

# Purified Dextranucrase from *Leuconostoc mesenteroides* NRRL B-640 Exists as Single Homogeneous Protein: Analysis by Non-denaturing Native-PAGE

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## Citation

R Purama, A Goyal. *Purified Dextranucrase from Leuconostoc mesenteroides NRRL B-640 Exists as Single Homogeneous Protein: Analysis by Non-denaturing Native-PAGE*. The Internet Journal of Microbiology. 2008 Volume 6 Number 1.

## Abstract

The extracellular dextranucrase from *Leuconostoc mesenteroides* NRRL B-640 was purified using polyethylene glycol (PEG-400) fractionation. A 25% (v/v) PEG-400 concentration gave dextranucrase with maximum specific activity of 9.2 U/mg with 16 fold purification in a single step. The purified enzyme by PEG-400 showed multiple protein bands on SDS-PAGE with one prominent band corresponding to the size 180 kDa (12). However, the same PEG-400 fractionated dextranucrase samples showed single, intact and homogeneous band when analyzed on non-denaturing native-PAGE. This showed that dextranucrase remains in single molecular form in the native state and shows multiple forms only under denaturing conditions when it is heated before loading and when it contained SDS or 2-mercaptoethanol.

## INTRODUCTION

*Leuconostoc mesenteroides* NRRL B-640 is known to produce extracellular dextranucrase that gives highly linear dextran (1,2). The linear dextran is water soluble and has numerous applications in pharmaceutical, food and fine chemical industries (3). Several attempts have been made to enhance the production of dextranucrase from *Leuconostoc mesenteroides* NRRL B-640 (4,5,6,7,8,9). Various methods have been reported for purification of dextranucrase. The purification methods such as salt, glycerol and alcohol precipitation, fractionation by polyethylene glycol, ultra-filtration, chromatography and phase-partitioning have been standardized and successfully used for purification of dextranucrase from different strains (10). Among various purification methods, polyethylene glycol (PEG) fractionation is a simple and rapid method for dextranucrase purification (11,12,13). Polyethylene glycols are nonionic hydrophilic polymers and are known to selectively precipitate proteins. They also have an advantage of being readily removed by dialysis. The purification by two-phase partitioning using different molecular weight PEGs have also been reported for dextranucrase from various strains of *Leuconostoc mesenteroides* (14,15,16,17).

The extracellular dextranucrase from *Leuconostoc mesenteroides* NRRL B-640 was purified using polyethylene glycol fractionation PEG 400. The purified dextranucrase

fractions confirmed their presence by synthesis of dextran, when run on non-denaturing SDS-PAGE gels for in-situ activity detection by Periodic Acid Schiff staining and the activity bands corresponded to the native and active form of the purified dextranucrase of approximately, 180 kDa molecular size, that appeared on the identical denaturing SDS-PAGE gels stained with Coomassie Brilliant Blue (12). The purified enzyme by PEG-400 showed multiple protein bands on SDS-PAGE with one prominent band corresponding to 180 kDa (12). In the present study the native state of purified enzyme by PEG-400 fractionation was analyzed by non-denaturing native polyacrylamide gel electrophoresis.

## MATERIALS AND METHODS

### THE MICROORGANISM AND REAGENTS

*Leuconostoc mesenteroides* NRRL B-640 was procured from Agricultural Research Service (ARS-Culture collection), USDA, Peoria, USA. The culture was maintained in modified MRS (sucrose in place of glucose) agar medium (18) as a stab at 4°C and sub cultured every 2 weeks. The ingredients required for the maintenance and enzyme production media were from Hi-Media Pvt. Ltd., India. All the chemicals required for reducing sugar estimation, protein estimation and buffer preparation were of highest purity grade commercially available. PEG400

(Ranbaxy Pvt. Ltd., India) was used for fractionation of dextranucrase.

## **PRODUCTION OF DEXTRANSUCRASE**

For enzyme production a loop of culture from modified MRS agar stab was transferred to 5 ml of sterile medium as described by Tsuchiya et al., 1952<sup>(19)</sup>. The cultures were grown at 25°C with 200 rpm for 12-16h. 1% of the culture inoculum was used for the enzyme production from *Leuconostoc mesenteroides* NRRL B-640. The culture broth was centrifuged at 10,000g for 10 min at 4°C to separate the cells. The cell free supernatant was analyzed for enzyme's activity and concentration.

## **DEXTRANSUCRASE ASSAY**

The assay of dextranucrase was carried out in 1 ml of a reaction mixture in 20 mM sodium acetate buffer, pH 5.4, containing 146 mM (5%) sucrose and using the cell free supernatant (10- 20 µl) for the enzyme source. The reaction mixture was incubated at 30°C for 15 min. The enzyme activity was measured by estimating the liberated reducing sugar<sup>(20,21)</sup>. Aliquots (0.2 ml), from the reaction mixture were analyzed for reducing sugar concentration. The absorbance was measured at 500 nm using a UV-visible spectrophotometer (Cary100 Bio, Varian Inc., USA) against a blank with D-fructose as a standard. One unit (U) of dextranucrase activity is defined as the amount of enzyme that liberates 1 µmol of reducing sugar per min at 30°C in 20 mM sodium acetate buffer, pH 5.4. The total protein content of the cell free supernatant was estimated by the method of Lowry et al. 1951<sup>(22)</sup>. Bovine serum albumin ranging from 25µg/ml to 500µg/ml concentration was used as a reference to plot a standard curve.

## **PURIFICATION OF DEXTRANSUCRASE BY PEG FRACTIONATION**

The polyethylene glycol PEG-400 pre chilled to 0°C was added to 200 ml cell free supernatant to obtain the final concentrations of 20, 25, 33, 40 and 50 (% v/v). The mixture was incubated for 12h at 4°C to allow the dextranucrase to precipitate. The mixture was centrifuged at 12,000g for 30 min at 4°C to separate the fractionated dextranucrase. The enzyme pellet was dissolved in 20 mM sodium acetate buffer (pH 5.4). These fractions were analyzed for dextranucrase activity and protein concentration and subjected to dialysis using 5 kDa cutoff membrane.

## **NON-DENATURING NATIVE -PAGE ANALYSIS OF PURIFIED ENZYME**

The non-denaturing native-polyacrylamide gel electrophoresis was performed with a vertical slab mini gel unit (BioRad, USA) using 1.5 mm thick gels, following the method described elsewhere<sup>(12)</sup> using only 7.5% (w/v) acrylamide resolving gel prepared in 0.125 M Tris-HCl pH 8.8. The protein samples were prepared in 0.0625 M Tris-HCl buffer (pH 6.8) containing glycerol, 5% (w/v) and 0.05% (w/v) bromophenol blue but with out 2-mercaptoethanol and SDS. The enzyme samples from all PEG-400 purified fractions were loaded on 7.5% acrylamide gel without heating. The electrophoresis was carried out using Tris-Glycine buffer (0.025M Tris and 0.192 M Glycine) pH 8.3 with a current of 2mA per lane. The protein bands were fixed with solution containing acetic acid (5%, v/v) for 5 min, then stained for 30 min with 0.25% (w/v) Coomassie Brilliant Blue, and destained by repeated washing using a solution containing 20% methanol and 10% (v/v) acetic acid. Although for native-PAGE markers are not used but the molecular mass marker (Myosin from Rabbit Muscle, 205000; Phosphorylase b 97400; Bovine serum albumin, 66000; Ovalbumin, 43000; Carbonic anhydrase, 29000 Da purchased from Genei, India) along with BSA sample with out boiling were added to the gel for reference.

## **RESULTS AND DISCUSSION**

### **DEXTRANSUCRASE FRACTIONATION BY PEG 400**

The culture broth obtained after 12 h growth of *Leuconostoc mesenteroides* NRRL B-640 was centrifuged and the cells were discarded. Dextranucrase purification by fractionation using PEG-400 ranging from 20 to 50% (v/v) was carried out from the cell free extract with a specific activity of 0.6 U/mg. The fractionation by 25% (v/v) PEG-400 gave the maximum specific activity of 9.2 U/mg with 16 fold purification and 3.8% overall yield in a single step (Table 1). The purification by higher concentrations of PEG-400 did not favor the enzyme activity and the increase in the PEG-400 concentration beyond 25% resulted in decrease of specific activity of dextranucrase (Table 1).

**Figure 1**

Table 1: Purification of dextransucrase by PEG-400

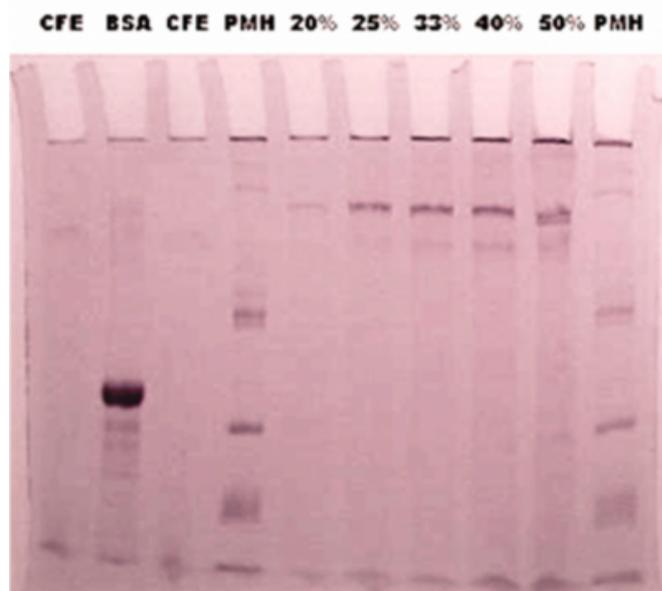
PEG-400 (%)	Volume (ml)	Enzyme activity (U/ml)	Total Units	Overall % Yield	Protein (mg/ml)	Total mg	Specific activity (U/mg)	Fold Purification
0	200.0	4.2	848.0	-	7.4	1474.0	0.6	-
20	5.3	3.8	20.0	2.4	0.58	3.1	6.5	11.3
25	5.8	5.5	32.1	3.8	0.60	3.5	9.2	16.0
33	7.8	3.4	26.1	3.1	0.61	4.8	5.5	9.5
40	10.8	2.2	23.5	2.8	0.65	7.0	3.4	5.8
50	15.9	1.7	27.3	3.2	0.76	12.1	2.3	3.9

### ANALYSIS OF PURIFICATION BY NON DENATURING NATIVE-PAGE

The dextransucrase samples obtained with PEG-400 fractionation were analyzed by SDS-PAGE to check the purity of dextransucrase. The results showed the presence of multiple protein bands however there was a prominent protein band of approximately 180 kDa molecular size obtained from all concentrations of PEG-400 as shown earlier (12). From SDS-PAGE results dextransucrase appeared to exist in multiple molecular forms, however, the same samples showed predominantly a single, intact and homogeneous protein band on non-denaturing native-PAGE (Fig. 3). This showed that dextransucrase remains in native state after it is purified and dialysed and shows multiple forms only in under denaturing conditions when enzyme sample was heated before loading and when it contained 2-mercaptoethanol. The non-denaturing native-PAGE of dextransucrase samples purified by higher than optimum concentration of 25% PEG-400 showed presence of other contaminating non-dextransucrase protein(s) with increasing protein band intensities (Fig. 3) which were not visible in SDS-PAGE, owing to low protein content and being denatured or proteolysed under denaturing conditions (12). The native-PAGE revealed that a concentration of PEG-400 lower than optimum (25%) fails to fractionate most of dextransucrase and so specific activity was low and the higher concentrations of PEG-400 fractionated also other proteins which accounted for decreased specific activity of dextransucrase (Table 1),

**Figure 2**

Figure 1: Non denaturing native -PAGE analysis of dextransucrase purified by PEG-400. (Lanes: CFE- cell free supernatant; BSA-Bovine serum albumin; PMH- Protein Marker High Mol. wt. 29- 205 kDa; 20%; 25%; 33%; 40% and 50% (v/v) PEG-400 fractions.



### ACKNOWLEDGEMENTS

The authors thank Indian Institute of Technology Guwahati, Guwahati, India, for providing experimental facilities and Ministry of Human Resource Development, Government of India, for providing a PhD fellowship to RKP.

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