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**Yuji Hatada · Nobuhiro Takeda · Kazumichi Hirasawa
Yukari Ohta · Ron Usami · Yasuhiko Yoshida
William D. Grant · Susumu Ito · Koki Horikoshi**

Sequence of the gene for a high-alkaline mannanase from an alkaliphilic *Bacillus* sp. strain JAMB-750, its expression in *Bacillus subtilis* and characterization of the recombinant enzyme

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Abstract A novel alkaline mannanase Man26A has been found in the culture of an alkaliphilic *Bacillus* sp. strain JAMB-750 and the optimal pH for the mannanase activity of the enzyme was around pH 10 (J Biol Macromol 4: 67–74, 2004). This optimal pH is the highest among those of the mannanases reported to date. The gene *man26A* coding the enzyme was cloned from the genomic DNA of strain JAMB-750 and sequenced. It encodes a protein of 997 amino acids including a signal peptide. The N-terminal half (Glu27–Val486) of the enzyme exhibited moderate similarities to other mannanases belonging to glycoside hydrolase family 26, such as the enzymes from *Cellvibrio japonicus* (37% identity), *Cellulomonas fimi* (33% identity), and *Bacillus* sp. strain AM-001 (28% identity). The C-terminal half was found to contain four domains. The first, second, third, and fourth domains exhibited similarities to the carbohydrate-binding module, the mannan-binding module, the *Homo sapiens* collagen type IX alpha I chain, and the membrane anchor region of Gram-positive surface proteins, respectively. Its recombinant mannanase was produced extracellularly using *Bacillus subtilis* as the host. The optimal pH for the mannanase activity of the recombinant enzyme was around pH 10. The enzyme was very resistant to surfactants, for example, SDS up to 2.0% (w/v).

Keywords Alkaliphile · Alkaline mannanase · Cloning · Recombinant enzyme

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Y. Hatada (✉) · Y. Ohta · S. Ito · K. Horikoshi
Japan Agency for Marine-Earth Science and Technology (JAMSTEC), 2-15 Natsushima, Yokosuka 237-0061, Japan
E-mail: hataday@jamstec.go.jp
Tel.: +81-46-867-9713
Fax: +81-46-867-9645

N. Takeda · K. Hirasawa · R. Usami · Y. Yoshida
Faculty of Engineering, Toyo University,
2100 Kuzirai, Kawagoe, Saitama 350-8585, Japan

W. D. Grant
Department of Microbiology and Immunology,
University of Leicester, Leicester, LE1 9HN, UK

Mannans are linear polymers, composed of the backbones of β-1,4-linked mannose (and glucose) units, the mannose residues often possessing α-1,6-galactose as side groups and acetylated at the O-2 and O-3 positions depending on origins. Mannan exists mainly in nature as galactomannan, found in the seeds of leguminous plants and the beans of carob trees, and acetylated galactoglucomannan, a principal component of the hemicellulose found in softwoods.

β-Mannanase (endo-1,4-D-mannanase, EC 3.2.1.78) is a hydrolase that catalyzes the random hydrolysis of β-1,4-mannosidic linkages in the main chain of mannans and it has been isolated from plants, fungi, and bacteria (Reese and Shibata 1965; Hashimoto and Fukumoto 1969; McCleary 1988). Many of the genes for the enzymes have been cloned and sequenced, and these enzymes have been shown to belong to either glycoside hydrolase (GH) family 5 or 26 (http://afmb.cnrs-mrs.fr/CAZY). β-Mannanase has many possible applications, for instance, in pulp bleaching, reduction of the viscosity of instant coffee, and for the clarification of fruit juices and wines (Wong and Saddler 1993). An alkaline mannanase was found for the first time by Horikoshi and his colleagues from an alkaliphilic *Bacillus* (Akino et al. 1987). Recently, alkaline mannanases were found to be effective additives in laundry detergents, helping to remove gum stains due to cosmetics and/or foods (WO 99/09128, WO 99/09133). More recently, Ma et al. (2004) reported an alkaline mannanase belonging to GH family 5 from an alkaliphilic *Bacillus*. We also have found a few members of the genus *Bacillus* that are alkaline mannanase producers. *Bacillus* sp. strain JAMB-602 was

found to produce an alkaline mannanase belonging to GH family 5 and having an optimal pH for activity at around pH 9 (Takeda et al. 2004a). Furthermore, *Bacillus* sp. strain JAMB-750 was found to produce an alkaline mannanase Man26A (formerly AmA) having an optimal pH for activity at around pH 10 (Takeda et al. 2004b), the highest pH optimum among those of other mannanases reported to date. In this report, we describe the sequencing of the gene (*man26A*) for the alkaline mannanase, Man26A, from the strain JAMB-750, the expression of *man26A* in *Bacillus subtilis* cells, and the characterization of the recombinant enzyme.

Bacillus sp. strain JAMB-750 was grown at 30°C for 1 day in a liquid medium (Takeda et al. 2004b). Genomic DNA was prepared from the strain as described by Saito and Miura (1963), digested with *Eco*RI and then ligated into the *Eco*RI site of plasmid pUC18 (Takara Bio). *Escherichia coli* HB101 (F′ *supE44 hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 leuB6 thi-1*) was used as the host for cloning of the mannanase gene. Transformants that appeared on the Luria–Bertani agar medium containing ampicillin (100 μg ml^{-1}) were overlaid with a soft agar medium composed of (w/v) 0.25% konjak mannan, 0.1% lysozyme, 0.7% agar, and 50 mM glycine–NaCl–NaOH buffer (pH 9.0). Transformants expressing mannanase activities were selected by staining the overlaid medium with Congo red as described before (Takeda et al. 2004a). The DNA insert of the recombinant plasmid which was obtained from a positive transformant was sequenced with custom oligonucleotide primers using an ABI Prism Big Dye Terminator Cycle Sequencing kit and an ABI 377 Sequencer (Applied Biosystems, Foster City, CA, USA). The DNA sequence of the gene has been deposited to DDBJ/EMBL/GenBank with the accession number AB128831. An open reading frame of 2,994 bp was found in the sequence. The *man26A* gene encodes a protein of 997 amino acids. A putative ribosome-binding site sequence of 5′-AAAGGAGG-3′ is present 6-bp upstream from the initiation codon. An inverted-repeat sequence was found 8-bp downstream from the stop codon. The free energy value of this sequence for a stem-loop structure was calculated to be −92.4 kJ mol^{-1}, which would be sufficient for the termination of transcription. As shown in Fig. 1, the N-terminal sequence Glu–Ser–Lys–Ile–Pro–Lys–Asp of the native enzyme purified from the culture of strain JAMB-750 agreed with the deduced amino acid sequence at the positions of 27–33, suggesting that the peptide from Met1 to Ala26 would be a signal peptide for the exo-production of the enzyme by strain JAMB-750. Database searches using FASTA (http://www.ddbj.nig.ac.jp) with the amino acid sequence deduced from the *man26A* gene showed that the N-terminal half (Glu27–Val486) exhibited moderate similarities to known mannanases belonging to GH family 26. For example, the identity between Man26A and a mannanase from *Cellvibrio japonicus* (CAA57670) was 37% in a 438-amino-acid overlap, a mannanase from *Cellulomonas fimi* (AF126471) was 33% in an 881-amino-acid overlap, a mannanase from *Bacillus* sp. strain AM-001 (AAA22586) was 28% in a 389-amino-acid overlap, suggesting that Man26A should be a member of the GH family 26. Two Glu residues are conserved well in all the mannanases belonging to GH family 26, and Bolam et al. (1996) proposed that these conserved Glu residues can be catalytic residues. Glu213

Fig. 1 Deduced amino acid sequence from the *man26A* gene. The deduced amino acid sequence of the *man26A* gene product is shown by the *single-letter code*. The N-terminal sequence of the purified enzyme is indicated by *triangles*. The amino acid sequence similar to the carbohydrate-binding module classified as family 27 is indicated with *dots* beneath the amino acid sequence. The amino acid sequence exhibiting similarity to the mannan-binding module (CBM23) is *underlined*. The (E/K)(P/S/L)GEEG repeat region is *double underlined*. The amino acid sequence exhibiting similarity to the membrane anchor region of Gram-positive surface protein is indicated with *double dots* beneath the residues. The presumed catalytic Glu residues are indicated by *circles*

and Glu323 (numbering in Man26A) were aligned with the conserved Glu residues, and they could be also the catalytic residues of this enzyme. The region at Gly553–Leu687 has a similarity (23% identity, 50% similarity) to the CBM27 (carbohydrate-binding module classed into family 27) of a mannanase (Man5) from *Thermotoga maritima* (Boraston et al. 2003). Boraston et al. (2003) reported that the CBM27 bound tightly to mannan but not to cellulose and xylan. The region at Ile700–Ala867 has a similarity (37% identity, 61% similarity) to the mannan-binding module (CBM23) of a mannanase from *C. fimi* that binds to soluble mannans but not to insoluble forms (Stoll et al. 2000). These binding-module domains of Man26A would facilitate mannan degradation by binding to mannan. Moreover, the region Glu873–Gly938 has a unique sequence having ten repeats of the six amino acids of (E/K)(P/S/L)GEEG. Homology searches revealed that the repeated sequence had similarities to *Homo sapiens* collagen type IX alpha I chain (AF036130-2) and GE-rich salivary gland protein (AY226454-1) from *Anopheles stephensi*. We have no information available on the region for localization of Man26A or effects on substrate binding. The C-terminal region Gln954–Lys993 exhibited a similarity (35% identity, 62% similarity) to the membrane anchor region of Gram-positive surface proteins consensus sequence (Guss et al. 1984; Hollingshead et al. 1986). This region of Man26A may have the function of localization of the enzyme on the surface of the producer. We are now trying to express mutant enzymes after deleting the domains of Man26A in order to examine each function of these domains, for example, binding ability to mannan, localization, and any effects on the relationship with the substrate of the enzyme.

A 4.0-kb DNA fragment between the upstream region and the terminator sequence of the *man26A* gene was amplified by PCR from the genomic DNA of strain JAMB-750 using LA *Taq* DNA polymerase and two primers A (5′-TCTTGGATCCACAATGTCAAAATCGTAACGAGC-3′) and B (5′-CGTTT GGATCCAGTCAATGGAATAGAC-3′), where *Bam*HI restriction sites were incorporated (underlined). The PCR product was digested with *Bam*HI and ligated into the *Bam*HI site of pHY300PLK. The plasmid constructed was introduced into a protease-deficient mutant of *B. subtilis* ISW1214 (Hatada et al. 2004). The transformant was cultured to express the mannanase activity, with shaking, in a medium composed of (w/v) 10% corn steep liquor (Nihon Syokuhin Kako, Shizuoka, Japan), 1% bonitomeat extract (Wako Pure Chemical), 0.1% yeast extract (Difco), 0.1% KH_2PO_4, 0.02% $MgSO_4{\cdot}7H_2O$, 0.05% $CaCl_2$, 7% maltose, and tetracycline (15 μg ml^{-1}) at 30°C. The recombinant alkaline mannanase exo-produced into the culture medium was purified 15.3-fold after anion-exchange chromatography and hydroxyapatite chromatography, essentially according to the method for native enzyme purification (Takeda et al. 2004b) with a specific activity of 36.3 U mg^{-1} and a final yield of 14.2%. The N-terminal amino acid sequence of the recombinant enzyme was Glu–Ser–Lys–Ile–Pro–Lys–Asp, corresponding to the amino acids Glu27–Asp33 of the deduced amino-acid sequence of *man26A* gene. The N-terminal sequence was identical to that of the native enzyme purified from the culture of strain JAMB-750 (Takeda et al. 2004b). The SDS-PAGE and activity staining of the purified enzyme gave a single band with a molecular mass of 130 kDa. This value is identical to that of the native enzyme produced by *Bacillus* sp. strain JAMB-750 (Takeda et al. 2004b), although larger than that estimated on the basis of its gene sequence. The calculated molecular mass of the recombinant enzyme was 107 kDa for Man26A. At present, we do not have an explanation for why such a discrepancy occurs between the estimated mass on SDS-PAGE and the theoretical value based on the *man26A* gene. Occasionally, differences of the values between those calculated from the sequence and estimated from the migration on SDS-PAGE have been seen, for example, in the case of a mannanase from a *Bacillus* (Yoshida et al. 1998). Yoshida et al. (1998) reported that the mannanase from *Bacillus circulans* K-1 contained sugars detected by a PAS staining test. However, both our native and recombinant enzymes were negative for PAS staining.

No significant differences in the physicochemical and catalytic properties were observed between the recombinant enzyme and the native enzyme. In detail, the pH optimum for the mannanase activity of the recombinant enzyme was observed at around pH 10 in 50 mM glycine–NaCl–NaOH. The enzyme was very stable at pH 6.0–10.5, retaining more than 80% of the original activity. The optimal temperature for the activity of the recombinant enzyme was around 55°C. The effects of the chemicals and cations on the activity of the recombinant enzyme were also the same as those of the native enzyme (Takeda et al. 2004b), for example, the mannanase activity was lost completely by 1.0 mM NBS, and Fe^{3+}, Fe^{2+}, Pb^{2+}, Hg^{2+}, and Cd^{2+} inhibited strongly the activity. The time course of the hydrolysis products from mannan extracted from ivory nuts was examined with 0.04 U ml^{-1} of the recombinant enzyme incubated at 40°C for up to 24 h. In the initial stage, the enzyme hydrolyzed mannan to generate manno-saccharides with various degrees of polymerization, suggesting that this enzyme was an endo-type mannanase. After incubation for 24 h, the main products were mannobiose and mannotriose.

The enzyme was very stable at high pH. In addition, we found that this enzyme was very stable to surfactants, such as TritonX-100, Tween 20, sodium dodecyl benzene sulfonate, and sodium alkane sulfonate (each at 0.5%). Remarkably, the enzyme was found to be stable to SDS at least up to 2.0% (Fig. 2). These stabilities of the enzyme are very important for detergent. In order to understand the mechanism of tolerance to high pH and high activity at high pH, the determination of the three-dimensional structure of the alkaline mannanase is clearly required. Now we are trying to crystallize the

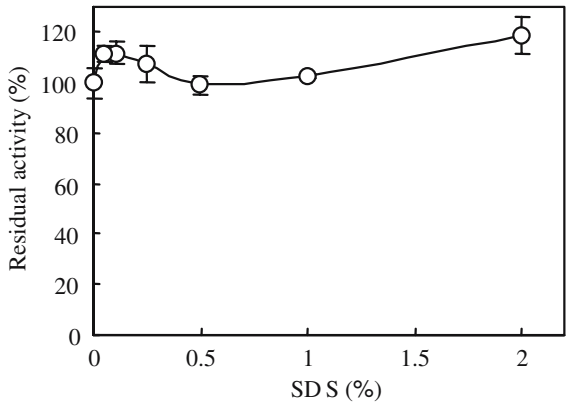

Fig. 2 Tolerance to SDS. The mannanases were incubated with up to 2.0% SDS at pH 7.5 in 25 mM Tris–HCl buffer and 20°C for 30 min. After 1 : 20 dilution, the samples were used for the measurement of the residual activity under the standard conditions of the assay

enzyme, and the resulting structure should be compared with that of the published mannanase (Hogg et al. 2001), which belongs to GH family 26 and has its optimal pH for activity at around seven.

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