Three kinds of extracellular glucosyltransferases from *Streptococcus mutans* 6715 (serotype *g*)

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In addition to the 1,3-α-D-glucan synthetase (pI 4.9) and the highly-branched 1,6-α-D-glucan synthetase (pI 3.9–4.1), *Streptococcus mutans* 6715 (serotype *g*) was found to secrete the third glucosyltransferase in multiple forms (pI 5.5–7.0), which exhibited 87% 1,6-α-bond-, 6% 1,3-α-bond- and 7% 1,3,6-branch-forming activities. The production of this enzyme was extremely enhanced when the organism was grown in Tween 80-supplemented medium. The 3 glucosyltransferases from the same organism were enzymatically and immunologically distinct from each other, and they were commonly found among the serotype *g* strains.

Streptococcus mutans · *Glucosyltransferase* *Glucan* *Tween 80*

1. INTRODUCTION

Streptococcus mutans is a major cariogenic organism to colonize on smooth tooth surface [1,2], which is closely correlated to the ability to synthesize adhesive water-insoluble polysaccharides from sucrose. The insoluble glucan has been considered to be synthesized by the cooperative action of two glucosyltransferases, 1,3-α-D-glucan synthetase and water-soluble glucan synthetase secreted by the organism [3–7].

The highly-branched 1,6-α-D-glucan synthetase (sucrose: 1,6-α-D-glucan 3-α- and 6-α-glucosyltransferase) [8] and the 1,3-α-D-glucan synthetase (sucrose: 1,3-α-D-glucan 3-α-glucosyltransferase) [9] have been purified from *S. mutans* 6715. However, both of the two distinct enzymes have required an exogenous primer or acceptor such as dextran for the full expression of their essential activities, indicating that the two enzymes alone could not be enough to synthesize the adhesive insoluble glucan in vivo. Therefore, it is reasonable to expect the secretion of another glucosyltransferase such as dextran-sucrase from the organism and the cooperative synthesis of the insoluble glucan by these 3 enzymes. However, no paper has been reported, proving that such 3 kinds of extracellular glucosyltransferases were actually excreted by a *S. mutans* strain.

Here, we describe the finding of the third glucosyltransferase in the culture supernatant of *S. mutans* 6715 that is enzymatically and immunologically distinct from the other two glucosyltransferases previously reported.

Abbreviations: IEF, isoelectric focusing; PAS, periodic acid-Schiff base

2. MATERIALS AND METHODS

2.1. *Growth of* S. mutans *serotype* g *strains*

S. mutans 6715 obtained from Dr H.D. Slade, Department of Oral Biology, School of Dentistry, University of Colorado Health Sciences Center (Denver CO) was grown with a 5% inoculum at 37°C for 13 h in a screw cap tube containing 10 ml of the FMC medium used in [10] supplemented with 0–1% Tween 80. *S. mutans* K1R from Dr H.D. Slade and OMZ 65 from Dr S. Hamada, Department of Dental Research, National Institute

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of Health (Tokyo) were grown in sucrose-free Todd-Hewitt Broth [6]. Cell-free culture supernatants were obtained by centrifugation and cells harvested were suspended in 1 ml of distilled water after washing twice with distilled water.

2.2. *Isoelectric focusing*

Analytical isoelectric focusing (IEF) was carried out on the gel as in [8,11], with 1% Triton X-100 [12]. An 8 μl amount of culture supernatant was directly applied to a filter paper on the gel surface. After the IEF, the gel was incubated at 37°C for 22 h in 5% sucrose to locate the activities of glucosyltransferases. Water-insoluble polysaccharides were directly located as white bands, and water-soluble ones were stained by the periodic acid-Schiff base (PAS) method [13].

2.3. *Enzymological characterization of glucosyltransferases on gel*

The effects of exogenous dextran on the glucan syntheses by glucosyltransferases and the extents of hydrolysis of the product glucans with exodextranase were examined on an isoelectrofocused gel. The enzyme mixture obtained from a preparative IEF, which contained all the glucosyltransferases (pI 3.9–6.0), was used. After the analytical IEF was run in triplicates, one of the gels was incubated at 37°C for 24 h in 5% sucrose. Another gel was incubated in 5% sucrose containing 12 μM dextran T10. The last gel was incubated in 5% sucrose and then treated with 10 μg exodextranase from *Penicillium funiculosum* at 37°C for 6 h in 100 ml of 0.1 M sodium acetate buffer (pH 5.5), as used in [8]. The activity stain was described above.

2.4. *Linkage analysis of glucan*

Glucans were synthesized as in [8] except for 4 days. The enzyme preparations used were the 7 fractions of pI 5.5–7.0 and the 1,3-α-D-glucan synthetase of pI 4.9, which were obtained by a preparative IEF. The purification and properties of the enzyme in multiple forms (pI 5.5–7.0) will be reported elsewhere. Each of the glucans was collected as a 75% ethanol precipitate [8]. The glucan synthesized by the 1,3-α-D-glucan synthetase was collected as a water-insoluble glucan by centrifugation. The partially methylated glucitol acetates derived from each glucan were analyzed as in [8], except for the use of Shimazu gas–liquid chromatograph GC-6A on a WCOT column of Silicone OV-101 (20 m × 0.25 mm).

2.5. *Double immunodiffusion*

Crude enzyme preparation (CEP) was prepared from 200 ml culture supernatant of *S. mutans* 6715 by the concentration and dialysis against 0.85% NaCl. A New Zealand white rabbit was immunized with a mixture of equal volumes of the CEP and Freund incomplete adjuvant by intravenous injection as in [14]. Double immunodiffusion was developed on 0.3% agarose gel as in [15].

3. RESULTS

3.1. *Enhanced glucosyltransferase production*

Tween 80 has been reported to enhance the total production of extracellular glucosyltransferases from *S. mutans* OMZ 176 (serotype *d*) [16] and *Streptococcus salivarius* and *S. mutans* 6715 [17]. Here, the similar effect of Tween 80 was also observed in 0.1% and 1% Tween 80-supplemented media, where the growth rate of *S. mutans* 6715 cells was not remarkably affected (table 1). The enhanced production of glucosyltransferases in the culture supernatant was further analyzed on the IEF gel (fig.1). The predominantly enhanced components were located at pI 5.5–7.0, which were strongly stained with the PAS reagent after the incubation of the gel in the sucrose solution (fig.1A, lanes 4 and 5). Two components of pI 5.5 and 5.8 were major among these activities. The appreciable enhancement was also observed in the 1,3-α-D-glucan synthetase (pI 4.9) (fig.1B, lanes 4 and 5). However, the production of the highly-branched 1,6-α-D-glucan synthetase (pI 3.9–4.1) was not affected in the presence of 0–1% Tween 80 (fig.1A, lanes 1–7). Similar results were also obtained in the cases of *S. mutans* K1R (*g*) (fig.1, lanes 6 and 7) and OMZ 65 (*g*) (not shown), which were grown in 0% and 1% Tween 80-supplemented Todd-Hewitt Broth.

3.2. *Specification of glucosyltransferases*

Exogenous dextran remarkably activated the highly-branched 1,6-α-D-glucan synthetase (pI 3.9–4.1) (fig.2, lane 2). The 1,3-α-D-glucan synthetase (pI 4.9) was also activated, as found by observing the intensified white band of 1,3-α-D-glucan (not shown). However, the activities at pH

Table 1

Effect of the concentration of Tween 80 on the growth and production of glucosyltransferases of *S. mutans* 6715

[Tween 80] (%)	Growth		Glucosyltransferase activity (units/ml of culture)	
	Final pH	Turbidity	Supernatant	Washed cell
0	4.56	3.20	0.116	0
0.001	4.58	3.17	0.105	0
0.01	4.53	3.28	0.127	0
0.1	4.52	3.30	0.309	0.0039
1	4.52	3.19	0.677	0.0158

Turbidity was measured at 550 nm. One unit of activity is defined as the amount of glucosyltransferase releasing 1 μmol reducing sugar from sucrose/min [12]

5.5–5.8 on the gel were not affected by the exogenous dextran (fig.2, lane 2).

Exodextranase significantly hydrolyzed the glucans formed at pH 5.5–5.8 on the gel, while the glucans at pH 3.9–4.1 and 4.9 were slightly hydrolyzed (fig.2, lane 3), suggesting that the glucosyltransferases of pI 5.5–5.8 synthesized 1,6-α-bond-rich glucans.

3.3. *Linkage analysis of glucan*

Glucans were synthesized from sucrose by using the several enzyme fractions obtained by a preparative IEF, and the linkage structures of the glucans were analyzed (table 2). All of the 7 fractions of pI 5.5–7.0, which were free from the other synthetases of pI 3.9–4.9 on the analytical IEF gel, similarly exhibited 1,6-α-bond-forming activity

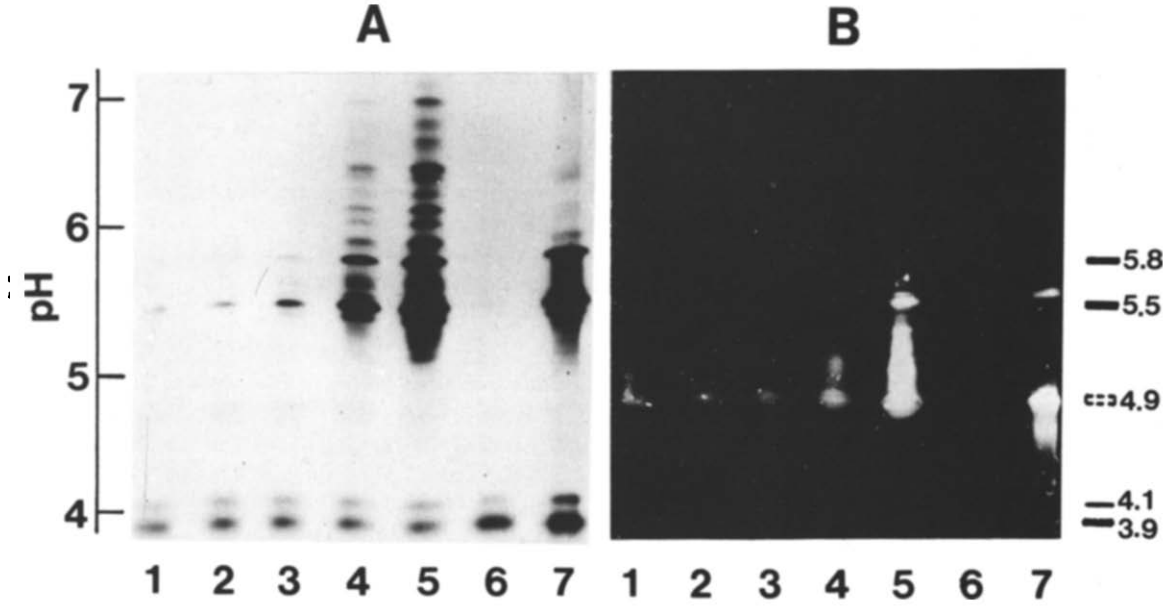

Fig.1. Activity patterns of extracellular glucosyltransferases from *S. mutans* 6715 and K1R developed on an IEF gel: (A) PAS stain for polysaccharide; (B) white precipitin band of polysaccharide. The illustration shows the characteristic bands of the serotype *g* glucosyltransferases; the appended numerals are their pI-value. The culture supernatants of *S. mutans* 6715 (lanes 1–5) and K1R (lanes 6,7) grown in 0% (lanes 1,6), 0.001% (lane 2), 0.01% (lane 3), 0.1% (lane 4) and 1% (lanes 5,7) of Tween 80-supplemented media were used.

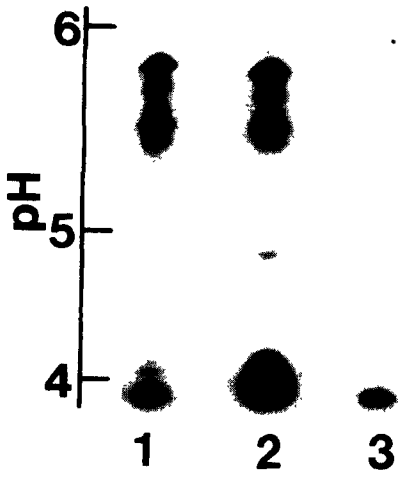

Fig.2. Enzymatic specificity of *S. mutans* 6715 glucosyltransferases. After isoelectric focusing of mixed 3 glucosyltransferases, the gels were incubated in 5% sucrose in the absence (lane 1) and presence (lane 2) of dextran T10. The other gel incubated in the absence of dextran T10 was further treated with exodextranase (lane 3).

predominantly (87%) with low 1,3-α-bond- and 1,3,6-branch-forming activities (6% and 7%, respectively). The 1,3-α-D-glucan synthetase of pI 4.9 had 92%, 1,3-α-bond- and 8% branch-forming

Fig.3. Double immunodiffusion of the purified glucosyltransferases from *S. mutans* 6715 against anti-6715 CEP serum. Each 2 μg (10 μl) of the purified enzymes of pI 5.5–7.0 (1), pI 4.9 (2) and pI 4.1 (3), and 10 μl of anti-6715 serum (center well) were used.

activities, that agreed with the finding in [9]. We had shown that the purified glucan synthetase of pI 4.1 possessed a relatively high extent of branch-forming activity (36%) with 64% 1,6-α-bond-forming activity [8]. Therefore, the glucosyltransferase under consideration in multiple forms (pI 5.5–7.0) was distinct at least in enzymatic action from the other two enzymes (pI 3.9–4.1 and pI 4.9).

3.4. *Double immunodiffusion*

These 3 enzymatically different glucosyltrans-

Table 2

Linkage analyses of glucans synthesized by the glucosyltransferase fractions from *S. mutans* 6715

pI-Value of major component in each fraction	Methylated glucitol acetate (mol%)			
	2,3,4,6-	2,4,6-	2,3,4-	2,4-
7.0	6.5	8.9	79.1	5.7
6.8	8.3	8.1	76.3	7.4
6.7	5.4	4.4	83.1	7.0
6.5	5.1	2.5	87.5	4.9
6.2	2.8	3.1	87.5	6.6
5.8	6.9	4.5	79.0	9.3
5.5	5.8	9.5	79.1	5.6
Av.	5.8	5.9	81.7	6.6
4.9[a]	3.6	88.1	0	8.2
4.1[b]	26.3	0	47.4	26.3

[a] Electrophoretically pure (M_r 180000)
[b] Cited from [8]

ferases were also immunologically clearly distinguished from each other by a double immunodiffusion test (fig.3). The fractions of pI 5.5–7.0 were antigenically perfectly identical with each other (not shown) and the fraction of pI 3.9 was antigenically identical with that of pI 4.1 (not shown).

4. DISCUSSION

This study demonstrates that the 3 kinds of glucosyltransferases are excreted from *S. mutans* 6715 (serotype *g*). The enzyme in multiple forms (pI 5.5–7.0) was enzymatically and immunologically distinguished from the previously reported glucosyltransferase (pI 3.9–4.1) with 36% branch-forming activity [8] and from the 1,3-α-D-glucan synthetase (pI 4.9) [9], and was characterized as the glucosyltransferase which exhibited 87% 1,6-α-bond-, 6% 1,3-α-bond- and 7% 1,3,6-branch-forming activities.

The glucans which were synthesized by the 3 glucosyltransferases were easily distinguishable from each other on the IEF gel. The transparent bulgy bands at pH 3.9–4.1, the white precipitin band at pH 4.9 and the slightly-opalescent bulgy bands at pH 5.5–7.0 on the gel were, respectively, weakly,negligibly and strongly positive to the PAS stain individually, possibly due to the 1,3,6-α-branch-, 1,3-α-bond- and 1,6-α-bond-rich structures. The activity of 1,6-α-bond-rich glucan synthetase (pI 5.5–7.0) was not affected by an exogenous dextran, while those of the other enzymes were extremely enhanced (fig.2). The extents of hydrolysis of the glucans with an exodextranase also depended on the structures of glucans on the gel (fig.2). Fructosyltransferase can be distinct from glucosyltransferases by using raffinose as a specific substrate of this enzyme [18]. These specifications could make it possible to easily identify the kinds of glucosyltransferases on the IEF gel. We have classified the IEF patterns of the serotypes *a–g* glucosyltransferases. All the patterns were the same within the strains of each serotype, but distinct from those of other serotypes (unpublished). The characteristic bands of the serotype *g* glucosyltransferases are illustrated in fig.1.

The glucosyltransferase (pI 5.5–7.0) enhanced in the Tween 80-supplemented medium exhibited high 1,6-α-bond-forming activity (table 2) and was not activated by an exogenous dextran. In contrast, the highly-branched 1,6-α-D-glucan synthetase (pI 3.9–4.1) and the 1,3-α-D-glucan synthetase (pI 4.9) required a primer for the expression of their activities [8,9]. The glucosyltransferases in the culture supernatant of *S. mutans* 6715 grown in the presence of the surfactant have not been activated by an exogenous dextran [17]. Therefore, it is reasonable to consider that the activities of the two primer-dependent enzymes (pI 3.9–4.1 and pI 4.9) were primed and further accelerated by the 1,6-α-bond-rich glucan synthesized by the third enzyme (pI 5.5–7.0). This enzyme may also act as one of the major members that build up the adhesive insoluble glucan.

It should be noted that the crude glucosyltransferases (pI 3.9–4.1 and pI 5.5–7.0) exist in multiple forms. For each enzyme, the isozymes of the lower pI-values were apt to change into those of the upper values during the purifications and the long time storage at 4°C. These phenomena were also found in the basic glucosyltransferase of *S. mutans* Ingbritt (serotype *c*) [12].

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