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Note

Isolation of oligosaccharides formed from sucrose by α-D-glucosyltransferases

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Glycosyltransferases occur widely and, depending on specificity, may transfer glycosyl groups between the same, or different, oligosaccharides, resulting in the synthesis[1] of novel oligosaccharides. Oligosaccharide reaction-products are usually purified by fractionation on activated charcoal[2], or by partition paper-chromatography[3], but these techniques do not lend themselves well to the isolation of a small proportion of oligosaccharide synthesized in the presence of a relatively high concentration of substrate. The separation of corn-syrup oligosaccharides on granulated hydroxylapatite[4] suggested that this might be a convenient method for the isolation of small amounts of oligosaccharides formed from sucrose by α-D-glucosyltransferases.

This Note describes the use of small columns of granulated hydroxylapatite to recover, quantitatively, the oligosaccharide components synthesized from sucrose by an α-D-glucosyltransferase of *Streptococcus mutans* and by a transferase in honey α-D-glucosidase.

EXPERIMENTAL

Materials. — D-Glucose, D-fructose, maltose, and sucrose were laboratory reagents. Isomaltose (6-*O*-α-D-glucosyl-D-glucose) was isolated from an acidic hydrolyzate of dextran, and maltotriose, from an enzymic hydrolyzate of amylose[5]. Kojibiose (2-*O*-α-D-glucosyl-D-glucose) and nigerose (3-*O*-α-D-glucosyl-D-glucose) were gifts from Dr. S. Chiba, Hokkaido University, and Dr. K. Matsuda, Tohoku University. [U-^{14}C]Maltotriose was purchased from Amersham Corporation, and [U-^{14}C]sucrose from New England Nuclear. Saccharides used as authentic standards were purified by paper chromatography.

Methods. — Granulated hydroxylapatite was prepared as described by Jenkins[4], and suspensions of the crystals in ethanol were allowed to settle in glass columns. The columns were washed with aqueous ethanol (usually, 90 or 95% ethanol).

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Oligosaccharide mixtures were dissolved in water, absolute ethanol was added to give the same concentration as in the wash solution, and a sample (~2 mL) was applied to the column, which was irrigated with the same ethanol solution. When a suitable volume had passed through the column, the ethanol concentration was changed in a stepwise manner, or by a decreasing, linear concentration-gradient in which the concentration changed from 90 to 70% over a volume of ~50 mL. Flow rates were from 5 to 10 mL per h, and 0.5–1.0 mL was collected in each fraction tube.

D-Glucose was determined by the D-glucose oxidase method[6], and total carbohydrate by the phenol–sulfuric acid method[7]. Total radioactivity was measured in

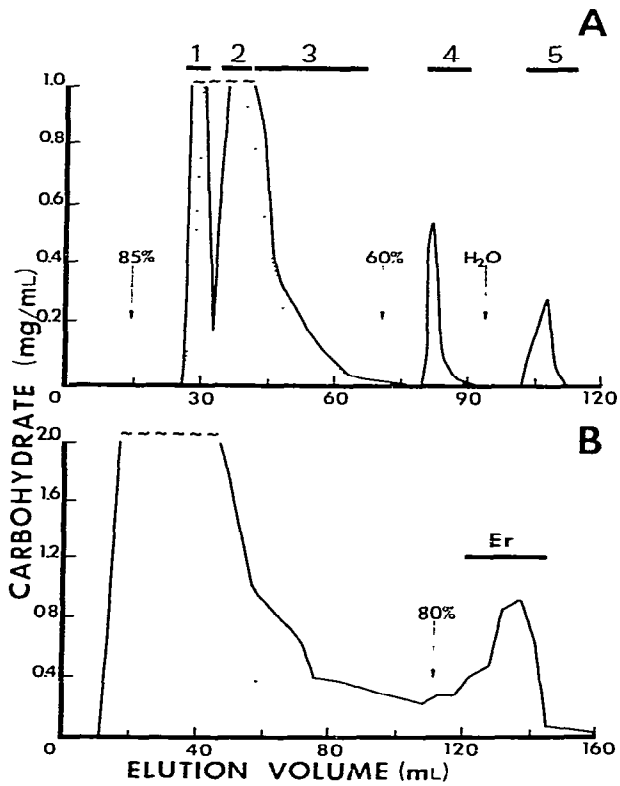

Fig. 1. Isolation, on hydroxylapatite, of oligosaccharides formed in small quantities by enzyme actions on sucrose. [A. Sucrose treated for 60 min (solid line; untreated, dotted line) with *S. mutans* glucosyltransferase, as described in the Experimental section, was applied in 90% ethanol solution (25 mg/2 mL) to a column (14 mL; 1.5 × 7.7 cm) of hydroxylapatite. The column was irrigated with 90% ethanol, and the ethanol concentration was changed in a stepwise manner as indicated by the arrows. The radioactivity in each fraction was measured, and the concentration of carbohydrate was calculated from the specific radioactivity of the sucrose substrate. The solid bars indicate the volume of eluate that was combined to give the numbered fractions. B. Sucrose treated with honey α-D-glucosidase, as described in the Experimental section, was applied in 90% ethanol solution (1.4 g/20 mL) to the column (14 mL; 1.5 × 7.7 cm) of hydroxylapatite. The column was irrigated with 90% ethanol solution, and 80% ethanol was applied as indicated by the arrow. Carbohydrate concentrations were determined by the phenol–sulfuric acid method. The solid bar indicates the elution volume in which the trisaccharide erlose (Er) was recovered.]

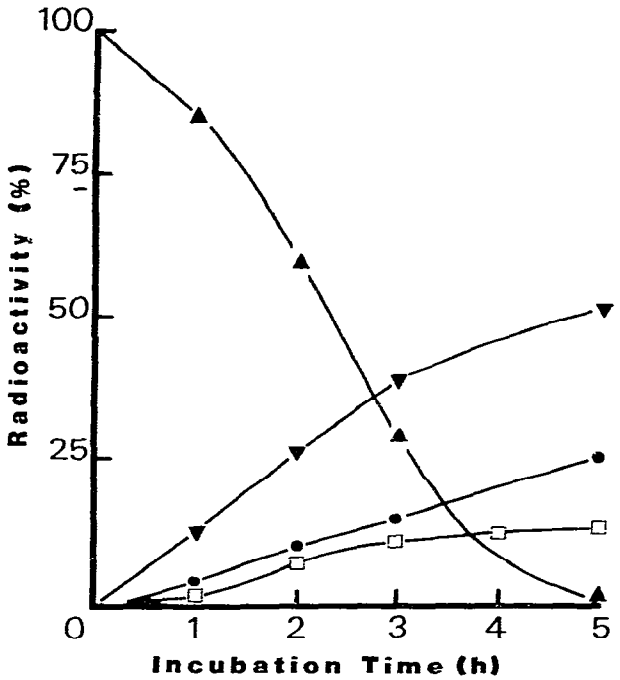

Fig. 2. Synthesis of oligosaccharides from [^{14}C]sucrose by *S. mutans* glucosyltransferase. {[^{14}C]-Sucrose was incubated for different times with *S. mutans* glucosyltransferase under the conditions described in the Experimental section. Samples of the reaction mixtures were applied to a hydroxylapatite column as in Fig. 1A, and the monosaccharide and sucrose components were eluted with 85% ethanol solution (56 mL). The column was then irrigated with water, to recover a mixture of leucrose and larger oligosaccharides. Radioactivity was measured in each fraction, and the percentage of the total radioactivity in monosaccharides released (▼), sucrose remaining (▲), and oligosaccharides synthesized (●), after the indicated times of reaction, was calculated. Radioactivity incorporated into ethanol-insoluble polysaccharide (□) was measured before the reaction mixtures were applied to the hydroxylapatite column.}

samples (50 μL) applied to squares (1 × 1 cm) of filter paper. The papers were dried under an infrared lamp, placed in vials, and counted in a scintillation spectrophotometer. Radioactivity in ethanol-insoluble, D-glucan fractions was similarly measured after papers to which samples had been applied had been washed in 70% ethanol solution. Descending paper-chromatography was conducted routinely on Whatman No. 1 papers, and 3 MM papers were used for preparative separations. Papers were irrigated with 6:4:3 (v/v) 1-butanol–pyridine–water, and sugar zones were made visible with alkaline silver nitrate reagent[8]. D-Fructose-containing sugars were located with urea–phosphate reagent[9].

Retention times of carbohydrate fractions on the hydroxylapatite column were measured in a Whatman Partisil-10 PAC analytical column (4.6 mm × 25 cm) in a high-performance, liquid chromatograph (Waters Associates, Model No. 244W). The solvent was 7:3 (v/v) acetonitrile–water and this was applied at a flow rate of 1 mL/min. Sugar fractions were detected with a differential refractometer (R401).

Enzyme reaction-mixtures. — *Streptococcus mutans* 6715–49 glucosyltransferase was prepared as described by McCabe and Smith[10], and enzyme (1.6 International Units) was incubated for different times at 37° with [^{14}C]sucrose (140 mg; 5.74 × 10^6 cpm) in reaction mixtures (10 mL) containing 50mM sodium phosphate and 0.02% sodium azide, pH 6.0. After incubation, each reaction mixture was frozen rapidly at −70°, and lyophilized. The residue was suspended in water, and insoluble material was removed by centrifugation. Supernatant solution (0.1 mL) was added to granulated hydroxylapatite (0.5 mL) that had been washed with 95% ethanol, and allowed to settle under gravity. Absolute ethanol (1.9 mL) was added to bring the concentration to 95%, and the suspension was applied to the top of a hydroxylapatite column that had been washed with 95% ethanol solution. The column was irrigated with ethanol and water solutions as indicated (see Figs. 1A and 2).

Honey α-D-glucosidase was prepared as described by Papadakis[11], and enzyme (0.5 International Units) was incubated for 64 h at 30° in a reaction mixture (20 mL) containing sucrose (2.4 g), 100mM sodium acetate solution, pH 5.7, and a few drops of toluene (to prevent bacterial growth). The mixture was heated for 15 min at 100°, to stop enzyme action, and the solution was lyophilized. An aqueous solution of the residue (2 mL; 1.4 g of carbohydrate) was added to granulated hydroxylapatite (5 mL) that had been washed with 90% ethanol and allowed to settle. Absolute ethanol (18 mL) was added, to bring the concentration to 90%, and the suspension was applied to a hydroxylapatite column pre-equilibrated with 90% ethanol. The column was irrigated as indicated in Fig. 1B.

RESULTS

Standard mixtures of oligosaccharides with sucrose were used, in order to test the ability of a hydroxylapatite column (4.4 mL; 0.7 × 11.5 cm) to separate the component sugars. A mixture (10 mg) containing equimolar amounts of D-glucose, D-fructose, sucrose, and maltotriose in 90% ethanol (2 mL) gave two carbohydrate peaks when the hydroxylapatite column was irrigated with 90% ethanol solution. The first peak contained D-glucose and D-fructose, and the second, pure sucrose. Pure maltotriose was eluted in a broad peak by a decreasing concentration gradient of ethanol (90→70%). About 90% of the total carbohydrate was recovered in the three carbohydrate fractions. In similar separations on the hydroxylapatite column, maltose, nigerose, and kojibiose were eluted, by 90% ethanol, in the same volume as sucrose, but isomaltose was more strongly absorbed and, like maltotriose, required the application of a decreasing ethanol concentration (90→70%) before it was eluted as a broad peak. A stepwise change to 80% ethanol as the irrigation solution desorbed the isomaltose as a sharp peak. The isomaltose preparation used contained small proportions of D-glucose and isomaltotriose, but, after fractionation on hydroxylapatite, the isomaltose moved as a single component on a paper chromatogram.

The loading capacity of the column was tested with an oligosaccharide mixture (100 mg) containing a small amount of [^{14}C]maltotriose (1 mg; 1.7 × 10^5 c.p.m.)

mixed with D-fructose (34 mg) and sucrose (65 mg). D-Fructose and sucrose were eluted by 90% ethanol solution as a single peak possessing a shoulder at the leading edge. Irrigation with 90% ethanol was continued until carbohydrate in the collected fractions decreased to an undetectable level. Less than 8% of the radioactivity applied to the column was desorbed by the 90% ethanol solution. Irrigation of the column with a decreasing concentration (90 → 70%) of ethanol, or with 60% ethanol solution, resulted in the recovery of ~94% of the [^{14}C]maltotriose. A good recovery was also obtained when [^{14}C]maltotriose (1 mg) alone was applied to the column, and we now use hydroxylapatite columns in a convenient method to purify small quantities of radiolabelled, neutral mono-, di-, and tri-saccharides.

The successful recovery of a small amount of maltotriose from artificial mixtures encouraged us to use a hydroxylapatite column (14 mL; 1.5 × 7.7 cm) to fractionate reaction mixtures in which small amounts of oligosaccharides had been enzymically synthesized from sucrose (see Figs. 1A and 1B). The fractionation of [^{14}C]sucrose (25 mg: 10^6 c.p.m.), before and after treatment for 60 min with *S. mutans* glucosyltransferase, revealed that the enzyme synthesized oligosaccharides that were eluted from the column by 60% ethanol solution and by water (see Fig. 1A). The relatively small sample-size, and the use of the larger column, ensured a good separation between the monosaccharide products and the sucrose substrate when the column was irrigated with 85% ethanol.

The eluate was combined as indicated, and concentrated, and portions of each concentrate were analyzed in a high-performance, liquid-chromatography column. The retention time of fraction 1 corresponded to that of glucose and fructose (473 s). Specific analysis of the fraction demonstrated that <10% of the monosaccharide was glucose. The oligosaccharides in fraction 5 were too large to be satisfactorily resolved in the high-performance column. Fractions 2 and 3 contained a single component having the same retention time as the sucrose substrate (523 s). Fraction 4 also contained a single component, which resembled sucrose in that it possessed a fructose moiety, but it was clearly differentiated from sucrose by its longer retention time (558 s) and by its ability to reduce copper under alkaline conditions. Experiments indicated that the oligosaccharide in Fraction 4 was leucrose, a disaccharide formed by the enzymic transfer of the D-glucosyl group of sucrose to O-5 of D-fructose. About 8% of the total leucrose was detected in the solution eluted between fractions 3 and 4, but, in another fractionation, in which the column was irrigated with a larger volume of 85% ethanol solution (110 mL), ~37% of the leucrose was released before the column was irrigated with 60% ethanol solution.

Irrigation of the column with the smaller volume (56 mL) of 85% ethanol solution, directly followed by irrigation with water, allowed the separation, and quantitation, of monosaccharide and oligosaccharide products after different times of incubation of [^{14}C]sucrose with the *S. mutans* glucosyltransferase. Fig. 2 illustrates the progressive release of [^{14}C]monosaccharides and synthesis of [^{14}C]oligosaccharides. Synthesis of ethanol-insoluble D-[^{14}C]glucan in the reaction mixtures was measured before the samples were fractionated on the column.

Sucrose solution incubated with honey α-D-glucosidase for 64 h was fractionated by preparative paper-chromatography, and from paper zones was extracted a D-fructose-containing oligosaccharide having an R_F value corresponding to that of the trisaccharide erlose (4-*O*-α-D-glucosylsucrose). The extract contained ~30% of sucrose, and a second separation on paper was needed in order to obtain a chromatographically pure trisaccharide. This trisaccharide (5 mg) was successfully separated from equimolar amounts of maltose and sucrose in a small hydroxylapatite column (not shown); therefore, a portion of the honey α-D-glucosidase reaction-mixture (1.4 g of carbohydrate) was applied to a larger column (14 mL; 1.5 × 7.7 cm). About 99% of the carbohydrate was eluted from the column by 90% ethanol solution, and a small carbohydrate peak was eluted with 80% ethanol (see Fig. 1B). The first peak contained fructose, glucose, and sucrose, but the carbohydrate in the small, second peak moved on a paper chromatogram as a single component that gave a positive reaction with a specific stain for fructose-containing sugars. It possessed an R_F value identical to that of the trisaccharide (erlose) previously isolated by paper chromatography. Although paper chromatograms of the fractions eluted from the column just before the 80% ethanol was applied (elution volume, 80–100 mL) showed the presence of erlose, it was estimated, based on the sensitivity of the fructose-positive spray reagent, that only ~5% of the total erlose had leaked from the column before the 80% ethanol was applied. Of the total carbohydrate in the D-glucosidase reaction-mixture, ~1.1% was recovered from the hydroxylapatite column as a pure trisaccharide fraction, compared to 0.8% recovered by preparative paper-chromatography.

DISCUSSION

Compared to charcoal-Celite and gel-filtration chromatography columns, the advantages of hydroxylapatite columns in the large-scale fractionation of oligosaccharide mixtures include high loading-capacity, high flow-rate, and ease of column regeneration[4]. Hydroxylapatite columns also provide a convenient, small-scale method for purifying neutral oligosaccharides and, more important, for quantitatively recovering minor products enzymically synthesized in oligosaccharide mixtures (see Fig. 1). An additional advantage of hydroxylapatite columns over gel filtration was the preferential adsorption of some sugars by hydroxylapatite, thus allowing separation of molecules of the same size. Thus, isomaltose in artificial mixtures was readily separated from kojibiose, maltose, nigerose, and sucrose (as described in the text), and leucrose was separated from a thirty-fold excess of sucrose in *S. mutans* glucosyltransferase reaction-mixtures (see Fig. 1A), to give a sample that, as analyzed in a high-performance, liquid-chromatography column, was 98% pure.

Separation, on hydroxylapatite, of the oligosaccharide products and sucrose substrate in *S. mutans* glucosyltransferase reaction-mixtures demonstrated that the synthesis of leucrose and other ethanol-soluble oligosaccharides exceeds, and probably precedes, the synthesis of ethanol-insoluble α-D-glucan when dextran or other acceptor

molecules are absent (see Fig. 2). Significant synthesis of oligosaccharides also occurred, albeit at a lower level, when acceptor dextran was present (not shown). This synthesis of oligosaccharides and, to a lesser extent, the release of D-glucose, explains why the release of reducing sugars from sucrose by *S. mutans* glucosyltransferase is greater than can be accounted for by the synthesis of ethanol-insoluble α-D-glucan alone[12].

Although increased loading of the hydroxylapatite column (4.4 mL; 0.7 × 11.5 cm) resulted in a loss in its capacity to separate the monosaccharide (D-fructose) and disaccharide (sucrose) components of an oligosaccharide mixture (100 mg), good resolution and recovery of the maltotriose component (1 mg) was obtained. This high resolving-power between the di- and tri-saccharide components allowed a quantitative recovery of a small amount of erlose (15 mg), synthesized in a sucrose–honey α-D-glucosidase reaction-mixture (1.4 g of total carbohydrate), to be accomplished in a single fractionation, despite gross overloading of the hydroxylapatite column (see Fig. 1B). Fractionation in the hydroxylapatite column required less than half the time needed for, and was much more convenient than, purification of the erlose by preparative paper-chromatography.

The results clearly demonstrate that oligosaccharides are directly synthesized in the action of *S. mutans* glucosyltransferase on sucrose, and establish that separation of oligosaccharides by hydroxylapatite column-chromatography will be a valuable adjunct to other methods used to analyze oligosaccharide mixtures, and to isolate and characterize reaction products formed by oligosaccharide-metabolizing enzymes in general.

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