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# Purification and properties of extracellular dextranase from a *Bacillus* sp.

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**Abstract**

Bacterial strains in the genus *Bacillus* were isolated from natural soil samples and screened for production of extracellular dextranases (E.C.3.2.1.11). One strain, determined by 16sRNA analysis as *Paenibacillus illinoisensis* exhibiting stable dextranase activity, was chosen for further analysis, and the dextranase from it was purified 733-fold using salt and PEG precipitations, two-phase extraction and DEAE–Sepharose chromatography with a total yield of 19%. The purified enzyme had three isoforms, with molecular masses of 76, 89 and 110 kDa and isoelectric points of 4.95, 4.2 and 4.0, respectively. The mixture of the three dextranase isoforms has a broad pH optimum around pH 6.8 and a temperature optimum at 50 °C. The N-terminal sequence (Ala–Ser–Thr–Gly–Lys) was identical between the isoforms. No sequence homology with the known dextranases in the protein databanks was found.


*Keywords:* *Bacillus*; Aqueous two-phase extraction; Dextranase; Enzyme

## 1. Introduction

Dextranase (α-1,6-D-glucan-6-glucanohydrolase; E.C. 3.2.1.11) catalyzes the hydrolysis of α-(1,6) linkages of dextran polysaccharides. Dextranases are of practical importance since these enzymes can depolymerise various troublesome microbial dextran deposits. *Streptococci* produce an exopolysaccharide (glycocalyx) composed predominantly of dextran. *Streptococcus mutans* and *S. sorbinus* produce tartar on teeth, which can be removed with dextranases. The enzyme dextranase has long been implemented in the manufacturing of blood substitutes obtained by controlled hydrolysis of dextran produced by *Leuconostoc mesenteroides*. Dextranases can be added to the mixture of hydrolytic enzymes in processes of sugar industry to eliminate difficult mucous residues. The efficiency of antibiotics is also enhanced by dextranases [1].

Fungal dextranases are the most extensively studied, but several bacteria also produce extracellular dextranases [2–5]. The genus *Bacillus* is the source of many different extracellular enzymes, especially of hydrolases of biopolymers, but little is known about *Bacillus* dextranases [6–9]. We isolated 23 dextranase-secreting *Bacillus* strains from natural soil

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samples. Dextranase from one of them was isolated. The pure enzyme contained three active isoforms with different molecular masses and isoelectric points. All isoforms had identical amino terminus, which was different from that of any known dextranase, and the identity suggests that the isoforms are formed by the degradation of the same enzyme from the carboxy terminus.

## 2. Materials and methods

### 2.1. Chemicals

Blue Dextran 2000 kDa, dextran 500 kDa, Sephadex G-75, G-150, G-200 and DEAE–Sepharose (Phast Flow) were purchased from Pharmacia (Uppsala, Sweden). Polyethylene glycol (PEG) 400, 4000 and 6000, and acrylamide and bis-acrylamide were the products of Fluka (Buchs, Switzerland), while dextran 67 kDa, PEG 8000, Remazol Blue R-250, lauryl sulphate sodium salt and a high-molecular-weight standard mixture for sodium dodecyl sulphate (SDS) gel electrophoresis were the products of Sigma (USA). Broad-range calibration kit pI (pH 3.0–10.0) and precast mini-polyacrylamide gels (PhastGel) IEF-3-9 were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden), and ammonium sulphate was from Merck (Germany). All other reagents were of analytical grade.

### 2.2. Screening methods for dextranase-producing Bacillus strains

Bacteria that could assimilate dextran were enriched from soil samples collected from different habitats within the whole territory of Russia. Two kinds of enrichment media were used for the screening. Medium 1 contained (w/v) 0.5% Dextran T 20, 0.1% $K_2HPO_4$ and 0.1% $KH_2PO_4$, pH 7.4. Medium 2 contained (w/v) 0.5% Sephadex G 75, 0.1% $K_2HPO_4$ and 0.1% $KH_2PO_4$, pH 7.4. A soil sample (about 0.3 g) was added into a 20 ml test tube with 5 ml of medium 1 or 2. After 7 days of incubation at 37 °C without shaking in aerobic conditions, the test tubes were heated in a water bath for 20 min at 80 °C. Petri dishes containing (w/v) 0.2% Blue Dextran, 0.3% corn steep liquor, 0.3% peptone, 0.3% yeast extract, 0.2% $K_2HPO_4$ and 0.2% $KH_2PO_4$, pH 7.4–7.6 were inoculated from the heated enrichment media. Dextran-degrading colonies showing white halos on a blue background were re-streaked until pure cultures were obtained. The gram-positive sporulating bacteria were identified as members of the genus *Bacillus*. One of the isolates, *Bacillus* sp., showing high dextranase activities in repeated cultivations was chosen for further analyses. The strain was further identified as *Paenibacillus illinoisensis* by a standard 16sRNA analysis.

### 2.3. Culture conditions for production of dextranase

Standard liquid culture medium for the production and purification of extracellular dextranase contained (w/v) 0.5% Dextran T-40, 0.89% $Na_2HPO_4$, 0.05% $KH_2PO_4$, 0.3% peptone, 0.3% yeast extract and 0.3% corn steep liquor. The pH of the medium was adjusted to 7.4–7.6 with 1 M NaOH before autoclaving. A single bacterial colony was diluted into this medium and incubated on a rotary shaker (200 rpm) at 37 °C for 3 days. Bacterial cells were removed by centrifugation for 20 min at 12,000 × *g* at 4 °C, and the supernatant was used for the purification of the enzyme.

### 2.4. Ammonium sulphate precipitation

The enzyme in the cell-free culture broth was concentrated by adding solid $(NH_4)_2SO_4$ (68 g/100 ml) on a magnetic stirrer at room temperature. After standing overnight at 4 °C, precipitated protein was collected by centrifugation at 12,000 × *g*, and the pellet was dissolved in distilled water.

### 2.5. PEG fractionation

The solubility of the enzyme obtained from $(NH_4)_2SO_4$ step was studied in different PEG solutions (PEG 400, 4000, 6000 and 8000) at pH values of 5.0–9.0. Buffers 50 mM sodium phosphate–25 mM citrate (pH 5.0–7.0) and 50 mM Tris–HCl (pH 8.0–9.0) were used. Different amounts of 50% (w/w) PEG in a buffer with a certain pH value were added to a protein extract (0.3 g) in a plastic micro centrifuge tube and the total weight of the content was adjusted to 1.5 g with the same buffer. The solution was then treated with a Vortex mixer for 30 s and centrifuged

at 1500 × *g* for 10 min. The supernatant was carefully taken off by a micropipette and the pellet was dissolved in 0.5 ml of buffer with the respective pH. The supernatant and dissolved pellet were analysed for the enzyme activity and protein content.

### 2.6. Interphase extraction

Two-phase systems were prepared by adding solid $(NH_4)_2SO_4$ at concentrations of 10, 20, 30, 40 and 50% (w/v) in the supernatants from PEG fractionations. The solution was then treated with a Vortex mixer for 1 min and centrifuged at 1500 × *g* for 10 min. The enzyme activities and protein concentrations of the upper and bottom phases and the interphase were measured. In the final selected extraction procedure, a 50% (w/w) PEG stock solution was added to obtain its final 20% (w/w) concentration, followed by Vortex mixing for 1 min and centrifuging at 3200 × *g* for 15 min at 17 °C. The pellet was discarded and 20 g of ammonium sulphate was added per 100 ml of supernatant to get the two phases.

After 1 min inversion, the mixture was centrifuged and the interphase formed was withdrawn. The interphase, enriched in dextranase activity, was collected and dissolved in an amount 10 times the weight of 50 mM Tris–HCl, pH 8.0. To form a new two-phase system, 20 g of $(NH_4)_2SO_4$/100 ml and 30 g of PEG 6000/100 ml were added to the diluted interphase solution. After mixing and centrifuging as above, the interphase was dissolved in 50 mM Tris–HCl (pH 8.0) and dialysed conventionally overnight against the same buffer and concentrated by a Centricon YM-30 (Millipore, USA).

### 2.7. Anion-exchange chromatography

The concentrated enzyme sample from the previous step was loaded onto a DEAE–Sepharose (Pharmacia, Sweden) column (1.0 cm i.d. × 20 cm) pre-equilibrated with 50 mM Tris–HCl, pH 8.0, and the chromatography was carried out at 4 °C with a Gradi Frac equipment (Pharmacia, Sweden). Proteins were eluted from the column with a linear NaCl gradient (0–360 mM) in 50 mM Tris–HCl, pH 8.0, at a flow rate of 0.5 ml/min. The fractions containing substantial dextranase activity were collected and concentrated with Centricon YM-30 (Millipore, USA).

### 2.8. Studies on affinity adsorption

A two-phase system containing 7% (w/v) dextran, 5% (w/v) PEG 6000 in 25 mM Tris–HCl buffer, pH 7.6, was prepared from aqueous stock solutions of 20% (w/w) dextran 500 kDa, 40% (w/w) PEG 6000, 100 mM buffer and water in a centrifuge tube. One millilitre of sample solution was added to get a total net weight of 4 g. After mixing for 15 s, the tube was centrifuged at 1500 × *g* for 10 min. The volumes of upper and lower phases were measured and then samples from both phases were taken for analyses of dextranase activity and protein. All procedures were undertaken at 4 °C.

Affinity material for extraction of the enzyme to the solid substrate was prepared by bisoxirane coupling of dextran 67 kDa to Sepharose-CL-4B [18]. One millilitre of culture broth was added to 200 mg of the affinity gel and then stirred for 4 h on an ice-water bath. After that, Sepharose was removed by centrifuging at 1500 × *g* for 5 min. The gel was treated with 5 M urea in a 1:1 proportion and centrifuged. The supernatant after affinity adsorption and the urea fraction were dialysed and analysed. Sephadex was also studied in a related way. Four milligrams of crude protein was added to 0.89 g of Sephadex G-200 in 50 ml of 50 mM phosphate–25 mM citrate buffer. The mixture was agitated on a magnetic stirrer for 2 h at 4 °C.

### 2.9. Protein and enzyme assays

Dextranase activity was measured with a method based on release of Remazol Blue R-250 dye from Sephadex G-150 (Blue Sephadex) as a result from the enzyme action [10]. A mixture of appropriately diluted enzyme (0.1 ml) and 0.5 ml of 50 mM sodium phosphate–25 mM sodium citrate buffer, pH 6.8 (25 °C), was added to a 0.4 ml of Blue Sephadex suspension (0.5% (w/v) in 50 mM sodium phosphate–25 mM sodium citrate, pH 6.8). The mixture was incubated at 50 °C for 15 min and centrifuged for 2 min at 10,000 × *g*. The absorbance of the supernatant was then measured at 595 nm. One unit of dextranase activity was defined as the amount of enzyme releasing 1 μg of the dye coupled to soluble dextrins per minute at 50 °C, considering that the absorbance value of 0.0312 ($A_{595\,nm}$) was equivalent to 1 μg/ml of Remazol blue R-250 [10]. The color of

Remazol blue was pH-independent in the pH range of 5–9.

Protein concentrations (mg/ml) were calculated by measuring absorbance at 260 nm and 280 nm followed by using the formula $1.55 \times A_{280} - 0.76 \times A_{260}$ according to Layne [11]. Bovine serum albumin was used as the standard.

### 2.10. PAGE and isoelectric focusing

Protein samples were separated on 8% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) gels according to Laemmli [12] with some modifications using a Protein II Mini-Cell device (BioRad, USA) followed by Coomassie Brilliant Blue staining. Dextranase activity was also analysed on an 8% acrylamide resolving gels containing 0.4% Blue Dextran (BD-SDS–PAGE) [13]. Dextranase activity was detected as white bands after incubating the blue gel at room temperature for 1 h in 20 mM Tris–HCl, pH 6.8, containing 0.5% Triton X-100, followed by a 2 h incubation at 37 °C in 50 mM sodium phosphate, pH 6.8. For nondenaturing conditions, the proteins were electrophoresed in 7.5–10% acrylamide gels [14].

Isoelectric focusing (IEF) was performed with a Pharmacia PhastGel System on precast acrylamide gels for an IEF range of pI 3–9. Protein Mixture pH 3–10 (Pharmacia, Sweden) was used as the pI standards. Enzymatic activities after IEF runs were estimated as follows: 8% acrylamide gel containing 0.4% Blue Dextran in 50 mM sodium phosphate, pH 6.8, was applied as a thin layer onto the unstained gels. After incubation at 37 °C for 1–2 h, white bands appeared. Their positions were correlated with the gels cut from the same electrophoresis runs but stained conventionally with protein dyes (Coomassie or silver stains).

### 2.11. N-terminal amino acid analysis

Dextranase bands were blotted from SDS–PAGE gel onto polyvinylidene difluoride membrane and were stained with Coomassie Brilliant Blue. Three protein bands which migrated at estimated sizes of 76, 89 and 110 kDa were excised and individually subjected to sequence analysis using Applied Biosystem Model 470 gas-phase protein sequencer equipped with an Applied Biosystems Model 120A PTH analyser.

## 3. Results

### 3.1. Isolation and identification of dextranase-producing microorganisms

Screening of more than 75 soil samples resulted in isolation of 23 bacterial strains with the ability to generate white halos on Blue Dextran on Petri dishes. The dextran-degrading strains were gram-positive motile rods, formed endospores and grew only under aerobic conditions. All the isolated strains had rather similar physiological and morphological properties: catalase (+); Voges-Proskauer test (−); fermentation of glucose, arabinose, mannitol, xylose and starch (+), liquefaction of gelatin (±), hydrolysis of casein (−), utilising of citrate (−), degradation of tyrosine and phenylalanine (−), egg yolk reaction (−), reduction of nitrate (+), formation of indol (−), requirement of urease (−), growth at pH 5.7 (±), growth at 5–7% of NaCl (−) and growth at 5–10 °C and 50–65 °C (−). The isolated strains were identified as *Bacillus circulans* in the genus *Bacillus* [15,16] and more precisely by 16sRNA analysis as *Paenibacillus illinoisensis*.

### 3.2. General purification strategy of dextranases

Dextranase is an industrially used enzyme and therefore purifications steps which can be normally easily scaled up were preferred.

While dextran is the substrate of dextranase, it also forms two-phase systems with PEG. Conceivably, it could be possible to purify dextranase by affinity adsorption either to a solid or liquid dextran phase. Sephadex (a cross-linked dextran) and dextran bound to agarose were studied in batch conditions. Only weak adsorption of dextranase from buffer solutions occurred with Sephadex G-200. Sepharose–dextran adsorbed up to 50% of activity in similar conditions but only 5% activity was recovered from 5 M urea detachment solution due to denaturation of the enzyme. Affinity extraction in phase systems composed of PEG 6000–Dextran 500 kDa showed a protein partition coefficient of 0.3. No dextranase activity was found in the upper phase. Enzyme could be detached from dextran with 5% trichloroacetic acid with 80% recov-

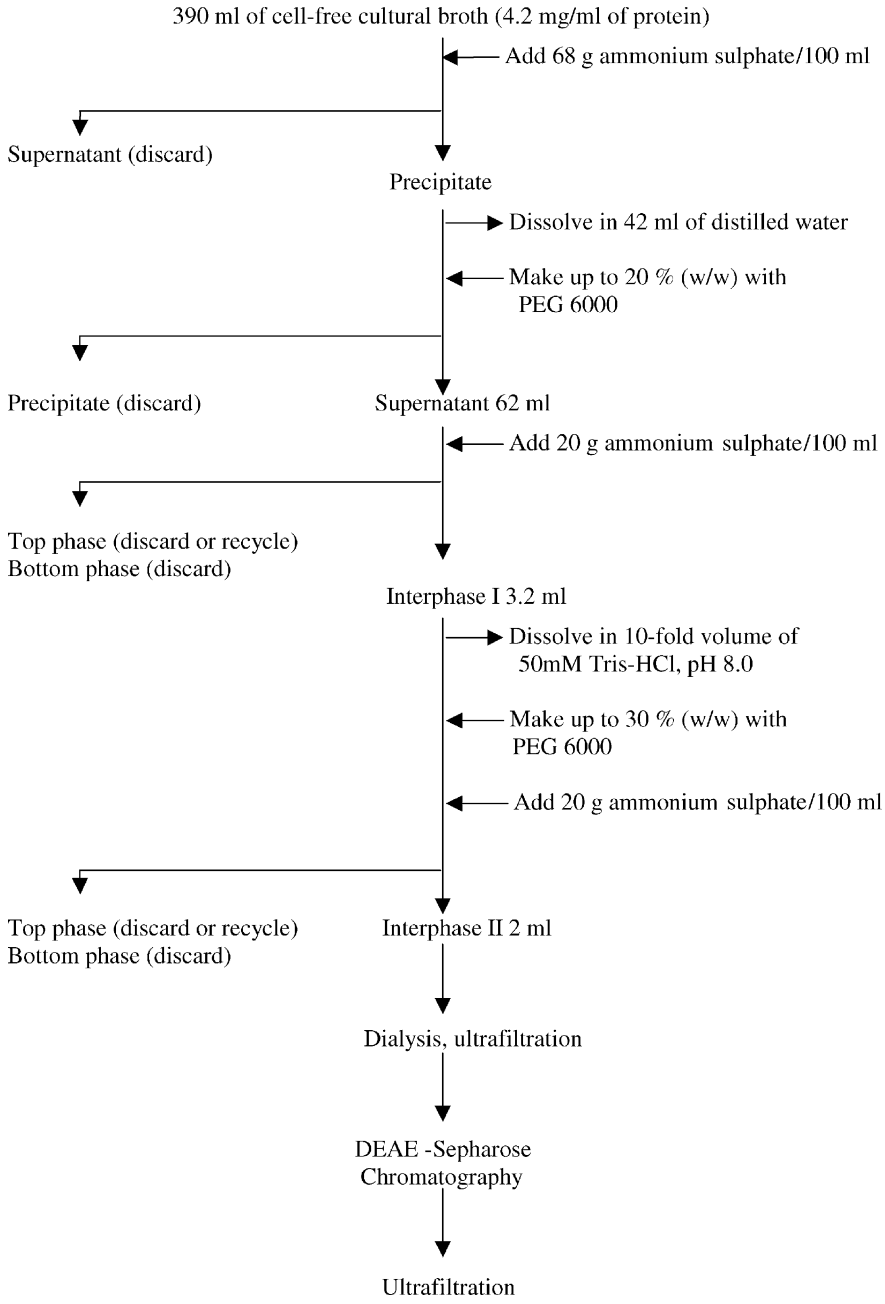


Fig. 1. Scheme for purification of dextranase.

ery but only 1.7-fold purification was obtained. None of these affinity systems was found adequately effective and robust for a large-scale usage. A special additional drawback is the risk of hydrolysis of dextran during the procedures. Therefore, the affinity step was not included in our purification protocol. The purification method was essentially based on precipitation and two-phase extraction, and the final purification

Table 1
Summary of the procedure for purification of dextranase from culture supernatant of a *Bacillus* sp.

| Purification step (ml) | Volume (ml) | Total protein (mg) | Total activity (U) | Specific activity (U/mg) | Yield (%) | Purification factor |
|---|---|---|---|---|---|---|
| Culture supernatant | 390 | 1638 | 175000 | 107 | 100 | 1 |
| Ammonium sulphate precipitation | 42 | 595 | 163000 | 270 | 93 | 3 |
| 20% (w/w) PEG 6000 supernatant | 62 | 168 | 151000 | 900 | 86 | 8 |
| Interphase I | 3 | 25 | 91000 | 3600 | 52 | 34 |
| Interphase II | 2 | 11 | 57500 | 5200 | 33 | 49 |
| Dialysis, ultrafiltration | 4 | 3.6 | 42500 | 11800 | 24 | 110 |
| DEAE–Sepharose chromatography | 16 | 0.43 | 33700 | 78500 | 19 | 733 |

was obtained with DEAE–Sepharose chromatography. The selected procedure is presented in Fig. 1.

### 3.3. Ammonium sulphate precipitation

Dextranase from the culture broth was initially concentrated by $(NH_4)_2SO_4$. Recovery of 93% was obtained at 68 g/100 ml while the purification of the enzyme was around 2–3-fold (Table 1). Allowing a lower recovery did not essentially improve the purification. Enzyme was fully stable when stored in ammonium sulphate precipitate for more than 1 year in a refrigerator.

### 3.4. PEG fractionation

For finding out the optimal conditions for purification of dextranase with PEG precipitations, analytical

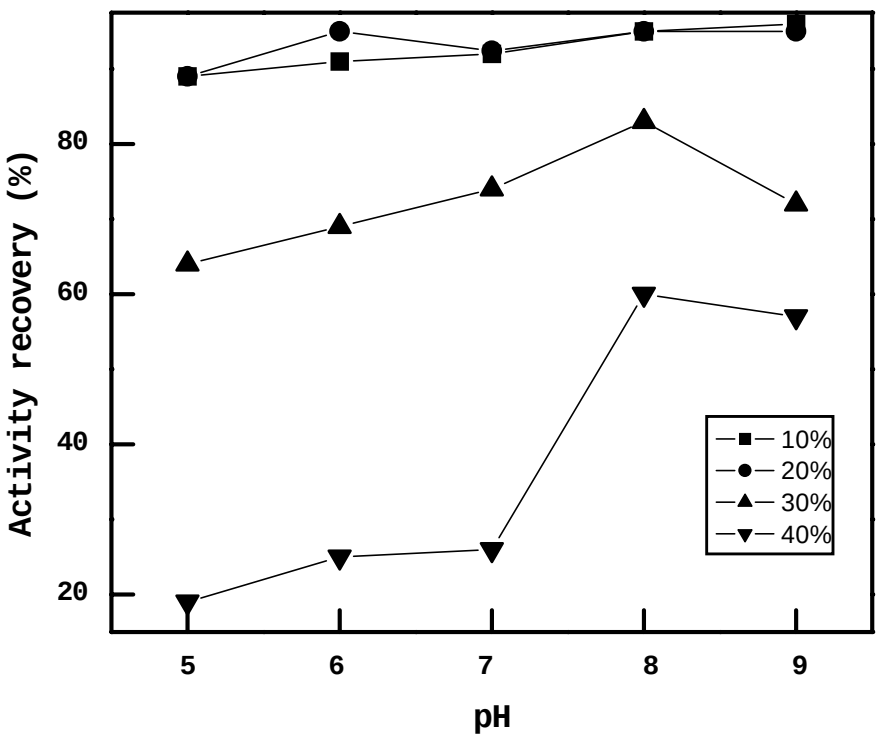


Fig. 2. Effects of pH on the activity recovery of dextranase (% of total) in supernatant of PEG 6000 at concentrations of 10, 20, 30 and 40%.

partitioning experiments were performed. Solubility of dextranase in PEG 4000, 6000, or 8000 was fairly similar and complete until about 20% (w/w) PEG, whereas the solubility in PEG 400 extends to about 50%. The purification factor was at its optimum around 20% of PEGs 4000–8000. Low viscosity of PEG 400 is convenient in practical work, but the purification factor was lower with it than with the other studied PEGs, and high amounts of PEG 400 (30–40%) are needed. Changing pH between 5 and 9 slightly affected the purification of dextranase with PEGs at concentrations 10–30% (w/w). The purification factor was from 1.8 to 3.5, with a tendency of being slightly higher at pH 8–9. The activity recovery varied from 89 to 95% for 10–20% PEG 6000 concentrations and from 64 to 83% for 30% PEG 6000.

The pH significantly affected the partitioning at 40% concentration of PEG 6000 (Fig. 2). The purification factor increased from 0.9 to 2.2 and activity recovery from 19 to 57% with increasing pH from 5–7 to 8–9. It is not conceivable that the net charge of the enzyme is changing drastically between pH 7 and 8, and therefore the most probable explanation is that Tris is solubilizing the proteins better than phosphate–citrate. This solubilization slightly favours dextranase over the other proteins. An analogous behaviour was found with the other high-molecular-weight PEGs. With PEG 400, related effects appeared at around 60% of ammonium sulphate.

PEG 4000, 6000 and 8000 did not differ essentially in the PEG fractionation. However, in the next, more effective step, PEG 6000 was slightly better than the other PEGs. To keep the total process simple, PEG 6000 was chosen. In these conditions, 93% recovery with 3.3-fold purification was obtained (Table 1).

### 3.5. Interphase adsorption

A two-phase system was created by taking the supernatant from the previous PEG 6000-fractionation

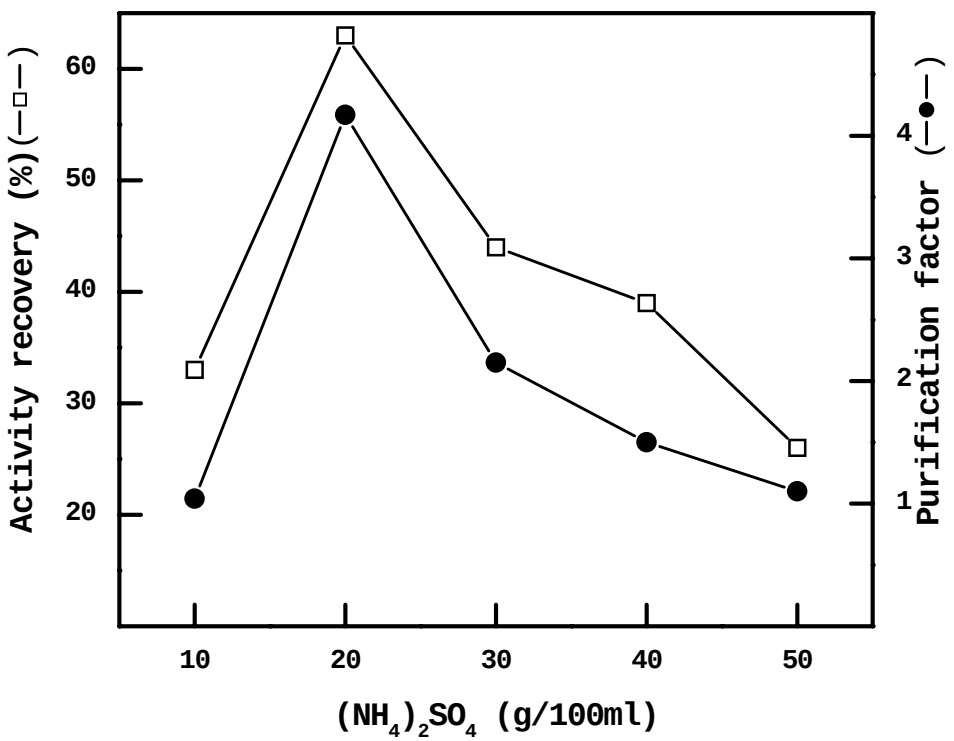


Fig. 3. Effects of $(NH_4)_2SO_4$ on the activity recovery (% of total) and purification factor of dextranase in the interphase of 20% (w/w) PEG 6000–$(NH_4)_2SO_4$ system in Tris–HCl, pH 8.0, at 20 °C.

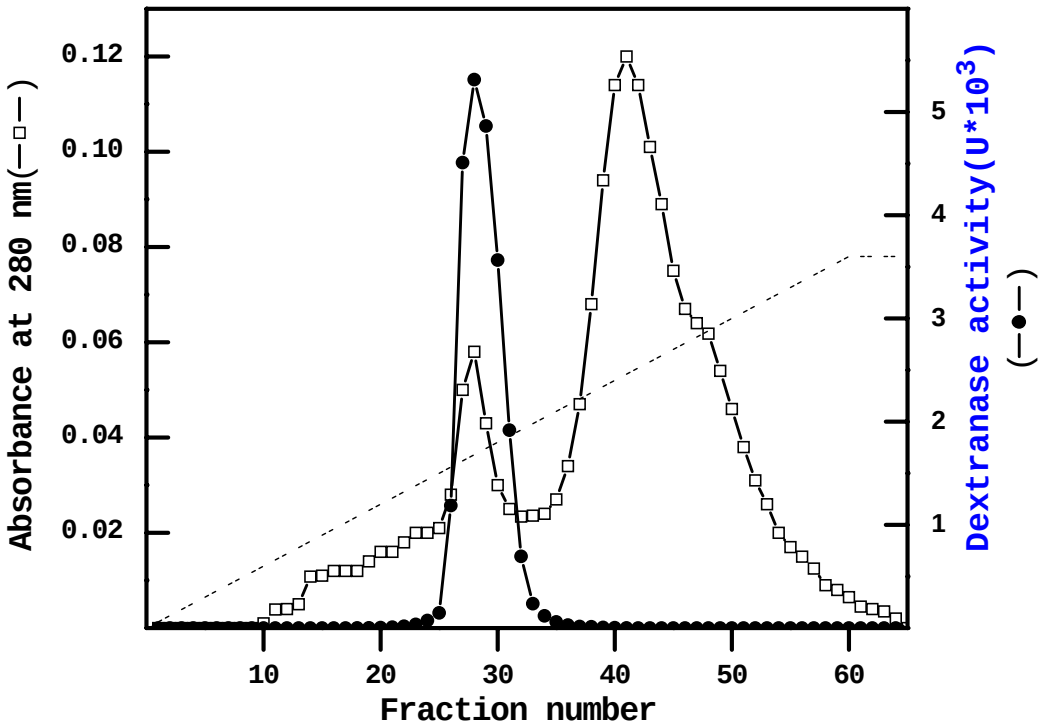


Fig. 4. Elution profiles for protein and dextranase activity on DEAE anion exchange chromatography. The absorbance values at 280 nm (protein) were redrawn from the recorder chart. Fractions of 2 ml were collected and measured for activity. The dashed line depicts the salt gradient (0–360 mM).

step by adding $(NH_4)_2SO_4$ from 10 to 50 g/100 ml. At 20% concentration, 60% of dextranase activity occurred in the interphase whilst the rest was in the upper PEG-phase. Thirty percent of the activity also occurred in the lower phase when 10% of $(NH_4)_2SO_4$ was used. When its concentration was increased to over 20%, the recovery decreased in the interphase and increased in the upper phase at the same time as the specific activity in the interphase decreased (Fig. 3). The optimum of 20% of $(NH_4)_2SO_4$ was chosen for further use. Repeating the interphase extraction in a slightly different way, the purification factor for the extraction was 5–6-fold (Table 1). The precipitations and extractions yielded the overall purification of about 50-fold from culture broth, with a recovery of 33% (Table 1).

### 3.6. DEAE–Sepharose chromatography

Preliminary experiments in batch conditions showed that dextranase is bound to DEAE–Sepharose at pH's above 7. To maintain the full capacity of the weak anion exchanger and relatively strong adsorption conditions (pI of dextranase 4–5), pH 8.0 was chosen for the chromatography. With a linear gradient from 0 to 360 mM NaCl, a single dextranase activity peak was detected (Fig. 4). Dextranase was thereby purified 733-fold with a recovery of 19% (Table 1). Surprisingly, the resolution of the DEAE–chromatography was not high enough for separation of the isoforms, although their isoelectric points differed by almost 1 pH unit. Attempts to use different NaCl gradients and pH's for resolving the isoforms failed.

### 3.7. Enzymatic properties of the dextranase

The pH optimum of the highly purified dextranase was broad, from 6.5 to 7.8 (Fig. 5). The activity appeared to be sensitive to the buffers used and apparent discontinuities occurred when individual buffers were applied separately. Fig. 5 suggested that even 2 or 3

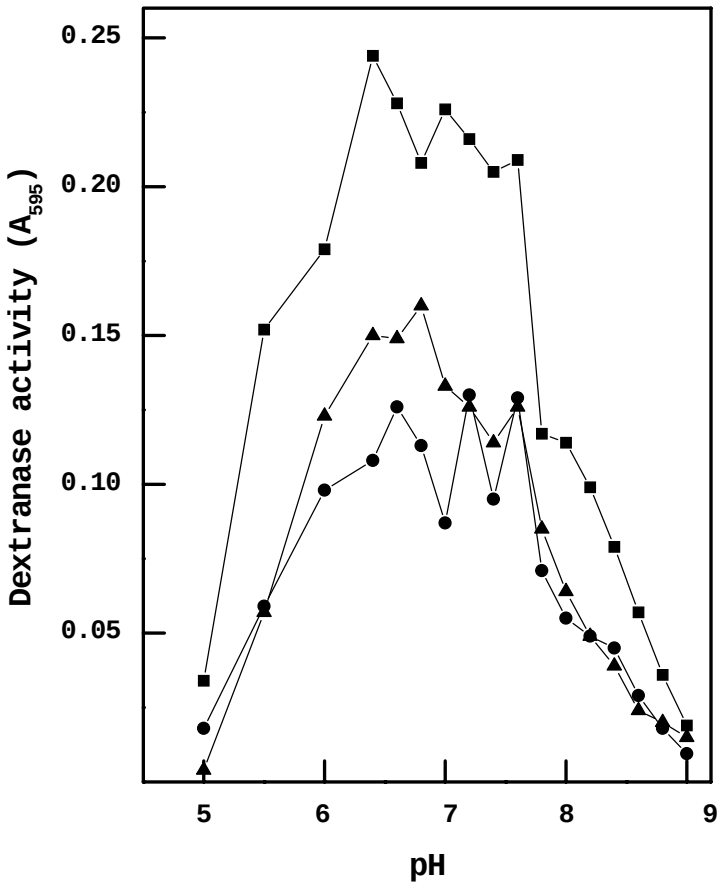


Fig. 5. The pH profile of dextranase activity (including three isoforms, see Fig. 7) was determined in a mixture of citric acid, phoshate, Tris base and glycin (final concentration of each buffer was 50 mM) in NaCl (final concentration 100 mM) by adjusting the pH with 1 M NaOH to a desired pH. Sephadex Blue G-150 substrate was dissolved in 0.9 ml of buffer of an indicated pH value to give a concentration of 0.5% (w/v). After the reaction, the mixture was centrifuged and the absorption was measured as in the standard assay. The color of Remazol Blue was pH-independent in the pH range of 5–9. The reaction was performed at 25 °C for 2 h (upper curve), 50 °C for 15 min (middle) and 25 °C for 1.5 h (lowest curve).

pH optima may occur. Because the enzyme contained three isoforms, their possible sensitivities to variations in the reaction conditions were searched for with changing the temperatures of incubation (Fig. 5). However, no clear conclusion can be drawn because the activity was buffer-dependent and because the principle of the assay method obviously involves limiting mass transfer steps, and further studies are necessary. The apparent temperature optimum was 50 °C as measured at pH 6.8, with the incubation time of 15 min (Fig. 6). The enzyme was stable at temperatures lower than 50 °C (Fig. 6) in the pH range of 5–9.

Homogeneity of dextranase was assessed with SDS–PAGE and BD-SDS–PAGE, as well as isoelectric focusing. The final purified enzymatic preparation was composed of three active bands with molecular masses of 110, 89 and 76 kDa (Fig. 7). The relative intensities of the protein and activity bands were approximately 1:0.5:0.25. Three bands with similar molecular weights were observed after separation of the proteins on non-denaturing gels conditions also. Isoelectric focusing (with protein and activity stainings) of dextranase showed isoelectric points of 4.0 (110 kDa), 4.2 (89 kDa) and 4.95 (76 kDa). The correlation with

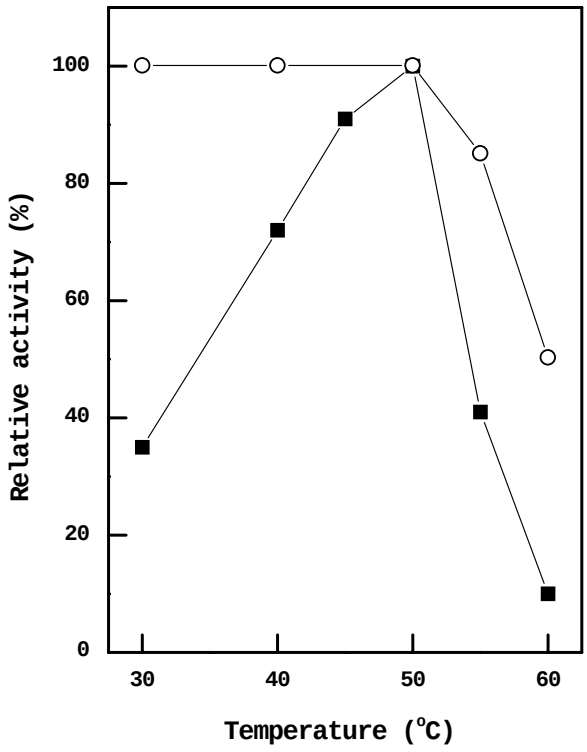


Fig. 6. Effect of temperature on activity and stability of dextranase. The apparent temperature optimum (■) of the enzyme was carried out by measuring the enzyme reaction for 15 min at temperatures between 30 and 60 °C in the citrate–phosphate assay buffer, pH 6.8 (calibrated at 25 °C). To study the thermal stability (○), the enzyme was incubated for 60 min at pH 6.8 at temperatures 30–60 °C, and then the remaining activity was assayed by the standard method.

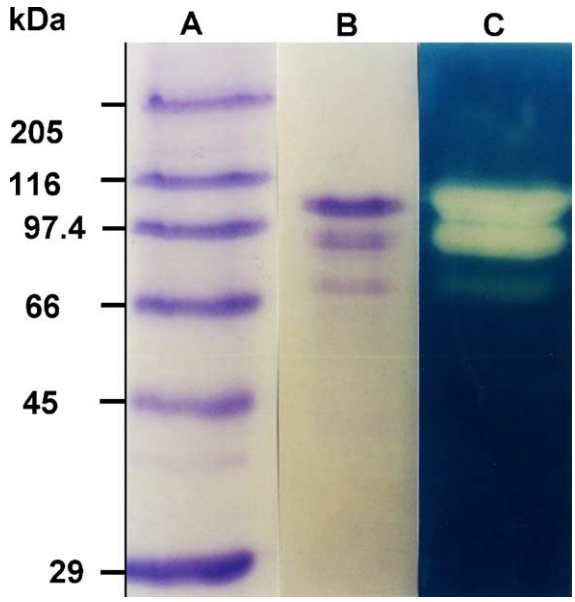


Fig. 7. Electrophoretic analysis of a protein sample after DEAE–Sepharose chromatography. Lane A represents SDS–PAGE standards of indicated molecular masses (on the left side). Lane B is protein sample (20 μg) stained with Coomassie Blue. A sample of 2 μg of protein was loaded on Lane C and electrophoresed with BD–SDS–PAGE (see Section 2). The protein was renatured and stained for dextranase activity. Activity was shown as a white band on blue background.

the pI and molecular masses were deduced on the basis of the intensities of the activity and protein bands. Samples from different purification stages of the enzyme showed approximately similar relative intensities of the isoforms. At least the first five N-terminal amino acids appeared to be identical (Ala–Ser–Thr–Gly–Lys) and unique among known dextranases.

## 4. Discussion

### 4.1. Purification protocol

Cells were removed with centrifugation and dextranase was precipitated from culture broth with $(NH_4)_2SO_4$ to get stable concentrated enzyme. In a large-scale process, these steps can be conveniently replaced by microfiltration and ultrafiltration, respectively. Dextranase was fairly soluble in PEG solutions, and a further purification was achieved by precipitating other proteins out from the supernatant. Adding $(NH_4)_2SO_4$ to this supernatant created a two-phase system resulting in purification with concentrating dextranase into the interphase, from where it was possible to collect with about 60% recovery. With once-repeated extraction in slightly different conditions, it was possible to obtain 49-fold purification with 33% recovery, calculated from the culture broth. The procedure of precipitation and two-phase extraction could be suitable for obtaining technical grade dextranase in a large scale. By proper equipment, even distinctly higher purification could be obtained by the interphase counter-current distribution [17]. The precipitations and two-phase extractions can be normally scaled up relatively easily. When the non-enzyme protein content in the preparation is sig-

nificantly reduced (as in Table 1, to 1/50 part), the chromatographic step is more feasible in a bulk scale.

Previously, a thermostable dextranase has been purified 25-fold by adsorption onto Sephacryl S-300 [18]. Dextran itself in an aqueous ethanol was used to adsorb dextranase with a 9-fold increase of specific activity [19]. We purified cyclodextrin glucanotransferase 100-fold in the scale of cubic metres by adsorption onto a Sepharose derivatized with the enzyme substrates at capacity of 30–40 g of enzyme absorbed onto 1 l of the sorbent [20]. However, the adsorption had to be carried out near 0 °C to prevent hydrolysis of the affinity ligands, and also because the low temperature favours affinity [21]. The positive results prompted us to study adsorption of dextranase onto Sephadex G-200 and onto Dextran 67 kDa coupled to Sepharose-CL-4B. Undoubtedly, affinity adsorption, especially to Sepharose–dextran, can be used successfully for purification of dextranase if an improved detachment from the sorbent could be solved. However, the adsorption capacity should be high enough to justify the use of expensive materials, and the purification must produce a highly purified enzyme with good recovery. Considering the risk of hydrolysis of the affinity matrix, the need for separation of substrate and enzyme after the purification and the relatively poor separation results, we concluded that affinity extraction or affinity adsorption are not favourable techniques for dextranase in a large scale.

A variety of conventional methods have been reported for the purification of dextranases from culture broths of microbes such as *Aspergillus*, *Chaetomium*, *Fusarium*, *Lipomyces*, *Paecilomyces*, *Penicillium*, *Arthrobacter* and *Streptococcus*. The number of steps and purification factors depend, as a rule, on the producer strain, composition of cultural broth and the desired use of the enzyme. Majority of the methods used $(NH_4)_2SO_4$ [2,22,23] or organic solvents [5] to precipitate proteins in the culture medium. Further purification involved a combination of chromatographic steps. The purification factor achieved ranged from 4 [24] to 202 [25].

### 4.2. Question of the dextranase isoforms

Multiple forms of dextranase have been reported for *S. mutans* and *S. sorbinus* [26,27]. Dextranase of *S. mutans* produced extracellular dextranases of 96, 108 and 167 kDa [26]. Dextranases of a recombinant *E. coli* had molecular masses from 160 to 260 kDa [27]. *Prevotella oralis* and *P. melaninogenica* also excreted multiple dextranases [28]. The polymorphism may be explained by endo- or extracellular proteolytic processing of dextranase. Igarashi et al. successfully inhibited breakdown of the 120 kDa enzyme in the culture fluids of *S. mutans* with a protease inhibitor [29]. Two active fractions have been obtained from culture broth of *Penicillium funiculosum* [30], as well as from that of *Aspergillus carneus* [2]. The molecular masses of *Penicillium notatum* dextranases were exceptionally low, 56 and 50 kDa [31].

While we have shown that *Bacillus* sp. has three isoforms, we did not find any significant changes in the activity pattern after cultivation of the bacterium in the presence of protease inhibitors PMSF and Pepstatin A at their different concentrations. Also, during various independent cultivations, the activity pattern remained unchanged. N-terminal parts of the three dextranases were identical. Thus, if the isoforms were a result of proteolysis, it must have taken place at the C-termini without any significant loss of activity. Such a cleavage is slightly implied by the lower activities (and smaller protein bands) in the smaller-molecular-mass dextranases, i.e. the largest form (110 kDa) could be sequentially cleaved to 89 and then to 76 kDa. For further studies of the formation of isoforms, immobiline electrofocusing [20] and C-terminal sequencing may be informative. The reason for abundance and remarkable variation of the dextranase isoforms among fungi and bacteria remains to be elucidated.

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