box under Ar atmosphere. The suspension was heated at 100°C for 10 h, and then lyophilized to remove hydrazine. The residue was dissolved in freshly prepared saturated $NaHCO_3$ solution (1 ml/mg glycoprotein), and *N*-reacetylated at room temperature by adding acetic anhydride (25 μl/ml $NaHCO_3$ solution), and then repeating addition of three similar portions of acetic anhydride at 15 min intervals. Thirty min after the last addition of acetic anhydride, the solution was applied to a column of Dowex 50-X8 (H^+, 200-400 mesh, 1 ml resin/ml $NaHCO_3$). The column was washed with five bed volumes of water. A few drops of octanol were added to the eluate. The eluate was then evaporated to dryness at 40°C using a rotary evaporator. Without further purification the residue was subjected to ABEE derivatization by the procedure described above.

Table 1. The molecular extinction coefficients at 304 nm of oligosaccharide-ABEE and the elution positions of oligosaccharide-ABEE relative to the elution positions of isomalto-oligosaccharides-ABEE as expressed as glucose units.

Oligosaccharides	E_{304}(A/M cm $^{-1}$)[a]	Glucose unit
N-acetylglucosamine	1.98×10^4	2.8
lactose	2.20×10^4	2.5
maltose	2.23×10^4	1.9
maltopentaose	2.29×10^4	3.8
maltohexaose	—	4.3
$GlcNAc_2$	—	4.9
$GlcNAc_3$	1.95×10^4	7.3
$GlcNAc_4$	2.09×10^4	9.7
$GlcNAc_5$	—	12.2
$GlcNAc_6$	—	14.8
Manβ1-4GlcNAc	—	3.9
Manβ1-4GlcNAcβ1-4GlcNAc	—	6.1
Manβ1-4GlcNAcβ1-4Manβ1-4GlcNAcβ1-4GlcNAc	—	9.3
Manα1-3Manβ1-4GlcNAc	—	4.6
Manα1-2Manα1-3Manβ1-4GlcNAc	—	5.6
Manα1-2Manα1-2Manα1-3Manβ1-4GlcNAc	—	6.6
lacto-*N*-fucopentaose II	—	6.5

[a] E_{304} is the molecular extinction coefficient at 304 nm, A absorbance and M the concentration in mole per liter.

Purification of ABEE Derivatives on a PRE-SEP C_{18} Cartridge

The ABEE derivative partially purified by ether extraction was dissolved in 2 ml of water. The solution was applied to a PRE-SEP C_{18} cartridge (Senetek PLC, Mountain View, CA, USA), which was pre-conditioned by washing with 5 ml of acetonitrile and 10 ml of water. The column was first eluted with water, collecting 2 ml of eluate in each tube. This was followed by measuring the UV absorbance spectrum in each tube. The elution was continued until no more UV absorbance could be observed. The column was eluted in a stepwise manner with water/acetonitrile, 95/5, 9/1, 4/1 by vol, and then acetonitrile in the same manner. The eluate was combined according to the ultraviolet characteristics and evaporated to dryness for further separations. The cartridge can be re-used at least 10 times by washing with acetonitrile and water as described.

Gel Permeation Chromatography

The chromatographic system used in this study was essentially the same as that described by Yamashita *et al.* [3]. Extensively de-fined Bio-Gel P-4 (-400 mesh) was packed into a water-jacketed 2.0 × 100 cm column (Umetani Seiki, Osaka, Japan). The column was kept at 50°C by circulating warm water in the jacket. The aqueous solution of the sample (1 ml) was injected on to the column *via* a switching valve fitted with a 1 ml loop using a syringe fitted with a membrane filter (GV, 0.22 μm, Millipore, Bedford, MA, USA). The column was eluted with de-gassed distilled water containing 0.01% sodium azide at a constant flow rate of 22.0 ml/h using a single plunger pump (NP-S-251U, Umetani Seiki).

The eluate was led into a UV spectrophotometric detector (Shimadzu SPD-6AV UV-VIS spectrophotometric detector) for continuous monitoring of the ultraviolet absorbancies at 304 nm. After passing through the sample cell, the eluate flowed into either a fraction collector or, when dual detection was desired, the refractive-index monitor (Shimadzu RID-6A) prior to collection. A dual-pen recorder, Unicorder U-228 (Nippon Denshi Kagaku), was used for simultaneous recording of both the ultraviolet and refractive index readings.

A water-jacketed 2.0 × 100 cm column containing Bio-Gel P-4 (200-400 mesh) was operated under the same conditions except for the flow rate (29.0 ml/h), as described above.

High Performance Liquid Chromatography

HPLC analysis was performed with a Shimadzu LC-6A liquid chromatograph equipped with a Rheodyne injection valve. Separation of ABEE derivatives of neutral oligosaccharides was carried out on a 0.4 × 25 cm column packed with TSK-GEL Amide 80 (silica based amide, 5 micron, Toyosoda, Tokyo, Japan) at 60°C. Elution was done using an acetonitrile-water mixture at a flow rate of 1 ml/min with a linear gradient as described in the figures. Oligosaccharides-ABEE were detected by UV spectrophotometric detector at 304 nm.

Results

Ultraviolet Absorption Characteristics of Oligosaccharides-ABEE

To examine the UV absorption characteristics, 20-100 mg of a number of different neutral sugars were derivatized with ABEE. The derivatives were purified by Bio-Gel P-4 (200-400 mesh) column chromatography and/or Iatrobeads column chromatography. The purity and the structures of the derivatives were confirmed by TLC, ^{13}C-NMR and/or FAB-MS.

As shown in Fig. 1, maltopentaose and $(GlcNAc)_4$ exhibited similar spectra giving absorption bands at 226 nm and 304 nm. No detectable spectra shifts were observed for any of the other oligosaccharides examined in this paper, including sialyllactose-ABEE (Fig. 1C). The molar absorption coefficients of the derivatives at 304 nm were determined and are shown in Table 1. The molar absorption coefficients did not vary according to the degree of polymerization of the sugar moieties but were sensitive to the type of residue at the reducing end. The ABEE derivatives with aldohexose as the reducing end sugar moiety gave slightly higher values than those of oligosaccharide derivatives with *N*-acetylglucosamine as the reducing end sugar moiety.

These results demonstrate that the derivatization of oligosaccharides with ABEE makes it possible to monitor quantitatively and at high sensitivity the elution of oligosaccharides from chromatographies without the interference of UV-absorbance of the eluting solvent.

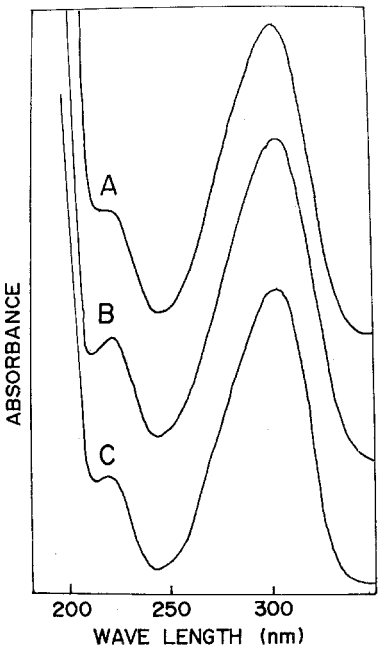

Figure 1. The ultraviolet absorption spectra of ABEE derivatives of (A) $GlcNAc_4$, (B) maltopentaose and (C) sialyllactose in aqueous solution. The spectra were recorded with a Shimadzu UV-260 spectrophotometer.

Derivatization Conditions

Effect of temperature (40°C, 60°C and 80°C), reaction time (30 min - 48 h) and the amounts of reagent (ABEE and $NaBH_3CN$) in the reductive amination were investigated using maltopentaose, $(GlcNAc)_4$ and sialyllactose. The extent of the reaction was estimated from quantification by spectrophotometric measurement at 304 nm. E_{304} ($A/M\ cm^{-1}$) of 2.2×10^4 was used for oligosaccharides having hexose at the reducing end and 2.0×10^4 for those having *N*-acetylglucosamine at the reducing end. The derivatization of maltopentaose was complete at 40°C and 60°C within a few hours. However, $(GlcNAc)_4$ was not derivatized quantitatively under these conditions. The recovery of $(GlcNAc)_4$-ABEE was 92% after heating at 80°C for 30 min and reached a maximum (94%) after heating at 80°C for 1.0 h. The amounts of ABEE and $NaBH_3CN$ could be reduced to 1/5 and 1/10 of those originally published [11], respectively. The lesser amounts of the reagents made it easy to treat the reagent mixture and to remove the excess ABEE by ether extraction.

In order to examine the stability of the sialic acid residue, sialyllactose-ABEE was treated with a reagent mixture at 80°C, for 30 min, 1 h, 1.5 h and 2.0 h. The reaction mixture was analyzed by paper electrophoresis for the detection of released lactose-ABEE. Less than 3% of total UV-absorbance was detected at the position of lactose-ABEE after heating for 30 min. However, the UV-absorbance found in the lactose-ABEE region was increased to 8%, 12% and 20% after heating for 1 h, 1.5 h and 2.0 h, respectively.

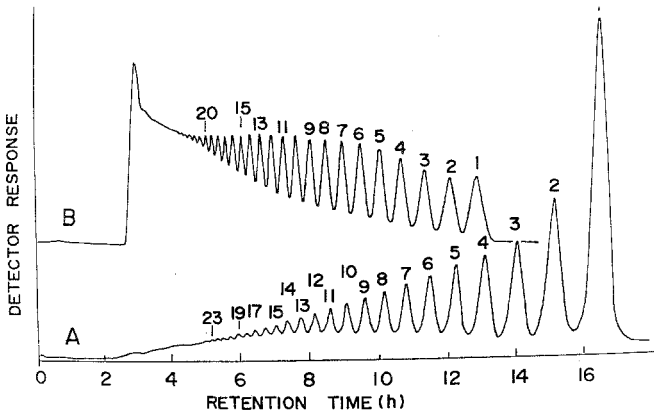

Figure 2. Chromatography of (A) isomalto-oligosaccharides-ABEE and (B) non-derivatized isomalto-oligosaccharides on a 2.0 × 100 cm column of Bio-Gel P-4 (-400 mesh). Isomalto-oligosaccharides (2.0 mg) were derivatized with ABEE by reductive amination under the conditions described in the Materials and Methods section. A solution (1 ml) containing 1/5 of the isomalto-oligosaccharides-ABEE and 8 mg of non-derivatized isomalto-oligosaccharides was injected. The column was eluted at 50°C in H_2O with a flow rate of 22 ml/h. Isomalto-oligosaccharides-ABEE and free isomalto-oligosaccharides were simultaneously detected with an ultraviolet spectrophotometric detector (304 nm) and refractive-index monitor, respectively. The degree of polymerization is indicated by the numbers over the peaks.

Summarizing these results we adopted the reaction conditions described in the Materials and Methods section. Under these reaction conditions, the recoveries of the ABEE-adducts from the oligosaccharides listed in Table 1 were 92-94%.

Bio-Gel P-4 Chromatography

The elution profile shown in Fig. 2A demonstrates that ABEE derivatives of isomalto-oligosaccharides which differ by only one hexose unit can be effectively separated on a 2.0 × 100 cm column of Bio-Gel P-4 (-400 mesh). The ABEE derivatives were retarded to some extent compared with non-derivatized isomalto-oligosaccharides (Fig. 2B). This effect may arise from the aromatic adsorption which is notable on Sephadex gels [21] Compared with the separation of non-derivatized isomalto-oligosaccharides and NaB^3H_4-reduced isomalto-oligosaccharides [3, 4], ABEE derivatives were more completely resolved on Bio-Gel P-4. A plot of the logarithm of the molecular weight versus the retention time for each of the ABEE derivatives of the isomalto-oligosaccharides mixture revealed a linear relationship, as shown in Fig. 3. Various oligosaccharides with known structures were analyzed on a single column and the results are presented in Table 1. *N*-Acetylglucosamine at the reducing end approximated 2.8 glucose units in size, whereas internal *N*-acetylglucosamine was equivalent to about 2.4 glucose units. Hexose residues behaved as 0.6 glucose units in malto-oligosaccharides and 1.0 glucose equivalent in mannose-containing oligosaccharides. The retention behavior of ABEE-derivatives on Bio-Gel P-4 was similar to that observed for tritium-labeled oligosaccharides [3, 4]. The results demonstrate that the size and species of the components of an oligosaccharide can be predicted by its retention behavior as an ABEE derivative on Bio-Gel P-4.

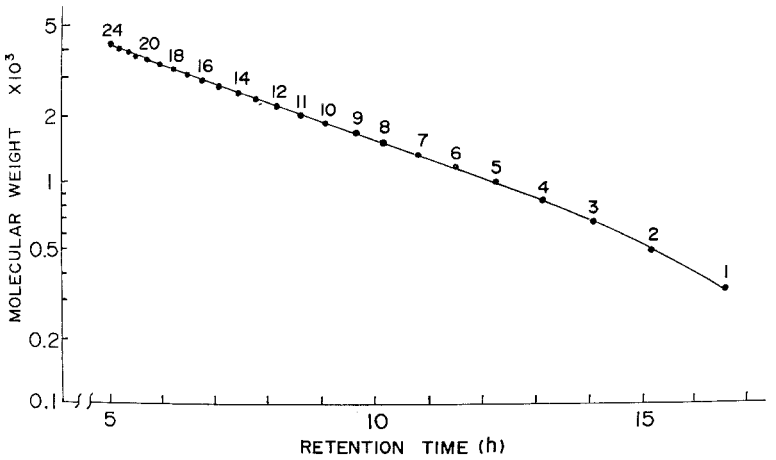

Figure 3. Plot of the logarithm of molecular weights of isomalto-oligosaccharides-ABEE versus the retention times. The data were obtained on a 2.0 × 100 cm column of Bio-Gel P-4 (-400 mesh). The numbers indicate the degree of polymerization.

To determine the sensitivity and concentration range in which oligosaccharides-ABEE may be quantified, aliquots containing from 50 pmol to 500 nmol of lactose-ABEE were injected and monitored at 1/10 of maximum sensitivity of the detector. As little as 50 pmol of lactose-ABEE was detected and the areas of the peaks showed a linear relationship to the molar ratio. In order to examine the chromatographic properties of the derivatives, the mixture of underivatized isomalto-oligosaccharides and their ABEE derivatives which was stored at 4°C was repeatedly analyzed on the same column. The chromatographic properties of ABEE derivatives relative to underivatized standard isomalto-oligosaccharides did not change significantly for over two years. For precise analytical purposes the injection of standard isomalto-oligosaccharides along with unknown ABEE derivatives is recommended.

Application to Glycoproteins

Release of Oligosaccharides and Coupling Reaction: Release of Asn-linked oligosaccharides from glycoproteins was performed by hydrazinolysis/*N*-reacetylation [19, 20]. The reaction mixture solution in saturated $NaHCO_3$ was applied to a column of Dowex-50 (H^+) and the column was washed with water. The combined washings and eluate were evaporated to dryness and the residue was coupled with ABEE under the conditions described above. The reaction mixture was diluted with water and extracted with ether. The aqueous layer was frozen and lyophilized.

Purification of ABEE Derivatives: ABEE derivatives obtained above showed very strong ultraviolet absorption bands in the range of 200-320 nm which interfered with the detection of oligosaccharides-ABEE at 304 nm on Bio-Gel P-4 chromatography and HPLC. The aqueous solution of the reaction mixture was applied to a PRE-SEP C_{18} cartridge and the

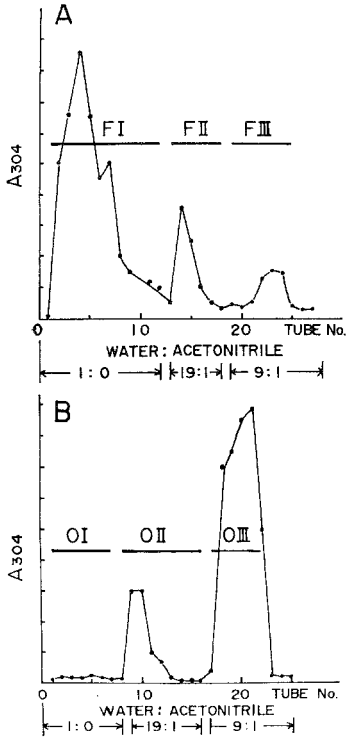

Figure 4. Elution profiles of ABEE derivatives of oligosaccharides, liberated from (A) fetuin and (B) hen ovomucoid by hydrazinolysis, on a PRE-SEP C_{18} cartridge. Fetuin and ovomucoid were subjected to hydrazinolysis and *N*-reacetylation and the liberated oligosaccharides were derivatized with ABEE. The derivatives were applied to a PRE-SEP C_{18} cartridge and the cartridge was eluted stepwise; with water, 5% and 10% acetonitrile in water; collecting 2 ml of eluate in each tube. The elution was monitored by UV spectrophotometric measurement at 304 nm. The cartridge was further eluted with 20% acetonitrile in water, and acetonitrile. The eluate contained non-sugar materials having UV absorbance at 275 nm.

cartridge was eluted in a stepwise manner with water, and water/acetonitrile mixtures, 95/5 and 9/1 by vol. The eluate was monitored at 304 nm. Fig. 4A shows the eluation profile of oligosaccharides-ABEE from fetuin. The materials recovered in F-I, F-II and F-III gave the same ultraviolet absorption spectra as those shown in Fig. 1. The cartridge was further eluted with water/acetonitrile, 4/1 by vol, and acetonitrile. The material eluted with water/acetonitrile, 4/1 by vol, contained non-sugar materials which had a strong UV absorption band at 275 nm. In some cases, small amounts of non-sugar materials were co-eluted with neutral oligosaccharides in the last few tubes of water/acetonitrile, 9/1 by vol.

When analyzed by high voltage paper electrophoresis, F-I and F-II moved towards the cathode and separated into a few components (Fig. 5). All the components were converted into neutral oligosaccharides by the treatment with neuraminidase from *Arthrobacter ureafaciens*. F-III moved to the position where all of the ABEE derivatives of

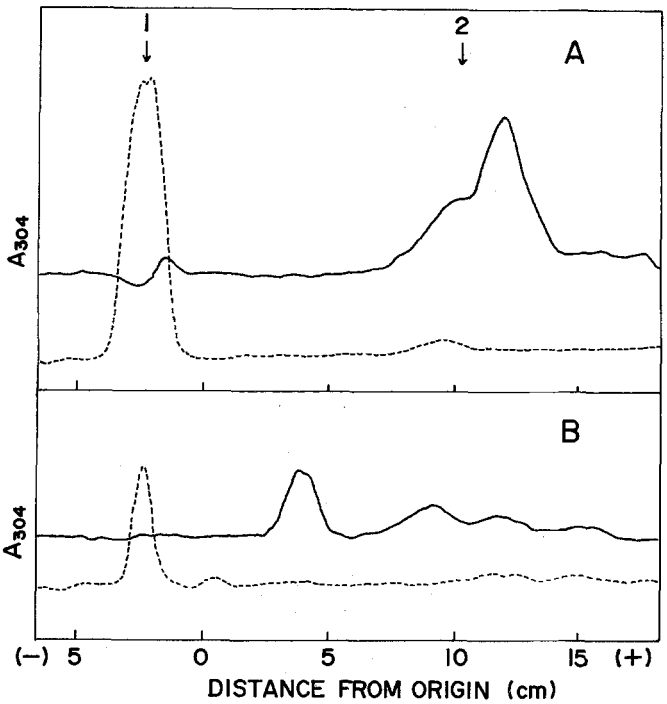

Figure 5. High voltage paper electrophoresis of F-I(A) and F-II(B) in Fig. 4. F-I and F-II were subjected to paper electrophoresis at pH 5.4, 76 V/cm for 70 min (—). F-I and F-II were treated with neuraminidase in 0.05 M sodium acetate buffer, pH 5.4 at 37°C for 20 h, and examined by paper electrophoresis (---). Arrows indicate the position to which lactose-ABEE (1) and sialyllactose-ABEE (2) migrated.

neutral oligosaccharides were located. The ratio of acidic oligosaccharides (sum of F-I and F-II) and neutral oligosaccharides (F-III) were very close to those of published values [22]. Oligosaccharides-ABEE from hen ovomucoid gave the elution profile shown in Fig. 4B. The major oligosacharides (O-III) moved to the position of ABEE derivaties of neutral oligosaccharides on high voltage paper electrophoresis as anticipated from the literature [23]. These results demonstrate that the crude ABEE derivatives of oligosaccharides obtained from glycoproteins can be freed from contaminants and separated into acidic and neutral oligosaccharides on a PRE-SEP C_{18} cartridge.

Quantification of Oligosaccharides-ABEE from Glycoproteins: 1.0 mg of asialofetuin was subjected to hydrazinolysis and *N*-reacetylation. Before coupling oligosaccharides with ABEE, 10 nmol of $(GlcNAc)_3$ were added as an internal standard. Oligosaccharides-ABEE were analyzed by Bio-Gel P-4 column chromatography (Fig. 6A). The ratio of peak area of asialofetuin-derived oligosaccharide (peak I) and $(GlcNAc)_3$ (peak II) was 5.0 : 1.0. Fetuin (MW 48 000) contains 14 residues of sialic acid per molecule and three asparagine-linked sugar chains are present in a molecule [24-26]. It was estimated from these data, together with the yield of derivatization of $(GlcNAc)_3$, that the yield of the ABEE-derivatized asialofetuin oligosaccharide was approximately 70% based upon the amount of starting glycoprotein.

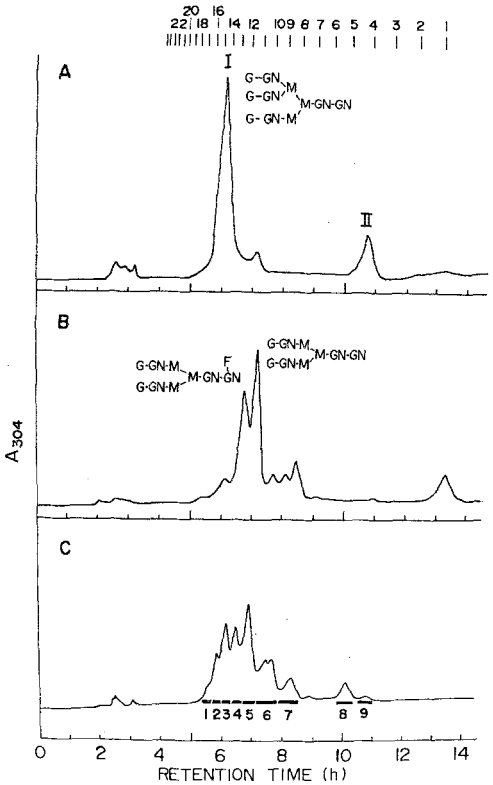

Figure 6. Chromatography of ABEE derivatives of oligosaccharides liberated from glycoproteins on a 2 × 100 cm column of Bio-Gel P-4 (-400 mesh).
(A) Asialo-fetuin (1 mg) was subjected to hydrazinolysis and *N*-reacetylation. The liberated oligosaccharides were mixed with 10 nmol of $(GlcNAc)_3$ and the mixture was derivatized with ABEE. The ABEE derivatives were purified on a PRE-SEP C_{18} cartridge. 1/5 of the sample was injected on the column. The elution was monitored at 1/40 of maximum sensitivity of the detector. I, oligosaccharide-ABEE from asialofetuin; II, $(GlcNAC)_3$-ABEE.
(B) ABEE derivatives of acidic oligosaccharides from hCG were pooled and digested with neuraminidase. The resulting neutral oligosaccharides were injected on the column.
(C) ABEE derivatives of neutral oligosaccharides from hen ovomucoid (O-III in Fig. 4) were injected on the column.
The conditions for operation of the column are the same as shown in Fig. 2. The arrows at the top of the figure indicate the position of non-derivatized isomalto-oligosaccharides (numbers indicate glucose units). Oligosaccharides from hen ovomucoid were fractionated as indicated (1 - 9) for HPLC analysis.

Bio-Gel P-4 Chromatography of Asn-linked Oligosaccharides: Fig. 6 shows the elution profiles of oligosaccharides-ABEE derived from glycoproteins. Analysis of the asialofetuin oligosaccharides showed tri-antennary and a small amount of bi-antennary compounds [22]. The desialylated oligosaccharides from hCG (Fig. 6B) gave the two peaks corresponding to bi-antennary compounds with or without a fucose residue, as anticipated from the literature [27]. The elution profile of ABEE derivatives of the neutral oligosaccharide fraction from hen ovomucoid (O-III in Fig. 4B) was similar to that of tritium-labeled oligosaccharides on Bio-Gel P-4 chromatography published by Yamashita *et al.* [23].

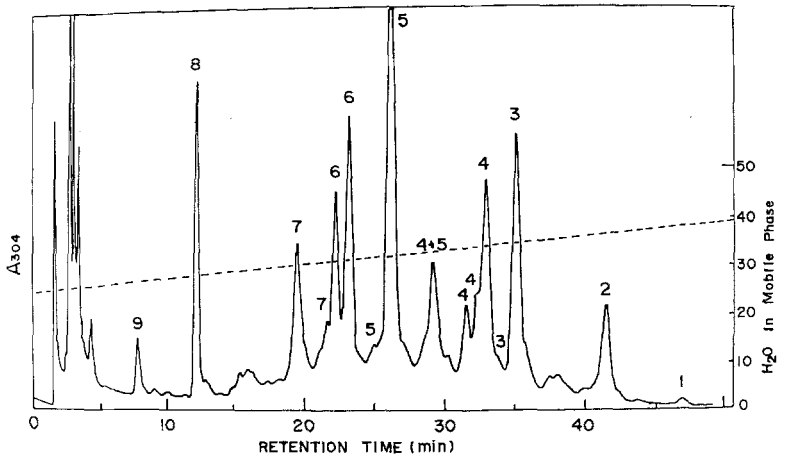

Figure 7. Analysis of ABEE derivatives of neutral oligosaccharides from hen ovomucoid on TSK-GEL Amide-80. The sample was prepared in the same manner as described in Fig. 6 and applied to the column equilibrated in acetonitrile/water, 73/27 by vol. The column was eluted with a linear gradient of acetonitrile/water, from 73/27 to 61/39 by vol, for 50 min. The numbers on top of the peaks indicate the fraction numbers in Fig. 6C.

HPLC Analysis of Hen Ovomucoid Oligosaccharides: The separation of ABEE derivatives of neutral oligosaccharides from hen ovomucoid on TSK-GEL Amide-80 (silica gel modified by organic amide) is shown in Fig. 7. The effective separation of more than 17 components was achieved within 50 min. Each fraction from Bio-Gel P-4 chromatography (Fig. 6C) was analyzed by HPLC and the results are shown at the top of the peaks in Fig. 7. Fractions 1, 2, 8 and 9 gave a single peak on HPLC. Fractions 3, 5, 6 and 7 gave two peaks and fraction 4 gave four peaks. These results, except for fraction 4, were consistent with those obtained by the combined use of Bio-Gel P-4 chromatography and paper chromatography which took more than a week for completion [23, 28]. Although fraction 4 gave a single peak on paper chromatography [28], it was separated into four components by HPLC. This fraction contains two galactose-containing tri-antennary compounds, two tetra-antennary compounds containing the GlcNAc1-6-Man-linkage and one tetra-antennary compound with no GlcNAc1-6Man linkage [28]. Based on the fact that the compound containing the (1-6)-linkage was retarded on the silica-NH_2 column in comparison with the (1-4)-linked analog [29], the separation of the components in fraction 4 on HPLC may be due to their structural features.

Discussion

The first step in the structural characterization of Asn-linked oligosaccharides is the release of oligosaccharides from glycoproteins by hydrazinolysis, or treatment with various kinds of endo-glycosidases. The released oligosaccharides are usually labeled with tritium [6] or fluorescent compounds such as 2-aminopyridine [7-9], or dansylchloride [10], for sensitive detection following chromatographic separation.

In this paper, we expanded on the suitability of ABEE as a UV-absorbing reagent for this purpose. Coupling reaction of ABEE with oligosaccharides proceeded almost quantitatively under mild conditions without significant release of sialic acid residues from acidic oligosaccharides. The derivatives can be stored at 4°C over several months without significant degradation. The crude oligosaccharides obtained by hydrazinolysis/*N*-reacetylation of glycoproteins could be derivatized with ABEE without pre-purification by paper chromatography [19] or gel permeation chromatography [9]. The crude oligosaccharides-ABEE were freed from non-sugar materials, which interfere with the following chromatographic analysis, by a simple fractionation on a PRE-SEP C_{18} cartridge. In addition, the PRE-SEP C_{18} cartridge separated oligosaccharides-ABEE into acidic and neutral oligosaccharides. The former can be separated further by high voltage paper electrophoresis, which may be due to the degree of sialylation. Overall recoveries of oligosaccharides-ABEE from glycoprotein were approximately 70%. In contrast with the tritium labeling and fluorescent labeling, our procedure described here required no special purification step.

Retardation of oligosaccharides-ABEE on Bio-Gel P-4 by hydrophobic interaction [21] resulted in better separation of Asn-linked oligosaccharides than that obtained for reduced oligosaccharides [3, 4]. Monitoring the elution by variable wavelength UV-detector is convenient compared to counting radioactivity or colorimetric quantification of the eluate.

ABEE derivatives of neutral oligosaccharides were also suitable for separation on HPLC using a TSK-GEL Amide-80 column. Combined use of Bio-Gel P-4 chromatography and HPLC for the separation of hen ovomucoid oligosaccharides was more effective than the combined use of Bio-Gel P-4 and paper chromatography [28]. Because UV-absorbance originating from solvents for chromatography gives no effect on the detection of oligosaccharides-ABEE at 304 nm, it is possible to obtained stable base line at high sensitivity range using gradient elution of various kinds of solvents. We could detect 50 pmol of oligosaccharides-ABEE on Bio-Gel P-4 chromatography and 4-5 pmol on HPLC. Coupling oligosaccharides with the hydrophobic compound, ABEE, may make it possible to use reverse-phase (ODS) column for HPLC separation of oligosaccharides-ABEE, which is now under investigation.

In summary, the present study together with that of Wang *et al.* [11] demonstrates that derivatization of oligosaccharides, released from glycoproteins by hydrazinolysis, with ABEE is a convenient alternative to tritium labeling and 2-aminopyridine amination for their separation, highly sensitive detection and structural characterization. We tested aniline, *p*-aminoacetophenone and *p*-anisidine as the other UV-absorbing compounds. Although all of these UV-absorbing derivatives gave similar resolution on Bio-Gel P-4, their low molar absorptivity at shorter wavelength (around 220 nm) was a problem with high sensitivity detection. In addition, the stability of these other derivatives was insufficient for analysis.

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