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PURIFICATION AND CHARACTERIZATION OF TWO ACTIVITIES OF THE INTRACELLULAR DEXTRANSUCRASE FROM *LEUCONOSTOC MESENTEROIDES* NRRL B-1299

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Summary

Dextransucrase (sucrose: 1,6-α-D-glucan 6-α-glucosyltransferase EC 2.4.1.5) activity of *Leuconostoc mesenteroides* NRRL B-1299 cells has been purified by adsorption on hydroxyapatite, followed by chromatographies on DEAE-cellulose and DEAE-Sephadex. The enzyme activity was readily separated into two principal forms of the enzyme, I and II, by DEAE-cellulose chromatography. Both enzymes I and II were purified to an electrophoretically homogeneous state in which the relative enzyme activities reached 32- and 14-fold of the original specimen, respectively. Molecular weights were 69 000 for the enzyme I and 79 000 for the enzyme II as determined by electrophoresis in sodium dodecyl sulphate-polyacrylamide, and no subunit structure was observed. Enzyme I had an optimum pH at 6.3—6.5 and exhibited a maximal activity at 45°C, while the optimum pH and temperature of enzyme II were pH 5.5—5.9 and 35—40°C. The K_m values of enzymes I and II were 10.7 and 250 mM, respectively. The effects of several metal ions, chemical reagents, and addition of various dextrans were also examined. Beside linear α-1,6-linkages, the polymer synthesized by the enzyme II contained lesser amount of α-1,2- and α-1,3-linkages, which seems to be a primary characteristic of the B-1299 dextran.

Introduction

Dextransucrase (sucrose: 1,6-α-D-glucan 6-α-glucosyltransferase EC 2.4.1.5), has been isolated from species of the genera of *Leuconostoc* and *Streptococcus* [1—3]. Although dextransucrase from NRRL B-512 or B-512F of *L. mesenteroides*, which synthesizes a linear α-1,6-linked dextran with about 5% of α-1,3-linked branch points, has been extensively studied [1,2,4,5], several strains of *Leuconostoc* and *Streptococcus* are known to produce two or

more dextrans with different structure and solubility [6—8]. Dextrans elaborated by *L. mesenteroides* NRRL B-1299, which were shown to have a highly branched structure with α-1,2- and α-1,3-linkages, are known to have different solubility and heterogeneous molecular weight distributions [9—12]. However, the mechanism of the formation of the non-α-1,6-linkages such as α-1,2-branch points remains to be clarified.

Our previous studies have shown that dextransucrase activity of the B-1299 strain occurred in extracellular and intracellular forms as well as cell-bound form. Furthermore, multiple forms of both the enzymes have been demonstrated by polyacrylamide-gel electrophoresis and DEAE-cellulose chromatography [6]. In the present paper, we report on the purification and some properties of the intracellular dextransucrase from this strain.

Materials and Methods

Materials

A standard dextran with 95% α-1,6- and 5% α-1,3-glucosidic linkages was kindly provided by Meito Sangyo Co., Japan. Dextran 10 and 110 were products of Pharmacia. Hydroxyapatite, prepared according to the method of Siegelman et al. [13], was equilibrated with 0.005 M sodium phosphate buffer (pH 5.4) before use.

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis (7.5% gel) at pH 7.5 was carried out according to the method described by Maurer [14]. The sodium dodecyl sulphate-gel electrophoresis for the estimation of the molecular weight of the enzymes was performed according to the procedure described by Weber and Osborn [15]. The following proteins were used as the markers; cytochrome *c* ($1.3 \cdot 10^4$), myoglobin ($1.78 \cdot 10^4$), ovalbumin ($4.6 \cdot 10^4$) and bovine serum albumin ($6.7 \cdot 10^4$, dimer $13.4 \cdot 10^4$).

Protein determination

Protein was determined by the method of Lowry et al. [16] or by the absorbance at 280 nm.

Enzyme assay

In standard assays, 0.25 ml of a suitably diluted enzyme solution was incubated with 1.0 ml of sucrose (125 mg/ml) in 0.2 M McIlvaine's buffer (pH 6.4) at 45°C for 15 min for enzyme I, and pH 5.6 at 35°C for enzyme II. Reactions were stopped by the addition of 0.2 ml of 1 M NaOH, followed by the addition of 5.0 ml of distilled water. Samples (1.0 ml) of the inactivated reaction mixtures were used for the determination of reducing sugars by the Nelson-Somogyi method [17,18]. One unit of enzyme is the same as described previously [6].

Preparation of the crude enzyme

Crude intracellular dextransucrase was prepared from the cells of *L. mesenteroides* strain NRRL B-1299. As described previously [6], 41.3% of the

enzyme activity of the cells was solubilized by successive treatments with disruption, lysozyme digestion, and deoxycholate extraction. The solubilized enzyme was then dialyzed against 0.005 M sodium phosphate buffer (pH 6.0) at 4°C.

Purification of the intracellular dextransucrase

All purification was carried out at 0—4°C. A slurry of hydroxyapatite (50 ml) was added to the dialyzed enzyme solution (272 ml). After continuous stirring for 20 min, the slurry was centrifuged at 8000 × *g* for 15 min and the supernatant was removed. The slurry was then extracted twice with 80 ml portions of 0.2 M sodium phosphate buffer (pH 6.0) for 20 min to elute the enzyme and then with 0.4 M buffer (150 ml). The extract with 67% of the enzyme activity, separated from the slurry by centrifugation, was dialyzed overnight against 0.005 M sodium phosphate buffer (pH 6.0).

The dialyzate obtained was applied to a DEAE-cellulose column (2.2 × 18 cm) pre-equilibrated with 0.005 M sodium phosphate buffer (pH 6.0). After adsorption of the enzyme, the column was washed with the equilibrating buffer (300 ml) and eluted with the same buffer with increasing NaCl concentration (linear gradient, 0.1—0.4 M), the effluents being collected in 10 ml fractions. Those fractions containing enzyme activity were pooled and concentrated about 10-fold with an ULVAC micropore ultrafiltrator with G-10T membrane. Then, two enzyme fractions obtained were dialyzed overnight against the same buffer.

Each enzyme was adsorbed on a DEAE-Sephadex A-50 column (1.6 × 21 cm) pre-equilibrated with 0.01 M sodium phosphate buffer (pH 6.0). After adsorption of enzyme I, the column was washed with 250 ml of the same buffer, then eluted with 250 ml of the buffer containing 0.2 M NaCl, and finally with 250 ml of the buffer containing 0.3 M NaCl. The fractions containing enzyme activity were pooled, concentrated with the ultrafiltrator as described above, and then dialyzed. Purification of enzyme II was carried out in the same way as enzyme I modifying the concentrations of NaCl in the eluting buffer (0.1 and 0.2 M NaCl).

Preparation of polysaccharides by purified dextransucrases

The reaction mixture contained 100 mg of sucrose/ml, 0.2 M of McIlvaine's buffer, and the purified enzyme (1.3—2.1 units/ml) in a final volume of 25 ml. After incubation for 24 h at each optimum pH and temperature as described above, the reaction was terminated by heating for 3 min at 100°C, and the digests were dialyzed against several changes of distilled water over a period of 72 h at 4°C. Then, the polysaccharides were obtained as freeze-dried powder.

Analyses of polysaccharides

The enzymatically synthesized polysaccharide was partially hydrolyzed with 0.2 M HCl for 2 h at 100°C. The hydrolyzate was neutralized with Ag_2CO_3, deionized by Amberlite IR-120 resin, and examined by paper chromatography. Acetolysis of the polysaccharide was carried out by the routine method as described previously [19]. Samples were dissolved in a mixture of

acetic acid, acetic anhydride, and sulfuric acid (16 : 24 : 3, v/v). Each solution was kept at 27°C for 72 h with occasional shaking, and then the reaction was terminated by pouring it into ice-cold water. Paper chromatography was carried out on Toyo No. 50 filter paper by double ascending method using a solvent system of *n*-butanol/pyridine/water (6 : 4 : 3, v/v). Sugar spots on the chromatograms were detected by aniline-hydrogen phthalate.

Results

Purification of the intracellular dextransucrase

A typical enzyme purification is summarized in Table I. Crude intracellular dextransucrase was solubilized from the cells of *L. mesenteroides* NRRL B-1299 by successive treatments with disruption, lysozyme digestion, and deoxycholate extraction. Water-insoluble dextran, precipitated concomitantly with the bacterial cells, was also partly solubilized by these treatments and the enzyme solution was slightly opalescent.

The dialyzed enzyme was directly adsorbed on hydroxyapatite and fractionated by stepwise elution with sodium phosphate buffer (pH 6.0). Major part of the enzyme activity was eluted with 0.2 M buffer with an overall recovery of 67.1%. A substantial amount of dextran could be removed by this step, since hydroxyapatite had no ability to adsorb dextran.

The intracellular enzymes I and II were clearly separated by chromatography on DEAE-cellulose as previously mentioned [6]. The first enzyme peak (isoenzyme II) appeared with 0.15 M NaCl concentration, and the second peak (isoenzyme I) with 0.3 M NaCl concentration.

Next, each enzyme, eluted from the DEAE-cellulose column, was concentrated by pressure dialysis and subjected to a column of DEAE-Sephadex A-50 pre-equilibrated with 0.01 M sodium phosphate buffer (pH 6.0). Both enzymes I and II were eluted as a single peak of activity (Fig. 1a,b). The fractions with enzyme activity were pooled and concentrated by pressure dialysis and then dialyzed against 0.005 M sodium phosphate buffer (pH 6.0). The recovery of the purified enzyme activity from the last purification step was 18% for enzyme I and 5% for enzyme II. Enzymes I and II were purified about 32- and 14-fold, respectively.

TABLE I

PURIFICATION OF INTRACELLULAR DEXTRANSUCRASE FORMS I AND II

Purification step	Volume (ml)	Total activity* (units)	Protein (mg)	Spec. activity (units/mg)	Yield (%)
1. Solubilization	272	372	241	1.54	—
2. Hydroxyapatite	150	249	21.9	11.39	67.1
3. DEAE-cellulose					
I	21.5	122.2	4.48	27.27	32.9
II	17.8	25.1	2.69	9.34	6.8
4. DEAE-Sephadex					
I	31.2	66.2	1.36	48.65	17.8
II	14.5	19.6	0.88	22.25	5.3

* All enzyme activities were measured at pH 6.0 and 40°C.

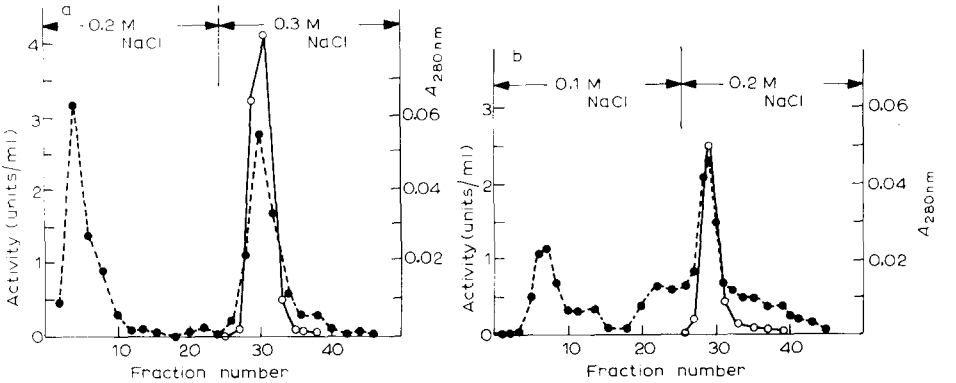

Fig. 1. Elution pattern of the intracellular dextransucrase from a DEAE-Sephadex A-50 column. ○———○, dextransucrase activity; ●- - - - - -●, protein. Fractions of 10 ml were collected and assayed for enzyme activity under the optimum conditions. Details are given in the text. (a) enzyme I; (b) enzyme II.

Purity of the enzyme preparations

Enzymes I and II yielded single protein bands, respectively, upon polyacrylamide-gel electrophoresis in a 7.5% gel at pH 7.5. Enzyme activity corresponded to the same position as the protein, and the mobility of enzyme I was somewhat higher than that of enzyme II. To estimate the molecular weight of enzymes I and II, sodium dodecyl sulphate-polyacrylamide-gel electrophoresis method of Weber and Osborn [15] was employed, and their molecular weights were calculated as 69 000 for I and 79 000 for II, respectively.

Effect of pH

Optimum pH range was between pH 6.3—6.5 for enzyme I, and between pH 5.5—5.9 for enzyme II (Fig. 2a) in 0.2 M McIlvaine's buffer. Enzyme I was stable at 4°C for 24 h in the pH range 6.2—6.9, and 50% of the activity was lost at pH 4.7 and 8.5. On the other hand, enzyme II was stable between pH 5.3 and 5.8, and 50% of the activity was lost at pH 4.6 and 7.2. Enzyme I was more stable than II at pHs above 7.0 (Fig. 2b).

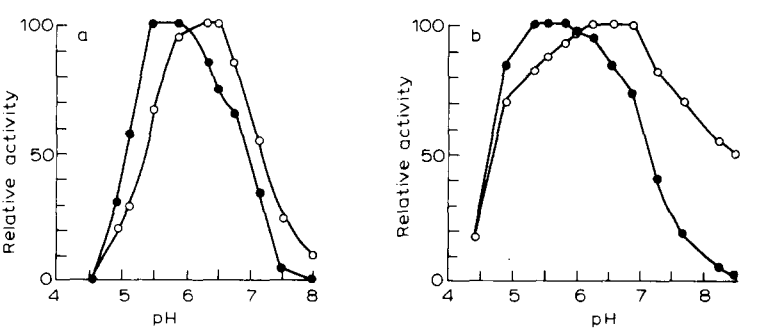

Fig. 2. Effect of pH and stability of intracellular dextransucrases I (○) and II (●). (a) pH-Activity curve: Enzyme activity was assayed at the pH indicated. Assay temperature was at 45°C for enzyme I and 35°C for enzyme II, respectively. (b) pH-stability curve: After kept at various pHs for 24 h at 4°C, each enzyme solution was adjusted to the optimum pH and residual activity was measured by the standard method.

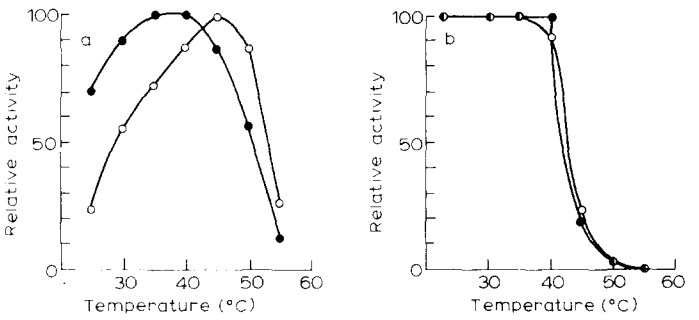

Fig. 3. Effect of temperature on the activity and stability of intracellular dextransucrases I (○) and II (●). (a) Temperature-activity curve: Enzyme activity was assayed at pH 6.4 for enzyme I and pH 5.6 for enzyme II, respectively. (b) Thermal stability curve: After incubation for 10 min at the temperature indicated, the remaining activity was assayed by the standard method.

Effect of temperature

Maximal activity of enzyme I was at 45° C and fell to 80% at 37 and 51° C (Fig. 3a). Enzyme I was stable at 35° C for 10 min, but about 80% of the activity was lost at 45° C (Fig. 3b). Enzyme II had a rather broad optimum temperature range at 35—40° C. Though the optimum temperature of enzyme II was lower than that of enzyme I, thermal inactivation commenced at around 40° C as in the case of enzyme I.

Kinetic properties

Under the standard assay conditions, the release of reducing sugar from sucrose by two isoenzyme preparations was linear for at least 40 min, and the velocity of the reactions was directly proportional to the concentration of the enzyme (data not shown). The effect of sucrose concentration on the velocity of the reaction with enzymes I and II was shown in Fig. 4a. Enzyme I was inhibited at higher sucrose concentration as in the case of the B-512F strain [20]. The K_m for enzyme I was determined to be 10.7 mM, and 250 mM for enzyme II (Fig. 4b,c).

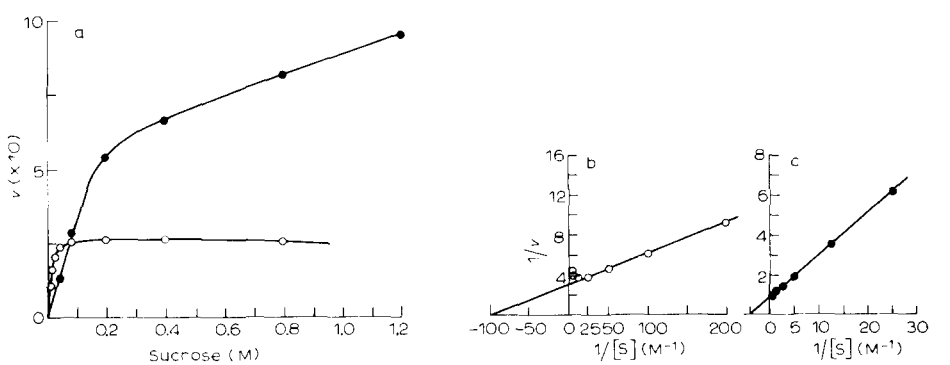

Fig. 4. Substrate saturation kinetics of intracellular dextransucrases I (○) and II (●). (a) Purified enzymes were assayed for 15 min under conditions described in Materials and Methods at the final concentrations of substrate indicated. Double-reciprocal plots of the same data for enzymes I (b) and II (c).

TABLE II

EFFECT OF VARIOUS METAL IONS ON THE ACTIVITY OF INTRACELLULAR DEXTRANSUCRASE FORMS I AND II

Metal ion (1 mM)	Remaining activity (%)	
	Enzyme I	Enzyme II
None	100	100
$CaCl_2 \cdot 2H_2O$	104	87
$CuCl_2 \cdot 2H_2O$	100	97
$MgCl_2 \cdot 6H_2O$	102	103
$MnCl_2 \cdot 4H_2O$	49	70
$FeCl_2$	113	102
$FeCl_3 \cdot 6H_2O$	95	61
$CoCl_2 \cdot 6H_2O$	115	114
$ZnCl_2$	96	93

Effects of metal ions and various reagents

The effects of several metal ions on the enzyme activity were examined (Table II). Both enzymes I and II were slightly activated by 1.0 mM Co^{2+}. Enzyme II was somewhat inhibited by Mn^{2+} and Fe^{3+} and enzyme I was inhibited by Mn^{2+}. As shown in Table III, both enzymes were strongly inhibited by 1.0 mM of sodium dodecyl sulphate and phenylmercuric acetate. However, EDTA and *o*-phenanthroline gave no significant effect on both enzymes.

Effect of dissociating agents

The purified enzymes were very sensitive toward dissociating agent guanidine-HCl, which caused about 60% of inhibition at 0.05 M (Fig. 5a). On the other hand, both enzymes gave different inactivation curve toward urea (Fig. 5b). While enzyme I lost 50% of its activity at 2.4 M of urea, enzyme II was inhibited to the same extent by 1.7 M of urea. The effect of Ca^{2+} and Mg^{2+} was also examined in the presence of urea, but no significant effects were observed.

TABLE III

EFFECT OF VARIOUS REAGENTS ON THE ACTIVITY OF INTRACELLULAR DEXTRANSUCRASE FORMS I AND II

Chemical	Concn of reagent (mM)	Remaining activity (%)	
		Enzyme I	Enzyme II
None	—	100	100
Iodoacetic acid	1	100	98
N-Ethylmaleimide	1	94	105
p-Chloromercuribenzoate	0.1	94	100
Phenylmercuric acetate	1	14	12
Sodium thioglycollate	1	96	96
L-Cysteine	1	104	97
Sodium dodecyl sulphate	1	24	7
Thiourea	1	107	109
o-Phenanthroline	1	85	80
EDTA	1	104	99

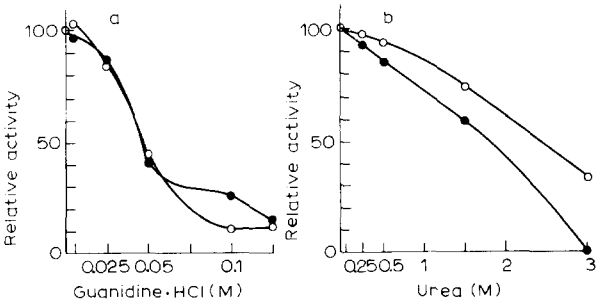

Fig. 5. Effect of dissociating agents on the activity of intracellular dextransucrases I (○) and II (●). Reaction mixture, containing each dissociating agent and the enzyme in a final volume of 1.0 ml, was pre-incubated for 10 min at 20° C and then 10% of sucrose (final concn.) was added to the mixture. After incubation for 15 min at each optimum temperature, the reaction was stopped by adding 0.2 ml of 1 M NaOH solution and the residual activity was measured by the standard method.

Dextransucrase activity in the presence of various dextrans

Effect of the coexistence of the primer dextrans in the various range of concentration on the enzyme activity was examined as shown in Fig. 6. Addition of the B-1299 water-soluble dextran evidently stimulated the activity of enzyme I, while no remarkable effect was observed on the activity of enzyme II (Fig. 6). Though the standard dextran, dextran 10 and 110 seemed to be less effective on this reaction, the water-soluble and -insoluble dextran recovered from the cell fraction of the B-1299 strain also stimulated the activity of enzyme I in proportion to the amount added, ranging 0—6 mg/ml.

Product analysis

The partial acid hydrolysate of non-dialyzable polysaccharides synthesized by both enzymes gave glucose and oligosaccharides of α-1,6-isomaltose series on the paper chromatogram (Fig. 7). Moreover, the acetolysate of the product

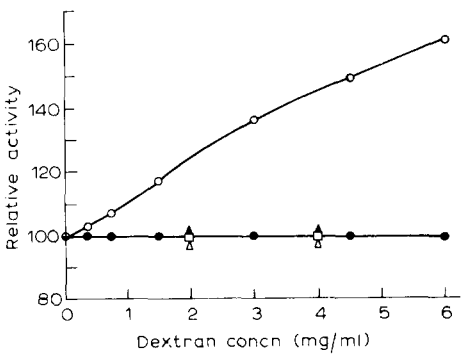

Fig. 6. Effect of various dextrans on the intracellular dextransucrase activity. Dextrans were added to the reaction mixture containing 1% of sucrose, 0.2 M McIlvaine's buffer and the enzyme (0.015—0.020 units). The reaction velocity was measured by the release of reducing sugars. ○, B-1299 dextran and enzyme I; □, standard dextran and enzyme I; △, dextran 10 and enzyme I; ▲, dextran 110 and enzyme I; ●, B-1299 dextran and enzyme II.

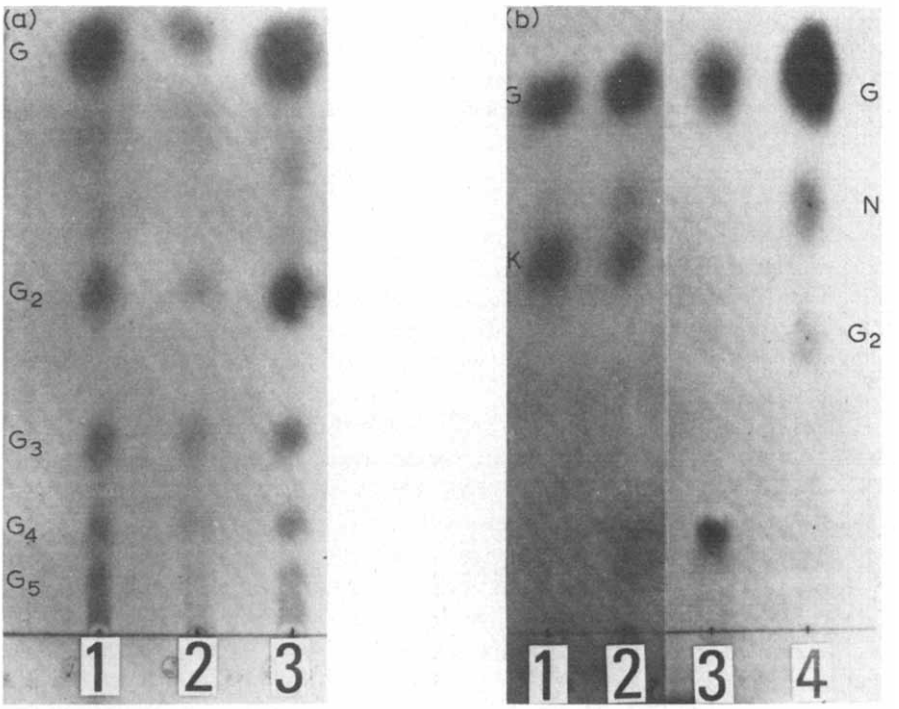

Fig. 7. Chromatograms of polysaccharide fractions after (a) partial acid hydrolysis and (b) acetolysis. Experimental details are described in Materials and Methods. (a) Partial acid hydrolysate. 1, Standard sugars of α-1,6-isomalto-oligosaccharides: G, glucose; G_2, isomaltose; G_3, isomaltotriose; G_4, isomaltotetraose; G_5, isomaltopentaose; 2, Polysaccharide fraction obtained by the pure enzyme I; 3, Polysaccharide fraction obtained by the pure enzyme II. (b) Acetolysate. 1, and 4, Standard sugars: G, glucose; K, kojibiose; N, nigerose; G_2, isomaltose; 2, Polysaccharide fraction obtained by pure enzyme II; 3, Polysaccharide fraction obtained by pure enzyme I.

with enzyme II gave glucose, α-1,2-linked kojibiose, and a small amount of α-1,3-linked nigerose (Fig. 7b). These results strongly suggest that the polymer synthesized by purified enzyme II are also endowed with the structural features of the native B-1299 dextran [9,10]. However, the polymer produced by enzyme I gave glucose and unidentified higher oligosaccharide (R_{Glc} 0.18) by acetolysis (Fig. 7b).

Discussion

The intracellular dextransucrase of *L. mesenteroides* NRRL B-1299 was partially solubilized by Hehre et al. [21] and Suzuki [22]. More recently, however, Smith has claimed the difficulty of this solubilization [23]. Our previous report has demonstrated that 41.3% of the total intracellular enzyme activity was solubilized from the cells, and two enzyme activities were separated from each other by disc gel electrophoresis or DEAE-cellulose column chromatography [6]. Further purification of these enzymes is shown in Table I. Of the purification steps, exclusion of the contaminated dextran, produced concomitantly in cultivation with sucrose, from the enzyme preparation is the

most difficult problem for inducible *Leuconostoc* dextransucrases [6,24]. But in the case of the constitutive enzyme such as *S. sanguis* [7] and *S. mutans* [8], this was favourably avoided by using glucose as a carbon source. Though the solubilized intracellular enzyme preparation of the B-1299 strain contained a large amount of water-soluble dextran, it was not adsorbed on hydroxyapatite and DEAE-cellulose column and was almost completely eliminated from the purified enzyme preparations.

The purified enzymes were homogeneous as judged by column chromatography and disc electrophoresis. In contrast to the extracellular enzymes, these intracellular enzymes gave a single active peak by rechromatography on DEAE-Sephadex A-50 column and could not be separated further. The purification resulted in 32-fold increase of specific activity of enzyme I and 14-fold increase of enzyme II activity, respectively.

Both enzymes I and II showed a single protein band corresponding to the enzyme activity by polyacrylamide-gel electrophoresis. The molecular weights were 69 000 and 79 000 for I and II, respectively, as determined by sodium dodecyl sulphate-gel electrophoresis. Moreover, both enzymes have no subunit structure or at least the subunits were not separated by this procedure.

Some characteristics of purified enzyme II were similar to those of other extracellular enzymes of *L. mesenteroides* [25,26]. All have optima near pH 5.0—5.5 and 30—35°C. In contrast to enzyme II, enzyme I has pH and temperature optima at pH 6.3—6.5 and 45°C, respectively. Moreover, the Michaelis constants of enzymes I and II differed from each other, with K_m values of 10.7 mM and 250 mM. The heat stability of enzymes I and II were similar to each other, but pH stability was different at pHs above 7.0. Ca^{2+} stimulated the extracellular enzyme activity of B-512 strain [27], but have not caused any effects on these enzymes (Table II). Various SH- and chelating reagents were without effect, in contrast to the extracellular enzymes, which were strongly inhibited by 1.0 mM of EDTA. However, urea gave different inactivation curves on enzymes I and II. Enzyme II was inhibited at lower concentrations of urea than enzyme I. These inactivation curves were similar to that reported of the extracellular enzyme of B-512F [28]. Furthermore, guanidine-HCl caused a more drastic inhibition than urea at a concentration of 1/10 order.

Structural analyses revealed that the polymer synthesized by enzyme II contained α-1,2- and α-1,3-glucosidic linkages in addition to α-1,6-linkages, which is the primary characteristic of the B-1299 dextran [9,10]. This polymer, therefore, seems to have essentially the same structure as that of the native dextran. The polymer produced by enzyme I also yielded α-1,6-oligosaccharides series on partial acid hydrolysis, and gave on acetolysis an unknown oligosaccharide with low R_{Glc} value (0.18), besides glucose. Although some structural features of these polysaccharides were shown by these results, more detailed structural studies are necessary for a firm conclusion to be drawn.

The activity of the highly purified dextransucrase of *S. mutans* [29,30] and *S. sanguis* [31] was increased by the addition of various dextrans. On the other hand, an increase in partially purified enzyme activity of the B-512 strain was not so distinct as in the case of the addition of maltose [32—35]. The addition of oligosaccharides such as maltose, isomaltose, and isomaltotriose also resulted in the stimulation of the reaction rate of the streptococcal en-

zymes [29,30]. In the present study, intracellular enzyme I of the two enzymes was stimulated by the addition of the B-1299 dextran, but standard dextran, dextran 10, and dextran 110 showed little stimulative effect. Although the molecular structure of the added dextran seemed to correlate with the stimulation, these effects were not so significant as in the case of the streptococcal enzymes. The mechanism of the enzyme activation remains to be clarified, in particular with respect to the reaction of oligosaccharides formation by the same enzyme (data not shown).

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