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Article

Purification and enzymatic properties of a highly alkaline mannanase from alkaliphilic *Bacillus* sp. strain JAMB-750

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An alkaliphilic *Bacillus* isolate (strain JAMB-750) was found to exoproduce a novel alkaline mannanase (AmA). AmA was purified to homogeneity (about 98% pure) as judged by SDS-polyacrylamide electrophoresis. The molecular mass was approximately 130 kDa. The hydrolysis patterns of various substrates indicated that the enzyme was an endo-type enzyme. The optimal temperature and pH for activity were 55°C and around 10, respectively. The optimal pH is the highest among mannanases reported to date. Highly alkaline mannanase is expected to have good wash performance in detergent solution and be effective in biobleaching of kraft pulp. Therefore, AmA is highly applicable to the paper and detergent industries, as well as the food industry.

Keywords:
Bacillus, alkaliphile, alkaline enzyme, mannanase

Mannan and heteromannans, such as locust bean gum, konjak mannan, guar gum, and ivory nut, are composed of a backbone of β-1,4-linked mannose (and glucose) units, which are often substituted with galactose and acetate residues depending on their origin [1]. For complete hydrolysis of these mannans, many mannolytic microorganisms synthesize the multiple mannolytic enzymes for cooperative actions. These enzymes include endo-β-1, 4-mannanase (mannanase; EC 3.2.1.78), β-mannosidase (EC 3.2.1.25), and enzymes that cleave side chain sugars from the mannan backbone, such as α-galactosidases (EC 3.2.1.22) and acetyl esterases (EC 3.1.1.6) [2]. Mannanase is a hydrolase that catalyzes the endo-type

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hydrolysis of β-1,4-mannosidic linkages in the main chain of naturally-occurring mannan and heteromannans [3]. Many mannanases have been found from various origins [4–8]. Some of the genes for the enzymes have been cloned and sequenced, and they are grouped into glycoside hydorolase families 5 and 26 (http://afmb.cnrs-mrs.fr/CAZY/).

Mannanases have many possible applications, for instance, for use in pulp bleaching, reduction of viscosity of instant coffee, and clarification of fruit juices and wines [9]. An alkaline mannanase was found by Horikoshi and his colleagues from alkaliphilic *Bacillus* sp. strain AM-001 for the first time [10]. And then, we found an alkaline mannanase from an alkaliphilic *Bacillus* isolate (strain JAMB-602) [11]. Recently, alkaline mannanase was found to remove gum stains of cosmetics and/or foods and thus be effective additives for laundry detergents (WO 99/09128–WO 99/09133). Therefore, alkaline mannanase is a promising enzyme and can be used in the detergent and paper industries, as well as in the food industry.

In this report, we describe the purification and properties of a highly alkaline mannanase (AmA) produced by *Bacillus* sp. strain JAMB-750 isolated from a sample of soils.

EXPERIMENTAL

Materials

Unless otherwise stated, all chemicals used were from Wako Pure Chemical. *N*-Bromosuccinimide (NBS) was obtained from Sigma. Iodoacetate, iodoacetamide, and 1-ethyl-3-(3-dimethyl-aminopropyl)carbonate were obtained from Nacalai Tesque. Dithiothreitol was purchased from Amersham Biosciences. Coomassie Brilliant Blue R-250 (CBB), SDS, EDTA, and EGTA are the products of Bio-Rad. D-Mannose (M1; Sigma), locust bean gum (Sigma), ivory nut (Megazyme), konjak mannan (Kanto Chemical), and guar gum (Sigma) were obtained from the manufacturers in parentheses. Manno-oligosaccharides [mannobiose (M2) through mannohexaose (M6)] are the products of Megazyme.

Bacterial strains and culture conditions

Strain JAMB-750 had been isolated from a sample of soils as a mannanase-producer in this laboratory. It was isolated at 30°C for several days on 1.5% (w/v) agar containing (w/v) 0.2% locust bean gum, 0.5% Polypepton S (Nihon Seiyaku), 0.5% yeast extract (Difco), 0.1% K_2HPO_4, 0.02% $MgSO_4{\cdot}7H_2O$, and 0.5% Na_2CO_3 (separately autoclaved). The isolate was grown at 30°C for 2 days in an alkaline medium composed of 0.2% (w/v) locust bean gum, 5% (v/v) Mieki (Ajinomoto), 0.2% (w/v) yeast extract, 0.5% (w/v) bonito meat extract (Wako Pure Chemical), 0.1% (w/v) K_2HPO_4, 0.1% (w/v) methionine (Kanto Chemical), 0.02% (w/v) $MgSO_4{\cdot}7H_2O$, and 0.5% (w/v) Na_2CO_3 (separately autoclaved). After removal of cells by centrifugation (10,000 × *g*, 10 min) at 4°C, the supernatant was used as the starting material for purification of the enzyme.

Enzyme assay

A suitably diluted solution of enzyme preparation was incubated routinely in 50 mM glycine-NaCl-NaOH buffer (pH 9.0) containing 0.4% (w/v) locust bean gum at 40°C for 30 min. Activity was expressed as the initial rate of hydrolysis by measuring the

release of the reducing ends using the 3,5-dinitrosalicylic acid procedure [12] with M1 as the standard. One unit (U) of enzymatic activity was defined as the amount of protein that produced 1 μmol of reducing sugar as M1 per min under the standard conditions of enzyme assay. Protein was determined using a DC-protein assay kit (Bio-Rad) with bovine serum albumin as the standard protein.

Purification of the alkaline mannanase

Enzyme purification was done at temperatures below 4°C. The centrifugal supernatant (3.58 liters) of the culture broth was treated with ammonium sulfate, and the fraction that precipitated at 30–60% saturation was collected. The precipitates formed were dissolved in a small volume of 10 mM sodium phosphate buffer (pH 7.0), and the solution was dialyzed against the same buffer overnight. The retentate was then applied to a column of DEAE-Toyopearl 650M (2.5 × 20 cm, Tosoh) that had been equilibrated with 10 mM sodium phosphate buffer (pH 7.0). The column was initially washed with 200 ml of 150 mM NaCl in the same buffer, and proteins were eluted with 1.0–liter linear gradient of 150 to 300 mM NaCl in the same buffer. The active fractions were combined and loaded directly onto a column of hydroxyapatite equilibrate with 10 mM sodium phosphate buffer (pH 7.0). The column was then washed with 200 ml of 50 mM sodium phosphate (pH 7.0), and proteins were eluted with 1.0–liter linear gradient of 50 to 125 mM sodium phosphate (pH 7.0). The active fractions were combined, concentrated, and exchanged with 5 mM Tris-HCl buffer (pH 7.5) by ultrafiltration on an Amicon Ultra PL-10 (Millipore). The resulting concentrate was used exclusively for further experiments as the final preparation of purified enzyme.

Electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed essentially as described by Laemmli [13] on slab gels [10% (w/v) acrylamide, 80 × 100 mm, 1.0-mm thick], and the gels were stained for protein with Coomassie Brilliant Blue R-250 (CBB, Bio-Rad). The molecular mass was estimated by SDS-PAGE with Precision Plus Protein Standards (Bio-Rad)

Activity staining

After SDS-PAGE, the gel was washed twice with 50 mM glycine-NaCl-NaOH buffer (pH 9.0) with gentle shaking to remove SDS. Onto the gel, the agar sheet containing 0.7% (w/v) agar, 0.25% (w/v) konjak mannan, and 50 mM glycine-NaCl-NaOH (pH 9.0) was overplayed, followed by incubation for 1 h at 40°C. The agar sheet was then soaked in a 0.1% (w/v) solution of Congo red for 10 min at room temperature and washed several times with 1% (w/v) NaCl.

Sequencing of amino-terminal regions

The enzyme sample was blotted on a polyvinylidene difluoride membrane (Applied Biosystems) that had been wetted with methanol. The N-terminal amino acid sequence of the protein was determined directly using a protein sequencer (model 476A, Applied Biosystems).

Analysis of products of hydrolysis of mannans and manno-oligosaccharides

Table 1. Summary of typical purification of AmA

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (-fold)
Culture supernatant	10600	924	0.087	100	1.0
Ammonium sulfate	540	486	0.90	52.6	10.3
DEAE-Toyopearl	16.2	220	13.6	23.8	156
Hydorxyapatite	2.1	83.1	39.5	9.0	454

Thin-layer chromatography (TLC) was used to identify products. Enzymatic hydrolysis of ivory nut, locust bean gum, guar gum, konjak mannan, and manno-oligosacchrides [0.4% (w/v) each] was carried out at 40°C in 50 mM glycine-NaCl-NaOH buffer (pH 9.0). At appropriate times, aliquots (5 µl each) were removed and developed by TLC (silica gel 60 plates, 10 × 20 cm, Merck) with 1-butanol-acetic acid-H_20 (2:1:1, v/v) as a solvent system. The sugar spots were detected by spraying with 10% (v/v) H_2SO_4, and heating. MI and manno-oligosaccharides from M2 through M6 were used as authentic standards.

RESULTS AND DISCUSSION

Taxonomic analysis of the isolate

Strain JAMB-750 occurs as Gram-positive rods 3.0–3.8 µm in length and 0.4–0.5 µm in width and is motile by means of peritrichous flagella and contains subterminal endospore. It is absolutely aerobic. The temperature range for growth is 13–40°C with an optimum of around 34°C. The pH range for growth is 7–11 with an optimum of pH 9. These results indicate that this isolate is a facultatively alkaliphilic *Bacillus*. The isolate is positive for catalase, oxidase, gelatinase, protease, DNase, and production of H_2S, and negative for amylase, reduction of nitrite and nitrate, and the producton of indole. It utilizes D-xylose, L-arabinose, cellobiose, D-fructose, D-galactose, D-glucose, glycerol, D-lactose, D-mannitol, D-mannose, maltose, and D-trehalose, but does not D-raffinose, L-rhamnose, *myo*-inositol and D-sorbitol. The major isoprenoid quinone type is MK-7, and

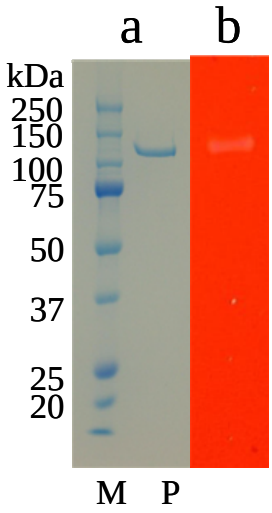

Fig. 1. Electrophoresis of purified AmA. (a) SDS-PAGE of the purified AmA (1 µg of protein) on a 10% (w/v) polyacrylamide gel is shown. The protein was stained with CBB (lane P). Protein mass markers (in kDa) are indicated on the left (lane M). (b) Activity staining of the enzyme after SDS-PAGE.

major fatty acids are *iso*-C15:0, and *anteiso*-C15:0. The G + C content of strain JAMB-750 genomic DNA was determined to be 36.2–38.4 mol% by the method of Tamaoka and Komagata [14]. To determine the phylogenetic position of strain JAMB-750, its 16S rDNA sequence was determined and analyzed using comparative sequence analysis against known 16S rDNA sequences. The16S rDNA sequence of the isolate had a closest match of 98.9% homology with that from *Bacillus alcalophilus* DSM485 ^{T}. The next highest similarity was with *Bacillus pseudoalcaliphilus* DSM8725^{T} (97.9% homology). Based on these taxonomic characteristics, strain JAMB-750 is closely related to *B. alcalophilus*. The GenBank/EMBL/DDBJ accession number for the 16S rDNA of the strain is AB128830.

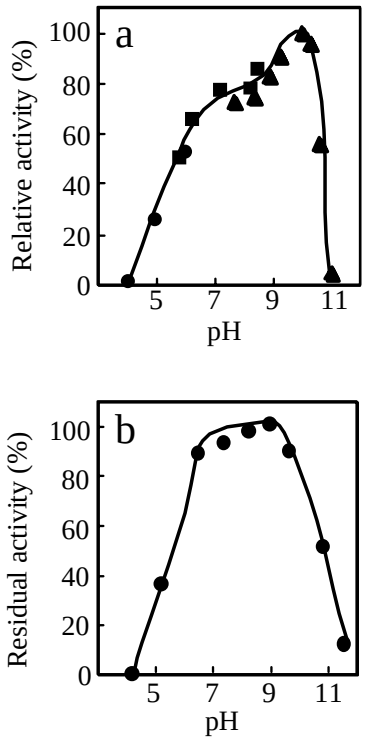

Fig. 2. Effects of pH on activity and stability**.** (a) The pH–activity curve of purified AmA (0.2 U/ml) is shown. The buffers (50 mM each) used were as follows: acetate, pH 5.6–6.1, closed circles; sodium phosphate, pH 6.3–8.5, closed squares; glycine-NaCl-NaOH, pH 7.7–10.9, closed triangles. The reactions were done at 40°C for 5 min. The values are shown in percentages of the maximal activity observed at pH 10, which is taken as 100%. (b) To assess the pH stability of AmA, the enzyme (0.3 U/ml) was preincubated at the indicated pH in 10 mM Britton-Robinson universal buffers at 40°C for 30 min. And then, samples (0.1 ml) were used for the measurements of the residual activity under the standard conditions of enzyme assay. The values are shown as percentages of the original activity, which is taken as 100%.

Purification of alkaline mannanase

When *Bacillus* sp. strain JAMB-750 was grown in an alkaline mannan medium, several extracellular mannanases were detected in the culture supernatant. Activity staining after SDS-PAGE showed that the major mannanase band had a molecular mass of 130 kDa (AmA). AmA was purified 454-fold, with a specific activity of 39.5 U/mg and a final yield of 9.0% (Table 1). The SDS-PAGE and activity staining of the purified enzyme gave a single band (approximately 98% pure) with an apparent molecular mass of 130 kDa, as shown in Fig. 1. The N-terminal amino acid sequence of AmA was Glu-Ser-Lys-Ile-Pro-Lys-Asp-X-Glu-Gly.

Effects of pH on activity and stability

The activity of AmA at various pH values was measured with locust bean gum (a galactomannan) as substrate. The enzyme showed high activity at alkaline side, with an optimum at pH 10, in 50 mM glycine-NaCl-NaOH buffer, as shown in Fig. 2a. This optimal pH value is the highest among those of other mannanase reported to

date [10,11]. Moreover, this is the first report of alkaline mannanase from *B. alcalophilus*. The pH stability of AmA was assessed by incubating at 40°C for 30 min at different pH values in 10 mM Britton-Robinson universal buffers, and the residual activity was measured by the standard conditions of enzyme assay. The enzyme was stable between pH 6.5 and pH 10 (Fig. 2b).

Effects of temperature on activity and stability

The optimal temperature of AmA was determined at various temperatures at pH 9.0 in 50 mM glycine-NaCl-NaOH buffer, as shown in Fig. 3a. The enzyme had an optimal temperature of 55°C. The thermal stability of AmA was examined after incubation at 40, 50, and 60°C in 5 mM Tris-HCl buffer (pH 7.5) for various periods. AmA was stable to incubation up to 40°C for 60 min. The first-order constants of irreversible thermoinactivation, k, at 50 and 60°C, were obtained by linear regression in semi-logarithmic coordinates. Each enzyme half-life was calculated using the equation $t_{1/2}=\ln 2/k$. The half-lives of AmA were 117 min at 50°C and 65 min at 60°C (Fig. 3b).

Effects of chemicals and cations

AmA (0.2 U/ml) was preincubated with chemicals or cations (1.0 mM each) at 40 °C for 30 min in 5 mM Tris-HCl buffer (pH 7.5). An aliquot (0.1 ml) was used to determine the residual activity under the standard conditions of enzyme assay. Iodoacetate, iodoacetamide, *N*-ethylmaleimide, dithiothreitol, 1-ethyl-3-(3-dimethyl-aminopropyl)carbonate, diethyl pyrocarbonate, EDTA, and EGTA did not essentially affect the AmA activity. NBS abolished the activity of AmA completely. The inhibition of some enzymes by NBS is caused by oxidation of Trp residues in proteins [15]. Therefore, tryptophan residue(s) may participate in the catalysis or maintain the structure of AmA. Cations (Cl forms) such

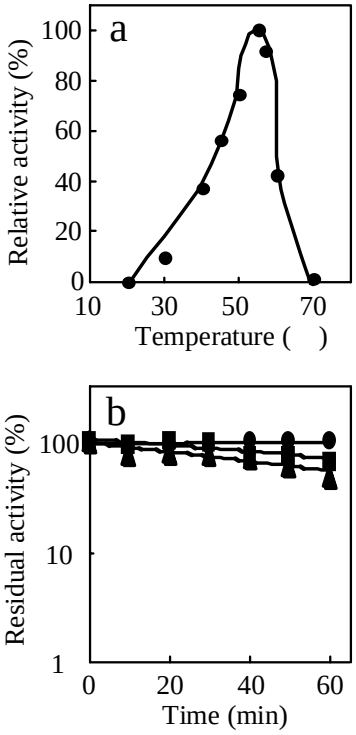

Fig. 3. Effects of temperature on activity and stability. (a) The temperature–activity curve of AmA (0.2 U/ml) at pH 9.0 in 50 mM glycine-NaCl-NaOH buffer is shown. The reactions were done at the indicated temperatures for 5 min. The values are shown in percentages of the maximal activity observed at 55°C, which is taken as 100%. (b) To assess the thermostability of AmA, the enzyme (0.3 U/ml) was heated at 40°C, 50°C or 60°C in 5 mM Tris-HCl buffer (pH 7.5). Aliquots (0.1 ml) were removed at different times and used for the measurements of the residual activity under the standard conditions of enzyme assay. The residual activities at 40°C (closed circles), 50°C (closed squares), and at 60°C (closed triangles) are shown. The values are shown in percentages of the original activity, which is taken as 100%.

as Fe^{3+}, Fe^{2+}, Pb^{2+}, Hg^{2+}, and Cd^{2+} inactivated the enzyme activity (60–100% inhibition). Under the same conditions Mn^{2+}, Ca^{2+}, Ni^{2+}, Mg^{2+}, and Co^{2+} were essentially without effect.

Analysis of hydrolysis products and substrate specificity

The relative activity toward locust bean gum, konjak mannann, ivory nut, and guar gum is approximately 100:100:60:30. The time courses of hydrolysis products from these natural mannans were examined at pH 9 and at 40°C for up to 17 h, as shown in Fig. 4a. AmA hydrolyzed these substrates to yield various oligosaccharides during the reactions. It could not hydrolyze M2 and M3, whereas it acted slightly on M4 and efficiently on M5

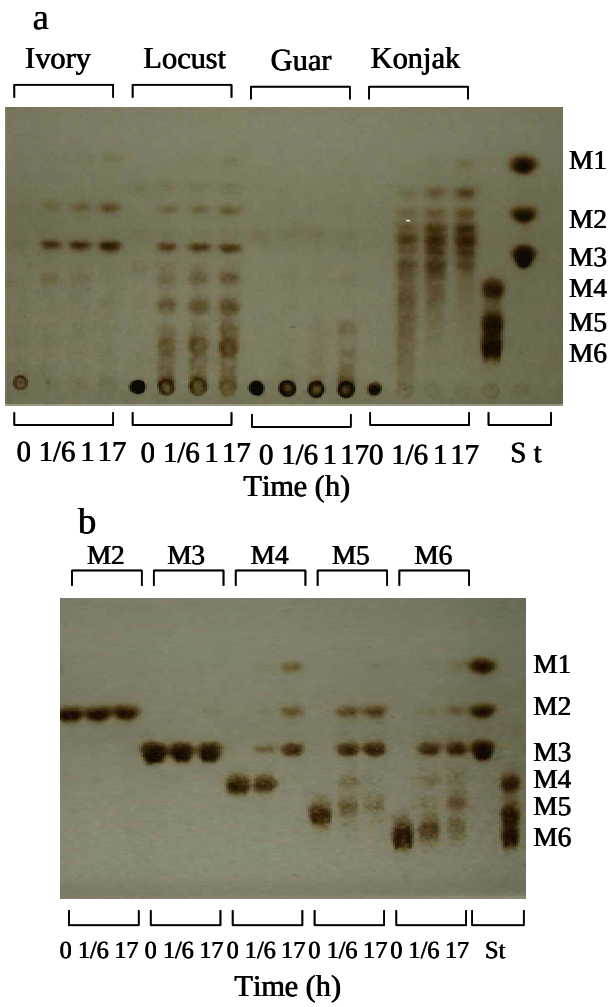

Fig. 4. TLC of the products of reaction by AmA. (a) The reactions were carried out at 40°C and at pH 9.0 in 50 mM glycine-NaCl-NaOH buffer with 0.05 U/ml of enzyme and 0.4% (w/v) ivory nut (Ivory), locust been gum (Locust), guar gun (Guar), and konjak mannan (Konjak). (b) The reactions were carried out at 40°C and at pH 9.0 in 50 mM glycine-NaCl-NaOH buffer with M2 through M6 as substrates [0.4% (w/v) each] and 0.05 U/ml of enzyme. The incubation times were 0 min, 10 min, and 17 h for all the reactions. At intervals, aliquots (5 μl) were withdrawn from the reaction mixtures and developed by TLC, as described in EXPERIMENTAL. Standard markers (St) are shown on the right.

and M6 to yield M3 as the major product and M2 as the minor product, as shown in Fig. 4b. These results show that AmA hydrolyzes mannan, manno-oligosacchrides larger than M4 and that it is an endo-acting enzyme. The hydrolysis pattern is essentially similar to that of alkaliphilic *Bacillus* sp. strain JAMB-602 [11], although the other enzymatic and physicochemical properties are quite different between the two enzymes.

Overall, AmA from *Bacillus* sp. strain JAMB-750 is very promising for use in the paper and detergent industries, because it is very active at high pH and stable at high temperature. Many nucleotide sequences of neutral mannanases from various microorganisms have been reported [16–19]; however, those of alkaline enzymes are only two to date [11,20]. We are now cloning and sequencing the gene for the highly alkaline AmA.

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