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Separation of glucooligosaccharides and polysaccharide hydrolysates by gradient elution hydrophilic interaction chromatography with pulsed amperometric detection

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ABSTRACT

Commercial glucooligosaccharide mixtures (Polycose) and polysaccharide hydrolysates (acid and enzymatic) were fractionated by hydrophilic interaction chromatography and observed by pulsed amperometric detection. Seven peaks were observed when 625 ng of glucose oligomers in Polycose were fractionated. The between-run precision of retention times (n = 10, 100 μg, 15 peaks) ranged from a relative standard deviation (R.S.D.) of 0.09 to 0.40%; between-run precision of peak areas (n = 10) for the same separations had values that ranged from 2.66 to 14.4%. Injection-to-injection time was 48 min. When polysaccharide hydrolysates were fractionated using a gradient program capable of resolving all of the oligosaccharide species, dextran-derived α-(1→6)-glucooligosaccharides were retained to a greater degree than amylose-derived α-(1→4)-glucooligosaccharides, which were retained to a greater degree than β-(2→1)-fructooligosaccharides derived from inulin. Excluding the peaks that eluted before glucose or fructose, 25 to 35 peaks were observed after fractionation of the hydrolysates. Differences in elution profiles were observed between acid and enzymatic hydrolysis products of the same polysaccharide as well as between hydrolysis products of different polysaccharides. In conjunction with high-performance size-exclusion chromatography, the method demonstrated the effect of preheating starch before hydrolysis with isoamylase.

INTRODUCTION

Hydrophilic interaction chromatography (HILIC) [1,2] describes the separation of polar molecules on a variety of hydrophilic bonded supports [3–12]. The mechanism by which carbohydrates are separated, as determined on silica bonded amine columns [7,11], appears to result from the partitioning of the carbohydrate between the amine-bound water layer and the mobile phase.

Glucose oligomers are used in medicine, biomedical research and the biotechnology industry. Consequently, a variety of chromatographic procedures have been devised for their separation and analysis. Size-exclusion chromatography has been used to fractionate oligomers of amylose, cellulose, pullulan and dextran [13]; oligomers with a degree of polymerization (DP) between 1 and 20 have been resolved, and differences in the retention times of α-(1→4)- and α-(1→6)-glucosidic linkages have been observed. Cation-exchange supports loaded with either Ag^+ or H^+ counterions have been used to fractionate degradation products in biomass hydrolysates [14], enzymatic starch digests [15], and malto-, cello-, galacturonic- and chitooligosaccharides [16] (oligomers ⩽ DP 14 were resolved). Glucooligomers have been separated on a C_{18}-bonded vinyl alcohol copolymers support using alkaline eluents, and oligomers up to DP 23 were resolved [17]. Chromatography of cellooligosaccharides on a silica-bonded C_{18} column resulted in the resolution of oligosaccharides up to DP 30, and 10 pmol of cellotetraose were detected electrochemically after

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passage through a cellulase-based enzyme reactor [18]. Glucooligosaccharides (up to DP 35) were resolved on a 3-μm amine-bonded silica support [10], and a polyamine-bonded polymer support was used to separate dextran oligosaccharides up to DP 8 [19]. High-performance anion-exchange chromatography resolved glucooligosaccharides and polysaccharides (DP $\geqslant$ 50) using alkaline eluents followed by pulsed amperometric detection [20]. The same methodology was used to analyze isoamylase digests of amylopectin from various sources, and plots of concentration versus DP served as chromatographic "fingerprints" [21].

In a previous study [22], 33 μg of a glucooligosaccharide mixture with added monosaccharide were fractionated by HILIC on a Protein-Pak 60 column and 19 peaks were resolved; fractionation and column reequilibration required 90 min. The objectives of the present study were (1) to decrease the fractionation time of glucooligosaccharides in Polycose and to submit to HILIC nanogram amounts of Polycose, (2) to determine the retention times of enzyme and acid hydrolysates of polysaccharides using identical gradient conditions, and (3) to demonstrate the potential of HILIC and high-performance size-exclusion chromatography (HPSEC) as chromatographic tools in the investigation of starch structure.

EXPERIMENTAL

Materials

The custom-packed Protein-Pak 60 column was from Waters (Milford, MA, USA). The Bio-Gel SEC-60 XL and guard columns were purchased from Bio-Rad (Richmond, CA, USA). Inulin (from chicory root), amylose (Type III, from potato), amylopeetin (from corn), starch (soluble ACS reagent, from potato), dextran (clinical grade, produced by ***Leuconostoc mesenteroides),*** trifluoroacetic acid (TFA), dextranase (EC 3.2.1.11, from *Penicillium* sp.), and isoamylase (EC 3.2.1.68, from ***Pseudomonas amyloderamosa)*** were purchased from Sigma (St. Louis, MO, USA). Acetonitrile (HPLC grade) and sodium hydroxide solution, 50% (w/w), were obtained from Fisher Scientific (Houston, TX, USA).

Hydrophilic interaction chromatography

Chromatography was performed on a Waters 860 system; the system configuration was described previously [22]. A Waters custom-packed Protein-Pak 60 column (150 mm × 7.9 mm I.D., 10 μm) was used to separate a commercially available mixture of glucooligosaccharides (Polycose) and acid and enzymatic hydrolysates of polysaccharides. A temperature control unit (Waters) was used to

TABLE I

GRADIENT ELUTION CONDITIONS FOR THE FRACTIONATION OF OLIGOSACCHARIDES

Oligosaccharides	Time (min)	Acetonitrile (%, v/v)	Water (%, v/v)
Glucose polymers from Polycose	0	67	33
	1	67	33
	8	60	40
	10	60	40
	17	55	45
	19	55	45
	27	50	50
	37	50	50
	38	67	33
	48	67	33
Oligosaccharides from acid and enzymatic hydrolysates of amylose, amylopectin, starch, dextran and inulin	0	67	33
	10	67	33
	65	50	50
	75	50	50
	76	67	33
	86	67	33

maintain the column temperature at 25°C. Samples (10–25 μl) were injected by a Waters Model 710 Wisp autoinjector and separated by gradient elution at 1 .O ml/min using two Waters Model 510 pumps; the gradient programs for the elution of the oligosaccharides are described in Table I. Solvents were sparged with helium and maintained in a helium atmosphere. Postcolumn 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 0.6 ml/min (helium, at 4.13 bar). After mixing, the peaks were detected with a Waters Model 464 pulsed electrochemical detector equipped with a gold working electrode. The potentials and time periods were set as follows: El was 0.1 V, E2 was 0.6 V, E3 was -0.6 V, T1 was 500 ms, T2 was 166 ms, and T3 was 83 ms. For all fractionations, the detector was set at 2.0 μA except where otherwise specified.

High-performance size-exclusion chromatography

Starch and isoamylase digests of starch were chromatographed on a Bio-Gel SEC-60 XL column (300 mm × 7.5 mm I.D., 13 μm) equipped with a guard column. Samples (10 μl) were injected and eluted isocratically at 1.0 ml/min; Milli-Q water was the mobile phase. Polysaccharides were detected with the Model 464 detector, and with the exception of the detector setting (5.0 μA), all other detector parameters were identical to those described for HILIC.

Partial acid hydrolysis

Amylose, amylopectin and starch (20 mg each) were mixed separately with 10 ml of 0.1 *M* TFA and hydrolyzed for 15.0 min at 100°C [10]; after hydrolysis, the solutions were centrifuged at 3600 g for 3.0 min to remove insoluble polysaccharide. The supernatant was lyophilized, and the dry material was weighed and redissolved in Milli-Q water to a final concentration of 10.0 mg/ml. Dextran (20 mg) was hydrolyzed with 10.0 ml of 0.3 *M* TFA for 30 min at 100°C [10]; after centrifugation and lyophilization of the supernatant as described above, the dry material was weighed and redissolved in Milli-Q water to a final concentration of 10.0 mg/ml. Inulin (20 mg) was mixed with 10.0 ml of 0.1 A4 TFA and hydrolyzed for 15 min at 30°C. This solution was centrifuged and the supernatant lyophilized as above; the material was weighed and redissolved in Milli-Q water to a final concentration of 10.0 mg/ml.

Enzymatic hydrolysis

Amylose, amylopectin and starch (100 mg each) were digested with isoamylase, with modifications, as previously described [21]. Briefly, the polysaccharides were suspended separately in 0.025 *M* sodium acetate buffer, pH 4.5, to a final concentration of 10.0 mg/ml. Isoamylase (35 000 U, 5.54 μg) was added and the mixtures were hydrolyzed for 16 h at 45°C. After hydrolysis, the mixtures were heated at 100°C for 5.0 min, then cooled to ambient temperature. The mixtures were centrifuged for 3.0 min at 3600 g, the supernatants were lyophilized, and the hydrolysates were resuspended in Milli-Q water to a final concentration of 10.0 mg/ml. In a separate experiment, starch was digested with isoamylase as stated above, except that before the addition of enzyme, the substrate was heated at 100°C for 5.0 min and then allowed to cool. The mixture was centrifuged for 3.0 min at 3600 g , lyophilized, and was resuspended in Milli-Q water to a final concentration of 10.0 mg/ml. In one set of substrate controls, one sample was heated for 5.0 min at 100°C while the other was not; both samples were then centrifuged (3.0 min, 3600 g), lyophilized, and resuspended to a final concentration of 10.0 mg/ml. In the second set, one sample was heated at 100°C for 5.0 min while the other was not; both samples were then incubated, without enzyme, for 16 h at 45°C. After centrifugation (3.0 min, 3600 g) and lyophilization of the supernatant, the dried material was resuspended in Milli-Q water to a final concentration of 10.0 mg/ml. Dextran (100 mg) was dissolved in 10.0 ml of 0.1 *M* potassium phosphate buffer, pH 6.0 and 50 μl of dextranase (50 μg, 10 U) was added. The mixture was heated for 15.0 min at 37°C [23], and the enzyme was deactivated by heating at 100°C for 5.0 min. After the mixture cooled, it was centrifuged (3.0 min, 3600 g), lyophilized, and resuspended to a final concentration of 10.0 mg/ml. After each aforementioned treatment, 10 μl of sample were submitted to HPSEC, 25 μl to HILIC.

Separation of a commercial glucose polymer mixture

Polycose was added to Milli-Q water to a concentration of 10.0 mg/ml then diluted to concentrations

of 2.5, 1.25, 0.625 and 0.0625 mg/ml. The solutions were injected separately onto the Protein-Pak 60 column and fractionated by the gradient program described in Table I. The detector sensitivity was set between 0.2 μA and 10.0 μA.

Precision studies

To determine reproducibility of the fractionation of glucooligosaccharides, five samples of the Polycose mixture were separated by HILIC on two consecutive days; the within-run (n = 5) and between-run (n = 10) precision of the retention times and peak areas were determined. A 10-μl volume containing 100 μg of the Polycose mixture was injected and fractionated using the gradient program described in Table I. The detector sensitivity was set at 10.0 μA.

Determination of retention times of oligosaccharides from polysaccharide hydrolysates

A 25-μl volume of the amylose (n = 3), dextran (n = 3), and inulin acid hydrolysates (3 hydrolysates, 1 injection) were fractionated by HILIC using the gradient program described in Table I. The detector sensitivity was set at 2.0 μA.

Statistical methods

Analysis of variance for repeated measures and

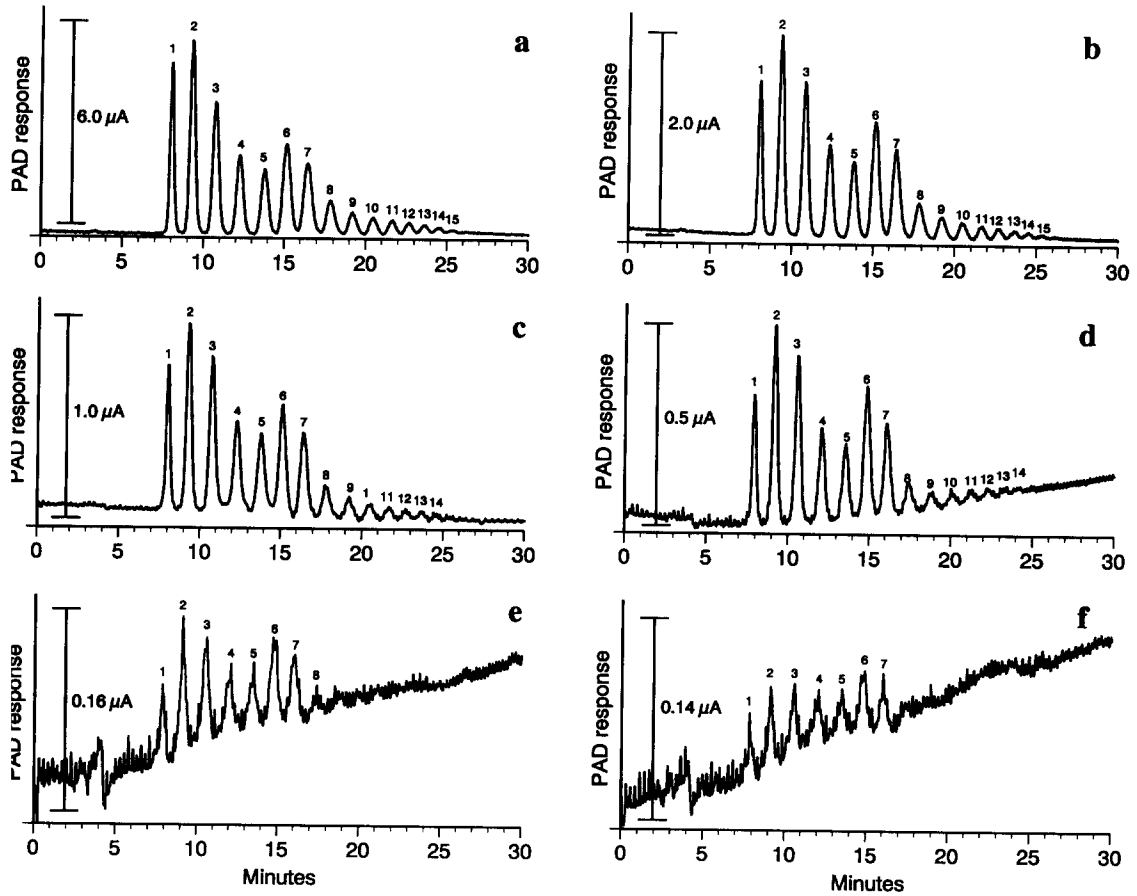

Fig. 1. The gradient elution HILIC fractionation of nanogram to microgram amounts of Polycose. The tracings depict the fractionation of Polycose in the following amounts: 100 μg (a), 25 μg (b), 12.5 μg (c), 6.25 μg (d), 1.25 μg (e) and 0.625 μg (f). The detector settings were 10.0 μA (a), 2.0 μA (b), 1.0 μA (c), 0.5 μA (d) and 0.2 μA (e and f). The gradient elution conditions are described in Table I, and the retention times of the peaks are listed in Table II. PAD = Pulsed amperometric detection.

trend analysis (BMDP2V statistical software package) were used to compare species (dextran, amylose, and inulin hydrolysates) across peaks. A multiple comparison procedure [24] was then used to isolate differences between species at specific peaks.

RESULTS AND DISCUSSION

Precision studies and chromatography of ng to μg amounts of glucose polymers

After separation of 100 μg (Fig. la) and 25 μg (Fig. 1 b) of Polycose by HILIC, 15 peaks were observed; injection of 12.5 μg (Fig. lc) and 6.25 μg (Fig. 1d) of material resulted in the detection of 14 peaks. Fractionation of smaller amounts of Polycose resulted in the detection of fewer peaks: 8 peaks for 1.25 μg (Fig. le), and 7 peaks for 625 ng (Fig. 1f). The within-run precision values of the retention times (Table II, n = 10, 100 μg, 15 peaks) were from 0.05 to 0.37% relative standard deviation (R.S.D.); the values for the between-run precision of the retention times were 0.09 to 0.40% R.S.D. (Table II). Values for the within-run precision for peak areas were from 1.14 to 6.19% R.S.D.; thirteen of the peaks had values less than 5.0% R.S.D. The between-run precision values of peak areas were from 2.66 to 14.4% R.S.D.; nine peaks had values less than 4.10% R.S.D. A previous report described the separation of glucose polymers on aqueous size-exclusion columns by HILIC [22]; of those columns, the Protein-Pak 60 (diol-bonded silica) resolved 19 peaks when 33 μg of a glucooligosaccharide mixture (Polycose) with added monosaccharides were fractionated by gradient elution; 90 min were required to separate the glucose polymers and reequilibrate the column (300 mm × 7.9 mm I.D.). In addition, the a factors (ratio of capacity factors) for the separation of DP 1 through DP 7 were larger than were required for baseline resolution. In the present study, a custom-packed Protein-Pak 60 column (150 mm × 7.9 mm I.D.) was used to shorten the analysis time, and an altered gradient program was used to decrease the α factors for the separation of DP 1 through DP 7. The initial starting condition was reduced from 70% aqueous acetonitrile [22] to 67% aqueous acetonitrile; the gradient slope was not significantly altered. The reduction in column length enabled the resolution of 15

TABLE II

WITHIN-RUN AND BETWEEN-RUN PRECISION OF RETENTION TIMES (t_R) AND PEAK AREAS FOR THE GRADIENT ELUTION SEPARATION OF GLUCOSE POLYMERS ON THE PROTEIN-PAK 60 COLUMN

Peak no.[a]	Within-run (n = 5)				Between-run (n = 10)			
	t_R (min)		Area (μV/s)		t_R (min)		Area (μV/s)	
	Mean	R.S.D. (%)	Mean	R.S.D. (%)	Mean	R.S.D. (%)	Mean	R.S.D. (%)
1	7.83	0.05	11 229 981	6.19	7.82	0.09	9 712 271	14.4
2	9.07	0.24	16357890	3.42	9.04	0.21	14 975 394	8.91
3	10.4	0.23	11 749 878	1.33	10.4	0.30	11 265 174	4.08
4	11.9	0.37	7 276 845	1.41	11.9	0.26	7 090 427	3.62
5	13.4	0.33	5 935 130	1.20	13.4	0.40	5 773 448	3.06
6	14.7	0.30	8 164 714	3.50	14.7	0.21	8 549 155	4.02
7	15.9	0.28	6 401 022	3.32	15.9	0.20	6 582 857	2.69
8	17.2	0.24	3 055 016	1.22	17.2	0.28	2 945 452	4.09
9	18.6	0.22	1968 951	1.14	18.6	0.38	1 854 749	2.66
10	19.7	0.22	1419 840	1.32	19.8	0.21	1311 617	8.23
11	21.0	0.21	1 053 027	2.85	21.0	0.24	986 847	5.88
12	22.0	0.20	765 165	1.96	22.0	0.19	745 510	3.99
13	23.1	0.19	508 647	1.95	23.0	0.14	491 572	3.41
14	24.0	0.19	292 153	5.32	23.9	0.18	300 886	6.00
15	24.7	0.18	150 921	4.95	24.7	0.17	157 175	6.81

[a] For peaks see Fig. la.

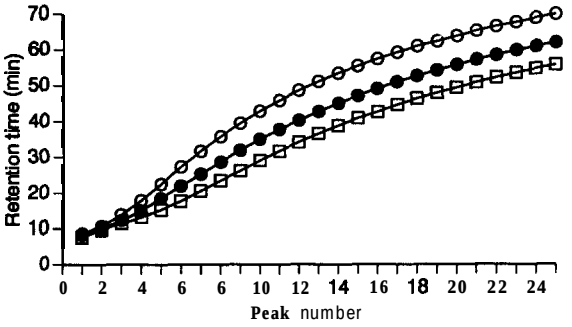

Fig. 2. Retention times of polysaccharide-derived oligosaccharides after HILIC. Oligosaccharides from dextran (○), amylose (●) and inulin (Cl) were prepared as described in the Experimental section and fractionated by gradient elution as described in Table I.

peaks within 26 min and an injection-to-injection time of 48 min. The lower starting concentration of acetonitrile reduced the α factors for the separation of DP 1 through DP 7 without sacrificing baseline resolution (Fig. 1). The gradient used for fractionation of glucooligosaccharides in Polycose (Table I) was also used to fractionate glucooligosaccharides and glucose polymers in hydrolysates of amylose, amylopectin, starch, and dextran (data not shown). Fructooligosaccharides fractionated with the same gradient, however, were not completely resolved (data not shown).

Retention times of polysaccharide hydrolysates

The retention times observed for the oligosaccharides and polysaccharides derived from polysaccharide hydrolysates are depicted in Fig. 2. The composition, bond type, and linkage position of the oligosaccharides studied are listed in Table III. The objective of this experiment was to determine the effect of composition and linkage position on the retention times of the different oligosaccharide and polysaccharide species. These retention times were determined using the same gradient elution program rather than an isocratic mobile phase, which requires inordinately long fractionation times for oligosaccharides of DP 10 to DP 30. The gradient depicted in Table I was chosen because it enabled resolution of the fructooligosaccharides obtained from acid hydrolysis of inulin; the gradient used for the fractionation of glucooligosaccharides in Polycose was not able to resolve the fructooligosaccharides in the inulin hydrolysate. For the oligosaccharides obtained from hydrolysates of amylose and dextran, the numbering of peaks was initiated with glucose (peak 1); for the oligosaccharides obtained from the hydrolysis of inulin, the numbering of the peaks was initiated with fructose (peak 1). The retention times of the oligosaccharides derived from the acid- and enzymatic hydrolysis of amylose, amylopectin and starch were identical; consequently, only the retention times of the amylose-derived oligosaccharides are depicted in Fig. 2. The R.S.D. of the retention times of the oligosaccharides derived from dextran, amylose, and inulin had values that ranged from 0 to 0.56% (80% <0.36), 0 to 0.82% (88% <0.30), and 0 to 0.60 (72% <0.38), respectively. Although peak number cannot unequivocally be equated with DP (except for DP 1 to DP 10 for α-(1→ 4)-glucooligosaccharides, data not shown), it is likely that they correspond. Even so, each species of oligosaccharide (all peaks within a series) clearly exhibited different retention times ($p < 0.001$) when fractionated using identical gradient elution conditions. Glucose (peak 1, Fig. 2) produced by hydrolysis of amylose and dextran had a retention time of 8.42 min, and fructose (peak 1, Fig. 2) produced by hydrolysis of inulin had a retention time of 7.65 min. Subsequent comparisons at

TABLE III

GLYCOSIDIC BOND TYPE, LINKAGE POSITION, AND COMPOSITION OF THE POLYSACCHARIDES STUDIED

Polysaccharide	Glycosidic bond type, linkage position and composition
Amylose	Linear α-(1→4)-glucopyranosyl units with few α-(1→6)-glucopyranosyl units
Amylopectin	Linear α-(1→4)-glucopyranosyl and branched a-(1→6)-glucopyranosyl units
Dextran	Linear a-(1→6)-glucopyranosyl units
Starch	Amylopectin-amylose (80:20)
Inulin	Linear β-(2→1)-fructofuranosyl units with terminal a-(1→1)-glucopyranosyl

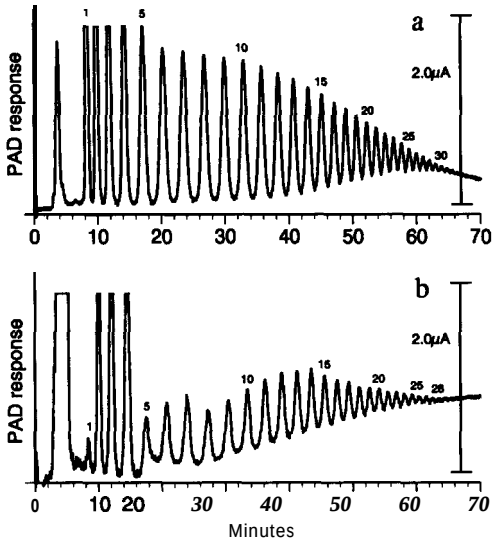

Fig. 3. HILIC of amylose hydrolysates. Elution profiles of the products of acid hydrolysis (a) and isoamylase hydrolysis (b). Hydrolysates were prepared as described in the Experimental section and chromatographed using the gradient elution program depicted in Table I.

specific peaks (for peaks 2-25) indicated differences between all three species ($p < 0.001$; p represents the probability value based on analysis of variance) at all peaks, ***i.e.,*** the retention time of any peak (2-25) was significantly different for all three species. The data clearly demonstrate that α-(1→6)-glucooligosaccharides (dextran) were retained to a greater degree than the a-(1→4)-glucooligosaccharides (amylose, amylopectin, and starch), which in turn were retained to a greater degree than β-(2→1)-fructooligosaccharides (inulin). When a-(1→4)- and α-(1→6)-glucooligosaccharides were separated on an amine-bonded silica column using an isocratic mobile phase composition of 57% (v/v) acetonitrile in water, similar results were obtained [10], ***i.e., a-(*** 1→6)-glucooligosaccharides were retained to a greater degree than were the α-(1→4)-glucooligosaccharides.

HILIC of the hydrolysates of amylose, amylopectin, dextran, and inulin

HILIC of the glucooligosaccharides obtained by partial acid hydrolysis of amylose (Fig. 3a) revealed 30 glucooligosaccharide peaks, whereas HILIC of the glucooligosaccharides obtained by isoamylase hydrolysis of amylose (Fig. 3b) revealed 28 glucooligosaccharide peaks. The chromatogram depicted in Fig. 3b was not expected; amylose is comprised of a mixture of linear, α-(1→4) linked molecules and molecules with a limited number of long-chain branches involving -(1 → 6) linkages [25]. Enzymatic hydrolysis with isoamylase should not, therefore, have resulted in the profile observed (Fig. 3b). When amylose was digested under similar conditions without enzyme (0.025 *M* sodium acetate, pH 4.5, 45°C, 16 h, data not shown), peaks 14 were produced to the degree observed in Fig. 3b; however, peaks 5-20 were present in minor amounts and did not resemble the profile in Fig. 3b. Although the amylose preparation was supposed to be essentially free of amylopectin, a comparison of peaks 5-28 in Fig. 3b with peaks 5-33 in Fig. 4b (isoamylase digest of amylopectin) reveals a similarity in the elution profiles. It is likely, therefore, that the amylose preparation contained amylopectin. In a previous study that used a silica-bonded amine column to fractionate partial acid hydrolysates of amylose, 32 peaks were resolved [10]. When anion-exchange chromatography was used to fractionate a mixture of short-chain amylose, approximately 46 peaks were resolved; maltodextrins pre-

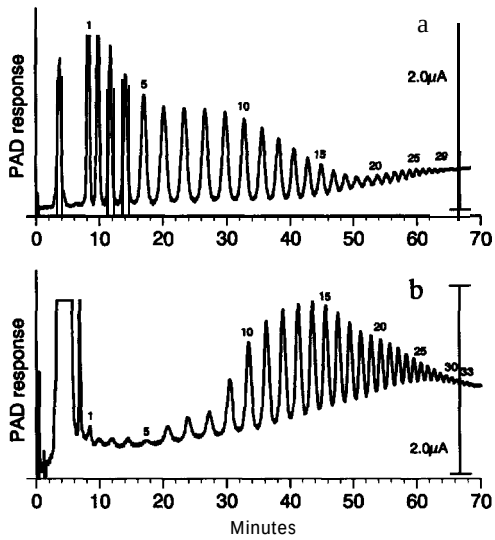

Fig. 4. HILIC of amylopectin hydrolysates. Elution profiles of the products of acid hydrolysis (a) and isoamylase hydrolysis (b). Hydrolysates were prepared as described in the Experimental section and chromatographed using the gradient elution program depicted in Table I.

pared from Amylo-Waxy maize were resolved into approximately 85 peaks [20]. The fractionation of amylose hydrolysates in this communication and on the silica-bonded amine column [10] used acetonitrile-water eluents, while the fractionation of the glucose polymers on the anion exchange column used alkaline eluents [20]. The greater solubility of glucose polymers in alkaline solution was responsible for the observation of the greater number of peaks observed after anion exchange analysis [20].

Fractionation of the partial acid hydrolysis products of amylopectin resulted in the separation of 29 peaks (Fig. 4a). Equal amounts of amylose and amylopectin were acid-hydrolyzed and fractionated using identical conditions; although the peaks were not quantitated, the amylopectin peak areas were smaller than the amylose peak areas, which indicated that the amylopectin was hydrolyzed less completely than the amylose. In addition, the relative intensity of peak areas for peaks 2-30 (Figs. 3a, 4a) was different for both hydrolysates. The elution profile of the fractionated enzymatic hydrolysates of amylopectin (Fig. 4b) was substantially different from the profile of the fractionated acid hydrolysates; a total number of 33 peaks were separated and the relative intensity of the peak areas of peaks

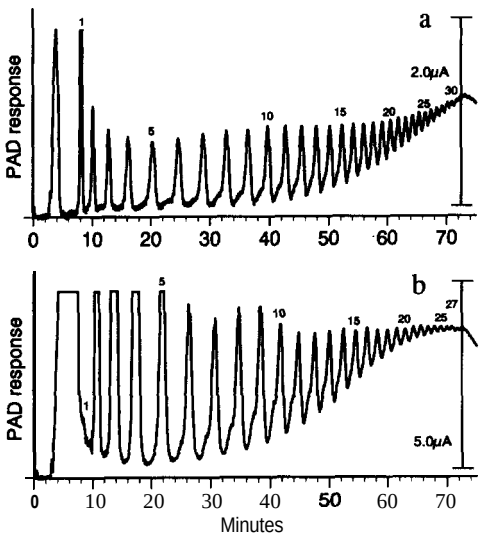

Fig. 5. HILIC of dextran hydrolysates. Elution profiles of the products of acid hydrolysis (a) and dextranase hydrolysis (b). Hydrolysates were prepared as described in the Experimental section and chromatographed using the gradient elution program depicted in Table I.

l-33 was different from that of the peak areas of peaks l-30 observed after acid hydrolysis. Anion-exchange chromatography with alkaline eluents was used to estimate the chain-length distribution of amylopectin obtained from various sources [21]. Fifty-five to sixty peaks were resolved; as was the case with glucose polymers derived from amylose [20], the enhanced solubility of the higher-molecular-mass polymers in the alkaline mobile phase resulted in the greater number of peaks [21]. Although only 33 peaks were observed after HILIC (Fig. 4b), the elution pattern (peaks l-33) closely resembled the patterns obtained after anion-exchange analysis [21].

Fractionation of the partial acid hydrolysis products of dextran resulted in the resolution of 30 peaks. When compared to the elution profiles of acid hydrolysates from amylose (Fig. 3a) and amylopectin (Fig. 4a), the profile of the dextran acid hydrolysates (Fig. 5a) was quite different; in general, smaller amounts (that is, lower relative intensity of peak areas) of the products represented by peaks l-9 were produced by acid hydrolysis of dextran. In addition. the differences in the relative intensity of peak areas for peaks 2-30 (Fig. 5a) were less pronounced than for the acid hydrolysates of amylose (Fig. 3a) and amylopectin (Fig. 4a). Twenty-seven peaks were resolved after fractionation of the dextranase hydrolysis products (Fig. 5b). In contrast to the results from acid hydrolysis, much less of peak 1 (glucose) was produced by enzymatic hydrolysis with dextranase. Chromatography of partial acid hydrolysates of dextran on silica-bonded amine [10] and anion-exchange [20] columns resulted in the resolution of approximately 28 and 40 peaks, respectively.

Inulin represents a group of polymers called "fructansa"; they are polymers comprised of β-(2→1)-D-fructofuranosyl units which contain terminal D-glucosyl residues [26]. The conditions for the partial acid hydrolysis of inulin were much milder than for the other polysaccharides. After fractionation of the hydrolysis products, 35 peaks were resolved; peak 1 was fructose. Including the three peaks that eluted before fructose, the total number of peaks observed was 38 (Fig. 6).

HILIC of starch hydrolysis products

The elution profile obtained after fractionation of the acid hydrolysates of starch (Fig. 7a) closely

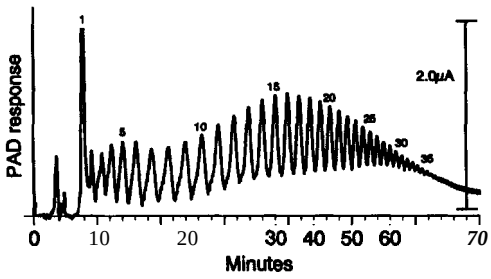

Fig. 6. HILIC of inulin acid hydrolysis products. Inulin was hydrolyzed as described in the Experimental section and the oligosaccharides were fractionated using the gradient elution program depicted in Table I.

resembled the elution profile of the amylose acid hydrolysates (Fig. 3a); a total of 30 peaks were resolved. Very little hydrolysis occurred when starch was digested with isoamylase for 16 h at 45°C (Fig. 7b). However, when starch was heated for 5.0 min at 100°C, cooled to ambient temperature, and then reacted with isoamylase, hydrolysis occurred (Fig. 7c). Potato starch contains approximately 80% amylopectin and 20% amylose [25], and the elution profile of the isoamylase digest of starch (Fig. 7c) resembled the elution profile obtained after fractionation of the isoamylase digest of amylopectin (Fig. 4b). Twenty-six peaks were observed (Fig. 7c). Starch granules are comprised of amorphous regions that are susceptible to hydrolysis and crystalline regions that are resistant to hydrolysis [27]. The crystalline regions may exist as a consequence of the presence of double helical chains formed between adjacent amylose molecules or adjacent clusters of chains in either the same or neighboring amylopectin molecules [27]. In addition, an amylopectin-rich zone may be located near the surface of the granule [27]. When potato starch in warm water was heated to approximately 70°C, amylose of relatively low DP was released and a high-molecular-mass β-amylolysis limit fraction could be extracted [28]; heating the starch enabled isoamylase hydrolysis, which might have occurred as a result of (1) hydrolysis of released species, or (2) hydrolysis of a newly exposed region of the heat-ruptured granule that contained amylopectin.

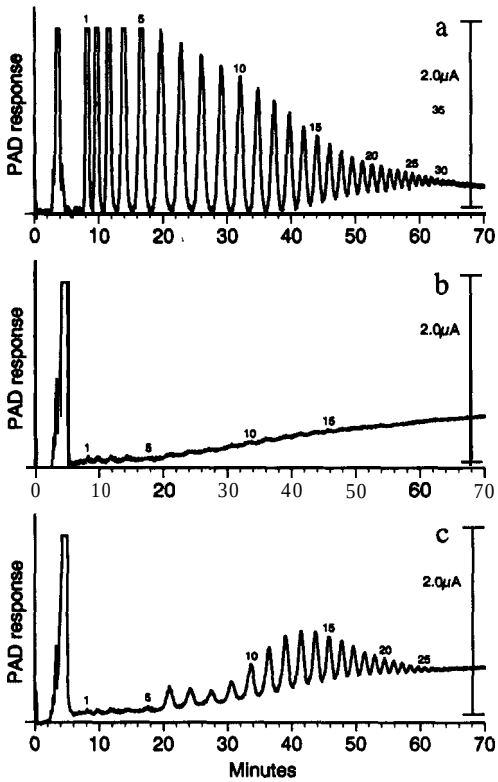

Fig. 7. HILIC of starch hydrolysates. Elution profiles of acid hydrolysate (a), isoamylase digest without preheating (b) and isoamylase digest with preheating (c). Hydrolysates were prepared as described in the Experimental section and chromatographed by the gradient elution program depicted in Table I.

HPSEC of starch and starch hydrolysis products

The Bio-Gel SEC-60 XL column reportedly exhibits a fractionation range of M_r 40 000 to 8 000 000 for polyethylene glycols, and its range was estimated at $M_r \leqslant 200$ 000 000 daltons for globular proteins [29]; the support is made up of a hydrophilic, hydroxylated polyether. When starch was left at ambient temperature for 5.0 min, then fractionated by HPSEC, one peak at 12.2 min was observed (Fig. 8a). However, after starch was exposed to 100°C for 5.0 min, the HPSEC elution profile revealed the presence of species that eluted at 4.34, 5.60, 6.77 and 12.1 min. (Fig. 8d). These products could represent the low-DP and high-DP species that were released when potato starch was heated at 70°C [28]. Fig. 8b represents the elution profile of starch after 16 h at 45°C, while Fig. 8e represents the elution profile of starch after it was heated at 100°C for 5.0 min, then at 45°C for 16 h. The elution profiles are identical. Heating (16 h, 45°C) caused the solubilization of the species depicted in Fig. 8b;

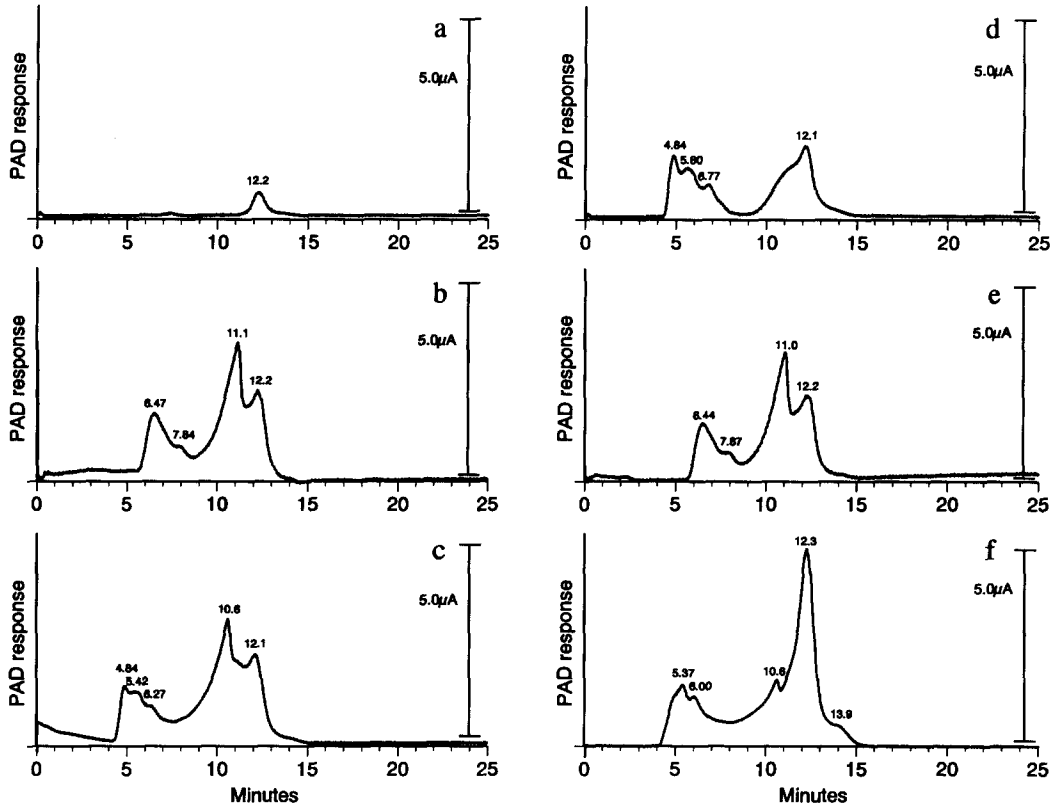

Fig. 8. HPSEC of starch and hydrolysis products. Fractionation of starch that was not preheated (a), starch that was not preheated, but was then heated at 45°C for 16 h (b), isoamylase digest of starch that was not preheated (c), starch that was preheated (d), starch that was preheated, then further heated at 45°C for 16 h (e) and isoamylase digest of starch that was preheated (f). Samples were prepared and chromatographed as described in the Experimental section.

these species do not, however, serve as substrates for isoamylase (Fig. 7b). Because the preheated starch produced the same elution profile after 16 h at 45°C (Fig. 8e) as did the starch that was not preheated (Fig. 8b), it is possible that the products released by heat rupture of the granule (Fig. 8d) are aggregates and that heating for 16 h at 45°C may cause dissociation and thereby result in the elution profile depicted in Fig. 8e. If so, then the products observed after heat pretreatment (Fig. 8d and e) are probably not substrates for isoamylase. When isoamylase was added to starch that was not preheated, and the mixture was then incubated, the elution profile changed (Fig. 8c), and species were observed whose molecular masses were apparently higher (4.84, 5.42 and 6.27 min). Although significant isoamylase hydrolysis did not occur (Fig. 7b), limited hydrolysis could have yielded the products observed (Fig. 8c). Addition of isoamylase to starch after the heat pretreatment (Fig. 8f), resulted in an elution profile that was different from those depicted in Fig. 8a and e. Aside from the alteration in retention times, the peak at 12.3 min had a much larger peak area than the peak with the same retention time in any of the other chromatograms (Fig. 8a and e). The peaks at 12.3 and 13.9 min could represent species that were formed by hydrolysis of accessible regions of the starch granule that contained amylopectin; other products included the oligosaccharides depicted in Fig. 7c. Although the interpretations of the data depicted in the elution profiles in Figs. 7 and 8 are not conclusive, this communication demonstrates the utility of HILIC and HPSEC as tools for investigating starch structure.

CONCLUSIONS

Nanogram to microgram amounts of glucooligosaccharides were fractionated within 26 min. The method and the conditions described for the fractionation of Polycose have potential use for the manufacturers of infant formulas. When the gradient program was altered to enable the resolution of oligo- and polysaccharides from different polysaccharide hydrolysates, the retention behavior of the column was significantly different for the different polysaccharide hydrolysates that were fractionated. Although the lower solubility of higher-molecular-mass glucose polymers in acetonitrile-water eluents may not allow the fractionation of polymers of DP $\geqslant$35–40, retention time precision and chromatographic resolution using different gradient programs demonstrate the utility of HILIC. Furthermore, these two chromatographic techniques, HILIC and HPSEC (with pulsed amperometric detection), will be powerful tools for the investigation of polysaccharide structure.

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