Stabilization of dextranucrase from *Leuconostoc mesenteroides* NRRL B-640

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Abstract Stabilization of dextranucrase from *Leuconostoc mesenteroides* NRRL B-640 with various stabilizers at different temperatures was studied. Dextranucrase was stable at lower temperatures (10–30°C) and lost the activity at above 30°C. The salts such as CaCl₂, CoCl₂ and MgCl₂ enhanced the dextranucrase activity. A 22% higher dextranucrase activity was obtained by 4 mM CoCl₂. The dextranucrase activity was lost by 50% at 1 mM EDTA. Urea denatured the enzyme and caused 45%, 90% and 98% loss of activity in 30 min when treated with 1 M, 3 M, and 5 M urea concentrations, respectively. Amongst the stabilizers Tween 80, glycerol, PEG-8000, dextran (500 kDa) and glutaraldehyde, Tween 80 provided the maximum stability at 30°C. In the presence of Tween 80 the enzyme lost only 8% activity at 30°C in 20 h but, it lost 65% of activity without any stabilizer. The enzyme lost 92% of activity within 4 days at 30°C and lost only 25% of activity at –20°C after 14 days.

Keywords Dextranucrase · *Leuconostoc mesenteroides* · Stabilization · Tween 80 · Urea

Introduction

The stability of enzymes is a critical issue in the biotechnology industry. Enzymes are sensitive to change in the environmental conditions such as temperature, pH and ionic strength. Both operational and storage stabilities affect the production of enzyme-based products. Stabilizing an enzyme normally means suppressing the unfolding of the protein and retaining the catalytic activity [1]. Stabilization of enzymes could be achieved by modification of the protein structure by protein engineering, chemical modification or by immobilization [2]. The enzymes that can withstand extreme conditions can be isolated from extremophiles. The stabilization can be achieved by the addition of additives like substrates, products, inhibitors, cofactors, metal ions, proteins, sugars and natural or synthetic polymers [1–5]. The use of enzymes for industrial purposes usually depends on their stability during isolation, purification and storage [3]. Stabilization by additives is the widely used procedure for various industrial enzymes [4, 6–8]. Dextranucrase is glucosyltransferase which synthesizes dextran, a glucopolysaccharide from sucrose. Dextranucrase is generally associated with the dextran. The removal of associated dextran from dextranucrase resulted in the loss of enzyme activity [8]. The dilution of the purified dextranucrase also resulted in the loss of activity [9]. Certain metal ions and additives have been reported to stabilize dextranucrase from some strains *Leuconostoc mesenteroides* [9, 10]. The Ca²⁺, Mg²⁺, Co²⁺ ions and stabilizers such as Triton X-100, Tween 80, glycerol, polyethylene glycols, dextran, glutaraldehyde and polyvinyl alcohols have been used for these enzyme systems [9, 10]. *Leuconostoc mesenteroides* NRRL B-640 was shown to produce dextranucrase that gives

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highly linear and soluble dextran [11]. Dextranucrase from *Leuconostoc mesenteroides* NRRL B-640 was purified [12] and the conditions for its assay were optimized [13]. However, there is no report on stabilization of dextranucrase from *Leuconostoc mesenteroides* NRRL B-640. In present study the effects of certain metal ions, stabilizers and different storage temperatures on dextranucrase of *Leuconostoc mesenteroides* NRRL B-640 were investigated.

Materials and methods

Enzyme preparation

Dextranucrase used in the present study was purified by a single step fractionation method using polyethylene glycol (PEG) 1500 as described earlier [12]. An ice cold 60% PEG-1500 solution in distilled water was added to cell free extract at 4°C, to get the final concentration 10% (w/v). The mixture was incubated at 4°C for 12 h to allow dextranucrase to fractionate. The mixture was centrifuged at 13200 g at 4°C for 30 min to separate the precipitated dextranucrase. The enzyme pellet was resuspended in 20 mM sodium acetate buffer (pH 5.4). The dextranucrase was subjected to dialysis using the same buffer and 5kDa cutoff membrane to remove any trace of PEG-1500. The purified dextranucrase obtained was analyzed for enzyme activity, protein concentration and purity by PAGE analysis.

Enzyme assay

The assay of dextranucrase was carried out in 1.0 ml of a reaction mixture in 20 mM sodium acetate buffer, pH 5.4, containing 146 mM (5%, w/v) sucrose and dextranucrase (0.55 mg/ml, 23 U/mg). The reaction mixture was incubated at 30°C for 15 min. Aliquots (0.2 ml), from the reaction mixture were analyzed for reducing sugar concentration. The enzyme activity was determined by estimating the liberated reducing sugar by Nelson-Somogyi method [14, 15]. The absorbance was measured at 500 nm using a UV-visible spectrophotometer (Cary 100 Bio, Varian, Inc., USA) against a blank using D-fructose as a standard. One unit (U) of dextranucrase activity is defined as the amount of enzyme that liberates 1 μ mol of reducing sugar per min at 30°C in 20 mM sodium acetate buffer, pH 5.4.

Effect of salts, denaturing agents and temperature on dextranucrase activity

The effects of CaCl_2 , CoCl_2 and MgCl_2 between 0–20 mM and EDTA between 0–6 mM concentrations were studied on dextranucrase activity. The assays were carried out in 1.0 ml

reaction mixture containing salt, enzyme and substrate. The activity was determined as described above. The effect of urea was studied by incubating enzyme with urea (0–5 M concentration) at 30°C for 30 min. Aliquots (10–20 μ l) were assayed for residual enzyme activity. For determining the thermostability, the enzyme was incubated at different temperatures (10–50°C) for 10 min and the aliquots (10–20 μ l) were assayed for residual enzyme activity in 1 ml reaction mixture containing 5% sucrose in 20 mM sodium acetate buffer, pH 5.4 by incubating at 30°C following the method as described above.

Effect of storage temperature and additives on stability of dextranucrase

Storage stability was studied by incubating the dextranucrase at different temperatures (0°C, 4°C and –20°C). The samples at different time intervals were analysed for dextranucrase activity. Aqueous solutions of dextran (500 kDa) and PEG-8000 and solutions of glutaraldehyde, glycerol, Tween-80 were added to dextranucrase solution (0.55 mg/ml, 23 U/mg specific activity) in sodium acetate buffer, pH 5.4 to obtain the final concentrations of 2 μ g/ml (w/v) dextran (500 kDa), 10 μ g/ml (w/v) PEG-8000, 0.1% (v/v) glutaraldehyde, 0.5% (v/v) glycerol and 10 μ g/ml (v/v) Tween 80, respectively, in a final volume of 0.6 ml. The enzyme with or without Tween 80 was incubated at three different temperatures –20°C, 4°C and 30°C for 14 days. The aliquots (10–20 μ l) were taken periodically for enzyme assay. All the experiments were carried out in triplicates and the results represent mean values with less than 2% of error.

Results and discussion

Effect of salts, denaturing agents and temperature on dextranucrase activity

The effect of metal ions on enzyme activity is an established fact with several enzymes. These solutes stabilize the catalytic activity of enzymes by stabilizing the three-dimensional protein structure. The metal ions Mg^{2+} and Ca^{2+} at lower concentrations exhibited a marginal increase in the enzyme activity of dextranucrase, whereas the increase in activity by Co^{2+} was highly significant. The addition of CaCl_2 and MgCl_2 at 1 mM concentration to the dextranucrase caused an enhancement of enzyme activity by 8% and 6%, respectively (Table 1A). The addition of 4 mM CoCl_2 to dextranucrase resulted in an increase in the enzyme activity by 22% (Table 1A). Further increase in the salt concentrations did not increase the enzyme activity. The addition of EDTA inhibited the enzyme activity and 50% enzyme inhibition was obtained with 1 mM EDTA (Fig. 1A). The greater extent of inhibition

Table 1 Effect of additives on dextransucrase

A. Effect of divalent metal ions				
	Dextransucrase + additive	Relative activity (%)		
1.	CaCl ₂ (1 mM)	108		
2.	MgCl ₂ (1 mM)	106		
3.	CoCl ₂ (4 mM)	122		
B. Half life ($t_{1/2}$) of dextransucrase and additive treated at various temperatures				
		*Half-life ($t_{1/2}$) h/d		
	Dextransucrase + additive	30°C	4°C	-20°C
4.	Dextransucrase	8.2h (0.34d)	14.3d	40d
5.	Dextransucrase + Tween 80	90h (3.75d)	35d	109d
6.	Dextransucrase + glycerol	12.1h	nd	nd
7.	Dextransucrase + PEG-8000	9.6h	nd	nd
8.	Dextransucrase + dextran (500 kDa)	9.1h	nd	nd
9.	Dextransucrase + glutaraldehyde	1.3h	nd	nd

nd -not determined; *Half-life was determined from the semi-logarithmic plots of Fig. 4 and Fig. 5

was observed with further increase in the concentration and saturation reached at 5 mM to a 30% residual activity. Similar results were reported for purified *Leuconostoc mesenteroides* B-512F dextransucrase where, the activity of enzyme was enhanced by the addition of Ca²⁺ and Co²⁺ and inhibited by EDTA [8, 10]. Urea displayed a deactivating effect on dextransucrase at all concentrations (Fig. 1A). The increase in time period of incubation of urea with enzyme had no further significant deactivating effect. However, with increase in the concentration of urea drastically affected the enzyme activity. The enzyme lost 45%, 70%, 90%, 95% and 98% of activity at 1, 2, 3, 4 and 5M urea, respectively (Fig. 1A). These results were different from those observed with urea effect on dextransucrase of *Leuconostoc mesenteroides* NRRL B-1299 where the complete loss of enzyme activity was achieved with 1M urea [16]. Dextransucrase exhibited a mesophilic nature, and stable up to 30°C, above which it rapidly lost the enzyme activity upon incubation for 10 min (Fig. 1B). Although, the dextransucrase showed an optimum temperature range for its assay between 30°C to 35°C, but enzyme was unstable in absence of its substrate sucrose at and above 35°C [13]. Similar results were reported for dextransucrase from *Leuconostoc mesenteroides* NRRL B1299 [7].

Effect of additives and storage temperature on stability of dextransucrase

The effects of various additives on the stability effect on dextransucrase were studied. The additive used did not have any effect on the enzyme activity when included in the assay except glutaraldehyde (data not shown). The residual activity of dextransucrase at 30°C at 20h was 92%, 44%, 38%, 36%, 32% and 6% with Tween 80, glycerol,

PEG 8000, dextran (500 kDa), control and glutaraldehyde, respectively (Fig. 2). The Tween 80 was the best stabilizer for dextransucrase at 30°C. Glutaraldehyde did not support the stability, rather it inhibited the enzyme activity (Fig. 2). Surprisingly, dextran (500 kDa) did not provide stability to the dextransucrase from *Leuconostoc mesenteroides* B-640, though it has been reported with other strains that dextran imparts stabilization to dextransucrase [17]. The other stabilizers glycerol, PEG-8000 displayed only a marginal or no effect at 30°C (Fig. 2).

The residual enzyme activity profile of storage stability at -20°C, 4°C and 30°C of purified enzyme are shown in Fig. 3. The enzyme lost its activity rapidly at 30°C after 1 day incubation with only retaining 32% residual activity. Storage of dextransucrase at 4°C showed a loss of only 10% enzyme activity after 6 days of incubation, whereas the enzyme lost 10% activity in 10 days when stored at -20°C (Fig. 3). Dextransucrase lost 95% activity within 4 days when stored at 30°C (Fig. 3). The addition of Tween 80 to dextransucrase provided stabilizing effect at all the three temperatures (Fig. 3). The loss of activity in presence of Tween 80 was 8% and 30% at -20°C and 4°C in 14 days as against the losses of 25% and 65% with corresponding controls which contained no Tween 80. Tween 80 provided significant stability to dextransucrase at 30°C up to 4 days, as the loss of the enzyme activity was only 32% in presence of Tween 80 as against 92% loss without Tween 80 in 4 days.

Half-life time of dextransucrase and additive-treated dextransucrase

The residual activity of dextransucrase was measured at various temperatures with respect to time with and without

Fig. 1 Effect of (A) urea and EDTA and temperature (B) on stability of dextransucrase. 0.55 mg/ml enzyme in 20 mM sodium acetate buffer (pH 5.4) was incubated with various concentrations of urea at 30°C for 10 min. For EDTA effect on enzyme activity no pre-incubation was done. Closed and open circles indicate the residual enzyme activity in presence of EDTA and Urea, respectively (A). Thermostability of dextransucrase was determined at different temperatures by pre-incubating for 10 min and assaying at 30°C in a 1 ml reaction mixture as described in methods (B).

additives. The enzyme deactivation followed first order rate kinetics. The half-life time ($t_{1/2}$) of dextransucrase and additives treated dextransucrase was calculated by assuming that the decay followed first order kinetics and were listed in Table 1B. The $t_{1/2}$ of enzyme increased from 0.34 d to 40 d with decreasing the temperature from 30°C to –20°C. This showed that the storage temperature used for dextransucrase can be –20°C. Amongst all the stabilizers used Tween 80 gave the maximum stabilization of dex-

Fig. 2 Effect of various stabilizers on dextransucrase activity at 30°C. 0.55 mg/ml dextransucrase in 20 mM sodium acetate buffer (pH 5.4) was pre-incubated with additives at 30°C. Open circles, enzyme without any additives (control); open rectangles, Tween 80; diamonds, glycerol; hourglass squares, PEG-8000; triangles down, dextran (500 kDa); half-filled circles, glutaraldehyde.

Fig. 3 Effect of storage stability of dextransucrase and Tween 80 treated dextransucrase at temperatures 30°C, 4°C and –20°C. 0.55 mg/ml dextransucrase in 20 mM sodium acetate buffer (pH 5.4) was pre-incubated with Tween 80 (10 µg/ml, final concentration) and aliquots were assayed for dextransucrase activity at 1 day intervals. Open circles, dextransucrase at 30°C; closed circles, Tween 80 added dextransucrase at 30°C; open squares, dextransucrase at 4°C; closed squares, Tween 80 added dextransucrase at 4°C; open diamonds, dextransucrase at –20°C; closed diamonds, Tween 80 added dextransucrase at –20°C.

transucrase with $t_{1/2}$ of 90 h at 30°C (Table 1B). A marginal increase in the $t_{1/2}$ (12.1 h) was observed with glycerol addition to dextransucrase at 30°C as compared to control (8.2 h). No significant increase in the $t_{1/2}$ of dextransucrase was observed with PEG-8000 (9.6 h) and dextran, 500 kDa (9.1 h) at 30°C. Surprisingly, $t_{1/2}$ of dextransucrase drastically decreased with the addition of glutaraldehyde ($t_{1/2}$ = 1.3 h) when compared to dextransucrase with no additive ($t_{1/2}$ = 8.2 h) at 30°C. The addition of Tween 80 to dextransucrase, incubated at three different temperatures (30°C, 4°C and –20°C) resulted in higher $t_{1/2}$ than with no Tween 80 (Table 1B). Taken together all these results it can be summarized that a temperature of –20°C and addition of Tween 80 can best serve for the stabilization of dextransucrase of *Leuconostoc mesenteroides* NRRL B-640 against activity losses.

It is important to obtain enzymes with high stability for their industrial applications. Dextransucrase is highly sensitive to pH and temperature. The thermo-stability results showed that dextransucrase is stable at lower temperatures (10–30°C) and loses rapidly the enzyme activity at temperatures higher than 30°C. Dextransucrase activity was enhanced by divalent cations (Ca^{2+} , Co^{2+} and Mg^{2+}). Co^{2+} showed an activation of the enzyme activity by 22%. EDTA showed an inhibitory effect on enzyme activity thus showing the requirement of metal ion for the catalytic efficiency of dextransucrase from *Leuconostoc mesenteroides* NRRL B-640. The enzyme activity loss was 98% with urea at 5M concentration when incubated for 30 min at 30°C. Amongst various stabilizers used Tween 80 provided the maximum stabilization to dextransucrase against activity loss at 30°C. Dextransucrase lost 92% activity within 4 days when stored at 30°C, whereas in the presence of Tween 80 this loss was only 33% in 4 days. The storage of dextransucrase at different temperatures showed that the enzyme retains 75% activity at –20°C in 14 days however, in presence of Tween 80 the enzyme retained 92% activity when stored at –20°C in 14 days. The results suggested that storage of dextransucrase at –20°C is better and Tween 80 can be used as a stabilizer for the enzyme which is an efficient method for preventing dextransucrase against the loss of enzyme activity.

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