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MILLIGRAM TO GRAM SCALE PURIFICATION AND CHARACTERIZATION OF DEXTRANSUCRASE FROM *Leuconostoc mesenteroides* NRRL B-512F

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ABSTRACT

A sequence of dextranase treatment, DEAE-cellulose chromatography, affinity chromatography on Sephadex G-200, and chromatography on DEAE-Trisacryl M has been optimized to give a dextransucrase preparation with low carbohydrate content (1–100 μg/mg protein) and high specific activity (90–170 U/mg protein) relative to previous procedures, in 30–50% yield. Levansucrase was absent after DEAE-cellulose chromatography, and dextranase was undetectable after Sephadex G-200 chromatography. The method could be scaled up to produce gram quantities of purified enzyme. The purified dextransucrase had a pH optimum of 5.0–5.5, a K_m of 12–16mM, and produced the same lightly branched dextran as before purification. The purified enzyme was not activated by added dextran, but the rate of dextran synthesis increased abruptly during dextran synthesis at a dextran concentration of ~0.1 mg/mL. The enzyme had two major forms, of molecular weight 177,000 and 158,000. The 177,000 form predominated in fresh preparations of culture supernatant or purified enzyme, whereas the amount of the 158,000 form increased at the expense of the 177,000 form during storage of either preparation.

INTRODUCTION

Dextransucrase[1–3] polymerizes the glucosyl moiety of sucrose to form dextran, an α-(1→6)-linked D-glucan having α-linked branches[3]. Dextransucrase and other glucansucrases (D-glucosyltransferases) are secreted by species of *Leuconostoc* and *Streptococcus*. Current research is focused on the streptococcal enzymes, because of their involvement in the etiology of dental caries[1,4]. However, purification of streptococcal glucansucrases usually suffers from low enzyme production[5] (0.1–0.2 U/mL culture) and from the presence of at least two glucansucrases and of substantial amounts of fructansucrase, dextranase, and invertase[1,3,4]. By contrast, *Leuconostoc mesenteroides* B-512F produces only one glucansucrase

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(dextransucrase)[2,3], and produces it in high amounts[6] (2.5–3.5 U/mL) with low levels of undesirable enzymes[3,7]. Specific activities of purified B-512F dextransucrase are high[7–9] (>50 U/mg), even though activity is measured at 25°, rather than at 37° as for the streptococcal enzymes.

Current procedures for purifying dextransucrase[7–9] from *L. mesenteroides* B-512F either have low yields or fail to remove important impurities, especially polysaccharide. Because dextransucrase from *Leuconostoc* species must be induced with sucrose, dextran is produced together with the enzyme. Glucansucrases from *Streptococcus* on the other hand are constitutive, resulting in purified preparations much lower in carbohydrate, although rarely, if ever, carbohydrate-free[4].

The goals of this study were to decrease carbohydrate in preparations of purified *L. mesenteroides* B-512F dextransucrase to the level found in purified preparations from *Streptococcus*, without sacrificing high yield, and to exploit the advantages of *L. mesenteroides* NRRL B-512F as a dextransucrase source by producing large amounts of dextransucrase with high specific activity. This was achieved by a combination of dextranase treatment, ion-exchange, and affinity chromatography. Properties of the enzyme have been determined in greater detail than previously[7].

EXPERIMENTAL

Materials. — Lyophils of *Leuconostoc mesenteroides* NRRL B-512F were obtained from the Northern Regional Research Center (Peoria, IL). *Penicillium funiculosum* dextranase (Grade I, chromatographically purified), DEAE-cellulose (medium mesh, 0.93 meq/g), DEAE-Sephacel, and dextran of various sizes, were from Sigma Chemical Co. (St. Louis, MO). DEAE-Trisacryl M was from LKB Instrument, Inc. (Gaithersburg, MD). Sephadex (40–120 μm dry-bead diameter for all types used), Sephacryl S-200, dextran T10, and dextran T2000 were from Pharmacia Fine Chemicals (Piscataway, NJ). Carbowax PEG 20,000 was obtained from Fisher Scientific Co. (Fair Lawn, NJ) and purified by precipitation from acetone with ether[10]. [U-^{14}C]Sucrose was purified by paper chromatography [two ascents at 85° in 6:1:1, (v/v/v) 1-butanol–pyridine–water on Whatman 3MM paper] before dilution with carrier sucrose. [*fructose*-U-^{14}C]Sucrose was from New England Nuclear.

Enzyme assays. — Assays were performed at 25° in 0.05M sodium acetate, pH 5.2, containing mM calcium ion and 1 mg/mL Tween 80. Levansucrase and total glycansucrase activities were measured by monitoring the incorporation of ^{14}C from 0.15M [*fructose*-U-^{14}C]sucrose or [U-^{14}C]sucrose, respectively, into methanol-insoluble polysaccharide[11]. One unit of enzyme is defined as the amount that will incorporate 1 μmol of D-glucose or D-fructose into polysaccharide in one min under these conditions. The amount of dextransucrase bound to Sephadex was taken to be the difference between the amount incubated with the Sephadex and the amount removed by buffer washes.

Dextranase was detected qualitatively by the production of isomaltooligosaccharides from dextran T200. Quantitatively, dextranase was determined by the increase in reducing value[12,13] using the same substrate.

Protein and carbohydrate analysis. — Protein was determined as bovine serum albumin by the method of Lowry *et al.*[14]. Precipitates caused by detergent[15] were removed by centrifugation. Carbohydrate was determined by the phenol–sulfuric acid method[16]. Polysaccharide composition was determined by acid hydrolysis[7] followed by thin-layer chromatography[17].

Electrophoresis. — SDS-gel electrophoresis was performed by the method of Laemmli[18] on 5 × 90 mm cylindrical gels (6% acrylamide). Protein was stained[19] with Coomassie Blue G–250. Dextransucrase and levansucrase activities were detected by incubation[20] with sucrose and Tween 80, followed by staining for polysaccharide by a periodic acid–Schiff procedure[21].

Dextransucrase production. — *Leuconostoc mesenteroides* NRRL B-512F was grown in sucrose medium as previously described[7], with the addition of 0.5–1.0 mg/mL Tween 80 to stabilize the enzyme[22].

Dextransucrase purification. — The procedure given here is for a one-liter culture. Modifications for larger cultures are described under Results and Discussion. Unless stated otherwise, all procedures were conducted at 4°. Buffers contained 1 mg/mL of Tween 80, 2mM $CaCl_2$, and 0.2 mg/mL of sodium azide.

After removing cells by centrifugation, 20 mg of lyophilized dextranase [made from a solution (pH 5.2) of Sigma dextranase containing 40 mg/mL of mannitol], which had 0.09 IU/mg at 4° and pH 5.2, was added to the 980 mL of culture supernatant, which was then dialyzed overnight against 21 L of 0.02M sodium acetate (pH 5.2), 0.05M NaCl. The dialysate (~1 L) was loaded onto a 1.5 × 35 cm DEAE-cellulose column equilibrated with the same buffer. The column was washed with 0.25 L of this buffer, and then with 1.9 L of 0.02M sodium acetate (pH 5.2), 0.2M NaCl. This was followed by 0.5 L of 0.02M imidazole-HCl (pH 6.7), 0.2M NaCl, and by a linear NaCl gradient (1.6 L; 0.2–1.0M) run over a period of 5 days.

The active fractions eluted from DEAE-cellulose (480 mL) were applied to a Sephadex G-200 column (4.2 × 14 cm) at 20–23°. The column was washed with 0.1 L of 0.02M imidazole-HCl (pH 6.7), 0.4M NaCl, and then with 0.2 L of 0.05M imidazole-HCl (pH 6.7) without NaCl, and finally eluted with the same buffer containing 3M urea.

The urea eluate from Sephadex G-200 (260 mL) was immediately applied to a 2.0 × 10-cm column of DEAE-Trisacryl M equilibrated with the same urea buffer, and then eluted with M NaCl while maintaining 3M urea in the eluent. For this elution, buffer containing urea was filtered through an Amicon PM10 membrane to remove fines, with Tween 80 added to the ultrafiltrate to a concentration of 1 mg/mL. Dextransucrase activity in the eluted fractions was determined after dialysis against 0.02M imidazole · HCl (pH 6.7), 0.2M NaCl to remove the urea. The terms "purified dextransucrase" and "purified enzyme" are used exclusively to denote dextransucrase after chromatography on DEAE-Trisacryl M.

RESULTS AND DISCUSSION

Production and purification. — Cultures from lyophils obtained from the Northern Regional Research Center produced surprisingly little enzyme (0.02 U/mL in the culture supernatant), considering reported values a hundred times higher (*e.g.*, by Jeanes[6]). After treating these cultures with nitrosoguanidine[23] and selecting colonies for high polysaccharide production, culture supernatants averaged 3–4 U/mL of dextransucrase (range 1.5–7 U/mL).

Tween 80, which increases the production of glucansucrases in *Streptococcus* species[24–26] and of exocellular bacterial enzymes in general[27], was included in the growth medium as a dextransucrase stabilizer[22], but did not increase dextransucrase production. Instead, enzyme yield began to decline between 0.3 and 1 mg/mL Tween 80, and bacterial growth between 3 and 10 mg/mL. The optimal concentration range for Tween 80 was 0.01–0.3 mg/mL.

Dextranase treatment and DEAE-cellulose chromatography. — Most of the purification occurred on DEAE-cellulose (Table I, Fig. 1). Nearly all of the carbohydrate (99.5%) and protein (98.4%) were removed. Levansucrase and most of the added dextranase were also removed at this stage. The remaining carbohydrate contained roughly equal amounts of D-mannose, D-glucose, and D-ribose, with a smaller amount of D-galactose (data not shown).

Unlike other dextransucrases successfully purified on DEAE-cellulose, yields for *L. mesenteroides* B-512F dextransucrase normally have been low[7]. However, we found that over a narrow range of conditions, DEAE-cellulose gave high yields (Table I). Lawford *et al.*[28] also purified B-512F dextransucrase on DEAE-cellulose, but did not report yield or more than minimally characterize the purified enzyme.

Critical requirements for high yields were dextranase treatment, to degrade the enzyme-associated dextran prior to DEAE-cellulose chromatography, and an optimal elution pH. Without dextranase treatment, up to 40% of the activity failed to bind to DEAE-cellulose or else eluted at low ionic strength ($I = 0.2$), and most of the enzyme that did bind could not be eluted.

Because of a protease impurity in the dextranase, the dextranase treatment was kept minimal (as indicated in the experimental section), and consequently the dextran present was not completely hydrolyzed. If dextranase treatment was too light, however, a second peak of dextransucrase activity appeared in the tail of the first activity peak. Increasingly lighter treatment caused increasingly more enzyme to be eluted at high elution volumes.

The maximum yield was at an elution pH of 6.8. Above pH 6.8, there was a slow decrease in yield caused by pH instability of the enzyme. The yield decreased rapidly between pH 6.6 and 6.0, and was a constant 7–9% from pH 6 to 5. The decrease below pH 6.6 was caused by tighter binding of the enzyme to the DEAE-cellulose. This was shown in a control experiment where M NaCl could only elute 8% of the bound activity at pH 5.0, while increasing the pH to 6.7 caused the elution of an additional 74%, for a total of 82%.

TABLE I

SUMMARY OF DEXTRANSUCRASE PURIFICATION

Step	*Volume after step (mL)*	*Dextransucrase*				*Protein*		*Specific activity (units per mg protein)*	*Carbohydrate*		*Levansucrase (units per mL)*
		Units per mL	*Total units*	*Step yield (%)*	*Overall yield (%)*	*mg per mL*	*Total mg*		*mg per mL*	*total mg*	
Cell removal	980	3.7	3626	100	100	2.7	2646	1.4	5.2	5096	0.08
Dextranase treatment and dialysis	1200	2.7	3240	89	89	1.1	1320	2.5	2.8	3360	0.04
DEAE-cellulose	480	5.9	2832	87	78	0.044	21.1	130	0.032	15.4	0[a]
Sephadex G-200	260	7.6	1976	70	54	0.045	11.7	170	0.006	1.56	0[a]
DEAE-Trisacryl M	74	24	1776	90	49	0.14	10.4	170	0.009	0.67	0[a]

[a]Levansucrase detection limit by use of [*fructose*-U-^{14}C]sucrose was ~1% of dextransucrase present. This percentage was not much lowered by increasing the specific activity of the [*fructose*-U-^{14}C]sucrose, because of increasing background and scatter in the methanol-wash assay procedure. Activity stains on SDS gels, which were more sensitive but less quantifiable, showed no remaining levansucrase in these preparations.

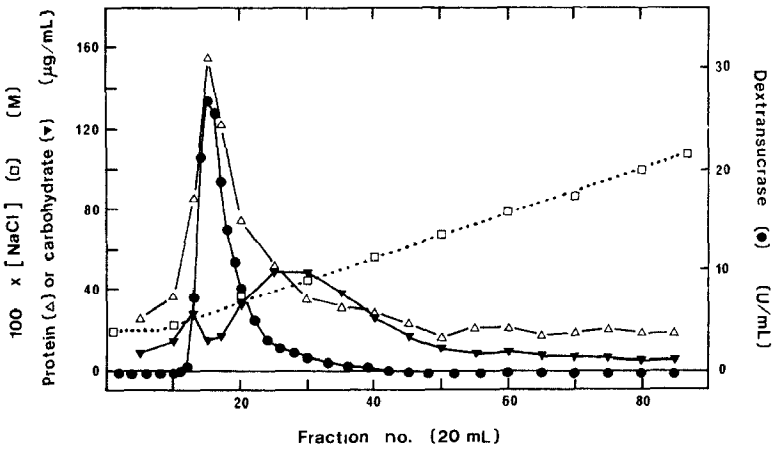

Fig 1. Chromatography of dextranase-treated culture supernatant on DEAE-cellulose. Before the first fraction shown in Fig. 1 was collected, the column was extensively washed with pH 5.2 and pH 6.7 buffers (20mM acetate or imidazole) containing enough NaCl to give ionic strengths of 0.06 and 0.2, respectively. Buffers contained 2mM $CaCl_2$, 1 mg/mL Tween 80, and 0.2 mg/mL sodium azide. Symbols: △, protein; ▼, carbohydrate; ●, dextransucrase activity; □, NaCl.

Affinity chromatography. — Sephadex has been found to be a good affinity matrix for glucansucrases from *Streptococcus*[29,30], but poor for those from *Leuconostoc*[8,28,31,32]. The latter is probably due to the endogenous dextran present in the *Leuconostoc* preparations. Removal of dextran from the B-512F culture supernatant by dextranase treatment and DEAE-cellulose chromatography greatly increased the fraction of the enzyme that bound to Sephadex G-200. If more than a trace of dextranase was present in the dextransucrase preparation applied to Sephadex, however, flow rates deteriorated during chromatography because of degradation of the Sephadex. For this reason, the DEAE-cellulose column was washed at pH 5.2 before eluting the dextransucrase at pH 6.7. This decreased dextranase in the enzyme preparation to an acceptably low level.

Chromatography on Sephadex G-200 removed most of the carbohydrate remaining after chromatography on DEAE-cellulose, as well as the remaining trace of dextranase (Fig. 2). Sephadex G-200 had the highest capacity for dextransucrase (~90 U/mL hydrated gel). Sephadexes G-150, G-100, G-75, and G-50 bound progressively smaller amounts of dextransucrase, and Sephadexes G-10, G-15, and G-25 did not bind significant amounts.

Urea and dextran were both effective at removing dextransucrase from Sephadex G-200 (Table II). The effect of pH in the range 5–8 on the desorption by urea was examined. Using 8M urea, the best yields were at pH 5.5–7.0. Yields declined slowly above pH 7.0 and rapidly below pH 5.5. The effect of urea concentration was studied at pH 6.5. On small trial columns (0.7 × 4 cm), none of the adsorbed activity was desorbed from Sephadex G-200 with M urea, 70–80% was desorbed at 2M urea, and 90–100% was desorbed at 3–8M urea. In practice, overall yields for the chromatography step on Sephadex G-200 were rarely >75%. The

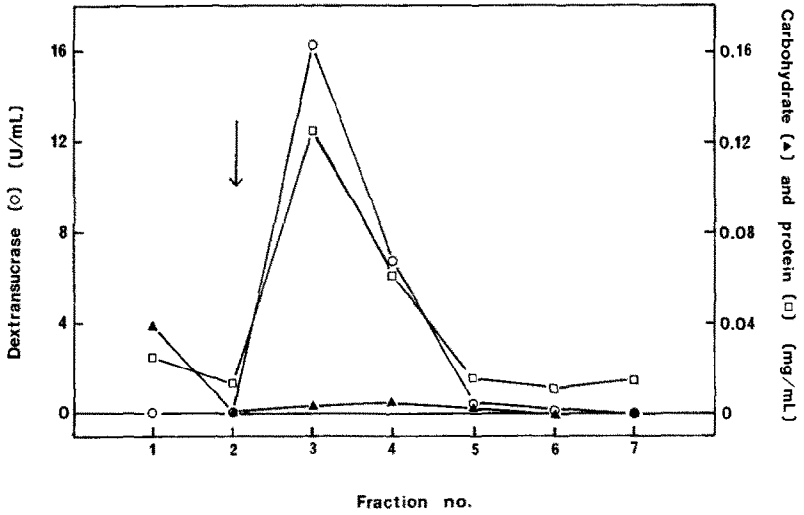

Fig. 2. Affinity chromatography on Sephadex G-200 of dextransucrase eluted from DEAE-cellulose. After applying the enzyme sample (480 mL), the column (4.2 × 14 cm) was washed with 0.05M imidazole (pH 6.7) containing 1 mg/mL Tween 80, 2mM $CaCl_2$, and 0.2 mg/mL sodium azide, and the activity was then eluted with the same buffer with the addition of 3M urea (arrow). Fraction 1 was 550 mL; fractions 2–7 were each 130 mL. Aliquots of each fraction were dialyzed and assayed. ○, dextransucrase; □, protein; ▲, carbohydrate.

dependence of desorption on urea concentration was inversely correlated with Neely's data on dextransucrase activity *vs.* urea concentration[33]. Because flow rates on Sephadex G-200 decreased with urea concentration, 3M urea was usually used.

Chromatography on *O*-(phenoxyacetyl)cellulose, hydroxylapatite, or octylamine-substituted Bio-Gel P-4 were less effective than chromatography on Sephadex G-200 in removing carbohydrate from the enzyme eluted from DEAE-cellulose. Sephacryl S-200, which like Sephadex is made from modified B-512F dextran, did not bind dextransucrase from dialyzed culture supernatant concentrate under conditions where Sephadex G-200 bound over 50%.

Chromatography on DEAE-Trisacryl M. — A final ion-exchange step on DEAE-Trisacryl M removed fines originating in reagent-grade urea and concentrated the enzyme. It also had the potential of eliminating traces of uncharged carbohydrate still associated with the enzyme or leached by urea from the Sephadex matrix. The specific activity, however, was not increased at this step.

Dextransucrase was eluted from DEAE-Trisacryl M as a sharp peak only if 3M urea was included in the eluent (Fig. 3). Without urea, the enzyme bound so tightly to the matrix that elution over several days with 1–2M NaCl eluted only 10–30% of the bound activity.

Carbohydrate was considerably decreased on DEAE-Trisacryl M only when urea was not used during enzyme elution. When urea was used, the eluted enzyme contained 0.03–0.06 mg of carbohydrate/mg protein. The lowest carbohydrate content thus far obtained, estimated at 0.7 μg carbohydrate/mg of protein, was for

TABLE II

DESORPTION OF DEXTRANSUCRASE FROM SEPHADEX G-200 BY VARIOUS COMPOUNDS

Addition to buffer[a]	*Yield (%)*[b]
Urea (8M)	89
Urea (8M) + 1M NaCl	66
Guanidine hydrochloride (6M)	72
Dextran[c] (10 mg/mL)	85
Maltose (0.5M)	2
None	2

[a]Buffer contained 2mM $CaCl_2$, 1 mg/mL of PEG 20,000, and 0.05M imidazole (pH 6.7). [b]Buffer (20 mL) was incubated for 12 h at 25° with a washed slurry of Sephadex G-200 (1.9 g) having 3.6 U of bound dextransucrase per g slurry. Bound dextransucrase was determined as described in the experimental section. Aliquots of the supernatant were dialyzed and assayed. [c]$\bar{M}_w$ 2 × 10^6.

a preparation eluted from DEAE-Trisacryl M without urea, and which had received a longer than normal dextranase treatment. This preparation, containing 28 mg of protein, had a specific activity of 170 U/mg of protein, and an overall yield of 31% relative to the culture supernatant.

Scale-up. — A 1-L culture gave 10.4 mg of purified enzyme (Table I). To produce larger amounts, scale-up was investigated. Growing the organism on the normal medium[7] at 25–28° in 25-L carboys without aeration or agitation (unsterilized medium for the final transfer, with a 10% inoculum) gave up to 7 U/mL in the culture supernatant. Cells were removed by a combination of centrifugation

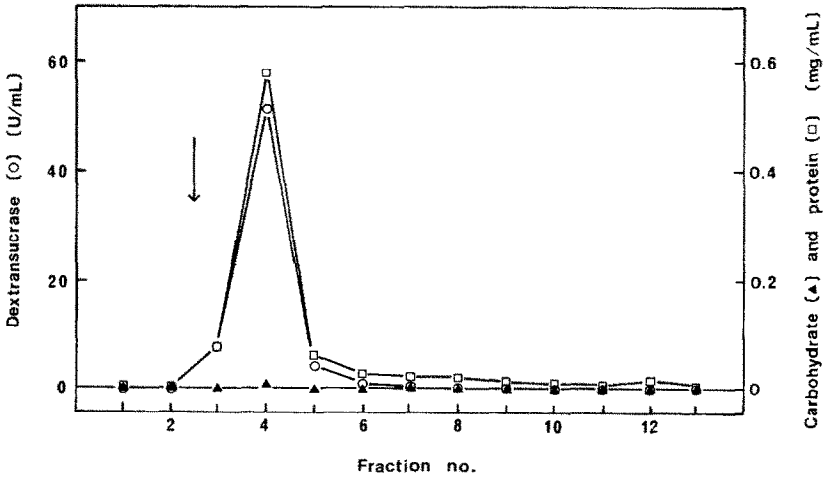

Fig. 3. Chromatography on DEAE-Trisacryl M of dextransucrase eluted from Sephadex G-200. Fractions 3 and 4 from the Sephadex G-200 column of Fig. 2 were applied to a column (2.0 × 10.0 cm) of DEAE-Trisacryl M equilibrated with the same buffer used to elute the enzyme from Sephadex G-200. Dextransucrase was eluted by adding M NaCl to the eluent (arrow) while keeping urea at 3M. Fraction 1 was 550 mL; fractions 2–13 were each 18 mL. Aliquots of each fraction were dialyzed and assayed. ○, dextransucrase; □, protein; ▲, carbohydrate.

and filtration with a Millipore Pellicon membrane-cassette system, which was also used to concentrate and dialyze the cell-free fluid.

Gram quantities of dextransucrase were purified on DEAE-cellulose columns of 1-L bed volume, run at either 4° or room temperature. Normal yields were 80–85% at either temperature. The enzyme eluted from DEAE-cellulose could be concentrated over an Amicon PM10 membrane in 90% yield to 800–900 U/mL. No loss of activity from the concentrate occurred during 6 months of storage at 4°, although small amounts of a fine precipitate developed. These concentrates have been stored frozen (−20°) for over a year with no loss of activity. Lyophilization of the concentrates resulted in 10% loss of activity.

Hundred-milligram amounts of dextransucrase have been purified in 70% yield on a 12 × 4 cm (diameter × height) Sephadex G-200 column. Sephadex G-200 could also be used batchwise at this step.

Enzyme properties. — The purified enzyme had a pH optimum of 5.0–5.5 (Fig. 4) and produced the same products with sucrose and with acceptors as the crude enzyme[34]. The purified enzyme produced a typical, lightly branched B-512F dextran, as determined by ^{13}C-n.m.r. spectroscopy and from the endodextranase hydrolysis-products of the dextran. The K_m value for sucrose was the same at all stages of purification (12–16mM). Neither the K_m nor the substrate inhibition constant (~1 M) for the purified enzyme were changed by the addition of 10 mg/mL of dextran T10.

After chromatography on DEAE-cellulose, progress curves for dextran synthesis were nonlinear (Fig. 5). The rate of dextran synthesis increased by a small but consistent 20–40% between dextran concentrations of 0.1 and 0.2 mg/mL. The increase was difficult to detect except at low enzyme or low sucrose concentrations, and did not take place in the presence of added dextran (Fig. 5C). The increase was observed whether or not the enzyme precipitate in the preparation (see discussion

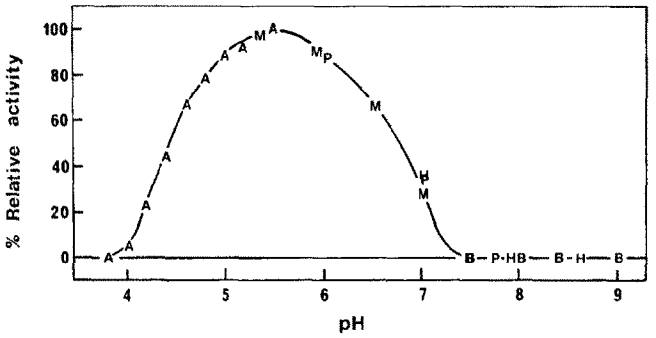

Fig. 4. pH–activity profile of purified dextransucrase. Dextransucrase eluted from DEAE-Trisacryl M was diluted 1:50 with buffer and assayed at 25°. Assay concentrations were mM $CaCl_2$, 0.1 mg/mL Tween 80, 0.15M sucrose, and 0.025M buffer. Buffers; A, sodium acetate; M, Mes (4-morpholineethanesulfonic acid); P, Pipes (1,4-piperazinediethanesulfonic acid); H, Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]; B, Bicine [*N*,*N*-bis(2-hydroxyethyl)glycine].

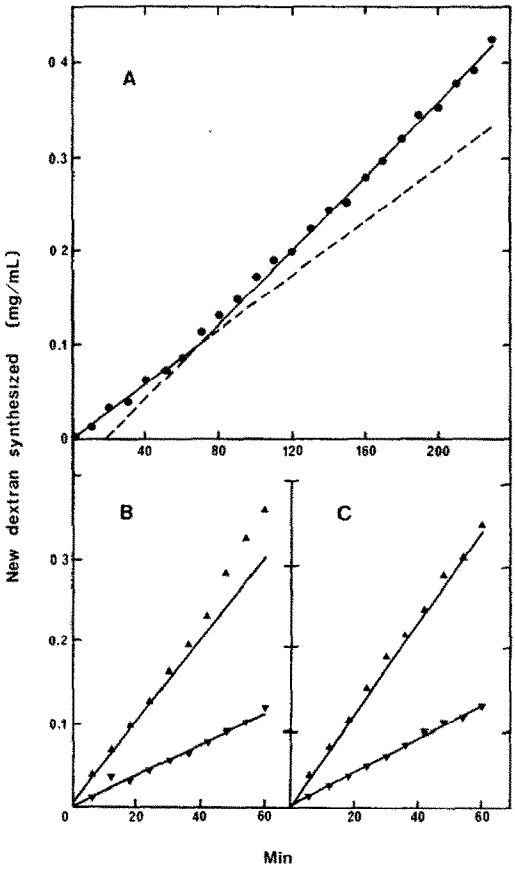

Fig. 5. Nonlinear dextran synthesis by purified dextransucrase. (A) Pre- and post-activation phases of dextran synthesis by enzyme eluted from DEAE-Trisacryl M. The mixture contained 13 mU/mL of dextransucrase and 150mM sucrose. The initial and final rates are shown by the two lines, which have been extrapolated (dotted portions) to show their difference more clearly.

(B) and (C) show the early part of the reaction. For (B) and (C), precipitate in the stock enzyme solution was dissolved with NaCl before assay. Mixtures contained 34 mU/mL of purified dextransucrase, 5mM (▼) or 50 mM (▲) sucrose, and no dextran T10 (B) or 10 mg/mL dextran T10 (C).

of stability later) was dissolved beforehand with salt or with a small amount of sucrose (Fig. 5B).

The enzyme was inhibited a relatively constant 10–15% by a 1–10 mg/mL range of dextran T10 or native B-512F dextran. The inhibition appeared to result from the prevention of the activation seen early in the reaction (see Fig. 5) in the absence of added dextran. Inhibition by dextran has rarely been observed for streptococcal glucansucrases[35], while activation is common[36]. For *L. mesenteroides* B-512F dextransucrase, activation has been found in some studies[8] but not in others[7].

Stability. — The activity in dialyzed culture supernatant was stable for at least 10 days at 4° and pH 5.0–6.8. Stability of the activity began to decrease sharply between pH 7.0 and 7.2.

The purified enzyme slowly lost activity at 4°, with the formation of a precipitate. Sucrose or NaCl dissolved the precipitate rapidly and completely, and dextran T2000 partially dissolved it. Storing the enzyme with M NaCl slowed the onset of precipitation, but did not prevent it indefinitely. Ready precipitation of highly purified dextransucrase has been observed by others[7,37,38], and these precipitates also could be dissolved by sucrose or dextran. The overall activity (solution plus precipitate) decreased during storage. Activity was lost from solution more rapidly than protein during precipitation.

At any stage before affinity chromatography on Sephadex G-200, the enzyme could be frozen or lyophilized with little or no loss of activity. After affinity chromatography, only dilute solutions could be frozen or lyophilized without loss. (Concentrated solutions with up to 1600 U/mL have been made.) Lyophilization caused less loss than freezing.

Lyophilization of dilute (2 U/mL) purified enzyme in sodium acetate buffer resulted in loss of most of the activity (Table III), whether the lyophilizate was reconstituted with water or buffer. The loss was prevented by dextran, but not by another stabilizer[22], PEG 20,000. The small apparent loss of activity in the presence of dextran was attributed to the inhibition by exogenous dextran already described. Loss of activity following lyophilization did not occur in the absence of dextran if the enzyme were lyophilized in the nonvolatile buffer Mes (4-morpholineethanesulfonic acid).

Purity. — SDS gel electrophoresis of culture supernatant (Fig. 6), followed by activity staining, showed two major and two minor bands with glycansucrase activity (gels B-D). The minor bands, at approximately 92,000 and 116,000 mol.wt., were identified as levansucrase, because they produced polysaccharide from raffinose, which is a substrate for levansucrase but not for dextransucrase[7,39]. The major bands, at 177,000 ±8,000 and 150,000 ±10,000 mol.wt., were dextransucrase.

The purified enzyme (gels E–H) lacked levansucrase, but had the same two dextransucrase bands as the culture supernatant, and frequently a minor one migrating just below the 177,000 mol.wt. form on SDS gels (clearest in gel F). The proportion of the 158,000 mol.wt. form of dextransucrase increased from merely a trace in fresh preparations to a large fraction of the total protein during storage of either the culture supernatant or the purified enzyme. An inactive band at 117,000 ±4,000 mol.wt. (gel F) also appeared in purified enzyme preparations during storage, as did increasing amounts of other inactive peptides of lower molecular weight (gels E and F). The molecular-weight changes during storage are probably due to proteolysis[40–42].

Carbohydrate concentrations in the fully purified enzyme were variable, as described here in the section on DEAE-Trisacryl M chromatography, but were close to the detection limit. Carbohydrate was detected in SDS gels of culture supernatant only at the tops of the gels, and not at all in gels of purified enzyme, even at heavy loadings (gels not shown).

TABLE III

LYOPHILIZATION AND RECONSTITUTION OF PURIFIED DEXTRANSUCRASE

Enzyme solution lyophilized[a]				*pH and yield after reconstitution*			
Buffer[b]	*Buffer pH*	*Stabilizer*	*Stabilizer concentration*	*Reconstitution with water*		*Reconstitution with buffer*[c]	
				pH[d]	*Yield (%)*	*pH*[d]	*Yield (%)*
Sodium acetate	5.2	None		7.0	27	5.4	29
Sodium acetate	5.2	Dextran T10	20 mg/mL	6.5	88	5.4	91
Sodium acetate	5.2	PEG 20,000	20 mg/mL	8.5	17	5.4	84
Mes[e]	5.5	None		5.7	100		
Mes	5.5	Dextran T10	2 mg/mL	5.7	91		
Mes	5.5	PEG 20,000	20 mg/mL	5.7	99		

[a]Enzyme eluted from DEAE-Trisacryl M (0.05 mL) was mixed with the buffer described (0.95 mL), lyophilized, and reconstituted with water or buffer (1 mL). [b]Buffers were 0.05M and contained 2mM $CaCl_2$, 0.2 mg/mL Tween 80, and 0.2 mg/mL sodium azide. [c]Buffer was 0.1M sodium acetate (pH 5.2), containing 4mM $CaCl_2$, 0.4 mg/mL Tween 80, and 0.4 mg/mL sodium azide. [d]pH After reconstitution. [e]4-Morpholineethanesulfonic acid

Fig. 6. Electrophoresis on SDS gels of dextransucrase fractions.

(A–D) Culture supernatant (400 μg protein). (A) Protein. (B)–(D) Dextransucrase and levansucrase activity: (B) gel soaked in 10% acetic acid, 25% 2-propanol after 6 h of incubation with sucrose and 10 mg/mL of dextran T10 (no bands could be seen against a dark background but the two visible here); (C) periodic acid–Schiff stain after 5-min incubation with sucrose; (D) periodic acid–Schiff stain after 3-h incubation with sucrose.

(E–H) Purified enzyme. (E) and (F): protein; (G) and (H): dextransucrase activity (periodic acid–Schiff stain). DS and LS mark the positions of the dextransucrase and levansucrase activities, respectively. The position of the inactive band (I) developing on storage of the purified enzyme is indicated by the dashes to the right of gels F–H. (E) Purified enzyme after storage for 1 week at 4° (100 μg of protein); (F) after storage for 8 months at 4° (70 μg of protein); (G) after storage for 8 months at 4° (70 μg of protein; 5 min incubation with sucrose); (H) after storage at 4° for 3 months (10 μg of protein; 15 min incubation with sucrose).

Summary. — Gram quantities of purified dextransucrase were made by carrying the method described here through DEAE-cellulose chromatography. Compared to previous purification procedures for B-512F dextransucrase, this enzyme had high specific activity (90–130 U/mg of protein) and low carbohydrate content (0.2–0.7 mg/mg of protein). The enzyme could be stored indefinitely without loss of activity at this stage. By adding an affinity chromatography step using Sephadex G-200, hundred-milligram amounts were made with less carbohydrate (0.1 mg/mg protein), in >50% overall yield, and with a higher specific activity (170 U/mg) than yet reported for B-512F dextransucrase. A final ion-exchange step using DEAE-Trisacryl M gave an overall yield of 30–50% when 3M urea was present in the eluent to weaken the binding of the enzyme to the DEAE-Trisacryl

M. When urea was omitted from the eluent, the dextransucrase eluted from DEAE-Trisacryl M did not have detectable carbohydrate (<1 μg/mg), but overall yields were generally less than 10%, because most of the enzyme could not be eluted.

Purification did not change the pH optimum or K_m value of the enzyme, but did result in an enzyme preparation that showed activation during dextran synthesis. Two major molecular-weight forms of the enzyme were found. The amount of the 177,000 mol.wt. form, which predominated in fresh preparations, decreased during aging, while the amount of the 158,000 mol.wt. form increased. This change in enzyme structure did not change the structure of the dextran synthesized; nor, when it occurred in the culture supernatant, did it change the amount of enzyme activity.

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