Purification and Properties of Extracellular Glucosyltransferases from *Streptococcus mutans* Serotype *a*

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Extracellular glucosyltransferases (sucrose: 1,6- α -D-glucan 3- α - and 6- α -glucosyltransferase) of *Streptococcus mutans* HS6 (serotype *a*) were purified from the culture supernatant by DEAE-Sephadex chromatography, ConA-Sephadex chromatography and chromatofocusing. The enzymes I and II with specific activities of 6.20 and 5.86 i.u. mg⁻¹, respectively, exhibited slightly different isoelectric points (pI 4.5 and 4.2) and the molecular weights were estimated to be 161 000 and 174 000, respectively, by SDS-PAGE. The enzymes had the same optimum pH of 5.5 and the same K_m values of 1.3 mM for sucrose and of 83 μ M-glucose equivalent for dextran T10. By double immunodiffusion test on agar, these enzymes were immunologically identical to each other. Analysis by GLC of the glucans synthesized *de novo* from sucrose by the enzymes (I and II) established that they were 1,6- α -D-glucans with 20 and 24.5 mol% 1,3,6-branch points, respectively. Both are therefore bifunctional enzymes.

INTRODUCTION

Streptococcus mutans is believed to play an important role in dental plaque formation on smooth tooth surfaces (Gibbons & Nygaard, 1968; Mukasa & Slade, 1973*b*). The cariogenicity of *S. mutans* is due to its ability to secrete the glucosyltransferases which convert sucrose to water-insoluble polysaccharide. The polysaccharide is formed by the cooperative action of a synthetase which forms a soluble glucan, a 1,3- α -D-glucan synthetase, a fructosyltransferase and polysaccharides (Carlsson, 1970; Mukasa & Slade, 1974; Schachtele *et al.*, 1975; Ciardi *et al.*, 1976).

We have previously purified highly-branched 1,6- α -D-glucan synthetase (pI 4.1) from *S. mutans* 6715 (serotype *g*) (Shimamura *et al.*, 1982) and a basic glucosyltransferase (pI 8.5) from strain Ingbritt (serotype *c*) (Mukasa *et al.*, 1982*b*). Both enzymes exhibited high 1,3,6- α -branch-forming activity (35 and 18 mol%) as well as 1,6- α -bond-forming activity, although they were immunologically unrelated. In contrast, one of the glucosyltransferases from *S. mutans* HS6 (serotype *a*) has been reported to be a dextranucrase which forms a glucan in which 94 mol% of the glucosyl residues are linked by 1,6- α -bonds (Fukui *et al.*, 1974*a*). Since the enzyme from serotype *a* strains is immunologically partially identical to the enzyme from serotype *g* strains (Fukui *et al.*, 1974*b*; Smith & Taubman, 1977), a highly-branched 1,6- α -D-glucan synthetase could also be expected to be excreted from serotype *a* strains.

In this study, we isolated two glucosyltransferases from *S. mutans* HS6 (serotype *a*) and found that the two enzymes are isozymes with slightly different molecular size and charge and that they are bifunctional enzymes with 1,6- α -bond- and 1,3,6- α -branch-forming activities.

METHODS

Strains and culture conditions. *Streptococcus mutans* HS6 (serotype *a*) was kindly provided by Dr H. D. Slade, Department of Oral Biology, School of Dentistry, University of Colorado Health Science Center, Denver, Colo.,

U.S.A. Cells were grown for 15 h at 37 °C in 12 l of the minimal defined medium developed by Fujiwara *et al.* (1978), supplemented with 0.025% (w/v) Tween 80. The cell-free culture supernatant was obtained by continuous centrifugation, as previously reported (Mukasa *et al.*, 1982b).

Purification of glucosyltransferase. The enzyme was purified at room temperature, approximately 20 °C, unless otherwise specified.

(1) *Ultrafiltration.* A mini-module equipped with four holotubes (1.2 m × 0.8 mm each, molecular weight limit 13000; Asahi Chemical Industry, Tokyo) was used under reduced pressure to concentrate 12 l of culture supernatant to 275 ml (Mukasa *et al.*, 1982b).

(2) *DEAE-Sepharose column chromatography.* After changing the buffer to 10 mM-sodium phosphate buffer (pH 6.8) using the mini-module, the concentrated enzyme solution was applied to a DEAE-Sepharose column (1.8 × 16.5 cm) equilibrated with the same buffer. The column was washed with 10 mM-sodium phosphate buffer containing 0.1 M-NaCl. The enzyme was eluted with a linear gradient of 0.1 to 0.3 M-NaCl in the same buffer (1 l) at a flow rate of 96 ml h⁻¹ and fractions of 5.8 ml were collected. The active fractions were pooled and concentrated to 10 ml at 0 °C.

(3) *Con A-Sepharose column chromatography.* After changing the buffer to 10 mM-sodium acetate (pH 5.0) containing 1 mM-CaCl₂, 1 mM-MgCl₂ and 1 mM-MnCl₂ at 0 °C as described above, the sample was absorbed on to a Con A-Sepharose column (1.8 × 31 cm) equilibrated with the same buffer and held in the column for 12 h at 4 °C (Shimamura *et al.*, 1982). The column was washed with 100 ml of the same buffer and the enzyme eluted with a linear gradient of 0 to 0.1 M-NaCl in 1 l of buffer at a flow rate of 40 ml h⁻¹; 5.5 ml fractions were collected. The elution was continued with 100 ml of 0.1 M-NaCl in the buffer, followed by 100 ml 1 M-NaCl in the buffer and finally by 100 ml 1 M-methyl- α -D-glucopyranoside in the buffer containing 1 M-NaCl. The active fractions were pooled and concentrated to 30 ml.

(4) *Chromatofocusing.* Chromatofocusing was done according to the Pharmacia instruction manual: 'Chromatofocusing with Polybuffer and Polybuffer Exchangers.' A Polybuffer exchangers 94 column (0.7 × 4.8 cm) was equilibrated with the starting buffer, 25 mM-piperazine/HCl (pH 5.5) containing 20 mM-NaCl. Portions (2 ml) of the enzyme solution in the starting buffer were applied to the column, and eluted with Polybuffer 74-HCl (pH 4.0) containing 20 mM-NaCl, at a flow rate of 25 ml h⁻¹. Immediately after the elution, the pH of the fractions was adjusted to 6 to 6.5 by adding 50 μ l of 1 M-sodium phosphate buffer, pH 6.5. The active fractions were collected and concentrated to 20 ml.

Enzyme assay. Glucosyltransferase activity was measured as previously reported (Mukasa *et al.*, 1979) with the following modifications. The reaction mixture contained 0.1 M-sodium phosphate buffer, pH 6.5, 41.7 mM-sucrose, 0.6 mg dextran T10, 0.01% (w/v) Merthiolate, 0.03% (w/v) Triton X-100 and 5 to 50 μ l enzyme solution in a total volume of 1.75 ml. The reaction was carried out at 37 °C for 15 h and stopped by heating at 100 °C for 5 min in a boiling-water bath. Total glucosyltransferase activity was determined by measuring the release of reducing sugar from sucrose as reported by Somogyi (1945), using glucose as a standard. One unit of total activity was defined as the amount of enzyme releasing 1 μ mol reducing sugar from sucrose per min at 37 °C.

Insoluble material in the reaction mixture was removed by centrifugation. Soluble glucan was then collected as a 75% (v/v) ethanol precipitate and its concentration determined by the phenol-sulphuric acid method (Dubois *et al.*, 1956), using glucose as a standard. One unit was the amount of enzyme catalysing the incorporation of 1 μ mol glucose from sucrose per min at 37 °C.

Analytical isoelectric focusing. A horizontal slab gel was used for analytical isoelectric focusing of the enzyme preparation as previously reported (Mukasa *et al.*, 1982a) except that the gel contained 0.1% (w/v) Triton X-100 to dissociate enzyme aggregates (Figures & Edwards, 1979). Glass-fibre filter paper pieces were put on the gel surface and 0.65 μ g of the enzyme was applied. Proteins were focused for 1 h at 18 °C and 100 V and subsequently for 3 h at 300 V. After focusing, the pH on the gel was measured directly with a surface electrode (SE1600 GC, Fuji Kagaku Keisoku, Tokyo, Japan) connected to a pH meter, and proteins were also stained with Coomassie brilliant blue R-250 (Mukasa *et al.*, 1982a). Glucosyltransferase activity was stained by the periodic acid-Schiff base method (Kapitany & Zebrowski, 1973) after the gel had been incubated for 15 h at 37 °C in 0.1 M-sodium phosphate buffer, pH 6.5, containing 1.5% (w/v) sucrose and 0.01% (w/v) Merthiolate.

SDS-PAGE. For SDS-PAGE by the method of Weber & Osborn (1969), 6.5 μ g of enzyme in 10 mM-sodium phosphate buffer (pH 7.2) containing 1% SDS, 5% (w/v) 2-mercaptoethanol, 25% (w/v) glycerol and 0.005% (w/v) Bromophenol blue was heated at 100 °C for 2 min. After electrophoresis at 5 mA per tube in 5% (w/v) polyacrylamide gels, protein in the gel was stained with Coomassie brilliant blue R-250 (Weber & Osborn, 1969). RNA polymerase B (5 μ g) was used as molecular weight standard. The glucosyltransferase activity was detected by the periodic acid-Schiff base method after incubating the gel for 15 h at 37 °C with sucrose in a reaction mixture containing 1% (w/v) Triton X-100 (Russell, 1979a, b).

Antiserum and serological procedure. The partially purified glucosyltransferase preparation, which synthesized water-soluble and -insoluble glucan, was obtained from *S. mutans* HS6 culture supernatant and injected intravenously into a rabbit with an equal volume of Freund's incomplete adjuvant (Mukasa & Slade, 1973a).

Double immunodiffusion tests on agar were as described by Ouchterlony (1958).

Determination of optimum pH and K_m values. The pH optimum was determined by measuring the enzyme activity in 0.1 M-sodium acetate buffer (pH 4.0 to 6.0) and 0.1 M-sodium phosphate buffer (pH 5.5 to 7.5).

The K_m value for sucrose was determined according to the method of Eisenthal & Cornish-Bowden (1974) by measuring the activity in 0.1 M-sodium acetate buffer, pH 5.5, containing 2.5 to 200 mM-sucrose in the presence of 0.5 mg dextran T10 ml⁻¹ at 37 °C for 1 h.

The K_m value for dextran T10 was estimated by measuring the activity in the reaction mixture containing 0 to 5.1 mg dextran T10 ml⁻¹. The values quoted represent the averages of results from triplicate experiments.

Linkage analysis of glucan. Glucan was synthesized by the purified enzyme preparation (130 µg) in 10 ml 0.1 M-sodium acetate buffer (pH 6.0) containing 5% (w/v) sucrose, 0.03% (w/v) Triton X-100 and 0.01% (w/v) Merthiolate at 37 °C for 5 d, precipitated with 3 vol. ethanol and washed three times as previously described (Shimamura *et al.*, 1982). The glucan was permethylated as described by Hakomori (1964) and the degree of permethylation was checked with an infrared spectroscope IR-400 (Shimazu, Kyoto, Japan).

The permethylated glucan was hydrolysed with 90% (v/v) formic acid followed by 0.25 M-sulphuric acid, reduced with sodium borohydride and acetylated with pyridine/acetic anhydride (1:1, v/v) as described previously (Shimamura *et al.*, 1982). The partially methylated alditol acetates were measured by a Shimazu GC-6A gas-liquid chromatograph, using a capillary column (20 m × 0.25 mm) which was wall-coated with Silicone OV-101 (Shimazu): the derivatives separated were identified by a Shimazu auto GCMS 6020 GLC-mass spectroscope, as previously reported (Shimamura *et al.*, 1982).

The extent of hydrolysis of glucan (5 mg) by dextranase (from *Penicillium funiculosum*, a kind gift from Dr T. H. Stout of Merck, Sharpe and Dohme Research Laboratories, Rahway, N.J., U.S.A.) was also determined as previously reported (Shimamura *et al.*, 1982).

Other assays. Protein was determined by the Lowry method using bovine serum albumin as a standard.

The carbohydrate content of proteins separated by SDS-PAGE was estimated by staining the gel by the periodic acid-Schiff base method, and the carbohydrate content of enzyme was estimated by comparing the intensity of the stained band with that of the glycoprotein, transferrin.

Materials. DEAE-Sepharose CL-6B, ConA-Sepharose, Polybuffer exchangers 94, Polybuffer 74 and Dextran T10 were purchased from Pharmacia. RNA polymerase B (*Thermus thermophilus*), Noble agar and Ampholines were supplied by Mitsubishi Yuka Co. Ltd, Tokyo, Japan, Difco and LKB Produkter AB (Bromma, Sweden), respectively. All other chemicals were of analytical grade.

RESULTS

Purification of glucosyltransferase

Streptococcus mutans HS6 (serotype a) glucosyltransferases, which synthesize soluble glucan from sucrose, were purified from the culture supernatant, as summarized in Table 1. Fructosyltransferase activity was negligible in this enzyme preparation (Mukasa *et al.*, 1979) and invertase activity was not detected, when analysed by analytical isoelectric focusing (Mukasa *et al.*, 1982a). DEAE-Sepharose chromatography effectively removed the other enzymes such as 1,3- α -D-glucan synthetase, and the fractions 32 to 106 contained two glucosyltransferases and extremely minor active components, as found by analytical isoelectric focusing (Fig. 1). The minor glucosyltransferases were removed by the successive step of ConA-Sepharose chromatography, although the two enzymes eluted in the same fractions. The two enzymes were effectively separated by chromatofocusing in the presence of 20 mM-NaCl. The faster- and slower-eluting glucosyltransferases were designated as I (fractions 13 to 16) and II (25 to 30), respectively.

Table 1. Purification of glucosyltransferases from *S. mutans* HS6 (serotype a)

Step	Total protein (mg)	Total activity (i.u.)	Specific activity (i.u. mg ⁻¹)	Recovery (%)	Purification (fold)
1. Ultrafiltration	289	510	1.76	100	1
2. DEAE-Sepharose	69.3	173	2.50	33.9	1.4
3. ConA-Sepharose	15.3	78.6	5.14	15.4	2.9
4. Chromatofocusing					
Enzyme I	2.5	15.5	6.20	3.0	3.5
Enzyme II	2.6	15.2	5.85	5.0	3.3

Fig. 1. (a) DEAE-Sepharose chromatography of crude *S. mutans* HS6 extracellular glucosyltransferases. Total glucosyltransferase activity of each fraction was estimated by measuring the rate of release of reducing sugar from sucrose. The horizontal bar represents the fractions pooled. (b) Isoelectric focusing patterns of the DEAE-Sepharose fractions. Bands of glucosyltransferase activity were stained with periodic acid-Schiff base as described in Methods.

Dextranase activity was not detected and carbohydrate contents were 1.6 and 1.5% (w/w) for the enzymes I and II, respectively.

Properties of glucosyltransferase

Electrophoretic properties. Each of the enzymes I and II was focused as a single sharp band of protein and activity (Fig. 2), and the pI values were estimated to be 4.5 for enzyme I and 4.2 for enzyme II. The enzymes also migrated as a single band of protein and activity during SDS-PAGE (Fig. 3). The molecular weights of the enzymes were estimated to be 161 000 and 174 000 for the enzymes I and II, respectively.

Immunological analysis. Each of the purified enzymes gave a precipitin band of antigenic identity against an anti-*S. mutans* HS6 glucosyltransferase serum (anti-GT serum) (Fig. 4). Glucosyltransferase (pI 4.1) from *S. mutans* 6715 (serotype g) was partially identical with the purified HS6-enzymes but basic glucosyltransferase (pI 8.5) from *S. mutans* Ingbritt (serotype c) did not cross-react with the anti-GT serum (Fig. 4).

Kinetic study. The pH optimum for both enzymes was 5.5. The K_m value for sucrose was estimated to be 1.3 ± 0.4 (S.E.) mM at pH 5.5 in the presence of 0.5 mg dextran T10 ml⁻¹. The enzymes I and II were similarly activated 6.2-fold by dextran T10 and the K_m value for dextran T10 was 83 ± 11 (S.E.) μ M-glucose equivalent at less than 0.16 mg dextran T10 ml⁻¹. However, this value shifted to 1.6 ± 0.2 (S.E.) mM-glucose equivalent at more than 0.16 mg dextran T10 ml⁻¹. The shift of K_m value for dextran T10 was also reported by Chludzinski *et al.* (1976) and Shimamura *et al.* (1982).

Fig. 2. Isoelectric focusing of the purified enzymes from *S. mutans* HS6 stained with Coomassie brilliant blue R-250 (a) and with periodic acid-Schiff base (b). Lane 1, enzyme I; lane 2, enzyme II.

Linkage analysis. Linkage analysis of the soluble glucans synthesized in the absence of dextran T10 gave the acetate derivatives of 2,3,4,6-tetra-, 2,3,4-tri- and 2,4-di-*O*-methyl-D-glucitol in the molar ratio of 1.11:3.87:1.00 and 1.24:2.83:1.00 for the glucans synthesized by the enzymes I and II, respectively, without other alditol acetates. If it is assumed that these glucans contain only single 1,3- α -linked residues attached to the branch points, these results indicate that the glucans synthesized by the enzymes I and II were 1,6-linked glucose polymers with 20 and 24.5 mol% of the residues substituted at C-3, respectively.

The glucan products of enzymes I and II were hydrolysed 33.9% (w/w) and 43.8% (w/w) by exodextranase, which was specific for the 1,6- α -D-glucosidic linkage and which hydrolysed dextran to an isomaltose unit (Chaiet *et al.*, 1970). Under the same conditions, commercial dextran T10 was completely hydrolysed. The partial hydrolysis of the glucans indicates the highly branched structure as previously reported (Shimamura *et al.*, 1982; Mukasa *et al.*, 1982*b*).

Fig. 3. SDS-polyacrylamide gel electrophoresis of the purified enzymes from *S. mutans* HS6 stained with Coomassie brilliant blue R-250 (a) and with periodic acid-Schiff base (b). Lane 1, RNA polymerase B as a molecular weight marker; lanes 2 in (a) and 1 in (b), enzyme I; lanes 3 in (a) and 2 in (b), enzyme II.

DISCUSSION

Two glucosyltransferases from *S. mutans* HS6 with different molecular weights and isoelectric points were purified and characterized. These enzymes exhibited the same K_m values for sucrose and for dextran T10 and the optimum pH values, and were immunologically identical. The molecular weights in the presence of SDS, K_m values for sucrose and for dextran T10,

Fig. 4. Immunodiffusion of purified enzymes against anti-GT serum. 1, enzyme I (3.9 μ g protein); 2, enzyme II (3.8 μ g); 3, basic glucosyltransferase from *S. mutans* Ingbritt (2.5 μ g); 4, glucosyltransferase from *S. mutans* 6715 (17.5 μ g); 5, anti-GT serum (30 μ l).

immunological properties against the anti-GT serum and optimum pH of the enzymes I and II were similar to those of glucosyltransferase (pI 4.1) from *S. mutans* 6715 (Chludzinski *et al.*, 1976; Shimamura *et al.*, 1982), while the values of isoelectric point of these enzymes were slightly but distinctly different from each other. Although no serological relationship between the basic glucosyltransferase (pI 8.5) from *S. mutans* serotype *c* and the enzymes from the serotypes *a* and *g* has been observed (Fukui *et al.*, 1974*b*; Smith & Taubman, 1977), the enzyme from the serotype *c* strain synthesized a highly branched glucan from sucrose, as did the enzymes from the serotypes *a* and *g* (Shimamura *et al.*, 1982; Mukasa *et al.*, 1982*b*). These results suggested that these enzymes differ from one another but probably play a similar physiological role.

The glucosyltransferases purified from *S. mutans* serotypes *a*, *c* and *g* synthesized soluble glucans which consisted of 17 to 36 mol% of 1,3,6-linked glucose residues and 64 to 83 mol% of 1,6- α -linked residues. A dextransucrase has been purified from *S. mutans* HS6, which synthesized the soluble glucan consisting of 94 mol% of 1,6- α -linked glucose residue and 6 mol% of 1,3- α -linked residues, as determined by the periodic acid oxidation method (Fukui *et al.*, 1974*a*). Although the isoelectric point of the dextransucrase has not been reported, the glucosyltransferase purified in the present study closely resembled the dextransucrase in properties such as molecular weight, pH optimum and K_m value for sucrose (17000, 5.75 and 2 mM, respectively). Therefore, these enzymes might be the same protein. Regarding the content of 1,3,6- α -glucose residue, the reason for the difference between the present and the earlier result of Fukui *et al.* (1974*a*) is not clear. Walker & Hare (1977) have partially purified two types of glucosyltransferases from *S. mutans* OMZ176 (serotype *d*) which catalysed soluble and insoluble glucan syntheses. The soluble glucan synthesized had a highly branched structure with 47 mol% 1,3,6- α -linked glucose residues and 53 mol% 1,6- α -linked residues (Hare *et al.*, 1978). The soluble glucans synthesized by crude enzyme preparations from 11 strains of *S. mutans* have ranged in content of 1,6- α -linkages from 64 to 81 mol% and of 1,3- α -linkages from 19 to 36 mol%, as determined by ^1H -NMR (Meyer *et al.*, 1978). A similar observation has been reported by Usui *et al.* (1975) using ^{13}C -NMR. These results agreed closely with the highly branched structure of the soluble glucans synthesized by the enzymes purified in the present study. When enzyme II synthesized the soluble glucan from sucrose in the presence of a sufficient amount of dextran T10 (6.3 mg ml $^{-1}$), the glucan contained 29 mol% 1,3,6- α -linked glucose residues (data not shown). Based on these results, the enzymes purified in the present study would be classified as bifunctional enzymes (sucrose: 1,6- α -D-glucan 3- α - and 6- α -glucosyltransferase), as previously reported by Shimamura *et al.* (1982) and Mukasa *et al.* (1982*b*).

ConA-Sepharose chromatography effectively removed contaminating glucosyltransferases (e.g. 1,3- α -D-glucan synthetase and other enzymes), although the enzymes I and II, whose values of isoelectric point were 4.5 and 4.2, respectively, were not separated from each other (Fig. 1). These enzymes, however, were effectively separated by chromatofocusing. A short column was used in order to avoid inactivation of the enzyme exposed to low pH during the fractionation. The enzymes I and II were highly purified with only 3.5- and 3.3-fold purification, respectively. This indicated that these enzymes comprised considerable portions of the extracellular proteins excreted by *S. mutans* HS6. To confirm the separation of the enzymes after chromatography, the fractions were examined by analytical isoelectric focusing, since this is a more suitable method than gel or SDS-gel electrophoresis to detect glucosyltransferases which are located over a wide pH range (Mukasa *et al.*, 1982a).

Streptococcus mutans HS6 produced several kinds of glucosyltransferases with different isoelectric points other than the enzymes purified in the present study (Fig. 1b). Details of the studies on these enzymes will be reported elsewhere. We suggest that for *S. mutans* serotypes a, d and g, enzymes such as 1,6- and 1,3,6- α -D-glucan synthetase (the enzyme now purified; Shimamura *et al.*, 1982), 1,3- α -D-glucan synthetase (Fukushima *et al.*, 1981; Fukui *et al.*, 1982), which existed as a high molecular weight enzyme complex with polysaccharide (Mukasa & Slade, 1974; Mukasa *et al.*, 1979) and probably glucanohydrolase (Germaine *et al.*, 1977) cooperate to synthesize adherent insoluble polysaccharides. Recently, the growth of glucan on the cell surface of *S. mutans* HS6 has been visualized by scanning electron microscopy and the glucan synthesized on the cell has been related to adherence of the cell to smooth surfaces (Tsumori *et al.*, 1982).

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