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Purification and Properties of Dextransucrase from *Streptococcus mutans*

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The dextransucrase (EC 2.4.1.5) activity from cell-free culture supernatants of *Streptococcus mutans* strain 6715 has been purified approximately 1,500-fold by ammonium sulfate precipitation, hydroxylapatite chromatography, and isoelectric focusing. The enzyme was eluted as a single peak of activity from hydroxylapatite, and isoelectric focusing of the resulting preparation gave a single band of dextransucrase activity which focused at a pH of 4.0. The final enzyme preparation contained two distinct, enzymatically active proteins as judged by assay in situ after polyacrylamide gel electrophoresis. One of the proteins represented 90% of the total dextransucrase activity and 53% of the total protein. The molecular weight of the enzyme was estimated by gel filtration to be 94,000. The temperature optimum of the enzyme was broad (34 to 42 C) and its pH range was rather narrow, with optimal activity at pH 5.5. The K_m for sucrose was 3 mM, and fructose competitively inhibited the enzyme reaction with a K_i of 27 mM.

Streptococcus mutans has been associated with dental caries in experimental animals (7, 14) and man (3, 6, 15). The cariogenic potential of this bacterium is thought to reside in its ability to produce water-soluble and water-insoluble extracellular dextrans from sucrose (8, 9, 13, 27). The *S. mutans* dextrans are made up of α-(1 $\rightarrow$ 6)-linked glucose molecules with a high and variable proportion of α-(1 $\rightarrow$ 3)-linked branch points (11, 17, 18). The heterogeneity of the *S. mutans* dextrans has been proposed (12) to be due to the production of multiple forms of the enzyme dextransucrase (EC 2.4.1.5). This enzyme occurs in a cell-associated (insoluble) state and in a cell-free (soluble) form (10, 20, 22). In one study (12), the soluble enzyme was fractionated into at least seven activities which demonstrated distinct physical properties. This observation and the importance of elucidating the exact mechanism of dextran synthesis by *S. mutans* seemed to warrant further investigation of the dextransucrase produced by this organism. In this communication, we report on the purification and properties of dextransucrase activity from *S. mutans* strain 6715.

MATERIALS AND METHODS

Bacteria and growth conditions. *S. mutans* strain 6715 was obtained from the Forsyth Dental Center, Boston, and was used throughout this investigation. Cells were grown anaerobically (Gas Pak system, BBL, Cockeysville, Md.) in 1% Trypticase soy broth (BBL) at 37 C. After overnight growth (approximately 0.3 optical density units at 600 nm), cultures were chilled on ice, and the bacteria were removed by centrifugation (10,000 $\times$ *g*, 10 min, 4 C). Residual cells and debris were removed by filtration (0.45 μm membrane filter, Millipore Filter Corp., Bedford, Mass.).

Dextransucrase assay. Detailed characteristics of the assay for dextran synthesis will be presented elsewhere (G. R. Germaine, C. F. Schachtele, and A. M. Chludzinski, submitted for publication). Diluted enzyme was added to a reaction tube containing (final concentrations): 50 mM sodium acetate buffer (pH 5.5); 7 mM NaF; 20 mM total sucrose containing about 7 mM L-[U-^{14}C]sucrose (3.35 Ci/mol, New England Nuclear Corp., Boston); and 20 μM dextran T10 (molecular weight 10,000, Pharmacia, Uppsala). At appropriate intervals during incubation at 37 C, 10-μliter volumes were pipetted onto Whatman 3MM filter disks (24 mm) and immediately submerged in a beaker of absolute methanol. After completion of an experiment, a further 15 min of soaking was allowed in the methanol. The methanol was decanted and replaced by fresh methanol in which the disks were soaked for another 15 min. Batch washing with methanol was repeated once more as above. The disks were then dried, submerged in scintillation fluid, and assayed for radioactivity as before (24). Isomaltodextrins larger than isomaltotetraose are detected by this assay method.

One unit of dextransucrase is that amount of enzyme causing the polymerization of 1 μmol of sucrose-derived glucose per min at 37 C.

Protein determination. Protein was measured by the method of Lowry et al. (19), with bovine serum albumin as a standard. Protein elution profiles of column effluents were obtained with an LKB Uvicord II (LKB Instruments, Bromma, Sweden) operating at 280 nm.

Ammonium sulfate precipitation. Dextransucrase was precipitated from culture supernatant at 4 C by adding solid ammonium sulfate (enzyme grade, Mallinkrodt Chemical Corp., St. Louis, Mo.), with stirring, to 55% of saturation. The precipitate was collected by centrifugation, dissolved in 0.01 M potassium phosphate buffer (pH 6.0, buffer P), and extensively dialyzed against the same buffer. Formation of a small precipitate was usually observed during dialysis. The precipitate was removed by centrifugation (10,000 × *g*, 20 min) prior to the next purification step.

HA chromatography. The dialyzed enzyme preparation was applied to a column (2.6 by 40 cm) of hydroxylapatite (HA) (HT, Bio-Rad Laboratories, Richmond, Calif.) previously equilibrated with buffer P. The column was washed with buffer P at a flow rate of 75 ml/h until no further protein was eluted (250 ml). A linear gradient of 0.01 to 0.15 M potassium phosphate buffer (pH 6.0) was used to elute additional protein. Fractions of 5 ml were collected at a flow rate of 35 ml/h. Those fractions containing dextransucrase activity were pooled and concentrated 10-fold by dialysis against 25% polyethylene glycol in buffer P.

IEF. A 3.2-ml preparation (4 mg of protein per ml) of the pooled, concentrated, HA-chromatographed enzyme activity was incorporated into the glycerol density gradient (10 to 60%) of a 110-ml capacity LKB isoelectric focusing (IEF) column. A pH gradient of 3 to 5 was established by using 1% (vol/vol) carrier ampholytes (LKB). Enzyme was focused with a constant applied voltage of 480 V for 48 h, after which fractions of 2 ml were collected. Before the assay for dextransucrase activity, the fractions were dialyzed against buffer P to remove glycerol.

Polyacrylamide gel electrophoresis. Slab polyacrylamide gel electrophoresis was conducted in the apparatus described by Studier (26), using the tris-(hydroxymethyl)aminomethane-hydrochloride buffer system of Laemmli (16) at pH 8.8. Electrophoresis was performed at room temperature for 3 to 5 h at a constant current of 17 mA per slab (9 by 13 cm). Proteins were visualized by fixing the gel in 50% (wt/vol) trichloroacetic acid, staining for 15 min with 0.1% (wt/vol) Coomassie brilliant blue in 50% (wt/vol) trichloroacetic acid, and destaining in 7% (wt/vol) acetic acid. Stained gels were scanned with a Joyce-Loebl MK III CS microdensitometer. Dextransucrase activity in the gels was detected by using the [^{14}C]sucrose assay mixture described above. Immediately after electrophoresis, the gel was washed for 2 h in 0.05 M sodium acetate buffer (pH 5.5) and then incubated for 3 h at 37 C in contact with the assay mixture. After the 3-h washing with water to remove nonpolymerized sugar, the gel was dried on Whatman 3MM filter paper. Kodak no-screen medical X-ray film was used for autoradiography.

Gel filtration. A column (2.6 by 40 cm) of Bio-Gel P-150 (BioRad), equilibrated with 0.05 M sodium acetate buffer (pH 5.5), was used to estimate the molecular weight of the enzyme obtained after HA chromatography. A 2-ml sample (5 mg of protein per ml) was chromatographed at a reverse flow rate of 12 ml/h, and 1.1-ml fractions were collected. Blue dextran (Pharmacia) was used to determine the void volume of the column. The proteins (and their molecular weights) used to standardize the column were: chymotrypsinogen (Pharmacia), 25,000; ovalbumin (Sigma Chemical Co., St. Louis, Mo.), 45,000; bovine serum albumin (Sigma), 67,000; and collagenase (Worthington Biochem. Corp., Freehold, N.J.), 79,000.

Chemicals and radioisotopes. Carbohydrates not previously mentioned were obtained from either Difco Laboratories, Detroit, Mich., or Aldrich Chemical Co., Milwaukee, Wisc. β-Mercaptoethanol was obtained from Eastman Kodak Co., Rochester, N.Y., and dithiothreitol was obtained from Sigma Chemical Co. Sucrose-[1-^{3}H]fructose (5.6 Ci/mmol) was obtained from New England Nuclear Corp. [U-^{14}C]Glucose (190 Ci/mol) and [U-^{14}C]fructose (185 Ci/mol) were obtained from ICN Nuclear Corp., Irvine, Calif.

RESULTS

Purification of dextransucrase. A typical enzyme purification is summarized in Table 1.

Ammonium sulfate precipitation of the enzyme gave a 36-fold purification with only a 5% loss in activity. Extensive dialysis of this en-

TABLE 1. *Purification of S. mutans 6715 dextransucrase*

Fraction	Volume (ml)	Total protein (mg)	Total activity (U)	Sp act (10^{-4} U/mg of protein)	Purification (fold)	Yield (%)
I. Culture supernatant	4,700	39,000	0.805	0.206	1	100
II. Ammonium sulfate	85	1,020	0.762	7.47	36	95
III. Dialyzed ammonium sulfate	130	637	0.555	8.71	42	69[a]
IV. Hydroxylapatite chromatography and concentration	4	12	0.240	200	970	30
V. Isoelectric focused	3.2	3.2	0.098	306	1,515	13

[a] 14% of the dextransucrase activity was in the form of an insoluble precipitate.

zyme preparation resulted in formation of a precipitate which contained approximately 14% of the dextransucrase activity.

The dialyzed enzyme preparation was chromatographed on HA (Fig. 1). No dextransucrase activity was found in the major protein peak eluted with 0.01 M buffer (fractions 25 to 90). The enzyme was eluted as a single symetrical peak with approximately 0.06 M potassium phosphate (fractions 145 to 160). No additional enzyme activity could be removed from the column by washing with up to 0.5 M buffer. Concentration of the fractions containing enzyme activity gave an overall purification of 970, with an overall recovery of 30%. Forty-three percent of the enzyme activity applied to the HA column was recovered.

Next, the concentrated enzyme from the HA step was subjected to isoelectric focusing (Fig. 2). Dextransucrase focused at a pH of 4.0 as a band with a small shoulder. The recovery of HA enzyme activity from the IEF step was approximately 42%. Fractions containing enzyme activity were pooled and dialyzed, as before, against 25% polyethylene glycol. The final enzyme preparation retained its activity during extended storage at either 4 C or −70 C. This procedure gave a 1,515-fold purification, with an overall yield of 13% (Table 1).

Polyacrylamide gel analysis of dextransucrase preparations. To evaluate the purity of the enzyme preparations, the HA and IEF fractions were subjected to polyacrylamide gel electrophoresis (Fig. 3). Microdensitometer tracings demonstrated the presence of at least 10 distinct protein bands in the HA enzyme preparation. The IEF preparation yielded 4 protein bands.

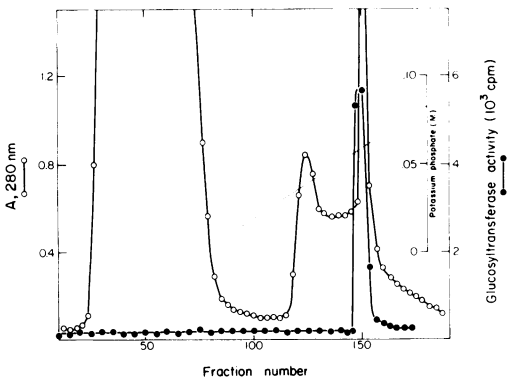

FIG. 1. *Elution profile of protein* (O) *and dextransucrase activity* (●) *from HA column. Enzyme activity is expressed as the counts* [^{14}C]*hexose polymerized in 60 min under standard assay conditions. Inset (dashed line) shows potassium phosphate gradient.*

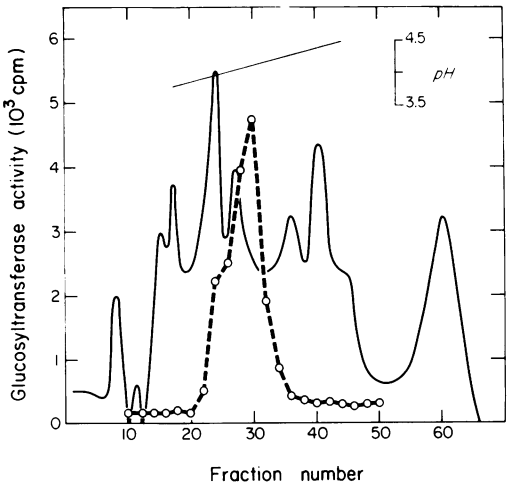

FIG. 2. *Isoelectric focusing of dextransucrase. Symbols:* O, *enzyme activity expressed as in Fig. 1;* —, *protein measured by absorbance at 280 nm. Inset demonstrates pH in region of enzyme activity.*

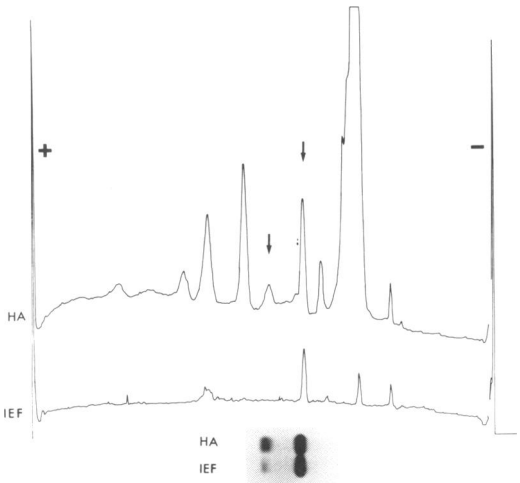

FIG. 3. *Microdensitometer tracings and enzyme activity analysis of polyacrylamide gels run with HA and IEF dextransucrase preparations. Migration was from right (cathode) to left (anode). Enzyme activity is indicated by bands in autoradiograph, and the arrows indicate the corresponding protein bands in the microdensitometer tracings.*

The location of dextransucrase was determined by incubating gels in the [^{14}C]sucrose reaction mix and determining the position of the [^{14}C]dextran product by autoradiography. Two distinct dextran-producing activities were present in both the HA and IEF preparations (Fig. 3). Microdensitometer tracings of the autoradiographs indicated that the ratio of major to minor activities was 3 in the HA preparation and 9 in the IEF preparation. The

bands visualized by autoradiography corresponded with two protein bands (marked by arrows in Fig. 3) in the HA enzyme preparation. A protein band corresponding only to the major enzyme activity was seen in the IEF preparation. The concentration of minor enzyme was too low to be detected by protein stain in the IEF preparation. Examination of the microdensitometer tracing of the protein pattern obtained with the IEF enzyme preparation indicated that the major enzyme activity comprised 53% of the total protein in the gel. This percentage is based on the assumption that all proteins in the gel stained equivalently.

Specificity of dextransucrase. *S. mutans* strain 6715 also produces alcohol-insoluble fructans from sucrose (25). The IEF enzyme preparation does not contain fructan-forming activity, however, since [U-^{14}C]fructose or sucrose-[1-^{3}H]fructose did not yield alcohol-insoluble polymers. These compounds supported only 0.25 to 0.4% of the amount of polymer obtained with [U-^{14}C]sucrose (data not shown). In addition, [U-^{14}C]glucose was not polymerized by the enzyme preparation. Thus, the polymer formed by the IEF preparation is derived exclusively from the glucosyl portion of sucrose. Levansucrase activity was lost at the ammonium sulfate precipitation step (data not shown).

Molecular weight. The molecular weight of HA-chromatographed enzyme activity was estimated by gel filtration (Fig. 4). The elution volume of the dextransucrase corresponded to a molecular weight of 94,000.

Effect of temperature. The effect of temperature on the rate of dextran synthesis by the IEF enzyme preparation was determined in an apparatus which gave a linear temperature gradient over the range 14.5 to 58 C. Enzyme

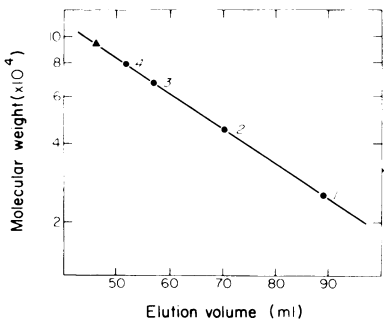

FIG. 4. *Molecular weight determination of dextransucrase by gel filtration. Symbols:* ▲, *dextransucrase;* ●, *standard proteins; 1, chymotrypsinogen; 2, ovalbumin; 3, bovine serum albumin; and 4, collagenase.*

reactions were initiated by addition of 10 μliters of enzyme preparation to 40 μliters of reaction mix pre-equilibrated to the appropriate temperature. Incubation was for 20 min. Note the broad temperature optima over the range of 34 to 42 C (Fig. 5a). Substantial loss of activity was observed at temperatures in excess of about 45 C. Other results (data not shown) indicated that thermal inactivation commenced around 50 C. This was determined by heat treatment of the enzyme, followed by cooling and assay at 37 C. Thus, the steep drop in activity observed at temperatures greater than 45 C (Fig. 5a) is most likely due to thermal inactivation. The insert to Fig. 5a gives an Arrhenius plot of the data between 14.5 and 34 C. The slope was calculated to be 10,500 calories per mol.

Effect of pH. The 20-min rate of dextran synthesis in a variety of buffers over a pH range of 3 to 8 was determined (Fig. 5b). The activity of the enzyme increased dramatically at pH values above 4.0 and reached a maximum at pH 5.5. Finally, the enzyme activity decreased as the pH increased to 8.0. Note that specific buffer effects were not observed. The rather dramatic increase in activity upon progression from pH 4.0 to 4.3 may be due to a change in state of the enzyme since its isoelectric point was found to be 4.0 (Fig. 2).

Kinetic properties. Under our standard assay conditions, dextran production by an IEF enzyme preparation was linear for at least 60 min, and the initial velocity was directly proportional to enzyme concentration over the range 20 to 200 μg of enzyme protein per ml (data not shown). The effect of sucrose concentration on the velocity of dextran formation is shown in Fig. 6. The saturation kinetics appear to be typically Michaelis-Menten in form, with a K_m of 3 mM (Fig. 6, inset).

Fructose inhibition. Fructose is liberated from sucrose during dextran synthesis. Accordingly, we investigated its effect on the rate of dextran synthesis (Fig. 7). Increasing concentrations of fructose caused a marked decline in dextran formation. At 250 mM, fructose decreased the rate of reaction by 80%. Inhibition by 30 mM fructose is of the competitive type (Fig. 7, inset), with a K_i of 27 mM.

Effect of various reagents. Inhibition of IEF enzyme activity by denaturants and sulfhydryl reagents was examined (Table 2). Urea (8 M) completely inhibited activity, as did sodium dodecyl sulfate at 3.5 mM (0.1%, wt/vol). Dithiothreitol caused 41% inhibition at 100 mM and 7% at 10 mM. The weaker reducing agent, β-mercaptoethanol, inhibited 17% at 120 mM.

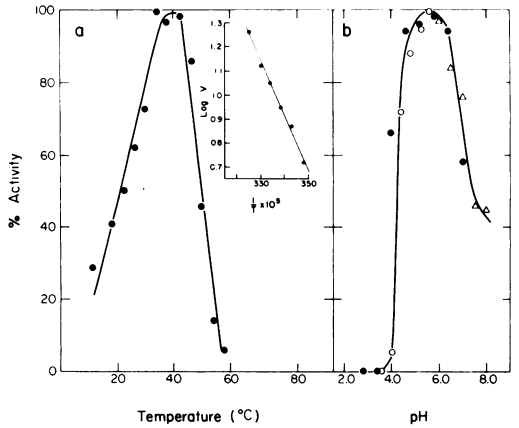

FIG. 5. *Temperature and pH optima of dextransucrase. (a) Dextransucrase activity was examined at the temperature indicated on the abscissa under standard assay conditions. (Inset) Arrhenius plot of dextransucrase velocity (nanomoles of hexose polymerized into dextran per milliliter per minute) at various temperatures. (b) Dextransucrase activity was assayed at the pH indicated on the abscissa. Assay conditions as above at 37 C, except that each buffer was present at a final concentration of 0.16 M. Symbols: ●, acetate; ○, citrate phosphate; △, phosphate.*

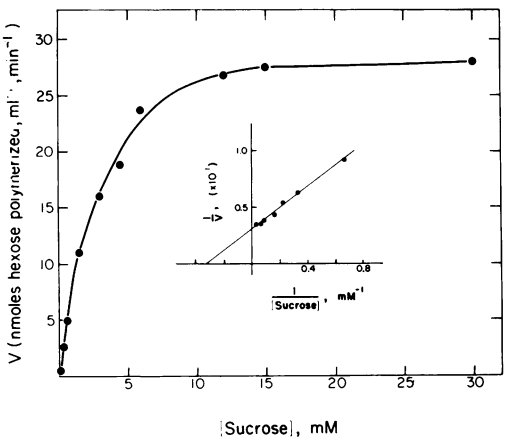

FIG. 6. *Sucrose saturation kinetics of dextransucrase. Isoelectric-focused enzyme was assayed for 20 min under conditions described in Materials and Methods at the final sucrose concentration indicated. Enzyme preparation was present at a final concentration of 80 μg of protein per ml. (Inset) Double reciprocal plot of same data; K_m = 3.0 mM.*

Both Ag^+ and Hg^{2+} at 1 mM exhibited rather potent inactivations of 94 and 35%, respectively. Iodoacetamide (1 mM), *p*-chloromercuribenzoate (0.2 mM), and *N*-ethylmaleimide (25 mM) had no effect on enzyme activity. Similarly, ethylenediaminetetracetic acid and sodium citrate at 1 mM were without effect, as were Ca^{2+}, Mg^{2+}, Mn^{2+}, NH_4^+, and Na^+ (data not shown).

DISCUSSION

Polyacrylamide gel electrophoresis of the IEF dextransucrase preparation indicated that at least four proteins were present (Fig. 3). The predominant protein band contained over 90% of the total enzyme activity and accounted for 53% of the total protein. A second minor dextransucrase activity was also detected; however, a corresponding protein band was only discernible in the HA enzyme preparation. The total purification procedure gave a 1,515-fold specific concentration, with an overall recovery of 13% (Table 1).

Neither HA chromatography (Fig. 1) nor gel filtration (data not shown) resolved the major and minor dextransucrase activities observed in the polyacrylamide gels (Fig. 3). The IEF results suggested the presence of a second enzyme activity since the focused peak of activity exhibited a shoulder (Fig. 2). In contrast, both HA chromatography and IEF of culture supernatants from *S. mutans* strain OMZ176 (12) yielded discrete, multiple peaks of enzyme activity. It should be noted that in the present study enzyme was eluted from HA in a very sharp peak at 0.06 M potassium phosphate, whereas, in previous studies with either *S. mutans* (12) or *S. sanguis* (2) the major dextransucrase activities were eluted step-wise at potassium phosphate concentrations of 0.2 to 0.5 M. In one instance, where linear gradient elution was employed (12), two minor activities were eluted with 0.08 and 0.12 M potassium

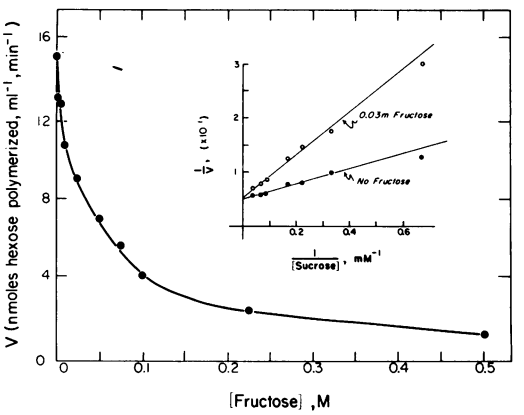

FIG. 7. *Effect of fructose on dextransucrase activity. Isoelectric-focused enzyme (66 μg of protein per ml) was assayed for 60 min with various concentrations of fructose. (Inset) Double reciprocal plot of 20-min data obtained in the presence (○) and absence (●) of 30 mM fructose.*

TABLE 2. *Effect of various reagents on dextransucrase activity*[a]

Reagent tested	Conc of reagent (mM)	Inhibition (%)
None[b]		0
Urea	8,000	99
Sodium dodecyl sulfate	35	98
	3.5	95
	0.35	5
	0.035	3
Dithiothreitol	100	41
	10	7
β-Mercaptoethanol	120	17
$AgNO_3$	1.0	94
$HgCl_2$	1.0	35

[a] Isoelectric-focused enzyme preparation (66 μg/ml) was assayed under standard conditions.

[b] 0.94 mmol of hexose was polymerized per ml per h.

phosphate, and the third major activity eluted at 0.17 M buffer. In our hands, continued development of the HA column with up to 0.5 M potassium phosphate failed to elute further significant quantities of enzyme activity (data not shown). Enzyme recovery from HA chromatography in the present study (43%) was similar to that obtained in the earlier work cited above (40 to 52%).

As already mentioned, the elution profile of isoelectric-focused enzyme suggested that, at most, two enzyme forms were present. The major activity focused at a pH of 4.0. It should be noted that the pH gradient of Fig. 2 was very shallow, ranging from pH 3 to 5. Isoelectric focusing in a gradient of pH 3 to 10 (data not shown) clearly showed that only one predominant activity was present with an apparent isoelectric point of 4.0. Thus, the multiple activities with apparent isoelectric points ranging from 4 to 6 observed by others (12) were not seen here.

We noted that the glycerol utilized as a stabilizing agent in the pH gradient markedly inhibited the enzyme reaction. Over 50% inhibition was achieved with only 7.5% (vol/vol) glycerol (data not shown). Thus, we found it necessary to dialyze each fraction from the IEF step prior to assay. The inhibition was only observed when the enzyme was assayed by measurement of methanol-insoluble radioactive dextran. When the enzyme was measured by determining release of reducing sugar (fructose), glycerol in excess of 30% (vol/vol) had no effect. Glycerol may act as an acceptor and thus terminate further dextran synthesis (4) but allow sucrose binding and hydrolysis. This observation is under further investigation.

Temperature (34 to 42 C) and pH (5.5) optima of this enzyme are similar to those reported for the dextransucrase activity of *S. bovis* (1), *S. sanguis* (2), *S. mutans* (12), and *Leuconostoc mesenteroides* (5).

The molecular weight of the active enzyme, determined by gel filtration, is approximately 94,000 (Fig. 4). Both 0.1% (wt/vol) sodium dodecyl sulfate and 8 M urea completely inhibited enzyme activity (Table 2). Urea at a concentration of 0.8 M caused 25% inhibition (data not shown). Strong reducing conditions (100 mM dithiothreitol) partially inhibited dextran synthesis. Both silver and mercury were highly effective inactivators (Table 2). Thus, it appears that some sulfhydryl integrity is required for enzyme function; however, more extensive studies are required to develop a structural concept of this enzyme.

The apparent disagreement of our data (presence of at most two dextransucrase activities) with data of others (12) who obtained multiple enzyme activities from *S. mutans* strain OMZ176 may be due, in part, to the presence or absence of enzyme-bound dextran. We observed, in agreement with others (20, 21), that addition of dextran stimulated enzyme activity of culture supernatant solutions (data not shown). However, substantial activity could be measured without dextran addition. This is also true of ammonium sulfate-precipitated and -dialyzed enzyme preparations. In marked contrast, after HA chromatography, dextran synthesis was primer (i.e., dextran) dependent (manuscript in preparation). Since HA is known for its ability to adsorb dextrans (23), the HA chromatography step most likely removed primer dextran molecules associated with dextransucrase. It seems possible that the buffer strength at which enzyme elutes from HA may also depend upon the size of its associated dextran. Indeed, for pure dextrans this is the case (23). In addition, perhaps the effectiveness of dextran dissociation from enzyme by HA is dependent upon dextran molecular weight. Thus, the level of contaminating sucrose in the culture media might be expected to affect the molecular weight of enzyme-associated dextran (20) and may affect the physical properties of dextransucrase. A brief mention of the role of culture media on the spectrum of enzyme activities appeared in one report (12).

Ebert and Brosche (4) have proposed that dextran synthesis by *L. mesenteroides* is carried out by one dextransucrase and that this enzyme alone is sufficient for formation of branched

dextrans. Additional characterization of the two dextransucrase activities reported in this paper, and analysis of their respective reactions, will hopefully give insight into the mechanism(s) by which *S. mutans* produces dextrans containing a high proportion of α-(1 $\rightarrow$ 3)-linked branch points.

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