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Separation of neutral mono- and oligosaccharides derivatized with ethyl *p*-aminobenzoate by high-performance liquid chromatography on an amine-bonded vinyl alcohol copolymer column

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ABSTRACT

A number of mono- and oligosaccharides derivatized with an ultraviolet-absorbing compound, ethyl *p*-aminobenzoate, were separated by high-performance liquid chromatography on an Asahipak NH2P-50 amine-bonded vinyl alcohol copolymer column. The derivatized mono- and oligosaccharides were sufficiently separated with isocratic elution and the separation efficiency for the derivatives was better than that for the underivatized counterparts. The column was remarkably stable relative to a conventional amine-bonded silica column and no decrease in the retention of the derivatives due to dissociation of the stationary phase was observed.

INTRODUCTION

High-performance liquid chromatography (HPLC) has been employed to separate oligosaccharides, and amine-bonded silica columns have mostly been used because of its high resolution and short analysis times [1–9]. However, the column has the disadvantage that considerable amounts of amine-bonded phase and silica itself dissociate into the mobile phase, which causes a gradual decrease in retention [10]. An amine-bonded silica column is frequently used with acetonitrile–water mobile phases, oligosaccharides usually being separated without derivatization [11]. Although measurements of refractive index and intrinsic ultraviolet (UV) absorption have been widely used for the detection of underivatized oligosaccharides, the sensitivity of these detection methods is low relative to that for oligosaccharides derivatized with UV-absorbing or fluorescent compounds [12,13].

In this paper, the separation of mono- and oligosaccharides derivatized with a UV-absorbing compound, ethyl *p*-aminobenzoate (EAB), on an Asahipak NH2P-50 stable amine-bonded vinyl alcohol copolymer column is described.

EXPERIMENTAL

Materials

Monosaccharides, dextran (200–300 kilodalton) and ethyl *p*-aminobenzoate (EAB) was purchased from Wako (Osaka, Japan). Malto-, isomalto-, cello- and N-acetylchitooligosaccharides were obtained from Seikagaku Kogyo (Tokyo, Japan). The isomaltooligosaccharide series were also prepared by partial acid hydrolysis of dextran according to the slightly modified method of Yamashita *et al.* [14]: to a Pyrex tube with a PTFE-lined screw-cap containing 0.5 g of dextran were added 5 ml of 0.2

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M HCl, followed by hydrolysis for 3 h at 100°C and the hydrolysate was passed through a Dowex 1-X8 (OH^-) resin column (5 cm × 1 cm I.D.) to remove the acid. N-Acetylchitooligosaccharides were further purified by gel permeation chromatography on a Bio-Gel P-4 (Bio-Rad Labs., Richmond, CA, USA) column (100 cm × 1.5 cm I.D.) equilibrated with water. Sodium cyanoborohydride ($NaBH_3CN$) was purchased from Aldrich (Milwaukee, WI, USA). All other chemicals and solvents were of analytical-reagent or HPLC grade.

High-performance liquid chromatography

The HPLC system consisted of the following components from Waters Assoc (Milford, MA, USA): a multi-solvent delivery system (Model M600), a universal injector (Model U6K), a variable-wavelength detector (Model 450) and a differential refractometer (Model 410), with a computing integrator (Model C-R6A) from Shimadzu (Kyoto, Japan). Separations were done on an Asahipak NH2P-50 column (250 mm × 4.6 mm I.D.) packed with an amine-bonded vinyl alcohol copolymer gel (5 μm) (Asahikasei, Tokyo, Japan), and the column temperature was kept at 25°C if not specified otherwise. The mobile phase was acetonitrile–water. All elutions were done isocratically at a flow-rate of 0.5 ml/min.

Derivatization of mono- and oligosaccharides with EAB

The procedure employed for labelling of mono- and oligosaccharides at their reducing end with EAB was done by the method of Wang *et al.* [15] with slight modification. To a Pyrex tube with a PTFE-lined screw-cap containing 10 μl of 0.3 *M* mono- and oligosaccharides were added 40 μl of 1.4 *M* $NaBH_3CN$ in distilled water, 350 μl of 0.6 *M* EAB in methanol and 40 μl of glacial acetic acid and the mixture was heated at 80°C. After 1 h, the reaction mixture was cooled and 1 ml of distilled water was added. The aqueous phase was extracted with four 1-ml volumes of chloroform to remove excess of EAB and the aqueous phase containing derivatized mono- and oligosaccharides was lyophilized.

RESULTS AND DISCUSSION

Although the main feature of the Asahipak NH2P-50 amine-bonded vinyl alcohol copolymer column appears to be the separation of oligosaccharides by size, as reported for an amine-bonded silica column [11], the column could be employed for the resolution of both underivatized and EAB-derivatized mono- and disaccharides. The HPLC elution profiles of underivatized mono- and disaccharides eluted with acetonitrile–water (65:35) are shown in Fig. 1; it was not possible to separate glucose from N-acetylchitobiose and maltose from cellobiose (Fig. 1A), or arabinose from N-acetylglucosamine

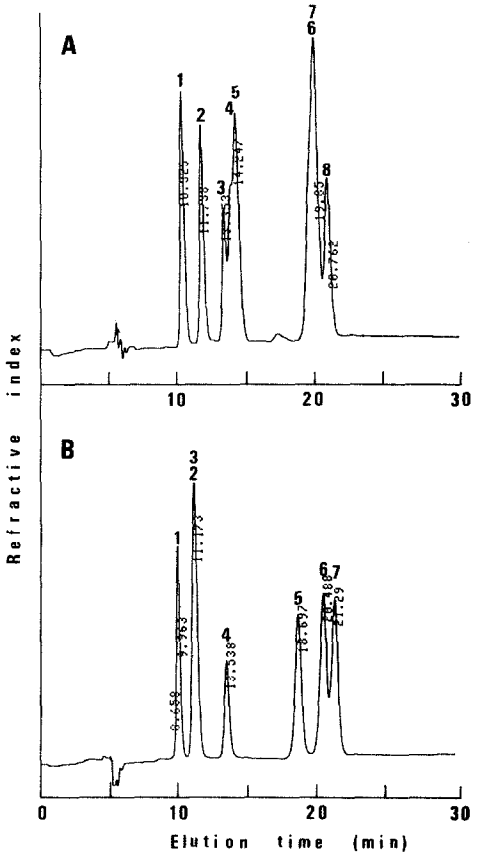

Fig. 1. Elution profiles of underivatized mono- and disaccharides. Peaks: (A) 1 = fucose; 2 = xylose; 3 = mannose; 4 = glucose; 5 = N-acetylchitobiose; 6 = maltose; 7 = cellobiose; 8 = isomaltose; (B) 1 = rhamnose; 2 = arabinose; 3 = N-acetylglucosamine; 4 = galactose; 5 = lactose; 6 = melibiose; 7 = gentiobiose. Column, Asahipak NH2P-50; mobile phase, acetonitrile–water (75:25); flow-rate, 0.5 ml/min.

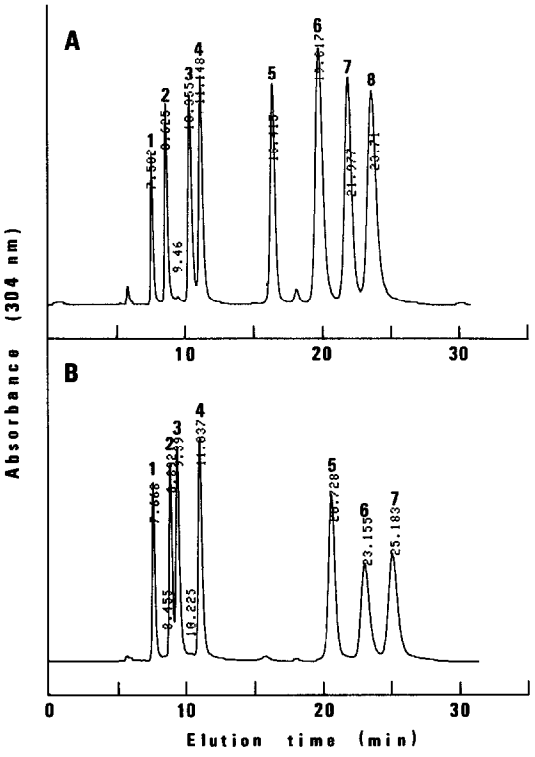

Fig. 2. Elution profiles of mono- and disaccharide EAB derivatives. Peaks: (A) 1 = fucose; 2 = xylose; 3 = mannose; 4 = glucose; 5 = N-acetylchitobiose; 6 = maltose; 7 = cellobiose; 8 = isomaltose derivatives; (B) 1 = rhamnose; 2 = arabinose; 3 = N-acetylglucosamine; 4 = galactose; 5 = lactose; 6 = melibiose; 7 = gentiobiose EAB derivatives. Column, Asahipak NH2P-50; mobile phase, acetonitrile–water (85:15); flow-rate, 0.5 ml/min.

(Fig. 1B). However, much greater resolution was obtained when the corresponding mono- and oligosaccharides were derivatized with EAB and eluted with acetonitrile–water (75:25) (Fig. 2A and B). The major mechanism for the retention of mono- and oligosaccharide EAB derivatives seems to be due to hydrogen bonding between the hydroxyl groups of the sugar residue and the amine group of the stationary phase [7,8]. However, as a substantial improvement in the separation of mono- and disaccharides was obtained by derivatization with EAB, a subtle interaction between the aromatic ring of EAB derivatives and the stationary phase appears to be responsible for the improved resolution.

The retention times of mono- and disaccharide EAB derivatives separated with two mobile phases are summarized in Table I. Monosaccharide EAB derivatives generally exhibited shorter retention times than disaccharide derivatives and were eluted in following 6-deoxyhexose (fucose and rhamnose), pentose (xylose and arabinose), N-acetylhexosamine (N-acetylglucosamine) and hexose (mannose, glucose and galactose) EAB derivatives, and this order was independent of the mobile phase used. As suggested in the HPLC of underivatized monosaccharides [10], the absence of a hydroxyl group at the C-6 position of 6-deoxyhexose EAB and pentose EAB appears to be primarily associated with the shorter retention times of these derivatives. On the other hand, disaccharide EAB derivatives were sufficiently separated with acetonitrile–water (85:15), and large increases in the retention times and peak widths were observed when the derivatives were eluted with acetonitrile–water (90:10).

The HPLC elution profiles of the isomalto-, malto- and cellooligosaccharide EAB series, which have a common constituent sugar (glucose) and different linkage types, are shown in Fig. 3. The isomaltooligosaccharide EAB series, which have an α-1–6-linkage (Fig. 3A) showed longer retention times than the maltooligosaccharide EAB series, which have an α-1–4-linkage (Fig. 3B), suggesting that the presence of an α-1–6-linkage in the structures seems to be correlated with the increase in the retention times, as reported for underivatized oligosaccharides [10]. In contrast, only minimal differences in the retention times were observed between the malto- and cellooligosaccharide EAB series (β-1–4-linkage), indicating that an anomeric configuration of the linkage is not likely to be responsible for changes in retention (Fig. 3C).

The HPLC elution profiles of the cello- and N-acetylchitooligosaccharide EAB series, which have a common β-1–4-linkage and different constituent sugars, glucose and N-acetylglucosamine, are shown in Fig. 4. The N-acetylchitooligosaccharide EAB series (Fig. 4B) exhibited considerably shorter retention times than the cellooligosaccharide EAB series (Fig. 4A). As the replacement of a hydroxyl group at the C-2 position with an acetoamide group is only the difference between the two oligosaccharide EAB series, the structural difference could be correlated with shift in the retention times, as indicated by Mellis and Baenziger [16].

Fig. 5 shows the HPLC elution profiles of mono-

TABLE I

RETENTION TIMES OF MONO- AND DISACCHARIDE EAB DERIVATIVES

EAB derivative	Structure	Retention time (min)	
		Acetonitrile–water (85:15)	Acetonitrile–water (90:10)
Monosaccharides			
Fucose	Fuc	7.6	10.3
Rhamnose	Rha	7.7	10.5
Xylose	Xyl	8.6	12.8
Arabinose	Ara	8.9	12.6
Mannose	Man	10.4	18.6
Galactose	Gal	11.6	20.9
Glucose	Glc	11.1	21.3
N-Acetylglucosamine	GlcNAc	9.4	16.4
Disaccharides			
Lactose	Galβ-1–4Glc	20.7	57.3
Melibiose	Galα-1–6Glc	23.2	63.3
Gentiobiose	Glcβ-1–6Glc	25.1	75.0
Cellobiose	Glcβ-1–4Glc	22.0	61.9
Maltose	Glcα-1–4Glc	19.8	50.0
Isomaltose	Glcα-1–4Glc	19.8	50.0
Isomaltose	Glcα-1–6Glc	23.7	69.2
N,N′-Diacetylchitobiose	GlcNAcβ-1–4GlcNAc	16.4	36.6

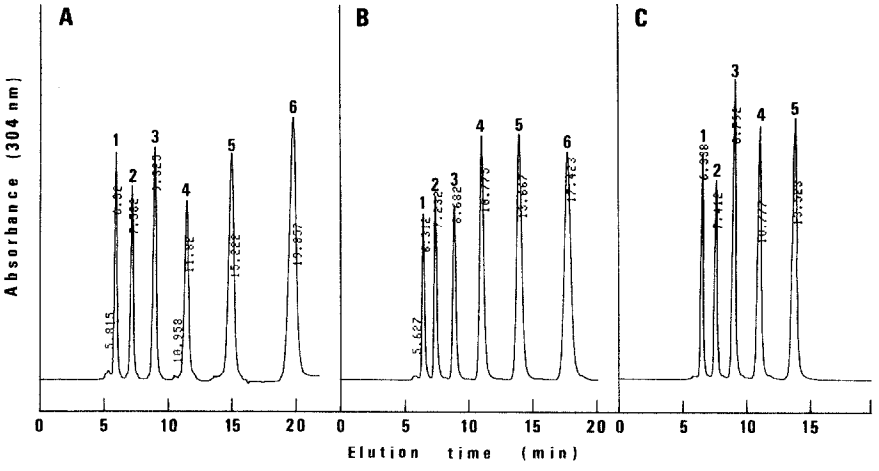

Fig. 3. Effect of linkage type on separation of oligosaccharide EAB series. (A) Isomaltooligosaccharide EAB series (Glcα-1–6Glc); (B) maltooligosaccharide EAB series (Glcα-1–4Glc); (C) cellooligosaccharide EAB series (Glcβ-1–4Glc). The number above each peak indicates the number of sugar residues present. Column, Asahipak NH2P-50; mobile phase, acetonitrile–water (70:30); flow-rate, 0.5 ml/min.

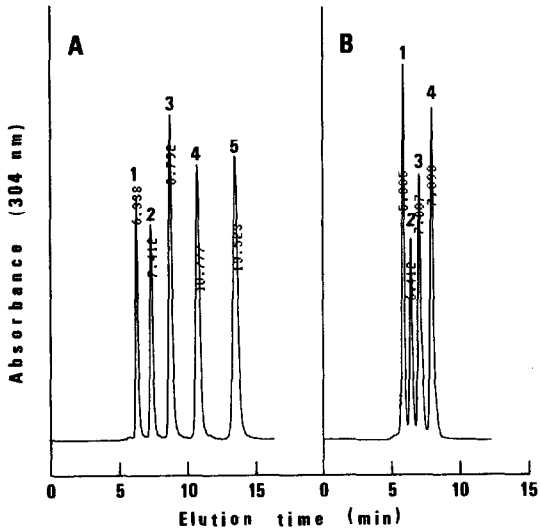

Fig. 4. Effect of constituent sugars on separation of oligosaccharide EAB series. (A) Cellooligosaccharide EAB series (Glcβ-1–4Glc); (B) N-acetylchitooligosaccharide EAB series (GlcNAcβ-1–4GlcNAc). The number above each peak indicates the number of sugar residues present. Column, Asahipak NH2P-50; mobile phase, acetonitrile–water (70:30); flow-rate, 0.5 ml/min.

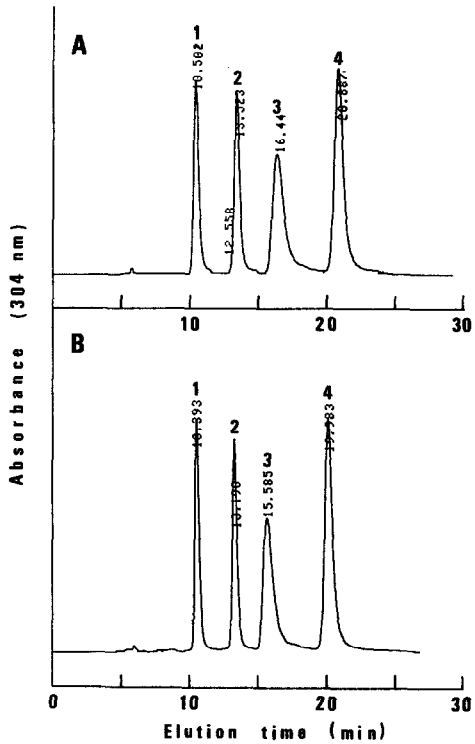

Fig. 5. Effect of temperature on separation of monosaccharide EAB derivatives: (A) 25°C; (B) 45°C. Peaks: 1 = rhamnose; 2 = arabinose; 3 = N-acetylglucosamine; 4 = galactose EAB derivatives. Column, Asahipak NH2P-50; mobile phase, acetonitrile–water (90:10); flow-rate, 0.5 ml/min.

saccharide EAB derivatives separated at different column temperatures. Although variations in the column temperature led to the different elution profiles in the HPLC of underivatized oligosaccharides [9,17], only slight differences in the elution profiles and the retention times of monosaccharide EAB derivatives were observed when operating temperature was increased from 25 to 45°C (Fig. 5A and B).

The HPLC profiles of underivatized and EAB-derivatized isomaltooligosaccharide series derived from dextran by partial acid hydrolysis are compared in Fig. 6. The underivatized isomaltooligosaccharide series having up to nine sugar residues were separated as single peaks with acetonitrile–water (65:35) in less than 50 min (Fig. 6A). Although a similar separation of the underivatized isomaltooligosaccharide series was achieved by HPLC on a C_{18}-bonded vinyl alcohol copolymer column, alkaline eluents were necessary to circumvent an undesirable separation of α- and β-anomeric peaks [18]. On the other hand, isomaltooligosaccharide EAB derivatives possessing up to thirteen or more sugar residues were well resolved with acetonitrile–water (65:35) (Fig. 6B). Isomaltooligosaccharides that was derivatized with EAB showed longer retention times than underivatized oligosaccharides in size fractionation on a Bio-Gel P-4 column [19], whereas isomaltooligosaccharide EAB derivatives showed lower retention times than the underivatized counterparts in HPLC on an Asahipak NH2P-50 column. The decrease in the retention times of the derivatives appears to be due to the interaction between the aromatic ring of EAB derivatives and the stationary phase.

Fig. 7 shows the dependence of the retention times of isomaltooligosaccharide EAB derivatives on the acetonitrile concentration in the mobile phase. At the highest acetonitrile concentration examined (70%), isomaltooligosaccharide EAB derivatives having nine sugar residues were well resolved in 50 min (Fig. 7A), whereas the derivatives with thirteen or more sugar residues were separated at a

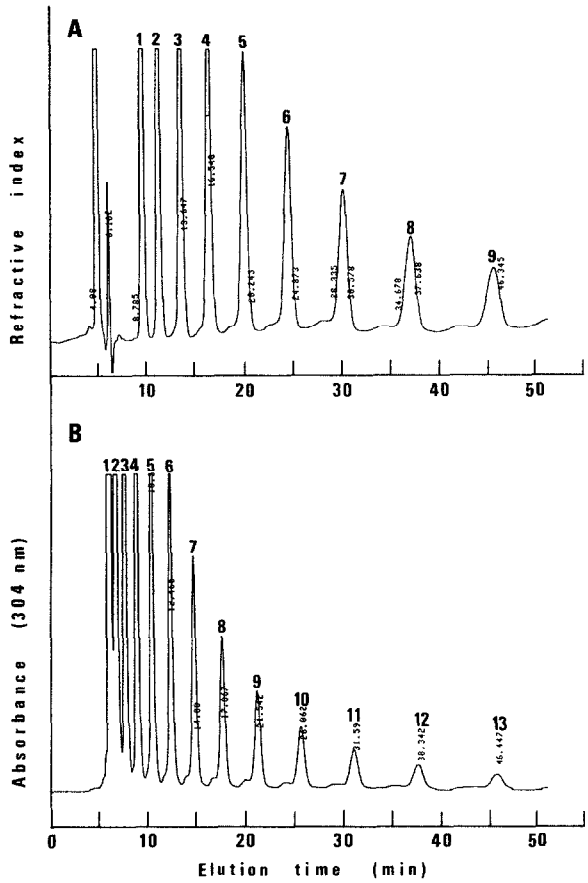

Fig. 6. Elution profiles of underivatized and EAB-derivatized isomaltooligosaccharide series, (A) before derivatization and (B) after derivatization with EAB. The number above each peak indicates the number of sugar residues present. Column, Asahipak NH2P-50; mobile phase, acetonitrile–water (65:35); flow-rate, 0.5 ml/min.

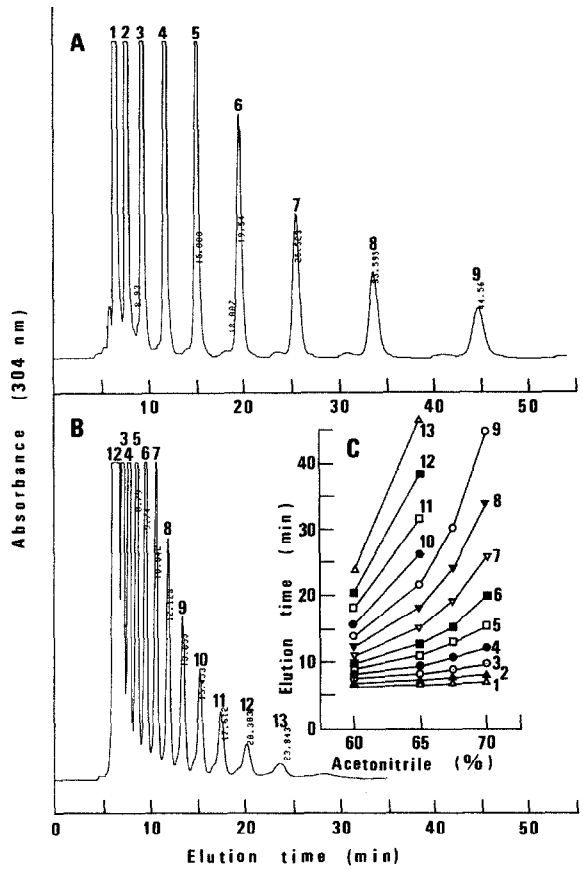

Fig. 7. Effect of acetonitrile concentration in the mobile phase on retention times of isomaltooligosaccharide EAB series, (A) 70% and (B) 60%, and (C) relationship between acetonitrile concentration and retention times of the derivatives. The numbers indicate the number of sugar residues present. Column, Asahipak NH2P-50; flow-rate, 0.5 ml/min.

60% acetonitrile concentration in 25 min (Fig. 7B). Further, the relationship between the retention times of the derivatives and acetonitrile concentration in the mobile phase is summarized in Fig. 7C; increasing concentrations of acetonitrile resulted in a regular increase in the retention times of the derivatives, and the increase in retention approximated to an exponential increase in the range of 60–70% acetonitrile.

In conclusion, mono- and oligosaccharides derivatized with EAB were well resolved with a simple isocratic method. Further, the resolution of the underivatized mono- and oligosaccharides was greatly improved by derivatization with EAB. The column was clearly stable in comparison with conventional amine-bonded silica columns, and no decrease in the retention times of the derivatives was observed during the course of this study. The stable Asahipack NH2P-50 column could be useful for the preparation not only of mono- and oligosaccharide EAB derivatives, but also of their underivatized counterparts, free from dissociation products.

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