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PRODUCTION, PURIFICATION, AND PROPERTIES OF DEXTRANSUCRASE FROM *Leuconostoc mesenteroides* NRRL B-512F*

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ABSTRACT

The production of dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F was stimulated 2-fold by the addition of 0.005% of calcium chloride to the medium; levansucrase levels were unaffected. Dextransucrase was purified by concentration and dialysis of the culture supernatant with a Bio-Fiber 80 miniplant, and by treatment with dextranase followed by chromatography on Bio-Gel A-5m. A 240-fold purification, with a specific activity of 53 U/mg, was obtained. Contaminating enzyme activities of levansucrase, invertase, dextranase, glucosidase, and sucrose phosphorylase were decreased to non-detectable levels. Poly(acrylamide)-gel electrophoresis of the purified enzyme showed only two protein bands, both of which had dextransucrase activity. These bands also gave a carbohydrate stain, indicating that the dextransucrase could be a glycoprotein. Acid hydrolysis, followed by paper chromatography, of the purified enzyme showed that the major carbohydrate was mannose. Concanavalin A completely removed dextransucrase activity from solution, confirming the mannoglycoprotein character of the enzyme. Dextransucrase activity was not altered by the addition of 0.008–4 mg/ml of dextran, but its storage stability was increased by the addition of 4 mg/ml of dextran. As previously shown by others, the activity of dextransucrase was decreased by EDTA, and was restored by the addition of calcium ions. Zinc, cadmium, lead, mercury, and copper ions were inhibitory to various degrees.

INTRODUCTION

Several species and strains of *Leuconostoc* and *Streptococcus* elaborate dextransucrase[1], an enzyme that synthesizes the polysaccharide, dextran, from sucrose. Since the discovery[2] of dextransucrase in 1941, it has gained importance because

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dextran and modified dextrans have found many industrial and medical uses[3,4]. Dextran formation may also be an unwanted occurrence, for instance, by *Leuconostoc* in the sugar-refining industry[5] and by *Streptococcus* in dental plaque in the oral cavity[6].

Leuconostoc mesenteroides B-512F dextransucrase has received wide attention[1-5] and the resulting dextran is produced commercially[4]. The B-512F dextransucrase is secreted in relatively large amounts into the culture supernatant solution with a minimum number and quantity of related contaminating enzymes, and it forms a high-molecular-weight, soluble dextran[3,4]. This may be contrasted with other strains of *L. mesenteroides*[7,8] and with the dental-plaque streptococci[9], which form both soluble and insoluble dextrans and elaborate more than one type of dextransucrase[9], together with relatively large amounts of such related, contaminating enzymes[10,11] as invertase and levansucrase. For these reasons, *L. mesenteroides* B-512F serves as an important model in studying the structure of dextran and the mechanism of dextran biosynthesis by dextransucrase. The biosynthesis of B-512F dextran has already been shown to have some interesting mechanistic features[12-14].

In the early years of the study of *L. mesenteroides* B-512F dextransucrase, relatively crude enzymes were prepared by alcohol precipitation of the culture supernatant solution[15-17]. Dextransucrase from this organism is an inducible enzyme that requires sucrose in the culture medium and results in copious amounts of dextran being formed in the culture supernatant. The precipitation of the enzyme from the culture supernatant by alcohol carries a significant amount of dextran with it. Braswell *et al.*[18] purified the alcohol precipitate by adsorption onto calcium phosphate gels; the eluted enzyme was free of dextran as judged by a serological test. In this instance, however, a serological test was not convincing evidence of a dextran-free enzyme, because B-512F dextran is a very weak antigen and gives poor serological reactions[19,20].

Ebert and Schenk studied the purification of dextransucrase extensively and developed a scheme that produced an enzyme apparently free of dextran[21]. However, their scheme was tedious and resulted in low yields and insoluble enzyme-precipitates. In none of the aforementioned studies were the preparations tested for contaminating enzyme activities.

Because an enzyme free of related, contaminating enzyme-activities was needed to study various aspects of its mechanism of action, we studied the production, purification, and properties of a B-512F dextransucrase by using modern techniques. A relatively simple, three-step procedure was developed; it resulted in a 240-fold purified enzyme that was free of levansucrase, invertase, dextranase, D-glucosidase, and sucrose phosphorylase activities.

EXPERIMENTAL

Materials. — *Leuconostoc mesenteroides* B-512F was obtained from the Northern Regional Research Laboratory (NRRL), Peoria, IL. [U-^{14}C]Sucrose,

[^{14}C]sucrose-[(U)-glucose], and [^{14}C]sucrose-[(U)-fructose] were purchased from New England Nuclear, Boston, MA. The agarose series of Bio-Gels, A-5m and so on, were purchased from Bio-Rad Laboratories, Richmond, CA. Dextranase (Type I) was purchased from Sigma Chemical Co., St. Louis, MO.

The B-512F dextran was prepared by using purified dextransucrase. Isomaltooligosaccharides were prepared by acid hydrolysis of B-512F dextran[22], and pure isomaltotetraose was obtained by preparative, paper chromatography of the dextran hydrolyzate[22].

Culturing of L. mesenteroides *B-512F.* — The organism was grown on the medium described by Hehre[23] and Tsuchiya *et al.*[24]. Cultures were incubated on a rotary shaker for 12 h at 25°. Successive transfers (10%) were made into larger volumes until 1 L was used to inoculate 10 L of medium in a New Brunswick fermentor that was incubated at 25° with agitation (150 r.p.m.) and aeration (0.5 L per 10 L of culture per min). Successive cultures were made by removing 9.5 L of the culture from the fermentor and adding aseptically 10 L of fresh medium. Cells were removed by centrifugation and the supernatant solution was used as the source of dextransucrase. For long-term preservation (for more than one month), cultures were maintained as lyophils[25] in double-strength, reconstituted, skim milk[26]. Agar slants of production medium were used when more-frequent transfers were required.

Enzyme assays. — Dextransucrase was assayed at 25° and pH 5 by a radiochemical method[27] by using 30 μL of appropriately diluted enzyme and 30 μL of 0.3M (10 μCi/mmol) [^{14}C]sucrose-[(U)-glucose]. Levansucrase was assayed similarly, except that 0.3M [^{14}C]sucrose-[(U)-fructose] was used. Aliquots were removed as a function of time and the initial velocity of polysaccharide formation was determined. One unit of enzyme (dextransucrase or levansucrase) is defined as the amount of enzyme that will incorporate 1 μmol of D-glucose or D-fructose into polysaccharide in 1 min. Dextranases were assayed by determining the reducing sugars released when incubated with dextran. Reducing values were measured by the alkaline ferricyanide method on the Technicon AutoAnalyzer[28].

Protein and carbohydrate determinations. — Protein was determined by the Folin–Lowry method[29] by using bovine serum albumin as a standard. Protein eluted from columns was estimated by absorbance measurements at 280 nm. Total carbohydrate was determined by the orcinol–sulfuric acid procedure adapted for use with the Technicon AutoAnalyzer[30,31].

Production of dextransucrase. — Samples (10 mL) were removed from the fermentor every h during a 12-h growth period. The turbidity of the sample was measured at 550 nm in a spectrophotometer, by using a 0.1-cm cuvette. The cells were removed by centrifugation and the pH of the supernatant was measured, dextransucrase and levansucrase* activities were determined after the pH of the

*The fructan produced by *L. mesenteroides* B-512F was examined by partial acid hydrolysis and by agar gel, double-diffusion, precipitin tests against concanavalin A[32]. Both experiments confirmed that the fructan was a levan and not an inulin[33].

supernatant solution had been adjusted to 5.0.

Purification of dextransucrase. — All of the purification steps were performed at 4°. The culture supernatant was dialyzed and concentrated against 20mM acetate and 3mM calcium chloride buffer, pH 5, by using a Bio-Fiber 80 miniplant (Bio-Rad Laboratories, Richmond, CA). Culture supernatant (10 L) was pumped continuously through the fiber bundle at a flow rate of 1200 mL/h. The buffer was allowed to flow through the miniplant jacket (area outside the fiber bundles) at a flow rate of 600 mL/h. After 12 h of dialysis, the supernatant solution was concentrated by continuously pumping the enzyme solution through the miniplant fibers at a flowrate of 150 mL/min with a partial vacuum of 50 cmHg outside the fibers. When the dialyzed culture supernatant had been concentrated approximately 40-fold, it was collected and the miniplant fibers were backwashed with two fiber-volumes (200 mL) of buffer; this backwash was added to the concentrate, which was labeled culture supernatant concentrate. Recoveries of 80–100% of the dextransucrase were obtained.

The culture supernatant concentrate (9.7 mL) was treated with 0.3 mL of Sigma dextranase (30 U/mL in 20mM acetate buffer, pH 5) for 40 h at 25° in a dialysis tube placed in 2 L of 20mM acetate + 3mM calcium chloride buffer, pH 5, which was changed 3 times during the course of the reaction. The digest was chromatographed on a 2.5 × 76 cm column of Bio-Gel A-5m which was eluted with 20mM acetate + 3mM calcium chloride buffer, pH 5, at a flow rate of 8.6 mL/h The dextransucrase fractions were pooled and concentrated by using an Amicon ultrafiltration cell with 1400 g/cm nitrogen gas and a PM-10 membrane. This concentrate (17 mL) is referred to as the A-5m concentrate and is considered purified enzyme.

Poly(acrylamide)-gel electrophoresis of purified dextransucrase. — The separating gel was 5% acrylamide and 0.25% Bis, with separation at pH 8 (ref. 34). The electrode buffer contained 6 g of Tris and 28.8 g of glycine in 1 L of distilled water[35]. Electrophoresis was performed in 6 × 100 mm tubes for 90 min at a constant current of 2.5 mA per tube. The gels were stained by three methods: Coomassie Blue for protein[36], periodate oxidation–Schiff base for carbohydrate[37], and dextransucrase activity by incubation in 100mM sucrose at pH 5 (ref. 38).

Reaction of purified dextransucrase with concanavalin A. — Purified dextransucrase (0.22 U/mL) was incubated with 25 mg of concanavalin A (Sigma Chemical Co.) in saturated sodium chloride, 3mM calcium chloride at pH 5.3 and for 30 min at 25°. After centrifugation, the supernatant solution was assayed for dextransucrase activity.

Detection of contaminating enzyme activities. — Contaminating enzyme activities in dextransucrase preparations were detected by incubating 10 μL of enzyme dextransucrase preparation containing 0.1 unit of dextransucrase activity with 10 μL of a specific substrate (each described later for the detection of individual enzymes) at 25°. After 24 h, the mixtures were examined by descending paper-chromatography on 23 × 56 cm Whatman 3 MM paper irrigated with 10:4:3 (v/v) ethyl acetate–pyridine–water for 18 h at 40° followed by the alkaline silver nitrate dip-procedure[39].

Levansucrase was detected by incubating the enzyme preparation with either [^{14}C]sucrose-[(U)-fructose], or with 0.3M raffinose. The presence of levansucrase was indicated by the formation of ^{14}C-labeled levan from the [^{14}C]fructose-labeled sucrose or by the formation of melibiose and polysaccharide from raffinose. *Glycosidase* or *invertase* activities were indicated by the formation of galactose or fructose from raffinose. *Dextranase* was detected by the formation of glucose, isomaltose, and isomaltotriose from isomaltotetraose. The presence of *sucrose phosphorylase* was indicated by the formation of D-fructose and α-D-glucosyl phosphate when incubated with 0.1M inorganic phosphate and 0.3M sucrose.

Effect of chelating agent and metal ions on the activity of purified dextransucrase.— The A-5m concentrate was assayed in the presence of EDTA [(ethylenedinitrilo)tetraacetic acid] at pH 5 (20mM acetate buffer). Before assaying and before the addition of EDTA, the enzyme was dialyzed at 4° against acetate buffer (pH 5) to remove exogenous ions. The chlorides of various divalent metal ions in 20mM, pH 5, acetate buffer were added to dialyzed enzyme or they were added to purified enzyme that had been preincubated for 90 min in 5mM EDTA. The enzyme preparations were then assayed at pH 5 in the presence of 5 mM metal ion.

Stability of purified dextransucrase. — Freshly prepared A-5m concentrate was stored at −15 and at 4° in the absence and presence of 4 mg/mL of B-512F dextran. Samples were taken every few days and assayed for dextransucrase.

Nature of the carbohydrate content of purified dextransucrase. — A 4-mL sample of the A-5m concentrate, having a carbohydrate content of 1.7 mg/mL (determined as glucose), was concentrated to 1 mL by evaporation *in vacuo*. Acid hydrolysis was conducted in sealed glass ampules with M trifluoroacetic acid for 90 min at 121°. The B-512F dextran was completely hydrolyzed to glucose under these conditions. After hydrolysis, the samples (0.5 mL) were taken to dryness and redissolved in 0.2 mL of distilled water. Aliquots (20 μL) were chromatographed by using the descending system described in the section on contaminating enzyme activities.

RESULTS

Production of dextransucrase. — Dextransucrase production was stimulated approximately 2-fold in the presence of 0.001–0.1% (w/v) calcium chloride (Fig. 1); and levansucrase levels were not affected by calcium ions until the concentrations were >0.05% (data not shown). The addition of 0.05% of calcium chloride evidently had no effect on cell growth, because the turbidity and acid production were the same in the presence or absence of calcium chloride (Fig. 1). Calcium levels >0.1% decreased dextransucrase production, and levels >0.2% adversely affected growth of the organism (data not shown). For optimal production of dextransucrase and minimal production of levansucrase, a concentration of 0.005% of calcium chloride was adopted. Dextransucrase was not produced until near the beginning of the stationary phase. At this point, the enzyme was secreted rapidly into the culture

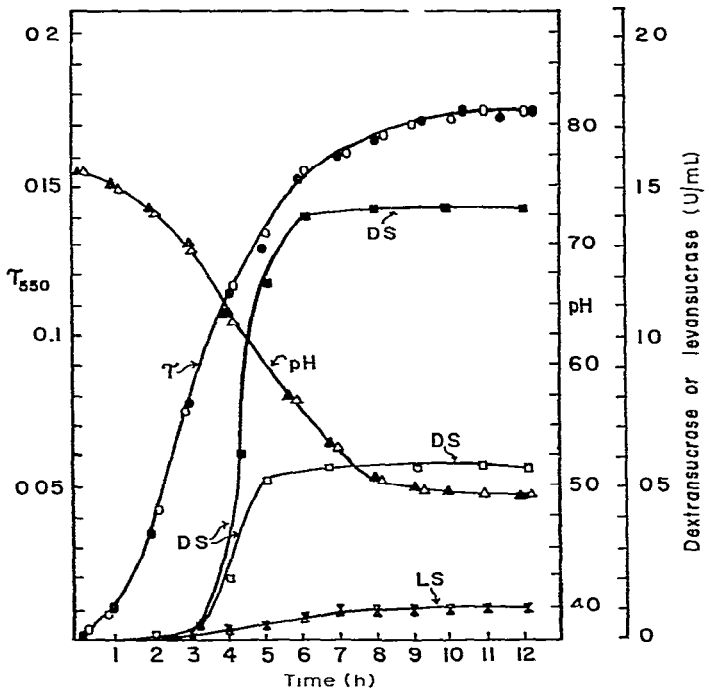

Fig. 1. Production of extracellular dextransucrase in cultures of *Leuconostoc mesenteroides* B-512F in the absence (open symbols) and in the presence (solid symbols) of 0.05% calcium chloride. ○, ● Turbidity (τ); □, ■ dextransucrase (DS); △, ▲ pH; and X, X levansucrase (LS).

medium and reached a maximum after about 3 h. These results were reproducible, and were repeated during several fermentation runs.

Purification of dextransucrase. — Table I gives the purification data for dextransucrase. Chromatography of the culture supernatant concentrate on Bio-Gel A-5m, before and after dextranase treatment, is shown in Fig. 2. Dextransucrase and levansucrase migrated with the void volume. After treatment with dextranase, however, the dextransucrase was retarded by Bio-Gel A-5m, and the levansucrase still migrated with the void volume. Furthermore, the added dextranase was retarded to a greater extent than dextransucrase, thus giving separation of dextransucrase from levansucrase and dextranase. This step gave an overall 240-fold purification, with a dextransucrase specific activity of 53 U/mg, and removed several contaminating enzymes from dextransucrase (see Fig. 3). Fig. 3 shows that the culture supernatant concentrate gave fructose, glucose, galactose, and melibiose from raffinose, and that the purified enzyme did not give any detectable products from raffinose, indicating the removal of levansucrase, invertase, and glycosidase activities from dextransucrase. Culture supernatant concentrate gave glucose, isomaltose, isomaltotriose, and isomaltopentaose from isomaltotetraose; the purified enzyme did not give any detectable products from isomaltotetraose, indicating the removal of glucosidase and transferase activities from dextransucrase. Culture supernatant concentrate gave glucose and a

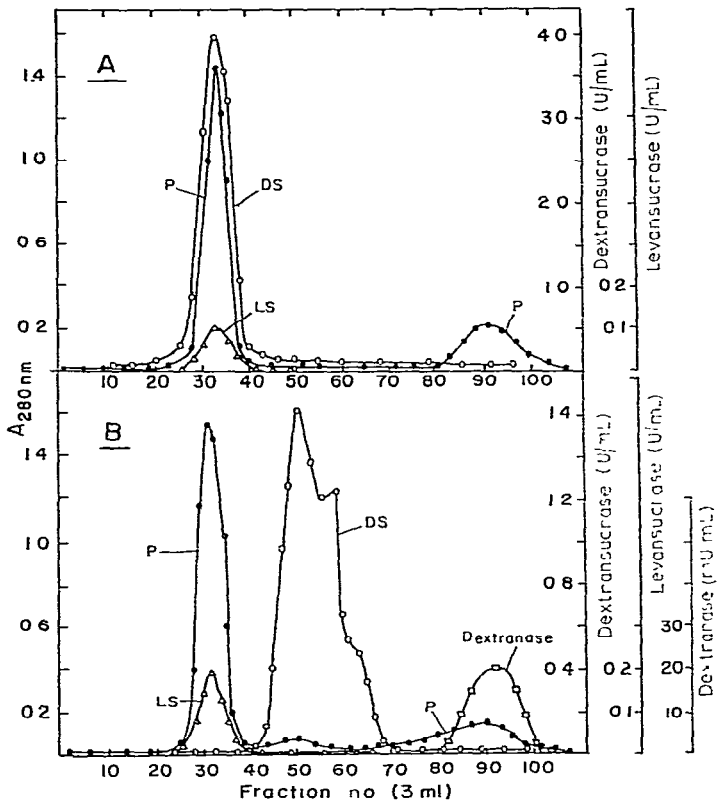

Fig. 2. Chromatography of the culture supernatant concentrate on Bio-Gel A-5m before (A) and after (B) dextranase treatment: –●–, A_{280nm} measurement of protein (P): –○– dextransucrase (DS), –▲–, levansucrase (LS), and –□–, dextranase.

trace of fructose from dextran, and the purified enzyme did not give any detectable products from dextran, indicating the removal of dextranase activity from dextransucrase. A control of culture supernatant concentrate (not shown) showed that it was chromatographically free of the observed products, indicating that the observed products were the result of reactions with the various substrates.

Table I shows that the purified dextransucrase was obtained in ~33% yield. and 99% of the protein and 84% of the carbohydrate had been removed from the starting material. Gel electrophoresis of the purified dextransucrase (Fig. 4) shows that all of the protein bands present (gels B, C, and D) had dextransucrase activity, and that they contained carbohydrate (gel A). The sample for gel D was applied to the gel immediately after purification on Bio-Gel A5m, and showed a single, fast-moving, active band near the dye front. The samples for gels A, B, and C were applied ~24 h after purification and showed an active, slower-moving band as well as the faster-moving band. The slow-moving band showed high dextransucrase activity relative to the fast-moving band.

TABLE I

PURIFICATION DATA FOR DEXTRANSUCRASE

Fraction	*Volume (mL)*	*Dextransucrase*			*Protein*			*SA*[a]	*PF*[b]	*Carbohydrate*			*LS*[c] (*U per mL*)
		U per mL	*Total U*	*Percent yield*	*Mg per mL*	*Total (mg)*	*Percent removed*			*Mg per mL*	*Total (mg)*	*Percent removed*	
Culture supernatant	9600	0.9	8640	100	4.1	39400	0	0.22	1	1.0	9600	0	0.02
Culture supernatant concentrate	425	19.0	8075	93.5	2.0	850	97.8	9.5	43.2	11.6	4930	48.6	0.5
A-5m concentrate[d]	17	3.8	2830	32.8	0.072	54	99.9	52.8	240	2.1	1564	83.7	0.0

[a]Specific activity (μmol of glucose incorporated into dextran per min per mg protein). [b]Purification factor. [c]Levansucrase. [d]Data were adjusted by a factor of 43.8 to account for the use of only 9.7 mL of culture supernatant concentrate to obtain the A-5m concentrate.

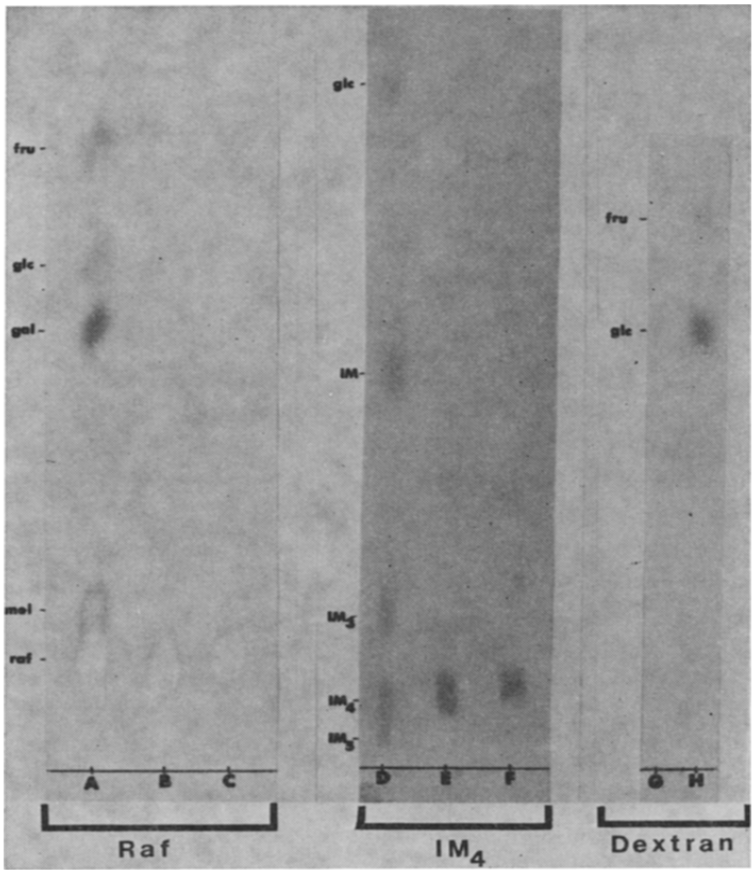

Fig. 3 Paper chromatograms of the products of the reaction of dextransucrase-purification fractions on raffinose, isomaltotetraose, and dextran; A, culture supernatant concentrate on raffinose; B, purified enzyme on raffinose; C, raffinose control; D, culture supernatant concentrate on isomaltotetraose; E, purified enzyme on isomaltotetraose, F, isomaltotetraose control; G, purified enzyme on dextran; H, culture supernatant concentrate on dextran.

Properties of dextransucrase. — Table II gives the mole percents of the products formed by the various purification fractions. All three of the fractions gave high yields of dextran. Purified dextransucrase (A-5m concentrate) gave 45.7% of dextran, 3.6% of leucrose, 0.5% of glucose, and 50.2% of fructose from sucrose. The purified enzyme produced only 0.5% of glucose, whereas the other fractions produced 1.5–2.9% of glucose.

The dextran produced by the purified enzyme had properties identical to those previously reported for B-512F dextran: it was a soluble, high-molecular-weight polysaccharide whose ^{13}C-n.m.r. spectrum (not shown) indicated 95% of α-D-(1→6) linkages and 5% of α-D-(1→3) branch linkages.

The storage stability at 4 and −15° of purified dextransucrase is shown in Fig. 5. The enzyme lost activity rapidly at 4° and lost ~60% of its activity at −15°

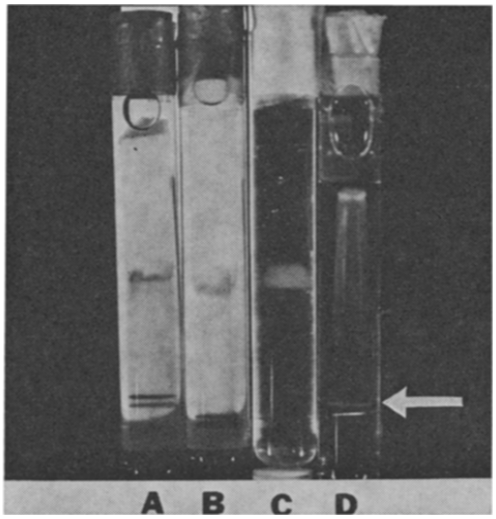

Fig. 4. Poly(acrylamide)-gel electrophoresis of purified dextransucrase: *gel A*, electrophoresis of a 24-h sample followed by periodate–Schiff base stain for carbohydrate; *gel B*, electrophoresis of a 24-h sample followed by Coomassie Blue stain for protein; *gel C*, electrophoresis of a 24-h sample followed by incubation in 100mM sucrose at pH 5 for 30 min, and *gel D*, electrophoresis of a 1-h sample followed by incubation in 100mM sucrose at pH 5 for 30 min. Gels A and B were photographed with transmitted light and gels C and D were photographed with stray light. The last band in gels A and B is the dye front. The arrow indicates the position of the fast-moving, dextransucrase band in gels C and D.

TABLE II

DISTRIBUTION OF PRODUCTS (MOLE PERCENT) FROM THE REACTION OF DEXTRANSUCRASE PURIFICATION FRACTIONS WITH [^{14}C]SUCROSE

Fraction	*Dextran*	*Leucrose*[a]	*Sucrose*	*Glucose*	*Fructose*
Culture supernatant	43.1	4.5	0	1.5	50.9
Culture supernatant concentrate	44.1	3.5	0	2.9	49.5
A-5m concentrate	45.7	3.6	0	0.5	50.2

[a]Leucrose is the disaccharide, α-D-glucopyranosyl-(1→5)-D-fructopyranose, formed by the reaction of D-fructopyranose with the D-glucopyranosyl–dextransucrase complex[14,49,50].

during a 20-day period (Fig. 5A). The addition of 4 mg/mL of dextran, however, stabilized the enzyme (Fig. 5B). The addition of 25% of glycerol had little effect on the storage stability (Fig. 5C).

The chromatographic analysis of the carbohydrate content of purified enzyme is given in Fig. 6. The principal monosaccharide formed was mannose, together with

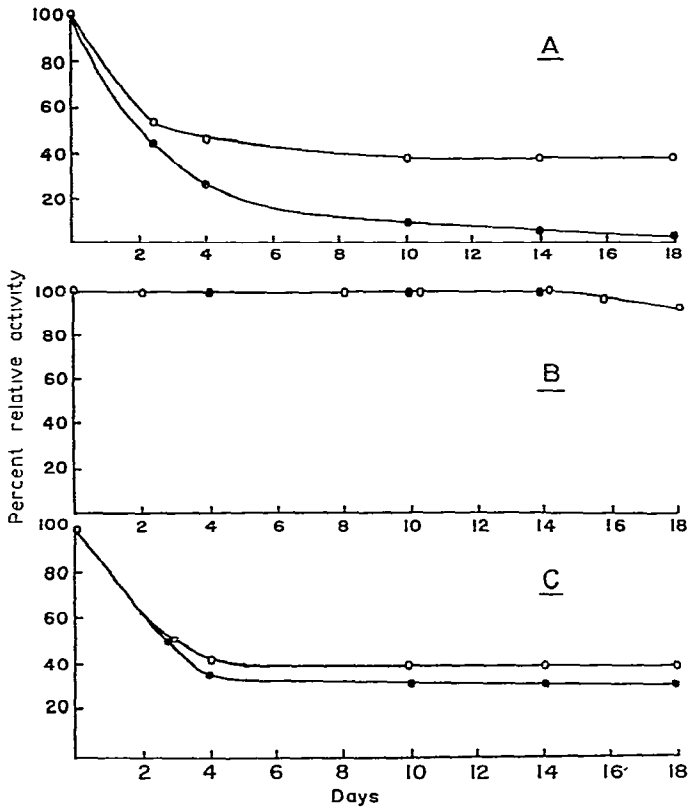

Fig 5. Storage stability of purified dextransucrase Open symbols, storage at −15°; solid symbols, storage at 4°. (A) pH 5 buffer; (B) pH 5 buffer + 4 mg/ml of dextran; and (C) pH 5 buffer + 25% (v/v) of glycerol

traces of glucose and galactose. The absence of any large amount of glucose demonstrates the absence or very low content of dextran in the purified enzyme. Incubation of the purified enzyme with concanavalin A removed all of the dextransucrase activity from solution.

Table III shows the effect of EDTA on the activity of the purified enzyme Inhibition of 50% was obtained with 12.5mM EDTA; greater inhibition could not be obtained with higher concentrations of EDTA, for example 25mM. The results of the addition of metal ions to the EDTA-treated enzyme are given in Table IV.

DISCUSSION

Attempts to purify the dextransucrase of *L. mesenteroides* B-512F began in our laboratory by using techniques that were effective in the purification of dextransucrase from other organisms, for instance, ammonium sulfate precipitation[8,40–42], and hydroxylapatite[18,42–45] and DEAE-cellulose chromatography[8,41,46,47].

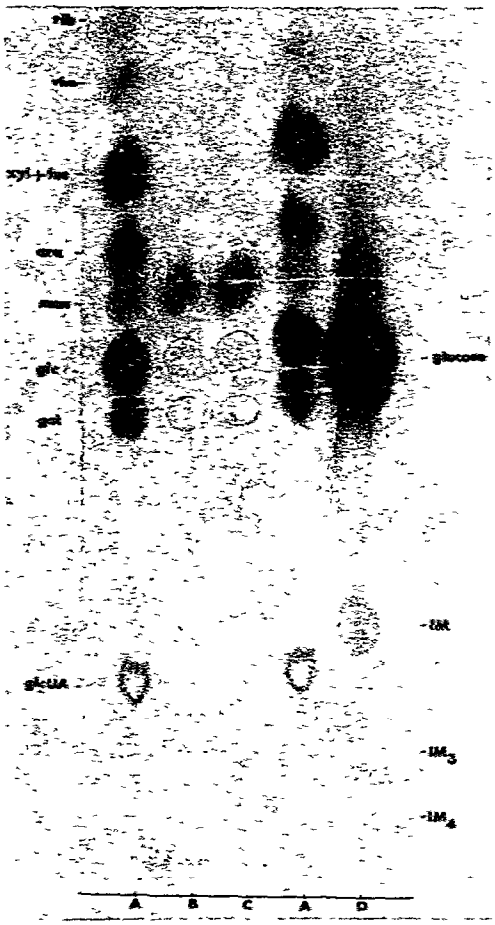

Fig. 6. Paper chromatogram of an acid hydrolyzate of the purified dextransucrase. A, mixture of monosaccharide standards; B and C, acid hydrolyzates of purified enzyme; and D, isomalto-oligosaccharide standards.

TABLE III

EFFECT OF EDTA ON THE PURIFIED DEXTRANSUCRASE

Concentration (mM) of EDTA	*Relative activity (%)*
0	100
0 25	91
1.25	68
2 50	62
6 25	54
12.50	50
25.00	50

TABLE IV

THE EFFECT OF DIVALENT-METAL IONS ON 5mM EDTA-TREATED, PURIFIED DEXTRANSUCRASE

Metal ion added (5mM)	*Relative activity[a] (%)*	*Relative activity[b] (%)*
none	56	78
Ca^{2+}	99	79
Mg^{2+}	40	74
Mn^{2+}	40	74
Sr^{2+}	68	56
Ba^{2+}	65	78
Zn^{2+}	0	17
Co^{2+}	0	65
Ni^{2+}	0	69
Cd^{2+}	0	39
Fe^{2+}	0	30
Hg^{2+}	0	0
Cu^{2+}	0	0
Pb^{2+}	0	0

[a]The metal ions (5mM) were added to enzyme that had been incubated with 5mM EDTA. The percent relative activity is based on the activity of purified, untreated dextransucrase. [b]The metal ions were added to EDTA-treated enzyme that had been dialyzed to remove EDTA. The percent relative activity also is based on the activity of purified, untreated dextransucrase.

We found that ammonium sulfate is completely ineffective in precipitating B-512F dextransucrase from a culture supernatant concentrate; 90% of the dextransucrase activity remained in solution when the concentration of ammonium sulfate was 80% (w/v). When culture supernatant concentrate was applied to Bio-Gel hydroxylapatite and eluted with a stepwise gradient of potassium phosphate, five fractions containing dextransucrase were obtained. All of these fractions were contaminated with levansucrase, invertase, and dextranase. Furthermore, the total yield of dextransucrase was $<10\%$. The low yield, the dilution of the dextransucrase activity, and the presence of contaminating enzymes contra-indicated the use of hydroxylapatite. When the culture supernatant concentrate was chromatographed on DEAE-cellulose, dextransucrase activity could not be eluted from the column, even at very high ionic strengths (for instance, 5M sodium chloride). Small proportions (10–20%) of the activity could be extracted from the upper third of the DEAE-cellulose with 2M sodium chloride buffered at pH 4 or 7, but the enzyme was not very stable at these pH values*, and enzyme precipitates were formed during dialysis to remove the salt. The precipitates were active and apparently highly purified, although levansucrase activity was still present. Dextransucrase and levansucrase did not bind to cation exchangers, *O*-(carboxymethyl)cellulose, or *O*-(phosphono)cellulose.

*The dextransucrase of *L. mesenteroides* B-512F has an extremely narrow pH-stability range, with an optimum[15,16] at pH 5.

Subsequent studies led to a relatively simple, three-step purification: the culture supernatant was dialyzed, and concentrated with a Bio-Fiber 80 miniplant, and the dialyzed concentrate was treated with dextranase in a dialysis tube. Finally, the dextranase-treated enzyme was chromatographed on Bio-Gel A-5m. The first purification step of dialysis and concentration gave a 43-fold purification, with a yield of nearly 100% of dextransucrase (Table I). Dextransucrase and levansucrase activities in the dialyzed concentrate migrated with the void volumes of the Bio-Gel A series, even Bio-Gel A-150m (Fig. 2A). This result indicated that the enzymes had a very high molecular weight or that they associate in large, molecular aggregates. Chromatography of the dialyzed concentrate on Bio-Gel A-5m resulted in ~120-fold purification. When treated with dextranase, the molecular size of dextransucrase was decreased and the enzyme was retarded on Bio-Gel A-5m, whereas levansucrase activity still migrated with the void volume (Fig. 2B). This difference resulted in the separation of dextransucrase from levansucrase. Chromatography on Bio-Gel A-5m of the dextranase-treated, culture supernatant concentrate gave a 240-fold purification of dextransucrase having a specific activity of 53 U/mg.

The decrease of the molecular size of dextransucrase after dextranase treatment indicated, because of a complex formed with dextran, that the enzyme in the culture supernatant concentrate is in a high-molecular-weight form. After being kept for a few days at 4°, however, the A-5m concentrate re-aggregated and again migrated with the void volume of a Bio-Gel A-5m column (data not shown). This aggregation could not have been caused by a complex with dextran, because the major part of the dextran had been removed by the dextranase treatment. Aggregation of dextransucrase seems to be a fairly general phenomenon. It has been observed for *L. mesenteroides* B-1299 and for *Streptococcus mutans* GS-5 dextransucrases[8,48]; both enzymes exist as monomers of 45,000 and 48,000 mol. wt. and as high-molecular-weight aggregates of the monomer.

Gel electrophoresis of the purified enzyme (A-5m concentrate) (Fig. 4) showed only two protein bands, both of which possessed dextransucrase activity. Only the fast-moving band was present when the sample was applied immediately to the gel. When the purified enzyme was allowed to stand before electrophoresis, a slower-moving band appeared and predominated. Qualitatively, the slower-moving band seemed more enzymically active than the fast-moving band. The fast-moving band could be a monomer of relatively lower molecular weight than the slow-moving, somewhat diffuse band, which is probably aggregates of the monomer.

The very high yield of dextran (45.7%, close to the theoretical amount of 50%), from sucrose by the purified enzyme is an indication of the high degree of purity of the preparation. Leucrose, a disaccharide [*O*-α-D-glucopyranosyl-(1→5)-D-fructopyranose] that is always found as a product in the reaction of dextransucrase with sucrose[49,50], was formed to the extent of 3.6%. Leucrose is formed by an acceptor reaction in which D-fructopyranose, a product of the polymerization reaction, displaces D-glucose from a D-glucosyl–dextransucrase complex[14]. The purified enzyme also gave 0.5% of glucose. The other fractions (culture supernatant and culture

supernatant concentrate) gave 1.5 and 2.9% of glucose, respectively. These latter, less purified, fractions produced glucose primarily by the action of levansucrase, whereas the glucose produced by the purified enzyme was not being formed by levansucrase (levan could not be detected by using [^{14}C]fructose-labeled sucrose), but was probably formed by hydrolysis of the glucosyl–dextransucrase complex[12,53].

The purified dextransucrase contained carbohydrate, as determined by the specific carbohydrate-staining of the electrophoresis gels (Fig. 4). Acid hydrolysis of the purified enzyme (Fig. 6) showed that mannose was the principal carbohydrate component, indicating that dextran had been significantly removed from the enzyme and that dextransucrase is a glycoprotein containing mannose. This hypothesis was confirmed by the complete removal of dextransucrase activity from solution by the addition of concanavalin A. Concanavalin A complexes with glycoproteins containing multiple, unsubstituted, nonreducing D-mannopyranosyl groups and does not complex with B-512F dextran[51,52].

We confirmed the findings of Brock Neely and Hallmark[54] that EDTA decreases the activity of B-512F dextransucrase (Table III) and that calcium ions restore the activity (Table IV) The maximum amount of inhibition that we could obtain with EDTA, however, was 50%, possibly because EDTA forms stronger chelate complexes at pH > 5. The addition of metal ions to the EDTA-treated enzyme (Table IV) showed that calcium was the only ion that completely restored the activity. This result, coupled with the 2-fold increase in dextransucrase activity in the culture medium supplemented with 0.05% of calcium chloride, suggests that dextransucrase is a calcium-metalloenzyme. This conclusion was also reached by Brock Neely and Hallmark[54] for B-512F dextransucrase and by Itaya and Yamamoto[55] for dextransucrase of *L. mesenteroides* IAM 1046.

The addition of other divalent-metal ions to the EDTA-treated enzyme either had no effect or produced further loss of activity. Complete loss of activity at low concentration (mM) of mercury, copper, and lead was expected because these ions are general enzyme poisons, but the complete inhibition by 5mM zinc ions when added to the EDTA-treated enzyme and 61% inhibition when added to the EDTA-treated and dialyzed enzyme are unusual. The addition of 25mM zinc ions to untreated enzyme gave 100% inhibition. Dialysis of the EDTA-treated enzyme gave an increase in the relative activity (56–78%) by apparently restoring some of the calcium that had originally been removed by the EDTA. Some 21% of the activity was irreversibly lost, however, as the addition of 5mM calcium ions did not restore the activity beyond 79%.

The addition of dextran (0.08–4 mg/ml) had no effect on the activity of the purified enzyme, but at 4 mg/ml, dextran stabilized the enzyme against losses of activity on storage (see Fig. 5). The failure of 25% (v/v) glycerol to stabilize the enzyme indicated that the stabilization by dextran was relatively specific. The failure of added dextran to stimulate the activity of the purified enzyme indicates that dextransucrase is not a primer-dependent enzyme[56].

Summarizing, we report here a purification scheme for *L. mesenteroides* B-512F

dextransucrase that gave a 240-fold purification with a specific activity of 53 U/mg. We demonstrated that the purified enzyme is free of contaminating enzyme activities of levansucrase, dextranase, invertase, glucosidase, and sucrose phosphorylase. We found that the enzyme is a calcium-metalloglycoprotein, containing D-mannose as the principal carbohydrate component, and that its activity is not stimulated by added dextran, but that it is stabilized by added dextran.

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