Purification and Properties of Extracellular Glucosyltransferase Synthesizing 1,3- α -D-Glucan from *Streptococcus mutans* Serotype *a*

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Extracellular 1,3- α -D-glucan synthase (sucrose:1,3- α -D-glucan 3- α -D-glucosyltransferase, EC 2.4.1.-) of *Streptococcus mutans* HS6 (serotype *a*) was purified from culture supernatant by ultrafiltration, DEAE-Sephadex chromatography and preparative isoelectric focusing. The enzyme had a molecular weight of 158 000 by SDS-PAGE and an isoelectric point of pH 5.2. The specific activity of the enzyme was 48.3 i.u. (mg protein)⁻¹. The K_m for sucrose was 1.2 mM and the activity was optimal at pH 6.0. The enzyme activity was stimulated about 20-fold in the presence of dextran T10. Glucan was synthesized *de novo* from sucrose by the enzyme and characterized as a linear 1,3- α -D-glucan by GC-MS.

INTRODUCTION

Strains of the cariogenic micro-organism *Streptococcus mutans* have the specific ability to form plaque on smooth tooth surfaces (Gibbons & Nygaard, 1968; Mukasa & Slade, 1973*b*). They secrete glucosyltransferases and, in some serotypes, fructosyltransferase (Carlsson, 1970) which synthesize insoluble, sticky polysaccharide from sucrose (Mukasa & Slade, 1974*b*; Ciardi *et al.*, 1976; Mukasa *et al.*, 1979). *Streptococcus mutans* 6715 (serotype *g*) secretes three kinds of glucosyltransferases (Shimamura *et al.*, 1983), and we have previously purified the highly-branched 1,6- α -D-glucan synthases from *S. mutans* 6715 (*g*), Ingbritt (*c*) and HS6 (*a*) (Shimamura *et al.*, 1982; Mukasa *et al.*, 1982*b*; Tsumori *et al.*, 1983*a*) which exhibited high 1,3,6- α -branch-forming activity as well as 1,6- α -bond-forming activity. 1,3- α -D-Glucan synthases have also been purified from *S. mutans* B13 (*d*) (Fukushima *et al.*, 1981) and 6715 (*g*) (Fukui *et al.*, 1982). The serotype *a* specific polysaccharide is immunologically partially identical with *d* and *g* antigens (Mukasa & Slade, 1973*a*), and the 1,3- α -D-glucan synthase from serotype *g* has also been shown to be immunologically related to the corresponding enzyme from serotype *a* by using a double immunodiffusion technique (Fukui *et al.*, 1983), although the two enzymes exhibited different pI values (Tsumori *et al.*, 1983*b*). Therefore, it was of interest to purify and characterize the 1,3- α -D-glucan synthase from *S. mutans* (*a*) in order to clarify the differences between these two enzymes. In this paper, we describe the purification and properties of 1,3- α -D-glucan synthase from *S. mutans* HS6 (*a*), which synthesizes a linear 1,3- α -D-glucan.

METHODS

Organism and culture conditions. *Streptococcus mutans* HS6 (serotype *a*) was the strain previously used (Tsumori *et al.*, 1983*a*). Cells were grown for 19 h at 37 °C in 5 l of chemically defined medium (Terlekyj *et al.*, 1975) with fructose instead of glucose and supplemented with 0.1% Tween 80 (Umesaki *et al.*, 1977; Wittenberger *et al.*, 1978). The optical density of the culture at 550 nm was 6.1. The cell-free culture supernatant was obtained by centrifugation for 30 min at 8900 g.

Purification of 1,3- α -D-glucan synthase. The enzyme was purified at 0 to 5 °C unless otherwise indicated.

(1) **Ultrafiltration.** Cell-free culture supernatant (5 l) was concentrated to 130 ml and equilibrated in 10 mM-Tris/HCl buffer (pH 7.4) containing 30 mM-NaCl by ultrafiltration through eight holotubes (1.2 m $\times$ 0.8 mm

each, molecular weight limit 13000; Asahi Chemical Industry, Tokyo, Japan) under reduced pressure (Mukasa *et al.*, 1982b).

(2) *DEAE-Sepharose chromatography.* The enzyme solution was applied to a DEAE-Sepharose column (1.8 × 35 cm) equilibrated with 30 mM-NaCl in Tris/HCl buffer (pH 7.4). The column was washed with 100 ml of the same buffer, followed by 300 ml of 90 mM-NaCl in the same buffer, and 1,3- α -D-glucan synthase was eluted with a linear gradient of 90 to 150 mM-NaCl in the same buffer (1 l) at a flow rate of 48 ml h⁻¹. The elution was followed by 1 M-NaCl in the same buffer and fractions of 4.1 ml were collected. All the fractions with 1,3- α -D-glucan synthase activity were pooled, concentrated, and the buffer was changed to 5 mM-sodium phosphate (pH 6.5).

(3) *Preparative isoelectric focusing.* This was done by the method of Vesterberg (1971) with some modifications. Briefly, the dense solution contained 60 ml 50% (w/v) glycerol, 2.25 ml Ampholine pH 4–6 and 1.25 g Triton X-100, and the less dense solution contained 60 ml distilled water, 0.75 ml Ampholine pH 4–6 and 1.22 g Triton X-100. Both solutions were pipetted into 24 test tubes and mixed carefully. The enzyme solution (16.5 ml) was mixed with 0.22 ml Ampholine pH 4–6 and 0.35 g Triton X-100 and then used to replace the less dense solution in test tube nos 11 to 17. These fractions were transferred into a column (LKB Ampholine column). The enzyme was focused at 800 V for 6 h, 1500 V for 5 h and 1950 V for 9 h, after which fractions of 1 ml were sucked out. Fractions were analysed by isoelectric focusing on polyacrylamide gel and selected fractions were pooled.

The second preparative isoelectric focusing was done as described above except that Ampholine pH 3.5–5.0 was used and the volume of the enzyme solution was 6.5 ml. Active fractions were also pooled.

Enzyme assay. Enzyme activity was determined as previously reported (Mukasa *et al.*, 1979) with the following modifications. The reaction mixture contained 0.1 M-sodium maleate buffer (pH 6.0), 41.7 mM sucrose, 0.01% (w/v) Merthiolate and 20 to 50 μ l enzyme solution with 0.6 mg dextran T10 in a total volume of 1.75 ml. The mixture was incubated at 37 °C for 4 h and the reaction stopped by heating at 100 °C for 5 min in a boiling water bath.

Reducing sugar-release activity was determined by measuring the release of reducing sugar from sucrose according to the method of Somogyi (1945), using glucose as a standard. One unit of activity was defined as the amount of enzyme releasing 1 μ mol reducing sugar from sucrose min⁻¹ at 37 °C.

Insoluble glucan was separated by centrifugation, and soluble glucan was collected by precipitation in 75% (v/v) ethanol. Glucan was measured by the phenol/sulphuric acid method (Dubois *et al.*, 1956), using glucose as a standard. 1,3- α -D-Glucan synthase activity was calculated by subtracting the added dextran T10 (0.6 mg) from the sum of insoluble and soluble glucans. One unit of 1,3- α -D-glucan synthase was the amount of enzyme catalysing the incorporation of 1 μ mol glucose into glucan from sucrose per min at 37 °C.

In order to measure a rate of glucan synthesis in the presence of dextran T10, 0 to 100 μ g dextran T10 was added to reaction mixtures (1.75 ml) to make up molar ratios of dextran T10 to enzyme of 0:1 to 1000:1.

Analytical isoelectric focusing. A horizontal polyacrylamide slab gel was used for analytical isoelectric focusing as described by Mukasa *et al.* (1982a) except that the gel contained 0.1% (w/v) Triton X-100. After focusing, the gel was incubated for 15 h at 37 °C in 0.1 M-sodium maleate buffer containing 3% (w/v) sucrose and 0.01% (w/v) Merthiolate. 1,3- α -D-Glucan synthase activity was revealed as a white band and other glucosyltransferase activities were detected by staining the glucan with periodic acid–Schiff reagent (Mukasa *et al.*, 1982a). Reducing sugar-release activity was detected in the gel with 2,3,5-triphenyltetrazolium chloride (Gabriel & Wang, 1969) after 2 h incubation in the same reaction mixture. Protein was stained with Coomassie brilliant blue R-250 (Vesterberg *et al.*, 1977) and the pH gradient profile across the gel was determined with a broad pI calibration kit (Pharmacia).

SDS-PAGE. The enzyme solution (0.5 μ g protein) was heated for 2 min in a boiling water bath, cooled to room temperature, and electrophoresis was carried out at 5 mA per tube for 6 h in 5% (w/v) polyacrylamide gel (Weber & Osborn, 1969). Proteins were stained with 0.2% (w/v) Coomassie brilliant blue R-250. High molecular weight protein standard (Bio-Rad) was used for molecular weight determination. For location of 1,3- α -D-glucan synthase, the SDS-gel was incubated with 3% (w/v) sucrose in 0.1 M-sodium phosphate buffer (pH 6.5) containing 1% (w/v) Triton X-100 (Russell, 1979), and the activity was revealed as a white band.

Antiserum and serological procedure. An antiserum against 1,3- α -D-glucan synthase was prepared from a male New Zealand White rabbit according to the method of Mukasa & Slade (1973b) with the modification that nine injections of 0.1 ml each (0.5 μ g protein) of the mixed solution were given during three weeks.

The double immunodiffusion test was as described by Ouchterlony (1958) except that 0.3% (w/v) agarose was used.

Determination of optimum pH and K_m values. The pH optimum was estimated by measuring the enzyme activity as described in the enzyme assay except that a buffer composed of 50 mM-sodium acetate, 50 mM-sodium maleate and 50 mM-sodium phosphate (pH 4.0 to 8.0) was used.

The K_m value for sucrose was estimated according to the method of Eisenthal & Cornish-Bowden (1974) by measuring the activity in 0.1 M-sodium maleate buffer, pH 6.0, containing 1 to 200 mM-sucrose in the presence of 0.25 mg dextran T10 ml⁻¹ at 37 °C for 90 min.

The K_m values for dextran T10 and the highly-branched 1,6- α -D-glucan which was synthesized by the purified enzyme (Tsumori *et al.*, 1983a) were determined by measuring the activity in the reaction mixture containing 0 to 0.5 mg dextran T10 or the synthesized glucan ml⁻¹. The values quoted represent the mean of values from triplicate experiments.

Linkage analysis of glucan. Glucan was synthesized by the purified enzyme (5 μ g) in 10 ml 0.1 M-sodium maleate buffer, pH 6.0, containing 5% (w/v) sucrose, 0.01% (w/v) Merthiolate and various amounts of dextran T10 at 37 °C for 66 h. Insoluble glucans were collected by centrifugation and washed three times with 5 ml distilled water. The 75% (v/v) ethanol precipitates were also collected, washed with 0.1 M-NaCl in 0.02 M-sodium phosphate buffer (pH 6.5) and dialysed against distilled water. The glucans were permethylated (Hakomori, 1964), hydrolysed, reduced and acetylated. The partially methylated glucitol acetates were analysed with a gas-liquid chromatograph (GC-6A, Shimadzu, Kyoto, Japan), equipped with a capillary column (20 m $\times$ 0.25 mm) which was wall-coated with Silicone OV-101. The separated derivatives were identified by GC-MS (auto GCMS 6020, Shimadzu; Shimamura *et al.*, 1982).

Other assays. Protein content was estimated by staining the gel after SDS-PAGE with Coomassie brilliant blue R-250 and scanning with a densitometer (Chromatoscanner CS-910, Shimadzu) using bovine serum albumin as a standard. Fructan was analysed with a gas-liquid chromatograph as described above, except that the permethylated fructan was hydrolysed by methanol and oxalic acid (Ebisu *et al.*, 1975). Protein content was also estimated by the Lowry method with bovine serum albumin as a standard.

Carbohydrate content was estimated as previously reported (Tsumori *et al.*, 1983a) except that the stained gel was scanned with a densitometer using the glycoprotein transferrin as a standard.

Materials. DEAE-Sepharose CL-6B, dextran T10 and pI markers were obtained from Pharmacia. Molecular weight markers and Ampholines were purchased from Bio-Rad and LKB, respectively. All other chemicals were of analytical or reagent grade.

RESULTS

Purification of 1,3- α -D-glucan synthase

1,3- α -D-Glucan synthase of *Streptococcus mutans* HS6 (serotype a) was purified from the culture supernatant, as summarized in Table 1. Fructosyltransferase activity was negligible in the purified enzyme preparation and dextranase activity was not detected. Other enzymes such as highly-branched 1,6- α -D-glucan synthase and 1,6- α -D-glucan synthase were mostly removed by the DEAE-Sepharose chromatography and the fractions which exhibited 1,3- α -D-glucan synthase activity were pooled (Fig. 1). Following two steps of preparative isoelectric focusing, the enzyme was recovered as a single peak of activity. The carbohydrate content of the purified enzyme was 6%.

Properties of 1,3- α -D-glucan synthase

Electrophoretic properties. The purified enzyme appeared as a single band of protein and activity after analytical isoelectric focusing and SDS-PAGE. A white band of 1,3- α -D-glucan synthase activity, which was not stained with periodic acid-Schiff reagent, coincided in position with that of reducing sugar-release activity stained with 2,3,5-triphenyltetrazolium chloride. The pI value of the enzyme was 5.2 and the molecular weight was estimated to be 158 000 by SDS-PAGE.

Table 1. *Purification of 1,3- α -D-glucan synthase from S. mutans HS6 (serotype a)*

Step	Total protein (mg)	Activity* (i.u.)	Specific activity (i.u. mg ⁻¹)	Recovery (%)	Purification (fold)
1. Ultrafiltration	556.4	3038	5.46	100	1
2. DEAE-Sepharose	13.6	116	8.50	3.8	1.6
3. Preparative IEF†					
1st	1.44	64.7	44.6	2.1	8.2
2nd	0.64	30.9	48.3	1.0	8.8

* 1,3- α -D-Glucan synthase activity was determined by measuring insoluble glucan synthesized.

† Preparative isoelectric focusing.

Fig. 1

Fig. 1. DEAE-Sepharose chromatography of crude glucosyltransferase preparation from *S. mutans* HS6 (serotype *a*). The reducing sugar-release activity (●) of each fraction which was eluted with a gradient of NaCl (○) was determined by measuring the rate of release of reducing sugar from sucrose. The horizontal bar represents the fractions pooled.

Fig. 2

Fig. 2. Immunodiffusion of the various glucosyltransferase preparations against antiserum to 1,3- α -D-glucan synthase from strain HS6 (serotype *a*) (centre well; 20 μ l). 1, Purified 1,3- α -D-glucan synthase from *S. mutans* HS6 (serotype *a*) (0.2 μ g protein); 2, partially purified 1,3- α -D-glucan synthase from strain 6715 (serotype *g*) (0.6 μ g); 3, crude enzyme preparation from strain B13 (serotype *d*) (180 μ g); 4, crude enzyme preparation from strain Ingbritt (serotype *c*) (270 μ g); 5, purified highly-branched 1,6- α -D-glucan synthase from strain HS6 (serotype *a*) (13.3 μ g).

Immunological analysis. The 1,3- α -D-glucan synthase was partially identical with the 1,3- α -D-glucan synthase from *S. mutans* 6715 (serotype *g*) and with the enzyme from strain B13 (serotype *d*) which formed a line of identity with the enzyme from strain 6715 (Fig. 2). A highly-branched 1,6- α -D-glucan synthase from strain HS6 (Tsumori *et al.*, 1983*a*), and an enzyme preparation from *S. mutans* Ingbritt (serotype *c*) did not react with the anti-1,3- α -D-glucan synthase serum (Fig. 2).

Kinetic studies. The enzyme activity was optimal at pH 6.0. A small amount of glucan (68% water-insoluble and 32% ethanol-insoluble) was synthesized with a large amount of reducing sugar in the absence of dextran T10. In the presence of dextran T10, the glucan synthesizing activity was stimulated about 20-fold, although the synthesis was still not comparable to the release of reducing sugar (Fig. 3). More than 96% of the glucan was water-insoluble after 12 h incubation with dextran T10. For a marked stimulation of the enzyme activity, more than a 300-fold concentration of dextran T10 in molar ratio to the enzyme was required (Fig. 4). The K_m value for sucrose was estimated to be 1.2 ± 0.5 (SE) mM at pH 6.0 in the presence of 0.25 mg dextran T10 ml⁻¹, and the K_m value for dextran T10 was 0.15 ± 0.04 (SE) mM-glucose equivalent. The glucan synthesized by a highly-branched 1,6- α -D-glucan synthase (Tsumori *et al.*, 1983*a*) also stimulated the enzyme activity in a similar way to dextran T10.

Linkage analysis. The acetate derivatives of 2,3,4,6-tetra-, 2,4,6-tri- and 2,3,4-tri-*O*-methyl-D-glucitol were obtained in the molar ratio 1:496:3 with a trace amount of 2,4-di-*O*-methyl-D-glucitol from the water-insoluble glucan synthesized in the absence of dextran T10. This indicated that the insoluble glucan was a high molecular weight linear 1,3- α -D-glucan containing less than 1 mol% of 1,6- α -linked glucose and 1,3,6- α -linked glucose residues. Glucans synthesized in the presence of various amounts of dextran T10 were also analysed. When the molar ratio of dextran T10 to the enzyme was less than 10 (3 μ g dextran T10 ml⁻¹), the structure of the glucan synthesized was similar to that of the glucan without dextran T10. At the molar ratio of 100 (30 μ g dextran T10 ml⁻¹), the glucan consisted of about 95 mol% of 1,3- α -linked glucose residue and 4.1 mol% of 1,6- α -linked glucose residue which almost corresponded to that of the exogenous dextran T10 (3.8 mol%).

Fig. 3

Fig. 3. Time course of 1,3- α -D-glucan synthesis in the absence (a) and presence (b) of dextran T10. Enzyme activity was determined by measuring the rate of release of reducing sugar (○) and synthesis of glucan (●).

Fig. 4

Fig. 4. Rate of glucan synthesis in the presence of dextran T10. 1,3- α -D-Glucan synthase activity was determined by measuring synthesis of glucan in the presence of various molar ratios of dextran T10 to enzyme.

DISCUSSION

The extracellular 1,3- α -D-glucan synthase was purified from *S. mutans* HS6 (serotype a) culture supernatant by DEAE-Sephadex chromatography and preparative isoelectric focusing. The molecular weight (158000) of the purified enzyme in the presence of SDS was lower than that of the 1,3- α -D-glucan synthase from *S. mutans* 6715 (serotype g) (180000; Fukui *et al.*, 1982) and its isoelectric point (pH 5.2) was clearly different from that of the strain 6715 enzyme [pH 4.3 (Ciardi, 1976) and 4.9 (Shimamura *et al.*, 1983)], although the K_m value for sucrose (1.2 mM) was similar to that of the enzyme from strain 6715 (Fukui *et al.*, 1982; Schachtele & Harlander, 1983). While the enzyme from strain HS6 showed an optimum for activity at pH 6.0, the enzyme from strain 6715 has been reported to show two pH optima (Fukui *et al.*, 1982; Schachtele & Harlander, 1983). In the presence of dextran T10, glucan synthesis by the strain 6715 enzyme was comparable to the release of reducing sugar (Fukui *et al.*, 1982), while glucan synthesis by the strain HS6 enzyme was considerably lower than the release of reducing sugars (Fig. 3). These enzymes were immunologically partially identical with each other (Fig. 2; Fukui *et al.*, 1983). Such differences between the enzymes from strains HS6 and 6715 may be due to their serotype specificities.

The purified enzyme synthesized a small amount of glucan with a large amount of reducing sugar in the absence of dextran T10 (Fig. 3), while both activities always appeared in the same position of the focused gel. More than 1 mM-Hg²⁺ simultaneously inhibited reducing sugar release and glucan synthesizing activities (data not shown), as reported by Mukasa *et al.* (1979). These indicated that the 1,3- α -D-glucan synthase also exhibited invertase activity especially in the absence of dextran T10. Exogenous dextran T10 not only stimulated the formation of longer, more insoluble glucans but also enhanced the glucan synthesizing activity of the enzyme relative to its invertase activity.

Dextran T10 and the branched glucan synthesized by the purified enzyme (Tsumori *et al.*, 1983a) both stimulated 1,3- α -D-glucan synthase activity. These observations indicate that the side chains attached by 1,3- α -glucosidic branch linkages do not necessarily stimulate 1,3- α -D-glucan synthase activity, and that a sequence of 1,6- α -linked glucose residues may be required for the stimulation of the enzyme activity (Hare *et al.*, 1978).

A 1,3- α -D-glucan synthase partially purified from *S. mutans* OMZ176 (serotype d) synthesized a 1,3- α -D-glucan containing 1,6- α -linked and 1,3,6- α -linked glucose residues (7 and 1 mol%,

respectively, by methylation analysis; Walker & Hare, 1977; Hare *et al.*, 1978). In the presence of an exogenous dextran, the purified enzyme from *S. mutans* 6715 (serotype g) synthesized a branched glucan consisting of 1,3- α -linked and 1,6- α -linked glucose residues (93 and 7 mol%, respectively; Fukui *et al.*, 1982) and a partially purified enzyme from strain 6715 also synthesized a branched glucan (Robyt & Martin, 1983). The structures were deduced by either NMR spectroscopy and controlled Smith degradation (Fukui *et al.*, 1982), or dextranase treatment (Robyt & Martin, 1983). Nevertheless, the strain HS6 (*a*) enzyme in the present study solely catalysed the formation of 1,3- α -glucosidic linkages regardless of the presence of exogenous dextran. The difference among these glucan structures may be due to serotype specificities of the enzymes. The enzyme purified in this study is a 1,3- α -D-glucan synthase (sucrose:1,3- α -D-glucan 3- α -D-glucosyltransferase, EC 2.4.1.-).

The glucosyltransferases of *S. mutans* are bound to the cell surface and synthesize insoluble glucan, resulting in the adherence of *S. mutans* to smooth surfaces (Gibbons & Nygaard, 1968; Mukasa & Slade, 1973*b*, 1974*a*). These enzymes exist as a high molecular weight complex (400000 to 2000000) and it has been suggested that they cooperate in the synthesis of the adhesive insoluble glucan (Mukasa & Slade, 1974*b*; Ciardi *et al.*, 1977; Mukasa *et al.*, 1979). Interestingly, we have recently found that *S. mutans* HS6 (serotype *a*) secretes three kinds of glucosyltransferase (Tsumori *et al.*, 1983*b*), as does *S. mutans* 6715 (serotype g) (Shimamura *et al.*, 1983). After purification of each enzyme, details of studies of cooperative interactions among these enzymes will be reported elsewhere.

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