Purification and Characterization of a Third Glucosyltransferase from *Streptococcus mutans* Serotype g

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(Received 7 April 1986; revised 9 September 1986)

Streptococcus mutans strain AHT (serotype g) secretes at least two glucosyltransferases with different pI values. A novel glucosyltransferase with a pI of 5.8 was purified 244-fold from the ammonium sulphate fraction by DEAE-cellulose chromatography, FPLC (Mono Q column, Pharmacia) and hydrophobic chromatography. The enzyme preparation gave a single protein band on analysis by both PAGE and SDS-PAGE, and did not form multiple protein bands detectable by IEF. The M_r was estimated to be about 130 000 by SDS-PAGE and about 135 000 by ultracentrifugal analysis. The apparent K_m value and pH optimum of the enzyme were 3.9 ± 0.2 mM (mean $\pm$ SD) and about 4.7, respectively. The enzyme synthesized water-soluble glucan from sucrose, and the glucan consisted of over 90 mol% 1,6- α -D-glucosidic linkages. The enzyme activity was not stimulated by primer dextran. Anti-enzyme serum produced a single precipitin band with the purified enzyme preparation, whereas it did not react with either of the other two known glucosyltransferases.

INTRODUCTION

It has been suggested by several authors that various strains of *Streptococcus mutans* secrete at least two glucosyltransferases synthesizing different products (Ciardi *et al.*, 1977; Fukushima *et al.*, 1981; Robyt & Martin, 1983; Koga *et al.*, 1983). One of these glucosyltransferases synthesizes a water-insoluble glucan, and the other, a water-soluble glucan. Shimamura *et al.* (1983) reported that there is a third glucosyltransferase existing in multiple form (pI 5.5–7.0) in *S. mutans* 6715 (serotype g) which is different from previously purified glucosyltransferase (Shimamura *et al.*, 1982). However, this enzyme has not yet been purified and characterized. We have also found a fourth glucosyltransferase isolated from *S. mutans* AHT (serotype g) (Takehara *et al.*, 1984), the characterization of which will be described elsewhere (N. Hanada & T. Takehara, unpublished).

In this report, we describe the purification and characterization of the third glucosyltransferase from *S. mutans* AHT.

METHODS

Materials. *S. mutans* AHT (serotype g, subculture AHT-k; Hamada *et al.*, 1978) was kindly provided by Dr M. Inoue, Kagoshima University Dental School, Kagoshima, Japan. An electrophoresis calibration kit (HMW), an IEF calibration kit, Pharmalyte, Octyl-Sepharose CL-4B, Dextran T10 (DT 10) and Dextran T2000 (DT 2000) were purchased from Pharmacia, alcohol dehydrogenase (EC 1.1.1.1; from yeast) from Sigma, catalase (EC 1.11.1.6; bovine) from Boehringer Mannheim, DEAE-cellulose (DE 52) from Whatman, Bio-Gel A-5m from Bio-Rad, Ultrogel Aca 34 from LKB, Freund's incomplete adjuvant from Difco, and Schiff reagent from Merck. All other chemicals were of analytical grade.

Abbreviations: FPLC, fast protein liquid chromatography; IEF, isoelectric focusing; PAS, periodic acid-Schiff.

Culture conditions. *S. mutans* AHT was grown anaerobically for 18 h at 37 °C in Trypticase, tryptose and yeast extract/glucose broth (Hamada & Torii, 1978). Screw-capped laboratory bottles were used to obtain twenty 5-litre batches (100 l).

Enzyme assay. Total glucosyltransferase activity was determined by measuring the amount of glucan synthesized in the presence of sucrose. An enzyme sample (0.9 ml), containing 0.1 M-sodium acetate buffer, pH 6.0, 5% (w/v) sucrose and 5 mM-sodium fluoride, was incubated at 37 °C for 18 h, and the total polysaccharides subsequently precipitated with 70% (v/v) ethanol and harvested by centrifugation (1500 g, 30 min). Glucan was quantified by a modification of the anthrone method (Halhoul & Kleinberg, 1972). For determination of enzyme kinetics, the incubation time was 60 min in the same reaction mixture, except that the buffer pH was 4.7. One unit (U) of glucosyltransferase activity was defined as the amount of enzyme catalysing the incorporation of 1 µmol glucose from sucrose into glucan per min in the reaction mixture described above.

The priming effect of glucan synthesized by glucosyltransferase was studied by measuring the activity in the reaction mixture containing 0–5 mg DT 10 ml⁻¹, 5% (w/v) sucrose and 5 mM-sodium fluoride.

Water-insoluble glucan synthetic activity was determined by measuring the amount of water-insoluble glucan synthesized in the presence of 5% (w/v) sucrose, DT 10 (5 mg ml⁻¹) and 5 mM-sodium fluoride.

Dextranase activity was assayed in the reaction mixture with DT 2000 (0.5 mg ml⁻¹) in 0.4 ml 50 mM-sodium acetate buffer, pH 6.0. Before and after incubation at 37 °C for 18 h, the amount of reducing sugar released was measured by the method of Park & Johnson (1949), using glucose as a standard, and the net increase of reducing sugar was calculated.

Purification. All procedures were carried out at 0–4 °C except for FPLC and HPLC. One hundred litres of culture liquor was centrifuged at 7000 g for 20 min and the cell-free culture supernatant was precipitated with ammonium sulphate at 50% saturation. The precipitate was then collected by centrifugation (11 000 g, 60 min), suspended in 20 mM-Tris/HCl buffer, pH 7.5, and dialysed against the same buffer.

The crude enzyme (540 ml) was applied to a DEAE-cellulose column (9 × 36 cm) equilibrated with the same Tris/HCl buffer until no absorbance at 280 nm was detected in the effluents, and then eluted with a linear gradient of 0–0.4 M-NaCl in 6000 ml 20 mM-Tris/HCl buffer, pH 7.5. This procedure was done three times. Active glucosyltransferase fractions were pooled for each peak and dialysed against Tris/HCl buffer, pH 7.5. The peaks were designated P-1 (pI 5.8), P-2 (pI 5.5), P-3 (pI 5.1) and P-4 (pI 4.2) according to their order of elution from the column (Takehara *et al.*, 1984). Each enzyme fraction was concentrated by ultrafiltration with Amicon PM 10 and used for further studies. P-1 solution (2 ml) was applied to an FPLC (Mono Q) column equilibrated with Tris/HCl buffer and washed with the same buffer. The column was then eluted with a linear gradient of 0–0.2 M-NaCl in 30 ml 20 mM-Tris/HCl buffer, pH 7.5. This procedure was done seventeen times. The enzyme activity contained in pooled fractions was concentrated using Immersible CX-30 (Millipore) and dialysed against 50 mM-sodium acetate buffer, pH 6.0.

Ammonium sulphate was added to the enzyme solution (2.4 ml) to 25% saturation. Then the enzyme was applied to a column of Octyl-Sepharose CL-4B (1.6 × 15 cm) equilibrated with the same buffer containing ammonium sulphate at 25% saturation. The enzyme was eluted with a linear gradient of ammonium sulphate at 25–0% saturation and 0–60% (v/v) ethylene glycol in the same buffer. This procedure was repeated twice. The active enzyme fractions were then pooled and concentrated using an Amicon PM 10 membrane and dialysed against 50 mM-sodium acetate buffer, pH 6.0.

PAGE. PAGE of the purified enzyme was done according to the method of Davis (1964). The sample (about 6 µg protein) was applied to a separation gel of 7.5% (w/v) acrylamide in 5 mM-Tris/glycine buffer (pH 8.4). After electrophoresis, the gel was stained with Coomassie brilliant blue R-250. For detection of enzyme activity, the gel was incubated for 18 h at 37 °C in 0.1 M-sodium acetate buffer, pH 5.2, containing 5 mM-sodium fluoride and 5% (w/v) sucrose. Carbohydrate was stained with the periodic acid-Schiff (PAS) reagent (Kapitany & Zebrowski, 1973).

SDS-PAGE. This was done according to the method of Weber & Osborn (1969) in order to determine the homogeneity and M_r of the purified enzyme. The run was made at pH 7.2 in a 7% (w/v) polyacrylamide gel containing 0.1% (w/v) SDS. The gel was stained for protein with Coomassie brilliant blue R-250.

IEF. IEF in a thin polyacrylamide gel layer was done as described by Mukasa *et al.* (1982), with some modifications. Polyacrylamide containing 4.4% (w/v) acrylamide, 0.14% (w/v) methylenebisacrylamide, 3.3% (v/v) Pharmalyte (pH 3–10) and 3.0% (v/v) Pharmalyte (pH 4–6.5) was covalently bound to a siliconized glass plate. The cathode and anode of the polyethylene glycol strips were soaked in 1 M-NaOH and 0.04 M-aspartic acid, respectively. A prefocusing run was done at 18 °C at 100 V for 1.5 h. After the prefocusing, enzyme solution was put on the gel surface and IEF subsequently done at 300 V for 3 h. After the focusing, the pH of the gel was measured directly with a surface pH electrode (SE1600GC, Fuji Kagaku Keisoku, Tokyo, Japan) and protein was also stained with Coomassie brilliant blue R-250.

Ultracentrifugal analysis. The purified enzyme fraction was loaded onto linear 10–30% (v/v) glycerol gradients, which were then centrifuged and fractionated as described by Newman *et al.* (1980). Alcohol dehydrogenase and catalase were used as M_r markers in the same gradients.

Protein assay. Protein concentration was measured by the method of Bradford (1976) with bovine serum albumin as a standard.

Glucan assay. Glucan produced by the purified enzyme was separated by 70% (v/v) ethanol precipitation as reported by Koga & Inoue (1978). Periodate oxidation and Smith degradation were done by the method of Hamilton & Smith (1956). Degraded products were analysed by HPLC (SCR 101N column, Shimadzu, Kyoto, Japan).

Antiserum against the purified enzyme. Purified P-1 (90 µg protein) was mixed with an equal volume of Freund's incomplete adjuvant and injected intradermally into a rabbit. The animal received three injections of antigen at intervals of one week and antiserum was collected from an ear vein 10 d after the final injection.

Immunodiffusion. Ouchterlony double-immunodiffusion analysis (Ouchterlony, 1958) was done in 1% (w/v) agarose gel.

Purification of the P-3 and P-4 enzymes. We purified both P-3 and P-4 in order to confirm whether the three glucosyltransferases separated by DEAE-cellulose chromatography were indeed immunologically different from each other.

The third peak eluted from the DEAE-cellulose column, which was the total glucosyltransferase activity fraction containing P-3, was rechromatographed on DEAE-cellulose and eluted with a linear gradient of 0–0.3 M-NaCl in 20 mM-Tris/HCl buffer, pH 7.5, in a total volume of 1000 ml. Water-insoluble glucan synthetic activity fractions were pooled and concentrated with Amicon PM 10. P-3 was again chromatographed on a Bio-Gel A-5m column and its pooled fractions were concentrated with Amicon PM 10. Further purification was done by using hydrophobic chromatography with Octyl-Sepharose CL-4B and FPLC (Mono Q column) in a similar manner to that used for the P-1 preparation. The last step of the purification procedures was done by using HPLC employing a TSK-gel G3000SW column (ToyoSoda, Tokyo, Japan) with 50 mM-sodium acetate buffer, pH 6.0, containing 0.1 M-NaCl and 0.02% (w/v) NaN_3 . Water-insoluble glucan synthetic activities were pooled and concentrated with Immersible CX-30 and dialysed against 50 mM-sodium acetate buffer, pH 6.0. This dialysed enzyme preparation was subjected to the gel precipitation reaction.

Total glucosyltransferase activity in the fourth peak, which contained P-4, was chromatographed on an Ultrogel AcA 34 column. Active fractions were collected and condensed using Amicon PM 10 as above, and subjected to immunodiffusion analysis.

RESULTS

Separation of the four glucosyltransferases

A typical chromatogram of glucosyltransferases on DEAE-cellulose is shown in Fig. 1. The crude solution was prepared from about 33 litres of culture solution and its volume reduced to 540 ml by ammonium sulphate precipitation. Glucosyltransferase activity was found in four peaks. Dextranase activity was observed neither in P-1 nor in P-2, but mostly in the P-4 fractions. From another run, four enzymes accounting for the total glucosyltransferase activity were also obtained from 100 litres of culture medium. The P-1, P-2 and P-4 fractions basically produced water-soluble glucan, whereas P-3 synthesized water-insoluble glucan.

Purification of the P-1 enzyme

After pooling and concentration of the first active peak obtained from the DEAE-cellulose column, it was subjected to FPLC (Mono Q column) (Fig. 2). The eluate was concentrated and loaded on an Octyl-Sepharose CL-4B column for hydrophobic chromatography (Fig. 3). The purification procedures are summarized in Table 1. The specific activity of the final preparation was 244 times greater than that of the crude ammonium sulphate fraction; the yield was 1.2%.

This purified P-1 synthesized water-soluble glucan. With a high concentration of this purified enzyme, clear water-soluble glucan was found as the sole product in the reaction mixture.

Homogeneity of the P-1 enzyme

Purified P-1 exhibited a homogeneous electrophoretic profile in PAGE (Fig. 4a) and in SDS-PAGE (Fig. 5). It was confirmed that the protein band was, indeed, the glucosyltransferase by the positive glucan stain obtained with PAS after incubation of the gel in sucrose solution (Fig. 4b).

Fig. 1. Separation pattern of *S. mutans* AHT extracellular glucosyltransferases on DEAE-cellulose. Chromatography of the ammonium sulphate fraction of glucosyltransferases was carried out on a DEAE-cellulose column (9×36 cm) as described in Methods. The flow rate was 246 ml h^{-1} and 30 ml fractions were collected. ●, Total glucosyltransferase activity; △, dextranase activity (expressed as the amount of reducing sugar equivalent to $\mu\text{g glucose ml}^{-1}$); ----, NaCl concentration. Fractions indicated by the horizontal lines were pooled.

Fig. 2. Elution profile of P-1 enzyme (P-1 fraction obtained from the DEAE-cellulose column, pI 5.8) on an FPLC (Mono Q) column. Experimental details are given in Methods. The flow rate was 1 ml min^{-1} and fractions of 1 ml were collected. ●, Total glucosyltransferase activity; ○, protein monitored by the absorbance at 280 nm; ----, NaCl concentration. Fractions indicated by the horizontal line were pooled.

Fig. 3. Elution profile of P-1 enzyme (pI 5.8) on an Octyl-Sepharose CL-4B column (1.6×15 cm). Experimental details are given in Methods. The flow rate was 25 ml h^{-1} and 5 ml fractions were collected. ●, Total glucosyltransferase activity; ○, protein monitored by the absorbance at 280 nm; ----, ammonium sulphate concentration; ----, ethylene glycol concentration.

Fig. 4. PAGE (7.5%, w/v, polyacrylamide) analysis of the purified P-1 enzyme (pI 5.8) (6 μ g protein). (a) Protein staining with Coomassie brilliant blue R-250; (b) activity staining with PAS reagent (see Methods).

Fig. 5. SDS-PAGE (7%, w/v, polyacrylamide) analysis of the purified P-1 enzyme (pI 5.8) (6 μ g protein). The enzyme was heated at 100 °C for 3 min in a solution which contained 10 mM-sodium phosphate buffer, pH 7.2, in the presence of 1% (w/v) SDS and 5% (v/v) 2-mercaptoethanol. (a) Purified P-1; (b) protein standards (1, thyroglobulin, M_r 330 000; 2, ferritin half unit, 220 000; 3, bovine serum albumin, 67 000; 4, catalase, 60 000; 5, lactate dehydrogenase, 36 000; 6, ferritin, 18 500).

Table 1. *Purification of the P-1 enzyme from S. mutans AHT*

Step	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U mg ⁻¹)	Purifica- tion (-fold)	Yield (%)
Ammonium sulphate	1620	562	6026	0.093	1	100
DEAE-cellulose	34	29	8.16	3.55	38	5.2
FPLC (Mono Q)	4.8	8.8	1.39	6.33	68	1.6
Octyl-Sepharose CL-4B	5	6.8	0.30	22.67	244	1.2

Physical and kinetic properties of the P-1 enzyme

P-1 did not form multiple protein bands in IEF and had a single pI of 5.8 (Fig. 6). Estimations of the M_r of this enzyme by two methods, SDS-PAGE and ultracentrifugation, yielded figures of about 130 000 and about 135 000 respectively. The reaction of the enzyme was linear with time up to 60 min at an optimal pH of 4.7, (determined in a pH range using 0.2 M-sodium acetate and 0.2 M-Tris/HCl buffer), and the optimal temperature was about 37 °C. The K_m for sucrose was 3.9 ± 0.2 mM (mean $\pm$ SD) under the reaction conditions mentioned above.

The enzyme activity was not stimulated by DT 10. Glucoside linkages of glucan from sucrose catalysed by this enzyme were examined by periodate oxidation and Smith degradation, and two linkages, 1,6- α -D and 1,3- α -D, with molar ratios of 91.5% and 8.6%, respectively, were found. No 1,4- α -D linkage was apparent.

Immunological properties of the P-1 enzyme

In order to have controls for the immunodiffusion analysis, P-3 was highly purified and P-4 was partially purified. The results of the gel precipitation studies with P-1, P-3 and P-4 against

Fig. 6. IEF analysis of the purified P-1 enzyme (pI 5.8). (a) Purified P-1; (b) protein standards (1, soybean trypsin inhibitor, pI 4.55; 2, β -lactoglobulin A, 5.20; 3, bovine carbonic anhydrase B, 5.85; 4, human carbonic anhydrase B, 6.55; 5, horse myoglobin – acidic band, 6.85; 6, horse myoglobin – basic band, 7.35; 7, lentil lectin – acidic band, 8.15; 8, lentil lectin – middle band, 8.45; 9, lentil lectin – basic band, 8.65).

Fig. 7. Double immunodiffusion of glucosyltransferases from *S. mutans* AHT against anti-P-1 serum. 1, Purified P-1 (pI 5.8, 1.2 μ g protein); 2, purified P-3 (pI 5.5, 1.8 μ g protein); 3, partially purified P-4 (pI 4.2, 14.9 μ g protein); 4, anti-P-1 serum.

rabbit anti-P-1 serum are shown in Fig. 7. After 12 h incubation, the anti-P-1 serum formed a single precipitin band against the purified P-1 but not against the other two enzymes. Serum from a rabbit which had not been injected with P-1 did not show any precipitation with the P-1.

DISCUSSION

The manifestation of cariogenicity in *S. mutans* depends on the nature of the glucan that is synthesized from sucrose by the extracellular glucosyltransferases produced by this organism. It is essential for *S. mutans* to possess mechanisms for synthesizing water-insoluble glucan with adhesive properties in order for the organism to adhere to the tooth surface. Certain

glucosyltransferases are responsible for synthesizing water-insoluble glucan. Without the cooperative action of these enzymes, it would not be possible for this organism to have any degree of adhesion. Each of the enzymes alone lacks the ability to produce adherence to the glass of a test tube (Fukushima *et al.*, 1981; Inoue *et al.*, 1982). Thus, their cooperative effect is a particular characteristic of *S. mutans*. For this reason, the function of each individual enzyme should be considered, rather than lumping them together as a single glucosyltransferase entity, when discussing the mechanism of caries formation.

We have previously isolated four enzymes from crude glucosyltransferase by DEAE-cellulose chromatography, and have determined their isoelectric points (Takehara *et al.*, 1984), immunological properties and some characteristics of the glucans they produce.

According to our findings, P-1, P-3 and P-4 are different proteins (see Fig. 7). P-1 did not form multiple protein bands in IEF (Fig. 6). The enzyme had a very strong auto-priming effect compared with P-4 (Takehara *et al.*, 1984). When either P-3 or P-4 was incubated with P-3 maintained at a constant level, the auto-priming ability of P-1 was 3.5 times as strong as that of P-4 (Takehara *et al.*, 1984). This suggests that P-1 is essential for glucan synthesis of *S. mutans* serotype *g*.

The low yield of P-1 (1.2%) leads to the question of whether this activity accounts for a significant proportion of the glucosyltransferase activity *in vivo*. However, the overall yield of primer-dependent glucosyltransferase (pI 4.1) activity obtained by Shimamura *et al.* (1982) was also low (3.8%), despite the addition of DT 10. This glucosyltransferase (pI 4.1) probably corresponds to P-4. The *in vivo* significance of P-1 relative to other enzymes is not known at present.

The existence of this enzyme, with a pI of 5.8, does not appear to be widely known. The review article by Ciardi (1983), and a recent paper of Furuta *et al.* (1985), made no mention of this enzyme. We therefore consider the purification procedures, and various physical, enzymic and immunological properties presented here of this newly discovered glucosyltransferase from *S. mutans* serotype *g* to be significant.

This work was taken from a dissertation submitted by Nobuhiro Hanada to the Graduate School of Kyushu Dental College in partial fulfilment of the requirements for the PhD degree.

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