PURIFICATION AND CHARACTERIZATION OF DEXTRANSUCRASE FROM \textit{LEUCONOSTOC MESENTEROIDES} NRRL B-1149

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ABSTRACT

\textit{Leuconostoc mesenteroides} NRRL B-1149 produces extracellular dextransucrase which in this study was purified using different concentrations of polyethylene glycol (PEG). The dextran produced by this enzyme is unique in that it contains \(\alpha-(1\rightarrow6)\) and \(\alpha-(1\rightarrow3)\) linkages which have clinical applications. The cell free supernatant with 0.9 U/mg enzyme specific activity was subjected to fractionation by PEG-400 and PEG-1500. The 33% PEG-400 gave dextransucrase with specific activity of 9.2 U/mg and 10 fold purification and the 15% PEG-1500 gave dextransucrase with maximum specific activity of 15 U/mg and 17 fold purification in a single step. The purified enzyme showed multiple molecular forms on denaturing SDS-PAGE with three prominent bands. The purified dextransucrase confirmed the presence of glucan, after in-situ activity detection by Periodic acid Schiff’s staining after running under denaturing SDS-PAGE. The three bands that appeared on denaturing SDS-PAGE stained with silver nitrate solution, corresponded to the three activity bands.

Keywords: dextran, dextransucrase, glucansucrase, \textit{Leuconostoc mesenteroides} NRRL B-1149

Introduction

\textit{Leuconostoc mesenteroides} produces an extracellular glucansucrase. Glucansucrase catalyse the synthesis of a variety of glucans containing mostly \(\alpha-(1\rightarrow6)\), \(\alpha-(1\rightarrow3)\), \(\alpha-(1\rightarrow4)\), and \(\alpha-(1\rightarrow2)\) linked D-glucosyl units (11, 14, 20). Depending on the structure of glucans, they are of three types viz. dextran, mutan and alternan. Dextranucrase produces a glucan which contains at least 50% of \(\alpha-(1\rightarrow6)\) osidic bonds within the main chain (1, 19, 24). Mutansucrase produces a water-insoluble glucan containing more than 50% of \(\alpha-(1\rightarrow3)\) glucosidic linkages, mainly associated with \(\alpha-(1\rightarrow6)\) linkages. This enzyme is produced by \textit{L. mesenteroides} NRRL B-523, B-1149 and several \textit{Streptococcus} strains (9, 13, 20). Alternansucrase synthesizes the alternan, which contains alternating \(\alpha-(1\rightarrow6)\) and \(\alpha-(1\rightarrow3)\) glucosidic linkages, with some degree of \(\alpha-(1\rightarrow3)\) branchings. The enzyme glucansucrase belongs to the family 70 except from amylosucrase which belongs to family 13 of glycoside hydrolases (4, 12, 14).

Several methods have been applied for efficient purification of dextransucrase as fractionation by polyethylene glycol, ultra-filtration, salt, glycerol and alcohol precipitation, chromatography and phase-partitioning. Polyethylene glycol mediated dextransucrase precipitation is known as an efficient method for the purification of dextransucrase from cell free supernatant (2, 8, 17, 18).

\textit{Leuconostoc mesenteroides} NRRL B-1149 is known to produce branched dextran with 52% \(\alpha-(1\rightarrow6)\) and 40% \(\alpha-(1\rightarrow3)\) linkages. Low dextran solubility in water is normally associated with the presence of large numbers of \(\alpha-(1\rightarrow3)\) linkages (5). In the present paper we report an efficient method of purification of dextransucrase from \textit{L. mesenteroides} NRRL B-1149 using polyethylene glycol. The crude dextransucrase in cell free supernatant and purified dextransucrase were identified and confirmed by Periodic Acid Schiff’s (PAS) staining procedure using sucrose as a substrate.

Materials and Methods

Microorganism and culturing

\textit{Leuconostoc mesenteroides} NRRL B-1149 was procured from Agriculture Research Service (ARS Culture Collection), USDA, Peoria, USA. The culture was maintained in modified MRS (sucrose in place of glucose) agar medium as a stab at 4°C and sub cultured every 2 weeks.
Production of dextransucrase
A loop of culture from 1.7% agar stab was transferred to 5 ml of sterile medium described by Tsuchiya et al. (22). The fermentation was allowed to proceed for 6-7 h at 28°C with 180 rpm. One percent of the culture inoculum was used for the enzyme production from L. mesenteroides NRRL B-1149. The enzyme was produced using the modification in the medium of Tsuchiya et al. (22) and the enzyme production medium contained (g/100 ml) sucrose, 4; yeast extract, 2; dipotassium phosphate, 2; MgSO4.7H2O, 0.02; MnSO4.4H2O, 0.001; FeSO4.7H2O, 0.001; CaCl2.2H2O, 0.001; NaCl, 0.001 and the pH was adjusted to 6.9 using 2N HCl before autoclaving (23). The culture broth was centrifuged at 10000 rpm for 10 min at 4°C to separate the cells. The supernatant was analyzed for enzyme activity and protein concentration.

Enzyme assay
The assay of dextransucrase was carried out in 1 ml of reaction mixture in 20 mM sodium acetate buffer, pH 5.4, containing 146 mM (5%) sucrose and using the cell free supernatant (20 µl) as 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 (15, 21). 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 (Cary 100 Bio, Varian Inc., USA) against a blank with D-fructose as a standard. One unit (U) of dextransucrase 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.

Protein estimation
The protein concentration of the cell free extract and purified enzyme was estimated by the method of Lowry et al. (7). Bovine serum albumin ranging from, 25 µg/ml to 500 µg/ml concentration was used to plot a standard curve.

Purification of dextransucrase by PEG fractionation
Ice cold polyethylene glycols (PEG-400, PEG-1500) were added to the 20 ml cell free supernatant to obtain the final concentrations of 25, 33, 40 and 50 (% , v/v). PEG-1500 was added to the cell free extract to get the final concentrations 15, 20, 25 and 30 % (w/v). The mixtures were incubated for 12 h at 4°C to allow the dextransucrase to precipitate. The mixture was centrifuged at 13,000 rpm for 30 min at 4°C to separate the dextransucrase. The pellet was resuspended in 20 mM sodium acetate buffer (pH 5.4). These fractions were analyzed for dextransucrase activity and protein estimation after dialysis.

SDS-PAGE analysis of purified enzyme
SDS-polyacrylamide gel electrophoresis was performed with a vertical slab mini gel unit (BioRad) using 1.5 mm thick gels, following the method of Laemmli (6). 7% (w/v) acrylamide for resolving gel and 4% (w/v) for stacking gel were used. The protein samples were prepared in 0.0625 M Tris–HCl buffer (pH 6.8) containing 2.3% (w/v) sodium dodecyl sulfate, 10% (w/v) glycerol, 5% (w/v) 2-mercaptoethanol and 0.05% (w/v) bromophenol blue. The enzyme samples purified from different molecular weight PEGs along with protein molecular weight marker were boiled and loaded on the gel. The running buffer containing 0.12% Tris, 0.56% Glycine and 0.5% SDS was prepared. The electrophoresis was carried out with a current of 2 mA per lane. The protein bands were fixed with solution containing 40% ethanol and 10% acetic acid and then stained with 0.1% silver nitrate in 0.02% formaldehyde solution. The protein bands were then developed by adding solution containing 2.5% sodium carbonate in 0.01% formaldehyde solution and then the reaction was stopped by 1.46% disodium salt of ethylenediaminetetraacetic acid. Molecular mass marker (Myosin from Rabbit Muscle, 205,000; Phosphorylase b 97,400; Bovine serum albumin, 66,000; Ovalbumin, 43,000; Carbonic anhydrase, 29,000 Da) purchased from Genei, India, were used as standard.

Non-denaturing SDS-PAGE analysis (sample buffer prepared without 2-mercaptoethanol and samples loaded without boiling) of purified enzyme was also carried out to check if the enzyme still showed three prominent bands which might confirm the presence of three forms of the enzyme.

Identification of dextransucrase by activity staining
The activity of dextransucrase was detected on 7% polyacrylamide gels run under SDS-denaturing conditions. The electrophoresis was performed on a mini gel unit (BioRad) using 1.5 mm thick gels, following the method of Laemmli (6). After run the gel was treated thrice (% , v/v) with a
solution containing 20 mM sodium acetate buffer, pH 5.4, 0.1% Triton-X-100 and 0.005% Calcium chloride for 20 min to remove SDS. Then the gel was incubated with 10% sucrose solution in 20 mM sodium acetate buffer, pH 5.4 for 10-12h (10, 23). Following incubation the gel was washed twice with 75% ethanol for 20 min and incubated in solution with 0.7% periodic acid in 5% acetic acid for 20 min at room temperature. The gel was then washed thrice with 0.2% sodium bisulfate in 5% acetic acid solution and finally stained with Schiff’s reagent (0.5% w/v basic Fuschin, 1% sodium bisulfite and 0.1N HCl until the discrete magenta bands appeared within the gel, which confirmed dextransucrase activity.

Results and Discussion
Purification of dextransucrase by PEG fractionation

*L. mesenteroides* NRRL B-1149 was grown for 6-7 h and the culture broth obtained was centrifuged and the cells were removed. The cell free supernatant containing extracellular dextransucrase was subjected to fractionation by PEG-400 and PEG-1500. Varied concentrations of two PEGs were used as described in methods. The Fig. 1 shows the specific activity profiles of dextransucrase purified with different concentrations of PEG-400 and PEG-1500. Fractionation with PEG-1500 gave dextransucrase with significantly higher specific activity than PEG-400. The specific activity of dextransucrase decreased with increasing the concentration of PEG-1500 from 15% to 30%, whereas, in the case of PEG-400 it increased with increase in concentration up to 33% (Fig. 1). The maximum specific activity and the extent of purification achieved with each PEG are listed in Table 1. The maximum specific activity with a final 15% (w/v) PEG-1500 was 15 U/mg giving 17-fold purification in a single step. A concentration of 33% (v/v) PEG-400 gave the enzyme with 9.2 U/mg specific activity of protein with 10.5 fold purification (Table 1). A lower percent of PEG was required for fractionating the dextransucrase with its increasing molecular weight. For example, a concentration of only 15% PEG-1500 and 33% PEG-400 gave maximum enzyme activity.

![Fig. 1. Purification of dextranucrase by PEG-400 and PEG-1500](image)

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Purification of dextranucrase by PEG-fractionation</th>
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<td>PEG (%)</td>
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<td>Fractionation by different concentrations of PEG-400</td>
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<td>25%</td>
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<td>Fractionation by different concentrations of PEG-1500</td>
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Analysis of enzyme purification by SDS-PAGE and confirmation by activity staining

The dextransucrase purified with different PEGs were also analyzed by SDS-PAGE to check the purity. The SDS-PAGE results of purification by both the PEGs at all concentrations showed the presence of multiple protein bands (Fig. 2).

Dextransucrase appears to exist in multiple molecular forms as the same samples showed three bands on denaturing SDS-PAGE after activity staining. The white bands were observed on the gels incubated in sucrose after 10-12h. These white bands turn to magenta color after PAS staining, which confirmed the presence of polysaccharide formed on polyacrylamide gels (Fig. 3). The PAS staining of the sucrose incubated gels showed that the three activity bands corresponded to the native and active form of the purified enzyme appearing on the denaturing gels stained with silver nitrate solution. These results are similar to those of other authors (11, 16, 23, 24). The samples from the purification of optimized concentrations of 33% of PEG-400 (Fig. 3, lane III) and 15% PEG-1500 (Fig. 3, lane VI), also showed 3 bands.

![Fig. 2](image1)  ![Fig. 3](image2)

Fig. 2. Denaturing SDS-PAGE of PEG purified enzyme, stained by silver nitrate solution. (I) Higher Ranged Molecular Weight Marker (29-205kDa); (II) Enzyme purified with 25% PEG-400; (III) with 33% PEG-400; (IV) with 40% PEG-400; (V) with 50% PEG-400; (VI) with 30% PEG-1500; (VII) with 25% PEG-1500; (IX) with 30% PEG-1500

Fig. 3. Denaturing SDS-PAGE of PEG purified enzyme, stained by PAS solution for GTF activity. (I) Higher Ranged Molecular Weight Marker (29-205kDa); (II) Enzyme purified with 25% PEG-400; (III) with 33% PEG-400; (IV) with 40% PEG-400; (V) with 50% PEG-400; (VI) with 15% PEG-1500; (VII) with 20% PEG-1500; (VIII) with 25% PEG-1500; (IX) with 30% PEG-1500

The optimized concentrations of both the PEGs giving highest in vitro dextransucrase activity were corroborated by the highest intensity of the bands obtained after activity staining (Fig. 3).

No white bands before PAS staining or the activity bands after staining appeared after the incubation of the SDS-PAGE gels run under denaturing conditions with raffinose (Fig. 4). However, the lanes showed only the native dextran associated with the fractionated dextransucrase samples from PEG-400. This confirmed that the purified fractions contain only dextransucrase and not levansucrase or inulansucrase.

![Fig. 4](image3)

Fig. 4. Denaturing SDS-PAGE of purified enzyme and confirmation for GTF activity by staining with PAS solution. A: Using rafinose as a substrate. B: Using sucrose as a substrate

Conclusions

The polyethylene glycol 1500 purification of dextransucrase from L. mesenteroides NRRL B-1149 from the cell free supernatant containing 0.9 U/mg enzyme activity. A final concentration of 15% PEG-1500 resulted in maximum specific activity of 15 U/mg in a single step. The results showed that, with high molecular weight of PEG resulted with higher specific activity of the fractionated dextransucrase. A lower concentration of higher molecular...
weight PEG gave higher enzyme activity. The purified dextranase exists in multiple molecular forms as shown by SDS-PAGE, which is similar to those from other strains as reported earlier and the same sample showed three discrete bands on denaturing SDS-PAGE. The enzyme was confirmed for dextranase by activity staining bands of the dextran produced, on the denaturing SDS-PAGE when incubated with sucrose. The three activity bands of dextranase corresponded to the native and active form of the purified enzyme existing in three molecular forms. To rule out the presence of inulansucrase or levansucrase the purified enzyme on the gels was incubated with raffinose which is a substrate for these enzymes. The gels did not show any activity bands upon staining. This confirmed the presence of only dextranase. The enzyme was maximally active at 30°C and pH 5.4. The structure and type of linkage analysis of dextran is being explored using NMR and FTIR spectroscopy. Preliminary results have shown that dextran produced by this strain contains α(1→6) linkage. Further studies are being done to produce purified dextranase from L. mesenteroides NRRL B-1149 in order to produce dextran and characterize it, so that it can be explored for commercial applications.

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