Improved gram-quantity isolation of malto-oligosaccharides by preparative HPLC *

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ABSTRACT

Malto-oligosaccharides up to a degree of polymerization of 8 were isolated in gram quantities by preparative HPLC from mild acid hydrolysates of cyclomaltoheptaose and cyclomaltooctaose [β-cyclo-dextrin (βCD) and γ-cyclodextrin (γCD), respectively]. Dynamax-60A NH_2 preparative aminopropyl silica gel columns (21.4 and 41.4 mm i.d.) and acetonitrile–water mobile phases were used. Isolation rates using the 41.4 mm i.d. preparative column approached 1 g of purified oligosaccharide per hour. The purity of isolated malto-oligosaccharides was at least 98% for dp 7 and dp 8 oligosaccharides derived from βCD and γCD, respectively.

INTRODUCTION

Malto-oligosaccharides are linear $(1 \rightarrow 4)$-linked α-D-glucopyranosyl-oligosac-charides that result from the acidic or enzymic hydrolysis of amylose, the predomi-nantly linear component of starch. Malto-oligosaccharides have become valued commodities due to their various uses[1] as food additives (sweeteners, flavor encapsulation agents, gelling agents, viscosity modifiers), fermentation feedstocks, synthons for pharmaceuticals and co-polymer plastics, experimental substrates for amylases, and standards for carbohydrate analysis. Improvements in methods used to purify individual or certain size classes of malto-oligosaccharides will dramati-cally impact research concerning the uses of these oligosaccharides.

Several methods are available for the analysis of malto-oligosaccharides (for reviews see refs 1 and 2). Of these methods, high-performance liquid chromatogra-phy (HPLC) offers the highest resolution of individual oligosaccharides over the

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widest range of oligosaccharide degree of polymerization (dp)[1,2]. Aminopropyl-bonded silica gel columns have been reported to have the highest capacity for carbohydrates among the HPLC columns available[2]. Hicks and Sondey[3] compared the utility of cation-exchange (H^+ and Ag^+), reversed-phase (C_{18}-silica gel) and aminopropyl silica gel columns for preparative HPLC purification of malto-oligo-saccharides. They observed that the aminopropyl silica gel stationary phase supported the highest sample load, highest oligosaccharide isolation rate and highest purity of isolated oligosaccharides from corn syrup solids[3].

Koizumi et al.[4] compared a semipreparative polyamine-bonded vinyl alcohol copolymer gel column with a preparative octadecylsilane column for the isolation of malto-oligosaccharides from short-chain amylose. These workers preferred the latter column, using a dilute methanol mobile phase at 33°C, since the solubility of malto-oligosaccharides in the acetonitrile–water mobile phase, used with the former column, was limited above dp 14 (ref. 4). Both columns required rechro-matography for the isolation of individual malto-oligosaccharides, which could not be isolated above dp 18 due to the insolubility of these oligosaccharides, even in water[4]. Unfortunately, information concerning column loading capacity, isolation efficiency or purity of the isolated fractions was not included.

Additional disadvantages associated with the use of corn syrup solids or short-chain amylose as the starting material for preparative HPLC purification of malto-oligosaccharides included the presence of trace amounts of isomalto-oligo-saccharides containing $(1 \rightarrow 6)$-linked α-D-glucose branches. A water wash period was required at the end of each run to remove the residual amounts of high dp oligosaccharides that could not be precipitated from corn syrup solids prior to injection[3]. Consequently, run times were longer, and the water wash period resulted in a decreased aminopropyl silica gel column lifetime due to increased silica bleed.

The preparation of malto-oligosaccharides from cyclodextrins (CDs) has been previously reported[5–8]. However, these procedures lacked an efficient chromato-graphic method that would allow for the rapid, high-resolution, large-quantity isolation of individual oligosaccharides present in hydrolysates. We describe such a chromatographic method in this report.

EXPERIMENTAL

Chromatography. —Instrumentation and methods used for preparative HPLC and analytical HPLC separations of malto-oligosaccharides have been described, in part, previously[3,9]. Normal-phase separations were performed using room temper-ature acetonitrile–water mobile phases and aminopropyl silica gel Dynamax-60A NH_2 (Rainin Instrument Co.) stationary phases. The analytical (4.6×250 mm) and preparative (21.4 and 41.4 mm i.d. $\times$ 250 mm) Dynamax-60A NH_2 columns were fitted with axial compression end-fittings that reduced stationary-phase voids formed from the leaching of silica gel by the mobile phase. The preparative HPLC

system (Gilson Medical Electronics, Inc.) included a Waters 403 preparative differential refractometer, while the analytical HPLC system (DuPont Instrument Co.) included an ultra-sensitive Erma ERC-7510 refractive index detector. Samples were first dissolved in water, then acetonitrile was added to make the solution 1 : 1 acetonitrile–water. Finally the samples were filtered (0.45 μm, Nylon 66) prior to injection. To confirm the chromatographic purity of the malto-oligosaccharide fractions purified by preparative HPLC, a high-performance anion-exchange chromatography pulsed amperometric detection (HPAEC-PAD) method (4 × 250 mm Dionex CarboPac PA1 column) was used[10].

The following formula was used to calculate peak resolution values[11] (R)

$$R = \frac{\Delta t}{1/2(w_1 + w_2)}$$

where Δt is the difference in the retention times for two peaks, and w the baseline peak width. Previously we observed that a resolution value of 1 allowed for the collection of peaks with 90% or greater purity[3].

Preparation of malto-oligosaccharides. —Malto-oligosaccharides up to dp 7 were prepared according to Nitta et al.[6]. βCD (a gift from American Maize Products Co.) was hydrolyzed in 0.2 N H_2SO_4 (50 g of βCD per 215 mL) for 1 h at 99°C, then cooled in ice–water, and refrigerated overnight. Following filtration to remove unhydrolyzed CD, the hydrolysate was neutralized by passing it through a weakly-basic, 100-mL Amberlite IRA-94 (Sigma Chemical Co.) anion-exchange column. Removal of the residual CD was accomplished by adding toluene (45 mL) to the hydrolysate[12] (which was contained in 250 mL of aqueous solution), shaking for 24 h at 25°C, and then removing the precipitated CD by centrifugation. The toluene extraction of the hydrolysate was then repeated. Finally the hydrolysate was deionized with a 10-mL Amberlite MB-3 (Sigma Chemical Co.) column (run at a flowrate of ~ 8 mL/min, which avoided adsorption of the malto-oligosaccharides) and then lyophilized.

The dp 8 malto-oligosaccharide was prepared by the procedure of French et al.[5], except that 9 g of γCD (a gift from American Maize Products Co.) was hydrolyzed in 0.01 N HCl (45 mL) for 2 h at 99°C. After cooling in an ice–water bath, the hydrolysate was neutralized by passing it through a 10-mL IRA-94 column. Unhydrolyzed CD was removed from the hydrolysate by toluene extraction as above, except that the first extraction lasted for 1 h and the second extraction lasted for 19 h. The recovered CD was then used as the starting material for subsequent hydrolyses. Following filtration, the hydrolysate was then evaporated under reduced pressure to dryness.

Purification of malto-oligosaccharides. — Routinely 500 mg (2.5 mL of 200 mg/mL in 1 : 1 acetonitrile–water) of the βCD hydrolysate was automatically injected on the 21.4 mm i.d. preparative Dynamax-60A NH_2 column. Like fractions were collected from several runs by an "intelligent" peak-sensing fraction collector and pooled. The pooled fractions were evaporated under reduced pres-

TABLE I

Malto-oligosaccharide isolation efficiency

Dp	Preparative HPLC peak resolution	Isolation rate (mg/h)	Purity (% by peak area)
3 [a]		55.5	91.9
4 [a]	1.4	79.6	91.4
5 [a]	1.0	79.8	91.8
6 [a]	1.2	75.8	92.6
7 [a]	0.9	255.1	98.0
8 [b]	0.6	996.3	99.9

[a] Dynamax 60A, NH_2 (21.4 mm × 30 cm), 11:9 acetonitrile–H_2O, 13 mL/min.
[b] Dynamax 60A, NH_2 (41.4 mm × 30 cm), 1:1 acetonitrile–H_2O, 40 mL/min.

sure to 100 mL and then filtered prior to lyophilization. Between 0.78 and 1.75 g (5.5–6 mL of 142–292 mg/mL in 2:3–9:16 acetonitrile–water) of the γCD hydrolysate was manually injected onto the 41.4 mm i.d. preparative Dynamax-60A NH_2 column. The dp 8 malto-oligosaccharide fractions were manually collected, pooled, and evaporated under reduced pressure to dryness. Mobile phases and flow rates for both preparative columns are listed in Table I. The chromatographic purity (based on peak area) of the fractions was evaluated with either an analytical Dynamax-60A NH_2 column or a CarboPac PA1 column. The malto-oligosaccharide dp value was determined by comparing retention times with standards previously isolated[3].

RESULTS AND DISCUSSION

Acid hydrolysis of β and γCD produced the malto-oligosaccharide mixtures shown in Fig. 1. After removal of unhydrolyzed CD, the βCD hydrolysate consisted of 43% dp 7 malto-oligosaccharide, while the γCD hydrolysate consisted of 72% dp 8 malto-oligosaccharide based on relative peak area from analytical Dynamax-60A NH_2 chromatograms. The milder conditions for γCD hydrolysis were selected so that dp 8 would predominate in the hydrolysate. More rigorous conditions were required for βCD hydrolysis since its glycosidic bonds have been reported to be 1.5 times more stable to acid hydrolysis (0.2 N HCl, 70°C) than γCD glycosidic bonds[7].

Under the conditions used here, the malto-oligosaccharide yield from βCD hydrolysis (24.16 g from 75.5 g βCD) was greater than that from γCD hydrolysis (6.4 g from 30.4 g γCD); however, the production of dp 7 and 8 malto-oligosaccharides per gram of the parent CD was virtually the same (14 vs. 15% of the original CD weight, respectively). Up to 70% yields of dp 7 malto-oligosaccharide from βCD have been reported using an inorganic acid in a nonaqueous medium for hydrolysis[8].
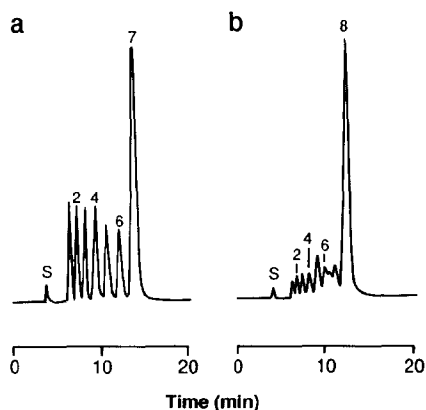
Fig. 1. Analytical-scale separation of malto-oligosaccharides present in (a) βCD and (b) γCD hydro-lysates on a Dynamax-60A NH$_2$ (4.6 mm i.d.) column. The dp value of each malto-oligosaccharide is indicated above the peaks (S = solvent). Chromatographic conditions included a 0.75 mL/min 1:1 acetonitrile–H$_2$O mobile phase with refractive index detection. The amounts of sample injected were (a) 0.2 and (b) 0.4 mg.

The βCD hydrolysate was automatically injected every 30 min on the 21.4 mm i.d. preparative column (Fig. 2a). The near-baseline peak resolution ($R \geq 0.9$) facilitated collection of purified dp 3–7 malto-oligosaccharides. Over a 21.5 h period, 21 g of the βCD hydrolysate was injected without significant loss of column performance. During this time, peak resolution was maintained as in Fig. 2a, and the column back-pressure rose from 67 to 78 bar. We also did not notice the development of any stationary-phase voids, which would have required tightening the axial compression column end fitting.

The chromatographic purity of the isolated dp 3–7 malto-oligosaccharides is demonstrated in Fig. 2b. The purity of individual malto-oligosaccharides based on relative peak area is given in Table I. Injection of isolated malto-oligosaccharides on the HPAEC-PAD system confirmed their high purity (data not shown). Inter-estingly, minor peaks were present in each malto-oligosaccharide HPAEC-PAD chromatogram that did not correspond to different dp malto-oligosaccharides based on retention times. The identity of these minor peaks is currently being investigated.

The 41.4 mm i.d. preparative column supported very high sample loads ranging from 0.8 to 1.75 g of the γCD hydrolysate per injection (Fig. 3a). During a 3 h period, 6.4 g of the γCD hydrolysate was injected on the 41.4 mm i.d. preparative column without any loss in performance. Although the dp 7 and 8 peaks were not well resolved ($R = 0.6$), dp 8 was collected approximately half way up the peak in order to ensure high purity of the isolated malto-oligosaccharide. The chromato-graphic purity of the isolated dp 8 malto-oligosaccharide is demonstrated in Fig. 3b, and the relative peak area purity is given in Table I. The high purity of this

malto-oligosaccharide was also confirmed by injection on the HPAEC-PAD system.

The β and γCDs were not completely resolved from the hydrolysate malto-oligosaccharides in analytical Dynamax-60A NH_2 chromatograms (data not shown). Hence, we ensured that our toluene extraction procedure removed all but trace amounts of these CDs from hydrolysates prior to preparative HPLC injection. Therefore, the resulting purified malto-oligosaccharides are essentially free of their parent CDs.

The 21.4 mm i.d. preparative column used in this study was a prototype model, while both the analytical and 41.4 mm i.d. columns were new, commercially
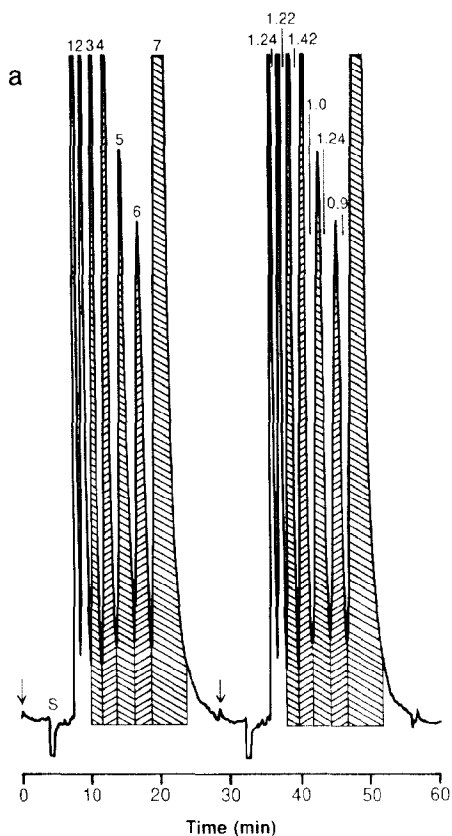

Fig. 2. (a) Automated, preparative-scale separation of malto-oligosaccharides (500-mg sample injected) from the βCD hydrolysate on a 21.4 mm i.d. Dynamax-60A NH_2 column. Malto-oligosaccharide dp values are indicated above the left-hand chromatogram peaks (S = solvent) and resolution values are indicated between the right-hand chromatogram peaks. These two chromatograms represent two consecutive injections. The hatched peaks were automatically collected. Chromatographic conditions included a 13 mL/min 11:9 acetonitrile–H_2O mobile phase with refractive index detection. (b) Chromatographic purity of malto-oligosaccharides purified by automated, preparative HPLC. Pooled fractions collected from the preparative HPLC column were injected on an analytical Dynamax-60A NH_2 column. See Fig. 1 for conditions (0.2-mg samples injected).
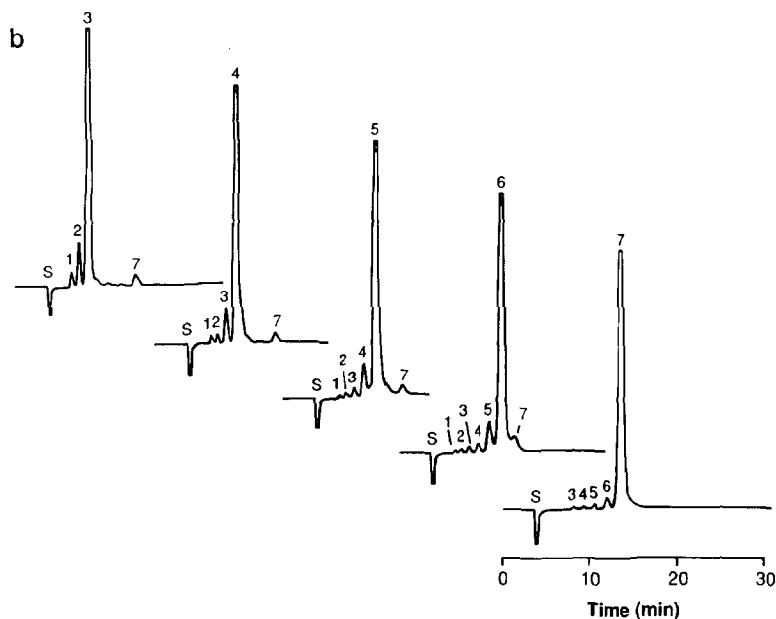
Fig. 2. (continued).

available models of the Dynamax-60A NH$_2$ column. In a previous study[13], we noticed that the prototype and new commercially available stationary phases required slightly different optimal mobile phase composition. Slight differences in the stationary phases could also explain why we found that 11 : 9 acetonitrile–water was the optimal mobile phase for the 21.4 mm i.d. column, and 1 : 1 acetonitrile–water was the optimal mobile phase for the analytical and 41.4-mm i.d. columns. Therefore, we expect that a 1 : 1 acetonitrile–water mobile phase would produce optimal malto-oligosaccharide normal-phase separations with all currently available Dynamax-60A NH$_2$ columns, regardless of size.

The Dynamax-60A NH$_2$ preparative columns provided an efficient means of isolating gram quantities of highly purified malto-oligosaccharides derived from cyclodextrins (Table I). More than 1 g of each oligosaccharide was isolated, which represented at least 59% recovery of the amount of each oligosaccharide injected on the preparative HPLC column. When the predominant oligosaccharide in each hydrolysate was isolated (6 g of dp 7 and 2.5 g of dp 8), the purity based on analytical Dynamax-60A NH$_2$ relative peak area was at least 98%. Up to 250 mg/h isolation rates were possible with the 21.4 mm i.d. column, while dp 8 was isolated at nearly a 1 g/h rate on the 41.4 mm i.d. column. These oligosaccharide isolation rates far eclipse the previous maximum rate reported for these columns (190 mg/h)[3].

We have now demonstrated a rapid, high-resolution, and high-capacity preparative HPLC system for the isolation of dp 2–8 malto-oligosaccharides. The use of
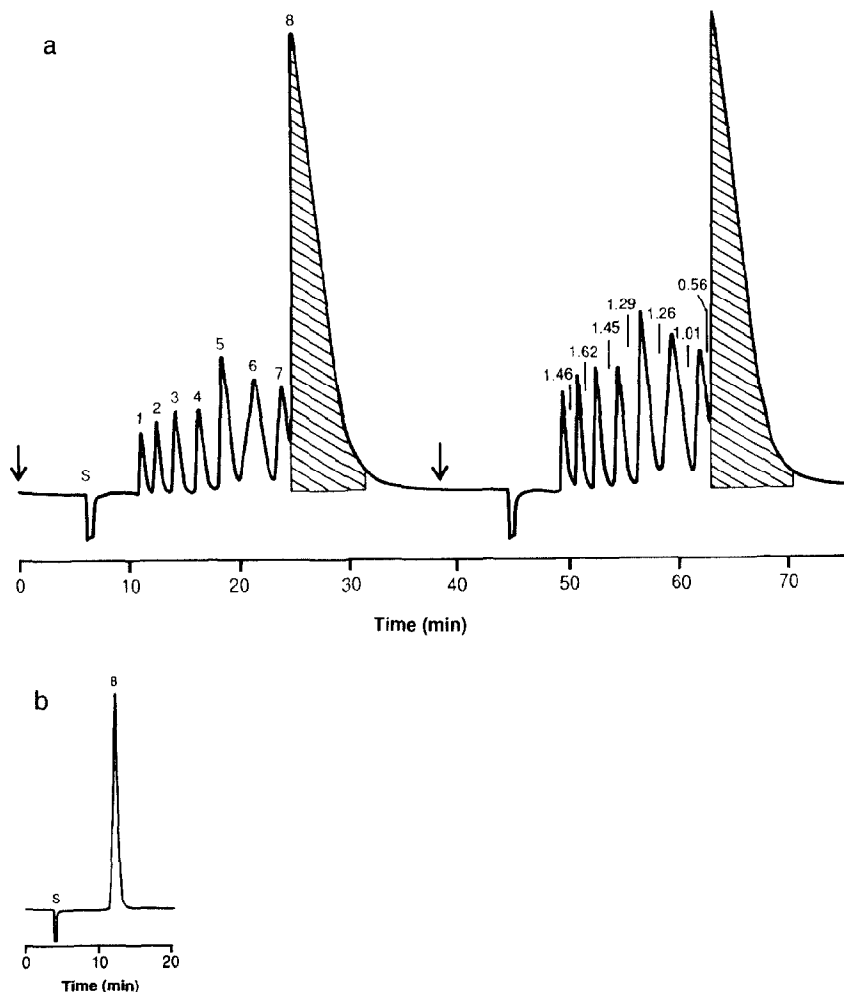
Fig. 3. (a) Consecutive manual injections (1.6 and 1.75 g) of the γCD hydrolysate on a 41.4 mm i.d. preparative Dynamax-60A NH$_2$ column. Conditions included a 40 mL/min 1:1 acetonitrile-H$_2$O mobile phase, otherwise see Fig. 2a. (b) Chromatographic purity of the dp 8 malto-oligosaccharide purified by preparative HPLC. See Fig. 1 for conditions (0.32-mg sample injected).

axial compression column end fittings and nondedicated preparative HPLC system components (pumps, injector, mixer, and detector) make the system more economical than previous dedicated preparative HPLC systems. This chromatographic method should also prove useful for the isolation of large quantities of malto-oligosaccharides up to dp 14, a limitation due only to oligosaccharide solubility in the acetonitrile–water mobile phase.

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