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IMPROVED PURIFICATION PROCEDURE FOR THE EXTRACELLULAR D-GLUCOSYLTRANSFERASE FROM *Streptococcus mutans* 6715

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ABSTRACT

The exocellular D-glucosyltransferase from *Streptococcus mutans* 6715 has been highly purified with minimal loss of enzymic activity. The organisms were cultured in trypticase soy-broth that had been treated with invertase and filtered through an ultrafilter fitted with a membrane having a cut-off molecular weight at 10,000. To the growth medium was added Tween 80, which prevented the enzyme from aggregating. The final step in the purification employed insoluble, streptococcal dextran as an affinity support. Two D-glucosyltransferase activities were detected, *viz.*, one that did not adsorb to the insoluble dextran and one that did. The enzymic fraction that had adsorbed to the insoluble dextran in the affinity column was strongly inhibited by added insoluble dextran.

INTRODUCTION

Streptococcus mutans produces extracellular D-glucosyltransferases that catalyze the formation of a D-glucan (dextran) from sucrose[1,2]. The synthesis of these polysaccharides *in vitro* from dietary sucrose appears to play an essential role in the attachment of the organisms to smooth tooth-surfaces. It is considered that the synthesis of that glucan leads to the subsequent formation of dental plaque and smooth-surface, dental caries[3–5]. In this respect, the purification and characterization of the extracellular D-glucosyltransferases have been the subject of considerable investigation. However, most purification schemes have been plagued by low yields, low specific activities, or the existence of the D-glucosyltransferase as an aggregate of high molecular weight[1,6–8]. We now describe a method for obtaining, in almost quantitative yield, a D-glucosyltransferase having a high specific activity and a "reasonable" molecular weight.

EXPERIMENTAL MATERIALS AND METHODS

Organisms and growth conditions. — *S. mutans* 6715 was grown in a modified, commercial, trypticase soy-broth (TSB, Baltimore Biological Laboratories). The modi-

fications were designed to eliminate sucrose and D-glucans of high molecular weight from the medium. TSB without dextrose was dissolved in distilled water (275 g.L^{-1}) and the pH of the broth was adjusted to 4.5 with glacial acetic acid. Yeast invertase (Sigma Chemical Co.; 30,000 I.U. per L) was added, and the mixture incubated for 2 h at 55°. The pH of the broth was then adjusted to 7.2 with 10M sodium hydroxide, any insoluble material was removed by centrifugation, and the supernatant liquor was filtered through an Amicon PM10 ultrafiltration membrane (to remove contaminating D-glucans). The ultrafiltrate was sterilized by passage through a bacterial filter (0.45 μm; Nalgene Plastic Co.). The final culture-medium was prepared by adding one volume of sterile broth to nine volumes of a pre-autoclaved, aqueous solution of D-fructose (1.11%) and Tween 80 (0.055%). The medium was inoculated with cells that had previously been subcultured once on TSB supplemented with D-mannitol (0.25%), and the culture was incubated for 16–18 h at 37°. Cells were then removed by centrifugation at 10,000 *g* in a continuous-flow centrifuge. The cell-free broth contained the D-glucosyltransferase activity.

Gel electrophoresis. — Gel electrophoresis was performed in cylindrical tubes containing 5% poly(acrylamide) gels, using a discontinuous buffer-system[9]. All gel solutions contained Tween 80 (1.0%), and the reservoir buffer contained 0.1% of Tween 80. In order to achieve sharp resolution of the AFF II preparation, dextran of low molecular weight (500 μg mL^{-1}; final conc.) was added to the sample prior to the electrophoresis; this was necessary only when the medium was completely free from sucrose or D-glucan. Areas of insoluble-D-glucan-synthesizing activity were made visible after electrophoresis by incubating gels in sodium phosphate buffer (0.2M, pH 6.0) containing sucrose (5.0%). Gels were scanned at 550 nm in order to record areas of polymer synthesis.

Enzyme assay. — D-Glucosyltransferase activity was assayed by one of the following methods. (*a*) One involved the incorporation of D-glucose-^{14}C(U)–sucrose

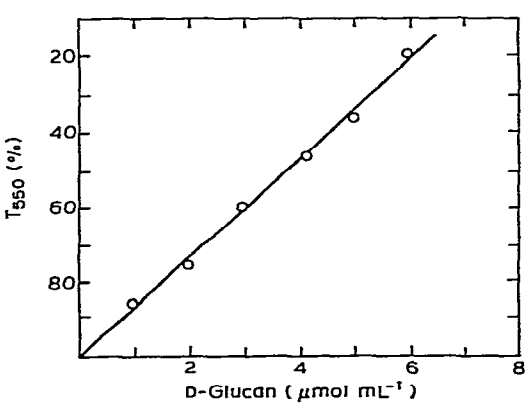

Fig 1. Correlation of T_{550}(%) with the amount of insoluble D-glucan produced by the D-glucosyltransferase. [D-Glucose-^{14}C(U)–sucrose (20mM, 2 μCi mmol^{-1}) was added to the enzyme preparation (step 1, 1.2 units of activity). Aliquots (1 mL) were removed, the insoluble D-glucan was filtered off, and the incorporation of radioactivity was determined. The T_{550}(%) of the assay mixture was simultaneously measured in a cuvet (1 cm).]

into a water-insoluble polymer by using a membrane-filter assay[10]. (*b*) The insoluble product was determined by measuring the change in transmittance (T) at 550 nm (see Fig. 1). (*c*) D-Glucosyltransferase activity was determined by the release of fluoride ion from α-D-glucopyranosyl fluoride[11]; one unit of enzymic activity synthesized one μmol of insoluble D-glucan from sucrose per min, or released one μmol of fluoride ion per min from α-D-glucopyranosyl fluoride at 25° in sodium phosphate buffer (50mM, pH 6.0). The protein concentration was determined by the Lowry method, using bovine serum albumin as the standard.

Source of materials. — Trypticase soy-broth without dextrose was purchased from Baltimore Biological Laboratory. Invertase, Tween 80, and dextran (M.W. 1×10^4) were purchased from Sigma Chemical Co. D-Glucose-^{14}C(U)–sucrose (0.6 mCi.mg^{-1}) was purchased from New England Nuclear Co. Gel-filtration materials were obtained from Bio-Rad Laboratories. The α-D-glucopyranosyl fluoride was prepared by the method described by Hall *et al.*[12]. All other chemical compounds used were of reagent grade.

PURIFICATION OF THE D-GLUCOSYLTRANSFERASE

Concentration of the broth D-glucosyltransferase. — The pH of the (culture) supernatant liquor was adjusted to 6.0 with 0.5M disodium hydrogenphosphate, and potassium chloride (70 g.L^{-1}) and sodium azide (200 mg.L^{-1}) were added. The broth was then concentrated 50-fold by using an Amicon "on-line" column effluent-concentrator (Amicon model CECl) equipped with a PM10 ultrafiltration membrane. The apparatus was so arranged that the broth was pumped across the membrane, and the non-filtrable effluent, which contained the D-glucosyltransferase activity, was returned to the stirred supply-reservoir. The filtrate, which contained broth components of low molecular weight, was discarded. After concentration had been achieved, the enzyme preparation was dialyzed against buffer that consisted of sodium phosphate buffer (50mM, pH 6.0) containing potassium chloride (1.0M) and 0.05% of Tween 80 (PKT buffer).

Gel-filtration chromatography. — The material from the first step (5 mL) was passed through a column (2.5 × 50 cm) of Bio-Gel A1.5m that had been pre-equilibrated with PKT buffer. The absorbance of the effluent was monitored at 280 nm, and fractions (7.2 mL) were collected. Each fraction was tested for enzymic activity by release of fluoride from α-D-glucopyranosyl fluoride[11] or by formation of insoluble D-glucan from sucrose. The active fractions were pooled, and concentrated to 5 mL by using an Amicon PM-10 ultrafilter.

Affinity chromatography on a column of Bio-Gel A1.5m–insoluble D-glucan. — Insoluble D-glucan was prepared by incubating the crude enzyme-preparation (5 mL) from the first step with 20mM sucrose for 2 h at 37°. The insoluble D-glucan was collected by centrifugation, and washed three times with PKT buffer (10 mL). The washed glucan (12 mg, wet weight) was mixed with enough Bio-Gel A1.5m to yield a final, packed volume of ~20 mL in a 1.5 × 11-cm column. The column was washed with

PKT buffer (100 mL) containing dextran of molecular weight 1×10^4 (1 mg.mL^{-1}) to remove any enzyme activity that might have adsorbed to the insoluble D-glucan during the D-glucan synthesis. The column was then washed with PKT buffer (100 mL), to remove the low-molecular-weight dextran. The material (5 mL) from the gel-filtration column (step 2) was applied to the column, and the column was washed with PKT buffer until no additional u.v.-absorbing material could be detected. The eluting buffer was then changed to PKT containing low-molecular-weight dextran (1 mg. mL^{-1}). Each fraction was assayed for enzymic activity. The enzymic activity that did not adsorb to the column was termed AFF I, and that which was eluted from the column with low-molecular-weight dextran was labeled AFF II.

RESULTS

Purification of the D-*glucosyltransferase.* — The results of the purification scheme are summarized in Table I. The enzymic activity was recovered in almost quantitative

TABLE I

SUMMATION OF THE PURIFICATION OF THE D-GLUCOSYLTRANSFERASE FROM *S. mutans* 6715

Preparation	*Volume (mL)*	*Protein*[a] *(mg)*	*Enzymic units*[b]	*Specific activity (units. mg*$^{-1}$*)*	*Degree of purification*	*Recovery (%)*
Culture supernatant-liquor	3,000	2,400	240	0.1	1	100
PM-10 concentrate	60	40	240	6.0	80	100
Bio-Gel A1.5m column (concentrated, active peak)	60	20	240	12.0	120	100
Insoluble D-Glucan–Bio-Gel A1.5m affinity column						
AFF I	180	12	20	1.66	16.6	8.3
AFF II	60	7	200	28.5	285	83

[a]Determined by the Lowry method, using bovine serum albumin as the standard. [b]Determined by fluoride release from α-D-glucopyranosyl fluoride, or by formation of insoluble D-glucan from sucrose, or both.

yield, as determined from the initial rate of formation of insoluble D-glucan from sucrose, and by the release of fluoride ion from α-D-glucopyranosyl fluoride. The initial concentration of the (culture) supernatant liquor resulted in an 80-fold purification, most probably due to the fact that the material of low molecular weight produced by the bacteria (material which reacts with the Lowry reagents, yielding large protein values) passed through the filter. Thus, any material retained during concentration by the PM10 membrane should consist of products of cellular growth that have molecular weights > 10,000, *viz.*, the D-glucosyltransferase. The D-glucosyltransferase activity was eluted from Bio-Gel A1.5m with a V_e/V_o of 1.55, indicating a molecular weight of $2.25–2.5 \times 10^5$ (see Fig. 2). The insoluble-D-glucan, affinity column (see Fig. 3)

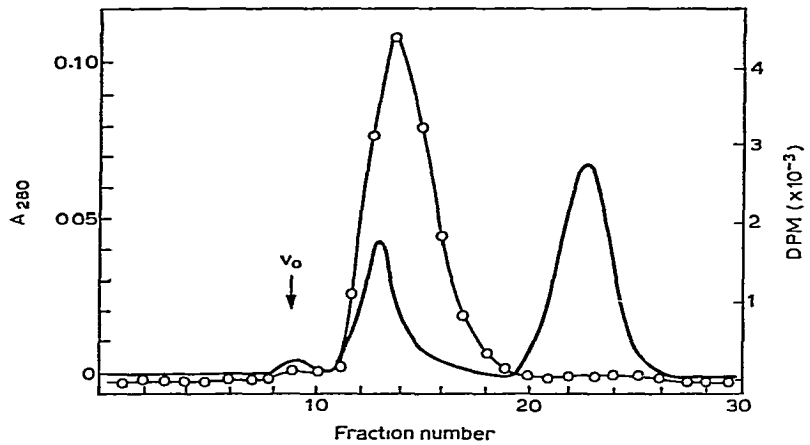

Fig. 2. Gel-filtration chromatography of D-glucosyltransferase activity on Bio-Gel A1.5m. [The PM-10 concentrate (5 mL) containing 20 units of D-glucosyltransferase activity was applied to the column. Protein concentration was determined by A_{280} (—), and the enzymic activity by the incorporation of D-glucose-^{14}C(U)-sucrose into insoluble D-glucan (-○-).]

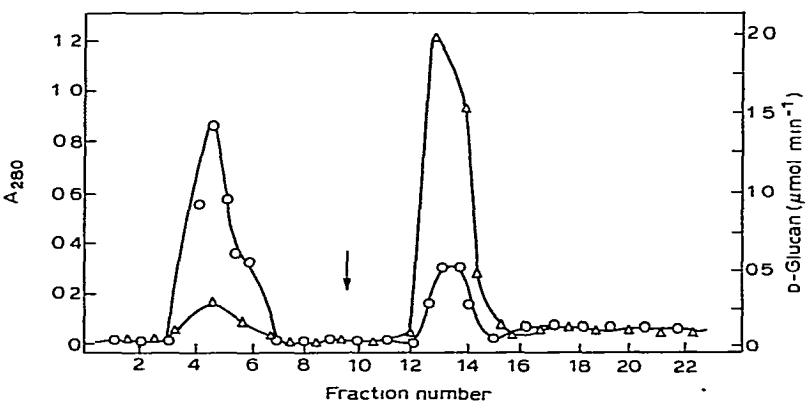

Fig. 3. Chromatography of the D-glucosyltransferase on a column of Bio-Gel A1 5m–insoluble dextran [D-Glucosyltransferase fractions from the A1.5m column were concentrated to 5 mL and applied to the column. The column was eluted with PKT buffer, followed by PKT buffer containing dextran (arrow); A_{280} (-○-), and insoluble-polymer formation (-△-).]

fractionated the enzymic activity into a fraction that demonstrated little affinity for insoluble dextran (AFF I, 10% of the transferase activity) and a fraction having affinity for insoluble D-glucan (AFF II, 85% of the transferase activity). The AFF II fraction was eluted from the affinity column before any appreciable amount of low-molcular-weight dextran appeared in the eluate, suggesting that the enzymic activity has a higher affinity for the soluble dextran than it does for the insoluble D-glucan. However, the small amount of soluble D-glucan in the AFF II preparation was sufficient to inhibit some of the synthesis of the insoluble D-glucan. The soluble D-glucan could be removed from the transferase by diafiltration through a PM30 membrane, resulting in the restoration of most of the synthetic activity as regards insoluble D-glucan.

It was found necessary to have Tween 80 and potassium chloride in the buffer in all of the steps. Elimination of both of these components from the elution buffer for the Bio-Gel A 1.5m column decreased the yield to ~10%. Elimination of potassium chloride from the concentration step decreased the yield by ~20%.

Gel electrophoresis of the D-*glucosyltransferase.* — A method for poly(acrylamide)-gel electrophoresis of the D-glucosyltransferase preparations has been developed in our laboratory[9]. Most D-glucosyltransferase preparations from cariogenic *Streptococci* are not resolved well by poly(acrylamide) gels[13]. However, when Tween 80 is included in all of the buffer systems, resolution of these activities can be achieved. The densitometric patterns of the D-glucosyltransferase are shown in Fig. 4. Fig. 4a shows the profile of the enzymic activities obtained for the A1.5m preparation; Figs. 4b and 4c give the profiles for the AFF I and AFF II preparations, respectively. The preparation from the A1.5m column exhibits at least three areas of activity, whereas the AFF preparations contain two areas of activity. The peaks having the highest mobility in each of the AFF preparations appear to be identical; however, they differ in their ability to bind to insoluble dextran. Although the AFF II preparation contains

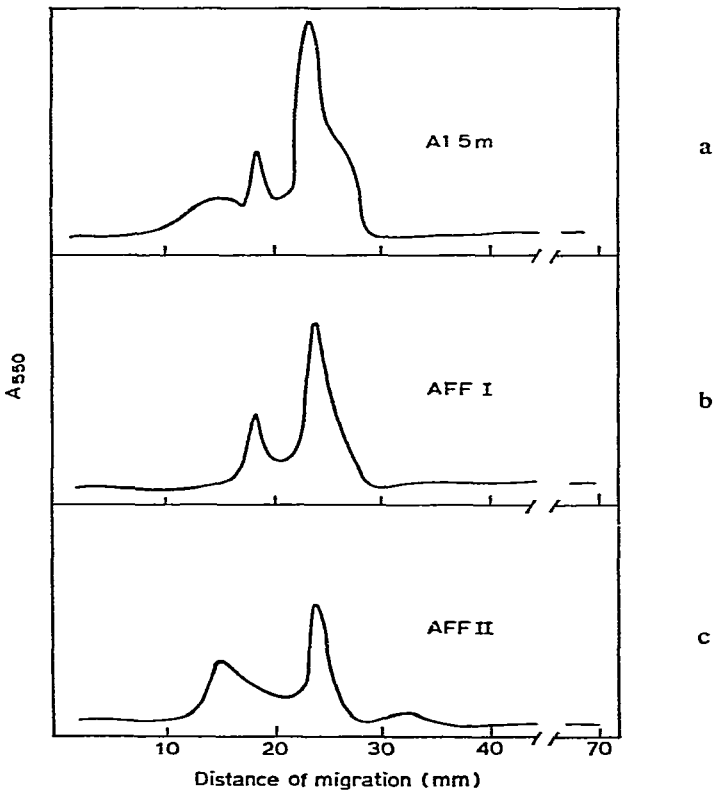

Fig. 4. Poly(acrylamide)-gel electrophoresis of D-glucosyltransferase preparations. [Poly(acrylamide)-gel electrophoresis was performed in the presence of Tween 80. After completion of the electrophoresis, the gels were incubated in sodium phosphate buffer (0.05M, pH 6.0) containing 5% of sucrose. Bands of insoluble polymer were recorded by scanning the gels at 550 nm.]

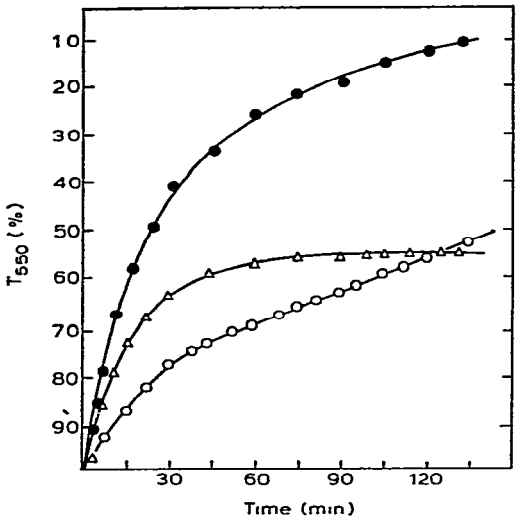

Fig. 5. Time course of synthesis of insoluble D-glucan by the D-glucosyltransferase preparations, as measured by change in % T. [The enzyme preparations were incubated with 20mM sucrose; A1.5m (-•-), AFF I (-○-), and AFF II (-△-).]

85% of the isolated activity, it seems to synthesize less polymer than AFF I during the 15-h incubation of the gels in sucrose. This is probably due to the adsorption, and then inhibition, of the AFF II preparation, as described in the following section.

Inhibition of AFF II D-glucosyltransferase by insoluble D-glucan. — The time course of formation of insoluble D-glucan from sucrose for the A1.5m and the AFF II preparations is shown in Fig. 5. The A1.5m preparation maintained a linear reaction-rate for ~15 min, at which time the rate decreased markedly. The AFF I and AFF II fractions exhibited distinctly different enzymic behavior. The AFF I preparation maintained a constant, initial rate for 30 min, followed by a lower rate, similar to that observed for the A1.5m preparation. The AFF I preparation maintained synthesis of insoluble D-glucan at the lower rate for ~6 h. In contrast, the AFF II preparation maintained a high initial rate for 15 min, and the rate then decreased to zero within 45 min. This cessation of synthesis was not due to attainment of chemical equilibrium, or to complete utilization of the sucrose. The addition of an excess of sucrose did not affect the amount of insoluble D-glucan formed. Addition of insoluble D-glucan to the AFF II preparation before addition of sucrose inhibited formation of insoluble D-glucan. The AFF I preparation was not affected by addition of insoluble D-glucan prior to the addition of sucrose.

DISCUSSION

A new scheme for the purification of *S. mutans* glucosyltransferase has been presented. The method makes use of a modified, trypticase soy-broth medium from which sucrose and polymers (M.W. ~10,000) have been eliminated. The nonionic

detergent Tween 80 was present in the culture medium, and was maintained at a concentration of 0.05% in all purification steps. Use of Tween 80 and potassium chloride permits almost quantitative recovery of D-glucosyltransferase activity throughout the purification. The Tween 80 also allows for the resolution of enzyme activities by poly(acrylamide)-gel electrophoresis. The addition of the Tween 80 to the culture medium caused increased production of the D-glucosyltransferase (data not shown), as has been noted recently[14,15]. Tween 80 was also necessary for elution of enzymic activity from Bio-Gel A1.5m in the lower-molecular-weight form; the detergent may disrupt intermolecular, hydrophobic interactions between enzyme molecules. McCabe and Smith[16] presented data showing the affinity of *S. mutans* D-glucosyltransferase for butyl-agarose, a support for hydrophobic chromatography.

The elimination of sucrose and D-glucans from the culture medium resulted in the isolation of an enzyme activity having a comparatively low molecular weight (compared to those of preparations obtained from untreated media[17]). This result indicates that either the contaminating D-glucan, or the sucrose from which the D-glucans can be synthesized, may cause interactions between enzyme molecules, resulting in aggregates having high molecular weight.

The isolation of a D-glucosyltransferase activity (AFF I) having no affinity for insoluble D-glucan permits an interesting speculation. The existence of an active enzyme of this form indicates that a high-affinity, dextran-binding site is not necessary for activity, suggesting that such a binding site may be distal to the enzymic site. Thus, the AFF I form of the enzyme may arise through some alteration of the dextran-binding form (AFF II). After enough dextran has been synthesized, either from sucrose or α-D-glucopyranosyl fluoride, the activity of the AFF II markedly decreases. It appears that the enzyme binds very strongly to the dextran, and thus loses its enzymic activity. The enzymic activity can be desorbed from the dextran formed by washing with the low-molecular-weight dextran. The AFF I synthesizes insoluble dextran, but is not inactivated by the dextran.

The total purification-scheme yields a D-glucosyltransferase (AFF II) having a high, specific activity and a combined recovery (AFF I and AFF II) that accounts for >90% of the original activity. The detection of two, electrophoretically different, activities in both AFF I and AFF II preparations indicates that further fractionation may be necessary in order to permit elucidation of the complete mechanism of the synthesis of D-glucan.

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