Immunological relationships between glucosyltransferases synthesizing insoluble glucan from *Streptococcus cricetus*, *Streptococcus sobrinus* and *Streptococcus downei*

HIDEAKI TSUMORI

Department of Chemistry, National Defense Medical College, 2, Namiki 3 chome, Tokorozawa, Saitama 359, Japan

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The $M_r$ values and isoelectric points of glucosyltransferases synthesizing insoluble glucan (GTF-Is) were determined, and the immunological relationships between them studied. The GTF-I enzymes were from *Streptococcus cricetus* (mutans group serotype $a$), *Streptococcus sobrinus* (mutans group serotypes $d$ and $g$) and *Streptococcus downei* (mutans group serotype $h$). By double immunodiffusion tests, the GTF-I enzymes from the three species possessed a common antigenic determinant; in addition, the GTF-I enzymes of serotypes $d$, $g$ and $h$ shared a further determinant. The GTF-I enzymes of *S. sobrinus* serotypes $d$ and $g$ and GTF-I enzymes were immunologically identical. The GTF-I enzymes of $S. sobrinus$ serotypes $d$ and $g$, and of *S. downei*, had an $M_r$ of 161 000 and isoelectric points of 4.8–4.9, while *S. cricetus* GTF-I had a lower $M_r$ (150 000) and a higher isoelectric point (5.2). This suggests that the *S. cricetus* GTF-I enzyme may lack a sequence of amino acids which include the determinant shared by *S. sobrinus* and *S. downei* GTF-I enzymes. Antibodies specific to the determinant shared by all four serotypes inhibited the homologous and heterologous enzymes by 94–100%.

Introduction

The formation of cariogenic dental plaque by the mutans groups of oral streptococci requires adherent insoluble polysaccharides (Gibbons & Nygaard, 1968; Mukasa & Slade, 1973b). Mutans streptococci synthesize these polysaccharides from sucrose by the cooperative action of glucosyltransferases (GTFs) (Carlsson, 1970; Mukasa & Slade, 1974; Ciardi et al., 1977; Mukasa et al., 1979; Fukushima et al., 1981; Ciardi, 1983). In *Streptococcus cricetus* (mutans group serotype $a$), *Streptococcus sobrinus* (mutans group serotypes $d$ and $g$) and *Streptococcus downei* (mutans group serotype $h$), three kinds of GTF are secreted: GTF-I, which synthesizes an insoluble and essentially linear 1,3-$\alpha$-glucan; GTF-S1, which synthesizes a soluble and highly 1,3,6-$\alpha$-branched 1,6-$\alpha$-glucan; and GTF-S2, which synthesizes a soluble 1,6-$\alpha$-glucan with small amounts of 1,3-$\alpha$-linked and 1,3,6-$\alpha$-branched glucose (Fukui et al., 1982; Shimamura et al., 1983; Tsumori et al., 1983b; Koga et al., 1983; McCabe, 1985; Mukasa, 1986). In *Streptococcus rattus* (mutans group serotype $b$) and *Streptococcus mutans* (mutans group serotypes $c$, $e$ and $f$), adherent insoluble polysaccharide is synthesized cooperatively by two GTFs: GTF-I, which synthesizes an insoluble glucan, and GTF-S, which synthesizes a soluble glucan; and probably fructosyltransferases, which synthesize an inulin-type fructan (Scales et al., 1975; Mukasa et al., 1982b, 1985, 1989; Kenny & Cole, 1983; Mukasa, 1986; Shimamura et al., 1987; Kumada et al., 1987; Tsumori et al., 1989).

The cell-surface polysaccharide antigens of *S. cricetus*, *S. sobrinus* and *S. downei* exhibit immunological partial identity (Mukasa & Slade, 1973a; Linzer & Slade, 1974; Iacono et al., 1975; Beighton et al., 1981; Okahashi et al., 1984), and although the three GTFs (GTF-I, GTF-S1 and GTF-S2) from one mutans group species are immunologically distinct (Shimamura et al., 1983; Tsumori et al., 1985b; Mukasa, 1986), corresponding GTFs from different mutans group species are immunologically related (Fukui et al., 1974, 1983; Tsumori et al., 1983a, 1985a, b).

In this study, GTF-I enzymes from the three mutans group species (four serotypes), which are immunologically related on the basis of cell-surface polysaccharide
antigens (Mukasa & Slade, 1973a; Linzer & Slade, 1974; Iacono et al., 1975; Beighton et al., 1981; Okahashi et al., 1984), were compared in detail, using specific antisera against the purified enzymes.

**Methods**

**Organisms and culture conditions.** *S. cricetus* strain HS6 (mutans group serotype a) and *S. sobrinus* strains B13 (mutans group serotype d) and 6715 (mutans group serotype g) were obtained from Dr H. D. Slade (Northwestern University Medical and Dental Schools, Chicago, Ill., USA). *S. downei* strain MFC28 (mutans group serotype h) was obtained from Dr R. R. B. Russell (Dental Research Unit, Royal College of Surgeons of England, Kent, UK). These strains were cultured in 21 synthetic medium (Terleckyj et al., 1975) with fructose instead of glucose, at 37°C for 6 h, in the presence of 0.05% Tween 80 (Umesaki et al., 1977; Wittenberger et al., 1978) and 0.1 mM-phenylmethylsulphonyl fluoride (Russell, 1981; Russell et al., 1983). When culture pH was 5.0–5.3, merthiolate was added to a final concentration of 0.01%, and the cells were removed by 30 min centrifugation at 5500 g and 5°C. OD550 of harvested cultures was 3.0–4.1.

**Purification of GTF-I.** GTF-I was purified from culture supernatant fluids as previously reported (Tsumori et al., 1989). Briefly, the culture supernatant fluids were dialyzed with two vols distilled water to adsorb the ion strength. The enzymes in the dialyzed fluids were adsorbed on 24 ml wet hydroxyapatite (Bio-Gel HT, Bio-Rad Laboratories) and eluted with 200 ml of a linear gradient of 10-40 mM-sodium phosphate buffer (pH 6.5). The active fractions were combined and diluted 6–8 times with distilled water. The enzymes in these preparations were adsorbed on 24 ml DEAE-Toyopearl (Tosoh Corporation) and eluted with 200 ml of a linear gradient of 0-200 mM-NaCl in 10 mM-sodium phosphate buffer (pH 6.5). The enzyme-containing fractions were combined and dialysed against 3 mM-sodium phosphate buffer (pH 6.5). The enzyme-containing fractions were pooled and stored at −40°C.

**Enzyme assay.** The enzyme activity was measured as described by Mukasa et al. (1979). The reaction mixture contained 0.1 M-sodium phosphate buffer (pH 6.5), 41.8 mM-sucrose, 0.34 mg dextran T10, 0.01% Merthiolate and 1–20 μl enzyme solution in a total volume of 1 ml, and was incubated at 37°C for 2–6 h. The enzyme activity was also measured in the presence of antiserum (0–20 μl). In this case, the enzyme preparations (0.0185 i.u.) were preincubated with or without antiserum for 10 min at room temperature before the addition of the other components. These were done with a single batch of each enzyme.

The release of reducing sugar from sucrose was measured by the method of Somogyi (1945), using fructose as a standard. One unit (i.u.) of reducing-sugar-release activity is defined as the amount of enzyme releasing 1 μmol of reducing sugar from sucrose per min at 37°C.

Insoluble glucan was collected by centrifugation and washed with distilled water. Soluble glucan was obtained as 75% (v/v) ethanol precipitates (Mukasa et al., 1979). These glucans were measured by the phenol/sulphuric acid method (Dubois et al., 1956), using glucose as a standard. One unit (i.u.) of glucosyltransferase activity is defined as the amount of enzyme catalysing the incorporation of 1 μmol glucose from sucrose into glucan per min at 37°C.

**Electrophoretic analyses.** Analytical isoelectric focusing (IEF) was done by the method of Mukasa et al. (1982a), in the presence of 0–5% Triton X-100, and calibrated using the Low pI Calibration Kit (Pharmacia). After focusing, the protein was stained with Coomassie Brilliant Blue R-250. GTF-I activity was detected as a white band after incubation of the focused gel in a sucrose-containing buffer at 37°C for 2–15 h. The band was not stainable by the PAS procedure, but was stained when the glucan was synthesized in the presence of the extraneous dextran T10. The pl values quoted are means from triplicate experiments.

SDS–PAGE was done by the method of Weber & Osborn (1969), and M, was estimated by using Bio-Rad high-molecular-mass marker protein standards (Bio-Rad Laboratories). Protein was stained with Coomassie Brilliant Blue R-250, and GTF activities were detected as described above, after incubation of the gel in the sucrose-containing buffer with 1% (v/v) Triton X-100 (Russell, 1979; Mukasa et al., 1982a). The M, values quoted are means from triplicate experiments.

**Immunological procedure.** Antisera against the purified GTF-I enzymes from mutans group serotypes a, d, g, and h strains were prepared by immunization of male New Zealand White rabbits as previously reported (Mukasa & Slade, 1973a). Double immunodiffusion tests were done by the method of Ouchterlony (1958) using 0.5% agarose gel.

The anti-*S. downei* GTF-I serum free of antibodies specific to the mutans group serotype h GTF-I-specific determinant was prepared as follows. Anti-*S. sobrinus* GTF-I serum (0.2 ml) was applied to an Immobilized Protein A (Pierce) column (0.3 × 1.4 cm), which had been equilibrated with 0.1 mM-sodium phosphate buffer (pH 7.2). The anti-*S. sobrinus* GTF-I column was washed 5 times with the same buffer (0.1 ml each) and loaded with 0.5 ml purified GTF-I from *S. downei*, then washed 5 times with the same buffer again (0.1 ml each). *S. downei* epitopes (the GTF-I determinant common to mutants group serotypes a, d, g, and h and the GTF-I determinant shared by serotypes d, g, and h) were thereby masked by the anti-*S. sobrinus* serum free of antibodies specific to the *S. downei* GTF-I antibodies adsorbed on the column. Anti-*S. downei* GTF-I serum (250 μl) was then applied to the column. Antibodies specific to *S. downei* GTF-I in the antiserum were adsorbed to the column by a, d, g, and h epitopes of the GTF-I. A double immunodiffusion test indicated that the unadsorbed fraction of the serum was then free of the *S. downei* GTF-I-specific antibodies.

**Results**

**Purification and electrophoretic properties of GTF-I enzymes**

The *S. cricetus*, *S. sobrinus* serotypes d and g and *S. downei* GTF-I enzymes were purified from culture supernatant fluids using three sequential steps: hydroxylapatite chromatography, DEAE-Toyopearl chromatography and preparative IEF. The isoelectric points of GTF-I enzymes were estimated to be 5.2, 4.8, 4.9 and 4.9, respectively (Fig. 1). These values were identical with those of GTF-I enzymes in the culture supernatant fluids (data not shown; Tsumori et al., 1983b). The M values of GTF-I enzymes from *S. sobrinus* serotypes d and g and *S. downei* serotype h strains were the same; estimated as 161000 by SDS-PAGE and higher than that for *S. cricetus* serotype a GTF-I enzyme (150000) (Fig. 2). These M values were also identical to those for GTF-I enzymes in culture supernatant fluids (data not shown).
Immunological relationships between GTF-I enzymes

The reactivity of the GTF-I enzymes with antisera raised against individual GTF-I enzymes purified from mutants group serotype $a$, $d$, $g$ and $h$ strains was investigated by immunodiffusion analysis. The $S. sobrinus$ serotype $d$ GTF-I was immunologically identical with the $S. sobrinus$ serotype $g$ GTF-I, showing completely fused precipitin lines (Fig. 3a–d). When reacted with an anti-$S. cricetus$ serotype $a$ GTF-I serum, the $S. sobrinus$ serotypes $d$ and $g$ GTF-I enzymes and $S. downei$ serotype $h$ GTF-I showed fused precipitin lines (Fig. 3a) without a spur. On the other hand, the $S. cricetus$ GTF-I and $S. sobrinus$ serotype $d$ GTF-I showed no fusion. However, the $S. sobrinus$ serotype $g$ GTF-I showed completely fused precipitin lines (Fig. 3a–d).
downei GTF-I formed precipitin lines with a spur with the anti-S. sobrinus serotype d GTF-I serum (Fig. 3b); furthermore, the S. cricetus GTF-I and S. sobrinus serotypes d and g GTF-I enzymes formed precipitin lines with a spur with the anti-S. downei GTF-I serum (Fig. 3d). This indicated that the GTF-I enzymes from mutans group serotype a, d, g and h strains had a common antigenic determinant and that the mutans group serotype d, g and h GTF-I enzymes shared an additional determinant.
**Effect of antisera on GTF-I activity**

The antisera raised against GTF-I enzymes from mutans group serotypes \( a, d, g \) and \( h \) inhibited the GTF-I activities from four strains by 95 to 100% (Table 1). Antiserum specific to the GTF-I determinant common to all four serotypes and the GTF-I determinant shared by serotypes \( d, g \) and \( h \) was prepared by removing serotype \( h \) GTF-I-specific antibodies from anti-\( S.\ downei \) GTF-I serum, as described in the Methods (Fig. 3e). Antiserum free of serotype \( h \) GTF-I-specific antibodies inhibited GTF-I activities by 94 to 100% (Table 1).

**Discussion**

GTF-I enzymes were purified from the culture supernatant fluids of \( S.\ cricetus \) (mutans serotype \( a \)), \( S.\ sobrinus \) (serotypes \( d \) and \( g \)) and \( S.\ downei \) (serotype \( h \), using conditions to prevent their proteolytic degradation (Russell, 1981; Russell et al., 1983), and were compared both immunologically and physicochemically. Double immunodiffusion tests revealed immunological relationships between GTF-I enzymes of strains from mutans group serotypes \( a, d, g \) and \( h \). The GTF-I enzymes from the four mutans group serotypes each contained species-specific determinants and a common determinant (Fukui et al., 1983; Tsumori et al., 1985a). The \( S.\ sobrinus \) serotype \( d \) and \( g \) GTF-I enzymes were immunologically identical with each other. In addition, the GTF-I enzymes from mutans group serotypes \( d, g \) and \( h \) strains shared an epitope which was not found in the \( S.\ cricetus \) GTF-I enzyme.

The GTF-I enzymes from serotypes \( d, g \) and \( h \) showed the same \( M_t \) (161 000) by SDS-PAGE and the isoelectric points (4.8–4.9) were similar. In contrast, the \( S.\ cricetus \) GTF-I enzyme had a lower \( M_t \) (150 000) and higher isoelectric point (5.2) than those of GTF-I enzymes from serotypes \( d, g \) and \( h \). The differences in the \( M_t \), values in the present and previous studies (Tsumori et al., 1985a) are due to the use of cylindrical gels in which the purified enzyme and \( M_t \) marker proteins were electrophoresed, separately. The lower \( M_t \) and the higher isoelectric point of the \( S.\ cricetus \) GTF-I may be due to the lack of a portion containing the determinant shared by the GTF-I enzymes of serotypes \( d, g \) and \( h \). It would be interesting to know the amino acid sequences to clarify the similarities among these enzymes.

\( S.\ cricetus \) (mutans group serotype \( a \)) and \( S.\ sobrinus \) (serotypes \( d \) and \( g \)) were classified as one taxonomic group on the basis of inhibition studies of GTF activities by anti-GTF sera (Smith & Taubman, 1977). GTF-I activities of individual serotype \( a, d, g \) and \( h \) strains have been previously shown to be inhibited by homologous and heterologous antisera (Mukasa & Slade, 1973b; Linzer & Slade, 1976; Fukui et al., 1983; Yamashita et al., 1989). In the present study, antiserum specific to both the common antigenic determinant of GTF-I enzymes from serotypes \( a, d, g \) and \( h \), and that shared by GTF-I enzymes from strains of serotypes \( d, g \) and \( h \), but free of the serotype \( h \) GTF-I-specific antibodies inhibited GTF-I activities of strains of serotypes \( a, d, g \) and \( h \) by 94–100% (Table 1). These results indicated that the portion of the GTF-I containing the common determinant was essential for glucan synthesis.

Monoclonal antibodies have been raised against the GTF-I enzyme from \( S.\ sobrinus \), and they inhibit GTF-I activity (Furuta et al., 1983; McCabe et al., 1987; Ochiai et al., 1990). Although monoclonal antibodies capable of specifically inhibiting GTFs would be powerful tools for dissecting the pathway of glucan synthesis in the mutans streptococci, polyclonal antibodies are still simple and useful tools for investigating the immunological properties of their antigens.

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**Table 1. Inhibition of GTF-I activities by anti-GTF-I sera**

<table>
<thead>
<tr>
<th>GTF-I purified from Serotype</th>
<th>S. cricetus HS6(a)</th>
<th>S. sobrinus B13(d)</th>
<th>S. sobrinus 6715(g)</th>
<th>S. downei MFe28(h)</th>
<th>S. downei MFe28(h)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>( S.\ cricetus ) HS6 ( a )</td>
<td>99</td>
<td>99</td>
<td>96</td>
<td>99</td>
<td>97</td>
</tr>
<tr>
<td>( S.\ sobrinus ) B13 ( d )</td>
<td>97</td>
<td>100</td>
<td>99</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>( S.\ sobrinus ) 6715 ( g )</td>
<td>96</td>
<td>98</td>
<td>98</td>
<td>99</td>
<td></td>
</tr>
<tr>
<td>( S.\ downei ) MFe28 ( h )</td>
<td>97</td>
<td>97</td>
<td>98</td>
<td>95</td>
<td>94</td>
</tr>
</tbody>
</table>

* Antiserum free of serotype \( h \) GTF-I-specific antibody was used.
† All assays were performed with a single batch of each purified enzyme; 0.0185 i.u. was used in each assay. The inhibition shown is the maximum recorded with up to 20 μl antiserum.


