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HIGH-SPEED LIQUID CHROMATOGRAPHY OF CELLODEXTRINS AND OTHER SACCHARIDE MIXTURES USING WATER AS THE ELUENT

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SUMMARY

Ion-exchange resin AG 50W-X4 (Ca^{2+}) separates the oligomers celloheptaose through glucose within 30 min when water is used as the eluent. The fractionation capabilities of this resin are a function of the procedures used in its preparation, packing, and operation. These procedures are described in detail. In addition to chromatograms showing separations obtained for cellodextrins and other saccharide mixtures, quantitative data relating concentrations of individual cellodextrin components to their respective peak areas are also presented.

INTRODUCTION

There are over 10^9 tons of "waste" cellulose generated annually in the United States[1]. This cellulose is a potentially renewable source of glucose syrups which are useful as a chemical feedstock and as a source of fermentation-derived chemicals[2]. In order to obtain data for design of a large-scale cellulose process, the kinetics of enzyme-catalyzed degradation of cellulose are being studied. This requires the ability to analyze for the hydrolysis products, which include the homologous series of cellodextrins, cellobiose through cellohaptaose, as well as glucose. Liquid chromatography (LC) appears to be the best approach.

Packings capable of separating various carbohydrates by LC may be divided into several groups according to the eluent used. Silica[3,4] and μBondapak[5–7] use acetonitrile–water while Bondapak AX/Corasil[5] and some ion-exchange resins[8–10] require alcohol–water as the eluent. IR-120[11] and DA-X4[12] need aqueous buffers while only water is used with IRA-400[13], some Dowex and Aminex resins[13–17], Bio-Gel P-2[18], and alumina[19]. Of these packings, Bio-Gel P-2 has been shown to give good separation of cellodextrins, although the separation takes 12 h per sample[20]. Chromatography of oligosaccharides is also possible on Dowex 1-X8 (Li^+) using ethanol–water as the eluent[8,10]. However, oligomers of high degree of polymerization tend to precipitate at the ethanol concentrations required for efficient separation[8].

Owing to the solubility properties of cellodextrins[21,22], an LC packing requiring only water as the eluent was chosen. This packing, AG 50W-X4 (Ca^{2+}), gives good fractionation of celloheptaose through glucose within 30 min.

MATERIALS

Cellodextrin mixtures were obtained by hydrolysis of Whatman CF-11 cellulose by the procedure of Miller *et al.*[23]. The mixtures were separated by preparative-scale LC. The identify of the individual cellodextrin components were verified using standard solutions of cellotriose, tetraose, pentaose, and hexaose.

Other sugars obtained commercially include glucose, lactose, fructose, cellobiose, galactose, mannose, arabinose, xylose, and sucrose. The water used in all experiments was distilled, deionized, and then ultrafiltered through an Amicon UM 2 membrane using a Model 402 cell (Lexington, Mass., U.S.A.).

The resin used was AG 50W-X4 (H^+) (particle size 20–30 μm, Bio-Rad, Griffin, Calif., U.S.A.). AG 50W-X4 is an analytical-grade cation-exchange resin.

EQUIPMENT

The liquid chromatograph was a Waters Assoc. (Milford, Mass., U.S.A.) ALC/GPC 200 series instrument with a UK 6 high-pressure injector and a differential refractometer detector. The detector was connected to a Spectra-Physics (Santa Clara, Calif., U.S.A.) Autolab I programmable integrator and an Omniscribe strip chart recorder. The pump in the ALC/GPC 200 was connected to an auxiliary solvent programmer (Waters Model 660). The refractometer was thermostated to 30° with a Haake Model FE circulating water-bath.

The resin was packed in an 61 cm × 8 mm I.D. stainless-steel column fitted with 10-μm inlet and outlet fittings (Waters Assoc.). The column, enclosed in a 25 mm I.D. glass jacket, was also thermostated with a Haake Model FE circulating water-bath.

Since degassed water was required for all runs, the water was maintained at 85–90° while being continuously stirred in a 1-l solvent reservoir flask. The water passed through a solvent reservoir filter (20–30 μm, Waters Assoc.) and then through a 1-m coil of 3.2 mm I.D. PTFE tubing before reaching the pump. The tubing, which was suspended in air of room temperature, served to cool the water to room temperature.

EXPERIMENTAL

Resin preparation

Before packing, the resin was converted to the Ca^{2+} form. In addition, resin fines were also removed.

AG 50W-X4 resin, 50 g, slurried in 200 ml of water, is added to 1.2 l of 2 *N* HCl in a 2-l vacuum flask. The mixture is very gently swirled to evenly distribute the resin and then placed under aspirator vacuum for 10 min. Following aspiration, the mixture is gently decanted into a 2000-ml graduate cylinder. The resin, which now has a yellow-white color, is allowed to settle. This takes 6 h or more.

After the resin has settled, some fines may still be suspended. These will appear either as a film floating on the top of the liquid or as very finely dispersed dust-like particles suspended in the liquid. To remove the fines all the liquid above the resin is siphoned off.

Next, the resin is resuspended in 200 ml of water by gently swirling. A volume of 2000 ml water is added in 300-ml increments while swirling and the resin is allowed to settle (6–8 h) followed by siphoning as before. This procedure is repeated twice.

After this, the resin is resuspended in 1 l of 0.5% (w/w) solution of $CaCl_2 \cdot 2H_2O$. The resin is allowed to settle (4 h), after which the calcium chloride solution is decanted. These steps are repeated a second time with a 2.5% $CaCl_2 \cdot 2H_2O$ solution and then a third time with a 5.0% solution. However, rather than decant the 5% calcium chloride solution after the third time, the resin is resuspended and the slurry is decanted into a 2-l flask. The flask is then placed in a water-bath and heated from room temperature to 80°. Once the slurry in the flask reaches 80° (approximately 1 h) it is held there for 30 min. Then the flask is allowed to stand at room temperature until its contents cool to room temperature (3–4 h). By this time the resin will have settled and it will be possible to decant the calcium chloride solution.

The resin is now resuspended in 1 l of distilled water to which two drops of Triton X-100 (Rohm & Haas, Philadelphia, Pa., U.S.A.) have been added. The suspension is poured into two 500-ml beakers and allowed to settle. The water is siphoned off. Note that the Triton serves to break up agglomerates of fines which might otherwise adhere to the larger resin particles and hence settle with the resin. Next 450 ml of water are added to each beaker in 150-ml increments with gentle swirling. Again the slurry is left to stand. Observe that a significant portion of the resin settles within 20–30 min. After this step is repeated several times, a sharply defined zone of finer particles moving down the beaker at the rate of *ca.* 0.1 cm/min should be noticeable. When the zone reaches a level within 1.5 cm above the bottom of the beaker, it (and the water above it) is carefully siphoned off without disturbing the resin already at the bottom. This step is repeated twice.

At this point, examination of the resin under the microscope at 256 × magnification will show a fairly uniform distribution of resin particles, devoid of fines (Fig. 1).

The procedure described above minimizes fracture of the resin and formation of fines. Our experience has shown AG 50W-X4 to be friable when shocked by sudden changes in temperature or calcium ion concentration. Decreased resolution, especially among higher-molecular-weight oligomers, and excessive operational pressure drop will result if a "shocked" resin is used.

Packing procedure

Two clean dry columns are connected as shown in Fig. 2a. At the top of the columns a 250-ml glass reservoir flask is attached with Tygon tubing. The 10-μm end-fitting at the bottom is closed off with an end-cap.

First the column assembly is filled with water. AG 50W-X4, prepared as above, is slurried in 150 ml of water, poured into the glass reservoir, and allowed to settle into the column. After all the resin has disappeared into the column (*ca.* 2 h), the reservoir is removed; an end-fitting is placed on the inlet, the pump is connected, and the cap on the bottom end-fitting is removed (Fig. 2b).

Using the solvent programmer the flow-rate is increased linearly over a 2-h period from 0 to *ca.* 1.2 ml/min. During the first hour the entire column apparatus is kept at room temperature. The water-bath is then turned on to heat the bottom column (Fig. 2b) from room temperature to 80°. During this phase of the packing

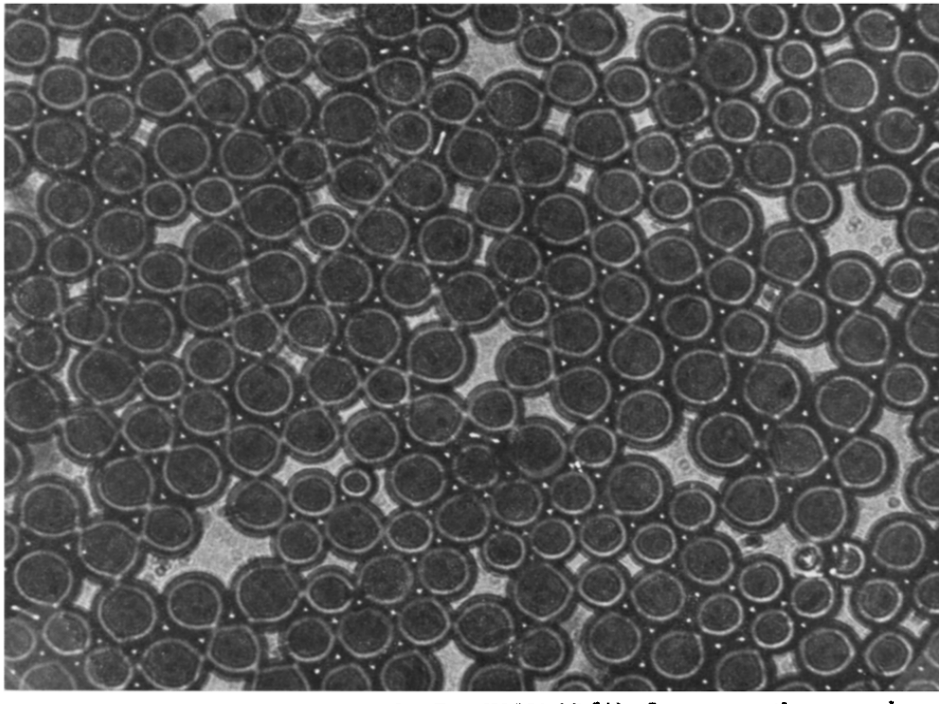

Fig. 1. Microphotograph (256 ×) of AG 50W-X4 (Ca^{2+}) after preparation procedure.

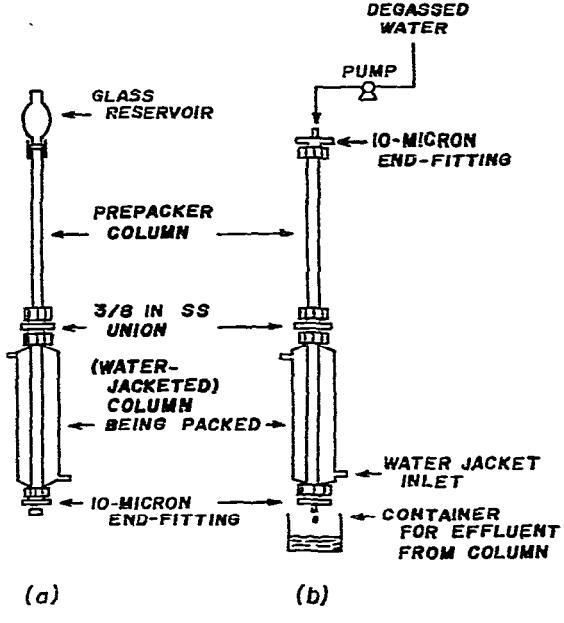

Fig. 2. Schematic diagram of column apparatus used for packing procedure. (a) Configuration for loading resin; (b) configuration during packing.

procedure the pressure must be monitored carefully. If the pressure reaches 2500–3000 p.s.i.g. prior to completing the flow program, the programmer is put into "hold" status and left there for the rest of the packing procedure. After final conditions are obtained, the column is left to pack for 12–14 h. The pressure during this period will typically be 2500 p.s.i.g. or less. Upon completion of packing, the solvent programmer should be set for "Reverse Program" using a 30-min time period. As soon as the flow stops, the bottom column is immediately disconnected and capped with a 10-μm end-fitting partially filled with resin. In the event that the column is not completely filled with resin at the termination of the packing procedure, more resin should be filled into the prepacker column and the procedure repeated.

Column operation

After packing, the column is connected in-line with the detector and heated for 2 h at 85°. Then, using Program No. 3 the flow-rate is increased from 0 to 0.5–0.6 ml/min over a 40-min period. The increase of pressure with flow-rate is sigmoidal. The greatest increase occurs typically between 0.5 and 0.6 ml/min. For the column discussed in this paper the pressure drop was initially 1500 p.s.i.g. when first started up. During seven days of continuous operation (24 h per day) the pressure slowly increased to and then leveled off at 2500 p.s.i.g.

After start-up the column is typically kept running 24 h per day at 85° and 0.5–0.6 ml/min during the work week. To shut down, the flow is programmed in the "Reverse" mode over a 40-min period using program No. 6.

If the column is allowed to cool during periods of inactivity, it must be re-heated to 85° for at least 6 h before flow is again initiated. Start-up is the same as described above.

Column efficiency

Using the resin preparation, packing, and operating procedures described above, a plate height of 0.10–0.12 mm will be obtained.

RESULTS AND DISCUSSION

The separation of cellodextrins celloheptaose through glucose is shown in Fig. 3. The peaks were identified using standards prepared from individual cellodextrin components. As can be seen from Fig. 4, the semi-logarithmic plot of the volume distribution coefficient D_v as a function of the number of glucose moieties is linear. D_v was computed by the method of Samuelson[24] using Maltrin (Grain Processing Company, Muscatine, Iowa, U.S.A.) to determine column void fraction (0.244). The refractometer attenuation (abbreviated "Attn."), eluent flow-rate, and sample size are indicated on the chromatogram. The quantities of cellodextrin represented by these peaks range from *ca.* 2 μg (100 μg/ml sample) for G_7 to *ca.* 20 μg (1 mg/ml) for G_4.

The area under the glucose and cellobiose peaks (as computed by the on-line integrator) as a function of concentration (Fig. 5) gives a linear relationship. Both glucose and cellobiose fall on the same line. The slope of the line (referred to as the response factor) is $2.6 \cdot 10^5$ area units per mg/ml. Similarly, the area response of cellotriose and cellotetraose is also linear with respect to concentration and the

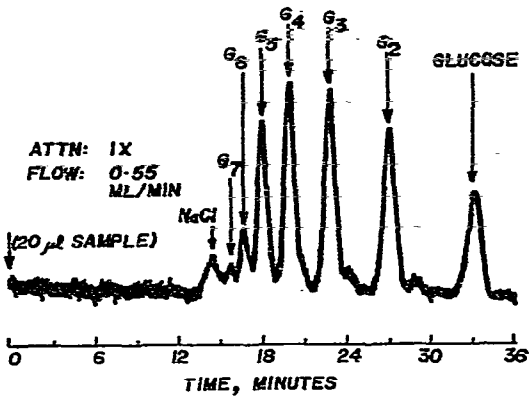

Fig. 3. Cellodextrin separation. (a) Chromatogram; G_2 = cellobiose; G_3 = cellotriose; G_4 = cellotetraose; G_5 = cellopentaose; G_6 = cellohexaose; G_7 = celloheptaose.

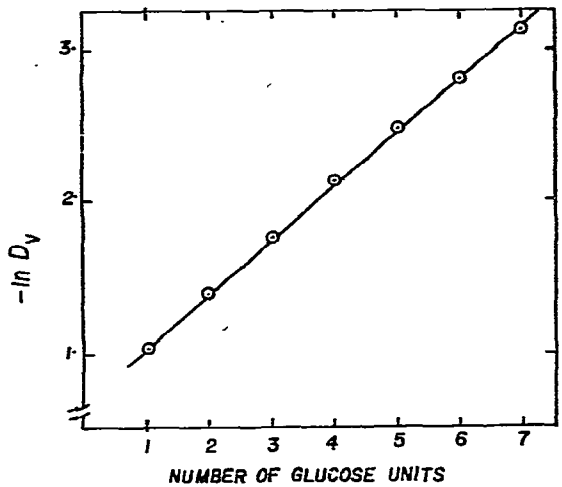

Fig. 4. Volume distribution plot.

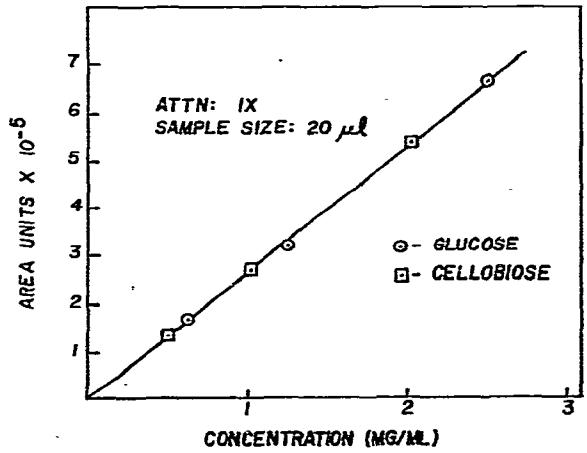

Fig. 5. Plot of integrated area as a function of glucose and cellobiose concentration.

response factors for these components are again *ca.* $2.6 \cdot 10^5$. Although the cellopentaose through celloheptaose fractions prepared in our laboratory were of insufficient purity to be able to carry out a rigorous calibration, it appears that their response factors are similar to those of the other cellodextrins. Therefore, we feel that the cellodextrins all have about the same area response on a mass basis. Thus, the column may be calibrated using glucose or cellobiose. This simplifies matters since glucose and cellobiose are available commercially while the other cellodextrins are not.

The reproducibility of analysis, based on multiple 20-μl injections of various concentrations of glucose and cellobiose, showed the relative sample-to-sample variation to be less than $\pm 3\%$ for concentrations as low as 100 μg/ml.

This column is capable of separating poly- and monosaccharide mixtures other than the cellodextrins. Included are lactose–glucose–galactose (Fig. 6a), sucrose–glucose–fructose (Fig. 6b), glucose–mannose–fructose (Fig. 7a), and glucose–xylose–arabinose (Fig. 7b).

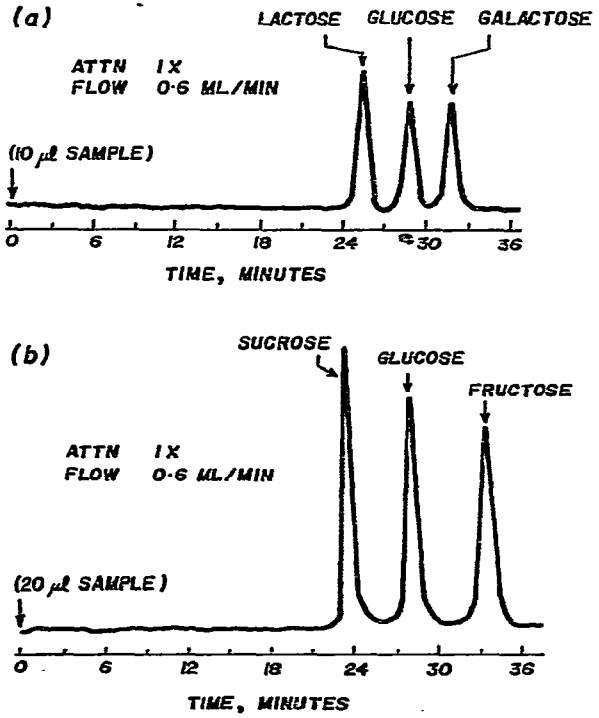

Fig. 6. Separation of mono- and disaccharides. (a) Lactose-glucose-galactose at concentrations of 2.4, 2, and 2 mg/ml, respectively. (b) Sucrose-glucose-fructose at concentrations of 2, 2, and 2 mg/ml, respectively.

Realistically, since many experiments require the use of buffers, it is necessary for the LC packing to be able to tolerate exposure to extraneous ions. During the course of its operation, the column has been exposed to various buffers without any deleterious effect. These include sodium succinate, Tris, sodium acetate, sodium

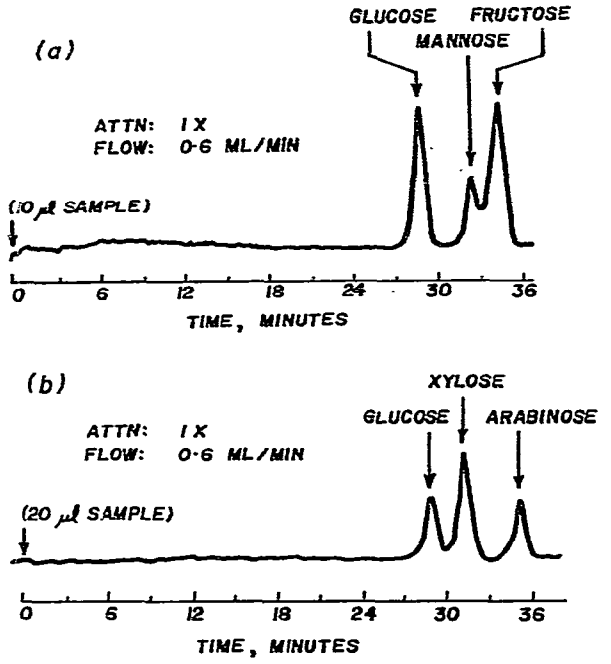

Fig. 7. Separation of monosaccharides. (a) Glucose–mannose–fructose at concentrations of *ca.* 3,3, and 3 mg/ml, respectively. (b) Glucose–xylose–arabinose at concentrations of 0.56, 1.13, and 0.56 mg/ml, respectively.

formate, citric acid, and sodium chloride. Their concentrations ranged from 1 to 100 m*M*. It should be noted, however, that if a buffer is present in a sample, care must be taken not to confuse the buffer peak with other components in the chromatogram.

The chromatograms in this report show the last peaks as eluting between 30 and 36 min. Since all the peaks in a chromatogram span only a portion of it, injections may be overlapped, thus reducing analysis time.

CONCLUSION

AG 50W-X4 gives a satisfactory separation of cellodextrins and other saccharide mixtures within 30 min. One of the resin's distinguishing features is that water is used as the eluent. This avoids the solubility problems encountered with other types of solvents. Moreover, water is cheap relative to other solvents, as well as non-toxic.

The resin has other advantages as well. It gives good fractionation of maltodextrins[7,25]. Resin and reagent costs for the column described are relatively low. If the column is properly prepared and packed, its resolution and pressure-drop characteristics are fairly stable. The AG 50W-X4 column discussed was operated continuously for 4 weeks without any detectable change in resolution.

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