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Purification and Characterization of an Endo-β-Mannanase from *Aeromonas* sp. F-25

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An endo-β-mannanase (1, 4-β-D-mannan mannanohydrolase, EC 3. 2. 1. 78.) was isolated from the culture fluid of strain no. F-25 of *Aeromonas hydrophila* subspecies anaerogenes, and purified about 440-fold by ammonium sulfate precipitation, and column chromatographies on DEAE-Sephadex A-50, Amberlite CG-50, Sephadex G-100, and hydroxylapatite. The final enzyme preparation was regarded as being homogeneous on polyacrylamide gel electrophoresis. The enzyme had a molecular weight of 24,000, pI of 5.6, pH optimum of 5.5, and was stable in a pH region of 5.0 to 9.0 and at temperatures below 45°C. The enzyme hydrolyzed at random the internal β-1, 4-linkages in mannotetraose and larger oligosaccharides, and in codium mannan, coffee mannan, konjac glucomannan and guar gum galactomannan to give various sizes of oligosaccharides. It did not act on mannobiose and mannotriose.

INTRODUCTION

β-Mannan, as well as cellulose and xylan, is an important structural component of plant cell walls. It is distributed in several seaweeds (Jones, 1950; Love and Percival, 1964), besides in terrestrial plant seeds such as ivory nut (Aspinall et *al.*, 1953) and coffee beans (Wolfrom et *al.*. 1961). For the structural analysis of this polysaccharide, β-mannanase should be a useful tool.

Recently we isolated a bacterium which secretes exo- and endo-β-mannanases into the culture fluid from natural habitat (Araki and Kitamikado, 1978, 1981). The exo-β-mannanase from the bacterium was a ***novel*** glycosidase that removes mannobiose residue successively from the non-reducing end of the β-mannan molecule (Araki and Kitamikado, 1982). This paper describes the purification and characterization of the endo-β-mannanase being another enzyme.

MATERIALS AND METHODS

Microorganism

The organism used was strain no. F-25 of ***Aeromonas*** *hydrophila* subspecies *anaerogenes* isolated from intestinal contents of rainbow trout ***Salmo*** *gairdnerii* in

1973,

Materials

Codium mannan (Love and Percival, 1964), coffee mannan (Wolfrom et al., 1961), konjac glucomannan (Sugiyama et al., 1972), and guar gum galactomannan (Heyne and Whistler, 1948) were prepared from *Codium fragile*, *coffee* beans, *Amorphophallus konjac*, and guar gum, respectively by the procedures reported previously. Konjac powder was obtained commercially.

Mannooligosaccharides were prepared by the method described in the previous paper (Araki and Kitamikado, 1982).

Enzyme assay

The reaction mixture consisted of 10 mg of codium mannan, 2.0 ml of 50 mM sodium acetate buffer, pH 5.5, and 0.5 ml of the enzyme solution. After incubation at 37°C for 10 min, the reaction was stopped by heating the mixture in a boiling water bath for 5min. Reducing sugar produced was then determined by the Somogyi-Nelson's method (Somogyi, 1952) and expressed as mannose. One unit of β-mannanase is defined as the activity that produces reducing capacity equivalent to 1 μmol of mannose per min under the above condition.

Purification of endo-β-mannanase

Step I. Preparation of culture fluid

The organism was cultured at 25°C for 3 days in Iiquid medium containing 1.0 % peptone, 0.1% yeast extract, 0.05 % $MgSO_4 \cdot 7\ H_2O$, 0.2 % K_2HPO_4, 0.05 % KH_2PO_4, 0.5 % NaCl, and 0.5 % konjac powder, pH 7.0. The culture was cenfrifuged at 15,000 g for 30 min. From 8,000 ml of liquid culture, 7,700 ml of clear culture fluid was obtained. All the remaining steps were conducted between 0 and 5°C, and centrifugations were carried out at 17, 000 *g* for 30 min.

Step 2. Ammonium sulfate precipitation

The culture fluid was adjusted to 75 % saturation with solid ammonium sulfate, and allowed to stand overnight. The precipitate was collected by centrifugation, and dissolved in 200ml of distilled water.

Step 3. Chromatography on DEAE-Sephadex A-50

The enzyme solution from the previous step was dialyzed against 10 mM sodium acetate buffer, pH 6.0, containing 2mM calcium acetate for 2 days. The dialyzed enzyme solution was applied to a column (2.2×42 cm) of DEAE-Sephadex A-50 equilibrated with the same buffer. After washing with 6 bed volumes of the same buffer, the column was eluted with 40 mM sodium acetate buffer, pH 6.0, containing 2 mM calcium acetate. The minor enzyme activity appeared in tube numbers 52 to 85 was due to the presence of exo-β-mannanase. The procedures of purification and characterization of the exo-enzyme was described in the previous paper (Araki and Kitamikado, 1982). The column was finally eluted with a continuous linear gradient between 500 ml of 40 mM sodium acetate buffer, pH 6.0, containing 2 mM calcium acetate and 500

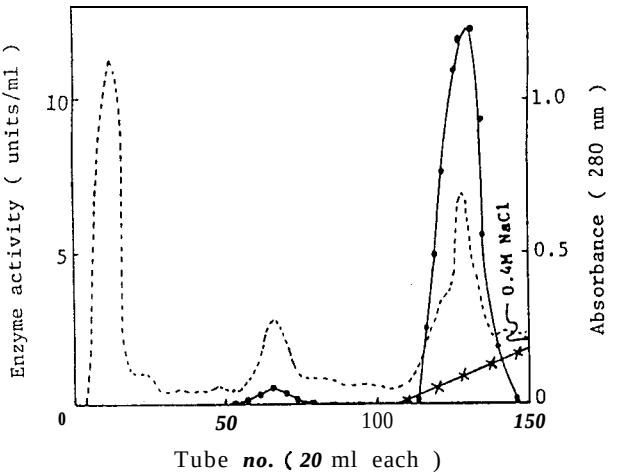

Fig. 1. Chromatography of endo-β-mannanase on a DEAE-Sephadex A-50 column. —•—, enzyme activity; - - -, absorbance at 280 nm ; —×—, concentration of NaCl.

ml of 0.4 M NaCl in the same buffer. The endo-β-mannanase emerged in tubes 116 to 140 as main enzyme fraction (Fig. 1). The endo-β-mannanase was further purified in the following steps.

Step* 4. *Chromatography on DEAE-Sephadex A-25

The endo-β-mannanase solution obtained above was dialyzed against 20 mM sodium acetate buffer, pH 6.0, containing 2 mM calcium acetate. The dialyzed enzyme was applied to a column (2 ×32 cm) of DEAE-Sephadex A-25 equilibrated with the same buffer. The column was eluted with a linear gradient between 400 ml each of 20 mM sodium acetate buffer, pH 6.0, containing 2 mM calcium acetate and 0.6 M NaCl in the same buffer. Active fractions were pooled and concentrated to 20 ml.

Step 5. ***Chromatography on Amberlite CG-50***

All the buffers used in this step contained 1 mM mercaptoethanol. The pooled enzyme solution was dialyzed against 20 mM sodium acetate buffer, pH 5.2, and then applied to a column (1.5 x20 cm) of Amberlite CG-50 equilibrated with the same buffer. The column was eluted a linear gradient between 250 ml of 10 mM sodium acetate buffer, pH 5.2, and 250ml of 1. OM acetate buffer, pH 6.2. Active fractions were combined and, after the addition of calcium acetate to a final concentration of 2 mM, concentrated to 6ml.

Step* 6. Gel *filtration on Sephadex G-100 column

The above enzyme solution was applied to a column (2.2~ 100 cm) of Sephadex G-100 equilibrated with 20 mM sodium acetate buffer, pH 6.0, containing 2 mM calcium acetate and 0.1 M NaCl. The column was eluted with the same buffer. Active fractions were pooled and concentrated to 6ml.

Step* 7. *Chromatography on hydroxylapatite

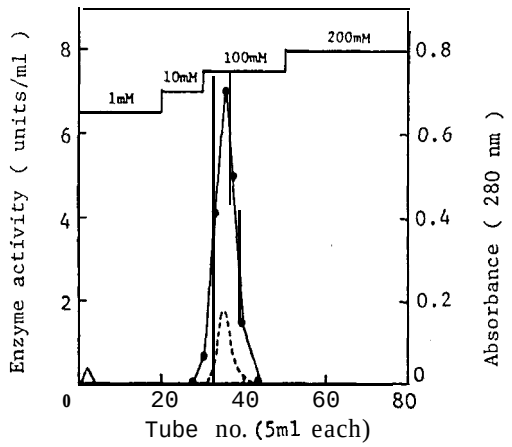

Fig. 2. Chromatography of endo-β-mannanase on a hydroxylapatite column. -•-, enzyme activity; - - - - -, absorbance at 280 nm; ——, concentration of phosphate buffer.

The enzyme solution from the previous step was dialyzed against 1 mM phosphate buffer, pH 6.8. The dialyzed enzyme was applied to a column (1.2 x 10 cm) of hydroxylapatite equilibrated with the same buffer. Elution was performed by a stepwise increase in the concentration of phosphate buffer (Fig. 2). Fractions showed the enzyme activity were pooled and dialyzed against 50mM sodium acetate buffer, pH 6.0. The dialyzed solution was used as the purified endo-β-mannanase.

Analytical methods

Polyacrylamide disc gel electrophoresis was routinely performed with 7 % gel in Tris-glycine buffer, pH 8.3, (Davis, 1964). Protein band was stained with Coomassie Brilliant Blue R-250.

Isoelectric focusing was carried out in a 110 ml column (Vesterberg and Svensson, 1966). Ampholine concentration was 1 % with a pH range from 5.0 to 7.0 in a sucrose gradient and the voltage was 600 volts. The run was performed at 2°C for 4% hr and fractions of 2 ml were collected.

Thin-layer chromatography of mannooligosaccharides was performed on Wako gel plate and developed with *n*-butano : isopropanol : water (50 : 25 : 20, v/v). The saccharides were visualized by spraying the plate with an anisaldehyde reagent.

RESULTS AND DISCUSSION

Purification and homogeneity of endo-β-mannanase

The purification and yield of the endo-β-mannanase are summarized in Table 1. The purified enzyme preparation gave a single protein band on polyacrylamide disc gel electrophoresis (Fig. 3).

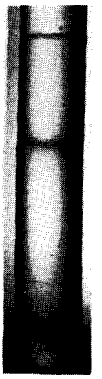

Fig. 3. Polyacrylamide gel electrophoresis of the final preparation of endo-β-mannanase. The purified enzyme was subjected to disc electrophoresis in 7.0 % polyacrylamide gel at pH 8.3. Approximately 50 μg of the enzyme was applied per tube. A current of 3 mA was supplied per tube and electrophored at room temperature. CBB, Coomassie brilliant blue R-250; (+), anode; (-), cathode.

Table 1. Purification of endo-β-mannanase from the culture fluid of *Aeromonas* sp. F-25.

Procedure	Total protein (mg)	Total activity (units $\times 10^{-3}$)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Culture fluid	38,300	17. 1	0.446	100	1
$(NH_4)_2SO_4$ precipitation	374	12.2	32.6	71.3	73.
DEAE-Sephadex A-50	134	9.3	69.4	54. 4	156
DEAE-Sephadex A-25	60. 4	7. 79	129	45. 6	289
Amberlite CG 50	17. 5	3. 22	184	18. 8	413
Sephadex G 100	4. 8	0.965	201	5. 61	451
Hydroxylapatite	2.7	0.532	197	3. 11	442

Characterization of endo-β-mannanase

Isoelectric point

The isoelectric point of the enzyme was pH 5.6 on isoelectric focusing (Fig. 4).

Molecular weight

The molecular weight of the enzyme was estimated on a column of Sephadex G-100 which had been calibrated with the standard proteins. From logarithmic plots of the molecular weights versus the elution volumes of the proteins, it was estimated to be 24,000 (Fig. 5).

Effects of pH

The optimal pH of the enzyme was determined by using 50mM sodium acetate (pH 3.2-6.0), phosphate (pH 5.0-7.0), and Tris-HCl (pH 7.0-8.0) buffers in the assay system. The maximal activity was observed at approximately pH 5.5 (Fig. 6). The stability of the enzyme at various pHs was studied by

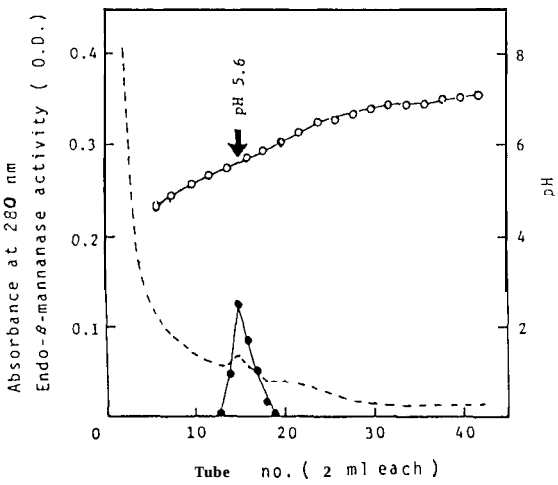

Fig. 4. Isoelectric focusing of endo-p-mannanase. —●—, enzyme activity; - - - - -, absorbance at 280 nm; —○—, pH.

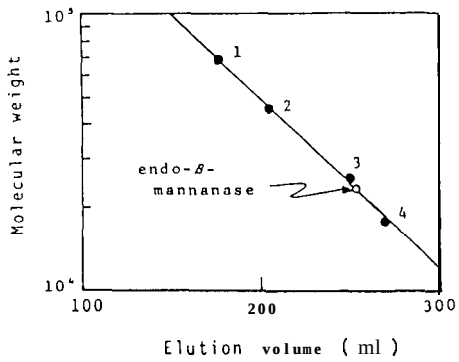

Fig. 5. Molecular weight determination of endo-p-mannanase by Sephadex G-100 column chromatography. 1, Bovine serum albumin (67,000) ; 2, Ovalbumin (45,000) ; 3, Chymotrypsinogen-A (25,003) ; 4, Myoglobin (17,800) .

incubating it in 50 mM sodium acetate (pH 3.2 6. 0), Tris-HCl (pH 7. 0-9. 0), and glycine-NaOH (pH 9.0-11) buffers at 20°C for 20 hr. Then, the remaining enzyme activity was assayed under the standard condition. The enzyme was stable between pH 5.0 to 9.0 (Fig. 7).

Effect of temperature

The enzyme was kept in 50mM sodium acetate buffer, pH 6.0, for 15 min at various temperatures. The enzyme retained full activity at temperatures up to 45°C, but lost its activity completely above 55°C.

Inhibitors

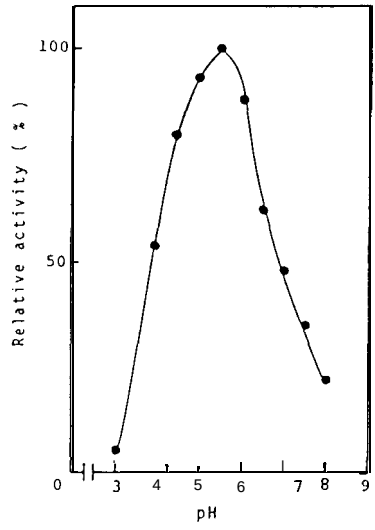

Fig. 6. pH-activity curve of endo-β-mannanase. pH 3.2-6.0, acetate buffer; pH 5.U-7.G, phosphate buffer; pH 7.0-8.0, Tris-HCl buffer.

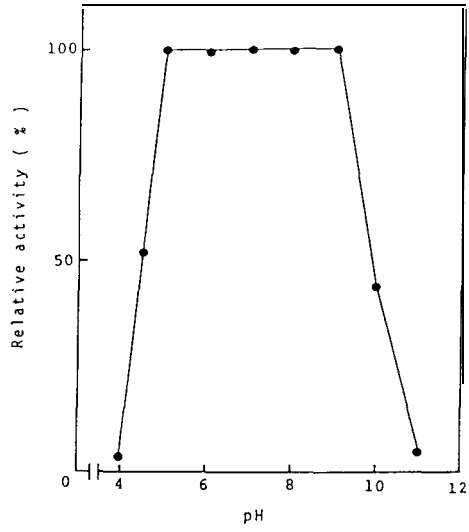

Fig. 7. pH-stability curve of endo-p-mannanase. pH 3.2-6.0, acetate buffer; pH 7.0-9.0, Tris-HCl buffer; pH 9.0-11.0, glycine-NaOH buffer.

The *effects* of metal ions and other materials on the enzyme were investigated. The activity of the enzyme was almost completely inhibited by 1 mM concentration of Ag^{+}, Hg^{2+}, and Cu^{2+}, while Pb^{2+}, Zn^{2+}, Fe^{3+}, and PCMB produced 13, 14, 77, and 25 % inhibition, respectively. Na^{+}, Mn^{2+}, Mg^{2+} and EDTA did not have any significant effects on the activity of the enzyme. Ca^{2+} stim-

ulated the action at the same concentration.

Action pattern of endo-β-mannanase

Action of the enzyme on natural substrates

The enzyme attacked codium and coffee mannans to give mannobiose, mannotriose, and larger oligosaccharides. The enzyme could also hydrolyze konjac glucomannan and guar gum galactomannan (Fig. 8).

Action of the *enzyme on several mannooligosaccharides*

The enzyme could act on mannotetraose and larger mannooligosaccharides but not on mannobiose and -triose. The hydrolysis products from manno-

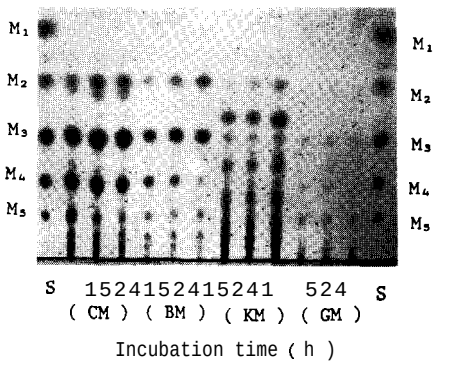

Fig. 8. Thin layer chromatogram of hydrolyzates of several β-mannanas with endo-β-mannanase: To 2 ml of 0.5 % CM, BM, KM or GM dissolved in 10 mM sodium acetate buffer, pH 5.5, was added 1 ml of the enzyme solution (1.0 unit), and the mixture was incubated at 37°C. M_1–M_5, mannose-mannopentaose ; S, standard; CM, codium mannan; BM, coffee mannan; KM, konjac glucomannan; GM, guar gum galactomannan.

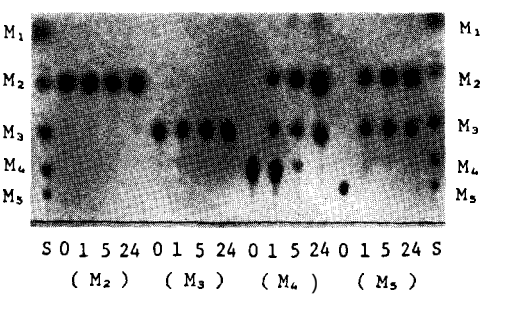

Fig. 9. Thin layer chromatogram of hydrolyzates of several mannooligosaccharides with endo-p-mannanase. The reaction mixture consisted of 0.5 ml of 10 mM sodium acetate buffer, pH 5.5, containing 1.0 % mannooligosaccharide and 0.5 ml of the enzyme solution (1 unit), and it was incubated at 37%. M_1–M_5, mannose-mannopentaose ; S, standard.

tetraose was mannose, mannobiose, and -triose. This shows that the enzyme attacks the β-1, 4-mannosidic linkage that was present apart two or three mannose residue from the non-reducing end in mannotetraose. Mannopentaose was hydrolyzed to mannobiose and -triose (Fig. 9).

Endo-β-mannanases have been detected from bacteria (Emi ***et al.,*** 1972), fungi (Reese and Shibata, 1965), and from higher plants (Shimahara ***et al., 1975).*** These endo-β-mannanases have been classified into three types on the basis of their specificities; the first type is capable of attacking mannooligosaccharides larger than mannobiose, e.g. the β-mannanase from *Aspergillus* ***niger*** (Yamazaki ***et al.,*** 1976), the second type attacking mannooligosaccharides larger than mannotriose, e.g. the β-mannanase from ***Bacillus subtilis*** (Emi ***et al.,*** 1972), and the third type attacking mannooligosaccharides larger than mannotetraose, e.g. the β-mannanase from *Rhizopus* ***niveus*** (Hashimoto and Fukumoto, 1969). The endo-β-mannanase from *Aeromonas* sp. F-25 acted on oligomers larger than mannotriose, and not on mannobiose and -triose. Thus, the enzyme was ascertained to be similar to the endo-β-mannanase from ***B.*** *subtilis,* and classified as 1, 4-β-D-mannan mannanohydrolase (EC 3. 2. 1. 78.).

Aeromonas sp. F-25 was found to produce a large quantity of endo-β-mannanase. The mannanase will be a powerful tool for determining structure? of β-mannans, and for preparing mannooligosaccharides of various sizes.

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