An Isomaltotriose-producing Dextranase from *Flavobacterium* sp. M-73: Purification and Properties

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An isomaltotriose-producing dextranase II, detected in the culture supernatant of *Flavobacterium* sp. M-73, was purified to an electrophoretically pure state. Successive chromatography on hydrophobic columns of Amberlite CG-50 and aminooctyl-Sepharose was very effective as the first step of purification. Further purification of the enzyme was performed by affinity column chromatography on isomaltotriose-Sepharose and preparative polyacrylamide gel electrophoresis.

The purified enzyme was shown to be a monomer and had a molecular weight of 114,000. Dextranase II was most active at pH 7.0 and 35°C. It was stable at 4°C for 24 hr over a pH range of 6.5~12.0 and up to 35°C on heating for 10 min. This enzyme had a strict specificity for consecutive α -1,6-glucosidic linkages and readily hydrolyzed clinical dextran and Sephadex gels. The degree of hydrolysis of clinical dextran was 31% expressed as apparent conversion into D-glucose. The amount of isomaltotriose in the hydrolyzate was determined to be 63%.

In a previous paper,¹⁾ we reported that two different types of dextranase, tentatively named dextranases I and II, were produced by a strain (M-73) belonging to a *Flavobacterium* species. The former enzyme (dextranase I) was purified to a satisfactorily pure state²⁾ and was characterized as a dextran α -1,2 debranching enzyme (the systematic name: dextran 2-glucosylhydrolase).³⁾ The latter enzyme (dextranase II) was shown to produce mainly isomaltotriose from various dextrans,¹⁾ but this enzyme was not examined further. The present paper describes the purification of and some properties of dextranase II.

MATERIALS AND METHODS

Materials. Clinical dextran (molecular weight, $75,000 \pm 25,000$) was provided by Meito Sangyo Co., Japan. This dextran contains 95% α -1,6-glucosidic linkages. Aminooctyl-Sepharose 4B was prepared by the method of Shaltiel and Er-El.⁴⁾

Microorganism and culture. The strain used in this study

(*Flavobacterium* sp. M-73) was the same as the one used in our previous study.¹⁾ The culture medium was the same as described in a previous paper,¹⁾ except that clinical dextran (0.5%) was used instead of B-1299 soluble dextran (0.3%). A stock culture of strain M-73 was inoculated into 10 ml of the culture medium and incubated for 48 hr at 30°C with shaking. The preculture was then transferred to 1 liter of the medium in 3 liter flasks and cultivated for 48 hr at 30°C on a reciprocating shaker.

Assay system for dextranase II. The standard assay system consisted of 0.5 ml of 0.5% clinical dextran solution in 50 mM phosphate buffer (pH 7.0) and 0.5 ml of suitably diluted enzyme solution. The mixture was incubated for 10 min at 30°C. The reaction was terminated by the addition of 1.0 ml of alkaline copper reagent, and the reaction mixture was assayed for reducing sugar by the Nelson-Somogyi method.^{5,6)} One unit of dextranase II was defined as the amount of enzyme that catalyzed the liberation of reducing sugar equivalent to 1.0 μ mol of D-glucose per min under the standard conditions.

Polyacrylamide gel electrophoresis. Analytical gel electrophoresis was carried out according to the method of Davis⁷⁾ with an acrylamide concentration of 7.5%. The electrophoresis was continued for 2 hr at 2 mA per column at 4°C. Preparative gel electrophoresis was carried out with

5% gel. Electrophoresis in the presence of sodium dodecyl sulfate (SDS) was carried out according to the method of Weber and Osborn⁸⁾ for 6 hr at 2 mA per column at room temperature. Protein was stained with Coomassie brilliant blue. Gel isoelectric focusing was performed according to the procedure described by Righetti and Drysdale⁹⁾ by a pH gradient with Ampholine (pH 3.0~7.0).

Analytical methods. Protein was determined by the method of Lowry *et al.*¹⁰⁾ or by absorbance at 280 nm. Total carbohydrate was determined by the phenol-sulfuric acid method¹¹⁾ and represented as D-glucose.

Paper chromatography. Ascending paper chromatography was performed on Toyo No. 50 filter paper at room temperature with 70% aqueous 1-propanol (v/v). Spots were detected by the alkaline silver nitrate dip-procedure.¹²⁾

Preparation of isomaltotriose-Sepharose 4B. Isomaltotriose-Sepharose 4B gel was prepared according to the method of Jeffrey *et al.*¹³⁾ Isomaltotetraose was coupled with ethylenediamine at 30°C for 48 hr and reduced with NaBH₄. The unreacted ethylenediamine was removed by gel filtration on a Bio-Gel P-2 column and the resulting isomaltotriose-aminoethane derivative was then coupled with CNBr-activated Sepharose 4B at 25°C for 16 hr.

RESULTS

Production and purification of dextranase II

The course of the production of dextranase

II was similar to that of the dextran α -1,2 debranching enzyme (dextranase I).²⁾ The activity of extracellular dextranase II reached a maximum about 36 hr after inoculation.

The culture broth (7 liters) was centrifuged at $10,000 \times g$ for 20 min. The supernatant was subjected to the following purification steps. All the purification steps were carried out at 4°C.

Step 1. Amberlite CG-50 column chromatography. The supernatant was adjusted to pH 4.2 with 0.5 M acetate buffer (pH 4.0) and applied to an Amberlite CG-50 column¹⁴⁾ (7×35.5 cm) previously equilibrated with the same buffer. The column was washed with 0.5 M acetate buffer (pH 4.0) and then eluted with 0.5 M acetate buffer (pH 5.5). Fractions, 400 to 600 ml each, were collected and the fractions containing dextranase II activity were pooled and dialyzed against 10 mM Tris-HCl buffer (pH 8.0). By this step, the specific activity of the enzyme was increased 7.7-fold over the culture supernatant (Fig. 1).

Step 2. Aminoethyl-Sepharose 4B column chromatography. The dialyzed solution from Step 1 was applied to an aminoethyl-Sepharose 4B column (2.5×12 cm) previously equilibrated with 10 mM Tris-HCl buffer (pH

FIG. 1. Representative Elution Pattern of Dextranase II from the Amberlite CG-50 Column.

The culture supernatant (12 units/250 ml) from *Flavobacterium* sp. M-73 was applied to the column (2.5×10 cm) and 4 ml fractions were collected. For preparative purposes, a larger column (7×35.5 cm) was used, as described in the text. Symbols: ●, enzyme activity (absorbance at 500 nm); ×, pH of acetate buffer; ----, absorbance at 280 nm.

FIG. 2. Elution Pattern of Dextranase II from the Aminoethyl-Sepharose 4B Column.

Symbols: ●, enzyme activity; -----, absorbance at 280 nm; -----, NaCl concentration.

8.0). The column was eluted with a linear gradient of 0~1.0 M NaCl in the same buffer (Fig. 2). The fractions with the enzyme activity were pooled and dialyzed against deionized water.

Step 3. Isomaltotriose-Sepharose 4B affinity column chromatography. The dialyzed solution from Step 2 was adjusted to pH 11.5 with 50 mM borate buffer (pH 11.5) and allowed to stand at 4°C for 24 hr. By this treatment, the concomitant α -1,2 debranching enzyme activity was completely inactivated. The enzyme solution thus treated was dialyzed against 10 mM Tris-HCl buffer (pH 8.0) and the dialyzed solution was applied to an affinity column (1.4 $\times$ 6.2 cm) of isomaltotriose-Sepharose 4B previously equilibrated with the same buffer containing 0.1 M NaCl. The column was washed with the same buffer used for equilibration and then eluted with Tris-HCl buffer (pH 8.0) containing 0.1 M NaCl and 0.1% clinical dextran (Fig. 3). Although a part of dextranase II activity was eluted at the breakthrough fraction, most of the enzyme

FIG. 3. Elution Pattern of Dextranase II from the Isomaltotriose-Sepharose 4B Column.

Symbols: ●, enzyme activity; -----, absorbance at 280 nm. The arrow indicates elution with 0.1% clinical dextran in 10 mM Tris-HCl buffer (pH 8.0) containing 0.1 M NaCl.

activity was biospecifically adsorbed to the affinity column through the ligand of isomaltotriose and was eluted with 0.1% clinical dextran solution.

Step 4. DEAE-cellulose column chromatography. The effluents with the enzyme activity were pooled and then applied to a DEAE-cellulose column (1.4 $\times$ 6.0 cm) previously equilibrated with 10 mM Tris-HCl buffer (pH 8.0). After washing with the same buffer, the column was eluted with a gradient of 0~1.0 M NaCl in the same buffer. The fractions with the enzyme activity were pooled and concentrated with a collodion bag (Sartorius membrane filter, collodion bag 12). By this procedure, the enzyme activity and clinical dextran were completely separated from each other.

Step 5. Preparative polyacrylamide gel electrophoresis. The concentrated enzyme solution from Step 4 was dialyzed against 10 mM Tris-HCl buffer (pH 8.0) and the dialyzed solution was subjected to preparative gel electrophoresis. The sliced gel sections (2 mm) were extracted with 0.5 ml of 10 mM Tris-HCl buffer (pH 8.0) for 24 hr at 4°C and the enzyme

FIG. 4. Analytical Gel Electrophoresis of Dextranase II.

a: Enzyme activity (top) and protein bands (middle: Lane #1) of the DEAE-cellulose fraction. The same fraction was analyzed by an acrylamide gel containing 0.1% clinical dextran (bottom: Lane #2).

b: Enzyme activity (top) and protein band (bottom) of the purified enzyme after the preparative gel electrophoresis step.

TABLE I. PURIFICATION OF DEXTRANASE II FROM *Flavobacterium* sp. M-73

Steps	Protein (mg)	Activity (units)	S.A. ^a (u/mg)	Purification (fold)	Recovery (%)
Broth sup	1430.0	336	0.234	1	100.0
Broth sup (pH 4.2)	—	161	—	—	47.9
Amberlite CG-50	65.8	119	1.81	7.7	35.4
Aminooctyl-Sepharose	3.34	67.2	20.1	85.9	20.0
pH treatment	—	65.2	—	—	19.4
IM ₃ -Sepharose ^b	—	50.4	—	—	15.0
DEAE-cellulose	1.13	38.5	34.0	145.3	11.5
Preparative PAGE ^c	0.28	10.1	36.3	155.1	3.0

^a Specific activity (units/mg protein).

^b Isomaltotriose-Sepharose 4B.

^c Polyacrylamide gel electrophoresis.

activity was assayed for each section. As the gel electrophoresis showed at least seven protein bands with overlapping enzyme activities (Fig. 4a), the main component with the highest activity (R_m 0.42) was extracted from the gel. The extracts were pooled and concentrated as above.

The purification steps of dextranase II are summarized in Table I. The finally purified dextranase II had a specific activity of 36 units per mg protein and was obtained in a final yield of 3%. The enzyme was purified 150-fold

over the culture supernatant.

Purity and molecular weight of dextranase II

As shown in Fig. 4b, the final enzyme preparation gave a single protein band and the dextranase activity coincided well with the position of the protein band on the gel. The molecular weight of this enzyme was estimated to be 116,000 by SDS-polyacrylamide gel electrophoresis (Fig. 5a) and 111,000 by gel filtration on Sepharose CL-6B column (Fig. 5b). According to the method of Hedrick-Smith,¹⁵⁾

FIG. 5. Determination of Molecular Weight of Dextranase II by SDS-Gel Electrophoresis (a), Gel Filtration on Sepharose CL-6B (b) and the Hedrick-Smith Method (c).

The molecular weight of standard proteins used in (a): 1, RNA polymerase β' , 165,000; 2, RNA polymerase β , 155,000; 3, rabbit muscle glycogen phosphorylase *b*, 98,400; 4, albumin (bovine), 67,000; 5, RNA polymerase α , 39,000; 6, chymotrypsinogen, 25,000. (b): 1, ovalbumin, 45,000; 2, albumin (bovine), 67,000; 3, aldolase, 158,000; 4, ferritin, 450,000. (c): 1, chymotrypsinogen, 25,000; 2, ovalbumin, 45,000; 3, albumin (bovine) monomer, 67,000; 4, dimer, 134,000; 5, trimer, 201,000.

the molecular weight of the enzyme was estimated to be 114,000 by electrophoretic analysis (Fig. 5c). Therefore, dextranase II was shown to have no oligomeric forms and the molecular weight was approximately 114,000 by three different methods. The isoelectric point of the enzyme was at pH 4.1 (data not shown).

Multiple forms of dextranase II

Electrophoretic analysis indicated that seven protein bands were detected from the enzyme preparation (Step 3) after the affinity column chromatography with isomaltotriose-Sepharose (Fig. 4a). The multiplicity of dextranase II was examined by gel electrophoresis with various gel concentrations. As shown in Fig. 6, seven proteins (A to G) were detected by staining with Coomassie blue. Molecular weights of these proteins were in the range of 131,000 (band A) to 97,000 (band G). Protein bands of C, D, E and F gave parallel lines (slope 8.2) indicating that these proteins differ in net charge but have almost the same molecular size (114,000) as defined by Hedrick-Smith.¹⁵⁾ When this preparation was subjected to affinity-gel electrophoresis with 7.5% gel containing 0.1% clinical dextran, a single pro-

FIG. 6. Effect of Gel Concentration on the Mobility of Dextranase II.

Symbols A to G correspond to the protein bands shown in the bottom gel. The average value of the slopes was 8.7 ± 1.0 .

tein band (R_m 0.32) was detected (Fig. 4a lane #2). These results suggest that all of these proteins are related with each other in mo-

FIG. 7. Effects of pH and Temperature on Dextranase II.

(a) Optimal pH. The enzyme activity was determined with 0.1 M McIlvaine buffer. (b) pH stability. ●, 50 mM McIlvaine buffer; ○, 50 mM borate buffer. (c) Optimal temperature. (d) Thermal stability.

lecular size and affinity for the dextran.

General properties of dextranase II

The following experiments were carried out with the finally purified preparation (Step 5). Dextranase II was most active at pH 7.0 (Fig. 7a) and was stable in the range of pH 6.5 to 12.0 at 4°C for 24 hr (Fig. 7b). The temperature optimum for the enzyme activity was at 35°C (Fig. 7c) and the purified enzyme was stable at temperatures below 35°C for 10 min (Fig. 7d). Table II shows the effects of metal ions and chemicals on the enzyme activity. The enzyme was strongly inhibited by Cu^{2+} , Hg^{2+} , and Zn^{2+} (2 mM), whereas Mg^{2+} , Mn^{2+} , and Ca^{2+} caused 54 to 22% of activation. Among the chemicals tested, EDTA showed 33% in-

TABLE II. EFFECTS OF METAL IONS AND CHEMICALS ON THE ACTIVITY OF DEXTRANASE II

Final concentration of metal ions (used in the chloride form) and chemical reagents was 2.0 mM, except for *p*-chloromercuribenzoate^a (0.2 mM).

Compound	Relative activity (%)	Compound	Relative activity (%)
None	100	Ca^{2+}	122
Zn^{2+}	18	Al^{3+}	97
Mn^{2+}	132	CH_2ICOOH	96
Mg^{2+}	154	<i>p</i> CMB ^a	89
Cu^{2+}	0	EDTA	67
Fe^{3+}	66	δ -Glucono-lactone	100
Co^{2+}	88	Sorbitol	95
Hg^{2+}	3	NaF	97

TABLE III. SUBSTRATE SPECIFICITY OF DEXTRANASE II

Dextranase II (0.02 units) was mixed with 0.1% of each substrate and 50 mM phosphate buffer (pH 7.0), and incubated for 24 hr at 30°C.

Substrate	Major linkage	Relative activity (%)
Clinical dextran	α -1,6	100
Glycogen	α -1,4	0
Soluble starch	α -1,4	0
Pachyman	β -1,3	0
β -1,6-Glucan	β -1,6	0
CM-Sephadex	α -1,6	0
DEAE-Sephadex	α -1,6	0
Pullulan	α -1,4, α -1,6	0
Sephadex G-15	α -1,6	10.2
Sephadex G-75	α -1,6	52.5
Sephadex G-200	α -1,6	69.2

hibition while D-glucono-1,5-lactone had no significant effect on the activity. Activation by Mg^{2+} was completely repressed by the addition of 5 mM EDTA (data not shown).

Substrate specificity of dextranase II

Several glucans containing various types of glucosidic linkages were digested with dextranase II, and the degree of hydrolysis of each substrate was determined. The results are summarized in Table III. Glucans without α -1,6-linkages were not hydrolyzed by this enzyme at all. Moreover, dextran derivatives such as DEAE-Sephadex and CM-Sephadex were not susceptible to dextranase II although cross-linked Sephadex gels were degraded to a large extent. Paper chromatography of the hydrolyzate of the clinical dextran with dextranase II showed that isomaltotriose was the major product (Fig. 8). Besides isomaltotriose, isomaltose, isomaltotetraose, and an unknown sugar were detected after 8 hr digestion. Based on the structure of the substrate clinical dextran, it is suggested that the unknown sugar may have one α -1,3 branched glucose on the linear isomaltotriose structure. The hydrolysis of the clinical dextran with dextranase II reached a maximum after 10 hr incubation and the degree of hydrolysis was 31.0% expressed as ap-

FIG. 8. Paper Chromatogram of the Digest of Clinical Dextran with Dextranase II.

STD Glc, D-glucose; IM₂, isomaltose; IM₃, isomaltotriose; IM₄, isomaltotetraose.

TABLE IV. OLIGOSACCHARIDE COMPOSITION IN THE HYDROLYZATE OF CLINICAL DEXTRAN WITH DEXTRANASE II

Sugar produced	Relative (%) as D-glucose	Relative molar ratio
Glucose	0.9	1
Isomaltose	0.4	0.2
Isomaltotriose	63.4	25.2
Branched tetraose	4.6	3.4
Isomaltotetraose	7.0	—
Higher oligomer	23.7	—
Total	100.0	—

parent conversion into D-glucose. The sugar component in the digest was analyzed by paper chromatography and the amount of isomaltotriose in the hydrolyzate was determined to be 63.4% (Table IV). The relative molar ratio of D-glucose, isomaltose, isomaltotriose, and tetrasaccharide mixture (total of branched-saccharide and isomaltotetraose) was calculated to be 1 : 0.2 : 25.2 : 3.4 (Table IV).

DISCUSSION

An attempt was made to purify dextranase II by affinity chromatography on Sephadex gel, but purification by this method was not successful. The susceptibility of Sephadex gel to dextranase II caused us to search for another affinity gel stable to this enzyme. For this purpose, we prepared three different types of affinity gels and tested their specificity for this enzyme. These gels are: Isomaltotriose-Sepharose 4B, prepared by coupling of CN-Sepharose 4B and isomaltotetraose^{13,16}; Isomaltotriose-hexamethylene-Sepharose 4B, prepared by carbodiimide coupling of aminohexyl-Sepharose 4B and isomaltotrionic acid¹⁷; and isomaltotriose-Sepharose 6B, prepared by treatment of Sepharose 6B and butanediol-diglycidylether (epoxy reagent)¹⁸ followed by coupling with isomaltotriose. Of these gels, the isomaltotriose-Sepharose 4B gel was most effective for the purification of dextranase II.

The final products from non-branched dextrans by dextranase are, in most cases, isomaltose and/or glucose.^{19,20} Only a few dextranses are reported to produce isomaltotriose as the major product. Sugiura *et al.*²¹ reported that an exo-dextranase from *Brevibacterium fuscum* produced isomaltotriose as the sole hydrolysis product. The degree of hydrolysis of a virtually non-branched dextran with this enzyme was 14% expressed as apparent conversion into D-glucose. The degree of hydrolysis of clinical dextran with our enzyme was 31% expressed as D-glucose and the content of isomaltotriose in the hydrolyzate was 63.4%. These results suggest that the *Flavobacterium* dextranase, dextranase II, yields isomaltotriose more effectively than the exo-dextranase from *Brevibacterium*. The action pattern and substrate specificity of the dextranase II will be reported in detail in our subsequent paper.

Another isomaltotriose-producing enzyme was reported by Pulkownik and Walker.²² This enzyme was an extracellular endo-dextranase produced by *Streptococcus mutans*

K1-R. The hydrolysis of virtually non-branched dextran with this enzyme was almost complete, but the soluble glucan of *S. mutans* was hydrolyzed only to a limited extent. The activity of this enzyme seems to be comparable to that of our enzyme, although general properties of these two enzymes are somewhat different from each other. Whereas the enzymes of *Brevibacterium* and *Streptococcus* are both extracellular enzymes, major part of the enzyme activity of dextranase II from *Flavobacterium* was retained in the cells as a cell-bound enzyme. Such association of the enzyme with the cells may be of great advantage in utilizing this enzyme as an immobilized preparation.

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