Purification and Properties of Extracellular Glucosyltransferase Synthesizing 1,6-, 1,3- α -D-Glucan from *Streptococcus mutans* Serotype *a*

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An extracellular glucosyltransferase (sucrose:1,6-, 1,3- α -D-glucan 3- α - and 6- α -D-glucosyltransferase, EC 2.4.1.-) of *Streptococcus mutans* HS6 (serotype *a*) was purified from culture supernatant by DEAE-Sepharose chromatography and preparative isoelectric focusing. The molecular weight measured by SDS-PAGE was 159000 and the isoelectric point was pH 4.9. The specific activity was 89.7 i.u. (mg protein)⁻¹ and the optimum pH was 6.0. The K_m value for sucrose was 4.9 mM and the enzyme activity was not stimulated by exogenous dextran T10. Glucan was synthesized *de novo* from sucrose by the purified enzyme and consisted of 49.1 mol% 1,6- α -linked glucose and 33.9 mol% 1,3- α -linked glucose, with 13.6 mol% terminal glucose and 3.3 mol% 1,3,6- α -branched glucose.

INTRODUCTION

Serotype *a*, *d* and *g* strains of the cariogenic micro-organism *Streptococcus mutans* secrete three kinds of glucosyltransferase; strains of serotypes *c*, *e* and *f* secrete basic glucosyltransferases and acidic fructosyltransferases (Carlsson, 1970; Ciardi *et al.*, 1976; Shimamura *et al.*, 1982; Mukasa *et al.*, 1982*b*; Tsumori *et al.*, 1983*b*). Serotype *b* strains also produce both glucosyl- and fructosyltransferases (Scales *et al.*, 1975; Tsumori *et al.*, 1983*b*). These enzymes exist as high molecular weight complexes (400000 to 2000000) and synthesize adhesive water-insoluble polysaccharide from sucrose by a cooperative action (Mukasa & Slade, 1974*b*; Ciardi *et al.*, 1977; Mukasa *et al.*, 1979). Highly-branched 1,6- α -D-glucan synthases have been purified from serotype *a*, *c* and *g* strains in our laboratory (Shimamura *et al.*, 1982; Mukasa *et al.*, 1982*b*; Tsumori *et al.*, 1983*a*); in addition, 1,3- α -D-glucan synthases have been purified from serotype *a*, *d* and *g* strains (Fukushima *et al.*, 1981; Fukui *et al.*, 1982; Koga *et al.*, 1983; Tsumori *et al.*, 1985). However, the third glucosyltransferase has yet to be isolated, purified and characterized from serotype *a*, *d* and *g* strains: hence the mechanism of synthesis of the adhesive insoluble glucan is not yet known. A glucosyltransferase has been partially purified from strain OMZ176 (serotype *d*) (Koga *et al.*, 1983) which might correspond to the third enzyme, although its properties were not adequately characterized. It seemed essential, therefore, to purify and characterize the third glucosyltransferase in order to establish the mechanisms of adhesive insoluble glucan synthesis by the three kinds of glucosyltransferases and so advance studies in the prevention of dental caries. In this study, we have purified this glucosyltransferase from *S. mutans* HS6 (serotype *a*) and shown it to be a bifunctional enzyme which synthesizes water-soluble 1,6-, 1,3- α -D-glucan.

METHODS

Organism and culture conditions. *S. mutans* HS6 (serotype *a*) was used throughout the present study (Tsumori *et al.*, 1983*a*). The batch culture method used was that reported previously (Tsumori *et al.*, 1985). The OD₅₅₀ of the culture at harvest was 6.1 and the cell-free culture supernatant was obtained by centrifugation for 30 min at 8900 *g*.

Purification of 1,6-, 1,3- α -D-glucan synthase. All steps were done at 0 to 5 °C unless otherwise indicated.

(1) *Ultrafiltration*. The culture supernatant (5 l) was concentrated to 130 ml and equilibrated with 30 mM-NaCl in 10 mM-Tris/HCl buffer (pH 7.4) as reported by Tsumori *et al.* (1985).

(2) *DEAE-Sepharose chromatography*. The concentrated enzyme solution was applied to a DEAE-Sepharose column (1.8 × 35 cm) previously equilibrated with 30 mM-NaCl in 10 mM-Tris/HCl buffer (pH 7.4). The column was washed with 100 ml of the same buffer containing 30 mM-NaCl. The enzyme was then eluted with 300 ml 90 mM-NaCl in the buffer at a flow rate of 48 ml h⁻¹ (Tsumori *et al.*, 1985). The eluate was concentrated to 24 ml and the buffer was changed to 5 mM-sodium phosphate (pH 6.5) by ultrafiltration (Mukasa *et al.*, 1982b).

(3) *Preparative isoelectric focusing*. This was done using a 110 ml LKB Ampholine electrofocusing column. The density gradient was formed manually (Vesterberg, 1971) with glycerol, and Ampholine pH 4–6, Ampholine pH 3.5–10 and Triton X-100 were used at concentrations of 0.98, 0.02 and 0.5%, respectively. The enzyme (24 ml) was focused at 800 V for 8 h, 1300 V for 2 h, 1500 V for 2 h and 1950 V for 12 h. Samples (1 ml) were sucked out and tested for activity, and active fractions were pooled.

The second preparative isoelectric focusing was done as described above except that 0.95% Ampholine pH 5–7, 0.05% Ampholine pH 3.5–5 and 2% Triton X-100 were used, and the volume of the enzyme solution was 3 ml.

Enzyme assay. Enzyme activity was routinely measured by the method of Mukasa *et al.* (1979) with the following modifications. The reaction mixture in a final volume of 1.75 ml contained 0.1 M-sodium phosphate buffer (pH 6.5), 41.7 mM-sucrose, 0.01% Merthiolate and 0.5 to 50 µl enzyme solution with or without 0.6 mg dextran T10. The reaction was done at 37 °C for 2 to 6 h and terminated by heating at 100 °C for 5 min. After insoluble materials had been removed, the reducing sugar released was measured by the method of Somogyi (1945), using glucose as a standard. One unit of activity (i.u.) was the amount of enzyme releasing 1 µmol reducing sugar from sucrose per min at 37 °C.

Soluble glucan was collected as a 75% (v/v) ethanol precipitate and measured by the phenol/sulphuric acid method (Dubois *et al.*, 1956), using glucose as a standard. One unit (i.u.) of 1,6-, 1,3- α -D-glucan synthase activity was defined as the amount of enzyme catalysing the incorporation into glucan from sucrose of 1 µmol glucose min⁻¹ at 37 °C.

Analytical isoelectric focusing. This was done as reported by Mukasa *et al.* (1982a) except that the gel contained 0.1% Triton X-100. After focusing, 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 (pH 3–10, Pharmacia). 1,6-, 1,3- α -D-Glucan synthase activity was detected by staining the gel with periodic acid–Schiff reagent after incubation of the focused gel for 15 h at 37 °C in 0.1 M-sodium phosphate buffer (pH 6.5) containing 3% (w/v) sucrose and 0.01% Merthiolate (Mukasa *et al.*, 1982a). After 2 h incubation of the gel in the same reaction mixture, reducing sugar released was detected with 2,3,5-triphenyltetrazolium chloride (Gabriel & Wang, 1969).

SDS-PAGE. The enzyme solution (4 µg protein) was subjected to SDS-PAGE according to Weber & Osborn (1969) using 5% (w/v) gel at 5 mA per tube constant current for 6.5 h. Gels were stained for protein with Coomassie brilliant blue R-250 and calibrated with Bio-Rad high molecular weight protein standards. The activity was detected with periodic acid–Schiff reagent after incubation of the gel in 0.1 M-sodium phosphate buffer (pH 6.5) containing 3% (w/v) sucrose and 1% Triton X-100 (Russell, 1979) and 0.01% Merthiolate.

Antisera and serological procedure. Antisera against 1,6-, 1,3- α -D-glucan synthase and 1,3- α -D-glucan synthase were produced in New Zealand White rabbits as previously reported (Tsumori *et al.*, 1985). An antiserum against highly-branched 1,6- α -D-glucan synthase was prepared according to Mukasa & Slade (1973a). Double immunodiffusion tests were done according to Ouchterlony (1958) except that 0.3% agarose was used.

Determination of pH optimum and K_m . The pH optimum was estimated by measuring the enzyme activity in 0.1 M-sodium acetate (pH 4.0 to 5.5), 0.1 M-sodium maleate (pH 5.0 to 7.0) and 0.1 M-sodium phosphate (pH 6.0 to 7.5).

The K_m for sucrose was estimated by measuring the activity in 0.1 M-sodium maleate buffer (pH 6.0) containing 1 to 100 mM-sucrose at 37 °C for 90 min according to the method of Eienthal & Cornish-Bowden (1974).

Linkage analysis of glucan. Glucan was synthesized by the purified enzyme (10 µg protein) in 10 ml 0.1 M-sodium maleate buffer (pH 6.0) containing 5% (w/v) sucrose and 0.01% Merthiolate for 71 h; it was collected as a 75% (v/v) ethanol precipitate and washed three times. The glucan (1 to 12 mg) was permethylated (Hakomori, 1964), hydrolysed, reduced and acetylated. The partially methylated glucitol acetate was analysed on a gas-liquid chromatograph (GC-6A, Shimadzu, Kyoto, Japan) on a WCOT column of Silicone OV 101 (20 m × 0.25 mm). The separated derivatives were identified by an auto GC-MS 6020 (Shimadzu; Shimamura *et al.*, 1982).

Other assays. Protein and carbohydrate contents were determined with a densitometer (Chromatoscanner CS-910, Shimadzu) as previously reported (Tsumori *et al.*, 1985). Protein content was also determined by the Lowry method with bovine serum albumin as a standard.

Fructan was analysed as described above, except that the permethylated fructan was hydrolysed according to Ebisu *et al.* (1975).

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

Fig. 1. Second preparative isoelectric focusing of the DEAE-Sepharose fraction from *S. mutans* HS6 (serotype *a*). ●, Enzyme activity, estimated by measuring the rate of reducing sugar-release from sucrose; ○, pH. The horizontal bar indicates the fractions pooled.

Table 1. Purification of 1,6-, 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	2069	3.72	100	1
2. DEAE-Sepharose	20.8	662	31.8	32	8.5
3. Preparative IEF†					
1st	2.4	205	85.4	9.9	23.0
2nd	1.0	89.7	89.7	4.3	24.1

* 1,6-, 1,3- α -D-glucan synthase activity was determined by measuring glucan synthesized.

† Preparative isoelectric focusing.

RESULTS

Purification of 1,6-, 1,3- α -D-glucan synthase

1,6-, 1,3- α -D-Glucan synthase of *S. mutans* HS6 (serotype *a*) was purified from the culture supernatant by DEAE-Sepharose chromatography and preparative isoelectric focusing as summarized in Table 1. 1,6-, 1,3- α -D-Glucan synthase was eluted from the DEAE-Sepharose column with 90 mM-NaCl in 10 mM-Tris/HCl buffer (pH 7.4), whereas other enzymes such as 1,3- α -D-glucan synthase and highly-branched 1,6- α -D-glucan synthase were not eluted under the same conditions (Tsumori *et al.*, 1985). The enzyme was further purified by two steps of preparative isoelectric focusing, and fractionated as a single active peak (Fig. 1). Fructosyltransferase and dextranase activities were not detected. The carbohydrate content of the purified enzyme was 1.5%.

Properties of 1,6-, 1,3- α -D-glucan synthase

Electrophoretic properties. The purified enzyme moved as a single band of protein and activity in SDS-PAGE and analytical isoelectric focusing (Fig. 2). The enzyme activity stained with periodic acid-Schiff reagent coincided in position with the release of reducing sugar stained with 2,3,5-triphenyltetrazolium chloride. The enzyme had an isoelectric point of 4.9 and a molecular weight of 159000 (Fig. 2).

Immunological analysis. 1,6-, 1,3- α -D-Glucan synthase, highly-branched 1,6- α -D-glucan synthase and 1,3- α -D-glucan synthase purified from strain HS6 (serotype *a*) formed precipitin lines against the corresponding antisera, but these enzymes were immunologically unrelated to each other (Fig. 3*a*). The 1,6-, 1,3- α -D-glucan synthase of strain HS6 was partially identical with the corresponding enzyme purified from strain 6715 (serotype *g*) and with the enzyme in the crude enzyme preparation of strain B13 (serotype *d*) (Fig. 3*b*).

Kinetic studies. The pH optimum was 6.0, and the K_m value for sucrose was 4.9 ± 1.7 (SE) mM at pH 6.0. The enzyme activity was not stimulated by exogenous dextran T10 in the range 0.01 to 2.0 mg ml⁻¹.

Fig. 2. Electrophoretic analyses of the purified glucosyltransferase from *S. mutans* HS6 (serotype *a*). (a) Analytical isoelectric focusing. The pI markers (1 to 2.5 µg protein; lane 1) and the purified enzyme (0.4 µg protein; lane 2) were stained with Coomassie brilliant blue R-250; the enzyme activity was stained with periodic acid-Schiff reagent (lane 3) and 2,3,5-triphenyltetrazolium chloride (lane 4) after the incubation of the gel in 3% (w/v) sucrose solution. (b) SDS-PAGE. The molecular weight markers (1 µg protein; lane 1) and the purified enzyme (4 µg protein; lane 2) were stained with Coomassie brilliant blue R-250; the enzyme activity was stained with periodic acid-Schiff reagent (lane 3).

Fig. 3. (a) Immunodiffusion of three kinds of glucosyltransferase against a mixture of the antisera to the corresponding enzymes (1,6-, 1,3- α -D-glucan synthase/highly-branched 1,6- α -D-glucan synthase/1,3- α -D-glucan synthase, 1.4:1:3.3, by vol.; centre well, 20 µl). 1, 1,6-, 1,3- α -D-glucan synthase (4 µg); 2, 1,3- α -D-glucan synthase (0.2 µg); 3, highly-branched 1,6- α -D-glucan synthase (6 µg). (b) Immunodiffusion of enzyme preparations obtained from different serotypes against antiserum to 1,6-, 1,3- α -D-glucan synthase (centre well, 8 µl). 1, 1,6-, 1,3- α -D-glucan synthase from strain HS6 (serotype *a*) (2 µg); 2, 1,6-, 1,3- α -D-glucan synthase from strain 6715 (serotype *g*) (1.4 µg); 3, crude enzyme preparation from strain B13 (serotype *d*) (330 µg).

Product analysis. The reaction mixture became viscous and somewhat opalescent during glucan synthesis and the synthesized product would not redissolve in water after precipitation with 75% (v/v) ethanol.

Linkage analysis of the glucan gave the acetate derivatives of 2,3,4,6-tetra-, 2,3,4-tri-, 2,4,6-tri- and 2,4-di-*O*-methyl-D-glucitol in the molar ratio 1.0:3.6:2.5:0.2. This indicated that the

Table 2. *Characteristics of the three kinds of glucosyltransferase purified from S. mutans HS6 (serotype a)*

D-Glucan synthase*	10 ⁻³ × Mol. wt	pI	K _m (mM)	pH optimum	Structure of glucan α -linked glucose (mol%)				Primer dependency
					Terminal	1,6-	1,3-	1,3,6-	
1,6-, 1,3- α - ^a	159	4.9	4.9	6.0	13.6	49.1	33.9	3.3	No
1,3- α - ^b	158	5.2	1.2	6.0	0.2	0.6	99.2	trace	Yes
Highly branched	I 161	4.5	1.3	5.5	18.6	64.7	0	16.7	Yes
1,6- α - ^c	II 174	4.2			24.5	55.8	0	19.7	

* References: a, this paper; b, Tsumori *et al.* (1985); c, Tsumori *et al.* (1983a).

glucan was composed of 1,6- and 1,3- α -linked glucose with some 1,3,6- α -branch points and was essentially different in structure from highly-branched 1,6- α -D-glucan, 1,3- α -D-glucan and dextran T10 (1,6- α -D-glucan).

DISCUSSION

1,6-, 1,3- α -D-Glucan synthase was purified from the culture supernatant of *S. mutans* HS6 (serotype *a*) by DEAE-Sepharose chromatography and preparative isoelectric focusing. Although the enzyme had previously been expected to be 1,6- α -D-glucan synthase (Shimamura *et al.*, 1983; Tsumori *et al.*, 1983*b*), the purified enzyme synthesized a soluble glucan consisting of 1,6- α -linked glucose (49.1 mol%) with a high quantity of 1,3- α -linked glucose (33.9 mol%). Therefore, the final enzyme preparation obtained in this study was suspected of containing a significant amount of 1,3- α -D-glucan synthase, and an attempt to separate the supposed 1,3- α -D-glucan synthase from this preparation was done by SDS-PAGE with a 50-cm-long polyacrylamide gel (data not shown). After the enzyme or enzymes had migrated about 45 cm, the gel was cut into 2 mm slices, extracted, and the extracts used to synthesize glucans from sucrose. The linkage analyses showed that the structures of the glucans formed were similar to that of the glucan synthesized by the purified enzyme (data not shown), indicating that the 1,3- and the 1,6- α -bond-forming activities of the final enzyme preparation were inseparable electrophoretically.

The three kinds of glucosyltransferase purified from strain HS6 were immunologically unrelated to each other (Fig. 3*a*). Considering this observation, the enzyme purified in the present study is most probably a bifunctional enzyme with 1,6- and 1,3- α -bond-forming activities (sucrose:1,6-, 1,3- α -D-glucan 3- α - and 6- α -D-glucosyltransferase).

Côté & Robyt (1982) have purified an alternansucrase from *Leuconostoc mesenteroides* NRRL B-1355 culture supernatant which synthesized an alternating 1,6-, 1,3- α -D-glucan. The alternan dissolved readily in water and was not hydrolysed by dextranase or 1,3- α -glucanase. In contrast, the glucan synthesized in the present study failed to redissolve in water after precipitation by ethanol (75%, v/v) and was hydrolysed up to 70% by dextranase (data not shown). These data indicated that the enzyme purified from strain HS6 was distinct from the alternansucrase of *L. mesenteroides*.

The HS6-enzyme was not stimulated by exogenous dextran T10 and the glucan synthesized was composed of 1,6- and 1,3- α -linked glucose. These observations are compatible with the known properties of the third glucosyltransferase from strain 6715 (serotype *g*) described by Shimamura *et al.* (1983), and those of the partially purified glucosyltransferase from strain OMZ176 (serotype *d*) which synthesized soluble glucan (Koga *et al.*, 1983). Although the purified enzyme from strain HS6 (serotype *a*) showed partial immunological identity with the corresponding enzymes from strains B13 (serotype *d*) and 6715 (serotype *g*) (Fig. 3*b*), its physical characteristics such as molecular weight and isoelectric point were significantly different from those reported by Shimamura *et al.* (1983) and Koga *et al.* (1983).

The highly branched 1,6- α -D-glucan synthase and 1,3- α -D-glucan synthase from strain HS6 are partially identical immunologically to the corresponding enzymes from serotypes *d* and *g* previously reported by Fukui *et al.* (1974; 1983) and Tsumori *et al.* (1983*a*; 1985). The 1,6-, 1,3- α -D-glucan synthases from serotype *a*, *d* and *g* strains were also partially identical with each other (Fig. 3*b*). These observations are compatible with the partial identity among cell wall polysaccharide antigens purified from *S. mutans* serotype *a* and *d* (*g*) strains (Mukasa & Slade, 1973*a*; Linzer & Slade, 1974). On the other hand, the three kinds of glucosyltransferase secreted from one strain are immunologically unrelated (Fig. 3*a*; Shimamura *et al.*, 1983). Properties of the three enzymes purified from *S. mutans* HS6 (serotype *a*) are summarized in Table 2. It would be interesting to know the amino acid sequences in order to clarify the differences among these enzymes.

Highly-branched 1,6- α -D-glucan synthase and 1,3- α -D-glucan synthase are stimulated by exogenous dextran T10 (Shimamura *et al.*, 1982; Fukui *et al.*, 1982), whereas 1,6-, 1,3- α -D-glucan synthase synthesized glucan regardless of the presence of dextran T10. Therefore, 1,6-, 1,3- α -D-glucan synthase may initially synthesize the soluble glucan which would successively stimulate the other two glucosyltransferase activities, resulting in the formation of adhesive insoluble glucan by the cooperative action of the three kinds of glucosyltransferases (Mukasa & Slade, 1974*b*; Ciardi *et al.*, 1977; Mukasa *et al.*, 1979), and in the adherence of *S. mutans* cells to smooth surfaces (Gibbons & Nygaard, 1968; Mukasa & Slade, 1973*b*, 1974*a*).

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