*Journal of Chromatography*, 319 (1985) 311–318
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17,314

# STRUCTURAL HETEROGENEITY OF CELLOOLIGOMERS HOMOGENEOUS ACCORDING TO HIGH-RESOLUTION SIZE-EXCLUSION CHROMATOGRAPHY

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(First received September 27th, 1984; revised manuscript received October 19th, 1984)

## SUMMARY

Acid-free cellooligomer mixtures (G3...G7) can be prepared efficiently by precipitation of cellooligomers with ethanol and 1-propanol after partial hydrolysis of cellulose with hydrochloric acid. The carbohydrate oligomers, homogeneous according to their molar mass, can be obtained in good yield by preparative high-resolution size-exclusion chromatography on Bio-Gel P4 using water as eluent.

However, the analysis of the separated cellooligomers by ion-exchange chromatography of their borate complexes makes it evident that, besides the cellodextrins, several other oligomers are present. The secondary components observed contain at least one monomeric unit that is structurally different from glucose.

## INTRODUCTION

The use of cellodextrins as definite substrates for the characterisation of different cellulolytic enzymes dates back to the work of Grassmann *et al.*[1] in 1933. In the 1950s and 1960s several authors published results on the fractionation of cellooligomers either as free sugars[2–4] or as their acetates with a subsequent deacetylation[4–6].

The best known method for preparation, used almost exclusively even today, is the one described by Miller *et al.*[3,4] in 1960. Mixtures of glucose oligomers obtained from partial degradation of cellulose by acetolysis and deacetylation or by hydrochloric acid hydrolysis are separated by ethanol–water gradient elution on stearic acid-treated charcoal–celite columns. But this classic method is not suitable for producing purified cellodextrins with a degree of polymerisation (DP) greater than 5.

Following the introduction of polyacrylamide and dextran gels, separation of cellodextrins on these supports was reported[7–10]. Streamer *et al.*[11] and Huebner *et al.*[12] carried out separation on silica gel columns using a partial aqueous eluent, but their results for higher oligomers were unsatisfactory. Huebner *et al.*[12] were also able to fractionate cellooligomers up to a DP of 7 on a cation-exchange resin in the calcium form in an all-aqueous system.

0021-9673/85/$03.30 

Our objective was to develop a method that would allow the preparation of cellodextrins, especially with a DP greater than 5 in sufficient amounts and with high purity employing a chromatographic separation technique on reusable resins. The method of choice was high-resolution size-exclusion chromatography (SEC) on a polyacrylamide gel with a narrow range of particle sizes and with water as eluent. Cellodextrins were checked for purity by the sensitive method of sugar borate chromatography[13,14].

## EXPERIMENTAL

### *Acid hydrolysis of cellulose*

Cellulose powder (70 g; Cellulose APX, Serva, Heidelberg, F.R.G.) was suspended in 700 ml of fuming hydrochloric acid ($\rho$ = 1.19 g/mol) and stirred at 25°C for 2–3 h. The mixture was then slowly added to 5 l of 1-propanol at room temperature. The precipitate was collected by centrifugation at 5000 *g* for 5 min and the pellet was resuspended in 1.5 l of technical grade ethanol. This "washing" step was repeated four times (the last time with 1-propanol) until the pH of the suspension reached pH 5–6. After a final centrifugation the precipitate was extracted twice with 2.5 l of distilled water and stirred at 25°C overnight. The insoluble material was removed by centrifugation and the soluble cellodextrins of the supernatant were concentrated by rotary evaporation and subsequently freeze-dried to give 3.5 g of cellooligomers.

### *Acetolysis of cellulose*

Depolymerisation of cellulose by acetolysis was carried out as described by Miller *et al.*[4].

### *Size-exclusion chromatography of cellooligomer mixtures*

The chromatographic set-up was assembled from commercially available components. Three water-jacketed Cheminert glass columns (two LC-2-43, 100 × 5 cm I.D., and one LC-2-13, 30 × 5 cm I.D., used as precolumn) were packed with polyacrylamide gel Bio-Gel P-4 (less than 400 mesh) (Bio-Rad Labs., Richmond, CA, U.S.A.) using the conventional slurry-packing technique. To give the desired narrow range of particle sizes the dry gel was specially wind-sieved; additionally fines were removed from the hydrated particles by several decantation steps. The three columns were connected by 3.2 × 1.5 cm PTFE tubing and eluted in a downward-upward-downward direction with double-distilled water that was continuously prefiltered through a 0.22-$\mu$m filter unit and supplied to a degassing reservoir kept at 80–90°C. Columns were thermostated at 65°C using a Haake N2 B circulating bath (Haake, Karlsruhe, F.R.G.). After equilibration the total column length amounted to 210 cm. The SEC system was calibrated using D-glucose (inner volume, $V_i$) and dextran 70 ($\bar{M}_w \approx 7 \cdot 10^4$ g/mol) (void volume, $V_0$). A Milton Roy Minipump (Milton Roy, Philadelphia, PA, U.S.A.) was used to provide a constant flow-rate of 220 ml/h (corresponding $\Delta p$ *ca.* 75 kPa), and a differential refractive index monitor (Knauer, No. 88.00, Berlin, F.R.G.) for detection. Detector cells were maintained at 30°C by means of a Haake FE2 water-bath. The sample was applied with an Altex rotary valve (Altex, CA, U.S.A.). The sample loop was made from 3.2 × 1.5 cm PTFE

tubing to give a volume of 50 ml. Sample concentrations were 10 or 20 mg/ml, depending on the method of preparation of the cellooligomer mixtures. Fractions from several runs, collected by an LKB fraction collector (Super Rac, LKB Instruments, Bromma, Sweden), were concentrated by rotary evaporation and lyophilisation.

*Anion-exchange chromatography of cellooligomer borate complexes*

Analyses of cellooligomers and cellooligomer mixtures were performed with an automated sugar analyser Biotronik ZA 5100 (Biotronik, Munich, F.R.G.) using a 30 × 0.6 cm I.D. glass column thermostated at 60°C and filled with a strong-base anion-exchange resin DURRUM DA-X4-20[13,14]. The carbohydrate–borate complexes were eluted with a two-step borate buffer followed by a regeneration and equilibration procedure. Buffer flow-rates were 1 ml/min.

Eluting buffer A: 0.2 *M* $H_3BO_3$, pH 8.15; 0.8 h
Eluting buffer B: 0.4 *M* $H_3BO_3$, pH 9.45; 1.8 h
Regenerating buffer: 10% (w/v) $K_2B_4O_7$; 0.6 h
Equilibrating buffer: 0.1 *M* $H_3BO_3$, pH 8.0; 0.8 h

Carbohydrates were detected after a post-column derivatisation with orcinol–sulphuric acid reagent by measuring the absorbance at 420 nm. Peak areas were evaluated with a computing integrator. Sample sizes typically were 100 μg for individual cellodextrins and 300 μg for cellodextrin mixtures.

The reducing or non-reducing properties of the cellodextrin components were investigated by using a second Biotronik sugar analyser LC 2000 with a different detection system. Contrary to the orcinol–sulphuric acid derivatisation, only reducing carbohydrates can be detected by this method which is based on the reduction of a bicinchoninate–$Cu^{2+}$–aspartic acid reagent.

*Total hydrolysis of cellodextrins*

Samples of cellooligomers prepared by SEC were hydrolysed with hydrochloric acid (1 mol/l) at 100°C for 1 h (1 mg sugar/ml acid). Reaction was terminated by neutralisation with sodium bicarbonate. Samples of 150 μg were analysed by sugar borate chromatography.

## RESULTS

*Preparation of acid-free cellooligomer mixtures*

For the preparation of acid-free cellooligomers by hydrochloric acid hydrolysis of cellulose it is necessary to remove the acid without producing great amounts of neutralisation products such as sodium chloride. A rapid and efficient method for producing acid-free cellooligomers is to precipitate the cellooligomers after partial hydrolysis of cellulose using ethanol or 1-propanol. Whereas the main hydrolysis products, glucose and cellobiose, remain solubilised in the acid–alcohol–water phase, cellodextrins (G3...G8) precipitate accompanied by insoluble cellulose. As the cellooligomers are insoluble in alcohol, the hydrochloric acid adsorbed on the solid phase of cellooligomers and cellulose can be extracted by repeated washing with 1-propanol or ethanol. The mixture of oligomers obtained after water extraction and freeze-drying is a colourless and neutral product. The yield of water-soluble cellodextrins obtained is *ca.* 5% of the cellulose material used.

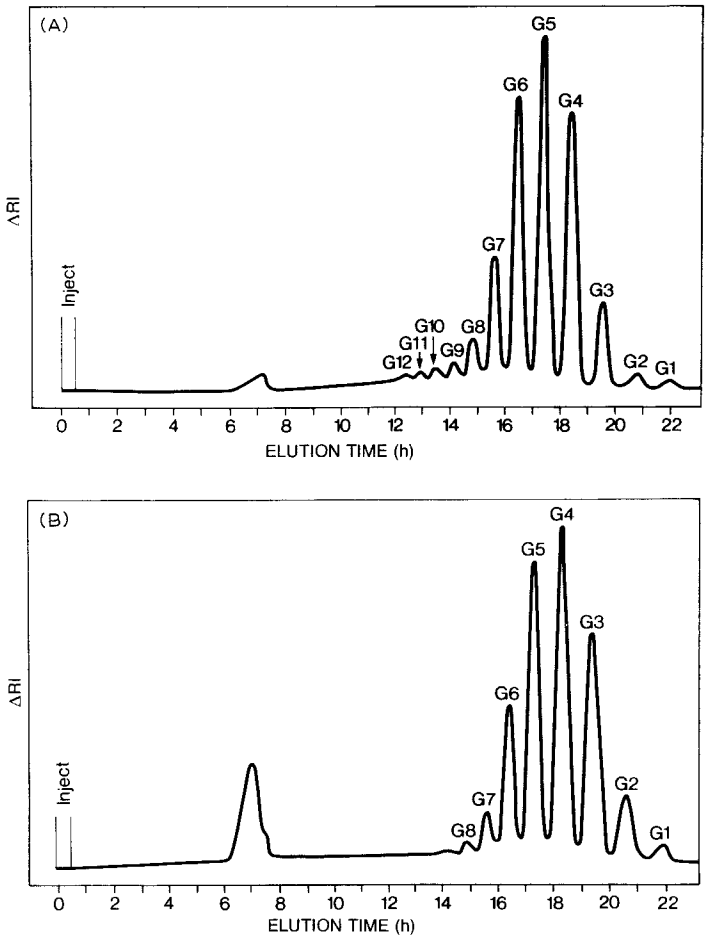


Fig. 1. Elution pattern of cellodextrin mixtures separated by size-exclusion chromatography. (A) Cellodextrins prepared by hydrochloric acid hydrolysis of cellulose. (B) Cellodextrins prepared by acetolysis of cellulose. Resin, Bio-Gel P4 (less than 400 mesh); temperature, 65°C; column, 210 × 5 cm I.D.; eluent, distilled water.

*Preparative separation of cellooligomers by SEC*

Typical elution patterns of cellooligomers separated by high-resolution SEC are shown in Fig. 1. An excellent separation efficiency is achieved on the Bio-Gel P4 columns at 60–65°C. Because of low band broadening and good selectivity the resolution is in the range 1–1.2 for each component, so that cellodextrins up to cellooctaose can be fractionated. Rechromatography of individual cellooligomers indicates products with uniform molar mass.

A plot of the negative logarithm of the distribution coefficient *versus* the degree of polymerisation of cellooligomers (Fig. 2) gives the same linear relationship as reported by various authors[9,10].

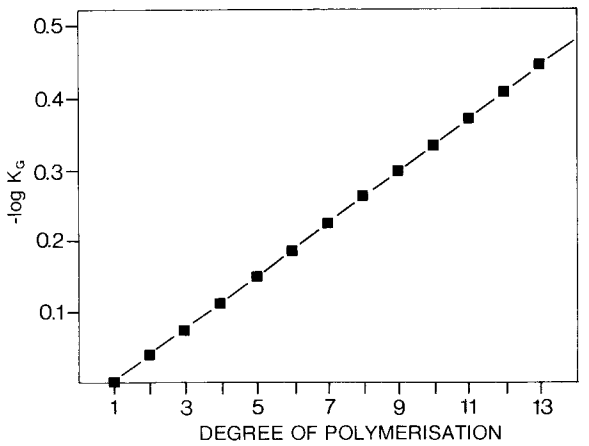


Fig. 2. Plot of $-\log K_G$ *versus* DP of cellodextrins. $K_G$ is defined as $(V_e - V_0)/V_i$ with $V_i = V_e^{glucose} - V_0$; where $V_e$, $V_0$, $V_i$ and $V_e^{glucose}$ are the solute elution volume, void volume, inner volume and glucose elution volume, respectively.

*Sugar borate chromatography*

Analyses of cellooligomers, homogeneous according to SEC, were performed by separating the oligomers as their borate complexes on an anion-exchange resin. Chromatograms obtained for cellodextrins (G3...G7) prepared from acid hydrolysis and for cellodextrins (G3...G6) prepared from acetolysis of cellulose are shown in Figs. 3A and B, respectively. The abbreviations G3, G4, G5, G6 and G7 are used to denote peaks corresponding to the members of the actual cellodextrin [$\beta(1\rightarrow4)$ glucose oligomer] series. The products purified by the method of Miller *et al.*[4] have the same elution times as those termed as G3...G7.

Sugar borate chromatography of individual cellooligomers obtained by acid hydrolysis of cellulose (Fig. 3A) reveals that, in addition to the cellodextrin peaks, at least three secondary components (termed a, b and c) are present. Another component starts to build up from cellohexaose onwards. Although sugar borate chromatography indicates that the carbohydrate components are different in their molecular structure, the molar mass of these products has to be the same or nearly the same as for the $\beta(1\rightarrow4)$ glucose oligomers.

Side component c makes up only 2% of the samples with no change in elution time detectable. Component b amounts to 8–9% of the samples independent of the degree of polymerisation. It shows the same tendency for shorter elution times with higher degrees of polymerization as occurs with the cellodextrin series itself. Component a also shows this reduction of elution times, but unlike b and c, it increases in amount as the degree of polymerization increases. Whereas the content in the cellotriose fraction is *ca.* 2%, it reaches 20% in celloheptaose. Component a was established to be non-reducing by analyses conducted on the second sugar analyser.

Total acid hydrolysis of cellooligomers (G3...G7) from acid hydrolysis yields *ca.* 96% glucose.

Fig. 3B shows chromatograms obtained from individual cellooligomers produced by acetolysis of cellulose. In this case there are a number of side components present in minor amounts that have elution times very close to those of the actual

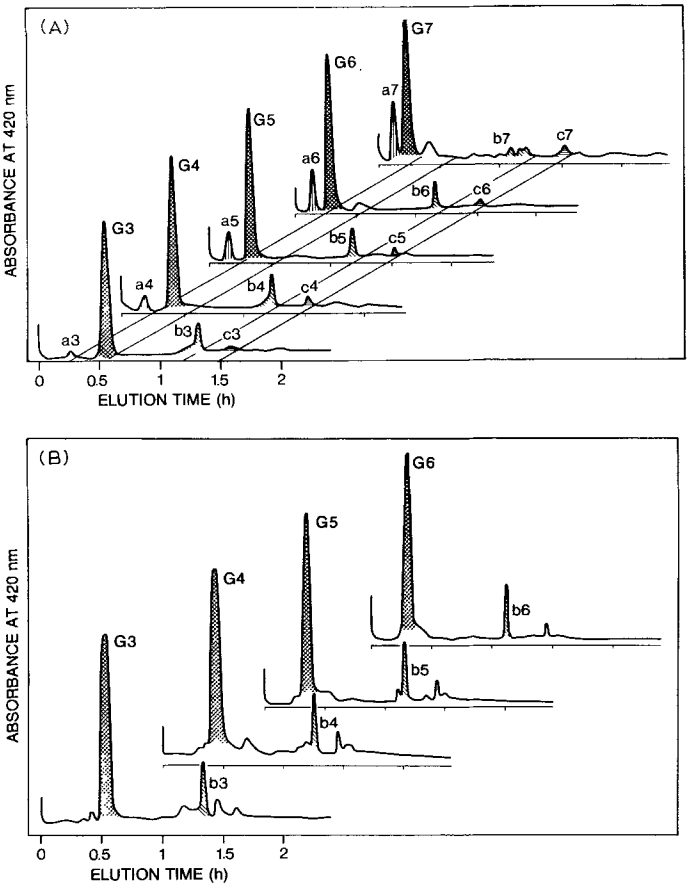


Fig. 3. Sugar borate chromatography of cellodextrins homogeneous according to SEC. (A) Cellodextrins (G3...G7) prepared by hydrochloric acid hydrolysis of cellulose. (B) Cellodextrins (G3...G6) prepared by acetolysis of cellulose. Anion-exchange resin Durrum DA-X4-20; eluent, two-step borate buffer; column, 30 × 0.6 cm I.D.; temperature, 60°C.

cellodextrin peaks. Component b makes up *ca.* 12% of the sample. The amount and the number of accessory components are clearly less in the case of cellodextrins produced by acetolysis of cellulose.

The composition of the cellodextrin mixture was not influenced by the type of alkanol used for the precipitation step. The composition of secondary components was independent of the temperature during the precipitation (0–20°C).

## DISCUSSION

The procedure of cellulose degradation by hydrochloric acid treatment as described[4], combined with a precipitating step using alkanols, is a rapid method for preparing water-soluble, acid-free cellodextrin mixtures without the need for a neutralisation step. Recently, Voloch *et al.*[15] described a precipitation method similar to that described here, but the primary hydrolysis step using hydrochloric acid in

place of sulphuric acid is more efficient, as reflected in the overall yield of cellodextrins (G3...G8). In comparison with the acid treatment of cellulose in an aqueous system, the procedure for preparing cellodextrins via acetylation of cellulose[4–6] is much more expensive and time-consuming. However, the advantage of this method is that the amount of oligomeric side components observed is reduced.

Although water-soluble oligomers can be fractionated with high-resolution SEC on Bio-Gel P4, each carbohydrate fraction with a defined molar mass contains components with different molecular structures. The sensitive analytical method of anion-exchange chromatography of the cellodextrin borate complexes shows that each cellodextrin fraction is accompanied by several related compounds with as yet unknown structures. It seems that the structural differences of these unknown oligomers in comparison with the known constitution of the cellodextrins are identical for each degree of polymerisation. In the case of hydrochloric acid hydrolysis, the amount of one of the unknown oligomers increased with increasing DP. The differences observed between the products of the hydrochloric acid hydrolysis and acetolysis of cellulose indicate that the formation of side components is dependent on the conditions of cellulose depolymerisation.

Cellooligomers prepared by either acid hydrolysis or acetolysis of cellulose are homogeneous according to chromatography on cation-exchange resins in their $Ag^+$ and $Ca^{2+}$ forms and on reversed-phase columns (unpublished results). Both types of resin are commonly used for fast HPLC of carbohydrate oligomers[12,15–18]. Thus these methods do not enable any separation of sugar oligomers with a constitution somewhat different from the structure of $\beta(1\rightarrow4)$ glucose oligomers. Attempted hydrolysis using amyloglucosidase indicates that no $\alpha(1\rightarrow4)$ or $\alpha(1\rightarrow6)$ glycosidic linkages are present.

$^{13}C$ NMR studies carried out at this stage with cellotriose and cellohexaose gave no information on the constitution of the secondary components. All the main peaks of the NMR spectra could be assigned and were in agreement with data available from the literature[19]. Several additional signals of very weak intensity were not identifiable.

For detailed kinetic studies on the cellulolytic hydrolysis of cellodextrins, it is necessary to use pure cellodextrins as substrates and to remove the components of unknown structure. Further purification of carbohydrate oligomers with an already homogeneous degree of polymerisation is achieved by affinity chromatography[20].

ACKNOWLEDGEMENTS

The authors thank K. Irrgang and Dr. K. L. Schimz for technical advice and for kindly performing the sugar analyses, and Dr. V. Wray (GBF, Braunschweig, F.R.G.) for running the $^{13}C$ NMR spectra.

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