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SIZE FRACTIONATION OF OLIGOSACCHARIDES BY LIQUID CHROMATOGRAPHY ON A CATION-EXCHANGE COLUMN

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SUMMARY

Oligosaccharides, labelled with 2-aminopyridine at their reducing ends, were satisfactorily fractionated according to the sugar sizes on Shodex RSpak DC-613, a cation-exchange resin column (Na^+ form), with water–acetonitrile as the eluent in the presence of sodium acetate or triethylammonium acetate buffer. For the fractionation of sugar samples, dextran hydrolyzates, chitin oligomers and oligosaccharide moieties of ovomucoid were used. The oligosaccharides were strongly adsorbed to the resin column with solvents containing less water and at lower temperature, and were eluted in order of increasing molecular size above the critical concentration of acetonitrile. Baseline separation of a dextran hydrolyzate up to oligomers having 20 glucose units was observed by gradient elution. The separation efficiency and elution pattern were investigated by changing the buffer concentration, mobile phase pH and temperature.

INTRODUCTION

For the structural elucidation of sugar chains of glycoproteins it may be essential to obtain the component oligosaccharides from the macromolecules, and inevitable to purify each oligosaccharide. Size fractionation of oligosaccharides may be useful for this purpose in the first step. Recently, this has been performed by a preparative gel filtration on a Bio-Gel P-4 column[1]. On the other hand, high-performance liquid chromatography (HPLC) is convenient, quick, accurate and quantitative for size fractionation of oligosaccharides. The following three separation modes have been used: (1) gel permeation using an ion-exchange resin[2–4], which shows poor separation for high-molecular-weight oligosaccharides compared to the following two modes; (2) reversed-phase partition[5–8], relating to hydrophobicity, not strictly to sugar size, and (3) normal-phase partition (amine adsorption)[9] using amine-bonded silica gel. This column basically with the solvent system of acetonitrile and water shows satisfactory separation for neutral oligosaccharides[10–15]. Especially, gradient elution using increasing water contents with the addition of buffer has enabled a good separation even of anionic oligosaccharides[16–18], the mechanism being called "ion suppression

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amine adsorption". However, this silica-based stationary phase is subject to deterioration and fouling, and not durable upon repeated analysis.

As a detection method for sugars in HPLC, refractivity has been widely monitored, but this shows low sensitivity and is susceptible to changes in solvent composition. Tritium labelling at the reducing end of the sugar chain shows high sensitivity[19], but one cannot utilize this specific facility at any time. Sensitive and selective post-column labelling methods have been devised[20], but it is difficult to recover the fractionated samples. Recently, Hase *et al.*[21] devised a highly sensitive detection method by pyridylamination at the reducing end of sugars. This method has been used for the structural analysis of oligosaccharides[22–24], and Hase *et al.*[25] reported that size fractionation of pyridylaminated (PA-) sugars was achieved by the ion suppression amine adsorption mode.

Samuelson and co-workers[26–29] have systematically investigated size fractionation of oligosaccharides or oligomeric sugar alcohols by open-column partition chromatography on ion-exchange resins in aqueous ethanol. They have reported that the sugars were eluted in order of increasing molecular size, and the distribution coefficients were influenced by the type of glycosidic linkage, and decreased with non-polar groups in the sugar molecule.

In this study, we investigate the usefulness of a cation-exchange polymer column according to the normal-phase partition mode for the purpose of size fractionation of PA derivatives of oligosaccharides prepared from dextran, chitin and ovomucoid.

EXPERIMENTAL

Chemicals

2-Aminopyridine was obtained from Tokyo Chemical Industry (Tokyo, Japan), and recrystallized from 1-hexane. Triethylamine was from Wako Pure Chemical (Osaka, Japan), and distilled once by flash evaporation. Anhydrous hydrazine and sodium cyanoborohydride were obtained from Aldrich (Milwaukee, WI, U.S.A.) and used without any pretreatment. Ovomucoid (trypsin inhibitor, Type III-0) was obtained from Sigma Chemical (St. Louis, MO, U.S.A.). Water was deionized and distilled before use. All other chemicals were of analytical reagent grade.

Preparation of oligosaccharides

Glucose (Glc) oligomers were prepared from dextran by partial acid hydrolysis[1] (0.1 *M* HCl, 4 h, 100°C), and excess of acid was removed on a Dowex 1-X8 (OH^- form) resin column. The hydrolyzate was applied on a Sephadex G-15 column (61 cm × 1 cm), eluted with distilled water at a flow-rate of 8–10 ml/h at room temperature, and high-molecular-weight Glc oligomers were pooled in the void volume fraction. Chitobiose, chitotriose, chitotetraose and chitopentaose were provided by Dr. E. F. Walborg (The University of Texas System Cancer Center, Science Park Research Division, Smithville, TX, U.S.A.). Oligosaccharides from ovomucoid were prepared by hydrazinolysis and sequential N-reacetylation for free amino groups according to the method of Takasaki *et al.*[30].

Pyridylamination of sugars

Aldehyde groups of the reducing ends of oligosaccharides were coupled with

2-aminopyridine by reductive amination with sodium cyanoborohydride according to the method of Hase *et al.*[21]. The following four samples were derivatized: (1) Glc oligomers from an hydrolyzate of 9 mg dextran; (2) high-molecular-weight Glc oligomers from an hydrolyzate of 64 mg dextran; (3) N-acetylglucosamine (GlcNAc) oligomers (mixture of 140, 99, 104, 102 and 118 nmol of GlcNAc, chitobiose, chitotriose, chitotetraose and chitopentaose); (4) oligosaccharide fraction of an hydrazinolyzate of 3.1 mg ovomucoid. Each sample was dissolved in 0.5 ml of a 2-aminopyridine solution (prepared by dissolving 1 g of 2-aminopyridine in 0.76 ml of concentrated hydrochloric acid) in a hydrolyzing tube (12 cm × 1.6 cm) with a PTFE screw cap, and warmed at 100°C for 13 min. After cooling, 25 μl of the reducing reagent (prepared by mixing 10 mg sodium cyanoborohydride, 20 μl of the 2-aminopyridine solution and 30 μl of water) were added to the tube, and warmed at 90°C overnight. The PA-sugars were separated from the reaction mixture by gel filtration on Sephadex G-15 column (61 cm × 1 cm) eluted with 10 m*M* ammonium acetate (pH 6.0) at a flow-rate of 8–10 ml/h at room temperature, and 2.5-ml fractions being collected. Fractions 6–16 were pooled, concentrated by evaporation and applied on a Dowex 50W-X2 (H^+ form) resin column (4.7 cm × 2 cm). After washing with 160 ml of water, PA-sugars were eluted with 100 ml of 3% aqueous ammonium solution, and concentrated by evaporation. Excess of 2-aminopyridine was removed by evaporation with repeated additions of triethylamine. The residue was dissolved in 250 μl of water, and used as a sample solution.

High-performance liquid chromatography

The following HPLC 800-series instruments (Japan Spectroscopic, Tokyo, Japan) were used for a high-pressure mixing gradient elution. From the two reservoirs containing different ratios of acetonitrile and water with an appropriate buffer, the solvents were delivered by two 880-PU pumps and passed through a 880-50 degasser. The two solvents were mixed in the programmed ratio at the solvent mixing module, and sent to the column at a flow-rate of 1 ml/min. The column temperature was regulated by a 860-CO column oven. After the sample injection by a Reodyne (Cotati, CA, U.S.A.) 7125 sample injector, a linear gradient elution was performed. The elution solvent and the sample solution were passed through a 0.45-μm membrane filter before use. A RSpak DC-613 column (15 cm × 0.6 cm), a highly cross-linked, sulphonated polystyrene resin in the sodium form (Showa Denko, Tokyo, Japan), was used. The samples were detected by a 820-FP spectrofluorometer, the excitation and emission wavelengths being 320 and 400 nm, respectively. The amount of PA-sugars was calculated from the peak area on the elution chromatogram by an 805-GI graphic integrator.

RESULTS AND DISCUSSION

Elution conditions for pyridylaminated sugars

PA derivatives of oligosaccharides were not eluted from the RSpak DC-613 column in acetonitrile–water solvents (from 9:1 to 4:6, v/v), indicating the strong adsorption of the PA-sugars to the resin. It is likely that PA-sugars protonated in this solvent displaced sodium ion from the cation exchanger. In an attempt to elute the PA-sugars, sodium acetate and triethylammonium acetate buffers were used. The

following two sections indicate elution conditions with respect to the column temperature (23, 40, 60°C), buffer concentration (25, 175 m*M*) and mobile phase pH (7.5, 8.5, 9.5).

Elution in the presence of sodium acetate

Sodium ion, the counter ion of this resin, was added to the elution solvent, at 25 m*M*, the concentration at its maximum solubility. The pH of the mobile phase was an important factor in the elution of PA-sugars. Below pH 6.7, PA-sugars were eluted considerably later or not eluted in acetonitrile–water (from 8:2 to 4:6, v/v). Therefore, the pH was adjusted to above 7.5 in order to suppress the protonation of the aminopyridyl group possessing pK_a 6.7. The samples were adsorbed to the column more strongly with solvents of lower water contents. The temperature had a considerable influence on the separation of PA-sugars, *i.e.*, at 23°C satisfactory elution was not obtained for the PA-Glc oligomers. Raising the column temperature led to a decrease in their retention times, and gave a higher separation efficiency. The oligosaccharides were eluted in order of increasing molecular size. When the water content was between 56 and 60% (v/v), PA-Glc oligomers were eluted as a single peak. This acetonitrile concentration is denoted the "critical concentration" as described by Havlicek and Samuelson[27–29]. When the water content was above 60% (v/v), the elution order was reversed, being in the order of decreasing molecular size.

Fig. 1 shows the elution pattern of PA-oligosaccharides where a linear gradient

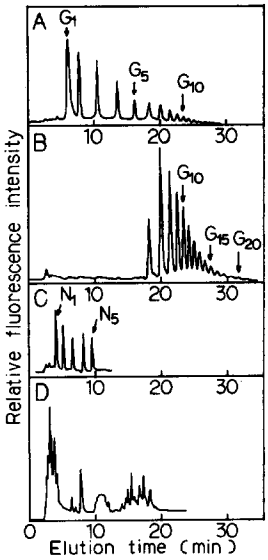

Fig. 1. Separation of pyridylaminated derivatives of oligosaccharides on an RSpak DC-613 column in the presence of sodium acetate. (A) PA derivatives of dextran hydrolyzate (0.1 μl); (B) PA derivatives of the high-molecular-weight fraction of a dextran hydrolyzate (PA-HMWF, 0.15 μl); (C) PA-GlcNAc oligomers (0.2 μl); (D) PA derivatives of ovomucoid oligosaccharides (PA-OM, 1 μl). The linear gradient elution from 24 to 54% water was performed in 20 min in the acetonitrile mobile phase containing 25 m*M* sodium hydroxide, adjusted to pH 7.5 with acetic acid. The column temperature was 60°C. G_1 is PA-Glc, G_5, G_{10}, G_{15} and G_{20} are PA-Glc oligomers of DP 5, 10, 15 and 20, respectively, and N_1 and N_5 are PA-GlcNAc and PA-chitopentaose.

elution was performed from 24 to 54% water in 20 min at 60°C, the pH of the mobile phase being 7.5. The gradient system was chosen so that all species might be well resolved and in an appropriate time. The boundary of the acetonitrile content was from the concentration at which PA-Glc and PA-isomaltose were well separated to the critical concentration. Baseline resolution of PA derivatives of dextran hydrolyzates containing 1–13 Glc oligomers was obtained (Fig. 1A). As the degree of polymerization (DP) of Glc increased, their peak areas decreased exponentially. Separation and detection of Glc oligomers up to DP 20 was observed from the high-molecular-weight fraction of dextran hydrolyzate (Fig. 1B). Moreover, the baseline resolution of GlcNAc oligomers, DP 1–5 was obtained (Fig. 1C). PA-GlcNAc oligomers possessed retention times about two-thirds those of PA-Glc oligomers of like DPs. Raising the pH of the mobile phase in the same elution system led to a decrease in the maximum detectable DP of PA-Glc oligomers. However, it did not affect the resolution of low-molecular-weight PA-Glc oligomers (Table I).

Ovomucoid is known to have highly heterogeneous oligosaccharides, its sugar size being 5–14 monosaccharide units[31–33]. In the gel filtration (Fig. 2), PA derivatives of oligosaccharide fractions of ovomucoids were separated into two main peaks. One was eluted at the void volume and the other at the retention time between PA-di- and trisaccharides. In HPLC (Fig. 1D), the elution profile of PA-ovomucoid oligosaccharides was constituted of several peaks, and their retention times were all shorter than that of PA-isomaltohexaose.

Elution in the presence of triethylammonium acetate

In the presence of buffered triethylamine (TEA), the elution pattern of PA-sugars was much influenced by its concentration. The results are as follows at lower (25 m*M*) and higher (175 m*M*) TEA concentrations.

TABLE I

COMPARISON OF RESOLUTION WITHIN THE DIFFERENT FOUR ELUTION CONDITIONS

Column: Shodex RSpak DC-613 (15 mm × 6 mm I.D.); elution solvent, water in acetonitrile-containing buffer; flow-rate, 1.0 ml/min.

Buffer	*pH*	*Temperature* (°C)	*Gradient* (water)	*Resolution*[a]		*Maximum* *DP*[b]	$\frac{t_R(N_5)^c}{t_R(G_5)}$
				$G_3 \leftrightarrow G_4$	$G_5 \leftrightarrow G_6$		
25 m*M* Na^+	7.5	60	24 → 54%/20 min	6.0	3.7	20	0.61
25 m*M* Na^+	8.5	60	24 → 54%/20 min	5.9	3.7	16	0.69
25 m*M* Na^+	10.0	60	24 → 54%/20 min	5.9	4.0	12	0.69
25 m*M* TEA	7.5	60	10 → 34%/24 min	3.3	1.5	11	0.73
175 m*M* TEA	7.5	60	10 → 24%/24 min	2.8	1.8	12	0.81
175 m*M* TEA	7.5	23	10 → 24%/24 min	2.6	2.2	16	0.89
175 m*M* TEA	8.5	60	10 → 26%/24 min	3.4	2.1	11	0.79
175 m*M* TEA	8.5	23	10 → 26%/24 min	3.7	2.5	15	0.98
175 m*M* TEA	10.0	60	14 → 32%/24 min	3.9	2.2	10	0.85
175 m*M* TEA	10.0	23	14 → 32%/24 min	4.5	2.3	14	0.94

[a] Calculated according to ref. 39. G_n represents the PA-Glc oligomer of DP = *n*.
[b] Maximum detectable DP of PA-Glc oligomer.
[c] Ratio of retention time for PA-chitopentaose to that for PA-isomaltopentaose.

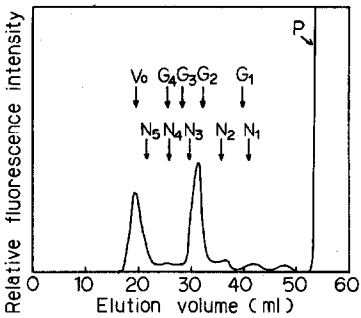

Fig. 2. Gel filtration of pyridylaminated oligosaccharides from ovomucoid. The pyridylaminated sample was applied to a Sephadex G-15 column (61 cm × 1 cm) equilibrated with 10 m*M* ammonium acetate, pH 6.0, and the column was eluted with the same buffer. The eluate was passed through the spectrofluorometer (820-FP), and PA-sugars were detected, the excitation and emission wavelengths being 320 and 400 nm, respectively. Arrows indicate the elution position of PA-sugars. G_1 is PA-Glc, G_2–G_4 are PA-Glc oligomers of DP 2–4, N_1 is PA-GlcNAc and N_2–N_5 are PA-GlcNAc oligomers of DP 2–5. V_0 = Void volume; P = 2-aminopyridine.

25 mM TEA concentration (pH 7.5). Raising the temperature led to a decrease of the retention time, and significant increase in the column efficiency. However, even with the lowest water content (10%), the baseline separation of PA-Glc and PA-isomaltose was not observed. PA-oligosaccharides were eluted in order of increasing molecular size above the critical concentration of acetonitrile (64–70%). Below it, the elution order was reversed. The gradient elution pattern is shown in Fig. 3. The detectable limit of PA-Glc oligomers was obtained only up to DP 12. PA-GlcNAc oligomers possessed retention times about two-thirds those of PA-Glc oligomers of like DPs. The elution profile of PA-ovomucoid oligosaccharides was constituted of several peaks, and their retention times were all shorter than that of PA-isomaltononaose.

175 mM TEA concentration. Raising the temperature led to a decrease in the retention time. In the isocratic elution at a water content of 10%, the number of theoretical plates was slightly increased. However, the resolution of PA-isomaltotriose and PA-isomaltotetraose was almost the same at 23, 40 and 60°C. PA-oligosaccharides were eluted in order of increasing molecular size above the critical concentration of acetonitrile (71–75%). In the gradient elution (water content from 10 to 24% in 24 min, pH 7.5), raising the temperature led to a decrease in both the resolution of PA-isomaltopentaose and PA-isomaltohexaose (2.2 at 23, 1.9 at 40 and 1.8 at 60°C) and the maximum detectable DP of PA-Glc oligomers (DP 12 at 23, 10 at 40 and 8 at 60°C). Fig. 4 shows the gradient elution pattern at 23°C. PA-Glc oligomers were detected up to DP 16. PA-GlcNAc oligomers possessed retention times about four-fifths those of PA-Glc oligomers of like DPs. The elution profile of PA-ovomucoid oligosaccharides was constituted of several peaks, and their retention times were all shorter than that of the PA-Glc oligomer of DP 13. Raising the pH of the mobile phase led to an alteration of the adequate gradient boundary and an increase in resolution, both at 23 and 60°C. However, it led to decrease in the maximum detectable DP of PA-Glc oligomers.

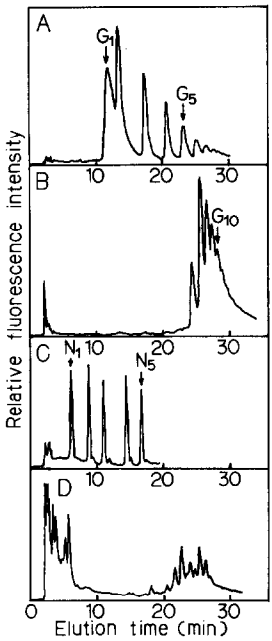

Fig. 3. Separation of pyridylaminated oligosaccharides on an RSpak DC-613 column in the presence of triethylammonium acetate. (A) PA-Glc oligomers (0.4 μl); (B) PA-HMWF (0.4 μl); (C) PA-GlcNAc oligomers (0.8 μl); (D) PA-ovomucoid oligosaccharides (4 μl). Conditions as in Fig. 1 except for the gradient (10 → 34%/24 min) and the mobile phase anion (25 m*M*).

A wide range of linearity was observed between the sample content and the peak area on the chromatogram, from the detection limit (about 2 pmol; signal-to-noise ratio S/N = 2) to about 25 nmol for PA-Glc (upper limit of fluorescence linearity of the detector used), and about 1 nmol for each PA-GlcNAc oligomer. At greater than 1 nmol, the PA-GlcNAc oligomer did not show linearity, suggesting its adsorption on this column at higher concentrations.

Sodium ion binding to the sulphonyl group of the resin was gradually replaced by protonated TEA with a decrease in retention time. After equilibration with the elution solvent (200 ml) in the column, the retention time for PA-Glc oligomers did not change significantly during the analysis in a day. The percentage deviation ($n = 5$) was calculated as 0.66 and 1.5% for the retention times of PA-Glc and PA-isomaltoheptaose in the elution system shown in Fig. 4. After elution of 500 ml solvent, the retention time was decreased by 6.6% for PA-Glc and 7.9% for PA-isomaltoheptaose, respectively, as often appears to be the case on amine-bonded phases[34]. However, this column was renewed by washing with diluted aqueous sodium hydroxide followed by water, and the column capacity was restored reversibly. Therefore, this column is superior to an amine-bonded silica column in stability and long-term durability.

Comparison of the elution systems

A comparison of the elution systems in size fractionation of PA-sugars with respect to the column efficiencies (resolution and maximum detectable DP) and

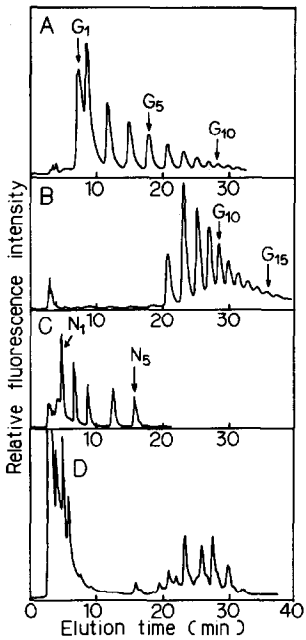

Fig. 4. Separation of pyridylaminated oligosaccharides on an RSpak DC-613 column in the presence of triethylammonium acetate. (A) PA-Glc oligomers (0.6 μl); (B) PA-HMWF (0.4 μl); (C) PA-GlcNAc oligomers (0.8 μl); (D) PA-ovomucoid oligosaccharides (4 μl). Conditions in Fig. 1 except for the gradient (10 → 24%/24 min), the mobile phase anion (175 m*M* TEA) and temperature (23°C).

separation characteristics between PA-Glc oligomers and PA-GlcNAc oligomers is shown in Table I. A good separation efficiency was obtained in the presence of sodium ions rather than in the presence of TEA. However, because of its non-volatility and low solubility in aqueous acetonitrile, this sodium acetate buffer is not suitable for preparative HPLC. The higher separation efficiency was obtained at higher temperature using the diluted TEA or sodium acetate buffer, as reported on an ion-exchange resin[35] and on an amine-bonded silica column[15]. Raising the pH of the mobile phase did not increase the separation efficiency. GlcNAc oligomers and ovomucoid oligosaccharides were eluted significantly earlier than Glc oligomers, similar to the amine adsorption mode where GlcNAc and fucose behaved like a 0.5 Glc unit[24]. On the other hand, at the high concentration of TEA, an higher separation efficiency was obtained at lower temperature. Raising the pH led to higher separation efficiency for low-molecular-weight PA-Glc oligomers, but lower efficiency for high-molecular-weight ones. Moreover, GlcNAc oligomers were eluted almost at the retention times corresponding to the Glc oligomers of the same DPs. Also, elutions of ovomucoid oligosaccharides were widely distributed, comparable to Glc oligomers.

The sulphonated polystyrene resin column used in this experiment (RSpak DC-613) has been used for the separation of monosaccharides, especially for anomers (in its sodium or calcium form)[36], and for monosaccharides present in glycoproteins (in its proton form)[37]. The separation mechanism on this column is regarded as a ligand-exchange interaction between water molecules in aquated metal ions and

hydroxyl groups in sugar molecules[38]. Alternatively, the partition of sugars between the mobile and stationary liquid phases can be considered[26–29]. In the oligosaccharide separation, the partition mechanism may be predominant on consideration of the reversal of the elution order below the critical concentration of acetonitrile.

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