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GEL CHROMATOGRAPHY OF OLIGOSACCHARIDES UP TO DP 60

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SUMMARY

Analytical and preparative gel chromatography have been used to separate oligosaccharides up to a degree of polymerization of 60 (fractionation range 180–10,000 daltons) using 40- and 47-μm polyacrylamide gel particles, respectively. Packing materials were obtained from commercial gels (Bio-Gel P-4 and P-6, −400 mesh) by an elutriation procedure. Columns were run at 60°C with water as eluent. The separation method has been applied to mixtures of malto-oligosaccharides, branched polymaltotrioses and xylo-oligosaccharides.

INTRODUCTION

Chromatography on polyacrylamide gel is one of the major analytical techniques for the separation and analysis of oligosaccharides. One of us previously described a method of fractionating malto-oligosaccharides up to a degree of polymerization (DP) of 15 on a Bio-Gel P-2 column at elevated temperature[1,2]. Several reports on the fractionation of oligosaccharides[3–7], oligosaccharide alditols[8] and uronic acid oligomers[9,10] using this procedure have since been published. Chromatography on Bio-Gel P-4 has been used for structural studies of glycoprotein-derived oligosaccharides[11,12]. Using polyacrylamide gels with different pore size, separations of oligosaccharides up to DP 25 were achieved.

Studies on the carbohydrate moiety of glycoproteins or glycolipids and the enzymic degradation of polysaccharides have made it necessary to introduce a gel chromatographic method that yields effective analytical and preparative separations of oligosaccharides containing more than 25 residues. We report here improved oligosaccharide separations up to DP 40 and 60 using carefully sized gel fractions of Bio-Gel P-4 and P-6, respectively.

EXPERIMENTAL

Chromatographic conditions

Bio-Gel P-4 (−400 mesh) and Bio-Gel P-6 (−400 mesh) were obtained from

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Bio-Rad Labs. Since small and uniform particle sizes lead to more efficient separations as predicted by theory[13], the gels were fractionated in warm deionized water (*ca.* 50°C) by repeated settling and decanting the fines until a sharp zone of settling gel particles was attained. The elutriation procedure yielded mean particle sizes of the Bio-Gel P-4 and P-6 fractions of 40 ± 4 and 47 ± 4 μm, respectively. Prior to packing the column, a 50% gel slurry was degassed under vacuum. The column packing procedure was the same as described earlier[2]. In order to obtain a close gel bed, the rigid Bio-Gel P-4 was packed at a flow-rate of 20 ml · cm^{-2} · h^{-1}. With the softer P-6 gel only a slightly higher flow-rate than is to be used during operation should be applied for column packing.

All experiments were carried out with one home-made and two commercial columns. The column system for the Bio-Gel P-4 procedure consisted of two water-jacketed Cheminert columns (LC-1-43; 109 × 2.54 cm I.D.) connected in series with a zero dead volume union, so that the gel bed was 201 × 2.54 cm I.D. In order to avoid additional peak broadening, low dead volume connections (Cheminert fittings and adapters; PTFE tubing, 0.3 × 1.6 mm) were used. The column used for the Bio-Gel P-6 chromatography was similar to that described earlier[2], except that the column dimensions were 210 × 1.8 cm I.D. All columns were coated using a solution of 5% dichlorodimethylsilane in *n*-hexane[14].

The columns were operated at 60°C with degassed water (80°C) as eluent. In most experiments the flow-rate of the P-4 columns was 55 ml · h^{-1} (≈ 1.4 bar) and of the P-6 column 25 ml · h^{-1} (≈ 1.3 bar). Milton Roy minipumps were used for column elution. The two Bio-Gel P-4 columns were eluted in an upwards–downwards direction. For long-term use of the Bio-Gel P-6 column, upward flow elution was found to be advantageous. In order to obtain extreme purity of the water used as eluent, a column (20 × 1.5 cm I.D.; Econo-Column, Bio-Rad Labs.) filled with AG 50 l-X8D mixed bed ion-exchange resin and activated charcoal (Bio-Rad Labs.) was fitted between the pump and the eluent reservoir. The eluent was further filtered through a 0.8-μm Milli-Fil filter unit (Type PF, Millipore).

In analytical runs 0.15–0.4 ml of a 1–2% aqueous solution was applied to the columns via a septum injector. For preparative work samples of 30–80 mg of carbohydrate were applied in 0.3–1.0 ml to the P-6 column, whereas 200–300 mg of carbohydrate were loaded in *ca.* 2 ml on the P-4 columns.

The carbohydrates were detected by their absorbance at 420 nm after an aliquot of the column effluent had reacted with orcinol–sulphuric acid reagent in an automated analysis system[2]. The detector was assembled from modular components: a peristaltic pump (Type PLG; Desaga, Heidelberg, G.F.R.), a 15-m glass reaction coil in a 95°C heating bath (Haake N2B), and a photometer with a recorder. Glass coils, fittings and acid-resistant pump tubings were obtained from Cenco or Technicon. In preparative runs the column effluent was passed through a refractive index detector (Types 51.78 and 51.88, Knauer).

Preparation of oligosaccharides

Malto-oligosaccharides were obtained by partial acid hydrolysis of amylose (potato type III, Sigma) under the following conditions: (A) 2 g of amylose were dissolved in 100 ml of boiling water and 100 ml of hot 0.5 *M* sulphuric acid were added. The resulting solution (0.25 *M* sulphuric acid) was kept boiling for exactly 13

min on a magnetic stirrer. The hydrolysate was cooled in an ice-bath and was immediately neutralized with solid barium carbonate, centrifuged and lyophilized. (B) Partial acid hydrolysis of amylose was carried out under the conditions described above with the exception that 100 ml of 0.25 *M* sulphuric acid were used (final sulphuric acid concentration 0.125 *M*).

Arabinoxylan from oat spelt (Roth, Karlsruhe, G.F.R.) was purified from contaminating α-glucan (*ca.* 16%) by successive treatment with α-amylase of *Bacillus subtilis* and amyloglucosidase of *Aspergillus niger* (Boehringer, Mannheim, G.F.R.). The glucan-free arabinoxylan was removed from the digest by precipitation with ethanol (3 volumes), washed twice and lyophilized. The purified arabinoxylan was suspended in warm water (*ca.* 60°C) and the soluble fraction was separated from insoluble arabinoxylan by centrifugation. The soluble arabinoxylan fraction contained 12% arabinose and 88% xylose and was used for the preparation of xylo-oligosaccharides. The purified arabinoxylan was partially hydrolysed in boiling 0.125 *M* sulphuric acid for 12 min. The procedure was the same as described for amylose under conditions B.

Branched polymaltotrioses were prepared by the action of pullulanase on pullulan. The incubation mixture (5.0 ml; 30°C) contained 500 mg of pullulan (Sigma) in 50 m*M* acetate buffer (pH 5.0) and 5 units of pullulanase from *Aerobacter aerogenes* (Boehringer). After incubation at 60 min the mixture was inactivated by heating, centrifuged and lyophilized.

Iodine staining of malto-oligosaccharides

Partial acid hydrolysates of amylose were prepared according to conditions A and B and were mixed in the ratio 1:1. A 30-mg amount of this mixture was fractionated on a column (210 × 1.8 cm I.D.) of Bio-Gel P-6. The column effluent was monitored with a refractometer and fractions of 2.6 ml were collected. The fractions were lyophilized and carbohydrates were dissolved in 200 μl of hot water. Iodine solution (100 μl, 0.01 *N*) was added for staining.

Other methods

Mono- and oligosaccharides obtained from the partial acid hydrolysis of arabinoxylan were identified as described[15]. The acid hydrolysate of arabinoxylan was also analysed by high-performance liquid chromatography (HPLC) on a column (90 × 0.9 cm I.D.; 85°C) packed with Aminex 50W-X4 (Ca^{2+}) (20–30 μm)[16]. The column was eluted with water and the effluent was monitored by an automated analysis system as described above.

RESULTS

Branched polymaltotrioses with a degree of polymerization ranging from 3 to 60 were separated on a Bio-Gel P-6 column within less than 20 h (Fig. 1). A good resolution is achieved within a molecular weight range of 180–10,000, which is due to a molecular weight difference of one trisaccharide unit. The oligomer series was prepared by enzymic hydrolysis of pullulan, which is a linear α-glucan produced by *Pullularia pullulans* and in which maltotriose and small amounts of maltotetraose units are linked by 1,6-glycosidic bonds[17]. The different polymaltotrioses were ob-

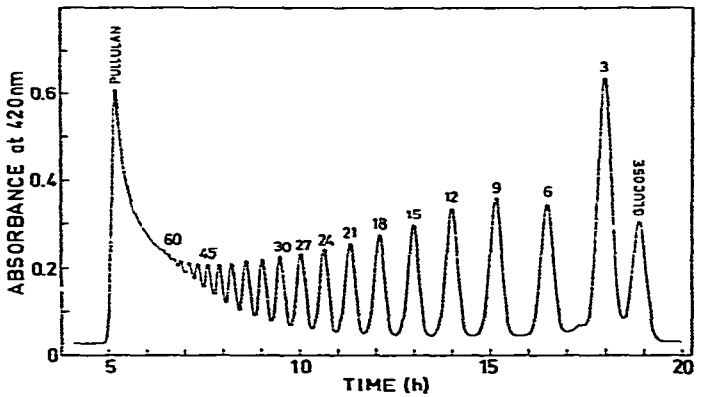

Fig. 1. Chromatography of glucose, maltotriose and different α-1,6-linked polymaltotrioses on Bio-Gel P-6. The column (gel bed, 197.6 × 1.8 cm I.D.) was eluted at 60°C with water at a flow-rate of 25 ml·h^{-1}. Sample size, 0.35 ml of a 2% solution. Oligosaccharides were prepared as described in the text and glucose was added as a marker. The numbers over the peaks indicate the DP.

tained by the action of pullulanase from *Aerobacter aerogenes*, which randomly hydrolyses the α-1,6-glycosidic linkages in pullulan.

A plot of the negative logarithm of the distribution coefficient (K_{av}) *versus* the degree of polymerization of polymaltotrioses (Fig. 2) yields the expected linear relationship with an almost constant increment of $\Delta - \log K_{av} \approx 0.05$ going from one member in this series to the next higher member. The results of linear regression analysis of $-\log K_{av}$ *versus* DP are given in the legend of Fig. 2.

The well known reaction of amylose and its degradation products to produce a colour with iodine has been studied extensively, but not many exact data exist on the effect of DP on colour formation. In order to demonstrate the effect of the chain

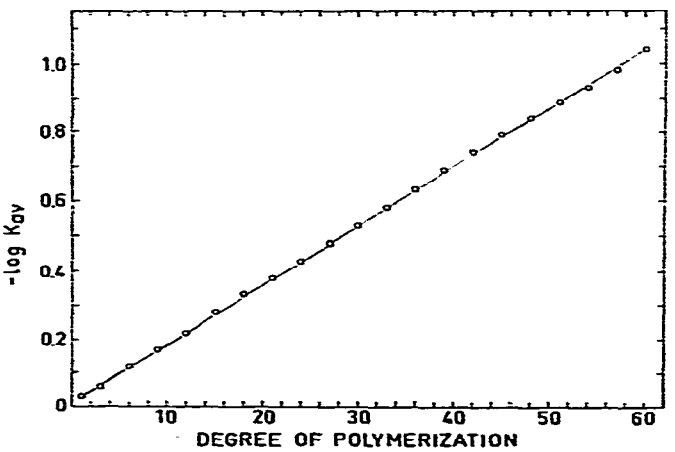

Fig. 2. Plot of $-\log K_{av}$ *versus* DP of α-1,6-linked polymaltotrioses. K_{av} is defined as $(V_e - V_0)/(V_t - V_0)$, where V_e, V_0 and V_t are the solute elution volume, void volume and total volume, respectively, of the gel mass in the column. V_0 = 124.6 ml was determined by chromatography of dextran 500. Chromatographic conditions as in Fig. 1. Linear regression analysis: slope, 0.0171; intersection with the ordinate, 0.0179; correlation coefficient, 0.9999.

length of malto-oligosaccharides on the colour of the carbohydrate–iodine complex, two amylose hydrolysates with different oligosaccharide size distributions (prepared according to conditions A and B) were mixed and fractionated on Bio-Gel P-6. Owing to the small increase in molecular mass, retention data for malto-oligosaccharides were obtained only for the first twenty members of this homologous series and separation to the baseline was incomplete using this column. From the resulting elution profile the K_{av} values were calculated. Using the first twenty members of the oligosaccharide series, linear regression analysis was performed. The linear relationship made it possible to calculate DP > 20 in this incompletely separated malto-oligosaccharide series from the elution volume. After staining the separated malto-oligosaccharides with iodine, the colour of the carbohydrate–iodine complex was correlated with the appropriate DP (Table I).

TABLE I

EFFECT OF CHAIN LENGTH OF MALTO-OLIGOSACCHARIDES ON THE COLOUR OF THE CARBOHYDRATE–IODINE COMPLEX

Degree of polymerization (DP)	*Colour of carbohydrate–iodine complex*	*Wavelength of maximum absorbance, λ_{max} (nm)**
2–18	None	—
19–24	Brown	460 (23)
25–29	Red	505 (27)
30–38	Red-violet	515 (31)
39–46	Blue-violet	545 (41)
>47	Blue	562 (59)

* The wavelength was measured at the DP given in parentheses.

Fig. 3 illustrates the separation of different series of homologous oligosaccharides on two coupled Bio-Gel P-4 columns. The lower chromatogram shows the separation of an amylose hydrolysate. Optimal resolution was obtained up to a chain-length of approximately 25 glucose units. With Bio-Gel P-4 columns branched polymaltotrioses were completely separated up to DP 40 (upper part of Fig. 3). Useful elution profiles of both hydrolysates were also obtained in half the time (10 h) on increasing the flow-rate to 100 ml · h^{-1} (column pressure *ca.* 2.5 bar). In preparative runs samples of 200–300 mg were fractionated on the coupled Bio-Gel P-4 columns and no loss in resolution was observed. Refractive index detection was used for this purpose. When the refractometer response measured as peak height is plotted as a function of carbohydrate concentration the resulting calibration graph shows that glucose and malto-oligosaccharides fall on the same line (data not shown).

Chromatography on Bio-Gel P-4 was used for the large-scale preparation of linear xylo-oligosaccharides. An elution profile of a partial acid hydrolysate of arabinoxylan is shown in Fig. 4. The arabinoxylan used in this study is characterized by the presence of arabinofuranose side-chains, which are extremely susceptible to acid hydrolysis. By mild acid treatment arabinose was completely removed from the higher oligosaccharides (X_4–X_n). As can be deduced from the HPLC results shown in the inset of Fig. 4, the di- and trisaccharide fractions contained mainly linear xylo-

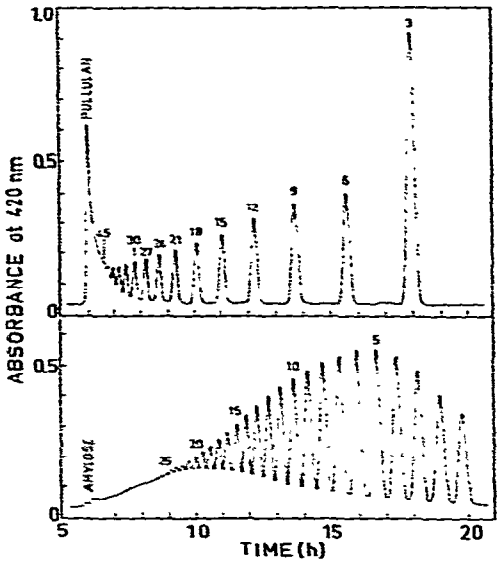

Fig. 3. Chromatography of oligosaccharides on Bio-Gel P-4. The upper figure shows the separation of α-1,6-linked polymaltotrioses from an enzymic digest of pullulan. The lower figure shows the elution profile of malto-oligosaccharides, which were obtained by partial acid hydrolysis of amylose according to conditions A as described under Experimental. Gel bed, 201 × 2.54 cm I.D.; column temperature, 60°C; eluent, water; flow-rate, 55 ml·h^{-1}. The numbers over the peaks indicate the DP.

oligosaccharides as well as minor amounts of arabinose-containing oligosaccharides. Confirmation that all isolated oligomers (X_4–X_n) are β-1,4-linked was obtained by degradation studies with highly purified β-xylosidase and xylanase preparations. UV spectra and enzymic degradation studies of the X_n peak revealed that this fraction contained lignin and small amounts of higher xylo-oligosaccharides.

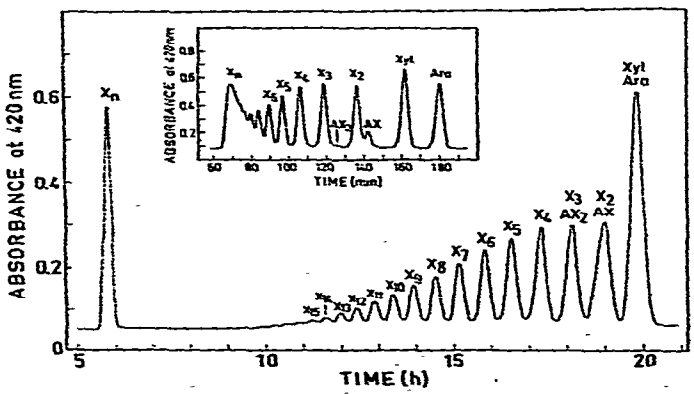

Fig. 4. Preparation of linear xylodextrins by partial acid hydrolysis of arabinoxylan and gel chromatography of the oligosaccharides on Bio-Gel P-4. The inset shows HPLC results for the same sample. Acid hydrolysis of arabinoxylan and peak identification are described under Experimental. Chromatographic conditions as in Fig. 3. Peaks: Xyl = xylose; Ara = arabinose; X_2, X_3, X_4, etc. = denote xylobiose, xylotriose, xylotetraose, etc.; AX = L-arabino-D-xylose; AX_2 = arabinoxylobiose; X_n = oligosaccharides with DP > 50.

DISCUSSION

We have performed oligosaccharide separations within a molecular weight range of 180–10,000 using 40 ± 4- and 47 ± 4-μm polyacrylamide gel particles with an appropriate pore size as column packings. The packing materials were obtained from commercial gels by an elutriation procedure.

Excellent separations of acetylated oligosaccharides containing up to 35 sugar residues have been achieved by reversed-phase liquid chromatography on bonded-phase silica[18] using an acetonitrile gradient (10–70% acetonitrile) as the eluent. However, the solubility of higher oligosaccharides in aqueous acetonitrile decreases as the DP increases and polysaccharides tend to bind irreversibly to the bonded phase packings. HPLC on cation-exchange resins in the silver[19] or calcium form[16,20] with water as eluent has been used successfully for the separation of mono- and oligosaccharides up to DP 15. For preparative purposes these HPLC techniques are not very useful, because with large sample amounts the resolution begins to deteriorate significantly, and hence long column lifetimes cannot be expected. Moreover, the cost of preparative columns packed with a high-performance support will limit this technique to analytical use.

Although major efforts were made to advance HPLC for the analysis of oligosaccharides, gel chromatography has remained an important technique for separating and isolating large oligosaccharides. The advantage of the system described in this paper over the many existing methods of analysis of oligosaccharide mixtures is the capability of separating oligomers up to DP 60, and as water is used as eluent at 60°C no solubility problems occur even with large oligomers. Moreover, the columns can be used for both analytical and preparative runs. Further advantages of the Bio-Gel techniques have been discussed earlier[1,2]. There is a disadvantage, however, in using the soft Bio-Gel P-6, as it tends to collapse at too high column pressures and high flow-rates should therefore be avoided.

The Bio-Gel P-4 columns have been used repeatedly for over 1 year and no deterioration of column performance was observed. Further particle size reduction of the relatively rigid Bio-Gel P-4 seems possible and would increase the column efficiency considerably.

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