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# Separation of glucose polymers by hydrophilic interaction chromatography on aqueous size-exclusion columns using gradient elution with pulsed amperometric detection

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## ABSTRACT

Maltooligosaccharides were submitted to hydrophilic interaction chromatography on three aqueous size-exclusion columns. When mobile phase compositions were 0% to 40% (v/v) acetonitrile in water, the chromatographic mechanism was by size exclusion on all three columns; at concentrations $\geq 50\%$ (v/v) acetonitrile, the carbohydrates were fractionated by partition chromatography ($0.88 > k' > 143$; where $k'$ is the solute capacity factor), and the order of elution was reversed. When maltooligosaccharides were eluted from the three columns using isocratic mobile phases in which the concentration of acetonitrile was varied from 50% to 75% (v/v), a negative linear relationship ($R^2 > -0.973$) existed between retention and solvent strength; retention increased as the polarity of the mobile phase was decreased. When the composition of the mobile phase was 65% acetonitrile in water, a correlation ($R^2 > 0.99$) was found in all three columns between the degree of polymerization and the retention of the oligosaccharide. With gradient elution, the Protein-Pak 60 column resolved N-acetylneuraminic acid, rhamnose, arabinose and a mixture of commercially available glucose polymers; the between-run precision of the retention times ($n = 16$) for the chromatography varied from 0.09 to 0.64% (relative standard deviation). The chromatography was applied to the analysis of enzyme-hydrolyzed starch digests.

## INTRODUCTION

The analysis of monosaccharides, non-protein-bound oligosaccharides, and glycoprotein-derived oligosaccharides is necessary in certain disciplines in both biomedicine and industry. Sulfonated polystyrene-divinylbenzene cation-exchange supports with various counter ions [1-6], reversed-phase columns with $C_{18}$ bound to silica [7] or polyvinyl alcohol [8] supports, and more recently, pellicular quaternary amine-bonded anion exchange supports [9–16] have been used to separate a variety of mono-, di- and oligosaccharides. Carbohydrates separated on the cation-exchange and reversed-phase supports were observed by refractive index detection [1–8], while those separated by anion exchange were observed by the more sensitive pulsed amperometric detection [9–16].

Carbohydrates have also been separated on columns that contain hydrophilic, polar-bonded phases. Mono-, di- and oligosaccharides have been separated on silica-bound amine [17–22], diol [20] and polyol [23] supports. A hydroxylated polymeric support was used to separate neutral oligosaccharides derived from glycoproteins [24]. A feature common to all these separations was the resolution of highly polar solutes on polar, hydrophilic bonded phases; the separations were performed with mobile phases of acetonitrile and water. Retention of the polar solutes increased with the volume percent of acetonitrile, and evidence suggested that carbohydrate separation occurred by partition [19,22]. At higher percentages of acetonitrile, proportionally

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more water was adsorbed to the hydrophilic group on the support than was present in the mobile phase, and the establishment of the static layer of water was required for partitioning and separation to occur (19,22).

Solutes other than carbohydrates have been separated on polar-bonded phases. Silica-bound polyol [23] was used to separate phenols and polyphenols with a mobile phase of hexane-methanol-tetrahydrofuran; when the mobile phase was phosphate buffer, however, proteins were separated by size exclusion. On chitosan (de-acetylated chitin) adsorbed to a silica support, amino acids, nucleotides, and dipeptides were separated by mobile phases of acetonitrile in water [25]. Binding of a hydrophilic group to a polystyrene–divinylbenzene support enabled separation of solutes by either reversed-phase or normal-phase chromatography [26]. Alpert has introduced the term "hydrophilic interaction chromatography" [27] to describe the previous polar-bonded phase separations [17–26], as well as the separation of amino acids, cyclodipeptides, phosphorylated amino acids, dipeptides, 3-hydroxyl-2-nitropyridyl-$\beta$-maltooligoglycosides, and oligonucleotides on polyhydroxyethyl-aspartamide-bound silica columns.

Our objectives in this study were to determine whether commercially available size-exclusion columns could function as hydrophilic interaction columns, and if so, to separate glucooligosaccharides using gradient elution and pulsed amperometric detection.

## EXPERIMENTAL

### *Materials*

Durapore hydrophilic filters (0.22 $\mu$m, 47 mm) and the Protein-Pak 60 column were from Waters (Milford, MA, USA). The TSK-G 2000 SW column was from Phenomenex (Torrance, CA, USA) and the G-Oligo-PW column was from TosoHaas (Philadelphia, PA, USA). All monosaccharides, disaccharides, maltooligosaccharides and starch were purchased from Sigma (St. Louis, MO, USA). Sodium acetate (HPLC grade), acetonitrile (HPLC grade) and sodium hydroxide solution (50%, w/w) were obtained from Fisher Scientific (Houston, TX, USA). Monitrol level II sera were purchased from American Dade (Miami, FL, USA).

### *Chromatography*

*Apparatus.* Chromatography was performed on a Waters 860 system; the host computer was a MicroVAX 2000 which employed the VMS operating system. DECnet software enabled multisystem networking via Ethernet connections. Connections were made to the Ethernet via a DEC transceiver, which was connected in turn to the Laboratory Acquisition and Communications/Enviroment (LAC/E) Module. Communication with the chromatography instruments was accomplished by IEEE interface (direct or via System Interface Module).

*Isocratic separations.* Three high-performance size-exclusion chromatography (HPSEC) columns were used to separate standard monosaccharides, disaccharides, and maltooligosaccharides: the Waters Protein-Pak 60 column (300 mm × 7.9 mm I.D., 10 $\mu$m), the Phenomenex TSK-G 2000 SW (300 mm × 7.6 mm I.D., 10 $\mu$m), and the TosoHaas G-Oligo-PW column (300 mm × 7.8 mm I.D., 6 $\mu$m). Samples (50$\mu$l) were injected by a Waters Model 710 WISP autoinjector; two Waters Model 510 pumps produced isocratic mobile phases which eluted the solutes at a flow rate of 1.0 ml/min. Solvents were sparged with helium and maintained in a helium atmosphere during all separations. Post-column eluate was delivered into a 3-way PTFE mixing tee and mixed with 0.5 *M* sodium hydroxide, which was delivered at a flow rate of 0.4 ml/min; helium, at 50 p.s.i., was used to deliver the sodium hydroxide solution to the mixing tee. After the eluate was mixed with sodium hydroxide, the peaks were detected with a Dionex pulsed amperometric detection (PAD) system (Sunnyvale, CA, USA). The potentials and time periods were set as follows: E1 was 0.05 V, E2 was 0.6 V, E3 was −0.6 V, T1 was 480 ms, T2 was 120 ms, and T3 was 60 ms. The sensitivity of the pulsed amperometric detector was set at 30 $\mu$A unless otherwise specified.

*Determination of capacity factors.* Separate stock solutions of α-D-glucose (DP 1; DP = degree of polymerization), maltose (DP 2), maltotriose (DP 3), maltotetraose (DP 4), maltopentaose (DP 5), maltohexaose (DP 6) and maltoheptaose (DP 7) were prepared at a concentration of 1.0 mg/ml, then diluted 1:9 with Milli-Q water (Millipore, Bedford, MA, USA). To determine the capacity factors of DP 1 through DP 7 on all three columns, 50 $\mu$l containing 5.0 $\mu$g of each individual standard was in-

jected and eluted isocratically; the mobile phase compositions were 0, 10, 20, 30, 40, 50, 55, 60, 65, 70 and 75% (v/v) acetonitrile in water.

Solutions of N-acetylneuraminic acid, α-L-rhamnose, α-L-fucose, D(+)-xylose, D(−)-arabinose, β-D-fructose, D(+)-mannose, α-D-glucose, D(+)-galactosamine, D(+)-glucosamine, D(+)-galactose, sucrose, lactulose, maltose and lactose were prepared at a concentration of 1.0 mg/ml, then diluted 1:9 with Milli-Q water. A 50-μl volume of each individual standard was chromatographed separately on both the Protein-Pak 60 and G-Oligo-PW columns. An isocratic mobile phase consisting of 75% acetonitrile in water, at a flow-rate of 1.0 ml/min, was used to elute the mono- and disaccharides.

*Chromatography of maltooligosaccharide mixtures.* A mixture of maltooligosaccharides (DP 1 through DP 10) was run on the three columns, with gradient elution; DP 1, DP 2 and DP 3 were each mixed to a final concentration of 0.25 mg/ml, and a standard mixture of DP 4 to DP 10 (powder) was mixed to a final concentration of 2.5 mg/ml. A 50-μl injection containing 12.5 μg each of DP 1, DP 2, DP 3, and 125 μg (total) of DP 4 through DP 10 was made on to each column. The gradient program for the separation on the Protein-Pak 60 column was 70% (v/v) acetonitrile in water for 5.0 min, a linear gradient to 50% in 30 min, held at 50% for 20 min. For the TSK-G 2000 SW column, the gradient program was 70% (v/v) acetonitrile in water for 15 min, a linear gradient to 65% in 15 min, a linear gradient to 50% in 30 min, held at 50% for 20 min. The gradient program for the separation of the maltooligosaccharides on the G-Oligo-PW column was identical to that used for the Protein-Pak 60 column, except that the final condition was held at 50% for 50 min.

*Separation of selected monosaccharides and glucose polymers.* The Protein-Pak 60 column was used to separate a commercially available mixture of glucose polymers (Polycose) to which were added selected monosaccharides; 100 μg of N-acetylneuraminic acid, 25 μg of rhamnose, 25 μg of arabinose and 7.0 mg of Polycose were dissolved in Milli-Q water to final concentrations of 0.12, 0.03, 0.03 and 8.23 mg/ml, respectively. The column was injected with 50 μl containing 1.45 μg each of rhamnose and arabinose, 5.85 μg N-acetylneuraminic acid and 411 μg Polycose, and the solutes were separated by gradient elution. The gradient program was 70% (v/v) acetonitrile in water for 10.0 min, a linear gradient to 50% in 30 min, held at 50% for 20 min.

To determine whether the separation was reproducible, eight samples of the Polycose and monosaccharide mixture were run consecutively on the Protein-Pak 60 column on each of two separate days; the within-run and between-run precision of the retention times of the monosaccharides and oligosaccharides were determined. Eighty min after injection, the column was allowed to equilibrate for 20 min at starting conditions, *i.e.*, 70% acetonitrile.

To demonstrate the sensitivity of detection and to optimize the separation, 10 μl containing 0.8 μg each of rhamnose and arabinose, 3.33 μg of N-acetylneuraminic acid and 33.3 μg of Polycose were injected on to the Protein-Pak 60 column. The gradient program consisted of 70% (v/v) acetonitrile for 1.0 min, a linear gradient to 50% for 39 min, held at 50% for 20 min.

*Separation of starch hydrolysates.* In one set of experiments, 0.9 ml of a 2.0 g/100 ml solution of starch was mixed with 0.1 ml of Monitrol level 2 serum containing 500 U/ml of α-amylase. The mixtures were allowed to incubate at 37°C for 4.0 h; at 1.0, 2.0, 3.0, and 4.0 h, the tubes were heated at 100°C for 2.0 min, then 50 μl containing 450 μg of hydrolysate was injected on to the Protein-Pak 60 column. The enzyme control consisted of adding 0.1 ml of Monitrol serum to 0.9 ml of water, while the substrate control consisted of adding 0.1 ml water to 0.9 ml of starch solution. The enzyme and substrate controls were incubated in the same manner as were the enzyme–substrate mixtures. The controls and incubation mixtures were separated by gradient elution. The gradient program was 70% (v/v) acetonitrile for 1.0 min, a linear gradient to 50% for 49 min, held at 50% for 20 min. The column was then equilibrated for 20 min with 70% (v/v) acetonitrile before the next injection.

In a separate set of experiments, 0.1 ml of Monitrol serum was added to 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 and 0.9 ml of a 1.0 g/ml solution of starch. Water was added to each solution to bring the volume to 1.0 ml. The solutions were incubated at ambient temperature for 16.0 h, then heated at 100°C for 2.0 min. A 50-μl volume was injected and separated by the gradient used for the starch hydrolysates.

RESULTS

The isocratic elution of maltooligosaccharides DP 1 through DP 7, with water as the mobile phase, are depicted for the Protein-Pak 60 (Fig. 1a), TSK-G 2000 SW (Fig. 1c), and the G-Oligo-PW (Fig. 1e) columns. Neither the Protein-Pak 60 nor the TSK-G 2000 SW columns resolved these oligomers by size exclusion. The G-Oligo-PW column, however, did give some resolution; the maltooligosaccharides DP 7, DP 6 and DP 5 eluted at 7.75 min, DP 4 at 7.96 min, DP 3 at 8.34 min, DP 2 at 8.82 min and DP 1 at 9.48 min. Although the Protein-Pak 60 and TSK-G 2000 SW columns did not resolve the components in the mixture under these conditions, chromatography of individual standards demonstrated that the maltooligosaccharides were separated by size exclusion, *i.e.*, they were eluted in descending size order from DP 7 through DP 1.

Chromatography of the maltooligosaccharides using a mobile phase composition of 65% (v/v) acetonitrile in water is shown for the Protein-Pak 60 (Fig. 1b), TSK-G 2000 SW (Fig. 1d) and G-Oligo-PW columns (Fig. 1f). All three columns exhibited retention behavior opposite to that observed for the

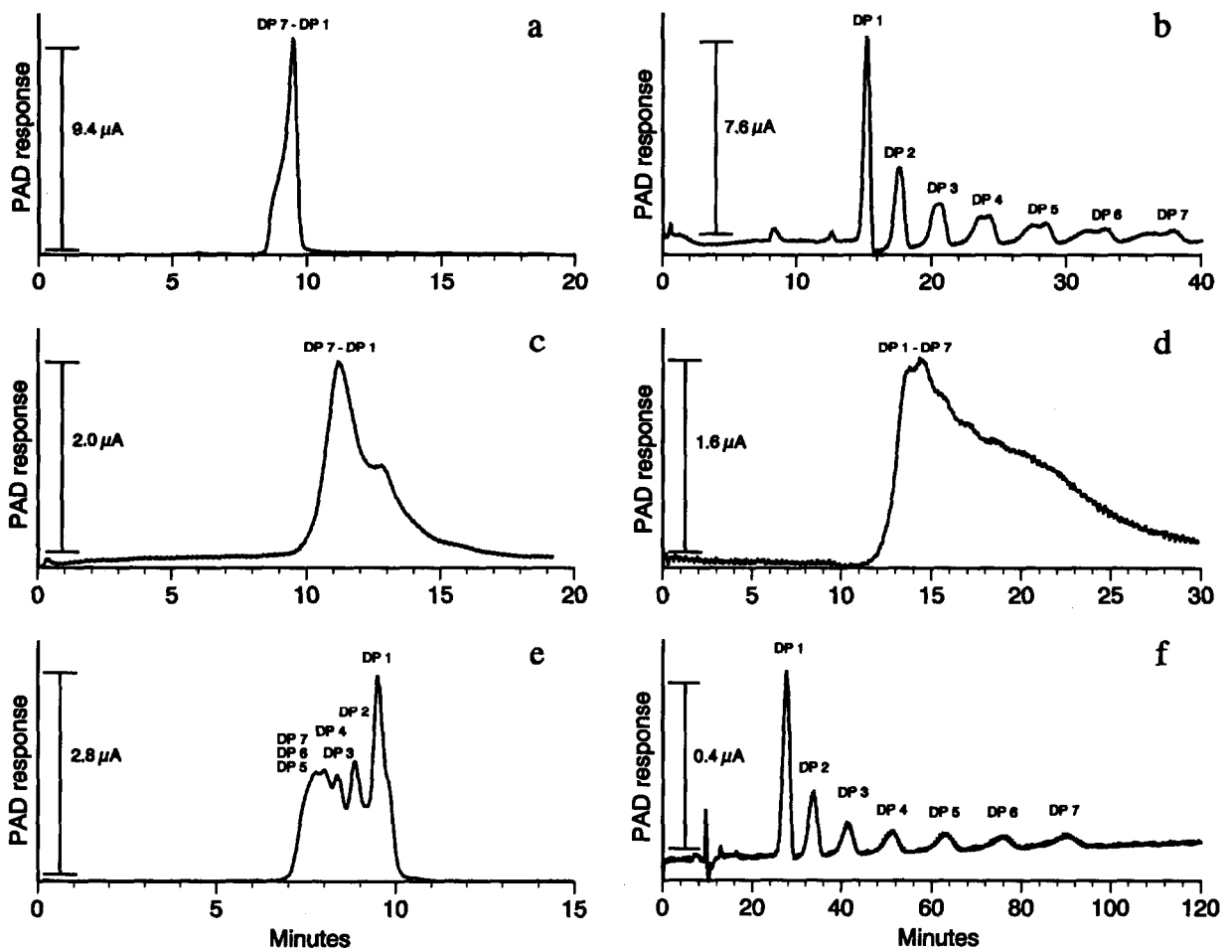


Fig. 1. Chromatography of glucose (DP 1) and maltooligosaccharides (DP 2 through DP 7) on HPSEC columns: using water as the mobile phase, the chromatography of DP 1 through DP 7 on the Protein-Pak 60 (a), TSK-G 2000 SW (c) and G-Oligo-PW (e) columns; using 65% (v/v) acetonitrile in water as the mobile phase, the partition chromatography of DP 1 through DP 7 on the Protein-Pak 60 (b), TSK-G 2000 SW (d), and G-Oligo-PW (f) columns.

size-exclusion separations; the order of elution was reversed. When the maltooligosaccharides were separated on the Protein-Pak 60 column under these conditions (Fig. 1b), their retention times were 15.1 (DP 1), 17.6 (DP 2), 20.6 (DP 3), 24.2 (DP 4), 28.4 (DP 5), 32.8 (DP 6) and 37.9 min (DP 7). In addition, DP 4 through DP 7 were separated into $\alpha$ and $\beta$ anomers. The TSK-G 2000 SW column (Fig. 1d) was unable to resolve the mixture, even though the maltooligosaccharides were retained and eluted in the order observed for the Protein-Pak 60 column. The maltooligosaccharides were separated in the same order on the G-Oligo-PW column (Fig. 1f) as on the Protein-Pak 60 column; they were retained, however, to a greater degree. The retention times were 27.7 (DP 1), 33.8 (DP 2), 41.3 (DP 3), 51.0 (DP 4), 63.0 (DP 5), 75.6 (DP 6), and 90.0 min (DP 7).

Table I lists the capacity factor ($k'$) values for the maltooligosaccharides DP 1 through DP 7 after elution from the three columns using mobile phase compositions where the percentage (v/v) of acetonitrile in water was 0, 10, 20, 30, 40, 50, 55, 60, 65, 70 and 75, respectively. For all three columns, chromatography with mobile phase compositions consisting of 0 to 40% (v/v) acetonitrile produced $k'$ values for the retention of DP 1 to DP 7 that were inversely proportional to chain length at each composition, *i.e.* the $k'$ values decreased as chain length increased (size exclusion). Conversely, at acetonitrile concentrations from 50 to 75% (v/v) the $k'$ values at each concentration were proportional to

TABLE I

THE EFFECT OF MOBILE PHASE COMPOSITION ON THE RETENTION OF MALTOOLIGOSACCHARIDES DP 1 THROUGH DP 7 ON THE PROTEIN-PAK 60, TSK-G 2000 SW AND G-OLIGO-PW COLUMNS

| | Capacity factor | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Acetonitrile in Milli-Q water (%, v/v) | | | | | | | | | | |
| | 0 | 10 | 20 | 30 | 40 | 50 | 55 | 60 | 65 | 70 | 75 |
| *Protein-Pak 60* | | | | | | | | | | | |
| DP 1 | 0.59 | 0.62 | 0.62 | 0.64 | 0.71 | 0.88 | 1.03 | 1.18 | 1.52 | 1.98 | 2.65 |
| DP 2 | 0.56 | 0.60 | 0.60 | 0.61 | 0.70 | 0.92 | 1.13 | 1.37 | 1.93 | 2.76 | 4.15 |
| DP 3 | 0.53 | 0.56 | 0.58 | 0.59 | 0.69 | 0.96 | 1.23 | 1.60 | 2.43 | 3.80 | 6.43 |
| DP 4 | 0.51 | 0.53 | 0.55 | 0.58 | 0.68 | 0.99 | 1.37 | 1.80 | 3.03 | 5.20 | 9.82 |
| DP 5 | 0.49 | 0.51 | 0.54 | 0.57 | 0.66 | 1.02 | 1.50 | 2.05 | 3.73 | 6.93 | 14.6 |
| DP 6 | 0.48 | 0.50 | 0.52 | 0.56 | 0.65 | 1.06 | 1.62 | 2.20 | 4.46 | 8.93 | 21.3 |
| DP 7 | 0.46 | 0.48 | 0.51 | 0.55 | 0.63 | 1.06 | 1.75 | 2.58 | 5.32 | 11.5 | 29.8 |
| *TSK-G 2000 SW* | | | | | | | | | | | |
| DP 1 | 1.03 | 1.05 | 1.08 | 1.12 | 1.19 | 1.31 | 1.79 | 1.74 | 2.06 | 2.54 | 2.94 |
| DP 2 | 1.03 | 1.05 | 1.07 | 1.12 | 1.17 | 1.32 | 1.87 | 1.70 | 2.15 | 2.86 | 3.77 |
| DP 3 | 1.01 | 1.04 | 1.06 | 1.11 | 1.17 | 1.34 | 1.97 | 1.86 | 2.41 | 3.53 | 5.08 |
| DP 4 | 0.99 | 1.01 | 1.05 | 1.10 | 1.17 | 1.36 | 2.03 | 2.04 | 2.76 | 4.34 | 6.96 |
| DP 5 | 0.98 | 1.00 | 1.03 | 1.09 | 1.16 | 1.39 | 2.13 | 2.19 | 3.08 | 5.32 | 9.45 |
| DP 6 | 0.97 | 1.00 | 1.03 | 1.08 | 1.16 | 1.41 | 2.19 | 2.42 | 3.54 | 6.33 | 12.2 |
| DP 7 | 0.96 | 1.00 | 1.02 | 1.08 | 1.16 | 1.41 | 2.24 | 2.62 | 3.72 | 7.49 | 16.0 |
| *G-Oligo-PW* | | | | | | | | | | | |
| DP 1 | 1.01 | 1.07 | 1.15 | 1.29 | 1.65 | 2.28 | 2.87 | 3.67 | 4.91 | 7.08 | 11.0 |
| DP 2 | 0.87 | 0.93 | 0.99 | 1.11 | 1.50 | 2.30 | 3.06 | 4.26 | 6.30 | 10.2 | 18.8 |
| DP 3 | 0.74 | 0.82 | 0.88 | 0.95 | 1.40 | 2.34 | 3.32 | 4.91 | 8.01 | 14.8 | 32.3 |
| DP 4 | 0.68 | 0.74 | 0.80 | 0.87 | 1.31 | 2.41 | 3.59 | 5.78 | 10.3 | 21.1 | 54.8 |
| DP 5 | 0.63 | 0.67 | 0.73 | 0.83 | 1.23 | 2.49 | 3.93 | 6.72 | 13.0 | 29.9 | 89.3 |
| DP 6 | 0.60 | 0.63 | 0.66 | 0.79 | 1.16 | 2.55 | 4.24 | 7.65 | 15.9 | 40.5 | 117 |
| DP 7 | 0.57 | 0.59 | 0.63 | 0.75 | 1.10 | 2.64 | 4.62 | 8.72 | 18.9 | 54.4 | 142 |

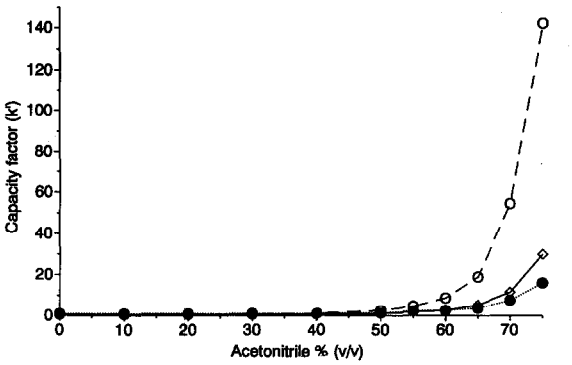


Fig. 2. The effect of mobile phase composition on the retention of maltooligosaccharide DP 7 on HPSEC columns. Maltoheptaose (DP 7) was applied to and eluted from the G-Oligo-PW (○), Protein-Pak 60 (◇) and the TSK-G 2000 SW (●) columns; isocratic mobile phase compositions consisting of 0, 10, 20, 30, 40, 50, 55, 60, 65, 70 and 75% (v/v) acetonitrile in water were used for elution on each column.

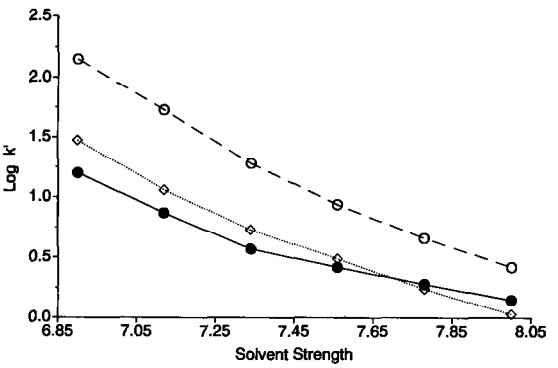


Fig. 4. The effect of solvent strength on the retention of oligosaccharide DP 7 when chromatographed by partition on HPSEC columns. DP 7 was eluted from the G-Oligo-PW (○), Protein-Pak 60 (◇), and TSK-G 2000 SW (●) columns using mobile phase compositions of 50, 55, 60, 65, 70 and 75% (v/v) acetonitrile in water.

chain length, *i.e.*, the $k'$ values increased for the retention of DP 1 to DP 7.

Fig. 2 further illustrates the trend that occurred for all three columns; at 50% (v/v) acetonitrile, the $k'$ values began to increase and continued to do so for concentrations of 60, 65, 70 and 75% (v/v). The only difference among the three columns was the degree of interaction at each concentration. The G-Oligo-PW column exhibited stronger interaction with the oligosaccharide at all solvent compositions than did the Protein-Pak 60 column; in turn, the Protein-Pak 60 column exhibited a greater interaction than did the TSK-G 2000 SW column.

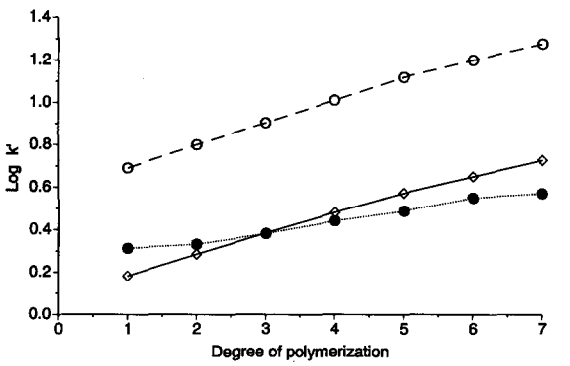


Fig. 3. The effect of the degree of polymerization of maltooligosaccharides on their retention when chromatographed by partition on HPSEC columns. Using a mobile phase composition of 65% (v/v) acetonitrile in water, DP 1 through DP 7 were applied to and eluted from the G-Oligo-PW (○), Protein-Pak 60 (◇), and TSK-G 2000 SW (●) columns.

For all three columns, when maltooligosaccharides DP 1 through DP 7 were eluted with a mobile phase composition of 65% (v/v) acetonitrile (Fig. 3), a linear relationship existed between the degree of polymerization of the maltooligosaccharide and log $k'$. The correlation coefficient ($R^2$) values were 0.998 for the G-Oligo-PW, 0.998 for the Protein-Pak 60, and 0.993 for the TSK-G 2000 SW columns. Chromatography of DP 1 through DP 7 on the G-Oligo-PW, Protein-Pak 60, and TSK-G 2000 SW columns at acetonitrile concentrations of 50, 55, 60, 65, 70 and 75% (v/v) produced a negative linear relationship ($R^2 > -0.973$, $p < 0.001$, for DP 1 to DP 7) between log $k'$ and solvent strength (Fig. 4). The total solvent strength, $S_T$, for each mixture of acetonitrile and water was calculated using the following equation (28): $S_T = \Sigma_i S_i -_i$, where $S_i$ is the solvent weighting factor and $-_i$ is the volume fraction of solvent in the mixture. The weighting factor was 10.2 for water and 5.8 for acetonitrile [28]. Increasing the polarity of the mobile phase resulted in decreased retention of all maltooligosaccharides on all three columns.

A maltooligosaccharide mixture comprised of DP 1 to DP 10 was separated by gradient elution on the Protein-Pak 60, TSK-G 2000 SW and G-Oligo-PW columns (Fig. 5). The separation of the mixture

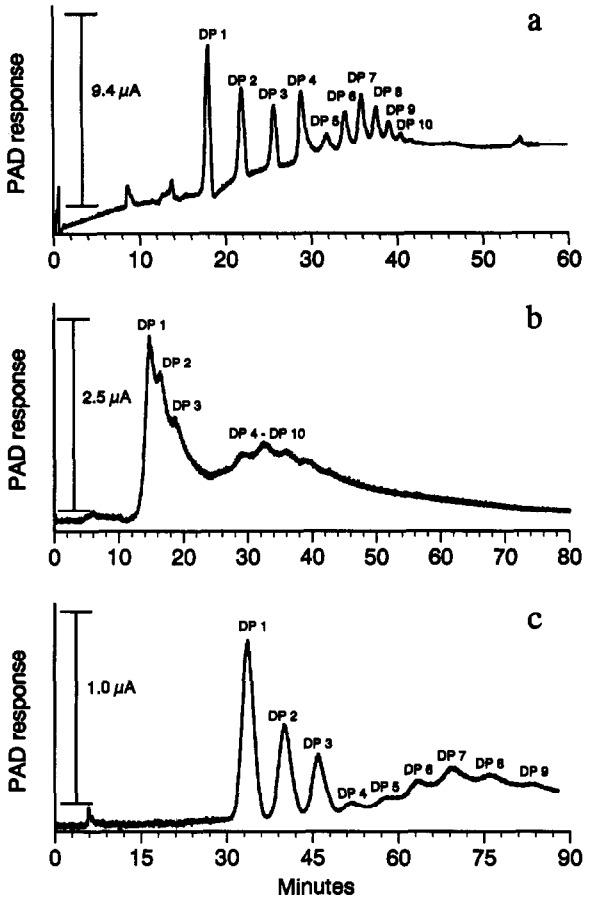


Fig. 5. Chromatography of standard maltooligosaccharides DP 1 through DP 10 on HPSEC columns by gradient elution: Protein-Pak 60 column (a), TSK-G 2000 SW column (b) and G-Oligo-PW column (c).

on the Protein-Pak 60 column was complete in 40 min; all peaks were resolved, and peak widths were narrow. The components were not resolved, however, on the TSK-G 2000 SW column, even when a shallow gradient was used for separation of the mixture. When the mixture was run on the G-Oligo-PW column, DP 1 through DP 9 were fractionated within 90 min; peak widths were wide, and DP 10 was not, therefore, observed.

Mono- and disaccharides exhibited similar retention behavior on the Protein-Pak 60 and G-Oligo-PW columns; however, differences did occur (Table II). Like the maltooligosaccharides, monosaccharides were retained longer on the G-Oligo-PW column than on the Protein-Pak 60 column. On the Protein-Pak 60 column, glucose and galactosamine were eluted at 18.1, glucosamine at 18.3 and galactose at 18.6 min; chromatography on the G-Oligo-PW column resulted in the early elution (after N-acetylneuraminic acid) of galactosamine (8.95 min) and glucosamine (9.49 min). On the Protein-Pak 60 column, glucose was eluted before galactose, and sucrose before lactulose; the converse occurred on the G-Oligo-PW column, *i.e.*, galactose was eluted before glucose, and lactulose before sucrose. Although there were differences in retention times, neither the Protein-Pak 60 nor the G-Oligo-PW column was suitable for the complete separation of all the mono- and disaccharides that were examined.

TABLE II

RETENTION TIMES ($t_R$) OF MONO- AND DISACCHARIDES SEPARATED ON THE PROTEIN-PAK 60 AND G-OLIGO-PW COLUMNS WITH A MOBILE PHASE COMPOSITION OF 75% (v/v) ACETONITRILE IN WATER

| Solute | $t_R$ (min) | |
|---|---|---|
| | Protein-Pak 60 | G-Oligo-PW |
| N-acetylneuraminic acid | 6.50 | 5.62 |
| α-L-Rhamnose | 14.4 | 23.1 |
| α-L-Fucose | 15.5 | 24.9 |
| D(+)-Xylose | 15.8 | 29.3 |
| D(−)-Arabinose | 16.3 | 29.2 |
| β-D-Fructose | 16.8 | 31.4 |
| D(+)-Mannose | 17.1 | 33.7 |
| α-D-Glucose | 18.1 | 38.2 |
| D(+)-Galactosamine | 18.1 | 8.95 |
| D(+)-Glucosamine | 18.3 | 9.49 |
| D(+)-Galactose | 18.6 | 37.7 |
| Sucrose | 21.2 | 45.7 |
| Lactulose | 22.1 | 43.6 |
| Maltose | 22.7 | 52.8 |
| Lactose | 23.5 | 52.8 |

A mixture of N-acetylneuraminic acid, rhamnose, arabinose and the glucose polymer mixture Polycose, was separated by gradient elution on the Protein-Pak 60 column (Fig. 6); Table III lists the retention times and identities of the peaks depicted in Fig. 6. The within-run precision (relative standard deviation, R.S.D., $n = 8$) of the retention times for the separation of the glucose polymer and monosaccharide mixture consisted of values from 0 to 0.26%; the between-run precision ($n = 16$) of the

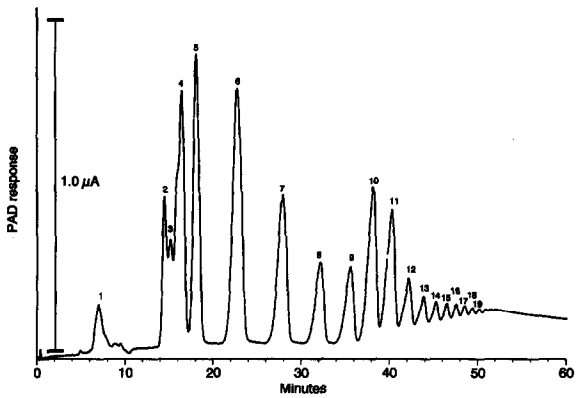


Fig. 6. The separation of selected monosaccharides and a commercial glucose polymer mixture by gradient elution on the Protein-Pak 60 column. The identity and retention times of the peaks are given in Table III.

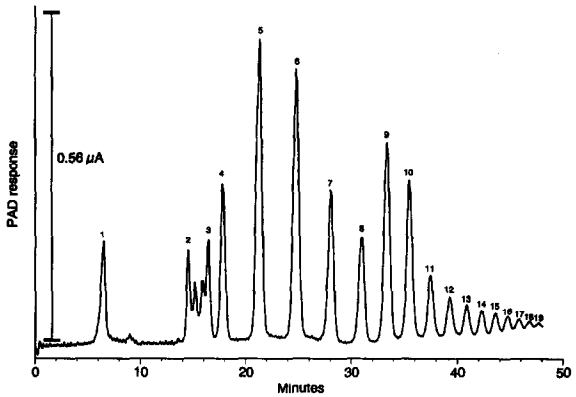


Fig. 7. Optimized gradient elution and detection of glucose polymers. The pulsed amperometric detector was in this case set at 1.0 μA full scale. The identity and retention times of the peaks are given in Table III.

retention times had values from 0.09 to 0.46% for 19 of the components and 0.64% for N-acetylneuraminic acid.

Fig. 7 depicts the optimized separation of the glucose polymer and monosaccharide mixture. The detector was set at 1.0 μA; the recorder at 0.56 μA.

TABLE III

WITHIN- AND BETWEEN-RUN PRECISION OF RETENTION TIMES FOR THE GRADIENT ELUTION SEPARATION OF SELECTED MONOSACCHARIDES AND GLUCOSE POLYMERS (Gp) ON THE PROTEIN-PAK 60 COLUMN

| Peak No. | Solute | $t_R$ | | | |
|---|---|---|---|---|---|
| | | Within-run | | Between-run | |
| | | Mean ± S.D. (min) | R.S.D. (%) | Mean ± S.D. (min) | R.S.D. (%) |
| 1 | N-Acetylneuraminic acid | 6.90 ± 0 | 0 | 6.92 ± 0.04 | 0.64 |
| 2 | Rhamnose | 14.4 ± 0.03 | 0.24 | 14.4 ± 0.03 | 0.24 |
| 3 | Arabinose | 16.3 ± 0.03 | 0.22 | 16.3 ± 0.05 | 0.31 |
| 4 | DP 1 | 18.0 ± 0.05 | 0.26 | 18.0 ± 0.08 | 0.43 |
| 5 | DP 2 | 22.5 ± 0.05 | 0.23 | 22.6 ± 0.10 | 0.46 |
| 6 | DP 3 | 27.8 ± 0.07 | 0.25 | 27.8 ± 0.10 | 0.37 |
| 7 | DP 4 | 32.0 ± 0.06 | 0.20 | 32.0 ± 0.10 | 0.30 |
| 8 | DP 5 | 35.4 ± 0.05 | 0.15 | 35.5 ± 0.08 | 0.23 |
| 9 | DP 6 | 38.0 ± 0.05 | 0.14 | 38.1 ± 0.08 | 0.20 |
| 10 | DP 7 | 40.2 ± 0.04 | 0.09 | 40.2 ± 0.06 | 0.15 |
| 11 | DP 8 | 42.0 ± 0.05 | 0.12 | 42.1 ± 0.08 | 0.18 |
| 12 | DP 9 | 43.7 ± 0.04 | 0.08 | 43.7 ± 0.06 | 0.14 |
| 13 | DP 10 | 45.1 ± 0.03 | 0.08 | 45.1 ± 0.06 | 0.14 |
| 14 | Gp peak 11 | 46.3 ± 0.06 | 0.14 | 46.3 ± 0.06 | 0.14 |
| 15 | Gp peak 12 | 47.4 ± 0.04 | 0.10 | 47.4 ± 0.04 | 0.13 |
| 16 | Gp peak 13 | 48.3 ± 0.04 | 0.10 | 48.4 ± 0.07 | 0.15 |
| 17 | Gp peak 14 | 49.2 ± 0.05 | 0.09 | 49.2 ± 0.05 | 0.09 |
| 18 | Gp peak 15 | 50.0 ± 0.00 | 0.00 | 50.0 ± 0.06 | 0.12 |
| 19 | Gp peak 16 | 50.7 ± 0.05 | 0.10 | 50.7 ± 0.07 | 0.14 |

The separation of rhamnose, arabinose, N-acetylneuraminic acid and Polycose was conducted by using a more shallow gradient than was used for the separations depicted in Figs. 5a and 6. Detector response increases in proportion to the% (v/v) of water in the mobile phase, and the decreased rate of

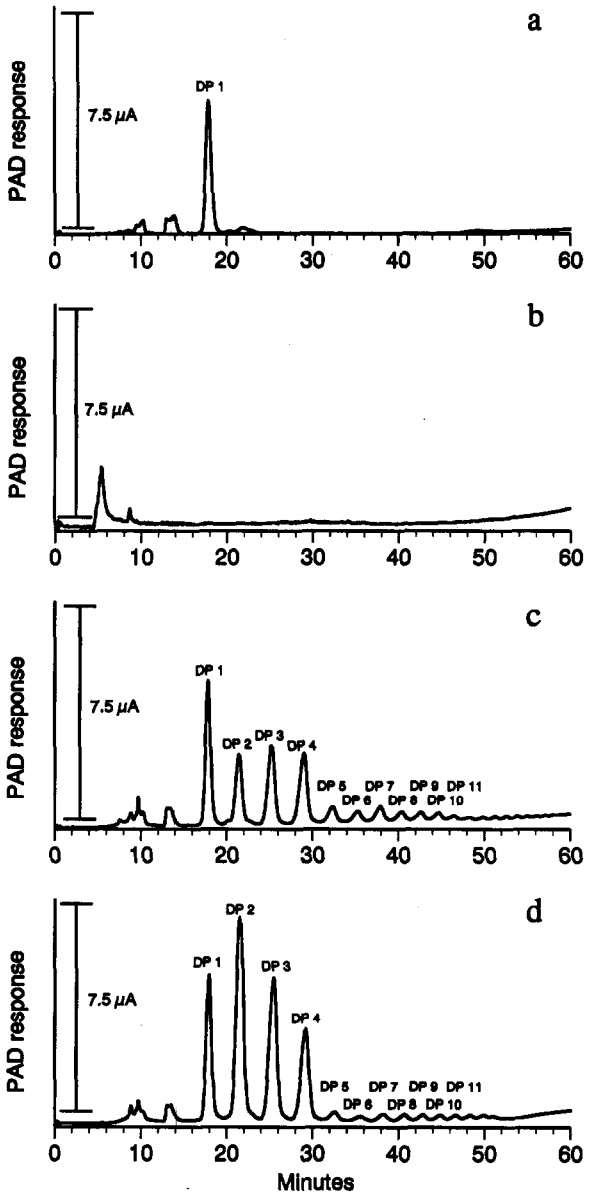


Fig. 8. The gradient elution chromatography of sera, starch and starch hydrolysates after incubation at 37°C. Monitrol serum (a), starch (b), starch hydrolysate (450 μg) after 1 h at 37°C (c) and starch hydrolysate (450 μg) after 4 h at 37°C (d) were injected after the treatment and eluted using the gradient conditions described in the Experimental section.

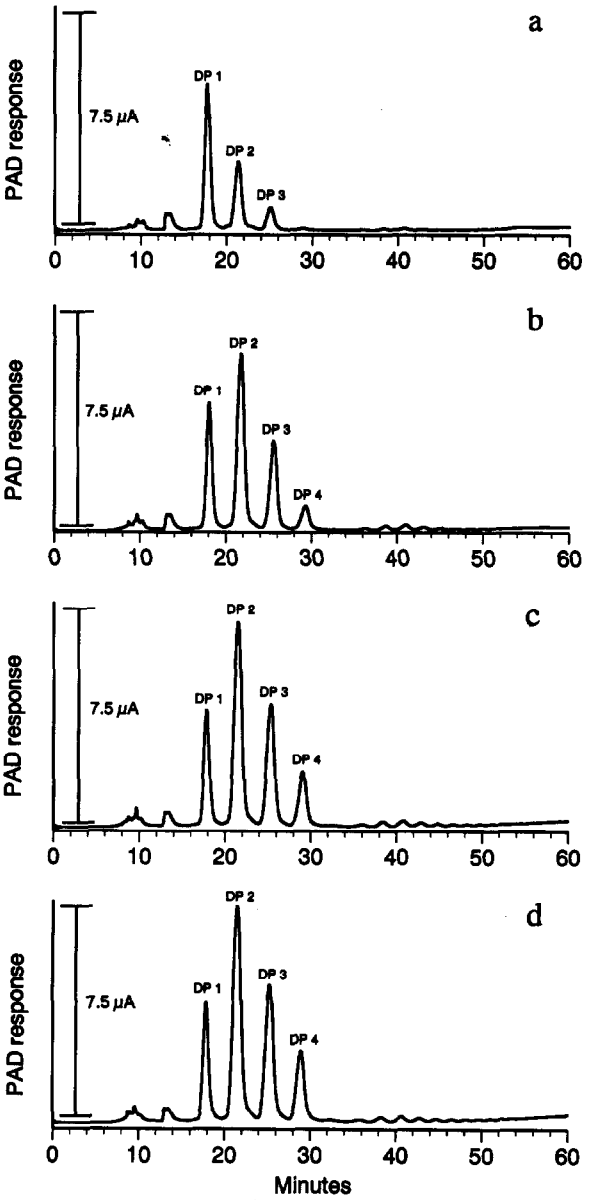


Fig. 9. The gradient elution chromatography of the products of starch digestion using different enzyme:substrate ratios. The chromatography of starch hydrolysates using enzyme:substrate ratios of (a) 1:1, (b) 1:4, (c) 1:7 and (d) 1:9 was performed after the treatment and by the gradient conditions described in the Experimental section. Starch hydrolysate was injected in amounts of 50 μg (a), 200 μg (b), 350 μg (c) and 450 μg (d).

water delivery resulted in a slower and more steady rise in the baseline than was observed in Figs. 5a and 6.

When starch was hydrolyzed with serum that contained $\alpha$- and $\beta$-amylase, oligosaccharides were produced. The chromatographic separations are depicted in Fig. 8. Monitrol serum contains glucose, which was eluted at 18.1 min (Fig. 8a); all other solutes detected in the serum were eluted before the glucose peak. Fig. 8b represents the nonhydrolyzed starch that was eluted in the void volume. The chromatograms in Fig. 8c and 8d represent the products of starch hydrolysis after 1 and 4 h at 37°C, respectively; the degree of hydrolysis can be easily visualized and shows that, under these conditions, maltose was the major product of hydrolysis. Altering the enzyme:substrate ratio to values of 1:1. 1:4, 1:7, and 1:9 and incubating at ambient temperature for 16 h produced the maltooligosaccharide patterns depicted in Fig. 9. Increasing the amount of substrate resulted in the increased formation of DP 2, DP 3 and DP 4.

## DISCUSSION

The Protein-Pak 60 column is a diol-bound silica support, the TSK-G 2000 SW column has hydrophilic groups bound to a silica support and the G-Oligo-PW column has hydrophilic groups bound to a polymeric support. With mobile phases containing from 0 to 40% (v/v) acetonitrile in water, maltooligosaccharides were fractionated by size exclusion on all three columns (Fig. 1a, c, e). Chromatography of the maltooligosaccharides with mobile phases containing from 50 to 75% (v/v) acetonitrile in water resulted in retention on the column and a reversal of the order of elution (Fig. 1b, d, e); at 50% (v/v) acetonitrile in water, all three size-exclusion columns became hydrophilic interaction columns.

Studies on the mechanism of retention of carbohydrates to amine-bonded silica supports, using mobile phases containing from 60 to 90% (v/v) acetonitrile in water, have demonstrated that as the percentage of acetonitrile in the mobile phase increases, the proportion of water bound to the support relative to that in the mobile phase increases [19,22]; as the amount of water bound to the column increases, the $k'$ value of various mono- and disaccharides increases, and separation results from the partitioning of the solutes between the two water phases.

At 50% (v/v) acetonitrile, all three columns, irrespective of support or bonded phase, retained the oligosaccharides; the only difference at compositions above 50% (v/v) acetonitrile was the degree of interaction between the oligosaccharide and the support (Fig. 2, Table I). Because the hydrophilic group bonded to the polymeric support on the G-Oligo-PW column cannot be identified, it is difficult to determine whether the bonded group or the polymeric support is responsible for the higher $k'$ values obtained for all of the oligosaccharides. However, neutral oligosaccharides separated on a hydroxylated polymeric support demonstrated the same trend as that observed for the G-Oligo-PW column, *i.e.*, very high $k'$ values and broad peaks at higher acetonitrile concentrations [24]. It is possible, therefore, that the support on the G-Oligo-PW column contributes to the increase in $k'$ values over those observed for either the TSK-G 2000 SW or Protein-Pak 60 columns.

Although data in the literature suggest that separation is due to partitioning of the carbohydrates between a static, adsorbed layer of water and the mobile phase [19,22], it is significant that all three columns retained the carbohydrates only when the concentration of acetonitrile was 50% (v/v) or greater. The amount of water bound to the three columns probably determined the value of $k'$, *i.e.*, the degree of interaction, but the polarity of the carbohydrate and its relationship to the polarity of the mobile phase may have provided the driving force required for interaction with the adsorbed layer of water.

At concentrations $\geqslant$ 50% (v/v) acetonitrile, a linear relationship existed between retention ($k'$) and the degree of polymerization of the oligosaccharide; a representative example, using a mobile phase composition of 65% (v/v) acetonitrile, is given in Fig. 3. This linear relationship was observed with the separation of deoxymonosaccharides, monosaccharides, disaccharides, and myoinositol, after separation on an amine-bonded column [22]; retention was related to the number of hydroxyl groups that could bond to hydrogen and to the calculated hydration number of the molecule.

Hydrophilic interaction chromatography is simi-

lar to normal-phase chromatography in that retention on the column ($k'$) increases as the polarity of the mobile phase decreases [27]. In normal-phase chromatography, a parameter called the solvent-strength weighting factor, $S_i$, can be used to describe the polarity of the mobile phase [28]. In our study, a linear relationship existed between the $k'$ value of DP 7 and the solvent strength of the mobile phase used for the separation (Fig. 4); this relationship held for all three columns when DP 7 was eluted with mobile phases having acetonitrile concentrations from 50 to 75% (v/v). Increases in the solvent strength of the mobile phase resulted in decreases in the values of $k'$ for the chromatography of DP 7 on all three columns.

Although all three columns were able to function as hydrophilic interaction columns, they exhibited different retention characteristics (Table I) and resolution capabilities (Fig. 5). Although the G-Oligo-PW column retained the oligosaccharides to a far greater degree than did the Protein-Pak 60 column, the resolution of DP 1 to DP 10 was much better on the Protein-Pak 60 column. The TSK-G 2000 SW column could not resolve the mixture even with a more shallow gradient. Gradient elution on the Protein-Pak 60 column (Fig. 5a) eliminated the separation of anomers that was seen when an isocratic mobile phase composed of 65% (v/v) acetonitrile was used in chromatography (Fig. 1b).

Both the Protein-Pak 60 and G-Oligo-PW columns were able to separate certain monosaccharides and to separate mono- from disaccharides, but neither column was suitable for the complete resolution of a mixture of mono- and disaccharides (Table II). Complete resolution did not occur even when 80% (v/v) acetonitrile was used; these columns were better suited to the separation of glucooligosaccharides, and the Protein-Pak 60 column gave the best resolution of the three columns tested. Twenty peaks were resolved by the Protein-Pak 60 column when a commercial glucose polymer mixture with added monosaccharides was applied. Although glucooligosaccharides with degrees of polymerization greater than 30 have been separated [16,21], the resolution of 20 peaks in this study was not a matter of separation capability, but probably a consequence of the composition of the starch-derived enzyme hydrolysate. Optimization of the gradient allowed the separation and detection of 33 $\mu$g of the Polycose mixture (Fig. 7). The peak widths were narrow and baseline resolution of the oligosaccharides occurred. The chromatography was also applied to the analysis of enzyme-catalyzed starch hydrolysis products (Figs. 8 and 9), and it enables one to see the effect of different conditions on the enzymatic hydrolysis of starch.

A significant feature of the gradient elution is its high degree of reproducibility (Table III); the column was equilibrated with 1.4 column volumes of starting mobile phase before each injection, and the between-run precision ($n$ = 16, 2 days) for the retention times of 18 peaks was less than 0.47% (R.S.D.).

The columns used in this study are polar-bonded supports that can be used either as size-exclusion or hydrophilic interaction columns. Of the three columns used, the Protein-Pak 60 column enabled superior resolution and reproducibility in the separation of glucose polymers. Pulsed amperometric detection allowed gradient elution and sensitive detection. The separations demonstrate the versatility and potential of hydrophilic interaction chromatography.

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## REFERENCES

1 S. A. Barker, B. W. Hatt, J. F. Kennedy and P. J. Somers, *Carbohydrate Res.*, 9 (1969) 327.
2 R. W. Goulding, *J. Chromatogr.*, 103 (1975) 229.
3 K. B. Hicks, P. C. Lim and M. J. Haas, *J. Chromatogr.*, 319 (1985) 159.

4 G. Bonn, *J. Chromatogr.*, 387 (1987) 393.
5 H. Derler, H. F. Hormeyer and G. Bonn, *J. Chromatogr.*, 440 (1988) 281.
6 K. B. Hicks and A. T. Hotchkiss, *J. Chromatogr.*, 441 (1988) 382.
7 N. W. H. Cheetham, P. Sirimanne and W. R. Day, *J. Chromatogr.*, 207 (1981) 439.
8 K. Koizumi and T. Utamura, *J. Chromatogr.*, 436 (1988) 328.
9 R. D. Rocklin and C. A. Pohl, *J. Liq. Chromatogr.*, 6 (1983) 1577.
10 D. C. Johnson and T. L. Polta, *Chromatogr. Forum*, 1 (1986) 37.
11 M. R. Hardy, R. R. Townsend and Y. C. Lee, *Anal. Biochem.*, 170 (1988) 54.
12 R. R. Townsend, M. R. Hardy, O. Hindsgual and Y. C. Lee, *Anal. Biochem.*, 174 (1988) 459.
13 L.-M. Chen, M.-G. Yet and M.-C. Shao, *FASEB J.*, 2 (1988) 2819.
14 M. R. Hardy and R. R. Townsend, *Proc. Natl. Acad. Sci. USA*, 85 (1988) 3289.
15 R. R. Townsend, M. R. Hardy, D. A. Cumming, J. P. Carver and B. Bendiak, *Anal. Biochem.*, 182 (1989) 1.
16 K. Koizumi, Y. Kubota, T. Tanimoto and Y. Okada, *J. Chromatogr.*, 464 (1989) 365.
17 J. C. Linden and C. L. Lawhead, *J. Chromatogr.*, 105 (1975) 125.
18 F. M. Rabel, A. G. Caputo and E. T. Butts, *J. Chromatogr.*, 126 (1976) 731.
19 L. A. Th. Verhaar and B. F. M. Kuster, *J. Chromatogr.*, 234 (1982) 57.
20 C. Brons and C. Olieman, *J. Chromatogr.*, 259 (1983) 79.
21 K. Koizumi, T. Utamura and Y. Okada, *J. Chromatogr.*, 321 (1985) 145.
22 Z. L. Nikolov and P. J. Reilly, *J. Chromatogr.*, 325 (1985) 287.
23 M. Verzele and F. Van Damme, *J. Chromatogr.*, 362 (1986) 23.
24 B. Bendiak, J. Orr, I. Brockhausen, G. Vella and C. Phoebe, *Anal. Biochem.*, 175 (1988) 96.
25 V. Carunchio, A. M. Girelli and A. Messina, *Chromatographia*, B23 (1987) 731.
26 Y.-B. Yang and M. Verzele, *J. Chromatogr.*, 387 (1987) 197.
27 A. J. Alpert, *J. Chromatogr.*, 499 (1990) 177.
28 C. F. Poole and S. A. Schuette, *Contemporary Practice of Chromatography*, Elsevier, Amsterdam, New York, 1984, p. 260.