Purification and Characterization of Extracellular Glucosyltransferase Synthesizing Water-insoluble Glucan from *Streptococcus rattus*

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An extracellular glucosyltransferase synthesizing water-insoluble glucan (GTF-I) was purified from the culture supernatant of *Streptococcus rattus* strain BHT (mutans serotype b) by hydroxylapatite chromatography, DEAE-Toyopearl chromatography and preparative isoelectric focusing. The $M_r$ of GTF-I was 155 000 by SDS-PAGE and the isoelectric point was pH 4.9. The specific activity, the optimum pH and the $K_m$ value for sucrose were 10.0 i.u. (mg protein)$^{-1}$, 6.5 and 2.4 mM, respectively. The enzyme synthesized a water-insoluble glucan consisting of 69.4 mol% 1,3-$\alpha$-linked glucose, 23.6 mol% 1,6-$\alpha$-linked glucose, 2.6 mol% 1,3,6-$\alpha$-branched glucose and 4.4 mol% non-reducing terminal glucose, and also a small amount (3% of the total glucan) of soluble glucan with 82.4 mol% 1,6-$\alpha$-linked glucose. The $M_r$ and pI values of purified GTF-I were identical with those of the enzyme in the culture supernatant.

INTRODUCTION

The mutans group of oral streptococci secrete two or more kinds of glycosyltransferases which synthesize adhesive water-insoluble polysaccharides from sucrose by cooperative action (Carlsson, 1970; Mukasa & Slade, 1974; Ciardi et al., 1977; Mukasa et al., 1979; Fukushima et al., 1981). These polysaccharides facilitate cell adherence to smooth hard surfaces (Gibbons & Nygaard, 1968; Mukasa & Slade, 1973b). The mutants group of oral streptococci was originally designated as *Streptococcus mutans*, which was divided into eight serological subspecies (Bratthall, 1972a; Perch et al., 1974; Beighton et al., 1981), but the subspecies of *S. mutans* have now been elevated to species level and classified as *S. cricetus* (mutans serotype a), *S. rattus* (b), *S. mutans* (c, e and f) and *S. sobrinus* (d, g and h) (Coykendall, 1974; Skerman et al., 1980; Moore et al., 1985). Three kinds of glycosyltransferases have been purified from *S. cricetus* and *S. sobrinus* (Shimamura et al., 1982, 1983; Fukui et al., 1982; Koga et al., 1983; Tsumori et al., 1983a, 1985a, b; Furuta et al., 1985; McCabe, 1985) and glycosyltransferases synthesizing water-soluble glucan (GTF-S) have also been purified from *S. mutans* (Mukasa et al., 1982a; Kuramitsu & Wondrack, 1983; Sato et al., 1984; Baba et al., 1986). Although isoelectric focusing (IEF) and two-dimensional electrophoretic patterns of glycosyltransferase activities from *S. rattus* are clearly different from those of *S. cricetus*, *S. mutans* and *S. sobrinus* (Tsumori et al., 1983b; Mukasa, 1986), there are few reports on the purification and characterization of GTF-S from *S. rattus* (Scales et al., 1975; Kumada et al., 1987), and a purification study of the glucosyltransferase synthesizing water-insoluble glucan (GTF-I) has not previously been reported.

Abbreviations: GTF-I, glucosyltransferase synthesizing water-insoluble glucan; GTF-S, glucosyltransferase synthesizing water-soluble glucan; ISG, water-insoluble glucan; SG, water-soluble glucan; IEF, isoelectric focusing; PAS, periodic acid/Schiff.
**S. mutans** is the commonest species in human dental plaque (Loesche & Grenier, 1976; Hamada et al., 1976; Fitzgerald et al., 1983). **S. rattus** has also been found in some samples of human dental plaque (Brathall, 1972b; Duany et al., 1972; Grenier et al., 1973; Ota et al., 1985). Therefore, **S. rattus** strains as well as **S. mutans** strains are presumably responsible for development of dental caries in the human mouth. Considering that **S. rattus** strains are the most prevalent in experimental rats, these strains may be the most suitable ones for studies on the prevention of dental caries in animal models. In this paper, we report on the purification and characterization of GTF-I from **S. rattus** strain BHT and also on the glucan synthesized by this enzyme.

**METHODS**

Organism and culture conditions. **S. rattus** BHT (serotype h) was previously used by Kumada et al. (1987). Cells were grown for 6 h at 37 °C in 171 of a chemically defined minimal medium (Fujiiwara et al., 1978) with fructose instead of glucose and 0.05% Tween 80 (Umesaki et al., 1977; Wittenberger et al., 1978) and 0.1 mM of the protease inhibitor PMSF (Sigma). The pH of the culture at harvest was 5.1 and its OD_{550} (after diluting sixfold) was 0.83. The cell-free culture supernatant obtained by centrifugation for 30 min at 5500 g at 5 °C was made 0.01% with respect to Merthiolate.

**Purification of GTF-I.** All of the buffers used during purification steps contained 0.1 mM-PMSF.

1. **Hydroxylapatite chromatography.** A 160 ml volume of wet hydroxylapatite (Bio-Gel HT; Bio-Rad), equilibrated with 10 mM-sodium phosphate buffer (pH 6.5) was added to culture supernatant previously diluted fourfold with distilled water (final volume 68 l) to decrease the ionic strength; the resulting suspension was stirred gently for 2 h. After the hydroxylapatite had adsorbed the enzymes, it was collected by decantation and overlaid on top of fresh hydroxylapatite previously packed into a column (3×6×3.0 cm) and equilibrated with the same buffer. The column was washed with the same buffer (200 ml) and the enzyme was eluted with 500 ml of a linear gradient of 10 to 400 mM-sodium phosphate buffer (pH 6.5), followed with 200 ml 1 M-sodium phosphate buffer (pH 6.5). The flow rate was 30 ml h^{-1} and 4-6 ml fractions were collected. The enzyme-containing fractions (60 ml) were combined and dialyzed eight times with distilled water.

2. **DEAE-Toyopearl chromatography.** The diluted enzyme preparation (480 ml) obtained above was applied to a DEAE-Toyopearl (Toyosoda Manufacturing Co.) column (1×40 cm) previously equilibrated with 10 mM-sodium phosphate buffer (pH 6.5). The column was washed with the same buffer (30 ml) and the enzyme was eluted with 300 ml of a linear gradient of 0 to 300 mM-NaCl in the same buffer. The flow rate was 60 ml h^{-1} and 2-6 ml fractions were collected. The enzyme-containing fractions were combined and dialyzed against 10 mM-sodium phosphate buffer (pH 6.5) at 0 °C.

3. **Preparative IEF.** This was done as previously described (Mukasa et al., 1985). Ampholine pH 3.5-5.0, Ampholine pH 3.5-10.0 and Triton X-100 were used at concentrations of 0.95%, 0.05% and 0.5%, respectively. The enzyme was focused at a constant power of 4 W for 2-7 h and then 8 W for 8-3 h at approximately 0 °C. The enzyme-containing fractions were pooled and stored at −40 °C.

**Enzyme assay.** The reaction mixture (final volume 1.0 ml) contained 0.1 mM-sodium phosphate buffer (pH 6.5), 41.8 mM-sucrose, 0.01% Merthiolate and various amounts of enzyme solution with or without dextran T10 (Mukasa et al., 1979). The mixture was incubated at 37 °C for 1 to 4 h. Water-insoluble polysaccharides were separated by centrifugation and soluble polysaccharides were collected as 75% (v/v) ethanol precipitate (Mukasa et al., 1979). Polysaccharides were measured by the phenol/sulphuric acid method (Dubois et al., 1956) using glucose as a standard and fructan was measured according to the method of Van Handel (1967), as described by Kumada et al. (1987), using fructose as a standard. The amount of glucan was obtained by subtracting the amount of fructan from the total amount of polysaccharides. One unit (i.u.) of GTF-I activity was the amount of enzyme catalysing the incorporation of 1 pmol glucose from sucrose into glucan min^{-1} at 37 °C.

Reducing-sugar-release activity was determined by measuring the release of reducing sugar from sucrose (Somogyi 1945), using fructose as a standard. One unit (i.u.) of reducing-sugar-release activity was the amount of enzymes releasing 1 pmol of reducing sugar from sucrose min^{-1} at 37 °C.

**Electrophoretic analyses.** Analytical IEF was done using a horizontal gel containing 0.5% Triton X-100 as previously reported (Mukasa et al., 1982b), and calibrated using a Pharmacia low pi calibration kit. Protein was stained with Coomassie brilliant blue R-250 after focusing. GTF-I activity was directly detected as a white precipitin band after incubation of the focused gel in a sucrose-containing buffer at 37 °C for 2 to 15 h. Other glycosyltransferase activities were also directly detected by staining the polysaccharides with PAS (Mukasa et al., 1982b). Reducing-sugar-release activity in the gel was also directly detected with triphenyltetrazolium chloride (Gabriel & Wang, 1969).

SDS-PAGE in a slab gel was done by the method of Mukasa et al. (1987). After SDS-PAGE, the gel was incubated in a sucrose-containing buffer with 1% (w/v) Triton X-100. Activity was detected as a white precipitin
band and the intensity of activity was estimated by scanning with a densitometer (Shimadzu CS-910) using purified GTF-I as a standard.

SDS-PAGE in cylindrical gels was done by the method of Weber & Osborn (1969) in a 5% (w/v) polyacrylamide gel for 5 h at 6 mA per tube using Bio-Rad high molecular mass protein standards. Glucosyltransferase activities were detected as above after incubation of the gel at 37 °C in the sucrose-containing buffer with the addition of 1% (w/v) Triton X-100 (Russell, 1979b; Mukasa et al., 1982b).

Immunological procedure. Antisera against GTF-I and the GTF-S were prepared from male New Zealand White rabbits as previously reported (Mukasa & Slade, 1973a). A double immunodiffusion test was done according to the method of Ouchterlony (1958) using a 0.5% agarose gel.

Determination of optimum pH and $K_m$ value. The optimum pH was estimated as described previously (Tsumori et al., 1985a). Enzyme activity was measured in 0.1 M-sodium phosphate buffer (pH 6.5) containing 0.5 to 100 mM-sucrose at 37 °C for 1 h; the $K_m$ value for sucrose was determined graphically according to the method of Eisenthal & Cornish-Bowden (1974).

Linkage analysis of glucan. Glucan was synthesized by the purified enzyme (24 μg) in 20 ml 0.1 M-sodium phosphate buffer (pH 6.5) containing 5% (w/v) sucrose and 0.01% Merthiolate at 37 °C for 13 h. The water-insoluble glucan (ISG) was collected by filtration and washed with distilled water. The soluble glucan (SG) was obtained as a 75% (v/v) ethanol precipitate and washed (Mukasa et al., 1979). The washed and lyophilized glucans were methylated (Hakomori, 1964), hydrolysed, reduced, acetylated and analysed by GLC (Shimamura et al., 1982). The composition of the ISG was also analysed by $^{13}$C NMR spectroscopy. The ISG (9.7 mg) was dissolved in 0.4 ml 0.5 M-NaOH containing 1% (w/v) sodium 3-(trimethylsilyl)-1-propane sulphonate (Merck). $^{13}$C NMR spectra were recorded at 67.9 MHz on a JEOL JNX-GX 270 spectrometer operating in the pulse-Fourier transform mode with complete proton-decoupling at 22 °C. The sample in a spinning sample tube of 5 mm outer diameter was pulsed with 90° radio-frequency pulses (7.2 μs pulse width). The spectrum was accumulated at 65,536 data points over a spectral width of 8 kHz and was recorded with 166,884 scans. Chemical shifts were measured with a digital resolution of 0.02 Hz and are reported in parts per million downfield from the internal standard of sodium 3-(trimethylsilyl)-1-propane sulphonate. Peaks were assigned according to previous reports (Colson et al., 1974; Gagnaire & Vignon, 1977; Ito & Schuerch, 1979).

Other assay. Protein was determined by the Lowry method with BSA as a standard. The protein and carbohydrate contents of the purified enzyme preparation were estimated by staining the gel after analytical IEF with Coomassie brilliant blue R-250 and with PAS (Kapitany & Zebrowski, 1973), respectively, and estimated by comparing the intensity of the bands with those of BSA and human transferrin (Sigma), respectively.

RESULTS

Purification of GTF-I

GTF-I was purified 47.6-fold with 1.8% recovery by hydroxylapatite chromatography, DEAE-Toyopearl chromatography and preparative IEF, as summarized in Table 1. Hydroxylapatite chromatography rapidly concentrated glycosyltransferases from the culture supernatant and DEAE-Toyopearl chromatography effectively separated GTF-I from other glycosyltransferases in the crude enzyme preparation (hydroxylapatite fraction) (Fig. 1a, b); preparative IEF then further removed contaminants effectively and gave a single active peak (Fig. 1c). Fructosyltransferase, dextranase and inulinase activities were not detected in the purified enzyme preparation. Carbohydrate was not detected.

Properties of GTF-I

Electrophoretic properties. The purified GTF-I preparation consisted of a main GTF-I species with trace amounts of other GTF-I species as found by analytical IEF (Fig. 2b, c). The $M_r$ of the

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<th>Table 1. Purification of GTF-I from S. rattus</th>
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<td>Culture supernatant</td>
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<td>Hydroxylapatite</td>
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major GTF-I was 155000 ± 2000 (mean ± sD of five experiments) by SDS-PAGE and the pI was 4.9 (mean of three experiments) (Figs 2 and 3). The protein band of the purified GTF-I corresponded to the white, ISG band, the PAS-stained band (Figs 2 and 3) and the reducing-sugar band (data not shown).

Immunological analysis. The GTF-I in the purified and the crude preparations from strain BHT (b) formed a single precipitin band with the homologous anti-GTF-I serum. The GTF-I and GTF-S were immunologically unrelated to each other (Fig. 4).
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Fig. 3. SDS-PAGE patterns of *S. rattus* GTF-I preparations. Protein was stained with Coomassie brilliant blue R-250 (a); ISG synthesized was detected as a white precipitin band (b); and polysaccharides synthesized by the enzymes were stained with PAS reagent (c). Lanes: 1, *M*, marker proteins (1.5 µg); 2, culture supernatant (3.1 µg for protein and activity); 3, hydroxylapatite fraction (12 µg for protein, 2.4 µg for activity); 4, DEAE-Toyopearl fraction (10 µg, 2 µg); 5, purified GTF-I (0.8 µg, 0.12 µg).

Fig. 4. Double immunodiffusion tests of the purified GTF-I and GTF-S to anti-GTF-I and anti-GTF-S sera. (a): 1, purified GTF-I (1.2 µg); 2, hydroxylapatite fraction (36 µg); and 3, anti-GTF-I serum (15 µl). (b): 1, purified GTF-I (1.2 µg); 2, purified GTF-S (3 µg); 3, anti-GTF-I serum (15 µl); and 4, anti-GTF-S serum (15 µl).

**Kinetic study.** The optimum pH of the GTF-I was 6.5 and the *K*ₘ value for sucrose was 2.4 mM (+0.1/-0.5 with a 77.3% confidence limit) at pH 6.5. Enzyme activity was increased by up to 80% in the presence of 50 µg dextran T10 ml⁻¹.

**Linkage analysis of glucan.** The purified GTF-I synthesized ISG (45.7%), SG (1.3%) and free glucose (53.0%) with the corresponding amount of fructose in the absence of a primer. The ISG
Fig. 5. $^{13}$C NMR spectrum of ISG synthesized by the purified GTF-I. 1,3-Glc, 1,6-Glc, 1,3,6-Glc and 1-Glc refer to 1,3-$\alpha$-linked glucose, 1,6-$\alpha$-linked glucose, 1,3,6-$\alpha$-branched glucose and non-reducing terminal glucose, respectively.

was converted into partially methylated alditol acetates. The acetate derivatives of 2,3,4,6-tetra-, 2,4,6-tri-, 2,3,4-tri- and 2,4-di-$O$-methyl $D$-glucitol were detected in the molar ratio 1:0:15:9:5:4:0,6, indicating that the ISG was composed of 69.4 mol% 1,3-$\alpha$-linked glucose and 23.6 mol% 1,6-$\alpha$-linked glucose with 2.6 mol% 1,3,6-$\alpha$-branched glucose and 4.4 mol% non-reducing terminal glucose. The $^{13}$C NMR spectrum showed 12 resonances in the region of $\alpha$-D-glucopyranosyl residues (Fig. 5). Six of them were identical with those of $S$. sobrinus 1,3-$\alpha$-D-glucan and the others coincided in position with those of dextran (1,6-$\alpha$-D-glucan) (data not shown). Signals were assigned as shown in Fig. 5, according to previous reports (Colson et al., 1974; Gagnaire & Vignon, 1977; Ito & Schuerch, 1979). The ISG was calculated to consist of 64.9 mol% 1,3-$\alpha$-linked glucose and 30.1 mol% 1,6-$\alpha$-linked glucose with 0.9 mol% of 1,3,6-$\alpha$-branch points and 4.0 mol% terminal glucose based on peak areas. These values were close to those obtained by methylation analysis as described above. Methylation analysis of SG synthesized by the GTF-I indicated that the SG consisted of 82.4 mol% 1,6-$\alpha$-glucan with 2.6 mol% 1,3-$\alpha$-linked glucose and 1.6 mol% 1,3,6-$\alpha$-branch points, which resembled the SG synthesized by the GTF-I purified from $S$. mutans strain Ingbritt (Mukasa et al., 1985).

**DISCUSSION**

In this study, the GTF-I was purified from $S$. rattus culture supernatant by hydroxylapatite chromatography, DEAE-Toyopearl chromatography and preparative IEF, although a trace amount of other GTF-I species, probably derived from the major GTF-I species (pI 4-9) by the action of extracellular proteases, was present in the purified GTF-I preparation (Fig. 2).

The $M_r$ (155,000) in the presence of SDS and the isoelectric point (4-9) of the purified GTF-I resembled those of the enzymes from $S$. mutans (Kenney & Cole, 1983; Kuramitsu & Wondrack, 1983). By double immunodiffusion tests the GTF-I from $S$. mutans and $S$. rattus were antigenically partially related to each other (Kuramitsu & Ingersoll, 1976; Russell, 1979a).

The $M_r$ and isoelectric point of the purified GTF-I were identical with those of the enzyme in the culture supernatant which synthesized ISG in analytical IEF and SDS gels (Figs 2 and 3), indicating that the GTF-I was not degraded by proteases during the purification steps.
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The GTF-I synthesized mainly an ISG containing a considerable amount (23.6 mol%) of 1,6-α-linked glucose, and also synthesized a small amount of SG which was mostly composed of 1,6-α-D-glucan, as found with the GTF-I species from S. mutans strain Ingritt (Mukasa et al., 1985). Analytical IEF and immunological analyses showed the homogeneity of this preparation (Figs 2 and 4).

The GTF-I enzymes purified from S. cricetus and S. sobrinus require a primer such as exogenous dextran T10 to synthesize ISG which is not adhesive and, in the absence of the primer, the enzymes exhibit invertase-like activity (Chludzinski et al., 1976; Fukui et al., 1982; Shimamura et al., 1983; Tsumori et al., 1985a). Two kinds of GTF-S, other than the GTF-I, are secreted by S. cricetus and S. sobrinus (Shimamura et al., 1983; Tsumori et al., 1983b; Mukasa, 1986). In vivo, these three kinds of glucosyltransferases cooperatively synthesize the sticky adhesive polysaccharides which facilitate plaque formation on smooth surfaces. In contrast, S. rattus and S. mutans secrete a GTF-I that synthesizes ISG with a small amount of SG and free glucose in the absence of the primer dextran T10 (Mukasa et al., 1985), and simultaneously secrete one GTF-S and some fructosyltransferases (Mukasa, 1986). Recently, Shimamura et al. (1987) reported that the fructans synthesized by the fructosyltransferases from S. rattus and S. mutans were of the inulin type and not the levan type, as determined by 13C NMR spectroscopy. These enzymes probably also take part in the synthesis of sticky adhesive polysaccharide which is analogous to those of S. cricetus and S. sobrinus (Mukasa, 1986). Therefore, two mechanisms of adhesive polysaccharide synthesis may exist among the mutans group of oral streptococci.

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