Carbohydrate Research, 134 (1984) 245–264
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

SPECIFIC AND NON-SPECIFIC AFFINITIES OF THE EXTRACELLULAR GLUCOSYLTRANSFERASE COMPLEX OF *Streptococcus mutans* 6715

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(Received October 18th, 1983; accepted for publication in revised form, February 22nd, 1984)

ABSTRACT

Glucosyltransferases (GTF) from different strains of streptococci exhibited different elution profiles when fractionated on insoluble-dextran affinity columns. The proportions of unadsorbed and adsorbed GTF were not related to their extent of stimulation by exogenous dextran, and GTF preparations exposed to, and freed from, clinical dextran prior to fractionation lost their ability to bind to the dextran columns. Different proportions of bound GTF were released by irrigation of columns with different concentrations of salt and clinical dextran, and the "specific" binding and release of GTF exhibited by a column possessing covalently linked, clinical dextran ligands was duplicated on a control column that did not possess the dextran ligands. These results, and the high affinity of GTF for hydrophobic alkyl (Shaltiel) ligands, demonstrate that ionic and hydrophobic properties of impure GTF aggregates may lead to erroneous characterization of the dextran affinity of some protein fractions. Fractionations on DEAE-Sepharose and on hydroxylapatite showed that the two dextran-dependant GTF activities (GTF-S and GTF-I) were present in the major enzyme fraction (*Streptococcus mutans* 6715) recovered from a Sephacryl S-200 affinity column. A minor, dextran-independent GTF was not adsorbed onto the Sephacryl column. The presence of SDS (0.005%) and Triton X100 (0.01%) stabilized GTF activity during gel filtration and improved the separation of GTF-S and GTF-I in hydroxylapatite fractionation of the highly aggregated enzyme. A comparable separation of the two enzyme forms on DEAE-Sepharose was achieved only if T10 dextran (10 mg/mL) was included with the detergent mixture in the column irrigant.

INTRODUCTION

Many microorganisms use sucrose to synthesize extracellular α-D-glucans ranging in structure from near-linear chains to highly branched and compact molecules[1]. The dual synthesis[2] of a water-soluble, predominantly (1→6)-linked glucan and a water-insoluble, predominantly (1→3)-linked glucan greatly affects the ability of *Streptococcus mutans* to form adherent plaque and to initiate dental

0008-6215/84/$ 03.00

caries at different sites on the tooth surface[3,4]. The enzymes responsible for these syntheses are known collectively as glucosyltransferases (GTF), and numerous reports describe the isolation of GTF preparations that synthesize α-D-glucans differing widely in structure. The distribution of at least four distinct GTF between seven serotypes of *S. mutans* has been reported[5]. Synergism between two GTF[2,6,7], one (GTF-S) synthesizing a water-soluble glucan and the other (GTF-I) a water-insoluble glucan, allows the synthesis of different glucan structures, so that the presence of different proportions of at least two GTF in impure enzyme preparations may account, in large part, for the apparent multiplicity of enzyme forms[8]. Recently a third, immunologically distinct GTF, which synthesizes a water-soluble glucan without requirement for dextran primer, was shown[9] to be secreted together with GTF-S and GTF-I by *S. mutans* 6715.

Electrophoretically homogeneous preparations of GTF-S[10] and GTF-I[11] have been isolated from *S. mutans* 6715, but enzyme recoveries were reported to be extremely low and in each purification other enzyme components were not recovered. Fractionation of GTF on a dextran affinity column provided a rapid method for partial purification of GTF in high yield[12,13] and apparent differences in the specific binding of GTF fractions held promise that affinity columns possessing α-(1→6)- or (1→3)-linked glucan ligands[14,15] might allow separations and quantitative recoveries of the specific GTF activities in the enzyme complexes elaborated by *S. mutans*. Non-catalytic glucan-(dextran)-binding proteins also have been purified on dextran affinity columns[15–17]. This communication describes fractionations, on dextran affinity columns, of GTF aggregates from a number of streptococcal strains and describes attempts to separate the different GTF activities in the high-molecular-weight aggregates by gel filtration and by hydrophobic and ion-exchange chromatography under various conditions.

EXPERIMENTAL

Materials and methods. — Sucrose was purchased from Fisher Scientific Company, and was dialyzed through a cellophane membrane to remove traces of dextran. [U-^{14}C]Sucrose was purchased from New England Corporation, clinical dextran (mol. wt. 60,000–90,000) from Nutritional Biochemical Corporation and T10 dextran (mol. wt. 10,000) from Pharmacia.

Hydroxylapatite (Bio-gel HTP) was purchased from Bio-Rad and Sephacryl S-200 from Pharmacia. Shaltiel hydrophobic chromatography kit I (agarose-Cn series) was purchased from Miles Laboratories, Inc. and ethylamine–Sepharose 4B was prepared as described by Shaltiel and Er-El[18].

Insoluble dextran–Bio-gel P2 columns (1 × 12 cm) were formed from suspensions of water-insoluble glucan and Bio-gel P2 beads as previously described[12]. A column possessing soluble-dextran ligands was prepared by conjugation of clinical dextran to epoxy-activated Sepharose 6B at pH 11.0 as described by Pharmacia. The excess of epoxy groups was removed by reaction with ethanolamine and epoxy

gel that had not been exposed to clinical dextran was treated with ethanolamine to provide a control column. Only ammonium sulfate GTF fractions were chromatographed on the insoluble-and soluble-dextran columns. After application of enzyme preparations, the columns were irrigated sequentially with the same buffer solution (~5 vol.) used for dialysis of the enzyme fraction and then with the same volume of suitable salt–dextran solutions to release bound enzyme.

For large-scale preparation of GTF from *S. mutans* 6715-49, Sephacryl S-200 was suspended and washed in 50mM sodium phosphate buffer, pH 6.0, containing 0.02% sodium azide. The slurry was poured into a column (8 × 7 cm) and washed exhaustively with the same phosphate–azide solution. Cell-free medium (up to 9 L) from cultures of *S. mutans* 6715-49 was made to 0.02% with sodium azide and up to 9 L of this solution (or 900 mL of a 50% saturated ammonium sulfate fraction of medium previously dialyzed against 50mM phosphate–0.02% azide solution) was applied and the column was washed with 3 vol. of the phosphate–azide solution. The bound GTF was released by elution of the column with 6M guanidine solution until the adsorption at 280 nM of eluted fractions decreased to zero. GTF activity in individually dialyzed column-fractions essentially paralleled protein concentration (adsorption at 280 nM), and therefore GTF preparations were obtained routinely by dialysis of the combined column-fractions that constituted protein peaks (see Fig. 1).

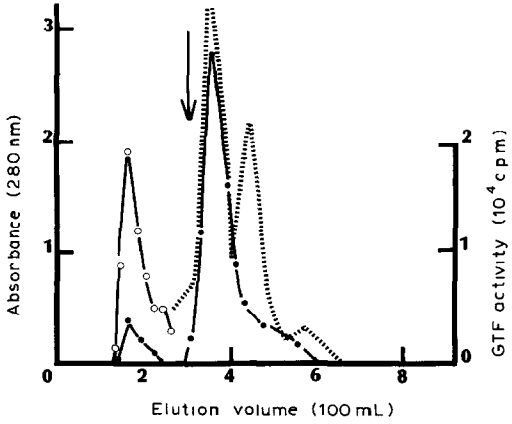

Fig. 1. Fractionation of GTF on a Sephacryl S-200 column. Cell-free culture media (9 L) was made to 0.02% with sodium azide and applied to the column (8 × 7 cm) at a flow-rate of ~500 mL/h. The column was washed with 50mM sodium phosphate–0.02% sodium azide solution, pH 6.0 (500 mL) and then irrigated with 6M guanidine solution, pH 6.0 (500 mL). The indicated elution volumes were measured from the application of the guanidine solution and the arrow indicates the volume in which the guanidine front appeared. Absorbance at 280 nm (closed circles) was measured in selected fractions and GTF activity (open circles) was measured in fractions not containing guanidine. Fractions containing guanidine and exhibiting significant absorbance at 280 nM were combined and dialyzed against 50mM sodium phosphate–0.02% sodium azide solution, pH 6.0. The interrupted line represents the absorbance (280 nM) of guanidine-eluted fractions obtained in the fractionation of a different preparation of GTF.

Ammonium sulfate fractions of extracellular glucosyltransferase were prepared as described previously[19]. Unless stated otherwise, GTF was assayed routinely by a paper-disc procedure[20]. For determination of the total glucan (water-soluble and water-insoluble) synthesized by GTF fractions, glucan was precipitated in the assay tubes by addition of ethanol to 70% concentration and the washed precipitates were dissolved in M sodium hydroxide solution. Measurements of the total glucan in the alkali solution by the paper assay method and by the phenol–sulfuric acid method were in good agreement. Conditions for the measurement of GTF inhibition after preincubation with oxidized clinical dextran were as previously described[19]. Fructose release in GTF reaction mixtures was determined by a copper-reducing method[21], total carbohydrate was determined by the phenol–sulfuric acid method[22], and protein by the Lowry method[23].

Electrophoresis. — Disc polyacrylamide gel electrophoresis (PAGE) was conducted on 5% gels essentially as described by Davis[24]. The gels were stained for protein with Coomassie Brilliant Blue R250 and GTF activity zones were made visible as white bands by incubation of duplicate gels for 18 h at 34° in sucrose solution (14 mg/mL) and 50-mM Tris–sodium acetate, buffer, pH 6.0, in the presence or absence of clinical dextran (1 mg/mL). For more accurate and sensitive GTF assays, gels were sliced, mashed, and incubated for 30 min at 37° with [^{14}C]sucrose (45×10^3 c.p.m./mg; 10 mg/mL) and 50mM sodium phosphate solution, pH 6.0, in the presence and absence of clinical dextran (150 μg/mL). At appropriate times, [^{14}C]glucan synthesis was measured by the paper-disc method.

Slab polyacrylamide gel electrophoresis was conducted on 2-mm gels in a Protein 16 cm, vertical electrophoresis apparatus (Bio-Rad Laboratories) equipped with an Ortec 4100 pulsed, constant power supply. Protein zones were made visible by a silver staining method[25], and zones of GTF activity were detected on duplicate gels immersed in 5% sucrose solution for 18 h. Enzyme (GTF-I) synthesizing water-insoluble glucan from sucrose was located by the appearance of a grey-white precipitate in the gel, and enzyme (GTF-S) synthesizing water-soluble glucan was located by a blue-white precipitate that appeared when the gel was transferred to 50% aqueous ethanol.

RESULTS

Adsorption of GTF to dextran affinity columns. — The proportions of GTF recovered from insoluble-dextran affinity columns (Table I) in unadsorbed (fraction I) and adsorbed (fraction II) components sometimes differed for different enzyme batches from the same organism (*S. mutans* 6715-49) and were highly dependent on the bacterial strain. Fractionations of the same batch of enzyme on the same dextran column were reproducible but, occasionally, new batches of insoluble dextran gave poor enzyme recovery, and these dextran preparations were discarded. The amount of enzyme produced by cells cultured in Todd–Hewitt broth was about one third of that in Trypticase–yeast extract broth, but the two enzyme

TABLE I

FRACTIONATION ON INSOLUBLE-DEXTRAN AFFINITY COLUMNS OF AMMONIUM SULFATE FRACTIONS OF GTF FROM DIFFERENT STRAINS OF *S. mutans*[a]

Strain	*GTF activity in fractions (% of total applied to column)*[b]	
	I	*II*
6715-49	2[c]	23
	2[c]	26
	42	25
	6	42
6715-13 wild type[d]	30	45
mutant 4	4	120
K-1R	30	8
AHT	0	46
	9	39
FA-1 (30)[e]	72	25
BHT (90)	115	0

[a]Ammonium sulfate fractions of impure enzyme were dialyzed against 50mM sodium phosphate–0.02% sodium azide solution, pH 6.0, and applied to columns (1 × 12 cm) equilibrated with the same solution. Columns were irrigated with ~5 vol. of the phosphate solution and then with about the same volume of phosphate solution containing clinical dextran (0.5 mg/mL). GTF activity in fractions was determined by the paper-disc assay. [b]Fraction I was not adsorbed to the column. Fraction II was adsorbed and was released by irrigation of column with clinical dextran solution (0.5 mg/mL). [c]Same enzyme batch on same column. [d]Wild type enzyme synthesized water-insoluble glucan from sucrose and mutant 4 enzyme synthesized mostly water-soluble glucan. [e]Numbers in parentheses indicate the presence of fructosyltransferase as a % of total glycosyltransferase activity.

preparations gave similar elution profiles on the same affinity column. Substitution of D-fructose for D-glucose in cultures of *S. mutans* strains 6715-49 and 6715-13 significantly changed neither the amount nor the elution profile of GFT activity (not shown).

GTF of a mutant strain[26] of *S. mutans* 6715-13 produced greater amounts of water-soluble glucan from sucrose (not shown) and was more completely adsorbed to the column than the enzyme preparation from the parent strain. However, enzyme preparations from *S. mutans* BHT, which synthesized only water-soluble glucan, exhibited no affinity for the dextran column (Table I). A stimulation of glucan synthesis by 6715-13 wild-type and mutant enzymes in the presence of soluble dextran, and a lack of stimulation for the BHT enzyme (not shown), are consistent with the different dextran-binding properties of the enzyme preparations (Table I), but this relationship between stimulation by soluble dextran and affinity for the dextran column did not hold for the unadsorbed enzyme component (fraction I) of wild-type 6715-13 enzyme which, despite its lack of affinity (Table I), was stimulated 100% in the presence of soluble dextran.

More enzyme (6715-49) was released (fraction III) from the insoluble-dextran column when the dextran concentration of the eluting solution was increased from 0.5 to 10 mg/mL, and almost all of the activity applied was recovered when the

TABLE II

SPECIFIC AND NON-SPECIFIC BINDING OF *S. mutans* GTF TO DEXTRAN AFFINITY COLUMNS[a]

Type of column	*Cell strain*	*GTF activity in fractions (% of total applied)*[b]		
		I	*II*	*III*
Insoluble dextran[c]	6715-49	6	41 (0.5 mg CD)	11 (10 mg CD)
		5	2 (2M KCl)	93 (2M KCl, 10 mg CD)
		28 (2M KCl)	62 (2M KCl, 10 mg CD)	—
	6715-13	17	58 (10 mg CD)	4 (2M KCl)
		5 (2M KCl)	80 (2M KCl, 10 mg CD)	—
Soluble dextran[d]	6715-13	4	32 (0.5 mg CD)	—
Control	6715-13	4	32 (0.5 mg CD)	—
		3 (M NaCl)	70 (M NaCl, 0.5 mg CD)	—

[a]Ammonium sulfate fractions of GTF were dialyzed against 50mM sodium phosphate–0.02% sodium azide solution, pH 6.0 in the presence or absence of high salt concentrations and were applied to columns equilibrated with the same solution. Columns were washed with ~5 vol. of the dialysis solution and then with the same volume of solution containing salt and clinical dextran. [b]Fractions were eluted from column with 50mM sodium phosphate solution, pH 6.0, containing clinical dextran (CD) and salt when indicated in parentheses. Concentrations of CD and salt are in mg/mL and molarities, respectively. [c]Mixture of insoluble dextran (*S. mutans* K-1R) and Bio-gel P2. [d]Clinical dextran covalently linked to epoxy-activated Sepharose 6B. Control column was inactivated without being exposed to dextran.

concentrated dextran solution was made 2M with respect to potassium chloride (Table II). If applied before (6715-49) or after (6715-13) the dextran solution, high salt concentrations released little of the adsorbed enzyme. The proportion of 6715-49 enzyme eluted in fraction I increased when the enzyme was preincubated, and the column saturated, with 2M potassium chloride solution, but this increase was not reproducible for other preparations and was not observed when 6715-13 enzyme was treated similarly.

Clinical dextran covalently bound to epoxy-activated Sepharose 6B apparently acted as an affinity ligand, and gave GTF separations similar to those of insoluble dextran–Bio-gel P2 columns (Table II). However, a control column of epoxy-activated Sepharose 6B, which had been neutralized without attachment of clinical dextran, also acted like the clinical-dextran and insoluble-dextran affinity columns, specifically releasing bound enzyme when irrigated with a solution of soluble dextran and releasing increased amounts of enzyme when the ionic strength of the dextran solution was increased. Elution profiles of GTF activity from the clinical dextran column and the control column were identical (not shown).

GTF (6715-49) in fractions I and II from the insoluble-dextran column (Table I) was not adsorbed when refractionated on a similar column (Table III). The GTF in fraction II did not bind even when the dextran content (0.5 mg/mL) was decreased to <25 μg/mL by ultrafiltration. Treatment of an ammonium sulfate fraction of GTF, by addition and removal of dextran (1 mg/mL) before it was applied to the insoluble dextran column, caused the enzyme activity to pass directly through

the column without binding. Similarly, GTF solubilized from an insoluble enzyme-complex formed by sucrose-grown cells[27] was poorly adsorbed to the affinity column, although soluble dextran (1 mg/mL) used in the solublization procedure had been removed. GTF released from a Sephadex G-50 column with 6M guanidine solution contained <25 μg polysaccharide/mL after dialysis, but it also failed to bind to the affinity column (Table III). In contrast, a dialyzed enzyme-fraction released by guanidine from a Sephacryl column (see Table IV) was adsorbed to a second Sephacryl column and 55% was released by irrigation with clinical dextran solution (0.5 mg/mL). In test-tube experiments (not shown), only 8% of the guanidine fraction from the Sephacryl column was adsorbed to Sephacryl beads after being exposed to, and removed from (ethylamine column) clinical dextran (0.5 mg/mL). This is compared to 45% adsorption of enzyme not exposed to clinical dextran but otherwise similarly treated.

In the absence of soluble dextran, the ammonium sulfate fraction of *S. mutans* 6715-49 GTF, and its components eluted in fractions I and II (Table I), synthesized mostly water-insoluble glucan. Although fraction I and fraction II (freed of dextran) exhibited no affinity for the insoluble dextran column, glucan synthesis by both fractions was stimulated several-fold in the presence of soluble dextran, this stimulation being two times greater for fraction II than for fraction I enzyme (not shown). Similar stimulations were obtained with the corresponding enzyme fractions (6715-13) eluted from the clinical-dextran and control epoxy-activated columns.

TABLE III

ADSORPTION OF *S. mutans* GTF PREPARATIONS ON AN INSOLUBLE-DEXTRAN (ID) AFFINITY COLUMN[a]

Source	*Treatment*	*GTF activity in fractions (% of total applied)*[b]	
		I	*II (0.5 mg CD)*
Fraction I (ID-column)	None	80	0
Fraction II (ID-column)	None	114	0
	Dextran removed[c]	65	0
Ammonium sulfate fraction	None	6	42
	Dextran added and removed[d]	60	0
Insoluble GTF from sucrose culture	Dextran added and removed[d]	75	20
Guanidine fraction from Sephadex G-50	Dialyzed	60	0
Guanidine fraction from Sephacryl S200 column (see Table IV)	Dialyzed[e]	0	55

[a]GTF fractions were treated as indicated and applied in 50mM sodium phosphate–0.02% sodium azide solution, pH 6.0 to columns (1 × 12 cm) which were equilibrated and irrigated as described in Table I. [b]See Table I. [c]Dextran (0.5 mg/mL) removed by ultrafiltration. [d]Dextran (1 mg/mL) removed by fractionation on butyl-Sepharose column. [e]Fraction from Table IV was reapplied to a similar Sephacryl column which was irrigated with dextran solution (0.5 mg/mL).

TABLE IV

ACTIVITY AND STABILITY OF GTF IN THE PRESENCE OF DISSOCIATING AGENTS AND ABILITY OF THESE AGENTS TO RELEASE ACTIVE ENZYME FROM SEPHACRYL S-200[a]

Agent (concentration)	*GTF activity (% of original)*		
	A	*B*	*C*
None	100	100	0
Guanidine (6M)	5	82	65
Sodium thiocyanate (3M)	3	55	18
Urea (8M)	16	136	7
Imidazole–citrate (M)	107	84	3
Triton X-100 (1%)	120	120	2
Brij 35 (1%)	100	100	
Ammonium sulfate (saturated)	30	100	
Lithium chloride (4M)		110	1
Sodium chloride (2M)	60	108	1
Sodium borate (0.4M)	120	118	1
SDS (1%)	3	5	
CPC (1%)	3	2	

[a]An ammonium sulfate fraction of GTF was dialyzed against 50mM sodium phosphate–0.02% sodium azide solution, pH 6.0. Enzyme activity was measured (paper-disc method) in the presence of the dissociating reagents (column A). The stability of enzyme exposed to the indicated concentration of dissociating agent at pH 6.0 and for 18 h at 4° was determined by measurement of enzyme activity after dissociating agents were removed by dialysis against 50mM sodium phosphate solution, pH 6.0 (column B). Portions of the dialyzed ammonium sulfate fraction of GTF were applied to Sephacryl S-200 columns which were washed with 5–6 vol. of the phosphate solution and then with solutions (pH 6.0) containing the indicated dissociating reagents. Total enzyme released was determined in fractions after dialysis against 50mM phosphate solution (column C).

Fractionation of GTF on Sephacryl S-200. — Use of Sephadex G-50 columns for GTF purification is limited by the instability of the dextran matrix to repeated irrigation with 6M guanidine solution. Sephacryl S-200, which has dextran-like sequences in its structure, also binds GTF and, because it is stable to guanidine, can be used repeatedly for enzyme fractionations. Of the reagents to which GTF was stable, guanidine was most effective for recovery of enzyme from Sephacryl columns (Table IV).

About 7% of GTF activity in a 50% saturated ammonium sulfate enzyme-fraction (*S. mutans* 6715-49) passed directly through a Sephacryl S-200 column and was not adsorbed when re-applied to a similar column. When cell-free culture-liquor (9 L) was applied to a large column, unadsorbed enzyme activity was not detected unless the effluent solution was concentrated about ten-fold (Amicon membrane PM 100). The concentrated effluent solution contained glucan (5 mg/mL) and ~10% of the total enzyme activity applied to the column. Irrigation of the large Sephacryl column with 6M guanidine solution released ~10% of the applied GTF before the guanidine front, and ~80% coincident with a major protein component that was eluted at and after the guanidine front (Fig. 1). In some

column fractionations, in which cell-free culture medium was applied, the shoulder on the trailing edge of the major protein peak was accentuated to give a composite peak (dotted line). When this occurred, the specific activity of enzyme in the leading portion of the composite peak (sp. act. 18) was three times higher than in the trailing peak and two times higher than that obtained as a single peak and shoulder (solid line, Fig. 1).

GTF in the concentrated effluent fraction did not require acceptor dextran for efficient synthesis of glucan from sucrose (see Table V, effluent) and, after separations on DE52 and 1,6-hexanediamine columns, this fraction yielded an enzyme preparation (representing about 1% of the original GTF activity in the culture medium) that no longer contained a detectable amount of glucan but still was independent of acceptor dextran. In contrast, the two GTF fractions (80–95% of total activity applied) released by guanidine from the Sephacryl S-200 column exhibited dependency on acceptor dextran for synthesis of glucan, although both rapidly catalyzed the release of fructose in the absence of acceptor dextran (Fig. 2, A and B).

Unlike the ammonium sulfate fraction of GTF, the major GTF fraction eluted from Sephacryl S-200 did not release fructose from raffinose, and did not release glucose or reducing sugars from clinical dextran. A radioactive poly-

TABLE V

SYNTHESIS OF GLUCAN BY GTF FRACTIONS AND INHIBITION BY 10% OXIDIZED DEXTRAN

Enzyme fractions	*Specific activity (units/mg protein)*	*Glucan synthesis*[a] *(μg/min/50 μL)*			
		+	−	*Maximum (μg/50 μL)*	*Inhibition (%)*
Ammonium sulfate	0.05	0.6	0.1	64	67
Sephacryl (Fig. 1)					
Effluent	—	1.3	1.3	300	0
Minor peak	1.2	1.6	0.3	50	85
Major peak	9	1.8	0.1	120	65
Major peak (i)[b]	18	7.5	0.2	130	—
(ii)[b]	6	1.0	0	90	—
DEAE[c] (not shown)					
Peak 1	14	2.3	0	70	90
Peak 2	9	3.3	0.2	240	40
Peak 1 + 2	—	1.5	—	162	—
Ethylamine[c] (Fig. 3 inset)					
Combined fractions	—	1.3	0.2	136	—
Late fractions	—	0.8	0.2	58	—

[a]Initial reaction rate in presence (+) and absence (−) of acceptor dextran and apparent maximum synthesis of glucan in the presence of acceptor were assayed by paper disc method. [b]Early (i) and late (ii) fractions in complex enzyme peak (dotted line, Fig. 1). [c]Fractionations of Sephacryl enzyme (major peak).

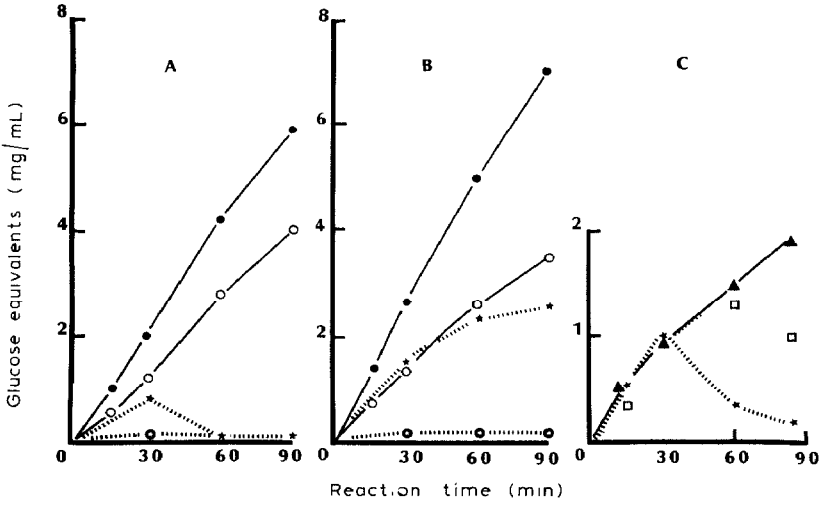

Fig. 2. Glucan synthesis and release of fructose from sucrose catalyzed by GTF fractions from a Sephacryl S-200 column. A and B: GTF (~0.2 IU/mL) in the minor (A) and major (B) fractions released by guanidine from the Sephacryl S-200 column (Fig. 1, solid line) were incubated with sucrose as described in the Experimental section. Release of fructose (circles) and synthesis of glucan (stars) in the presence (solid symbols) and absence (open symbols) of clinical dextran were determined at intervals. C: The minor GTF fraction used in A (about 0.2 IU/mL) was incubated with sucrose in the presence of clinical dextran and the amount of glucan was measured by (1) the filter paper-disc technique (stars) (2) after filtration of the ethanol precipitate on Millipore filters (squares) and (3) after solubilization of the ethanol precipitate in M sodium hydroxide (triangles).

saccharide, produced by this enzyme fraction from [^{14}C]sucrose in the presence of clinical dextran, was isolated and treated with the enzyme fraction in the absence of [^{14}C]sucrose, but hydrolysis of the incorporated ^{14}C-radiolabel did not occur. These tests showed that the Sephacryl enzyme fraction did not contain invertase, dextranase, or other hydrolytic activities capable of degrading its glucan products. Also, the stability of the Sephacryl GTF enzyme at 55°, compared to a rapid loss of activity (75%) of the ammonium sulfate enzyme when incubated for 5 min at 55°, indicated that proteolytic activity also was removed in the Sephacryl fractionation.

The paper-disc and the ethanol-precipitation (not shown) assays of glucan synthesis gave similar results in the absence of acceptor dextran. In the presence of acceptor, the paper-disc assay indicated that the amount of glucan synthesized by some GTF preparations (Fig. 2A) rose to a maximum and decreased, whereas the ethanol-precipitation method showed a continual increase in the total amount of glucan synthesized (Fig. 2C).

Affinity of GTF for aliphatic ligands. — GTF associated with the major protein component from the Sephacryl column was adsorbed on columns containing Shaltiel hydrophobic ligands (aliphatic amines) but adsorbed enzyme was not released by irrigation of the columns with dextran solution (0.5 mg/mL). Enzyme bound to hexylamine–, butylamine–, and ethylamine–Sepharose 6B columns was recovered on irrigation with 0.1M sodium chloride solution in 23, 40, and 75% yield,

respectively. GTF was adsorbed irreversibly to larger hydrophobic ligands and was not released when columns were irrigated with solutions of Triton X-100 (up to 1%) or high salt concentration. Indeed, direct irrigation of a charged ethylamine column with M sodium chloride resulted in loss of all of the bound enzyme activity, whereas irrigation with 0.1M solution released 75% of the enzyme (Fig. 3) with thirty-fold concentration and two to three-fold purification (18–23 units/mL). The GTF-activity peak eluted by a concentration gradient (0–0.02M) of sodium chloride was asymmetric (not shown) and refractionation of the leading portion of this asymmetric peak on the ethylamine–Sepharose column again yielded an asymmetric peak when the column was eluted with a more gradual salt gradient (Fig. 3, inset).

PAGE of dextran treated GTF preparations. — Disc-gel electrophoresis of the major protein peak from the Sephacryl column (solid line, Fig. 1) showed that GTF activity was associated with three of several minor protein bands (Fig. 4A). The intensities of two major, enzymically inactive protein bands were much decreased when the enzyme preparation was fractionated on an ethylamine column, and the middle activity-band (highly dependent on acceptor dextran) was lost (not shown). Preincubation of the Sephacryl enzyme-fraction with clinical dextran decreased the electrophoretic mobilities of the enzyme components (Fig. 4C) more than did preincubation with sucrose (E), but the largest decrease in mobility occurred after preincubation with dextran and sucrose (F). Preincubation with 10% oxidized dextran caused 65% inhibition of the enzyme, but decreased its elec-

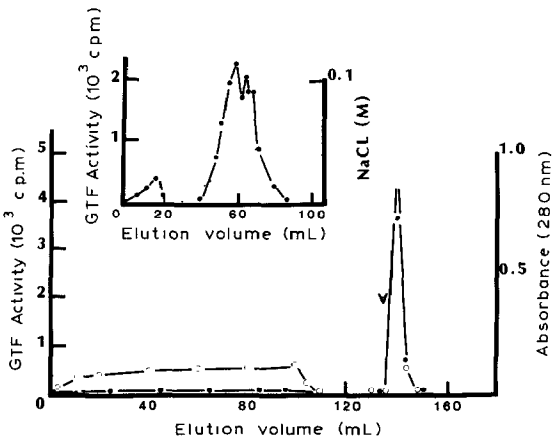

Fig. 3. Fractionation of GTF on an ethylamine–Sepharose 4B column. A solution (100 mL) of the major GTF component recovered from the Sephacryl S-200 column (Fig. 1, solid line) was applied in 50mM sodium phosphate–0.02% sodium azide solution, pH 6.0, to an ethylamine–Sepharose 4B column (1 × 7 cm). The column was washed with the phosphate–azide solution (30 mL) and adsorbed enzyme was released by irrigation of the column, at the point indicated by the arrow, with solution containing 0.1M sodium chloride. When a concentration gradient of sodium chloride (0–0.2M) was used, the activity peak released was asymmetric (not shown). Refractionation of the leading half of this asymmetric peak on an ethylamine column irrigated with a more-gradual salt gradient (interrupted line) is shown in the inset. GTF activity and absorbance (280 nm) are indicated by solid and open circles, respectively.

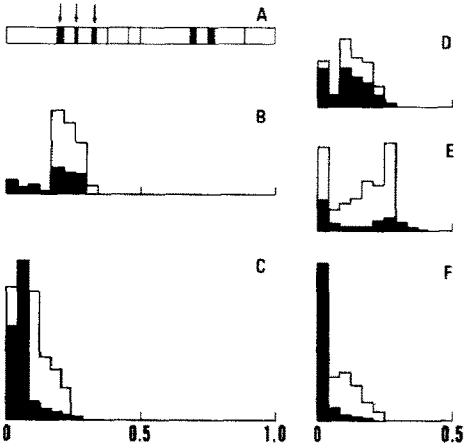

Fig. 4. Electrophoresis of the major GTF fraction released from a Sephacryl S-200 column. Enzyme was subjected to PAGE after preincubation (37° for 30 min) with (A and B) 50mM sodium phosphate buffer; (C) clinical dextran (150 μg/mL); (D) 10% oxidized clinical dextran (150 μg/mL); (E) sucrose (14 mg/mL); (F) sucrose (14 mg/mL) and dextran (150 μg/mL). In A, protein bands were stained with Coomassie Blue and zones of GTF activity were detected as white bands on a duplicate gel immersed for 10 h at 25° in sucrose solution (14 mg/mL). In B–F, gels were sliced and mashed slices were incubated with [^{14}C]sucrose in the presence (open bars) and absence (closed bars) of clinical dextran (150 μg/mL). Bar heights indicate the relative enzyme activities in gel slices.

trophoretic mobility (D) no more than did preincubation with clinical dextran (C).

Gel-filtration of GTF recovered from ethylamine–Sepharose column. — A single, high-molecular-weight component (~2 × 10^6 daltons) was eluted when combined active fractions from the ethylamine column (Fig. 3) were applied to a Sepharose CL-4B column, but enzyme activity in fractions from the trailing edge of the GTF-activity peak released from the ethylamine column by a salt concentration gradient (Fig. 3, inset), was eluted as a low-molecular-weight component in fractions corresponding to the total volume of the gel column. Large losses of enzyme activity (90%) in the gel fractionations resulted in a much decreased specific activity. About 80% of the applied enzyme activity was recovered from Bio-gel 0.5M and Bio-gel 5M columns, but enzyme was eluted without purification in fractions at, or immediately after, the exclusion volumes of the gels. Gel filtration of the ethylamine–GTF fraction on Bio-gel 0.5M in 6M guanidine solution resulted in irreversible loss of activity, although 90% of the original activity was recovered when guanidine was removed (dialysis) from enzyme solution not subjected to gel filtration. Elution profiles of these gel fractionations are not shown.

The enzyme was stable to Brij 35 and Triton X100 (1%), but the presence of Triton X-100 (0.1%) did not significantly affect the ethylamine fractionation and neither detergent (0.1%) affected the gel filtrations. In the absence of detergent, GTF was eluted from a Sepharose 6B column (Ve, Fig. 5A) with 90% loss of activity but, in the presence of a mixture[28] of sodium dodecyl sulfate (0.005%) and Triton X-100 (0.01%), it was stabilized and its elution volume was increased. Under

these conditions, about ten times more enzyme was recovered (88%) and the size of the enzyme aggregate apparently decreased from about 2×10^6 to 6×10^5 daltons. When high-molecular-weight, ^{14}C-labelled dextran was added to GTF before fractionation in the presence of mixed detergent, almost all enzyme activity was eluted with a [^{14}C]dextran–GTF complex in the void volume (Fig. 5A). On refractionation under identical conditions, the [^{14}C]dextran–GTF complex was again eluted in the void volume (Fig. 5B), but if T10 dextran (mol.wt. 10^4) was included in the detergent solution, the complex was largely dissociated and most of the GTF activity was eluted in the same volume as was enzyme not treated with [^{14}C]dextran (Fig. 5A).

The mixed detergents had little effect on dextran-dependent activity, but increased dextran-independent activity of the ethylamine–enzyme fraction two-fold. After gel filtration of the ethylamine–enzyme in the detergent mixture, the ratio of dextran-dependent and -independent activities (in the presence of mixed detergent) was increased from 0.7 to 2.4 and endogenous polysaccharide was decreased from 16 μg to 10 μg/unit of enzyme activity.

Ion-exchange chromatography of GTF preparations. — Components of the GTF activity in the ethylamine column fraction (sp. act. 23) were not separated by

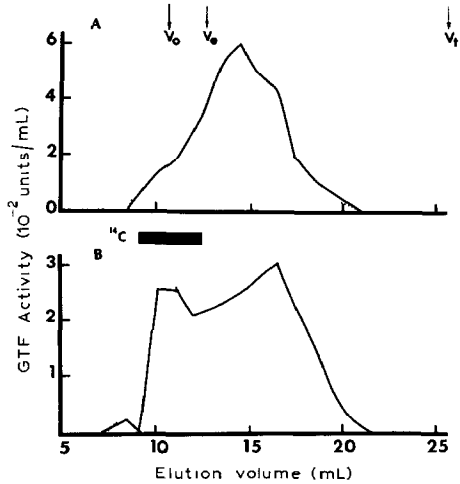

Fig. 5. Fractionation of GTF on Sepharose 6B in the presence of detergents and high- and low-molecular-weight dextrans. GTF fraction from the ethylamine–Sepharose column was fractionated in 50mM sodium phosphate solution pH 6.0 containing 0.005% SDS and 0.01% Triton X-100 on a column (1.1 × 33 cm) previously saturated with the same detergent solution. A; enzyme solution (2 mL, 8 IU/mL) was fractionated in the presence of mixed detergent (solid line) and a second sample, to which high-molecular-weight [^{14}C]dextran was added (0.5 mg/mL), was similarly fractionated (interrupted line). B; excluded fractions of the [^{14}C]dextran–enzyme complex in A were refractionated under the same conditions with (solid line) and without (interrupted line) addition of low-molecular-weight T10 dextran (3 mg/mL) to the detergent–buffer solution. The arrows indicate the void volume (V_o) and the total volume (V_t) of the column. Ve represents the volume in which the peak of GTF activity was eluted by buffer containing neither detergents nor dextran. The solid bar indicates the range of fractions in A and B that contained [^{14}C]dextran.

repeated column chromatography on DEAE-Sepharose 6B, but a partial separation was obtained for the major GTF fraction recovered from Sephacryl S-200 (not shown). The first GTF component (sp. act. 14) released by a sodium chloride concentration-gradient synthesized water-insoluble glucan from sucrose in the presence of soluble acceptor dextran, whilst the second component released (sp. act. 9) synthesized mostly water-soluble glucan. Table V summarizes properties of some of the enzyme fractions recovered in Sephacryl-, DEAE- and ethylamine-column fractionations. With the exception of the effluent fraction from Sephacryl, enzyme fractions required the presence of acceptor dextran for rapid glucan synthesis and catalyzed the release of fructose in the absence of acceptor (not shown). In the presence of dextran acceptor, the release of fructose was about two times faster than was glucan synthesis (see also Fig. 2A and 2B). Enzyme fractions that synthesized the more water-insoluble product (not shown), and apparently yielded smaller maximum syntheses in the filter-paper assay, were more inhibited by 10% oxidized dextran (Table V). Fructose release in the absence of acceptor dextran, however, was not inhibited by the oxidized dextran.

Two enzyme components in the major Sephacryl fraction were partially separated on a hydroxylapatite column irrigated with a phosphate concentration gradient (not shown), but the order of their elution was the reverse of that from DEAE-Sepharose. In both separations <40% of the total enzyme activity applied was recovered. A single fractionation of GTF on DEAE-Sepharose did not increase

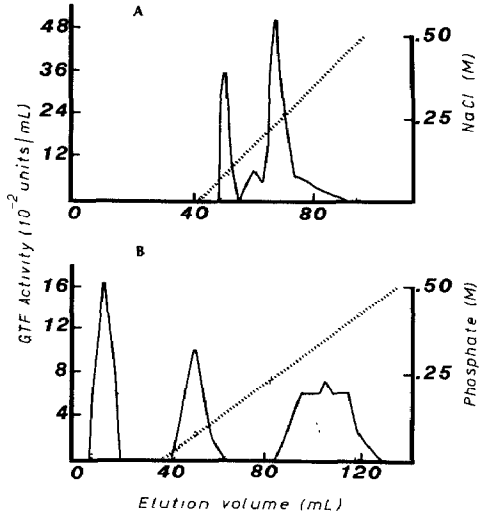

Fig. 6. Fractionation of GTF on DEAE-Sepharose 6B and on hydroxylapatite columns. The major GTF component from Sephacryl S-200 was fractionated on DEAE-Sepharose 6B (A) and on hydroxylapatite (B) columns saturated with 20mM sodium phosphate solution containing 0.005% SDS and 0.01% Triton X-100 (thin interrupted line). The fractionations were repeated with the same buffer–detergent solutions containing 10 mg of T10 dextran per mL (solid lines). Sodium chloride and phosphate concentration gradients applied to the columns are indicated by thick interrupted lines.

the specific activity of the enzyme, but three fractionations gave a progressive increase in specific activity of the major enzyme fraction (sp. 9, 18, and 23 units/mg protein) and a decrease in total activity (40, 24, and 12%). The loss of activity invariably was accompanied by a decrease in the capacity of the enzyme fraction to synthesize water-insoluble glucan. Electrophoresis (PAGE) of enzyme fractions isolated from the ion-exchange columns gave multiple bands of enzymically active and inactive protein (not shown).

Fractionation of the GTF complex was not improved when 0.05% Brij 35 (non-ionic detergent) was included in the solution used to irrigate the DEAE column, and in the presence of an SDS (0.005%) and Triton X-100 (0.01%) mixture, <25% of the total GTF activity applied to the DEAE-column was eluted at a low level over the range of the salt gradient (Fig. 6A). Slab gel (4% polyacrylamide) electrophoresis in the presence of the mixed detergent solution indicated that protein was eluted in this fractionation in the form of polydisperse aggregates progressively decreasing in their electrophoretic mobility, and that the greater part of the enzyme activity was associated with the slower moving aggregates (not shown).

When T10 dextran (mol. wt. 10^4) was included with the detergent mixture in the irrigation solution, GTF activity was recovered from the DEAE column in two

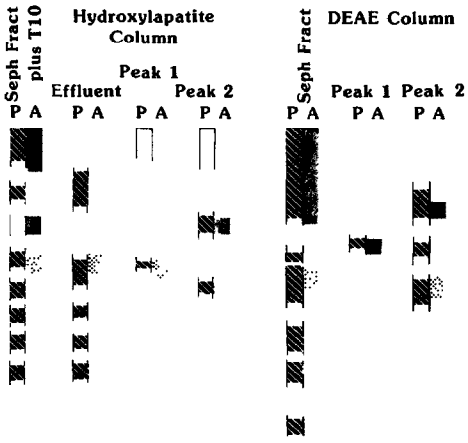

Fig. 7. Polyacrylamide slab gel electrophoresis of hydroxylapatite- and DEAE-column fractions of GTF in the presence of mixed detergents. Sephacryl S-200 GTF (Seph Fract), with (plus T10) or without T10 dextran (10 mg/mL), and enzyme fractions separated on hydroxylapatite or DEAE-Sepharose 4B columns in the presence of mixed detergents and T10 dextran (Fig. 6), were placed in wells of a vertical 4% polyacrylamide slab gel (2 mm thick). The gel was run at 12.5 V/cm (25 mA) for ~3 h in 25mM Tris–190mM glycine buffer, pH 8.3, containing 0.01% Triton X-100 and 0.005% SDS. The left half of each gel was used to detect protein with a silver stain and the right half of the gel, in which duplicate samples of the enzyme were placed in wells, was used to detect enzyme activity as described in the Experimental Section. The origin and cathode is at the top of the diagram in which protein bands (P) and enzyme zones (A) are paired for wells containing the same enzyme preparations. P: hatched areas, heavy protein staining; open lines, streaking of protein stain. A: fine, dark stippled area, water-insoluble glucan product (GTF-I); coarse, light stippled area, water-soluble glucan product (GTF-S).

fractions (Fig. 6A). Electrophoresis in the presence of the mixed detergents revealed that the first fraction eluted contained a single protein component (GTF-I) that catalyzed the synthesis of water-insoluble glucan from sucrose (Fig. 7). The second fraction contained three protein components, of which two were enzymically active. The slowest moving component synthesized a water-insoluble product, but its electrophoretic mobility was lower than that of the enzyme in the first fraction, whereas the fastest moving protein band (GTF-S) synthesized a water-soluble glucan and possessed a higher mobility than the enzyme in the first fraction.

GTF activity was recovered in three fractions (65% total activity) when Sephacryl enzyme was fractionated on hydroxylapatite in the presence of the detergent mixture, and again resolution of the fractions was improved in the presence of T10 dextran (Fig. 6B). The effluent fraction contained multiple protein bands (Fig. 7), one of which was associated with a single enzymic activity that synthesized water-soluble glucan (GTF-S). The same enzyme activity (GTF-S) was associated with a major protein component in the first eluted of the bound fractions, but a different enzyme component (GTF-I) was associated with the major protein band in the second fraction released from the column.

There were differences for different enzyme batches, but the resolution of the two GTF components in the Sephacryl S-200 fraction invariably was improved when fractionations were conducted in the presence of the mixed detergents and T10 dextran. The concentration of T10 dextran in enzyme fractions (10 mg/mL) interfered with the GTF assay, but control experiments on a standard enzyme solution in the presence and absence of T10 dextran indicated that enzyme recovery was higher when hydroxylapatite column fractionations were conducted in the presence of low-molecular-weight dextran.

DISCUSSION

Stimulation by soluble dextran of the glucan-synthesizing activity of GTF aggregates indicates a specific affinity of the catalytic protein(s) for dextran structure, but does not accurately predict that the GTF will bind to dextran ligands of affinity columns (Tables I and III). The reason may be that the intrinsic affinity of the GTF components is partly, or completely, obscured by glucan–protein and protein–protein interactions that form the enzyme aggregate[29]. The enhanced release of adsorbed GTF resulting from an increase in the ionic strength of the dextran solution used to irrigate dextran-affinity columns (Table II) is consistent with the participation of ionic (protein–protein) and specific (glucan–protein) interactions in the formation of the enzyme aggregate and in its binding to the column.

Aggregation and composition of GTF complexes vary with the conditions of growth of the source organism. Thus, aggregation is decreased by growth in the presence of Tween 80 and invertase-treated Trypticase–soy broth[13], and by substitution of D-fructose for D-glucose as carbon source in a chemically defined

medium[30,31]. Carbon source and growth conditions also influence cell adherence[32] and the production of lipotechoic acid[33,34], a macromolecule that induces aggregation of GTF[35] and that has been detected as a component of a high-molecular-weight GTF aggregate. The presence of different amounts of this amphipathic molecule would influence the ionic and hydrophobic properties of GTF aggregates and endow otherwise identical enzymes with different binding characteristics on affinity columns (Tables I–III). Also, the ratio of GTF-S and GTF-I changes significantly with the rate of cell growth[36], and the presence of Tween 80 dramatically increases production of a dextran-independent GTF[9]. The different elution patterns of enzyme prepared from different batches of *S. mutans* 6715-49 (Table I), thus may reflect changes in the composition and state of aggregation of the GTF complex in response to small differences in conditions of growth.

The identical elutions of GTF activity from a dextran–ligand column and a control column lacking the dextran ligand (Table II) demonstrate that affinity for dextran is not necessarily involved in enzyme adsorption, and raises a question as to the extent to which dextran affinity is responsible for adsorption of GTF aggregates to water-insoluble dextran fractions[27,37] or to dextran affinity columns[12–17]. Clearly, components of a GTF aggregate should not be characterized as "dextran-binding" solely on the basis that they are adsorbed to dextran–ligand columns, even if they are released in an apparently specific manner by irrigation with dextran solution. The hydrophobic properties of GTF aggregates that bind them to Shaltiel aliphatic ligands (Fig. 3) also may be responsible, at least in part, for their apparent affinity for some dextran columns. Thus, dissolution of hydrophobically bound aggregates by dextran (Tables I and III), by salt (Fig. 3), or by a combination of salt and dextran (Table II) then might release different proportions of GTF and other proteinaceous components that do not necessarily possess intrinsic affinities for dextran.

The lack of dextran affinity of a GTF fraction released by guanidine from a Sephadex G-50 column, compared to the corresponding, dextran-free fraction from Sephacryl S-200 (Table III), may result from saturation of the enzyme-binding sites by traces of dextran solubilized from the Sephadex matrix. Similarly, the presence of residual dextran in GTF preparations previously exposed to dextran may explain their apparent loss of affinity (Table III), but modification or loss of binding sites (either hydrophobic or dextran-specific) might equally well result from the significant increase in aggregation that is induced by clinical dextran (Fig. 4).

The similar aggregation of GTF exposed to either 10% oxidized dextran or clinical dextran (Fig. 4) indicates that aggregation is not responsible for enzyme inhibition by oxidized dextran[19], whilst the different extents of this inhibition (Table V) point to differences in the enzymic composition of the GTF fractions. The inability of oxidized dextran to inhibit fructose release by enzyme fractions acting in the absence of acceptor dextran confirms that inhibition is directed against the acceptor reaction in glucan synthesis[19]. However, the direct relationship of the extent of inhibition to the ability of enzyme fractions to synthesize water-insoluble

glucan (not shown), and its inverse relationship to maximum glucan synthesis as determined by the paper-disc assay (Table V), suggest that glucan synthesis by the GTF-I component may be more susceptible to inhibition. The lower inhibition of glucan synthesis by the DEAE peak 2 fraction (Table V) then would be consistent with its higher content of GTF-S which, after an extended reaction period, catalyzes glucan synthesis in the absence of acceptor dextran. Details of the "de novo" glucan synthesis by GTF-S purified from this fraction have been reported[38].

The disparity between values obtained for glucan synthesis by the paper-disc and ethanol-precipitation methods (Fig. 2) results from conversion of water-soluble dextran acceptor into insoluble and adherent glucan. This represents a serious defect when the routine paper-disc assay is used for reactions in which insoluble glucan is synthesized. To ensure that all insoluble glucan is transferred to the paper discs, it first must be solubilized by addition of alkali to the mixture or to the ethanol-precipitated product (Fig. 2).

Dissociation of the partially purified GTF-complex from *S. mutans* 6715 by the detergent mixture (Fig. 5) is identical to that reported for a GTF-complex from *S. sanguis*[28]. The detergent effect seems to be restricted to protein–protein interactions, because it does not prevent the formation of a high-molecular-weight dextran–GTF complex (Fig. 5), and it increases the apparent dextran-independent activity of the GTF preparation, presumably by increasing the priming efficiency of endogenous glucan. Disruption of protein interactions probably also is responsible for the improved fractionation of the enzyme complex (Fig. 6b) in the well-known separation of GTF-S and GTF-I components of the enzyme complex on hydroxylapatite[36,39]. The additional disruption of glucan–protein interactions by T10 dextran is required in order to obtain a comparable separation of the enzyme components on DEAE-Sepharose (Fig. 6a).

GTF-S and GTF-I in the *S. mutans* 6715 enzyme-complex both bind to dextran affinity columns, although the minor dextran-independent component[9] remains in the effluent fraction from a Sephacryl S-200 column. Release of adsorbed enzyme by clinical dextran solution does not separate the two components and gives a low yield (Table I) and an irreversible change in the dextran-binding properties of the recovered enzyme-fraction (Table III). Although irrigation with guanidine solution ensures higher yields of enzyme[16,37], it also fails to separate the GTF-S and GTF-I components (Fig. 1). The stability of Sephacryl under these conditions has the advantage over other dextran columns in that multiple fractionations may be conducted on the same column without deterioration in its performance. Sephacryl S-200 has been used, in conjunction with the hydroxylapatite column, for purification of the GTF activities of *S. mutans* 6715 only, but the good yield (55–60% of total activity) and near-homogeneity of enzyme components (Fig. 7) obtained suggest that, with further refinement, the two-step column procedure may be useful for isolating representative amounts of GTF components in enzyme aggregates elaborated in complex growth media by other serotypes of *S. mutans*, and by other strains of streptococci.

ACKNOWLEDGMENTS

We thank Donald Nuessle for his expert technical assistance in these studies. This work was supported by grant number DE-03118 from the National Institute of Dental Research.

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