Carbohydrate Research, 42 (1975) 325–338

PURIFICATION AND CHARACTERIZATION OF A GLYCOSYLTRANSFERASE COMPLEX FROM THE CULTURE BROTH OF *Streptococcus mutans* FA1

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(Received March 3rd, 1975; accepted for publication, March 26th, 1975)

ABSTRACT

The extracellular glycosyltransferases from *Streptococcus mutans* FA1 were purified by using the following procedures: ammonium sulfate precipitation, poly-(acrylamide) gel filtration, DEAE-cellulose chromatography, and agarose-gel filtration. The dextransucrase and levansucrase activities were purified 350- and 500-fold, respectively, and the ratio of the two activities remained almost constant throughout the purification. Both enzymes have a pH optimum of 6.0, a K_m for sucrose of 55mM, and isoelectric points of 3.7 and 4.6. The enzymes are inactivated by repeated freezing and thawing, but retain partial activity even after heating at 100°. The enzyme preparation contains a carbohydrate moiety which does not appear to be either bound levan or dextran.

INTRODUCTION

The extracellular polysaccharides synthesized from sucrose by *Streptococcus mutans* are thought to be involved in dental-plaque formation. This relationship to plaque appears to be mediated by the synthesis of either levans or dextrans, or both, when the organisms are exposed to dietary sucrose. These polysaccharides are believed to act by providing a mechanism whereby the bacteria can adhere to the tooth surface[1–3]. Thus, the roles of the enzymes responsible for the production of these polymers are of obvious interest.

The glycosyltransferase from the cariogenic streptococci have been purified from the culture broth[4,5] and extracted from whole cells[6,7]. Some of these preparations also contain invertase-like activity[8,9]. The present report describes the purification of a glycosyltransferase of high molecular weight from *Strep. mutans* FA1 which contains a levansucrase [(2→6)-β-D-fructan: D-glucose 6-fructosyltransferase, EC 2.4.1.10] and a dextransucrase [(1→6)-α-D-glucan: D-fructose 2-glucosyltransferase, EC 2.4.1.5]. The two activities were not resolved from each other during the purification procedures.

MATERIALS

Streptococcus mutans FA-1 was obtained from Dr. R. J. Gibbons, Forsyth Dental Center, Boston, Mass., and was maintained on Eugonagar. Broth cultures were grown anaerobically at 37° in trypticase soy-broth containing 0.25% of D-glucose (Baltimore Biological Laboratories, Baltimore, Maryland). Permeation gels (P-60 and A-15m) were obtained from Bio-Rad Laboratories, Richmond, California, and DEAE-cellulose was obtained from Schleicher and Schuell, Inc., Keene, New Hampshire. The D-glucose oxidase reagent and dextran were purchased from Sigma Chemical Company, St. Louis, Missouri. The D-glucose oxidase was reconstituted with 10μM silver nitrate, which inactivates the invertase commonly found in the preparation[10]. Ampholytes (pH 3–10 and 3–6) and the electrophoretic column used were obtained from LKB Produktor, Rockville, Maryland. Filtration and concentration apparatus and membranes were purchased from Amicon Corp., Lexington, Massachusetts. All other chemicals were of reagent grade. Radioactive sucroses (sucrose-[D-fructose-*1*-^{3}H] 5.09 Ci/mmol and sucrose-[D-glucose-U-^{14}C] 242 mCi/mmol) and scintillation fluid were purchased from New England Nuclear, Boston, Mass.

METHODS

Measurement of enzymic activity. — The enzyme preparation (4.5 ml) and sucrose (0.5 ml, 50%) were incubated at 37° in phosphate buffer (pH 6.0, 50mM). Aliquots (0.5 ml) of the enzymic reaction-mixtures were removed, and added to sodium hydroxide (50mM, 2.0 ml) to terminate the reaction. These samples were immediately analyzed for total reducing power[11] and for D-glucose by D-glucose oxidase-peroxidase. The boiling time for the analysis for total reducing sugar was increased from the usual 10 min to 45 min. D-Fructose gives a molar absorbance equal to that of D-glucose with the longer boiling-time. Standard solutions were prepared from equimolar amounts of D-glucose and D-fructose, and these were treated as the unknowns. Column eluates were analyzed in a similar way, except that 0.5 ml of the column eluate was incubated with sucrose (5%, final concentration) for 20 min, prior to analysis for D-glucose and reducing sugar. The D-glucose determination was used as a measure of levansucrase activity, and dextransucrase activity was determined from the difference between total reducing sugar and amount of D-glucose. The rate of the reaction was linear for 20 min under the standard incubation-conditions. One unit of enzymic activity was defined as that amount of levansucrase (dextransucrase) that will release 1.0 μmole of D-glucose (D-fructose) from sucrose in 50mM buffer (pH 6.0) when incubated for one min at 37°.

Polysaccharide determination. — The polysaccharide concentration was measured after exhaustive dialysis of the sample by the phenol–sulfuric acid method[12], with dextran as the standard. Levan was determined by the sulfuric acid–20% cysteine assay[13], with *Streptococcus salivarius* SS2 levan as the standard. The amount of hexulose (levan) was subtracted from the amount of total polysaccharide to give the

amount of dextran. The synthesis of both polymers was also monitored by measuring the incorporation of uniquely labeled sucrose (labeled in either the D-glucose or D-fructose moiety) into polymer. The method employed the chromatographic separation of labeled sucrose from polymer, and measured the labeled materials remaining at the origin[14].

Isoelectric focusing. — The enzyme preparation from the P-60 column was subjected to isoelectric focusing (500 V, 5°, 24–36 h) according to the method of Vesterberg and Svensson[15] in an LKB (Ampholine) column (110 ml), with a pH gradient of either 3–10 or 3–6 and a glycerol density-gradient. Fractions (1.7 ml) from the column were collected by gravity feed, the pH determined (5°), and the fractions dialyzed against glycine (1%) and then against phosphate buffer (50mM, pH 6.0).

Protein determination. — The protein concentration of the enzyme preparations was measured either by absorbance at 280 nm or by the method of Lowry *et al.*[16], with bovine serum albumin as the standard.

RESULTS

Isolation of glycosyltransferases. — Step 1. Ammonium sulfate (189 g/litre) was slowly added to cold (5°), cell-free broth from a 15–18 h culture. The mixture was constantly stirred (5°, 12–24 h), and sediment removed in a continuous-flow cen-

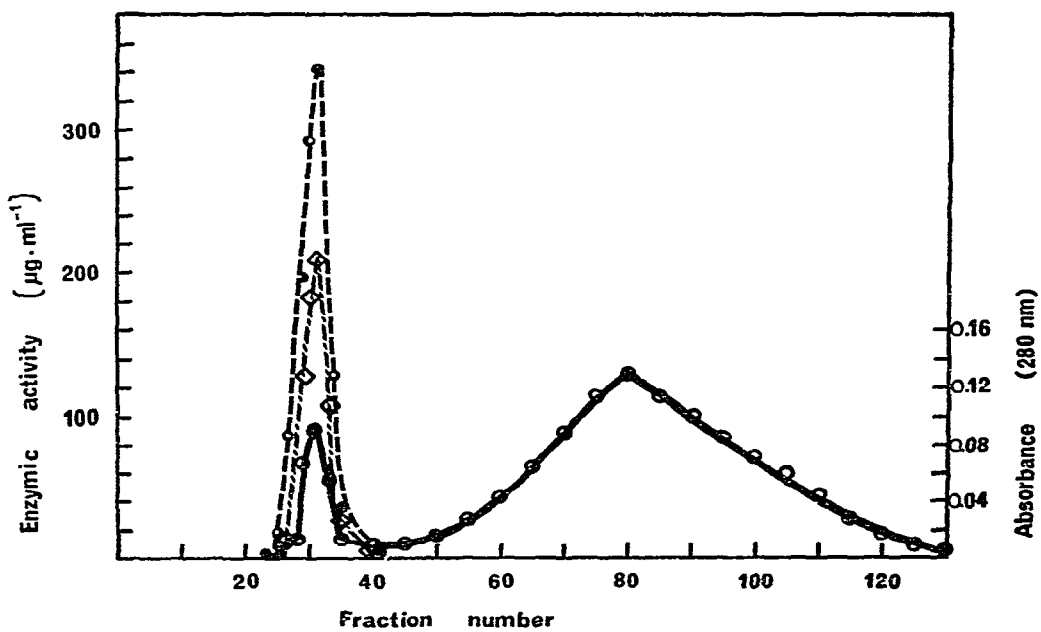

Fig. 1. Elution profile of the enzyme (precipitated with ammonium sulfate) through a P-60 column (5 × 45 cm). [Fraction vol., 7.4 ml per tube; absorbance (280 nm), ——●——; total reducing power (μg/ml), — — ● — —; D-glucose released (μg/ml), - - ◇ - - -.]

trifuge; the pellet was found to be inactive, and was discarded. A second addition of ammonium sulfate (53 g/litre) was made to the solution, and the mixture was stirred (5°, 12–24 h), and recentrifuged by using a continuous-flow apparatus. The inactive, supernatant liquor was discarded, and the pellet was recovered and dialyzed exhaustively (50mM phosphate buffer, pH 6.0). The material that did not dissolve was removed by centrifugation, and discarded.

Step 2. The dialyzed solution was concentrated in an Amicon positive-pressure cell having a PM10 membrane, and was applied to a P-60 column (5×45 cm) and eluted with phosphate buffer (pH 6.0, 50mM). Glycosyltransferase activity (release of reducing sugar and D-glucose) was found only in the void volume of the column (see Fig. 1).

Step 3. The enzymically active fractions were reconcentrated in an Amicon cell (PM10 membrane), and applied to a column of DEAE-cellulose (see Fig. 2). A linear

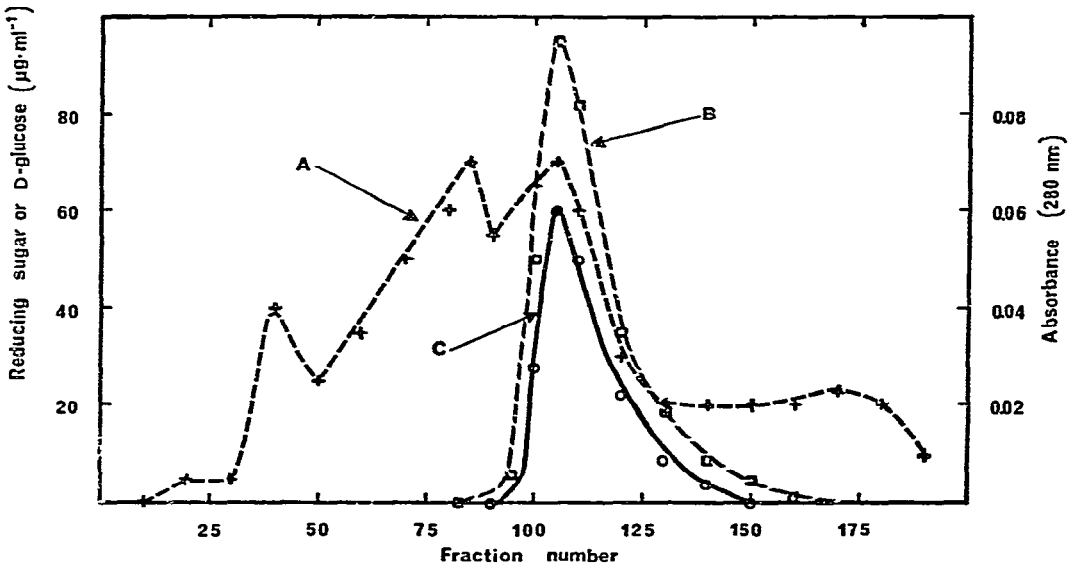

Fig. 2. Elution profile of the concentrated peak from P-60, through a column (2.5×42 cm) of DEAE-cellulose. [The column was washed with water (230 ml), and then a linear gradient (0–0.75M) of NaCl was used to elute the protein. Fraction vol., 3.2 ml per tube; A, absorbance at 280 nm —+—+—; B, total reducing power (μg/ml) —□—; C, D-glucose released (μg/ml) —○—]

gradient of sodium chloride (0–0.75M, 500 ml of each) in phosphate buffer (pH 6.0, 50mM) eluted only one enzymically active peak at a concentration of ~0.4M sodium chloride.

Step 4. The active fractions from the DEAE-cellulose column were concentrated in an Amicon cell, and applied to a column of Bio-Gel A-15 m (see Fig. 3). Two enzymically active peaks were found to be eluted within the inclusion volume of the column, *viz.*, fractions I (50–80) and II (85–95). The fractions from each peak were combined, tested for polysaccharide-synthesizing ability, and found to have the same

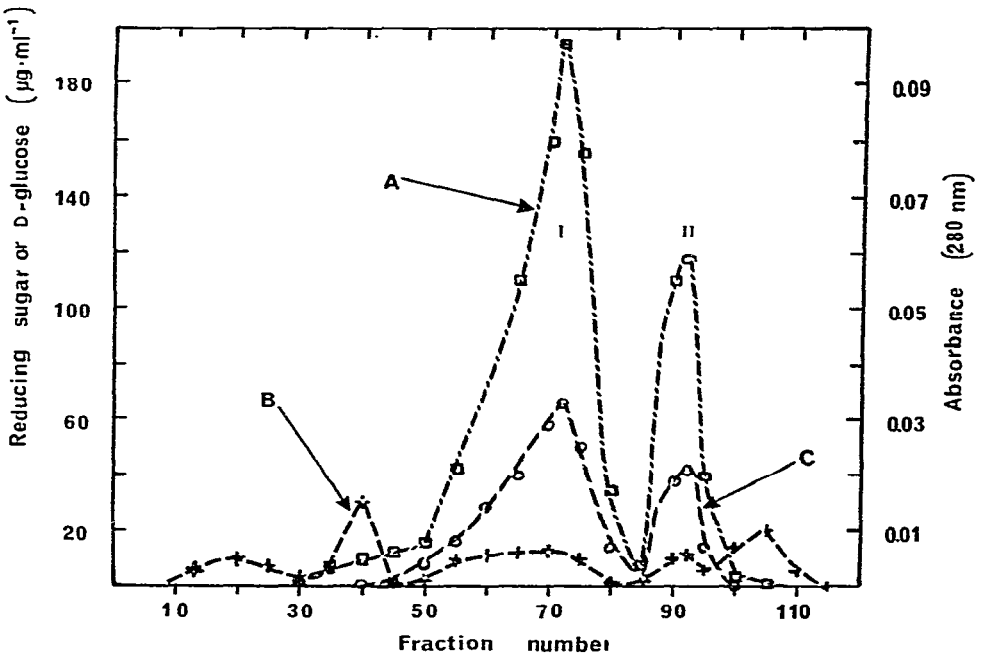

Fig. 3. Elution profile of the active peak from the DEAE-cellulose column through a column (2.5 × 45 cm) of A-15m. [Fraction vol., 1.9 ml per tube; A, total reducing power (μg/ml) ----□--; B, absorbance (280 nm) ±—+—;C, D-glucose released (μg/ml)——○—.]

levan/dextran (L/D) ratio. Thus, the enzymic activities were routinely obtained by measuring D-glucose and reducing sugar released from sucrose, and the same L/D ratio was found by determining the amount of levan and dextran formed, following exhaustive dialysis. The protein readily aggregated and precipitated during concentration in the filtration cell; presumably, the two peaks may represent two molecular sizes of the aggregated protein. This protein was excluded from a P-300 gel filtration, indicating a molecular weight >800,000. However, homogeneity studies of the preparation by disc gel-electrophoresis were hampered, because the protein did not enter the "running" gels (5%) during electrophoresis.

The data for the purification of the transferases are given in Table I. During purification, the L/D ratio rises somewhat, from 1.29 to 1.85. At no time in this purification was a fraction isolated that was found to be monospecific in enzymic capabilities.

The syntheses of the dextran and levan were confirmed by using sucrose labeled in either the D-glucosyl or the D-fructosyl group. The enzyme preparation purified on DEAE-cellulose was incubated with the labeled sucrose (~30 pmole) along with carrier sucrose (100mM, final conc.). Aliquots (10 μl) of the incubation mixture were applied to the thin-layer plates, the plates were developed, and the radioactivity of the origin of the chromatograms was determined (see Table II).

TABLE I

SUMMATION OF THE PURIFICATION PROCEDURE FOR THE BROTH GLYCOSYLTRANSFERASE

Procedure	*Volume* (ml)	*Protein* (mg/ml)	*Dextransucrase*			*Levansucrase*			*L/D*
			Enzyme concentration (*U/ml*)	*Total units* (*U*)	*Specific activity* (*U/mg*)	*Enzyme concentration* (*U/ml*)	*Total units* (*U*)	*Specific activity* (*U/mg of protein*)	
Cell-free broth	15,300	2.7	0.18	2,755	0.07	0.24	3,600	0.09	1.29
$(NH_4)_2SO_4$ precipitate	320	0.95	3.0	960	3.2	3.6	1,151	3.79	1.18
P-60 chromatography	730	0.1	0.6	438	8	1.0	720	10	1.25
DEAE-cellulose chromatography	240	0.04	0.9	216	22.5	1.55	372	39	1.78
A-15m chromatography, peak I	192	0.024	0.59	115	25	1.12	216	46.8	1.87
peak II	164	0.01	0.24	39	24	0.44	72	44.1	1.84

Almost the same L/D ratio was observed based on the incorporation of labeled precursors into polymers as was observed with the release of monosaccharide. The L/D ratio varied as the concentration of sucrose was varied from 3 to 146mM (see Table III). The enzyme preparation was incubated with various concentrations of sucrose for 16 h, and the material was exhaustively dialyzed, and then analyzed for non-dialyzable carbohydrate. The total amount of each of the polymers also varied with the sucrose concentration.

TABLE II

INCORPORATION OF SPECIFICALLY LABELED SUCROSE INTO LEVAN AND DEXTRAN BY THE GLYCOSYLTRANSFERASE

Time (*min*)	*Radioactivity at origin of chromatogram*				*L/D*[a]
	^{3}H-D-*Fructose*		^{14}C-D-*Glucose*		
	(*c.p.m.*)	(*pmole*)	(*c.p.m.*)	(*pmole*)	
30	2880	0.77	748	1.54	1.03
45	3959	1.06	914	1.89	1.18
60	5063	1.35	1131	2.34	1.23
90	7451	2.00	1543	3.19	1.36

[a]Normalized to 50 pmole of labeled sucrose per assay.

TABLE III

EFFECTS OF CONCENTRATION OF SUCROSE ON SYNTHESIS OF POLYSACCHARIDE

Sucrose concentration (*mM*)	*Non-dialyzable carbohydrate*[a] (*mg/ml*)	*Levan* (*hexulose*)[b] (*mg/ml*)	*Dextran* (*by difference*) (*mg/ml*)	*L/D*
146	4.4	3.3	1.1	3.0
117	3.9	3.1	0.8	3.9
88	3.7	3.0	0.7	4.3
59	3.5	2.9	0.6	4.8
44	2.7	2.4	0.3	8.0
39	2.4	2.1	0.3	7.0
3	1.0	0.8	0.2	4.0

[a]Phenol–sulfuric acid. [b]Cysteine (20%)–sulfuric acid.

Enzyme properties

pH optimum. — The dependence of the reaction on pH was studied over the pH range of 4–8 by using phosphate–citrate buffers. The activity range was rather broad, and centered around pH 6.0 (see Fig. 4).

Michaelis–Menten constant. — The rate of the release of reducing sugar was proportional to the time (20 min) and to the protein concentration (to 75 μg/ml). The K_m constant for sucrose was determined to be 55mM (see Fig. 5).

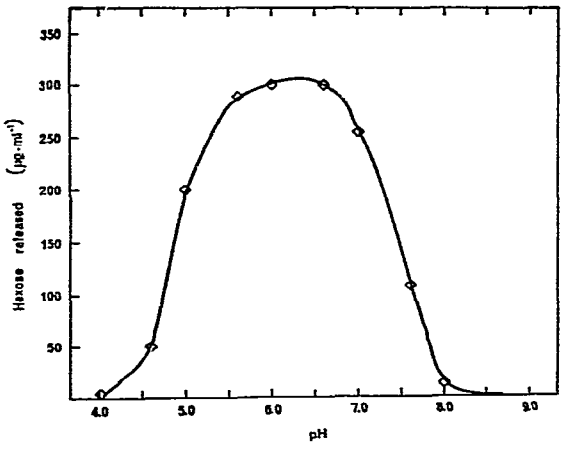

Fig. 4. The effect of pH on the glycosyltransferase activity of the enzymic preparation from the P-60 column, as measured by the increase in total reducing power.

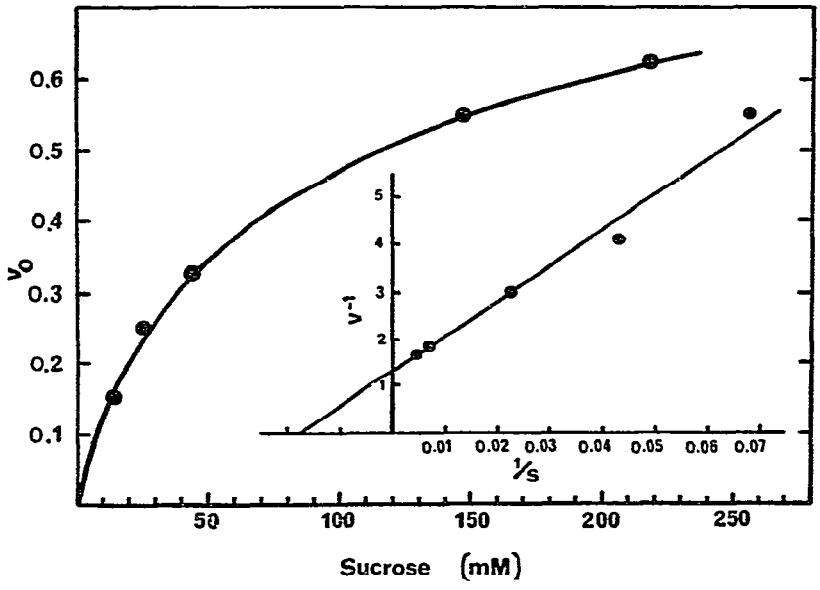

Fig. 5. Kinetics of the glycosyltransferase incubated with sucrose in phosphate buffer (pH 6.0, 50mM) at 37°. [Initial velocity is in nmole of reducing sugar released from sucrose per min. Insert: double reciprocal plot of the data.]

Effects of cations and anions on enzymic activity. — Because many divalent cations precipitate with the phosphate in the buffer, several other buffer systems were examined. Cacodylate and Tris-HCl (50mM, pH 6.75) could replace phosphate without a loss of activity, whereas imidazole buffer (50mM, pH 6.75) caused a 40% lessening in activity. Various chlorides and nitrates (salts) were added to the enzyme

(purified on DEAE-cellulose) that had first been dialyzed against cacodylate buffer. Sucrose was then added, and the mixture was incubated for 45 min at 37°. The reaction was terminated by the addition of alkali, and the amount of total reducing sugar was measured and compared to the control (see Table IV). There was an

TABLE IV

THE EFFECT OF VARIOUS CATIONS ON THE ACTIVITY OF THE GLYCOSYLTRANSFERASE IN CACODYLATE BUFFER

Metal ion	*Millimolarity*			
	1.6	*4.0*	*8.0*	*16*
	Activity remaining (%)			
None	100	100	100	100
Ag^+, Hg^{2+}, Cu^{2+}	0	0	0	0
Cd^{2+}	24	7	0	0
Pb^{2+}	24	21	15	13
Zn^{2+}	35	18	9	0
Ni^{2+}	47	35	29	26
Mn^{2+}	48	30	13	0
Fe^{2+}	59	32	15	9
Co^{2+}	65	50	35	24
Ca^{2+}, Mg^{2+}	100	100	100	100
Na^+, K^+, NH_4^+	100	100	100	100

inhibition of the enzymic activity by all of the divalent ions tested, except for Ca^{2+} and Mg^{2+}. None of the metal ions stimulated the activity. A series of anions was also examined in the same general way as for the cations, except that phosphate buffer was employed (see Table V). The hexametaphosphate, (ethylenedinitrilo)tetraacetate

TABLE V

THE EFFECT OF VARIOUS ANIONS ON THE ACTIVITY OF THE GLYCOSYLTRANSFERASE

Ion	*Millimolarity*			
	1.6	*4.0*	*8.0*	*16*
	Activity remaining (%)			
None	100	100	100	100
F^-, NO_4^-, SO_4^{2-}	100	100	100	100
CN^-	100	94	89	69
I^-	100	93	87	72
Pyrophosphate	100	100	100	100
Trimetaphosphate	100	100	100	100
Hexafluorophosphate	100	100	100	100
EDTA	86	79	73	69
Hexametaphosphate	41	37	34	31

(EDTA), and cyanide inhibited the reaction, which may be the result of chelation or complexation of a metal ion normally associated with the enzyme. The other phosphate derivatives were tested because they have been used in various studies on dental caries[17], but they showed no effect on the enzymic activity.

Isoelectric point. — Isoelectric focusing was examined as a possible means of separation of the two enzymic activities. Two enzymically active peaks were observed at pH 4.6 (major) and 3.7 (minor), and they yielded similar L/D ratios, namely, 3.0 and 1.7, respectively (see Fig. 6). This technique did not appear to resolve the activities, and was not therefore employed in the purification procedure.

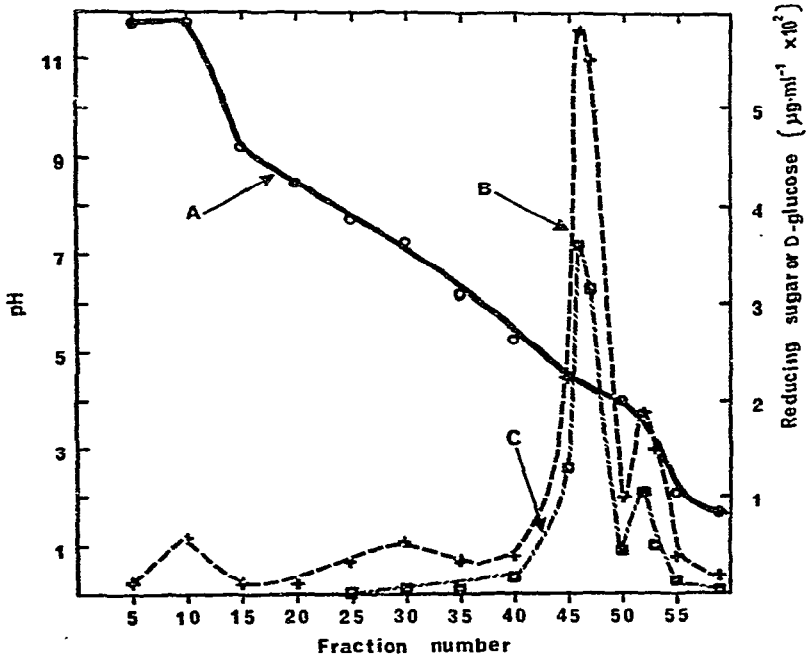

Fig. 6. Isoelectric-focusing profile of the glycosyltransferase isolated by the P-60 column. [Protein concentration (not plotted) follows the enzymic activity exactly. Fraction vol., 1.7 ml per tube; A, pH, ——○——; B, total reducing power (μg/ml) ±——±——; C, D-glucose released (μg/ml) - - -·-□-·-]

Thermal stability. — The stability of the purified enzyme preparation to various means of storage was studied in a series of freezing–thawing and repeated lyophilization experiments. After the initial activity had been determined, the enzyme was either frozen and then thawed, or was freeze-dried and then reconstituted with water. Both activities were more stable to repeated lyophilizing than to freezing–thawing (see Fig. 7), but neither method was completely satisfactory.

The lability to heat inactivation was studied by heating the enzyme preparation (purified on DEAE-cellulose) to various temperatures for either 10 or 30 min. The

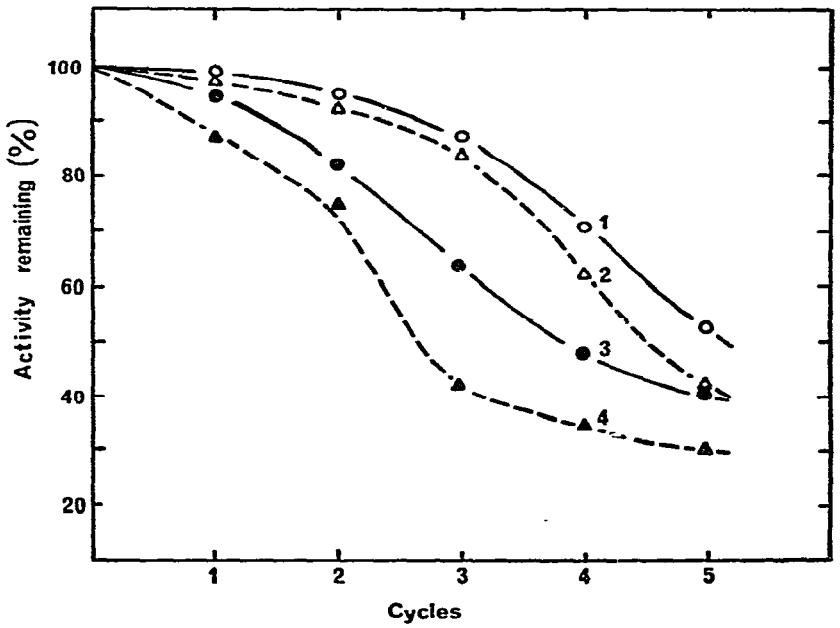

Fig. 7. The inactivation of the glycosyltransferases by thawing and by repeated lyophilization. [Curve 1, the dextransucrase (DS), and curve 2, the levansucrase (LS) activities subjected to lyophilization. Curves 3 and 4, the DS and LS activity subjected to freeze–thaw, respectively.]

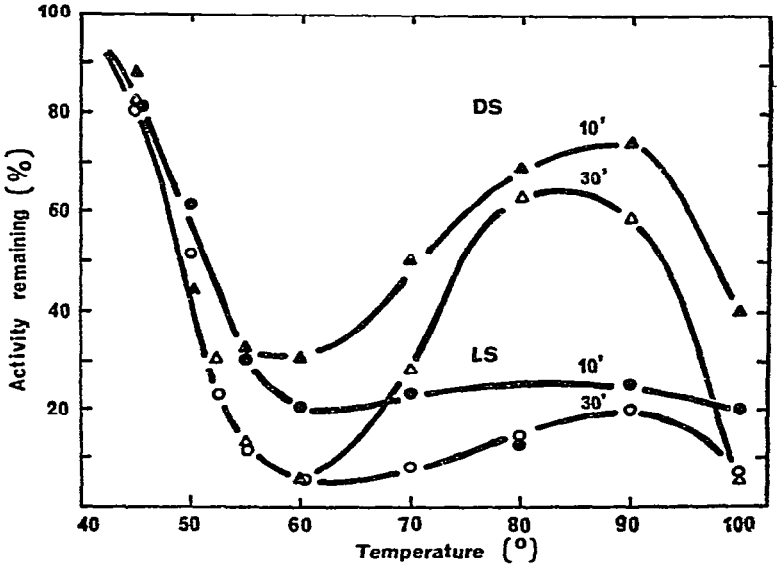

Fig. 8. The inactivation of the glycosyltransferase activities subjected to various temperatures for either 10 or 30 min. [The enzyme preparation was then incubated with sucrose for 30 min at 37°, and assayed for activity. DS activity after being heated for 10 min (▲) or 30 min (△); LS activity after being heated for 10 min (●) or 30 min (○).]

solution was cooled to 37°, incubated for 30 min with sucrose, and then assayed by the release of D-glucose and the total reducing sugar. The enzymic activity decreased as the temperature was increased to ~60° (see Fig. 8). As the temperature was raised from 60°, the dextransucrase activity increased markedly, while the levansucrase remained about constant. Both activities were present, even when the material was heated for 10 min at 100°. Reactivation by heat of a thermally inactivated protein has been termed the "Arrhenius effect", and has been reported[18] for staphylococcal α-toxin.

Glycoprotein nature of the enzyme preparation. — As there was the possibility of either a dextran or a levan association with the enzyme preparation, direct carbohydrate analysis of the purified enzyme was performed. The phenol–sulfuric acid assay revealed that the preparation contained 41% of carbohydrate (expressed as glucose). Paper-chromatographic analysis of acid hydrolyzates revealed two compounds—one migrating like a hexose and the other like a pentose. Glucose accounted for 41%, and galactose for 5.6%, of the carbohydrate, as enzymically determined in hydrolyzates. Sialic acid (4.7%) and 6-deoxyhexose (3.8%) were also detected, by the thiobarbituric acid assay[18] and the 3% cysteine–sulfuric acid assay[13], respectively. Hexuloses were not detected in the unhydrolyzed enzyme. Addition of concanavalin A (0.15–1 mg/ml) to the purified enzyme did not cause visible turbidity (precipitation), or inhibition of enzymic activity; this behavior suggests that this enzyme, like the *Strep. mutans* HS-6 dextransucrase[4], is a glycoprotein, and that it has little, if any, primer dextran or levan bound to it.

DISCUSSION

The enzyme(s) responsible for the extracellular synthesis of dextrans and levans have been purified from the spent culture-broth of *Strep. mutans* FA1. As the levan- and dextran-sucrase activities could not be separated from each other during the purification, it is possible that some form of association exists between them. Mixed-function sucrases have been isolated containing levansucrase and dextransucrase[4], and dextransucrase and invertase[9]. Kuramitsu[6] extracted from intact cells (by 1M sodium chloride) a dextransucrase of high molecular weight; the apparent molecular weight of the enzyme increased when the sodium chloride was removed by dialysis. Preliminary work with the preparation used in this study has shown the presence of an invertase activity; this activity becomes predominant after the polymerization activity has stopped. The possibility that there is but one enzyme having the three activities on sucrose, although remote, has not been excluded.

The enzyme preparation has a high molecular weight as judged by its behavior on filtration columns of poly(acrylamide) gel. The enzyme was excluded from a P-300 gel-filtration column, indicating a molecular weight >800,000. The large size of the enzyme molecule gives some credence to the idea of an enzyme aggregate. The protein precipitated from solution during concentration in a positive-pressure concentration-cell. Neither urea (0.8M) nor sodium chloride (1M) could dissolve the precipitate to

yield an active sucrase. The sucrase complex isolated by Mukasa and Slade[4] and Kuramitsu[6] also showed a very high molecular weight as determined by gel filtration. The low solubility and high molecular weight of the enzyme hindered the use of poly(acrylamide) disc electrophoresis for studying the homogeneity of the enzyme. Slight penetration of the enzyme into the electrophoresis gel had also been reported by Mukasa and Slade[4].

The dextransucrases from *Strep. mutans* have been found to differ in two important ways. First, antisera prepared against the *Strep. mutans* HS-6 (subtype *a*) crossreact with the dextransucrase from subtype *d*, but not[20,21] with *b* or *c*. This indicates that the sucrases from the various subtypes may differ in either chemical composition or physical structure. Second, the elution patterns of crude preparations of the dextransucrase on Sepharose 6B also differ, as between each of the four subtypes, indicating a diversity in molecular size or shape[20].

The properties of the glycosyltransferase from FA1 strain (subtype *b*) used in this study augment the results of the studies of the other subtypes. The pH optimum of the complex is ~6.0, and is similar to that for strain HS-6 (subtype *a*)[9] and strain 6715 (subtype *d*)[5]. The K_m for sucrose is 55mM, a value comparable to that of 2mM for the dextransucrase[9] and 20mM for invertase[9] of strain HS-6, and 3mM for the dextransucrase[5] of strain 6715. The isoelectric points of the two fractions from the isoelectric-focusing column are 3.7 and 4.6, values similar to that[6] of 4.0 for strain 6715. The L/D ratio is affected by the concentration of sucrose and by the temperature, making critical comparisons among crude preparations from various strains difficult. The enzymic activity was not activated by divalent ions, and was inhibited by divalent-metal ions. A metal ion may be associated with the complex, as EDTA and hexametaphosphate inhibit the activity.

The presence of carbohydrate in the enzyme preparation has been reported by Mukasa and Slade[4], as well as in this report, and it may be a general characteristic of the extracellular sucrases. Levan does not appear to be bound to the enzyme, as fructose could not be detected along with the protein by direct analysis. D-Glucose comprises only 41% of the total carbohydrate, and the enzymic activity was not affected by concanavalin A. There is no requirement for a dextran or a levan primer with this enzyme, although other preparations are stimulated by dextrans[9,21]. The likelihood of contamination by a polysaccharide has not been completely dismissed.

The unique heat-stability is much different from that to be expected from results with similar proteins[5]. An increased activity with an increase in temperature has been reported[18] for staphylococcal α-toxin, but has not yet been reported for a dextransucrase.

ACKNOWLEDGMENTS

This research was supported by funds provided by Villanova University and N.I.D.R. grant DE-03101. Predoctoral fellowships supported W.R.S. (N.D.E.A.) and L.W.L. (N.A.S.A.).

REFERENCES

1 R. J. GIBBONS AND R. J. FITZGERALD, *J. Bacteriol.*, 98 (1969) 341–346.
2 R. J. GIBBONS AND M. NYGAARD, *Arch. Oral Biol.*, 13 (1968) 1249–1262.
3 R. B. PARKER AND H. R. CREAMER, *Arch. Oral Biol.*, 16 (1971) 855–862.
4 H. MUKASA AND H. D. SLADE, *Infect. Immun.*, 10 (1974) 1135–1145.
5 A. M. CHLUDZINSKI, G. R. GERMAINE, AND C. F. SCHACHTELE, *J. Bacteriol.*, 118 (1974) 1–7.
6 H. KURAMITSU, *Infect. Immun.*, 10 (1974) 227–235.
7 S. M. GARSZCZYNSKI AND J. R. EDWARDS, *Arch. Oral Biol.*, 18 (1973) 239–251.
8 M. M. MCCABE, E. E. SMITH, AND R. A. COWMAN, *Arch. Oral Biol.*, 18 (1973) 525–531.
9 K. FUKUI, Y. FUKUI, AND T. MORIYAMA, *J. Bacteriol.*, 118 (1974) 796–804.
10 J. CARLSSON, *Caries Res.*, 4 (1970) 97–113.
11 M. SOMOGYI, *J. Biol. Chem.*, 160 (1945) 61–73.
12 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 262–271.
13 Z. DISCHE, *Methods Carbohyd. Chem.*, 1 (1962) 488–494.
14 E. J. WHITAKER AND J. R. EDWARDS, *Arch. Oral Biol.*, 19 (1974) 1237–1238.
15 O. VESTERBERGS AND H. SWENSSON, *Acta Chem. Scand.*, 20 (1966) 820–834.
16 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265–275.
17 N. D. GILMORE, *J. Publ. Health Dent.*, 29 (1969) 188–207.
18 J. P. ARBUTHNOTT, J. H. FREER, AND A. W. BERNHEIMER, *J. Bacteriol.*, 94 (1967) 1170–1177.
19 L. SVENNERHOLM, *Methods Enzymol.*, 6 (1963) 459–462.
20 K. FUKUI, Y. FUKUI, AND T. MORIYAMA, *Infect. Immun.*, 10 (1974) 985–990.
21 R. T. EVANS AND R. J. GENCO, *Infect. Immun.*, 7 (1973) 237–241.