Comparative Study of *Streptococcus mutans* Extracellular Glycosyltransferases by Isoelectric Focusing

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Extracellular glycosyltransferases from 17 strains of *Streptococcus mutans* were examined by analytical isoelectric focusing. Three kinds of glucosyltransferase: highly-branched-1,6- α -D-glucan synthetase, 1,3- α -D-glucan synthetase and 1,6- α -D-glucan synthetase, were excreted from serotype *a*, *d* and *g* strains. The enzymes of serotype *a* strains were distinguishable from those of serotypes *d* and *g* by differences in their pI values. Serotype *c*, *e* and *f* strains excreted basic glucosyltransferase and acidic fructosyltransferase. Serotype *b* strains also excreted the glucosyl- and fructosyltransferases, but the pI values were different from those of the enzymes from the other serotypes. Thus, *S. mutans* strains could be divided into four groups by analytical isoelectric focusing of glycosyltransferases which corresponded well to the four genetic groups.

INTRODUCTION

Streptococcus mutans is a cariogenic micro-organism which secretes glucosyltransferases and, in some serotypes, fructosyltransferase (Carlsson, 1970; Schachtele *et al.*, 1975; Ciardi *et al.*, 1976). Highly-branched-1,6- α -D-glucan synthetases have been purified from *S. mutans* 6715 (serotype *g*; pI 4.1) (Shimamura *et al.*, 1982) and HS6 (serotype *a*; pI 4.2 and 4.5) (Tsumori *et al.*, 1983). A basic glucosyltransferase (pI 8.5) (Mukasa *et al.*, 1982*b*) and an acidic fructosyltransferase (pI 4.6) (Carlsson, 1970) have been purified from *S. mutans* serotype *c* strains. 1,3- α -D-glucan synthetases have also been purified from *S. mutans* B13 (serotype *d*) (Fukushima *et al.*, 1981) and strain 6715 (Fukui *et al.*, 1982). By the cooperative action of these enzymes, a sticky, water-insoluble polysaccharide is synthesized from sucrose which is considered to be a major factor in the accumulation of *S. mutans* on smooth tooth surfaces (Gibbons & Nygaard, 1968; Mukasa & Slade, 1973*c*).

Strains of *S. mutans* have been classified into seven serotypes on the basis of cell wall polysaccharide antigens (Bratthall, 1972; Perch *et al.*, 1974). Certain similarities among these serotypes have been reported based on DNA–DNA hybridization (Coykendall, 1974), SDS–PAGE of whole cell proteins (Russell, 1976) and biochemical traits (Shklair & Keene, 1974).

In this paper, we have analysed the distribution of extracellular glycosyltransferases among the seven *S. mutans* serotypes by analytical isoelectric focusing over a wide pH range. Four patterns were obtained that support the four groups of serotypes. (A preliminary report of this work was given at the 54th Annual Meeting of the Japanese Biochemical Society, Sendai, Japan, October, 1981).

METHODS

Strains and culture conditions. The strains of *S. mutans* used in this study were: HS6, E49, OMZ61 (serotype *a*); FA1, BHT (*b*); Ingbritt, C67-1, MT6R (*c*); B13 (*d*); LM7, MT703 (*e*); SE11, OMZ175 (*f*); 6715, K1R, OMZ65, AHT (*g*). Strains HS6, OMZ61, FA1, BHT, B13, 6715 and K1R were kindly provided by Dr H. D. Slade (Department of Oral Biology, School of Dentistry, University of Colorado Health Science Center, Denver, Colo.).

Strain C67-1 was a gift from Dr T. Ogawa (Department of Microbiology, Josai Dental University, Saitama, Japan). The other strains were kindly provided by Dr S. Hamada (Department of Dental Research, National Institute of Health, Shinagawa-ku, Tokyo, Japan).

With the exception of strains FA1, BHT, SE11 and OMZ175, bacteria were grown for 10 to 15 h at 37 °C in a chemically defined medium (10 ml) (Terleckyj *et al.*, 1975) supplemented with 0.1% (w/v) Tween 80 for the strains of serotypes *a*, *d* and *g* and with 1% (w/v) Tween 80 for the strains of serotypes *c* and *e*. *Streptococcus mutans* FA1, BHT, SE11 and OMZ175 were grown for 16 h at 37 °C in Todd-Hewitt broth (1 litre) treated with invertase and fortified with fructose and salts (Mukasa *et al.*, 1979). The cultures were all harvested at late-exponential phase when the pH reached 5.0. The turbidity of the cultures at 550 nm was between 4.0 and 5.0. The cell-free culture supernatants obtained by centrifugation for 15 min at 1200 *g* were used directly as glycosyltransferase sources for the strains of serotypes *a*, *d* and *g*. For serotypes *c* and *e*, the cell-free culture supernatants were concentrated to 1 ml with a mini-module equipped with a holotube (1.2 m × 0.8 mm, molecular weight limit: 13000, Asahi Chemical Industry, Tokyo, Japan) under reduced pressure (Mukasa *et al.*, 1982*b*). For strains FA1, BHT, SE11 and OMZ175, 40% (v/v) ethanol precipitates obtained from the cell-free culture supernatants were suspended in 20 ml distilled water, and the insoluble materials were removed by centrifugation for 15 min at 38000 *g*.

Analytical isoelectric focusing. A horizontal, polyacrylamide slab gel containing 1% (w/v) Triton X-100 was used for analytical isoelectric focusing (Mukasa *et al.*, 1982*a, b*). Enzyme solution (1 to 10 µl) was applied with a piece of glass fibre filter paper. After the focusing, the gel was incubated in 0.1 M-sodium phosphate buffer, pH 6.5, containing sucrose (43.9 mM), dextran T10 (0.34 mg ml⁻¹) and merthiolate (0.01%, w/v) for 15 h at 37 °C. Raffinose (43.9 mM), recrystallized twice from 60% (v/v) ethanol, was used to indicate fructosyltransferase activity (Carlsson, 1970). Polysaccharides, which accumulated in the gel during incubation, were stained by the periodic acid-Schiff base method (Kapitany & Zebrowski, 1973) omitting the first fixing step which uses 12.5% (w/v) TCA.

Materials. Yeast invertase (390 i.u. ml⁻¹ at 20 °C) was obtained from Wako Pure Chemical Industry, Osaka, Japan. Ampholines and Dextran T10 were purchased from LKB and Pharmacia, respectively. All other chemicals were of analytical or reagent grade.

RESULTS

Extracellular glycosyltransferases from *S. mutans* were isoelectrofocussed on a horizontal slab gel. After incubating the focused gel for 15 h at 37 °C in a sucrose solution, highly-branched-1,6- α -D-glucan synthetase activities were detected as transparent, bulgy bands and 1,3- α -D-glucan synthetase activities as white opaque bands on the gel.

Figure 1 shows focused patterns of our collection of purified glycosyltransferases from *S. mutans* strain 6715 (pI 4.1, Shimamura *et al.*, 1982; pI 3.9, unpublished), Ingbritt (pI 8.5, Mukasa *et al.*, 1982*b*) and HS6 (pI 4.2 and 4.5, Tsumori *et al.*, 1983) and a fructosyltransferase (pI 4.6, Mukasa *et al.*, 1982*b*). These enzymes were clearly distinguishable from each other in the gel according to their pI values. The focused pattern of glycosyltransferases from HS6 (*a*) grown in Todd-Hewitt broth was almost identical with the pattern from the same strain grown in a chemically defined medium (Fig. 2, lanes 1 and 2). The pattern of enzymes from Ingbritt (*c*) was also unaffected by growth conditions (lanes 5 and 6). However, as previously reported (Shimamura *et al.*, 1983), the excretion of 1,6- α -D-glucan synthetase from HS6 (*a*) was remarkably enhanced with increasing concentrations of Tween 80 (lanes 3 and 4). Since excessive excretion of this enzyme complicates a comparative study of glycosyltransferases, the lower concentration (0.1%, w/v) of Tween 80 was added to the growth medium for serotypes *a*, *d* and *g* in this study. For serotypes *c*, and *e*, the higher concentration (1%, w/v) of Tween 80 was added to the medium, since enhancement of the excretion of glycosyltransferase was not so marked (Fig. 2, lane 7).

Glycosyltransferases from serotype a, d and g strains

The focused patterns of glycosyltransferases from eight strains of the serotypes *a*, *d* and *g* are shown in Fig. 3. Highly-branched-1,6- α -D-glucan synthetases were detected at pH 4.2 and 4.5 (serotype *a* strains), at pH 4.0 and 4.2 (*d*) and at pH 3.9 and 4.1 (*g*) (Fig. 3*a*). 1,3- α -D-glucan synthetases were also detected at pH 5.2, 4.8 and 4.9 for serotypes *a*, *d* and *g*, respectively (Fig. 3*b*). After incubation in a sucrose solution and staining with the periodic acid-Schiff base method, another kind of glycosyltransferase from 6715 (*g*) was detected on the gel at pH 5.5 and

Fig. 1. Isoelectric focusing patterns of *S. mutans* purified glycosyltransferase activities stained using the periodic acid-Schiff base method. Lanes 1 to 8: *S. mutans* HS6 (serotype *a*) glucosyltransferase, pI 4.5; HS6 glucosyltransferase, pI 4.2; 6715 (*g*) glucosyltransferase, pI 4.1; 6715 glucosyltransferase, pI 3.9; mixture of the purified glucosyltransferases from *S. mutans* HS6 and 6715; Ingbritt (*c*) basic glucosyltransferase, pI 8.5; Ingbritt fructosyltransferase, pI 4.6; mixture of the basic glucosyltransferase and fructosyltransferase.

5.8. The enzyme was not activated by exogenous dextran and excretion into the culture medium was greatly enhanced by Tween 80. From analysis of its product, the enzyme was identified as a 1,6- α -D-glucan synthetase (Shimamura *et al.*, 1983). 1,6- α -D-glucan synthetases were also detected at pH 4.8, 4.9 and 5.6 from serotype *a*, and at pH 5.4, 5.6 and 5.8 from serotype *d* (Fig. 3*a*). Thus, the focused pattern of the enzymes from the serotype *d* strain was very close to those of serotype *g* strains (Fig. 3*a*), and the patterns of serotype *a* strains were clearly distinguishable from those of serotypes *d* and *g* (Fig. 3*a*). The focused pattern of AHT (*g*) was intermediate between the pattern of serotype *d* and that of serotype *g*. The highly-branched-1,6- α -D-glucan synthetase was focused in the same position as that of serotype *d* (Fig. 3*a*), the 1,3- α -D-glucan synthetase band coincided with those of the other strains of serotype *g* (Fig. 3*b*), and the focused bands of 1,6- α -D-glucan synthetase coincided in position with those of both serotypes *d* and *g*. No fructosyltransferase activity was detected under the assay conditions employed.

Fig. 2. Effect of growth conditions on isoelectric focusing patterns of glycosyltransferases from *S. mutans* serotypes *a* and *c*. Crude enzyme preparations were used from HS6 (lanes 1 to 4) and Ingbritt (lanes 5 to 7) grown in modified Todd-Hewitt broth (lanes 1 and 5) and grown in chemically defined medium containing 0% (lanes 2 and 6), 0.1% (w/v) (lane 3) and 1% (w/v) (lanes 4 and 7) Tween 80. Pieces of glass fibre filter paper were put on the gel surface and the enzyme solutions were applied to them (□).

Glycosyltransferases from serotype b strains

The focused patterns of glycosyltransferases from serotype *b* strains are shown in Fig. 4. The staining of the focused gel revealed two or three active bands (pI 4.0, 4.6 and 4.8) using sucrose as substrate (Fig. 4*a*). Fructosyltransferase activity was identified using raffinose as substrate. The position of the fructosyltransferase band corresponded to that of the sucrose-active band at pH 4.0. These active bands did not coincide in position with the purified enzymes from serotypes *a*, *c* and *g* (Fig. 4).

Glycosyltransferases from serotype c, e and f strains

Figure 5 shows the focused patterns of glycosyltransferases from the seven strains of serotypes *c*, *e* and *f*. All strains produced basic glucosyltransferase (pI 8.5–8.6) and acidic fructosyltransferase (pI 4.5–4.6), as reported previously from Ingbritt (*c*) (Mukasa *et al.*, 1982*b*). The pronounced tailing of fructosyltransferase bands on gels could be due to insufficient dissociation of aggregated forms by Triton X-100 (Fig. 5, lanes 2, 3 and 5 to 7). The other glycosyltransferase activity bands were detected in multiple forms at pH 7 to 8 (Fig. 5) and most of these components disappeared during prolonged storage at 4 °C.

Fig. 3. Isoelectric focusing patterns of glycosyltransferases from *S. mutans* serotypes *a*, *d* and *g*: (a) stained using the periodic acid-Schiff base method; (b) white opaque bands of 1,3- α -D-glucan. Lanes 1 to 9: mixture of the purified glycosyltransferases from *S. mutans* HS6 and 6715, crude enzyme preparations from HS6 (a), E49 (a), OMZ61 (a), B13 (d), AHT (g), 6715 (g), K1R (g) and OMZ65 (g), respectively.

Fig. 4. Isoelectric focusing patterns of glycosyltransferases from *S. mutans* serotype *b*. Activities were stained using the periodic acid-Schiff base method: (a) sucrose as a substrate; (b) raffinose as a substrate. Lanes 1 to 4: mixture of the purified glycosyltransferases from *S. mutans* HS6 and 6715, crude enzyme preparations from FA1 and BHT, and mixture of the purified basic glycosyltransferase and fructosyltransferase from *S. mutans* Ingbritt, respectively.

DISCUSSION

Extracellular glycosyltransferases of different *S. mutans* strains were separated on horizontal slab gels by isoelectric focusing. The nature of the enzymes and their pI values were determined by comparison with previously purified enzymes; namely highly-branched-1,6- α -D-glucan synthetase, 1,6- α -D-glucan synthetase, 1,3- α -D-glucan synthetase, basic glycosyltransferase and fructosyltransferase from *S. mutans* serotypes, *a*, *c* or *g*. Recently, we found a 1,6- α -D-glucan synthetase in multiple forms (pI 5.5 and 5.8) from 6715 (Shimamura *et al.*, 1983), and this enzyme was also used as one of the glycosyltransferase markers. Highly-branched-1,6- α -D-glucan synthetase, 1,6- α -D-glucan synthetase and 1,3- α -D-glucan synthetase were immunologically distinct from each other (Shimamura *et al.*, 1983). All of the serotype *d* and *g* strains excreted highly-branched-1,6- α -D-glucan synthetase, 1,6- α -D-glucan synthetase and 1,3- α -D-glucan synthetase with slight differences of pI values among *d* and *g* strains. Guggenheim & Newbrun (1969) obtained by preparative isoelectric focusing, pI 4.24, 5.00 and 5.65 components

Fig. 5. Isoelectric focusing patterns of glycosyltransferases from *S. mutans* serotypes *c*, *e* and *f* stained using the periodic acid-Schiff base method. Lanes 1 to 8: mixture of the purified basic glucosyltransferase and fructosyltransferase from *S. mutans* Ingbritt, crude enzyme preparations from Ingbritt (*c*), C67-1 (*c*), MT6R (*c*), LM7 (*e*), MT703 (*e*), SE11 (*f*) and OMZ175 (*f*), respectively.

of 1,3- α -D-glucan synthetase from OMZ176 (*d*), of which the pI 5.00 component is compatible with the pI 4.8 1,3- α -D-glucan synthetase in this study. Bulkacz & Hill (1977) also detected pI 4.6, 5.0 to 5.2 and 5.6 components of glucosyltransferases from strain 6715. It is impossible to correlate these with the three kinds of glucosyltransferases in this study, since their enzymological properties have not been described. Recently, *S. mutans* serotype *h* was isolated from the dental plaque of monkeys (Beighton *et al.*, 1981). Since immunological properties and SDS-PAGE patterns have shown that serotype *h* strains were closely related to strains of genetic group III (i.e. serotypes *d* and *g*), it is possible that the glucosyltransferases from serotype *h* strains might exhibit a focused pattern similar to those of serotypes *d* and *g*.

Serotype *a* strains also excreted highly-branched-1,6- α -D-glucan synthetase (pI 4.2 and 4.5), 1,3- α -D-glucan synthetase (pI 5.2) and 1,6- α -D-glucan synthetase which focused at pH 4.8, 4.9 and 5.6 (Fig. 3*a*). There are certain differences between serotype *a* and serotype *d* and *g* glucosyltransferases: the 1,3- α -D-glucan synthetase from serotype *a* had a higher pI value (5.2) than those of serotypes *d* and *g* (4.8 and 4.9, respectively), while 1,6- α -D-glucan synthetase from serotype *a* had lower pI values (4.8 and 4.9) than those of serotypes *d* and *g* (5.4 to 5.8).

Streptococcus mutans serotype *b* showed a unique focused pattern of a glucosyltransferase (pI 4.6 and 4.8) and a fructosyltransferase (pI 4.0) (Fig. 4). A high molecular weight (> 800 000) enzyme aggregate with glucosyl- and fructosyltransferase activities was isolated by Scales *et al.* (1975). The two activities were resolved during PAGE in the presence of Tween 80 (Figures &

Edwards, 1979) and these correspond to the glucosyltransferase (pI 4.6) and the fructosyltransferase (pI 4.0) separated by isoelectric focusing in the present study. The glucosyltransferase activity was detected as a transparent bulgy band on the gel in this study. Therefore, the glucan synthesized could be expected to contain a considerable amount of 1,6- α -D-glucose residues. Although serotype *b* strains could synthesize insoluble polysaccharide during growth in sucrose-containing media, white opaque bands due to 1,3- α -D-glucan synthetase activity were not detected on the gels. Therefore, the insoluble polysaccharide could have been synthesized either by an undetectable amount of 1,3- α -D-glucan synthetase or by the cooperative action of the glucosyltransferase and the fructosyltransferase.

A basic glucosyltransferase (pI 8.5) and an acidic fructosyltransferase (pI 4.5) have been purified from strain Ingbritt (*c*) (Fig. 1, lanes 6 and 7) (Mukasa *et al.*, 1982*b*). The other strains from serotypes *c*, *e* and *f* also produced the basic glucosyltransferase (pI 8.5 to 8.6) and the acidic fructosyltransferase (pI 4.5 to 4.6) (Fig. 5, lanes 2 to 7). Activity bands focused in the range of pH 7 to 8 disappeared during prolonged storage at 4 °C and during the purification steps, possibly by changing into the basic glucosyltransferase, as suggested by Mukasa *et al.* (1982*b*). The glucosyltransferases have been shown to be antigenically identical within serotypes *c*, *e* and *f* (Russell, 1979). Therefore, one kind of basic glucosyltransferase is probably excreted in multiple forms with different pI values from the strains of serotypes *c*, *e* and *f*, but the reason for the different pI values is unknown. Mohan *et al.* (1979) showed that soluble and insoluble glucans were synthesized by interconvertible forms of a glucosyltransferase from serotype *c* strains; the aggregated form of the enzyme synthesized 1,3- α -D-glucan in the presence of 1.55 M-ammonium sulphate and the dissociated form synthesized 1,6- α -D-glucan (Newman *et al.*, 1980). It is not clear which enzyme focused on the gel in the present study would correspond to the enzyme that they prepared.

Serotype *c*, *e* and *f* antigens are composed of glucose and rhamnose (Linzer *et al.*, 1976; Hamada & Slade, 1976; Hamada *et al.*, 1976), *b* antigen contains rhamnose and galactose (Mukasa & Slade, 1973*a*), and *a*, *d* and *g* antigens contain galactose and glucose (Mukasa & Slade, 1973*b*; Linzer & Slade, 1974; Iacono *et al.*, 1975). The *a* antigen is immunologically partially identical with *d* and *g* antigens. Coykendall (1974) has divided *S. mutans* into four genetic groups and also reported that *S. mutans a* and *d* (*g*) strains were genetically partially identical. Relationships between *S. mutans* serotypes have been reported as $d > a \gg b > c$ based on the electrophoretic mobility of mannitol-1-phosphate dehydrogenase (Brown & Patterson, 1972), $a = d = g \gg b > c = e = f$ based on antigenic similarity of glucosyltransferases (Smith & Taubman, 1977), and $a, b, d \gg c, e, f, g$ according to a comparative study of intracellular invertases (Tanzer *et al.*, 1977). The data presented here show that the four groups are also apparent from isoelectric focusing patterns of extracellular glycosyltransferase activity.

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