Isolation of a Pure Dextranase from *Penicillium funiculosum*

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A dextranase, produced by *Penicillium funiculosum*, was purified 1,000-fold to yield the enzyme which was demonstrated by gel electrophoresis and electrofocusing to be a homogeneous protein. The purification method included acetone partition, ammonium sulfate fractionation, gel filtration, iron defecation and precipitation, and diethylaminoethyl-cellulose chromatography. The pure enzyme was also obtained by preparative gel electrophoresis. Gel-permeation chromatography indicates a molecular weight of 41,000. An isoelectric pH of 4.6 was established by electrofocusing. A 1-mg amount of the enzyme hydrolyzes a dextran substrate to yield 27,000 isomaltose reducing units in 2 hr.

It was recently found that dextranase may be useful in preventing dental caries by dissolving plaque-forming dextrans deposited on teeth by bacteria (1, 4, 5). The extracellular dextranase was produced in broth cultures of *Penicillium funiculosum* NRRL no. 1768 supplemented with dextran and assimilable sources of nitrogen (10). The methods used to isolate an electrophoretically pure dextranase from this culture and some characteristics of the enzyme are the subject of this report.

MATERIALS AND METHODS

Materials. Dextran 100 (Sigma Chemical Co., St. Louis, Mo., lot 105B-2490), a linear polymer of 155,000 molecular weight, was used as the assay substrate. Diethylaminoethyl (DEAE)-cellulose (Cellex D, Bio-Rad Laboratories, Richmond, Calif.) having a labeled ion-exchange capacity of 0.7 meq per g was prepared for use by successive washing on a filter funnel with 5% NaOH, water to pH 8, and 1.0 N HCl to pH 3 and was finally neutralized to pH 7. Bio-Gel resins, P-6, P-10, P-100, and P-150, 50 to 100 mesh (Bio-Rad Laboratories), were equilibrated in water or buffer for 24 hr before use.

Dextranase assay. A unit of dextranase is defined as the amount of enzyme required to release, from the substrate (dextran), 1 mg of reducing sugar (calculated as isomaltose monohydrate) after 2 hr of contact at 40 C in 0.1 M acetate buffer (pH 5.1). The method of measuring reducing sugar was essentially that of Somogyi (8). The unit thus obtained is similar to that described by Tsuchiya et al. (10).

Solid content. Process solution samples were dried in aluminum dishes at 105 C for 4 hr to determine the solid content.

Gel electrophoresis. Analytical disc electrophoresis by the method of Ornstein (7) was run in 7% polyacrylamide gels prepared from cyanogum CN41 and pH 8.9 tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (0.37 M) buffer. The current was maintained at 4 ma per tube, and the run was stopped when a bromphenol blue marker reached the bottom of the gel. The gels were stained with Coomassie Brilliant Blue R-250 for visual observation of protein.

Preparative electrophoresis was carried out on a running chamber (6 cm in diameter) containing 7% polyacrylamide gel in an apparatus similar to that described by Brownstone (2), modified by elimination of the continuous elution system and casting the gel directly in the running chamber. The gel solution was prepared by dissolving 11.2 g of recrystallized acrylamide, 280 mg of recrystallized methylene-bis-acrylamide, and 100 mg of ammonium persulfate in 160 ml of pH 8.4 buffer (0.05 M glycine, 0.015 M Tris). After filtration and brief vacuum degassing, 50 µl of *N*,*N*,*N*,*N*-tetramethyl-ethylenediamine (TEMED) was gently mixed in and the solution was poured carefully into the gel holder. The bottom was closed with a Saran membrane, and the whole container was placed on a flat, level metal surface. The Saran membrane was removed after 4 hr and the gel chamber was mounted in the apparatus. The system was equilibrated overnight with buffer at 4 C. Runs were carried out at 4 C.

Isoelectrofocusing. An LKB 8101 isoelectrofocusing column of 110-m1 capacity (LKB Instruments, Inc.) was used to analyze samples of the highly purified dextranase and a 50% pure dextranase (isolated without the iron treatment). Stabilization against convection was achieved by using a density gradient prepared stepwise from one dense and one less dense solution (11). A 3% carrier ampholyte was selected to give a pH gradient between pH 4 and 6. Preparation
of the solutions and of the density gradient was performed as described in the preliminary instruction sheet and its addendum supplied by LKB Instruments. The anode solution was placed at the bottom, and the cathode solution was placed at the top. The samples, 32 and 47 mg, respectively, replaced the less dense solutions in fractions 10 through 15. Electrofocusing was performed for 24 hr with a potential of 1,000 v at 5 C. After the current equilibrium was established, hexane was pumped into the apparatus to empty the gradient at a constant rate. The optical density of the emerging gradient was measured at 280 nm automatically with an Isco model 222 ultraviolet analyzer. Portions (2 ml) were collected, measured for pH, and assayed for dextranase activity.

Dextranase purification. A broth culture of P. funiculorum NRRL no. 1768 supplemented with dextran (9) was grown in 550-liter fermentors for 144 hr. The fermentation medium contained 1% Amber BYF 300, 1% dextran (molecular weight, 5 × 10^6 to 40 × 10^6), 0.5% K_2HPO_4, and 0.1% MgSO_4·7H_2O in 450 liters. The fermentation was maintained under aeration and agitation at 28 C with a controlled pH 6.3 to 6.5. One thousand liters of broth was clarified by centrifugation and evaporated 20-fold at pH 5 under vacuum (below 37 C); the concentrate was clarified by centrifugation and treated with one-half of its volume of cold acetone while maintaining a temperature of 0 C. After standing for 18 hr, the bottom layer was discarded. The top layer (52 liters) was treated with 26 liters of acetone at 0 C. The resultant mixture was allowed to stand at 0 C for 12 hr and the clear top layer was discarded. The oily residue which contained the dextranase was taken up in 5 liters of water and filtered, and the filtrate was evaporated at 10 C to 2 liters.

Two liters of acetone product was desalted at 25 C on a column (33 by 76 cm) containing 65 liters of wet Bio-Gel P-6. The column was developed with water at 0.5 liter per min and the effluent was monitored by a Meeco recording refractometer. Both the refractive index and dextranase activity peaks started at 16 liters of effluent and the dextranase activity ended after 23 liters.

The Bio-Gel-rich effluent (8 liters) was treated with 1,600 g of ammonium sulfate, the mixture was agitated for 2 hr, and the inactive centrifuged solids were discarded. The liquor was further treated with 1,600 g of ammonium sulfate. After stirring for 2 hr, the dextranase-rich precipitate was centrifuged, dissolved with water to make 2 liters of solution, and desalted as above on the same Bio-Gel P-6 column. The dextranase-rich effluent was obtained in 8 liters.

The dextranase solution from the second Bio-Gel P-6 column was adjusted to pH 7.5, treated with 120 ml of FeCl_3 solution containing 11 mg of ferric ion per ml (final pH 4.2), and allowed to remain at 4 C for 18 hr. The iron precipitate was centrifuged, washed with 0.5 liter of water, and discarded. The combined filtrate and wash were adjusted to pH 7.5, treated with 120 ml of the above ferric solution, adjusted to pH 5, and held at 4 C for 18 hr. The slurry was then centrifuged, the precipitate was washed with water, and the sedimanted solids were extracted with three successive 100-ml portions (0.5 hr of agitation) of 0.2 M phosphate buffer at pH 7.

The combined centrifuged phosphate liquors were evaporated to 30 ml, clarified, and desalted on a column (1.9 by 89 cm) containing Bio-Gel P-10, with water at a rate of 2 ml per min. The most active fractions (61 to 160 ml) were combined and adjusted to pH 7, and the dextranase was adsorbed on a DEAE-cellulose column (1.9 by 70 cm). The column was then treated at 2 ml per min in succession with 200 ml of water, 400 ml of 0.1 M sodium chloride, and finally with 500 ml of 0.2 M sodium chloride. The effluent, monitored at 280 nm with an Isco model 222 ultraviolet analyzer, was collected in 20-ml fractions. Fractions 30 through 33 (Fig. 1) contained the peak activity and coincided with the ultraviolet adsorption maximum. Ultrafiltration of a sample of the combined fractions showed the high-molecular-weight solids to be 360 mg.

### Results

Purified dextranase. The isolation procedure, summarized in Table 1, gave a 1,000-fold purification of dextranase from centrifuged culture broth to final product with a direct yield of 2.6%. A single Coomassie Blue-stained band of 0.45 mobility (bromophenol blue = 1.0) was observed when 0.2 mg of the purified enzyme in 50 μl was run on analytical gel electrophoresis (Fig. 2). The ultraviolet spectrum (Fig. 3) has a maximum at 280 nm ($E_{280}^\text{max} = 20$) and a minimum of 250 nm. Electrofocusing yielded a single protein peak in the pH 4 to 6 range with an isoelectric point at pH 4.55 ± 0.05 (Fig. 4). Ultracentrifugation at 20 C and at 180,000 × g (C, 0.2 mg per ml) gave one sharp sedimentation boundary at 4.7 Svedberg units. Assayed in the presence of excess Dextran 100, 1 mg of dextranase released 27,000 isomaltoolose units. The amino acid composition of acid-hydrolyzed dextranase (Table 2) indicates

![Fig. 1. Chromatographic pattern of dextranase on DEAE-cellulose with 400 ml of 0.1 M and 500 ml of 0.2 M NaCl. Symbols: C, dextranase activity; fraction volume, 20 ml.](http://aem.asm.org/Downloaded from)
TABLE 1. Purification procedure for a dextranase from P. funiculosum

| Procedure                              | Vol | Enzyme activity | Total solids | Specific activity units/mg of solids |
|----------------------------------------|-----|-----------------|--------------|-------------------------------------|
| Centrifuged culture broth              | 1,000 | $380 \times 10^4$ | 16,000 | 24 |
| Evaporated broth                       | 50  | $290 \times 10^4$ | 16,000 | 18 |
| Precipitate from 35% acetone           | 2   | $100 \times 10^4$ | 930  | 107 |
| Bio-Gel P-6 effluent                   | 8   | $42 \times 10^4$  | 90   | 470 |
| Ammonium sulfate precipitate and 2nd Bio-Gel P-6 effluent | 8  | $34 \times 10^4$ | 14 | 2,400 |
| Ferric chloride (120 ml) pH 4.2 filtrate | 9  | $28 \times 10^4$ | 4   | 7,000 |
| Ferric chloride (120 ml) pH 5 filtrate  | 9.7 | $0.6 \times 10^4$ | 2.5 | 240 |
| Ferric chloride precipitate in phosphate buffer | 0.3 | $20 \times 10^4$ |  |  |
| Bio-Gel P-10 effluent                  | 0.1 | $14 \times 10^4$ | 0.71 | 20,000 |
| DEAE-cellulose-rich fraction           | 0.08| $9.8 \times 10^4$ | 0.36 | 27,000 |

Fig. 2. Photograph of stained gel worms after electrophoresis of various dextranase samples. (1) Seventy-four per cent pure dextranase after Bio-Gel P-10; (2) pure dextranse; (3) 9% pure dextranase after second Bio-Gel P-6 filtration; (4) 26% pure dextranase after iron defecation.

that aspartic acid is present in the highest concentration followed by serine, glycine, isoleucine, valine, threonine, and glutamic acid. Microanalysis gave 15.7% nitrogen, indicating that the enzyme is not a glycoprotein.

Molecular-weight determination. Good correlation between the logarithm of the molecular weight of polypeptides or proteins and their elution volumes in gel filtration has been demonstrated by several workers (3). A series of experiments comparing dextranase to proteins having published molecular weights are summarized in Fig. 5 and permit assignment of a molecular weight of 41,000 for the purified enzyme.

Dextranase stability. Maximum stability of dextranase in aqueous solutions is observed at pH 5 to 7. Such solutions are stable at 4°C for at least 1 year but are rapidly inactivated above 55°C. In the presence of solvents (e.g., 50% aqueous methanol, 30% aqueous acetone), a 9% loss of enzyme activity was noted in 24 hr at 4°C. At 25°C, the same solutions lost 25 and 35% of their activity, respectively, in the same period of time.

Mercuric salts quickly inactivated dextranase in proportion to the mercury present. The enzyme activity was partially restored by the addition of 2-mercaptoethanol in a final concentration of 5% (Table 3). Although the addition of NaCl (1% final concentration) did not restore the activity of the mercury-inactivated enzyme, the presence of 1% NaCl in dextranase solutions prevented mercury salt inactivation.

FIG. 3. Ultraviolet spectrum of pure dextranase in water (concentration, 0.3 mg per ml).
dextranase containing 120 mg of solid. This solution was carefully pumped onto the gel column (described previously). The current was limited to 20 mA while the charge entered the gel to avoid convection mixing, after which buffer circulation was started and the current was raised to 50 mA (140 v). After 1.5 hr, the tracer dye was at the bottom of the gel plug and a refractile zone could be observed about 2 cm below the top of the gel. The gel was extruded from its container, and a thin vertical slice of gel was removed and stained with Coomassie Blue. A deeply stained band coincident with the refractile zone was observed along with several faintly stained bands ahead of the main zone. A taut wire was used to slice the entire gel plug horizontally into 1.5-mm sections, and each gel slice was forced through a syringe barrel along with 20 ml of buffer to produce a slurry of coarse gel granules. After 16 hr at 5°C, Aqueous solutions of pure dextranase at concentrations of the order of 12 mg/ml have been handled routinely without loss of activity. Lyophilization of solutions of highly purified enzyme, however, yielded solids which were not completely soluble in water at concentrations of 1 mg/ml and which showed some 30% loss of enzyme activity.

**Preparative electrophoresis.** The dextranase used for preparation of the pure enzyme by electrophoresis was estimated to be 80% pure and showed six protein-stained bands by analytical gel electrophoresis. A trace of bromophenol blue was added to 3 ml of ultrafiltered electrolyte-free dextranase containing 120 mg of solid. This solution was carefully pumped onto the gel column (described previously). The current was limited to 20 mA while the charge entered the gel to avoid convection mixing, after which buffer circulation was started and the current was raised to 50 mA (140 v). After 1.5 hr, the tracer dye was at the bottom of the gel plug and a refractile zone could be observed about 2 cm below the top of the gel. The gel was extruded from its container, and a thin vertical slice of gel was removed and stained with Coomassie Blue. A deeply stained band coincident with the refractile zone was observed along with several faintly stained bands ahead of the main zone. A taut wire was used to slice the entire gel plug horizontally into 1.5-mm sections, and each gel slice was forced through a syringe barrel along with 20 ml of buffer to produce a slurry of coarse gel granules. After 16 hr at 5°C, Aqueous solutions of pure dextranase at concentrations of the order of 12 mg/ml have been handled routinely without loss of activity. Lyophilization of solutions of highly purified enzyme, however, yielded solids which were not completely soluble in water at concentrations of 1 mg/ml and which showed some 30% loss of enzyme activity.

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**Figure 4.** Electrophocusing of purified dextranase in a pH 4 to 6 ampholyte gradient.

**Figure 5.** Gel permeation chromatography of dextranase on Bio-Gel P-150 (1.1 by 150 cm), 0.05 M phosphate (pH 5.0) containing 0.1 M KCl, O; Bio-Gel P-150 (1.1 by 150 cm), 0.05 M Tris-hydrochloride (pH 8.0) containing 0.1 M KCl, Δ; Bio-Gel P-100 (1.1 by 130 cm), 0.05 M acetate (pH 4.0), ●. (1) Dextranase; (2) L-aminooxidase (molecular weight, 62,000); (3) bovine serum albumin (molecular weight, 67,000); (4) ovalbumin (molecular weight, 44,500); (5) pepsin (molecular weight, 35,500); (6) trypsin (molecular weight, 24,000); (7) α-chymotrypsin (molecular weight, 22,500); (8) cytochrome c (molecular weight, 13,000).

**Table 2.** Amino acid analysisa of dextranase

| Amino acid     | 10⁻⁴ M/mg | Residues per moleculeb |
|----------------|-----------|------------------------|
| Lysine         | 0.29      | 12                     |
| Histidine      | 0.21      | 8                      |
| Arginine       | 0.19      | 8                      |
| Tryptophan     | 0.13      | 5                      |
| Aspartic acid  | 1.20      | 48                     |
| Threonine      | 0.59      | 24                     |
| Serine         | 1.00      | 40                     |
| Glutamic acid  | 0.58      | 23                     |
| Proline        | 0.51      | 20                     |
| Glycine        | 0.85      | 34                     |
| Alanine        | 0.48      | 19                     |
| Cystine        | 0.04      | 2                      |
| Valine         | 0.60      | 24                     |
| Methionine     | 0.17      | 7                      |
| Isoleucine     | 0.61      | 24                     |
| Leucine        | 0.36      | 14                     |
| Tyrosine       | 0.40      | 16                     |
| Phenylalanine  | 0.38      | 15                     |

a Beckman-Spinco automatic amino acid analyzer.
b Calculated on the basis of a molecular weight of 41,000.

**Table 3.** Effect of mercury and 2-mercaptoethanol on dextranase

| Original dextranase (units/ml) | Molar ratio (Hg/dextranase) | Remaining dextranase (units/ml) | Addition of mercaptoethanol (units/ml) |
|-------------------------------|-----------------------------|---------------------------------|----------------------------------------|
| 12,500                        | 1,000                       | Less 125                        | 1,000                                  |
| 12,500                        | 100                         | 1,640                           | 4,250                                  |
| 12,500                        | 10                          | 6,200                           | 11,250                                 |
the slurries were centrifuged and the supernatant liquids were subjected to analytical electrophoresis. The center cut of the refractile band showed a single stained protein and contained 49% of the dextranase charged. The leading cut of the refractile zone contained 24% of the dextranase and showed four faster moving protein bands. The trailing cut of the refractile band yielded 20% of the dextranase and contained one slower moving protein band.

**DISCUSSION**

A study of the effect of selected metal ions on dextranase has been carried out to determine whether some loss of enzyme in the purification methods could be due to metal complexing or inactivation. It was found that, whereas mercury inactivated dextranase, only the ferric ion of the common metals tested significantly precipitated the enzyme from broth or purified solutions. Salts adversely affected precipitation efficiency so that the iron procedure was performed on enzyme solutions which had first been desalted by gel filtration. Iron treatment was most effectively carried out in two stages. The first increment of iron added (final pH 4.2) precipitated impurities in preference to dextranase, leaving a threecold increase in enzyme purity in the centrifuged liquor. Neutralization of the liquor followed by further addition of ferric ion (final pH 5.0) precipitated essentially all of the dextranase. The iron-dextranase complexing mode of action was not determined, although it was found that the enzyme did not adsorb or complex with freshly prepared ferric hydroxide. Dextranase may be released from its iron complex with various salts preferably at pH 7. Phosphate buffer works exceedingly well and was used in the dextranase isolation.

Dextranase was completely precipitated from crude enzyme concentrates of low solids content (25 mg/ml) by treatment with 40 g of ammonium sulfate per 100 ml of solution. Minimal amounts of dextranase were precipitated with half this concentration. At higher solids concentration (200 to 300 mg/ml), dextranase was essentially all precipitated with 25 g of ammonium sulfate per 100 ml of solution.

Attempts were made to crystallize very pure dextranase by the method of Jakoby (6), in which a cold (2°C) mixture of an enzyme plus successively lowered concentrations of ammonium sulfate solutions are equilibrated and centrifuged and the supernatants are slowly warmed to room temperature yielding a crystalline product. However, with purified dextranase, no crystalline product was obtained.

**FIG. 6.** DEAE-cellulose batchwise adsorption of dextranase versus pH. Starting concentration, 40,000 units per ml; total, 1.6 × 10^4 units; 0.25 g of DEAE-cellulose. Symbols: ●, dextranase assay; △, optical density. Material tested is the supernatant of the DEAE-cellulose absorbate.

**FIG. 7.** Electrophoresing of 50% pure dextranase in a pH 4 to 6 ampholite gradient. Symbols: Solid line, optical density; line with solid circles, dextranase assay; dashed line, pH.

The adsorption of dextranase on DEAE-cellulose was adversely affected by the presence of extraneous ions so that the enzyme was first desalted by gel filtration. The optimum for dex-
Tranase adsorption on the anion exchanger was at pH 7.0 with little adsorption at pH 4.2 (Fig. 6). The enzyme was readily eluted with 0.2 M salt in a sharp peak, whereas 0.1 M salt removed ultraviolet-absorbing impurities (Fig. 1). Other DEAE-cellulose chromatograms equilibrated with either 0.1 M Tris-hydrochloride or 0.1 M phosphate, both at pH 7.4, gave distribution coefficient \( K_d \) values of 2.6 and 1.3, respectively. The \( K_d \) was calculated by the formula developed by Wheaton and Bauman (12): \( K_d = Ve/Vo/Vi \), where \( Ve \) is the enzyme elution volume and \( Vo \) and \( Vi \) are the void and intrastitial volumes of the exchanger, respectively. The lower \( K_d \) value for the phosphate buffer is expected because of its multivalence.

The electrophoretically pure dextranase obtained by preparative gel electrophoresis was comparable in enzyme activity, ultraviolet absorption, and electrophoretic mobility to the pure enzyme obtained by the multistep large-scale procedure. Although dextranase was not obtained crystalline, homogeniety was indicated by disc gel electrophoresis and also by the technique of electrofocusing which involves separation of proteins by differing isoelectric points and has extraordinary resolving power (9). Electrofocusing of the 50% pure dextranase, prepared without iron treatment, yielded a minor peak of dextranase activity at pH 4.1 and a trace at pH 4.9 (Fig. 7). This indicates the presence of dextranase isozymes in the culture broth.

The molecular weight of 41,000 for dextranase correlated well on gel filtration with three different buffer systems, although columns five times longer might give a more accurate determination. One must keep in mind that anomalous results may be obtained with proteins if they are not globular, if they form complexes with the gel, or if they dissociate into subunits.

A 30% increase in reducing sugar was obtained when a clinical grade dextran (molecular weight, 100,000 to 200,000) obtained from Nutritional Biochemicals Corp. (control no. 4271) was used as a substrate in place of the Sigma dextran. Since dextranase cleaves the \( \alpha-1,6 \)-glucosidic linkage in dextrans, the size of the dextran, degree of branching, microbial origin, etc., all may have an effect on the amount of isomaltose formed under the same experimental conditions.

The amino acid analysis for sulphydryl groups in dextranase was low and a question may therefore be raised as to whether this group is actually present in the enzyme. The inactivation effect of mercury ion and the restoration of enzyme activity by 2-mercaptoethanol is reasonable evidence that at least one sulphydryl is present and is essential for enzyme activity.

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