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Purification and Characterization of Thermostable β-Mannanase from a *Bacillus* sp. YA-14

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Thermostable β-mannanase from *Bacillus* sp. YA-14 was purified by acetone precipitation, CM-cellulose, Sephadex G-100 and hydroxyapatite column chromatography from culture supernatant. The final enzyme preparation appeared to be homogeneous on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). β-Mannanase appeared to be a monomeric protein with a molecular weight of 67,000 daltons. The optimal pH and temperature of the enzyme reaction were pH 6.0 and 75°C, respectively. The enzyme was stable at a pH range of 6.0 to 9.0 and at temperatures between 45 and 85°C. The kinetic constants of β-mannanase as determined with a galactomannan (locust bean) as substrate were a Vmax of 25 unit/ml and a Km of 1.1 mg/ml. The enzyme had only limited activity on galactomannan substrate. It was suggested that β-mannanase activity is limited by the number of branched α-galactose residues.

The hemicelluloses are the second most abundant polysaccharides in nature, and they seem to be linked to lignin in wood (10). The major constituents of hemicellulose are the hetero-1,4,-β-D-xylans and the hetero-1, 4,-β-D-mannans (galactoglucomannans and glucomannans). The heteroxylans are found mainly in grasses, cereals, and hardwood (angiosperms). The mannans are more abundant in softwoods (gymnosperms, O-acetyl galactomannans), but they are also found in hardwoods (glucomannans) (5, 6). Softwoods from which the majority of pulps are derived contain as much as 15 to 20% hemicellulose in the form of galactomannan (19). Hemicellulases having substrate specificities for galactomannan constituents would make excellent candidates for use in the enzymatic bleaching of soft wood pulps. Since pupling is best carried out at elevated temperatures thermostable hemicellulases could offer significant advantages over hemicellulases from mesophiles in terms of their higher intrinsic stability and catalytic efficiencies at such elevated temperatures.

β-Mannan consists of a backbone of mannose residues, but it may also contain glucose residues. α-D-Galactose and acetyl residues are attached to the backbone in galactoglucomannan (6). The enzymatic hydrolysis of β-mannans is accomplished by reaction with β-D-mannanase (1, 4,-β-D-mannan mannohydrolase; EC 3.2.1.78), which attacks the backbone to generate small oligomannose residues and β-D-mannosidase (β-D-mannoside mannohydrolase; EC 3.2.1.25). β-Mannanases have been isolated and characterized from bacterial, fungal, and plant sources (1-4, 8-9, 12, 15, 16, 20). Most of the mannosacchrides (MOS) which are produced by β-mannanase are used in the food and feed processing industries.

It was reported that mannooligosaccharides are useful being of the best growth factors for *Bifidobacterium* sp. and *Lactobacillus* sp. (11) which are important in maintaining our normal intestinal condition. For the application of these saccharides in the fields of pharmaceuticals and food stuffs as well as in the enzymatic bleaching of pulp in the manufacture of paper, we have isolated a β-mannan-hydrolyzing bacterium. As a result of screening, *Bacillus* sp. YA-14, which was known to produce pectinase, xylanase, β-xylosidase, protease and amylase (24-27) was selected as a potent strain. This strain produced significant amounts of extracellular β-mannanase when cultured in an alkaline pH media containing locust bean gum as a carbon source (17). This article describes the purification and characterization of thermostable extracellular β-mannanases from *Bacillus* sp. YA-14.

MATERIALS AND METHODS

Cultivation of Bacterial Strain

Bacillus sp. YA-14 was cultivated in a 100 ml Erlenmyer flask containing 10 ml of medium composed of 1% locust bean gum, 1% polypeptone, 0.5% NaCl, 0.1%

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K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$ and 0.2% Na_2CO_3 at 37 °C. After shaking the culture for 1 day, the culture medium was inoculated into a 5 liter Erlenmyer flask containing 1 liter of the same medium. The culture then was incubated at 37°C for 16 h on a rotatory shaker. Culture broth was collected by centrifugation and used in the experiment.

Materials

Locust bean gum, guar gum, mannan from *S. cerevisiae*, chitin, xylan, dextran and xanthan gum were obtained from Sigma. Co. Gel matrices for the column chromatographies were purchased as follows; CM-cellulose from Whatman, England, Sephadex G-100 from Pharmacia, Sweden and hydroxyapatite from Tosoh, Tokyo. Other chemicals used were of first grade and available commercially.

Enzyme Assay

A reaction mixture containing 5 mg of locust bean gum in 0.5 ml of water, 0.4 ml of 0.1 M Na-acetate buffer (pH 6.0) and 0.1 ml of the enzyme solution suitably diluted with water was incubated at 75°C for 5 min. The amount of reducing sugar liberated was determined by the method of Somogyi-Nelson (18, 19). One unit of β-mannanase activity was defined as the amount of enzyme which releases 1 mol of reducing sugar, as equivalent to D-mannose, per minute under the above conditions.

Determination of Protein Concentration

Protein concentration was measured by the method of Lowry *et al.* (14) using bovine serum albumin as a standard.

Purification Procedure

The extracellular β-mannanase released into the culture supernatant of *Bacillus* sp. YA-14 was purified at 4°C by the following procedure. The supernatant obtained by centrifuging a 1 liter culture broth was added to 3 liters of cold acetone and allowed to stand overnight at 4°C. After the supernatant was separated and discarded by centrifugation, the precipitate was dissolved in 0.01 M Na-acetate buffer (pH 5.0) and used as a source of crude enzyme.

Step 1. CM-cellulose cation-exchange chromatography. The crude enzyme was put on a column (4× 19 cm) of CM-cellulose preeqilibrated with a 0.01 M Na-acetate buffer (pH 5.0). After the column was washed with the same buffer, the enzyme was eluted with a linear gradient from 0 to 0.5 M NaCl in the same buffer at a flow rate of 1 ml/min, and the volume of each fraction was 10 ml. Most activity was eluted at 150-300 mM NaCl and the corresponding fractions were pooled for the next step.

Step 2. gel filtration chromatography on sephadex G-100. The combined fraction from step 1 was concentrated to 1ml in Amicon filtration apparatus (MW cut off; 30,000) followed by centrifugation at 20,000×g for 30 min to remove the denatured proteins. The supernatent solution was put on a column (2.3×64 cm) of Sephadex G-100 preequilibrated with a 0.01 M Na-acetate buffer (pH 5.0). The enzyme was eluted with the same buffer at a flow rate of 0.5 ml/min, and the volume of each fraction was 5 ml.

Step 3. hydroxyapatite column chromatography. The sample from step 2 was loaded into a hydroxyapatite column (1.8×13 cm) preequilibrated with 0.01 M Na-phosphate buffer (pH 6.8). Elution was continued with Na-phosphate gradient from 0 to 0.3 M. Most activity was eluted at 50-150 mM Na-phosphate at a flow rate of 1 ml/min, and the volume of each fraction was 2.5 ml.

Polyacrylamide Gel Electrophoresis

Denaturing polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (13) using 10% acrylamide gel. Proteins were stained with Coomassie Brilliant Blue R-250.

Determination of Molecular Weight

The molecular weight of native β-mannanase was determined by HPLC using gel filtration column. Phosphorylase b (97 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa) were used as standards.

RESULTS AND DISCUSSION

Purification of the β-Mannanase

Chromatography of a culture supernatant which was precipitated with acetone and dissolved in a buffer on CM-cellulose yielded one major peak of β-mannanase activity, which was eluted at 0.3 M NaCl (Fig. 1A). The peak was subjected to chromatography sequentially on Sephadex G-100 and Hydroxyapatite columns, and in each case, the active fraction was eluted as a single peak (Fig. 1B, 1C). The purified enzyme was homogenious as judged by SDS-PAGE (Fig. 2). Apparent molecular weight of the β-mannanase determined by SDS-PAGE was estimated to be 67,000 Da. The monomeric nature of the enzyme was confirmed by TSK gel G3000 SW HPLC on a molecular sieve calibrated with standard proteins (Fig. 3). The final preparation obtained from a hydroxyapatite column showed specific activity of 1984 unit/mg protein under the assay conditions described in "Materials and Methods". Typical results of the purification procedure were summarized in Table 1. *Bacillus* sp. AM-001 has been reported to produce three types of β-mannanases with molecular weights of 58,000, 59,000, and 42,000, respectively (5). The β-mannanase from *Bacillus stearothermophilus* has been reported to produce a dimeric enzyme (162 kDa) composed of subunits having identical molecular weight (12). The β-mannanase from *Streptomyces lividans* and *Caldocellum saccharolyticum* had a molecular weight of 36 and 39

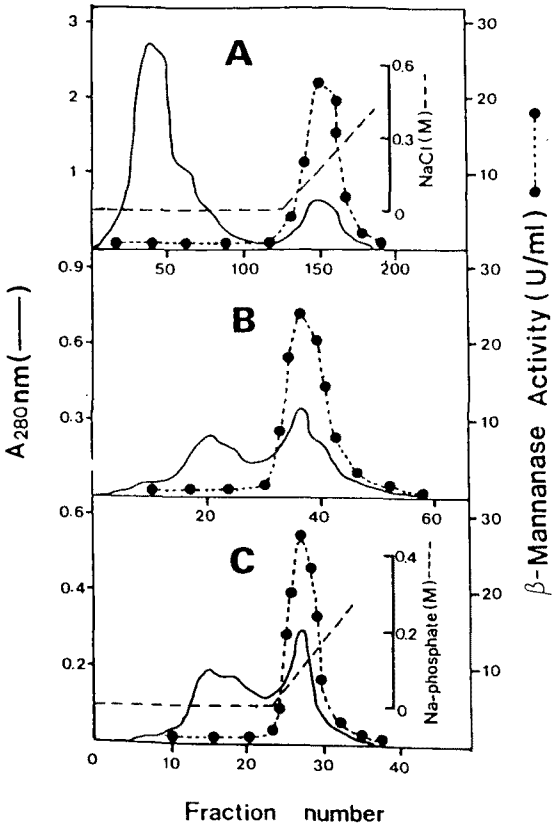

Fig. 1. Elution profiles of β-mannanase on column chromatography during its purification.
A, CM-cellulose column; B, Sephadex G-100 column; C, Hydroxyapatite columns. The activity of β-mannanase was assayed for β-mannan (locust bean) hydrolysis.

kDa, respectively (4, 8). The molecular weight of β-mannanse from *Bacillus* sp. YA-14 was somewhat larger than that of other β-mannanase except the enzyme of *B. stearothermophilus*.

Effect of pH

The enzymatic characteristics of the β-mannanase were studied with the purified enzyme. β-mannanase activities were measured from pH 4.0 to 12.0 using various buffer solutions; 0.05 M sodium acetate buffer (pH 4-6), sodium phosphate (pH 6-8.5), and glycine-NaOH (pH 8.5-12) buffers. The enzyme was active within a pH range of 6.0-9.0 with a maximum pH of 6.0 as shown in Fig. 4. The stability of the enzyme at various pH levels was studied by incubating it in various buffers at 40°C for 20 min. The remaining β-mannanase activity was then assayed under standard conditions. The enzyme was fairly stable at pH values ranging from 5.0 to 12.0. β-Mannanases from *Bacillus* sp. AM-001 (1) were most active at pH 8.5-9.0 and stable at pH 7.0-9.0 for 30 min. The pH profile of β-mannanase from *B. stearothermophilus* showed a broad plateau corresponding to maximal activity between pH 5.5 and 7.5 (21). The β-

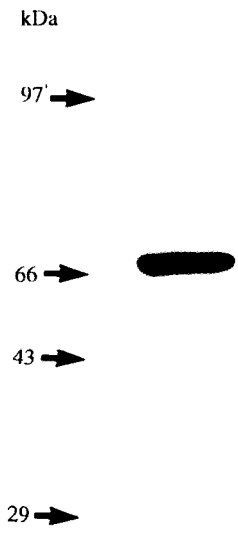

Fig. 2. SDS-PAGE of the purified β-mannanase.
β-mannanase purified from *Bacillus* sp. YA-14 was subjected to 10% SDS-polyacrylamide gel and stained with Coomassie Blue. Arrows indicate molecular weight standards from top to bottom, phosphorylase b (97 kDa), bovine serum albumin (66 kDa), chicken egg albumin (43 kDa) and carbonic anhydrase (29 kDa).

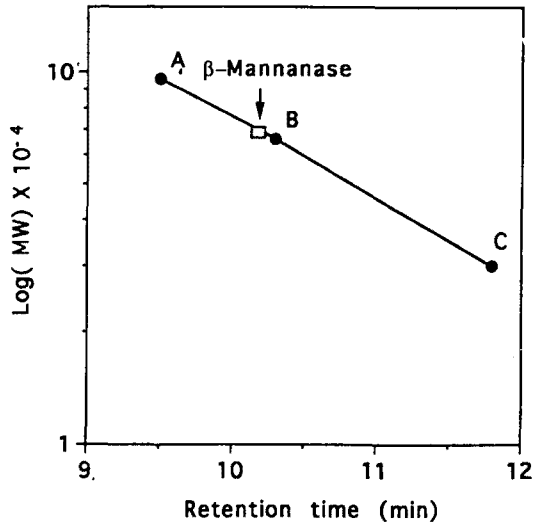

Fig. 3. Determination of molecular weight of β-mannanase by gel filtration.
A, phosphorylase b (97 kDa); B, bovine serum albumin (66 kDa); C, carbonic anhydrase (29 kDa).

Table 1. Summary of purification of β-mannanase from *Bacillus* sp. YA-14.

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Culture supernatant	2,852	21,460	7	1	100
Acetone precipitate	353	18,450	52	6.9	86
CM-cellulose	66	10,300	156	20.7	48
Sephadex G-100	2.7	4,520	1,694	225.3	21
Hydroxyapatite	0.65	1,290	1.984	263.8	6

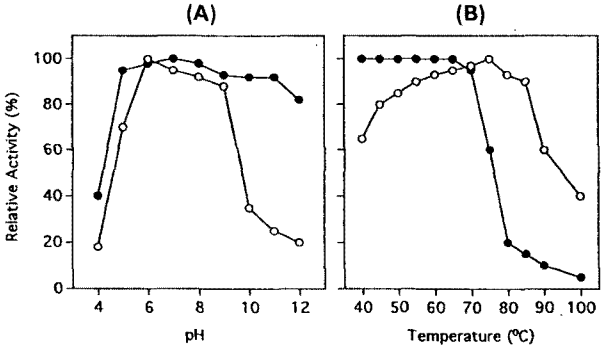

Fig. 4. Effects of pH (A) and temperature (B) on the activity and stability of the purified β-mannanase.
The enzyme was first assayed at various pHs (○; A) and temperatures (○; B). Next, the enzyme was assayed again after incubation of the reaction mixture at various pHs (●; A) for 1 h and temperatures (●; B) for 20 min.

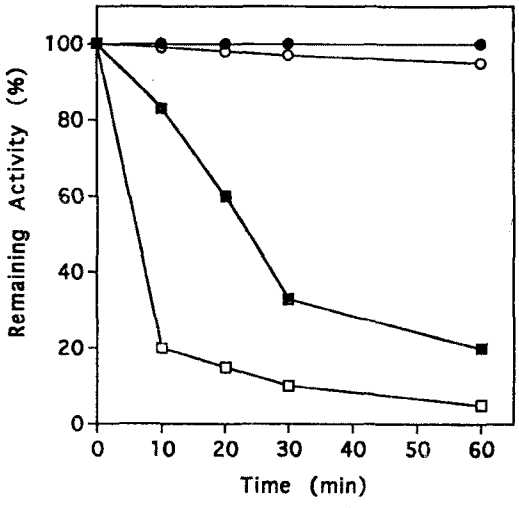

Fig. 5. Thermostability of the purified β-mannanase.
The enzyme solutions were incubated at 65 (●), 70 (○), 75 (■), 80 (□) °C. The remaining activities of heat treated enzymes were measured at 75°C.

mannanases having stability at broad pH range like β-mannanases from *Bacillus* sp. have not hitherto been reported.

Effect of Temperature

β-mannanase activities were kept in 50 mM sodium

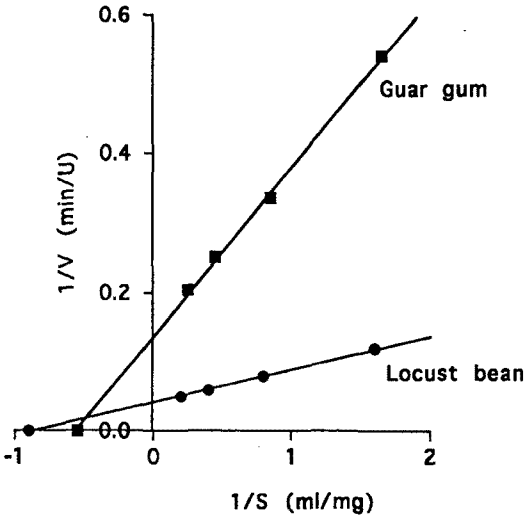

Fig. 6. Lineweaver-Burk plot of β-mannanase activity as a function of concentration of substrate.

acetate buffer, pH 6.0, at various temperatures ranging from 40 to 100°C. The enzyme was active between 45 and 85°C with an optimum temperature of 75°C as shown in Fig. 4. The thermostability of the enzyme was measured after incubation for 20 min at each temperature. The enzyme retained full activity at temperatures up to 70°C. The remaining activities as a function of heat treatment time are shown in Fig. 4. Mannanase activity was stable at 70°C for 1 h. At 75°C, remaining activity decreased to 80% of initial activity within 60 min. β-Mannanase from *Bacillus* sp. AM-001 was most active at 60-65°C and stable up to 50-60°C for 30 min. β-Mannanase from *B. stearothermophilus* was completely stable at 65°C for 24 h (21). Although thermostability of β-mannanase from *Bacillus* sp. YA-14 is not so excellent as that of β-Mannanase from *B. stearothermophilus*, the enzyme purified from *Bacillus* sp. YA-14 is thought to be more stable at high temperature than one of other β-mannanase reported.

Enzymatic Kinetics

The kinetic constants of the purified β-mannanase were determined under assay conditions of pH 6.0-75°C and using the Lineweaver-Burk method. The apparent Km values for locust bean and guar gum were 1.1 mg/ml

Table 2. Substrate specificity of β-mannanase.

Substrate (1%, w/v)	Relative activity (%)
Locust bean gum	100
Guar gum	36
Mannan from *S. cerevisiae*	0
Chitin	0
Xylan	0
Dextran	0
Xanthan gum	0

and 1.7 mg/ml, respectively. Vmax values for for locust and guar gum were 25 unit/ml and 7.7 unit/ml.

Substrate Specificity.

β-Mannanase specificity for β-1,4 mannosidic linkages is consistent with hydrolysis of locust bean gum and guar gum and resistance to enzymatic hydrolysis by mannan from *S. cerevisiae*, which has α-1,6, and α-1,2, and α-1,3 mannosidic linkages (28) (Table 2). The lower mannanase activity towards guar gum as a substrate compared with mannanase activity assayed in the presence of locust bean gum supports the hypothesis that the enzyme activity is limited by the number of branched α-galactose residues (9). Recently, we reported that the crude β-mannanase prepared from *Bacillus* sp. YA-14 acted as an endo-enzyme. From the preliminary hydrolysis studies carried out with purified enzyme, the similar result as mentioned above was obtained (data not shown).

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