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Characterization of Extracellular Glucosyltransferase Activity of *Streptococcus mutans*

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The extracellular glucosyltransferase activity of *Streptococcus mutans* GS-5 has been resolved into two non-overlapping fractions after gel filtration chromatography on Bio Gel A-15 columns. The activity eluting in the void volume, fraction A, was highly aggregated and synthesized both soluble and insoluble glucans. The activity retarded by the resin, fraction B, synthesized only soluble glucan. Almost all of the extracellular glucosyltransferase activity was eluted in the void volume when the cells were grown in Todd-Hewitt medium. However, most of the activity migrated as the lower-molecular-weight species when cells were grown under conditions which inhibit insoluble glucan formation. The activities in both fractions had identical temperature and pH optima as well as similar K_m values for sucrose. Fraction A synthesized both α-1,3- and α-1,6-linked glucans, whereas fraction B catalyzed α-1,6-glucan formation. Fraction B has been purified to near homogeneity and is also aggregated with a subunit molecular weight of 45,000. The properties of the glucosyltransferases in both fractions are discussed in terms of the role of the enzymes in both soluble and insoluble glucan formation.

Streptococcus mutans has been implicated as a significant factor in the development of human dental plaque and subsequent caries formation (22). The adherence of this microorganism to smooth tooth surfaces is dependent on the conversion of dietary sucrose to insoluble glucans mediated by enzymes possessing glucosyltransferase (EC 2.4.1.5) activity (12). This organism is capable of converting sucrose to water-soluble as well as water-insoluble glucans. Characterization of these products revealed that the soluble glucans contain glucose primarily in α-1,6-linkages, whereas insoluble glucans possess a high degree of branching involving α-1,3-linkages (4).

Glucosyltransferases from several strains of *S. mutans* have been purified and characterized (5, 8, 13, 19). In several of these studies (8, 13, 19) evidence for multiple forms of the enzyme activity was demonstrated. It was observed that glucosyltransferase activities of relatively high molecular weights catalyzed the formation of insoluble glucans, whereas soluble glucans were synthesized in the presence of enzymes with lower molecular weights. Furthermore, a number of different fractions containing glucosyltransferase activity could be resolved from each other on the basis of differences in their isoelectric points (13). Although glucosyltransferases from *S. mutans* synthesizing soluble glucans have been previously purified (5, 8), the enzyme or enzymes catalyzing insoluble glucan formation have not been comparably purified or characterized. Furthermore, the relationships between the various glucosyltransferases demonstrated in a single strain of *S. mutans* have not yet been clarified. Therefore, to better understand the mechanism of glucan formation in *S. mutans* and the role of multiple glucosyltransferase activities in this process, the present investigation was initiated.

The extracellular glucosyltransferase activity from *S. mutans* human cariogenic strain GS-5, serotype *c* (2), was resolved into fractions catalyzing primarily soluble or insoluble glucan formation. Both fractions were further purified, and the activity synthesizing soluble glucans was purified to near homogeneity. The enzymatic and physical properties of the two enzyme fractions have been investigated and discussed relative to the mechanism of glucan formation by *S. mutans*.

MATERIALS AND METHODS

Growth of the organism. *S. mutans* GS-5 was maintained as previously described (15). For routine extracellular enzyme isolation, 0.10 ml of the stored culture was initially inoculated into 5 ml of brain heart infusion medium (Difco) and incubated for 18 h at 37 C. The cell suspension was then inoculated into 600 ml of 1% glucose-0.05% dextran T10-Todd-Hewitt (Difco) medium and incubated for 18 h at

37 C. The cells were centrifuged at 1,500 × g for 10 min at room temperature and resuspended in 150 ml of fresh Todd-Hewitt medium containing dextran T10. The cell suspension was placed in dialysis tubing and incubated in 4 liters of the same medium for 9 to 11 h at 37 C.

Enzyme assays. Glucosyltransferase activity was determined by a modification of the radioisotope incorporation procedure described by Robrish et al. (21). The standard incubation mixture contained 0.10 M potassium phosphate buffer (pH 6.0), 20 μM dextran T10, 2.9 mM [^{14}C]glucose-sucrose (0.017 μCi/μmol), water, and enzyme in a total volume of 0.5 ml. The reaction mixtures were incubated for 1 h at 37 C, and the synthesis was terminated by the addition of 5 ml of methanol for total glucan synthetic activity or by heating at 100 C for 5 min for insoluble glucan-forming activity. The methanol-treated samples were filtered through 2.4-cm glass-fiber filters and washed three times with 5 ml of methanol. The heated samples were filtered directly through glass-fiber filters and washed three times with 3 ml of 0.9% sodium chloride and once with 3 ml of methanol. The dried filters were suspended in 10 ml of toluene–2,5-diphenyloxazole–1,4-bis-(5-phenyloxazolyl)benzene scintillation fluid and counted in a Packard model 3385 scintillation counter. Soluble glucan formation was calculated as the difference between total glucan synthesis and insoluble glucan formation. For assaying the activity present on gels, the gel slices were incubated for 18 h at 37 C in the standard reaction mixture containing 1.8 mM sodium azide to inhibit microbial growth and treated as described above for total glucan synthetic activity.

One unit of glucosyltransferase activity is defined as the amount of enzyme required to incorporate 1.0 μmol of glucose from sucrose into glucan polysaccharide per min under standard assay conditions.

Fructosyltransferase (EC 2.4.1.10) activity was assayed in a standard incubation mixture containing 0.10 M potassium phosphate buffer (pH 6.0), 1.4 mM [^{3}H]fructose-sucrose (1.8 μCi/μmol), enzyme, and water in a total volume of 0.5 ml. After incubation for 1 h at 37 C the samples were treated as outlined above for total glucosyltransferase activity. The addition of a soluble glucan-fructan mixture synthesized by fraction A (see below) as a possible primer for the enzyme did not stimulate the fructosyltransferase activity of the samples examined in this investigation.

The conversion of soluble [^{14}C]glucan to insoluble glucan was determined in reaction mixtures containing 0.06 M potassium phosphate buffer (pH 6.0), 1.4 μg of [^{14}C]glucan (0.41 nCi/μg of glucose), 36 mM sucrose, water, and enzyme in a total volume of 0.7 ml. After incubation at 37 C for 1 h, the reaction mixtures were treated and assayed for insoluble glucan formation as described earlier.

Protein was measured by the procedure of Lowry et al. (17) utilizing human serum albumin as the standard protein.

Cellular adherence to glass surfaces. The ability of different enzyme preparations to catalyze the adherence of *S. mutans* cells to glass surfaces was measured by a modification of the procedure of Olson et al. (20). The enzymes were incubated for 18 h at 37 C in glass tubes (13 by 100 mm) with 0.025 M potassium phosphate buffer (pH 6.0), approximately 10^{9} boiled GS-5 cells (heated at 100 C for 30 min), 2% sucrose, and sufficient 0.9% sodium chloride-0.2% sodium azide to bring the total volume to 2.0 ml. After incubation at an angle of 30°, the tubes were treated as previously described (16) for measurement of cellular adherence. Under these conditions there was no detectable celluar adherence in the absence of the added enzymes.

Gel electrophoresis. Polyacrylamide gel electrophoresis was carried out utilizing BioPhore (Bio-Rad Laboratories) 4% gels essentially as described by Davis (6), except that no sample or stacking gels were utilized and the samples mixed with glycerol were layered directly onto the separating gels.

Electrophoresis on sodium dodecyl sulfate (SDS)-4% polyacrylamide gels was carried out as described by Weber and Osborn (25), except that all samples were heated at 65 C for 30 min in the presence of 1.0 mM ethylenediaminetetraacetic acid-0.10 M mercaptoethanol-1% SDS before layering onto the gels. Coomassie blue was utilized to detect protein bands on the gels (7), whereas detection of glycoprotein was carried out utilizing the Alcian blue stain (24).

Isoelectric focusing on 4% polyacrylamide gels utilizing pH 3.5 to 10 carrier ampholytes (LKB Instruments) was performed essentially as described previously (1) at 4 C. The gels were sliced into 0.5-cm sections and either assayed for glucosyltransferase activity as described above or the slices were suspended in 0.5 ml of water for 18 h at 4 C for pH determinations.

Isolation of soluble [^{14}C]glucan. Soluble [^{14}C]glucan was synthesized in a reaction mixture containing 0.05 M potassium phosphate buffer (pH 6.0), 200 μg of dextran T10, 10 mU of purified fraction B (754 mU/mg), 0.7 mM [^{14}C]glucose-sucrose (0.15 μCi/μmol), and 0.9% sodium chloride-0.02% sodium azide in a total volume of 2.0 ml. After incubation at 37 C for 18 h the solution was mixed with 0.3 g of sucrose and added to a Bio Gel P-100 gel filtration column (2.5 by 30 cm) previously equilibrated with 0.01 M potassium phosphate buffer (pH 6.0)-0.2% sodium azide (PBA buffer). The fractions (10 ml) were assayed for radioactivity, and the [^{14}C]glucan fractions eluting in the void volume were pooled and concentrated through Amicon UM-2 ultrafilters. The [^{14}C]glucan could be hydrolyzed by dextranase (EC 3.2.1.11) but not by a fungal α-1,3-glucanase (EC 3.2.1.84), indicating that the radioactivity product was essentially a linear α-1,6-linked glucan.

Enzyme purification. The cell suspension from the Todd-Hewitt-dextran T10 medium grown in dialysis bags was centrifuged at 20,000 × g for 10 min at 4 C, and the supernatant fluids were utilized as the source of the extracellular glucosyltransferase activity (Table 1). The supernatant fluids were next treated with solid ammonium sulfate (50 g/100 ml) and allowed to stand for 18 h at 4 C. The resultant precipitate was collected by centrifuging at 20,000 × g for 10 min and dissolved in PBA buffer.

TABLE 1. *Purification of glucosyltransferase activity*

Fraction	Vol (ml)	Activity[a] (mU/ml)	Total activity (mU)	Protein (mg/ml)	Recovery (%)	Sp act (mU/mg)
Supernatant fluid	150	11.97	1,796	13.3	100	0.9
Ammonium sulfate precipitate	14.5	59.1	857	3.4	48	17.4
Concentrated fraction A (Bio Gel A-15 chromatography)	0.9	38.3	34	0.18	4	213
Concentrated fraction B (Bio Gel A-15 chromatography)	5.5	89.6	497	11.8	28	7.6
Concentrated fraction A (hydroxylapatite chromatography)	1.1	4.9	5.4	0.005	0.3	985
Concentrated fraction B (hydroxylapatite chromatography)	27.8	20.4	567	0.027	32	756

[a] Activity represents the total glucosyltransferase activity determined in the presence of exogenous dextran T10 primer.

The activity precipitated by ammonium sulfate was then applied to a Bio Gel A-15 column (2.5 by 60 cm) previously equilibrated with PBA buffer. Development of the column was carried out at room temperature utilizing PBA buffer, and 10-ml fractions were collected. The activity eluting in the void volume (Fig. 1) was designated as fraction A, whereas that eluting later was defined as fraction B. Fraction A was concentrated by passage through an Amicon UM-10 ultrafilter, whereas the enzyme from fraction B was concentrated after precipitation with ammonium sulfate (50 g/100 ml).

The concentrated enzymes were then individually applied to Bio Gel HTP hydroxylapatite columns (2.5 by 30 cm) previously equilibrated in 0.01 M potassium phosphate buffer (pH 6.0). The enzymes were eluted utilizing a 400-ml linear gradient from 0.01 to 1.0 M potassium phosphate buffer (pH 6.0) at room temperature (Fig. 2). Fractions (5 ml) were collected and assayed for total enzyme activity. The glucosyltransferase activity of fraction B eluted very late during development at high potassium phosphate buffer concentrations in two distinct peaks. Most of the activity eluted in the slower migrating peak and was utilized as the source of purified fraction B in this study. The other fraction with lower activity had kinetic properties similar to the major fraction. In some preparations the low-activity fraction constituted only a small percentage of the total eluted activity. The activity of fraction A also eluted from hydroxylapatite columns in two peaks at the same positions as found for fraction B. However, the total recovery of activity from fraction A after hydroxylapatite chromatography was very low (Table 1). Furthermore, no significant insoluble glucan-synthesizing activity could be detected in the fractions eluted. Thus, it appears that all of the insoluble glucan-synthesizing activity as well as most of the soluble glucan-synthesizing activity of fraction A could not be recovered from these columns despite extensive purification by this procedure.

Materials. Sucrose-[U-^{14}C]glucose (212 mCi/mmol) and sucrose-[U-^{3}H]fructose (4.75 mCi/μmol) were obtained from New England Nuclear Corp.

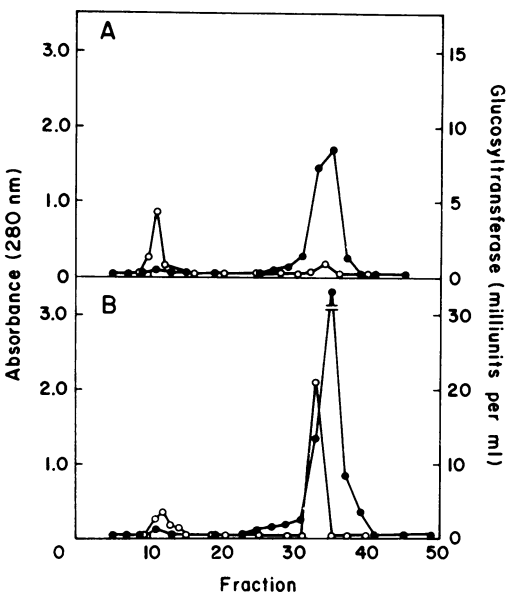

FIG. 1. *Effect of dextran T10 on gel filtration chromatography of glucosyltransferase activity. Cells of S. mutans were grown in Todd-Hewitt-1% glucose medium (4 liters) in the absence (A) and presence (B) of dextran T10 as described in Materials and Methods. The extracellular enzymes were precipitated with ammonium sulfate and chromatographed on Bio Gel A-15 columns as described earlier. Symbols: ●, absorbance (280 nm); ○, glucosyltransferase activity.*

Dextran T10 was a product of Pharmacia Fine Chemicals. Bio Gel A-15 resin, HTP hydroxylapatite resin, polyacrylamide P-100 resin, and BioPhore precast polyacrylamide gels were obtained from Bio-Rad Laboratories. Dextranase, concanavalin A, and oyster glycogen were products of the Sigma Chemical Co., and the α-1,3-glucanase from *Cladosporium resinae* was kindly donated by E. T. Reese (Univer-

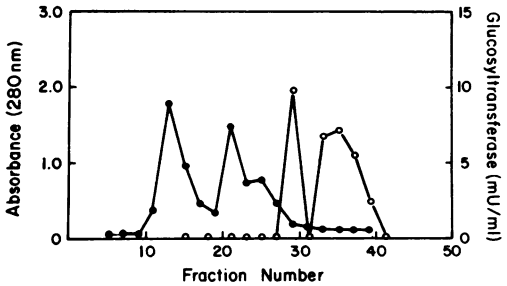

FIG. 2. *Chromatography of fraction B on hydroxylapatite columns. Fraction B was absorbed onto a hydroxylapatite column and eluted with a linear gradient from 0.01 to 1.0 M potassium phosphate buffer (pH 6.0), as described in Materials and Methods. Symbols: ●, absorbance (280 nm); ○, glucosyltransferase activity.*

sity of Massachusetts). *Escherichia coli* β-galactosidase (EC 3.2.1.23) was obtained from the graduate students in the Microbiology Techniques course (Northwestern University Medical School). All other chemicals and reagents were obtained from readily available commercial sources.

RESULTS

In vivo alteration of the size distribution of glucosyltransferase activity. Previously, the extracellular glucosyltransferase activity from *S. mutans* GS-5 was shown to migrate as a single high-molecular-weight species after chromatography on Bio Gel A-15 gel filtration columns (11). Likewise, in the present study it was observed that most of the extracellular enzyme, fraction A, migrated in the void volume after passage through Bio Gel A-15 columns (Fig. 1A). However, the more sensitive radioactive assay system employed in this investigation also revealed glucosyltransferase activity, fraction B, which eluted after the void volume. These observations suggested the possibility that the activity migrating in the void volume might consist of aggregates containing glucosyltransferase activity. Since glucosyltransferases are known to bind readily to glucans (10, 19) such an aggregate could be composed of the enzyme together with glucan products synthesized in the presence of trace amounts of sucrose detected in complex media (18). The formation of insoluble glucans might be expected to accelerate the aggregation process.

When cells were grown in the presence of dextran T10 (average molecular weight, 10,000), a poor acceptor for insoluble glucan formation (see below), there was a marked shift in the elution pattern of the enzyme activities from the high-molecular-weight species to the lower-molecular-weight form (Fig. 1B). Furthermore, the addition of yeast invertase (EC 3.2.1.26) to the growth medium also resulted in the majority of the extracellular glucosyltransferase activity migrating as the lower-molecular-weight species.

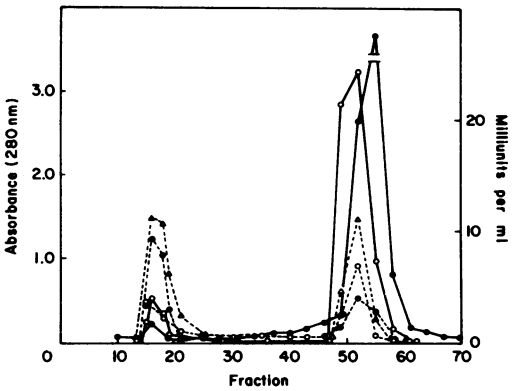

FIG. 3. *Effects of glucan hydrolases on extracellular enzyme gel filtration patterns. S. mutans was grown in Todd-Hewitt-1% glucose (4 liters) in the presence or absence of the indicated hydrolase. The extracellular enzymes were precipitated with ammonium sulfate and chromatographed on Bio Gel A-15 columns as previously described. Symbols: ●——●, absorbance (280 nm) of cells grown in the absence of hydrolases; ●---●, enzyme activity in the absence of hydrolases; ○---○, enzyme activity in the presence of dextranase (1 mg); △----△, enzyme activity in the presence of α-1,3-glucanase (1 mg); ○——○, enzyme activity in the presence of dextranase (1 mg) plus α-1,3-glucanase (1 mg).*

The fructosyltransferase activity present in the extracellular medium of *S. mutans* (3) was shown to be located almost exclusively in fraction A. The trace amounts of this activity present in fraction B were resolved from the glucosyltransferase activity of this fraction after hydroxylapatite chromatography (unpublished data).

Since the glucans synthesized by *S. mutans* are composed primarily of α-1,3- and α-1,6-linked glucose units, the inclusion of glucanases specific for these linkages in the growth medium might also inhibit the formation of high molecular aggregates of the glucosyltransferases. The results of such an experiment (Fig. 3) indicated that a combination of dextranase, α-1,6-glucanase, and fungal α-1,3-glucanase is much more effective than either enzyme alone in preventing aggregation. However, the presence of either enzyme alone does increase the relative amount of glucosyltransferase activity found in fraction B.

In vitro alteration of the molecular weight of glucosyltransferases. The high-molecular-

weight fraction A, isolated after Bio Gel A-15 chromatography, was also treated directly with the glucanases to determine whether any alterations in size distribution could be detected (Fig. 4). Treatment of fraction A with either dextranase or α-1,3-glucanase alone produced no significant shift in the molecular weight profile of the enzyme activity. However, the incubation of fraction A with both glucanases did yield activities which appear to migrate as lower-molecular-weight species. However, in this case the conversion of glucosyltransferase activity originally present in an aggregated form to activity of lower molecular weight was not nearly as great as the alteration observed in vivo.

Comparative properties of fractions A and B. A comparison of the two forms of glucosyltransferase activity separated by Bio Gel A-15 chromatography revealed that insoluble glucan synthesis was catalyzed exclusively by fraction A, whereas soluble glucans were produced by both fractions (Table 2). That fraction A alone was capable of synthesizing insoluble glucans was also indicated by the observation that the enzyme from fraction A, but not fraction B, catalyzed the adherence of cells of *S. mutans* to glass surfaces in the in vitro adherence assay (Fig. 5). Soluble glucan synthesis in both fractions was stimulated by the addition of dextran T10 as an exogenous acceptor source. However, the stimulation of soluble glucan synthesis by addition of dextran T10 to fraction A was not nearly as great as the observed with fraction B. The activity in fraction A was stimulated no more than twofold by the addition of dextran

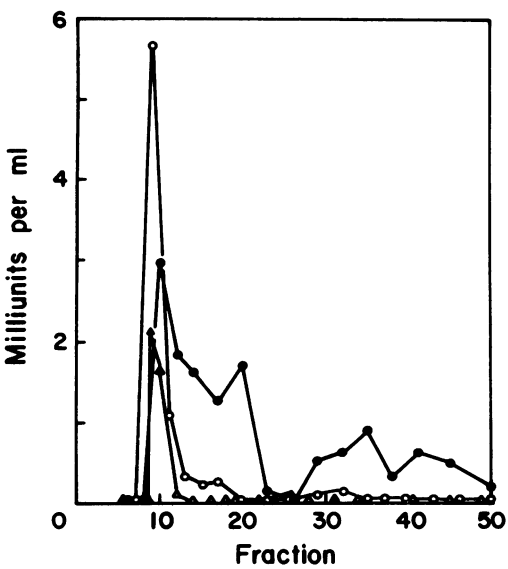

FIG. 4. *Effects of glucan hydrolases on the gel filtration properties of fraction A. Fraction A (79.3 mU), isolated after Bio Gel A-15 chromatography, was incubated with the indicated glucanases for 1 h at 37 C and rechromatographed on Bio Gel A-15 columns as previously described. Symbols: △, enzyme activity after treatment with α-1,3-glucanase (1 mg); ○, enzyme activity after treatment with dextranase (1 mg); ●, enzyme activity after treatment with dextranase (1 mg) plus α-1,3-glucanase (1 mg).*

TABLE 2. *Comparative properties of glucosyltransferases from fractions A and B*[a]

Property	Fraction A	Fraction B
Soluble glucan formation (mU/mg)	52.0	0.74
Soluble glucan formation plus dextran T10 (mU/mg)	122.3	4.6
Insoluble glucan formation (mU/mg)	65.1	0.02
pH optimum	6.0	6.0
Temperature optimum	37 C	37 C
K_m for sucrose (mM)	1.7	2.1
Gel filtration on Bio Gel A-15 columns	Eluted in void volume	Eluted after void volume

[a] Fractions A and B were obtained after chromatography on Bio Gel A-15 columns as described in Materials and Methods.

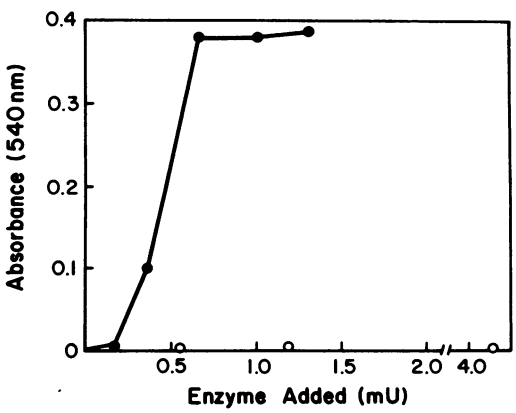

FIG. 5. *Cellular adherence catalyzed by fractions A and B. Adherence was measured as described earlier utilizing fraction A (595 mU/mg) and fraction B (4.6 mU/mg). All values have been corrected for adherence in the absence of sucrose. Symbols: ●, fraction A; ○, fraction B.*

T10, whereas that in fraction B was stimulated as much as 12-fold. The addition of dextran T10 had no significant effect on the synthesis of insoluble glucan by either fraction.

Despite the marked difference in the nature of the products synthesized by each fraction, the kinetic parameters of both fractions were similar (Table 2). The pH and temperature optima as well as the K_m for sucrose were essentially the same for both fractions.

The apparent molecular weights of both fractions are markedly different (Fig. 1). Fraction A eluted in the void volume of the Bio Gel A-15 columns, indicating that the molecular weight of this enzyme fraction is extremely large and the enzyme is highly aggregated. The synthesis of large amounts of both soluble and insoluble glucans by this fraction in the absence of exogenous acceptors (Table 2) suggests that endogenous acceptor glucan molecules are present in this fraction. Furthermore, the addition of concanavalin A to fraction A yielded marked aggregation, whereas a similar addition to fraction B produced no visible aggregation. This suggests that branched glucans are present in fraction A but not in fraction B.

When the glucosyltransferase activity of fraction B was isolated after hydroxylapatite chromatography and rechromatographed together with *E. coli* β-galactosidase (molecular weight, 510,000) on Bio Gel A-1.5 columns, it was observed that the glucosyltransferase activity now eluted in the void volume of the column prior to the appearance of β-galactosidase. This indicated that the enzyme purified from fraction B possesses a molecular weight much greater than 510,000. The molecular weight of the purified glucosyltransferase of fraction B was not determined more precisely, since its large size suggested that this activity must also exist in an aggregated form after purification. This aggregation apparently occurs during purification procedure, since the elution pattern of this enzyme on Bio Gel A-15 columns (Fig. 1) prior to the hydroxylapatite step indicates a much lower molecular weight then estimated for the purified enzyme.

Enzymatic characterization of the products synthesized by fractions A and B. To further characterize the two enzyme fractions, the glucans synthesized by each fraction were examined for their susceptibility to α-1,3- and α-1,6-glucanases (Table 3). The soluble glucan synthesized by fraction A appears to be primarily α-1,6-linked, since the presence of dextranase in the reaction mixture inhibited soluble glucan synthesis by 78%. The insoluble glucan product appears to be predominately α-1,3-linked, since the presence of the α-1,3-glucanase inhibited soluble glucan synthesis by 83%. Although the presence of dextranase inhibited insoluble glucan formation by 41%, this does not accurately measure the contribution of α-1,6-linkages to the insoluble glucans, since the commercial preparation of dextranase utilized in this investigation is contaminated with a small amount of α-1,3-glucanase activity (unpublished data). Fraction B catalyzes the formation of a product which is primarily α-1,6-linked, since dextranase addition inhibits product formation by almost 92% compared to the 24% inhibition in the presence of the α-1,3 glucanase.

TABLE 3. *Enzymatic hydrolysis of the glucans synthesized by fractions A and B*[a]

Fraction	Additions	Glucan (counts/min per reaction mixture)	
		Insoluble	Soluble
A	None	1,084	865
	Dextranase	641	190
	α-1,3-Glucanase	186	964
B	None		2,175
	Dextranase		178
	α-1,3-Glucanase		1,660

[a] Fractions A and B were isolated after gel filtration chromatography on Bio Gel A-15 columns as described earlier. Glucan synthesis was measured as described in Materials and Methods, except that no dextran T10 was added as a primer source. The glucanases were added at a concentration of 100 μg per reaction mixture. Each value has been corrected for control samples incubated in the absence of enzyme.

Polysaccharide acceptors for soluble and insoluble glucan formation. It was also of interest to determine whether the two enzyme fractions differed in their relative abilities to utilize different polysaccharides as primers for glucan formation. The polysaccharides dextran T10 (α-1,6-linked glucan), nigeran (α-1,3- and α-1,4-linked glucan), and glycogen (α-1,4-linked glucan) were evaluated as potential acceptors for soluble and insoluble glucan formation (Table 4). Fraction A, which can synthesize both soluble and insoluble glucans in the absence of exogenous acceptors, could utilize nigeran and glycogen as primers for insoluble glucan formation. In contrast, nigeran inhibited soluble glucan formation by this fraction, whereas dextran T10 and, to a lesser extent, glycogen could serve as acceptors for soluble glucan formation. Soluble glucan formation

TABLE 4. *Effects of exogenous primers on soluble and insoluble glucan synthesis*

Additions	Fraction A (counts/min per reaction mixture)[a]		Fraction B (counts/min per reaction mixture)[a]	
	Soluble glucan	Insoluble glucan	Soluble glucan	Insoluble glucan
None	236	154	289	0
Nigeran	16	257	393	144
Dextran T10	524	167	1,360	7
Glycogen	303	224	395	2

[a] Fraction A (100 mU/mg) and fraction B (1,790 mU/mg) were assayed as described in Materials and Methods. Polysaccharide primers were added at concentrations of 100 μg per reaction mixture. Control values determined in the absence of enzyme have been subtracted for each sample.

by fraction B was markedly stimulated by dextran T10, whereas both nigeran and glycogen produced much lower levels of stimulation. This fraction, which synthesizes only soluble glucan in the absence of exogenous primer, catalyzes the synthesis of much more soluble glucan in the presence of dextran T10. Dextrans of higher molecular weight also act as efficient acceptors for soluble glucan formation catalyzed by this fraction (unpublished data). Interestingly, this fraction also catalyzes the incorporation of a small but significant amount of glucose from sucrose into the nigeran primer. Since nigeran has low solubility in water, this incorporation is primarily into a water-insoluble product.

Conversion of soluble to insoluble glucans. A recent communication indicated that part of the insoluble glucan fraction synthesized by *S. mutans* consists of linear chains of α-1,6-linked glucose units containing single α-1,3-glucose residues at various branch points along the chain (K. K. Tung, K. J. Kovach, K. J. Pierre, and R. S. Davis, Fed. Proc. 33:1452, 1974). This suggested the possibility that soluble α-1,6-linked glucans synthesized by the glucosyltransferase of fraction B might serve as acceptors for insoluble glucan formation. Furthermore, Fukui et al. (8) cited preliminary unpublished data indicating that the soluble glucans could serve as precursors for insoluble glucan formation. To determine whether soluble glucans synthesized by fraction B could be converted to an insoluble product in the presence of fraction A, ^{14}C-labeled soluble glucan synthesized by fraction B was isolated as described in Materials and Methods. The conversion of this soluble glucan to an insoluble product did take place to a limited extent and required the presence of both fraction A and sucrose (Table 5). In contrast, fraction B could not catalyze the synthesis of insoluble glucan from the labeled soluble primer in the presence of sucrose.

Polyacrylamide gel electrophoresis of fractions A and B. When the glucosyltransferases from fractions A and B were electrophoresed on 4% polyacrylamide gels, all of the detectable proteins were present at the origin of the gels (Fig. 6). When parallel gels were assayed for enzyme activity, all of the glucosyltransferase activity of fraction B was found at or near the origin (Fig. 7). However, the partially purified enzyme from fraction A isolated after Bio Gel A-15 chromatography yielded three separate peaks of activity: one at or near the origin and two other smaller peaks of activity migrating into the gels. Protein bands corresponding to these later two peaks could not be detected on the gels after Coomassie blue staining.

SDS-polyacrylamide gel electrophoresis. Since the results utilizing gel filtration chromatography as well as polyacrylamide gel electrophoresis suggested that the glucosyltransferases isolated from the two fractions exist in aggregated structures, attempts were made to disassociate the enzymes into smaller units. When both fractions were electrophoresed on SDS-4% polyacrylamide gels it was observed that the major protein bands of both fractions now migrated into the gels (Fig. 6). Fraction A, isolated after gel filtration chromatography,

TABLE 5. *Soluble glucan as acceptor for insoluble glucan formation*[a]

Additions	Insoluble glucan formation (counts/min per reaction mixture)
[^{14}C]soluble glucan	0
Fraction A (13.4 mU) + [^{14}C]soluble glucan	3
Fraction A (13.4 mU) + [^{14}C]soluble glucan + sucrose	218
Fraction B (5.4 mU) + [^{14}C]soluble glucan	0
Fraction B (5.4 mU) + [^{14}C]soluble glucan + sucrose	2

[a] Fractions A (595 mU/mg) and B (7.6 mU/mg) were incubated with [^{14}C]soluble glucan, prepared as described in Materials and Methods, and the radioactivity converted to water-insoluble product was measured as previously described. Each value has been corrected for a control value of [^{14}C]soluble glucan alone (41 counts/min per reaction mixture).

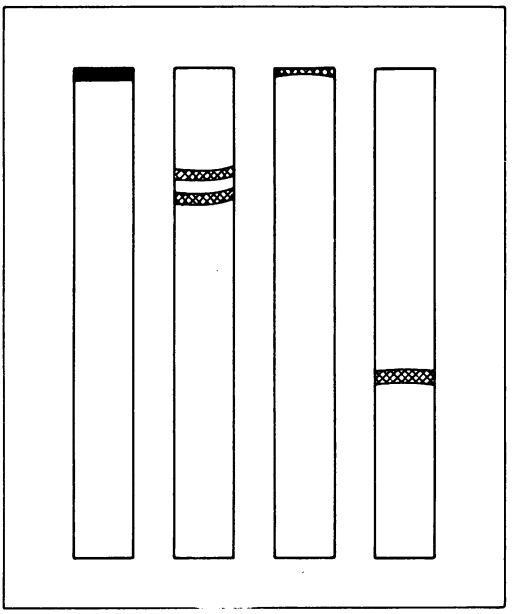

FIG. 6. *Polyacrylamide and SDS-polyacrylamide gel electrophoresis of fractions A and B. Fraction A, purified after Bio Gel A-15 chromatography, and fraction B, isolated after hydroxylapatite chromatography, were treated as described in Materials and Methods. From left to right: fraction A (56 μg) on 4% polyacrylamide gels, fraction A (28 μg) on SDS-4% polyacrylamide gels, fraction B (14 μg) on 4% polyacrylamide gels, and fraction B (27 μg) on SDS-4% polyacrylamide gels.*

yielded two major protein bands with mobilities corresponding to molecular weights of 190,000 and 235,000, respectively (Fig. 8). Both protein bands also contained polysaccharide, as detected by the Alcian blue stain. Several very faint protein bands of lower molecular weight could also be detected on the gels after protein staining. The glucosyltransferase of fraction B purified through the hydroxylapatite step yielded a single visible protein band on SDS-polyacrylamide electrophoresis which corresponded to a molecular weight of approximately 45,000 (Fig. 8).

Isoelectric focusing on polyacrylamide gels. The enzymes of both fractions A and B could also be resolved into multiple components after isoelectric focusing on 4% polyacrylamide gels (Fig. 9). The enzyme from fraction A yielded two peaks of activity after isoelectric focusing in a pH 3.5 to 10 ampholyte gradient. One peak of activity resided near the origin where the pH of the gel was measured as 4.3. This activity might correspond to the highly aggregated enzyme which does not migrate into 4% polyacrylamide gels (Fig. 7A). The other fraction migrated to a position corresponding to a pH of approximately 6.2. When the enzyme from fraction B was focused on polyacrylamide gels, two peaks of activity were also observed. The major peak of activity was found at pH 6.5, whereas the less active fraction focused at pH 4.3 near the origin. Thus, even though fraction B appears to be highly purified, as indicated on polyacrylamide and SDS-polyacrylamide gels (Fig. 6), the enzyme activity can be further resolved into at least two distinct fractions after isoelectric focusing.

DISCUSSION

As observed with other strains of *S. mutans* (8, 13, 19), the extracellular glucosyltransferase activity from human cariogenic strain GS-5 can be resolved into fractions synthesizing primarily soluble or insoluble glucans. In contrast to the enzymes from strain HS-6 (8, 19), glucosyltransferase activity synthesizing soluble glucans could be readily separated without overlap from the activity synthesizing insoluble glucans after a single passage through gel filtration columns. Insoluble glucans are synthesized exclusively by the activity which migrates in the void volume of Bio Gel A-15 columns, and

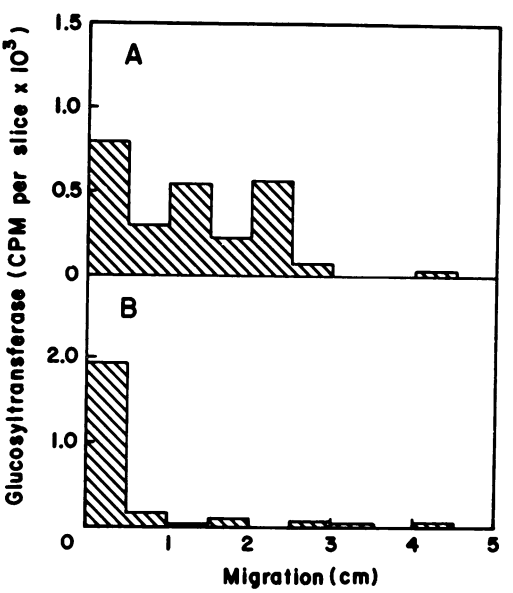

FIG. 7. *Glucosyltransferase activity of fractions A and B on polyacrylamide gels. Fraction A (0.5 mU), purified from Bio Gel A-15 columns, and fraction B (0.15 mU), purified after hydroxylapatite chromatography, were electrophoresed on 4% polyacryalmide gels; the gels were cut into 0.5-cm slices and assayed as described in Materials and Methods. A, fraction A; B, fraction B.*

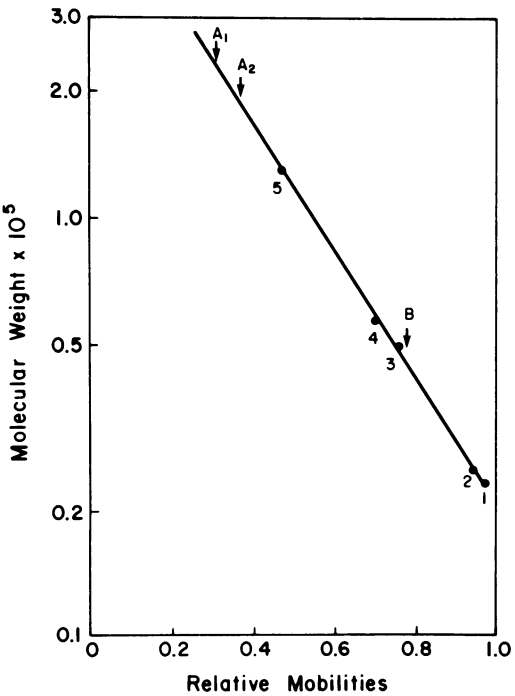

FIG. 8. *Molecular weight estimation of proteins in fractions A and B by SDS-4% polyacrylamide electrophoresis. Fractions A and B, described in the legend to Fig. 6, were electrophoresed as previously described. The molecular weight standards are: 1, trypsin; 2, human gamma globulin light chain; 3, human gamma globulin heavy chain; 4, pyruvate kinase; 5, E. coli β-galactosidase. A1, heavier major band from fraction A; A2, lighter major band from fraction A; B, single band from fraction B.*

the enzyme appears to be highly aggregated (Fig. 1). When strain GS-5 is grown in Todd-Hewitt-glucose medium, almost all of the activity is observed in this fraction. Fukui et al. (9) also observed that essentially all of the extracellular glucosyltransferase activity of another serotype *c* strain of *S. mutans*, Ingbritt, migrated through Sepharose 6B gel filtration columns as high-molecular-weight aggregates.

The results of this study suggest that the high-molecular-weight aggregate, fraction A, is composed of glucans and at least three enzyme activities: glucosyltransferase-synthesizing insoluble glucans, glucosyltransferase-synthesizing soluble glucans, and fructosyltransferase activity. The presence of glucan in the aggregates is suggested by the glucosyltransferase activity observed in the absence of exogenous primer. That the glucans present in these aggregates contain branching (α-1,3-linkages) is suggested by the observation that this fraction is agglutinated by the addition of the plant lectin concanavalin A (23). Furthermore, enzyme activity can be isolated primarily in a lower-molecular-weight form, fraction B, under growth conditions which do not favor insoluble glucan formation: the presence of dextran T10 in the growth medium (Fig. 1B) and the inclusion of both dextranase and α-1,3-glucanase in the growth medium (Fig. 3). Dextran T10 does not appear to serve as an effective primer for insoluble glucan formation (Table 4) in the presence of endogenous primer. In addition, the presence of this α-1,6-glucan in the extracellular medium continuously may inhibit insoluble glucan synthesis when the enzymes are initially secreted in culture and no endogenous acceptors for insoluble glucan formation have been synthesized. The presence of glucosyltransferase activity in aggregates containing insoluble branched glucans suggests that the Todd-Hewitt-glucose medium utilized in this investigation may be contaminated with sucrose, as has been suggested previously (18). This is further indicated by the observation that most of the glucosyltransferase activity exists in the lower-molecular-weight form when invertase is added to the growth medium to hydrolyze endogenous sucrose and when the cells are grown in a chemically defined medium (unpublished data).

The observation that most of the glucosyl-

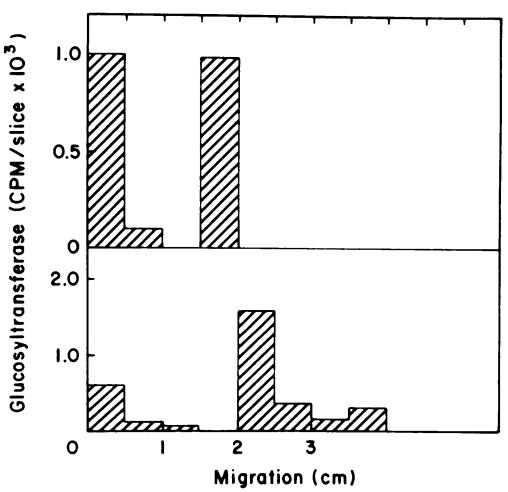

FIG. 9. *Glucosyltransferase activity of fractions A and B separated on isoelectric focusing polyacrylamide gels. Fraction A (1.8 mU), isolated after Bio Gel A-15 chromatography, and fraction B (0.82 mU), isolated after hydroxylapatite chromatography, were focused on 4% polyacrylamide gels, cut into 0.5-cm sections, and assayed as previously described. Top, fraction A; bottom, fraction B.*

transferase activity occurs as high-molecular-weight aggregates when cells are grown in the absence of dextran T10 suggests that these cells might possess relatively high levels of cell-associated activity. These aggregates may cosediment with the cells during centrifugation and be measured as cell-associated activity. One might predict that cell-associated activity would be lower when cells are grown in the presence of dextran T10. Recent results (W. Janda and H. Kuramitsu, unpublished data) have verified these predictions. In a representative experiment, cells grown in the absence of dextran T10 contained three times as much cell-associated glucosyltransferase activity (0.63 mU/10^8 cells) as cells grown in the presence of dextran T10 (0.2 mU/10^8 cells).

When the isolated high-molecular-weight aggregate, fraction A, is incubated with dextranase and α-1,3-glucanase, only a small portion of the enzyme is converted to the lower-molecular-weight species (Fig. 4). This indicates that the insoluble glucan matrix of the aggregate is not highly susceptible to the action of these hydrolases. This is further suggested by the observation that the glucanases inhibit glucan synthesis when present during glucan formation (Table 3) but are not effective when added after the polysaccharides have been synthesized.

A comparison of the kinetic properties of the two fraction (Table 2) revealed similar K_m values for sucrose and similar pH and temperature optima. Fraction A appears to contain a relatively large amount of endogenous primer, since the addition of dextran T10 stimulated soluble glucan synthesis no more than twofold in contrast to the much greater stimulation noted with fraction B. Fraction A also catalyzed the formation of insoluble glucan without exogenous primer addition, indicating the presence of an endogenous source of primer for insoluble glucan formation. When fractions A and B were incubated together, the formation of both soluble and insoluble glucans was always less than the sum of the individual activities of each fraction. Therefore, the inability of fraction B to synthesize insoluble glucan was not merely a reflection of the absence of necessary primer. The utilization of dextranase and α-1,3-glucanase (Table 3) suggested that fraction A synthesized both α-1,6- and α-1,3-linked glucans, whereas fraction B catalyzed the formation of α-1,6-linked glucans. However, since the glucanases were present throughout the incubation period, it is not clear whether these enzymes acted primarily on the endogenous primer, the nascent product, or on both types of molecules. Thus fraction B corresponds to the glucosyltransferase-synthesizing soluble glucans purified previously from strains HS-6 (8) and 6715 (5) of *S. mutans*. However, fraction A could transfer glucose units from sucrose onto an α-1,6-linked primer (Table 4) and, conversely, the glucosyltransferase of fraction B could utilize an α-1,3-containing glucan to a limited extent as a primer. This suggests that the glucans synthesized in vivo by the extracellular enzymes of *S. mutans* may contain varying amounts of both kinds of glucosidic linkages. Several previous reports (4; Tung et al., Fed Proc. 33:1452, 1974) indicated that both the soluble and insoluble glucans isolated from cultures of *S. mutans* do contain both kinds of linkages in varying amounts.

The glucosyltransferase from fraction B has been purified to near homogeneity after passage through hydroxylapatite columns (Fig. 6). The molecular weight of the purified enzyme could not be measured accurately because of its apparent large size after purification. However, the utilization of SDS-polyacrylamide gel electrophoresis suggests that the enzyme is composed of subunits with a molecular weight of 45,000. This is much smaller than the estimate of 170,000 daltons made for the analogous enzyme from strain HS-6 (8). However, this later estimate was obtained after SDS-polyacrylamide electrophoresis of samples which apparently were not heated at an elevated temperature (65 C in the present investigation). The heat treatment of the samples significantly altered the relative mobilities of the glucosyltransferases on SDS-polyacrylamide gels (unpublished data). The molecular weight of the soluble glucan-synthesizing enzyme from strain 6715 was estimated as 94,000 after gel filtration chromatography (5). Thus, assuming that the analogous enzymes from the different serotypes of *S. mutans* are structurally related, it would appear that the soluble glucan-synthesizing enzymes might be composed of subunits with a molecular weight of approximately 45,000. It is of interest that the glucosyltransferases of *Leuconostoc mesenteroides* NRRL B-1299 are composed of monomers with a molecular weight of 42,000 (14). The results from the electrofocusing on polyacrylamide gels indicated that the purified enzyme from fraction B contains two components with different isoelectric points. Thus result may indicate that the purified enzyme might contain two species composed of the same monomeric structure but with different oligomeric structures. It is also noteworthy that the purified enzyme from strain GS-5 eluted from hydroxylapatite columns at a much higher potassium phosphate

concentrations than the comparable enzymes from strains HS-6 (8, 19) and 6715 (5). Since hydroxylapatite columns are known to bind glucans very well, this difference may reflect the presence of glucans in fraction B at higher levels than present in the enzymes from the other two strains. This property might also explain the aggregated nature of the purified enzyme from fraction B.

Purification of the glucosyltransferase activity present in fraction A has been hampered by the highly aggregated nature of this fraction and by the poor recovery of enzyme activity upon further chromatographic manipulation (Table 1). It appears that almost all of the activity is not recovered after hydroxylapatite chromatography even when the phosphate concentration is raised to 1.0 M. Furthermore, the activity recovered from the columns is highly primer dependent and does not synthesize insoluble glucan. Since the nature of the endogenous primer for insoluble glucan synthesis has not been clearly defined, it is not yet possible to determine whether this primer-dependent activity recovered from hydroxylapatite columns represents soluble glucan-synthesizing activity of fraction A or the insoluble glucan-synthesizing enzyme which has been resolved from its endogenous primer. Since the activity recoverable from fraction A after hydroxylapatite chromatography is eluted from the column at positions corresponding to the elution pattern of fraction B, it is possible that the eluted enzymes from both fractions are identical. This would suggest that the high-molecular-weight aggregate of fraction A might also contain the enzyme activity isolated in fraction B. However, the limited yield of the eluted enzyme from fraction A after hydroxylapatite chromatography has made a direct comparison with the enzyme from fraction B not feasible at this time. In addition, it is not yet possible to determine directly whether the insoluble glucan-synthesizing enzyme of fraction A corresponds to the protein of molecular weight 190,000 or 235,000 detected as the major protein bands after SDS-polyacrylamide electrophoresis. Additional experiments designed to isolate the glucosyltransferase activity from fraction A in greater yield and higher purity, in order to test these possibilities, are in progress in this laboratory.

Since the glucosyltransferase(s) responsible for insoluble glucan synthesis has yet to be purified and characterized, the detailed mechanism of insoluble glucan synthesis remains unclear. However, the isolation by Guggenheim et al. of a glucosyltransferase fraction from *S. mutans* which synthesizes primarily α-1,3-linked glucans (4) suggests the presence of at least two distinct glucosyltransferase in *S. mutans*: one which incorporates glucose into α-1,3-linkages and another with α-1,6-specificity. Furthermore, earlier (13) and more recent immunological studies (9) suggest the presence of at least two antigenically distinct glucosyltransferase activities in *S. mutans*. Thus, the glucosyltransferase activities resolved in the present study may correspond to the two distinct enzyme activities. Experiments are currently in progress to examine the properties of the purified enzyme fractions immunologically to gain further evidence in this regard.

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