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Properties of Dextransucrase from *Leuconostoc mesenteroides* Isolated from Sikhae

RHEE, SUK HYUNG AND CHERL-HO LEE*

Department of Food Technology, College of Agriculture, Korea University, Seoul 136-701, Korea

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Studies on the optimum conditions for dextran production and the properties of dextransucrase (DS) were performed with *Leuconostoc mesenteroides* from Sikhae and *Leuconostoc mesenteroides* NRRL B-512(F). Dextransucrases were partially purified by lyophilization of the culture supernatant and subsequent gel chromatography on Bio-Gel A-5(m). The storage stabilities of Sikhae DS and B-512(F) DS were decreaed by the addition of dextranase. The optimum conditions for the enzyme stability were pH 5 and below 30°C. The B-512(F) DS lost the activity at pH 4, while Sikhae DS had 30% of the activity at the same pH. The activity of DS was decreased by EDTA, confirming the metalloprotein character of the enzymes, and was restored by the addition of calcium ions. Concanavalin A completely removed the activity of DSs, confirming the glycoprotein character of the enzymes.

Dextransucrase (DS) [EC 2.4.1.5, α-(1→6) D-glucan: D-fructose 2-glucosyltransferase] catalyzes the production of glucans linked predominantly by α-(1→6) bonds (dextrans) by transfer of glucosyl residues to growing polymer chains (6).

Leuconostoc mesenteroides B-512(F) dextransucrase (B-512(F) DS) has received wide attention and the resulting dextran is produced commercially (4, 5, 15). The B-512(F) DS is secreted in relatively large amounts into the culture supernatant solution, and it forms a high-molecular-weight soluble dextran (4). This may be contrasted with other strains of *L. mesenteroides* (2, 7) and with the dental-plaque *Streptococcus* sp. (14), which form both soluble and insoluble dextrans and elaborate more than one type of DS, together with relatively large amounts of such related contaminating enzymes as invertase and levansucrase (13). For these reasons, *L. mesenteroides* B-512(F) serves as an important model in studying the structure of dextran and the mechanism of dextran biosynthesis by DS.

Dextrans from *L. mesenteroides* are generally produced by batch fermentation, with part of the sucrose substrate being used for bacterial growth and part being converted by the excreted enzyme to dextran, although cell-free culture filtrates may be employed (7).

The commercial production and use of native dextrans and their derivatives have been reviewed (10). There have been great interest in dextran from its use in various food products as a conditioner, stabilizer, bodying agent, and other related uses in which it can replace natural gums.

Robyt and Walseth (13) studied the purification of DS extensively and developed a scheme that produced an enzyme apparently free of dextran. However, their scheme was tedious and resulted in low stability.

Accordingly, our objective was to synthesize dextran in a cell-free system by a relatively simple procedure. The present paper describes the method of partial purification of DS from *L. mesenteroides* and compares the enzyme properties of the strains of NRRL B-512(F) and Sikhae.

MATERIALS AND METHODS

Materials

L. mesenteroides from Sikhae was isolated from flatfish Sikhae (Korean traditional fermented fish product) in our laboratory (17). *L. mesenteroides* NRRL B-512(F) was obtained from the Korean Federation Culture Collection (KFCC), Seoul, Korea. The agarose gel of Bio-Gel A-5(m) was purchased from Bio-Rad Laboratories,

*Corresponding author

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Richmond, CA, USA. Dextranase (1,6-α-D-Glucan-6-glucanohydrolase; EC 3.2.1.11) was pruchased from Sigma Chemical Co., St. Louis, MO, USA.

Culturing of *L. mesenteroides* NRRL B-512F and *L. mesenteroides* from Sikhae

The organisms were grown on the medium described by Jeanes (6) (Table 1).

A 5 liter jar fermenter (SY-500, Shin-young Engineering Co. LTD., Korea) was used for enzyme production. The pH was maintained at 6.8±0.1 with 10 N NaOH by using a peristaltic pump. The temperature was 25℃, aeration rate 200 *ml* per 2000 *ml* of culture per min and agitation rate 150 rpm. Cells were removed by centrifugation and the supernatant solution was used as the source of DS.

Growth Measurements

Samples (5 *ml*) were removed from the fermenter every hour during a 12 h growth period. The turbidity of the sample was measured at 590 nm in a spectrophotometer (DU-64, Beckman). The cells were removed by centrifugation and the pH and the DS activity of the supernatant were measured.

Enzyme Assay

DS activity was determined under the conditions described by Hehre (1) by using the Somogyi method (16). One DS unit (DSU) is defined as the amount of enzyme which converts 1 mg of sucrose to dextran (liberation of 0.52 mg of reducing power, calculated as fructose) in 1 hour at 30℃.

Protein was determined by Lowry's method (9), using bovine serum albumin (BSA) as a standard, or by measuring the absorbance of the enzyme solution at 280 nm.

Table 1. Composition of medium for *L. mescnteroides*

Ingredient	Medium A	Medium B	Medium C
Sucrose	2.0	2.0	2.0
Difco yeast extract	0.5	0.5	0.5
Difco tryptone	0.25	0.25	
K_2HPO_4	0.25	0.5	
KH_2PO_4			2.0
Agar	2.0		
R-salts			0.5
pH		7.2	7.2

*R-salts; 4% $MgSO_4 \cdot 7H_2O$ + 0.2% $MnSO_4 \cdot H_2O$ + 0.2% $FeSO_4 \cdot 7H_2O$ + 0.2% NaCl

*Medium A; Composition of maintenance medium

*Medium B; Composition of medium for the first stage of inoculum build-up

*Medium C; Composition of medium for successive stages of inoculum build-up and enzyme production

Partial Purification of Dextransucrase

All of the purification steps were carried out at 4℃. The culture supernatant was concentrated by lyophilization (Freeze dryer 8, Labconco.), which was labeled as cell-free concentrate. The cell-free concentrate was diluted with 20 mM acetate buffer, pH 5.0, containing 3 mM $CaCl_2$, which was chromatographed on a 2×87 cm column of Bio-Gel A-5(m) that was eluted with 20 mM acetate buffer, pH 5.0, containing 3 mM $CaCl_2$, at flow rate of 18 *ml*/h. The DS fractions were labeled as A-5(m) eluent and considred as partially purified enzyme.

Removal of Dextran from Dextransucrase

The A-5(m) eluent (4.85 *ml*) and cell-free concentrate were treated with 0.15 *ml* of dextranase (30 U/*ml* in 20 mM acetate buffer, pH 5.0) for 24 h at 30℃ in a dialysis tube placed in 2 liter of 20 mM acetate buffer, containing 3 mM $CaCl_2$, pH 5.0, which was changed 3 times during the course of the reaction.

Measurement of pH and Thermal Stability

The dextranase-treated enzyme was kept at various pH for 24 h at 4℃ and then adjusted to the optimum pH and the residual activity was measured. After incubation for 10 min at various temperature, the dextranase-treated enzyme was adjusted to the optimum temperature and the residual activity was assayed.

Measurement of the Effects of Chelating Agent and Metal Ions

The dextranase-treated enzyme was assayed for the DS activity in the presence of EDTA [ethylenediamine tetraacetic acid] at pH 5.0 (20 mM acetate buffer). The effects of metal ions on the DS activity were measured both in the presence and absence of EDTA. The enzyme was dialyzed at 4℃ against 20 mM acetate buffer, pH 5.0, to remove the ions produced in the enzyme preparation before the addition of EDTA. The chlorides of various divalent ions in 20 mM acetate buffer, pH 5.0, were added to dialyzed DS and non-dialyzed DS that had been incubated for 90 min in 5 mM EDTA. The enzyme preparations were then assayed in the presence of 5 mM metal ion.

Reaction of Dextransucrase with Concanavalin A

Dextransucrase was incubated with 25 mg of concanavalin A for 30 min at 25℃. After centrifugation, the supernatant solution was assayed for DS activity.

RESULTS AND DISCUSSION

Production of DS

L. mesenteroides from Sikhae and *L. mesenteroides* NRRL B-512(F) were grown in Medium C (Table 1), and the changes in DS activity, turbidity and pH were

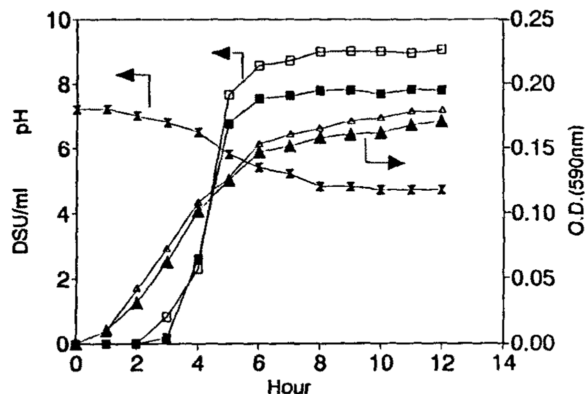

Fig. 1. Growth curve of *Leuconostoc mesenteroides* from Sikhae and *Leuconostoc mesenteroides* NRRL B-512(F).

–■– Sikhae dextransucrase Activity, –□– B-512(F) dextransucrase Activity, –▲– Sikhae Turbidity, –△– B-512 (F) Turbidity, –x– Sikhae pH, –x– B-512(F) pH

monitored (Fig. 1).

DS activity and turbidity reached maximum in 8 hours. Despite the high phosphate buffer concentration, the pH dropped from 7.2 to 4.8 in 8 hours (Fig. 1). The enzyme was produced in near the beginning of the stationary phase. At this point, the enzyme was secreted rapidly into the culture medium and reached a maxium at 6 h.

A cultural factor critically affecting enzyme yields is close control of pH of the growing culture at about pH 6.8. The optimum pH for DS production was pH 6.8~7.0 for both strains, but in this pH range, the enzyme was unstable (8). Koepsell (8) showed that DS was stable at pH 5.0~5.2. So these organisms were cultured for 8 hours in the maintenance of pH 6.8+0.1 with 10 N NaOH and then centrifuged for the removal of cells and adjusted to pH 5.0 with H_2SO_4.

Partial Purification of DS

Table 2 gives the purification data for Sikhae DS and B-512(F) DS. Chromatography of the culture supernatant concentrate on Bio-Gel A-5(m) is shown in Fig. 2 and 3.

DS activity was shown at void volume (tube no. 27). This result indicated that DS had a very high molecular weight or that DS was associated in large molecular aggregates. Kobayashi *et al.* (7) reported that the molecular weight of DS secreted by *L. mesenteroides* NRRL B-1299 was 48,000 Dal. Robyt *et al.* (13) studied the purification of DS from levansucrase (LS) by using dextranase. When the cell-free concentrate was treated with dextranase in a dialysis tube and the dextranase-treated enzyme was chromatographed on Bio-gel A-5(m), the retention time was retarded appearing at tube No. 45 and the DS activity significantly decreased. The retardation of elution time after dextranase treatment indicated that the enzyme in the cell-free concentrate was in a high-molecular-weight form complexed with dextran. After being kept for 5 days at 4℃, however, the dextranase-treated cell-free concentrate re-aggregated and migrated with the void volume of a Bio-Gel A-5(m) column. This fraction was found to lose most of the activity. This aggregation could not have been caused by a complex with dextran, because the major part of the dextran had been removed by the dextranase treatment. Same phenomenon was also observed from *L. mesenteroides* B-1299 DS (7). We didn't attempt to remove LS, because LS activity was no more than 10% of DS activity in Jeanes's medium (13), and the absence of dextran didn't stabilize DS (Fig. 4).

Gel chromatograms show that tube no. 60~70 were reacted with sucrose solution and then were detected

Table 2. Purification data for dextransucrase from two strains of *L. mesenteroides*

Fraction	Volume (m*l*)	Dextransucrase DSU/m*l*	Total DSU	% yield	Protein mg/m*l*	Total (mg)	% removed	S.A[1]	P.F[2]
Leuconostoc mesenteroides from Sikhae									
Culture supernatnat	1937	10.17	19699	100	4.21	8155	0	2.42	1
Cell-free concentrate	484	34.60	16744	85	16.68	8074	1.0	2.07	0.86
A-5(m) eluent	132	0.82	108	0.55	0.07	9.24	99.9	11.7	4.83
Leuconostoc mesenteroides NRRL B-512(F)									
Culture supernatant	1920	8.56	16435	100	4.10	7872	0	2.09	1
Cell-free concentrate	444	31.50	13986	85	17.40	7726	1.9	1.81	0.87
A-5(m) eluent	148	2.50	370	2.25	0.13	19.24	99.7	19.23	9.2

[1]S.A; Specific Activity (DSU/mg protein)
[2]P.F; Purification Fold

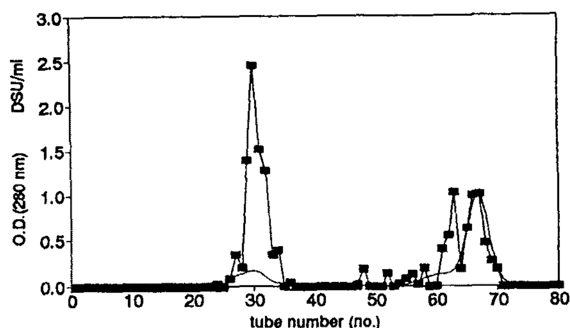

Fig. 2. Gel chromatography of Sikhae dextransucrase (Bio Gel A-5(m), 2×87 cm, flow rate; 18 ml/h).
— Protein (280 nm), –■– Dextransucrase activity

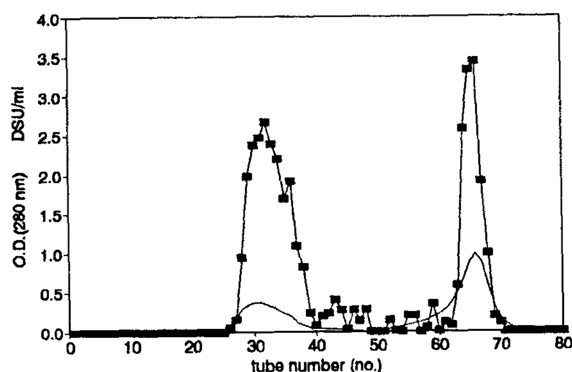

Fig. 3. Gel chromatography of B-512(F) dextransucrase (Bio Gel A-5(m), 2×87 cm, flow rate; 18 ml/h).
— Protein (280 nm), –■– Dextransucrase activity

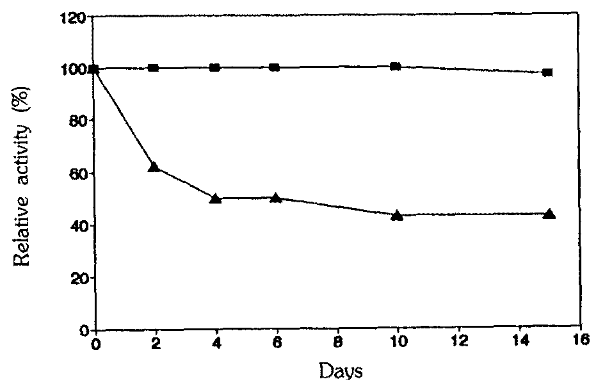

Fig. 4. Effect of dextran on dextransucrase.
–■– Dextransucrase, –▲– Dextransucrase+Dextranase

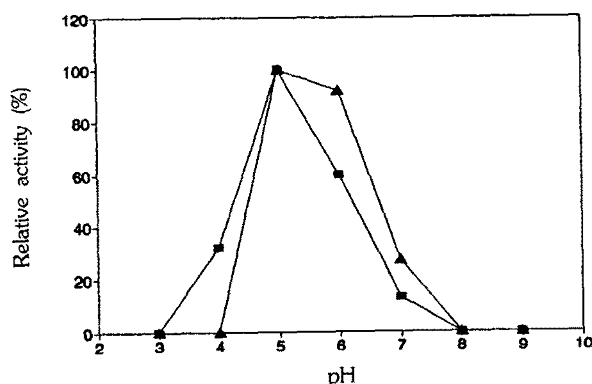

Fig. 5. Effect of pH on dextransucrase.
–■– Sikhae dextransucrase, –▲– B-512(F) dextransucrase

by Somogyi method. But the product was not viscous. We think that these fractions didn't contain DS but invertase and fructose produced in DS production. So DS activity of these fractions is referred to pseudo-DS activity.

Attempts to purify DS were made by using techniques that were effective in the purification of DS from other organisms, for instance, ammonium sulfate precipitation (2, 7, 14) and DEAE-cellulose chromatography (2, 7). We found that ammonium sulfate is completely ineffective in precipitating Sikhae DS and B-512(F) DS from cell-free concentrate; 95% of DS activity remained in solution when the concentration of ammonium sulfate was 90% (w/v). When the cell-free concentrate was chromatographed on DEAE-cellulose, DS activity could not be eluted from the column, even at very high ionic strengths (for instance, 5 M sodium chloride).

Subsequent studies led to a relatively simple purification; the culture supernatant was lyophilized, and the lyophilized concentrate was chromatographed on Bio-Gel A-5(m).

Effect of Dextran on the Storage Stability of DS

The storage stability at 4℃ of partially purified Sikhae DS and B-512(F) DS is shown in Fig. 4. DS didn't lose activity at 4℃ during a 15 day period. The dextranase-treated DS, however, lost activity rapidly at 4℃. Therefore, the cell-free concentrate was lyophilized, which had been stored at −21℃.

General Enzymatic Properties

In order to remove the stabilizing effect of dextran, DSs were treated with dextranase.

Similarly to the papers so far published (1, 2,15), DS showed its optimum pH at 5.0 and was stable in temperatures lower than 30℃ (Fig. 5 and 6). However, Sikhae DS was more stable than the B-512(F) DS at pH 4.0. This property is interesting for food application, for example, in controlling the rheological properties of lactic beverages by dextran formation.

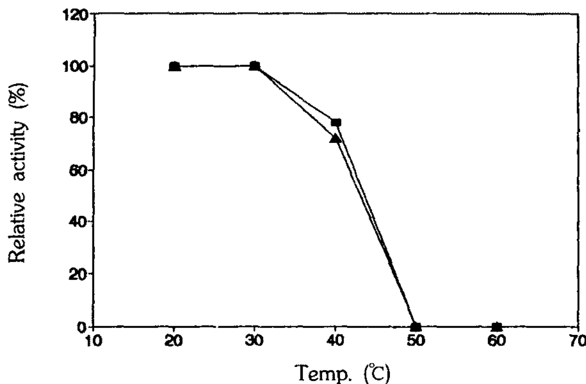

Fig. 6. Effect of thermal stability on dextransucrase.
—■— Sikhae dextransucrase, —▲— B-512(F) dextransucrase

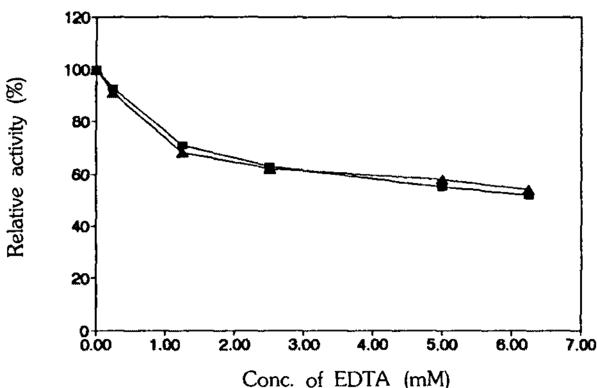

Fig. 7. Effect of EDTA on dextransucrase.
—■— Sikhae dextransucrase, —▲— B-512(F) dextransucrase

Effects of EDTA and Divalent Metal Ions

Fig. 7 shows the effect of EDTA on the activity of DS. Inhibition of 50% was obtained with 6.25 mM EDTA. The results of the addition of metal ions to the EDTA-treated enzyme are given in Fig. 8.

We confirmed the findings of Neely and Hallmark (11) that EDTA decreases the activity of DS and that calcium ions restore the activity. The maximum amount of inhibition that we could obtain with EDTA, however, was 50%, possibly because EDTA forms stronger chelate complexes at pH>5. The addition of metal ions to the EDTA-treated DS (Fig. 8) showed that calcium was the only ion that completely restored the activity. This result suggests that DS is a calcium-metalloenzyme. This conclusion was also reached by Neely and Hallmark (11) for B-512(F) DS and by Itaya and Yamamoto (2) for DS of *L. mesenteroides* IAM 1046.

The addition of other divalent-metal ions to the EDTA-treated enzyme either had no effect or produced further

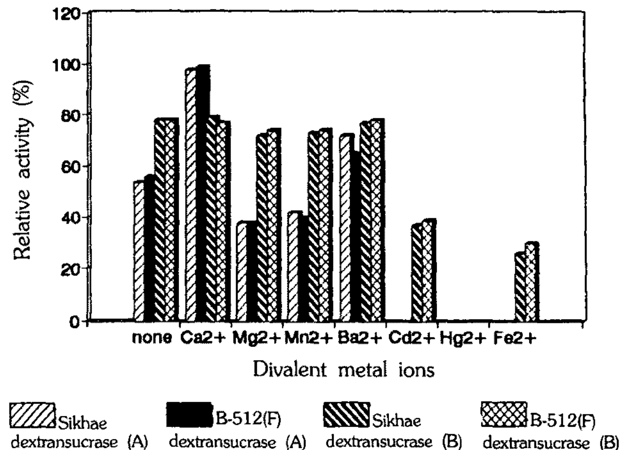

Fig. 8. Effects of divalent metal ions on dextransucrase.
(A) The metal ions were added to enzyme that had been incubated with 5 mM EDTA.
(B) The metal ions were added to EDTA-treated enzyme that had been dialyzed to remove EDTA.
*The percent relative activity is based on the activity of EDTA-untreated dextransucrase.

loss of activity. Complete loss of activity at low concentration of mercury was expected because this ion is generally considered as enzyme poison. Dialysis of the EDTA-treated enzyme gave an increase in the relative activity by apparently restoring some of the calcium that had originally been removed by the EDTA. Some 20% of the activity was irreversibly lost, however, as the addition of 5 mM calcium ions didn't restore the activity beyond 80%.

Effect of Concanavalin A on the Activity of DS

Dextransucrases incubated with 25 mg of concanavalin A for 30 min at 25℃ showed complete loss of the activity. Concanavalin A from *Canavalia ensiformis* (Jack Bean) complexes with glycoproteins containing multiple, unsubstituted, nonreducing α-D-mannosyl or α-D-glucosyl residues (12). Robyt *et al.* (13) showed that mannose was the principal carbohydrate component of DS by the paper chromatography of acid hydrolyzed DS, indicating that DS was a glycoprotein containing mannose. This hypothesis was confirmed in this experiment by showing the complete removal of DSs' activity by the addition of concanavalin A.

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