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Cloning and Characterization of a Novel Mannanase from *Paenibacillus* sp. BME-14

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A mannanase gene (*man26B*) was obtained from a sea bacterium, *Paenibacillus* sp. BME-14, through the constructed genomic library and inverse PCR. The gene of *man26B* had an open reading frame of 1,428 bp that encoded a peptide of 475- amino acid residues with a calculated molecular mass of 53 kDa. Man26B possessed two domains, a carbohydrate binding module (CBM) belonging to family 6 and a family 26 catalytic domain (CD) of glycosyl hydrolases, which showed the highest homology to Cel44C of *P. polymyxa* (60% identity). The optimum pH and temperature for enzymatic activity of Man26B were 4.5 and 60°C, respectively. The activity of Man26B was not affected by Mg^{2+} and Co^{2+}, but was inhibited by Hg^{2+}, Ca^{2+}, Cu^{2+}, Mn^{2+}, K^{+}, Na^{+}, and β-mercaptoethanol, and slightly enhanced by Pb^{2+} and Zn^{2+}. EDTA did not affect the activity of Man26B, which indicates that it does not require divalent ions to function. Man26B showed a high specific activity for LBG and konjac glucomannan, with K_m, V_{max}, and k_{cat} values of 3.80 mg/ml, 91.70 μmol/min/mg protein, and 77.08/s, respectively, being observed when LBG was the substrate. Furthermore, deletion of the CBM6 domain increased the enzyme stability while enabling it to retain 80% and 60% of its initial activity after treatment at 80°C and 90°C for 30 min, respectively. This finding will be useful in industrial applications of Man26B, because of the harsh circumstances associated with such processes.

Keywords: *Paenibacillus* sp., mannanase, glycosyl hydrolase family 26, carbohydrate binding module 6, thermal stability

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Hemicelluloses are the second most abundant heteropolymers present in nature, and are only exceeded by cellulose. The major component of the hemicellulose fraction of soft woods and seeds of leguminous plants is mannan [10], which is a complex molecule composed of linear mannan, glucomannan, galactomannan, and galactoglucomannan. In glucomannan, mannose residues are interspersed by β-1,4-linked glucose, and single galactosyl residues are α-1,6-linked to the mannan backbone in galactomannan.

β-Mannanase (E.C. 3.2.1.78) catalyzes the random hydrolysis of β-1,4 mannosidic linkages in β-1,4-mannan, glucomannan, and galactomannan; however, this process is greatly affected by the extent and pattern of substitution on the mannan backbone. Mannan oligosaccharides released from mannans can be used as energy, feed, and food sources. Mannanase is also used in several industrial processes, including the extraction of vegetable oils from leguminous seeds and reduction of the viscosity of coffee extracts during the manufacture of instant coffee [12]. Furthermore, the synergistic action of mannanase and xylanase is effective for prebleaching of softwood pulp, thereby reducing the environmental pollution associated with chemical agents commonly used in the pulp and paper industry [16]. Because of its wide applications, mannanases derived from fungi, bacteria, plants [5], and even mollusks [21] have been extensively studied in recent years. These enzymes have been shown to belong to either glycosyl hydrolase family 5 or 26. However, few organisms that produce mannanase have been isolated from the sea, which is the largest region on Earth and has abundant and novel resources that have yet to be discovered.

Here, we report the cloning, purification, and characteristics of a mannanase (Man26B) produced by the sea bacterium *Paenibacillus* sp. The removal of CBM was found to result in a mannanase with high heat stability, which indicates that it has the potential for widespread application.

MATERIALS AND METHODS

Strains and Vectors

Paenibacillus sp. BME-14 was isolated from the Xiamen shallow sea and analyzed based on its 16S rDNA sequence. *E. coli* DH5α and *E. coli* BL21 (DE3) were used as hosts for gene cloning and protein expression, respectively. Plasmid pGEX-6P-1 was used for the expression and purification of Man26B and Man26Bcd.

Materials and Chemicals

Locust bean gum (LBG), sodium carboxymethycellulose, barley glucan, oat spelts xylan, birchwood xylan, and laminarin from *Laminaria digitata* were purchased from Sigma. Konjac glucomannan was purchased from Wuhan Qingjiang Konjac Co., Ltd. A GST Bind Purification Kit was purchased from Novagen. An AxyPrep DNA Purification Kit was purchased from Axygen. All other chemicals used in this study were of analytical grade.

Inverse PCR

Genomic DNA was extracted from *Paenibacillus* sp. BME-14 and then digested with the restriction enzyme, *Hin*dIII. Next, the fractions were ligated onto themselves to form a circle, which was used as the template for inverse PCR. The primers for inverse PCR were BM (5'-TAATGTATGATCGATTCACGAACTT) and BF (5'-ACTTCACTGAAATTGGTTGTTTCAT). After PCR, the product was purified by gel electrophoresis and sequenced.

Sequence Analysis

The ORF coding mannanase was identified using Softberry (http://linux1.softberry.com/berry.phtml) to evaluate the sequences returned by a BLASTX search of the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/), after which multiple sequence alignment was performed using ClustalW. The promoter was screened for by using Softberry. The amino acid sequence was analyzed to predict the signal peptide using SignalIP (http://www.cbs.dtu.dk/services/SignalP/), and to evaluate the function of the protein using the tool of InterProScan.

Gene Cloning and Expression

Based on the results of sequencing and analysis, the following primers were designed to clone the mannanase gene without the signal peptide: BMF, 5'-CATGGATCCATTATATTTGAAGCGGAAGAT; BMR, 5'-CGCGTCGACTTAATACTCATTTTTCCAAT (underlined nucleotides indicate the *Bam*HI and *Sal*I restriction sites, respectively). After PCR, the products were double-digested with *Bam*HI and *Sal*I, purified, and then ligated into the vector pGEX-6p1, which resulted in the addition of an N-terminal GST tag. This product was then transformed into *E. coli* DH5α. Positive transformants were identified by functional screening on locust bean gum (LBG) medium. To induce the expression of mannanase, the recombinant plasmids were transformed into the expression host, *E. coli* BL21 (DE3).

Purification of Enzymes

The strain containing the recombinant plasmid was incubated in LB liquid medium supplemented with 100 μg/ml ampicillin at 37°C overnight and then transferred to fresh LB medium (1:100) that contained ampicillin. When the OD_{600} reached 0.6, isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.2 M to induce the expression of mannanase. After induction for 5 h at 28°C, the cells were harvested by centrifugation, washed, resuspended in PBS buffer (0.8% NaCl, 0.02% KCl, 0.142% Na_2HPO_4, 0.027% KH_2PO_4; pH 7.4), and disrupted using a French Cell Press. Next, the cell-free extract was obtained by centrifugation (12,000 rpm, 30 min) and the targeted protein was then purified by conducting the following procedures at 4°C. The supernatant passed through a glutathione Sepharose 4B column that had been equilibrated with PBS buffer, after which the unbound proteins were eluted using PBS buffer. Next, 10 μl of 3C protease stock solution was diluted with the same buffer and added to the column overnight to cleave the enzyme from GST bound with glutathione. Finally, 2 ml of PBS buffer was used to elute the enzyme, which was then mixed with an equal volume of glycerol and stored at −80°C.

Protein Measurement and Zymogram Analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) was conducted to determine the purity and the molecular mass of the enzyme using the method described by Laemmli [14]. Native polyacrylamide gel electrophoresis was performed similarly but without SDS. A protein quantification kit with bovine serum albumin as the standard was used to determine the protein concentration. For the zymogram analysis, native polyacrylamide gel containing 0.25% LBG was prepared and the whole electrophoresis process was carried out at 4°C. The gel was then incubated in the citrate-phosphate buffer (pH 4.5) at 50°C for 30 min. Later, the clear zones that corresponded to the enzyme activity were visualized by Congo red staining method [6]. Briefly, the gel was stained in 0.25% Congo red solution for 30 min, and then washed in 1 M NaCl solution for three times.

Enzyme Assays

The mannanase activity was assayed using the 3,5-dinitrosalicylic acid method [17]. The standard assay reaction mixture contained 0.5% (w/v) locust bean gum (LBG) supplemented with citrate-phosphate buffer (pH 4.5) and enzyme, and the final volume was 0.1 ml. The reaction mixture was incubated at 60°C for 30 min and one unit (U) of enzyme activity was defined as the amount of enzyme producing 1 μmole of mannose per minute under the assay conditions.

The effects of pH on mannanase activity were evaluated at pH 3.5–10.0 under standard assay conditions at 40°C using the following buffers: citrate-phosphate buffer (pH 3.5–0.8) and glycine-NaOH buffer (pH 6.7–8.2). The optimal temperature of mannanase activity was examined at temperatures ranging from 20°C–80°C at 5°C intervals in citrate-phosphate buffer (pH 4.5). The thermal stability of the enzyme was determined by pretreating the samples at temperatures ranging from 20°C–70°C at 10°C intervals for 30 min and then measuring the residual activities under standard conditions. Half-lives of thermal inactivation of enzymes ($T_{1/2}$ values) were determined by plotting the residual activities of enzymes after incubation at 60°C, 70°C, 80°C, and 90°C for various time intervals.

Effects of Various Reagents

The effects of chemical compounds and metal ions on the activity of Man26B were determined by measuring the activities of Man26B under standard conditions in the presence of various additional reagents. The activity obtained using the reaction system without any additional regent was taken to be 100%.

Substrate Specificity

The substrate specificity was determined by assay using the following polysaccharides as substrates: LBG, konjac glucomannan, sodium

carboxymethycellulose, barley glucan, oat spelts xylan, birchwood xylan, and laminarin from *Laminaria digitata*. To determine the kinetic parameters, locust bean gum was used in the concentration ranges of 1–10 mg/ml and the enzymatic activity was assayed under standard conditions. The data were plotted according to the Lineweaver–Burk method.

Effect of CBM

The region of the catalytic domain (*man26Bcd*) was amplified using the following primers: MCF (5'-AGCGGATCCCATCAAATCAATGCA TCACT); BMR (5'-CGCGTCGACTTAATACTCATTTTTCCAAT). The amplicons were then cloned into the pGEX-6p-1, expressed in *E. coli* BL21(DE3), and purified using the same method used to purify the Man26B. The biochemical properties of Man26Bcd were also evaluated using the same methods used to evaluate Man26B.

Nucleotide Sequence Accession Number

The DNA sequence described in this study has been deposited into the GenBank database under Accession No. GQ250044.

Results

Strain Identification

The comparison of the 16S rDNA sequence (Accession No. GQ920787) from the strain BME-14 with that in GenBank showed a nucleotide identity of 99.9% with *Paenibacillus provencensis* strain 4401170 (Accession No. AF395033.1), 98.3% with *Paenibacillus urinalis* strain 5402403 (Accession No. EF212892.1), 98.1% with *Paenibacillus* sp. J16-10 (Accession No. AM162327.1), 97.8% with *Paenibacillus* sp. 7–5 (Accession No. AM043868.1), and 97.3% with *Paenibacillus xylanilyticus* (Accession No. EU249590.1). Thus, strain BME-14 was classified into the genus *Paenibacillus*. The distance tree created by the neighbor-joining method also showed the same classification (data not shown).

Gene Cloning and Sequence Analysis

According to the results of the sequenced insert that was obtained by genomic library construction of the cellulase gene, there was fragment homology to β-mannanase except for an endoglucanase gene described previously [6]. A 2-kb fragment was obtained by inverse PCR and sequenced, and then assembled with the known fragment. The integrity of the fragment was then analyzed by a BLASTX search, which revealed the presence of a putative mannanase region. Overall, the results revealed that the ORF was composed of 1,428 bp encoding a preprotein of 475 amino acid residues with a predicted molecular mass of 53 kDa. The initiation codon, ATG, was preceded at a spacing of 8 bp by a potential ribosome-binding sequence (AAGGAGG). This sequence was a perfect complement to a sequence found at the 3' end of the *E. coli* 16S ribosomal RNA (3'-UUCCUCC-5'), which is known to play a crucial role in bringing the 30S ribosome to the initiator codon. No transcription terminator sequence was found downstream of the stop codon (TAA) when the sequence was analyzed using the Findterm program in Softberry (http://linux1.softberry.com/berry.phtml).

Amino Acid Sequence Analysis

Analysis of the amino acid sequence using SignalP revealed that the N-terminal amino acid sequence of Man26B has features of a typical signal peptide that are characteristic of extracellular enzymes. The predicted signal peptide cleavage site was between A29 and A30. Removal of the signal peptide yielded a mature protein that had a predicted Mr of 50 kDa. Amino acid sequence analysis conducted using InterProScan revealed that Cel9P contained two distinct domains. Specifically, the region between residues 35 and 151 of Man26B was a carbohydrate binding module (CBM) belonging to family 6 that was found to be most similar to Cel44C of *Paenibacillus polymyxa* (49% homology) [4]. Following this region was a family 26 catalytic domain (CD) of glycosyl hydrolases, which showed the highest homologies to Cel44C of *P. polymyxa* (65% homology), ManA of *Dictyoglomus thermophilum* (56% homology) [8], ManA of *Caldicellulosiruptor* Rt8B.4 (56% homology) [7], endo-1,4-beta-mannanase of *Rhodothermus marinus* (48% homology) [19], and Man26B of *Clostridium thermocellum* (43% homology) [13]. One of the most highly conserved regions among family 26 enzymes was an aromatic amino-acid-rich region with the consensus sequence WFWWG [3], which was found in Man26B by the multiple sequence alignment using ClustalW (Fig. 1).

Purification and Biochemical Properties of Man26B

To clone the complete *man26B* gene in *E. coli*, primers were designed to amplify the complete ORF of *man26B*

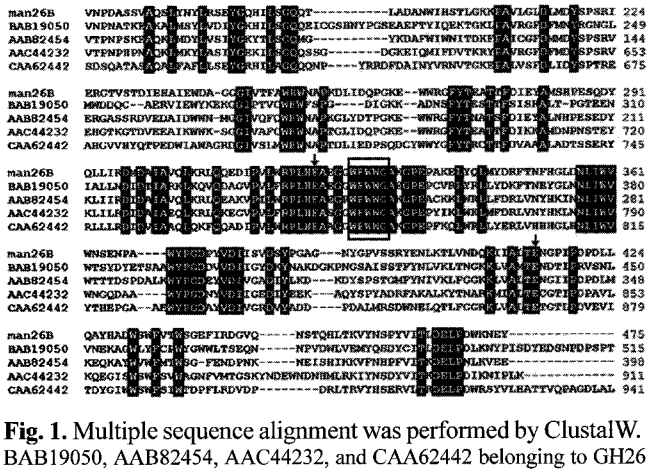

Fig. 1. Multiple sequence alignment was performed by ClustalW. BAB19050, AAB82454, AAC44232, and CAA62442 belonging to GH26 were from *Clostridium thermocellum*, *Dictyoglomus thermophilum*, *Caldicellulosiruptor saccharolyticus*, and *Rhodothermus marinus*, respectively. Identical amino acids are shaded. The most highly conserved region is labeled by the box. The putative catalytic residues are labeled with an arrow.

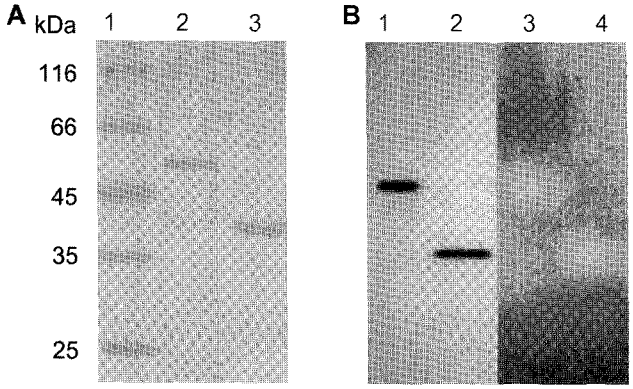

Fig. 2. Gel analyses of the purified Man26B and Man26Bcd.
A. 10% SDS-PAGE analyses lane 1 is a protein maker, lane 2 is the purified Man26B, and lane 3 is the purified Man26Bcd. **B.** Zymogram analysis. Lanes 1 and 2 represent the native polyacrylamide gel electrophoresis of the purified Man26B and Man26Bcd, respectively. Lanes 3 and 4 represent the zymograms of the purified Man26B and Man26Bcd, respectively.

encoding mature mannanase by PCR, using genomic DNA as the template. The *man26B* gene was cloned into expression vector pGEX-6p1 and the resulting plasmid, pGEX-man26B, was transformed into *E. coli* BL21 (DE3). The recombinant Man26B mannanase was then purified to electrophoretic homogeneity from obtrite *E. coli* cells harboring pGEX-man26B using the GST-tag (Fig. 2A). Through the zymogram analysis, a clear zone that corresponded to the single band of Man26B on the native polyacrylamide gel was observed. This indicates our purified enzyme is most likely a monomeric mannanase. The final specific activity of the enzyme was found to be 145.90 U/mg when LBG was used as the substrate under standard conditions.

The purified mannanase was active at a broad pH range of 4–8.5, and the optimal pH for activity was 4.5 (Fig. 3A). However, the activity of the mannanase decreased sharply at pH values below 4 or above 8.5. Man26B was active at temperatures between 35°C and 65°C, with the optimal activity occurring at 60°C. These findings were universal among the mannanases. The activity of Man26B decreased dramatically at 70°C, with only 40% of the initial activity being retained (Fig. 3B). Thermostability experiments revealed that the enzyme was very unstable, even at temperatures as low as 20°C, where it retained only 55% of its initial activity (Fig. 4). Further experiments showed that the $T_{1/2}$ values of the enzyme at 70°C and 60°C were 7 min and 12 min, respectively.

The effects of metal ions and chemical compounds on Man26B are shown in Table 1. The mannanase activity was unaffected by EDTA and Mg^{2+}, enhanced by Pb^{2+} and Zn^{2+}, and inhibited slightly by Ca^{2+}, Na^{+}, Mn^{2+}, and β-mercaptoethanol, whereas it was strongly inhibited by Hg^{2+}.

The purified mannanase was examined for its ability to hydrolyze various substrates under the standard conditions

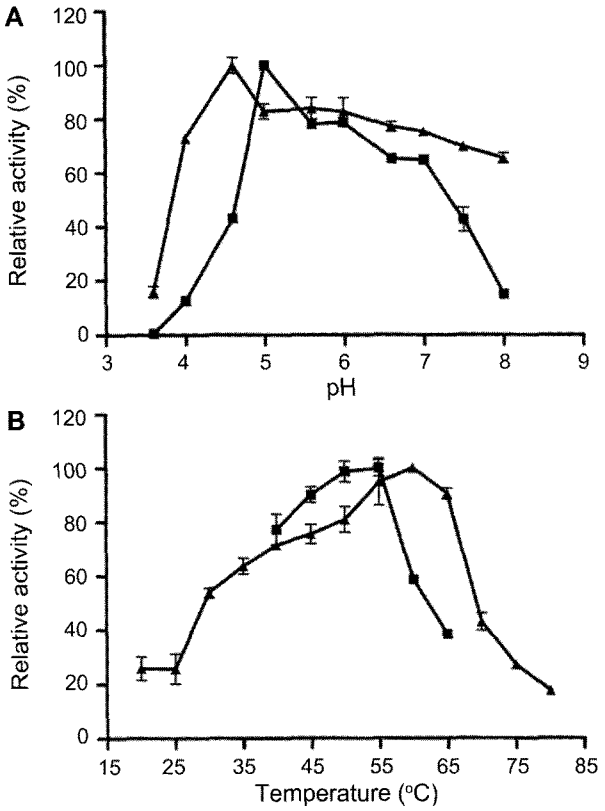

Fig. 3. Effects of pH and temperature on the activities of Man26B (▲) and Man26Bcd (■).
A. The pH assay was performed in citrate-phosphate buffer containing 0.5% LBG at pH values ranging from 3.5 to 8. The maximal activity was taken to be 100%. **B.** Effect of temperature on the activity of Man26B (▲) and Man26Bcd (■). Assay was performed in 0.2 M citrate-phosphate buffer containing 0.5% LBG at pH 4.5 and various temperatures. The maximal activity was taken to be 100%.

(Table 2). The results revealed that the highest specific activity occurred when LBG was used as the substrate (mannose:galactose ratio, 4:1), whereas 80% of the LBG

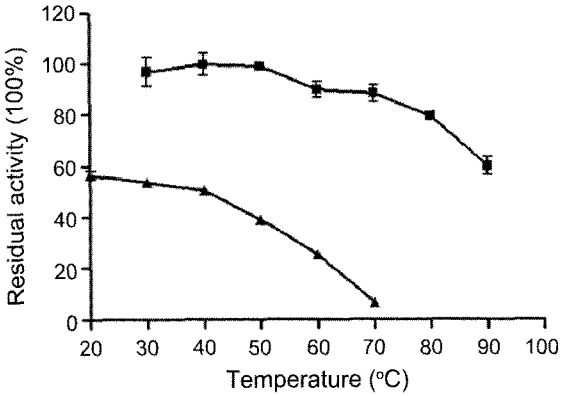

Fig. 4. Effect of temperature on the stability of Man26B (▲) and Man26Bcd (■).
The enzyme was pretreated at various temperatures for 30 min, after which the activity was measured under the optimum conditions. The activity of the untreated enzyme was taken to be 100%.

Table 1. Effects of metal ions and reagents on the activity of Man26B.

Reagent	Concentration (mmol/l)	Relative activity[a] (%)[b]
Control	-	100
Ca^{2+}	1	82.8±6.9
Mg^{2+}	1	98.9±11.5
Cu^{2+}	1	75.7±1.8
Mn^{2+}	1	62.8±4.3
Co^{2+}	1	106.7±6.0
Pb^{2+}	1	127.9±8.9
Hg^{2+}	1	4.0±1.1
Zn^{2+}	1	116.9±1.9
K^{+}	1	62.2±5.2
β-Mercaptoethanol	1%	53.9±4.6
EDTA	1	107.2±3.8

[a]Assay was performed under the optimum condition.
[b]Standard deviations are shown behind the relative activities.

activity was retained when konjac glucomannan was used as the substrate (mannose:glucose ratio, 1.5:1). No reducing sugars were detected when sodium carboxymethycellulose, barley glucan, oat spelts xylan, birchwood xylan, or laminarin were used as substrates. Taken together, these data demonstrate that Man26B is a mannanase with narrow substrate specificity. Specifically, these results indicate that the enzyme does not hydrolyze other polysaccharides and that it specifically cleaves the β-1,4-glycosidic linkages between mannopyranosyl residues. The kinetic parameters (K_m, V_{max} and k_{cat}) of Man26B were determined on LBG at 60°C, which revealed that the K_m, V_{max} and k_{cat} were 3.80 mg/ml, 91.70 μmol/min/mg protein, and 77.08/s, respectively.

Effect of CBM

The CD of Man26B (Man26Bcd) with a theoretical molecular mass of 37 kDa was purified to homogeneity (Fig. 2A). A clear zone showing the mannanase activity was produced on a zymogram, confirming the purified Man26Bcd as most possibly a monomer. The optimal activity of Man26Bcd was obtained at pH 5 (Fig. 3A) and 55°C (Fig. 3B). The specific activity of Man26Bcd was 146.1 U when LBG was used as the substrate, which was nearly the same as that of Man26B. However, Man26Bcd was very thermally stable, as indicated by it retaining approximately 90%, 80%, and 60% of its initial activity after treatment for 30 min at 70°C, 80°C, and 90°C, respectively (Fig. 4). Further experiments showed that the $T_{1/2}$ values of the enzyme at 60°C, 70°C, 80°C, and 90°C were 210 min, 130 min, 70 min, and 45 min, respectively.

Table 2. Substrate specificity of Man26B.

Substrate	Specific activity (U/mg)
Locust bean gum	145.90±2.91[a]
Konjac glucomannan	116.46±1.29
Oat spelts xylan	<0.01
Birchwood xylan	<0.01
Barley glucan	<0.01
CMC-Na	<0.01
Laminarin	<0.01

[a]Standard deviations are shown behind the specific activities.

Discussion

To date, there is only one mannanase gene having been cloned from the *Paenibacillus* sp., which belonged to glycosyl hydrolase family 26 and was found to be part of a multidomain glycosyl hydrolase (Cel44C) [4]. However, the properties of this mannanase were not evaluated. In the present study, we cloned and characterized a mannanase from *Paenibacillus* sp. BME-14. Based on the substrate specificity of Man26B, the enzyme was classified as an endo-β-1,4-mannanase that displayed no detectable activity against other polysaccharides.

Based on sequence analyses and hydrophobic cluster analysis [11], the catalytic domains of glycosyl hydrolases have been classified into 115 families (http://www.cazy.org/fam/acc_GH.html). To date, only β-mannanases belonging to family 5 or 26 have been identified. The putative CD of Man26B, which was identified by gene sequence comparisons, displayed up to 65% homology with other mannanases belonging to family 26. Some mannanases contain carbohydrate-binding domains or other domains in addition to the catalytic domain, such as Man26A from *Cellulomonas fimi*, which has a mannan-binding domain, a S-layer homology domain, and a domain of unknown function [19]. The present study revealed that Man26B possesses a CBM6 domain preceding the CD26, which is a mannan-binding domain that was first identified in the N-terminus of Man26A from *Cl. thermocellum* strain YS [9]. Furthermore, multiple-sequence alignment revealed that the putative catalytic residues in Man26B, E322, D377, and E414 were highly conserved among GH26 family members, with E322 and E414 located at the C-terminal end of strands β-4 and β-7, respectively [18].

Some biochemical properties of different mannanases are listed in Table 3. Compared with other mannanases, Man26B showed the highest activity in a relatively acidic pH condition; therefore, it has the potential for use in feed because it can withstand the conditions of the intestinal tract. Likewise, in comparison with other homologous mannanases, the optimal temperature of Man26B was lower and the enzyme was more labile, even at 20°C. However, Man26Bcd, which was composed of the catalytic domain alone, was most active at 55°C. Although this was similar to the activity of Man26B, the thermostability of Man26Bcd

Table 3. Biochemical properties of mannanases from various microorganisms.

Microorganism	Enzyme	Optimum temperature (°C)	Thermostability (half-life)	Optimum pH	kDa	Reference
Paenibacillus sp. BME-14	Man26B	60	7 min at 70°C	4.5	55	This work
Paenibacillus sp. BME-14	Man26Bcd	55	130 min at 70°C	5.0	37	This work
Clostridium thermocellum	Man26BC	75	NR[a]	7.0	55	[13]
Dictyoglomus thermophilum	ManAD	80	5.4 min at 90°C	5.0	40	[8]
Caldicellulosiruptor Rt8B.4	ManAC	60–65	NR[a]	6.0–6.5	NR[a]	[7]
Rhodothermus marinus	ManAR	85	50 min at 90°C	5.4	46	[19]

[a]Not reported.

was much greater. The half-life of Man26Bcd at 70°C was nearly more than 20 times of that of Man26B. Previously, Araki *et al.* [1] reported that the catalytic module of xylanase alone exhibited higher thermal stability than when it was attached to CBM22. Similarly, studies conducted by Wang *et al.* [20] revealed that truncation of the CBM of an endo-β-1,4-glucanase from *Bacillus subtilis* JA18 increased the half-life of the enzyme at 65°C by three times. It has been suggested that the increased thermal stability of these truncated enzymes may result from the higher enzyme refolding efficiency after denaturation and the larger enzyme fractional polar surface area with added hydrogen bonding density to water. Notwithstanding that these descriptions could partially explain the enhanced thermal stability of Man26Bcd, it is believed to be the first time a truncated mannanase with high thermal stability is found. Nevertheless, the results of the present study indicate that Man26Bcd could be applied as a thermostable enzyme in processes such as biobleaching of softwood pulps.

The metal chelator EDTA had no effect on the activity of Man26B at a concentration of 1 mM. These results suggest that Man26B does not require metal ions for activity or stability, which is similar to ManA from *Dictyoglomus thermophilum*. β-Mercaptoethanol inhibited the activity of Man26B significantly. This indicates that there may be some thiol groups existing around the active sites of Man26B. Hg^{2+} strongly inhibited the activity of Man26B, possibly due to binding with Try residue(s) or carboxyl group(s) of the amino acid(s) [15]. Generally, the amino acids located at active sites participating in the catalytic action as nucleophilic and acid-base catalysts were Glu or Asp, such as E212 and E320 in Man26A from *Pseudomonas fluorescens* [2], and the corresponding sites were E322 and E414 in the present study. Taken together, these findings suggest that the majority of mannanases may be inhibited by the addition of Hg^{2+}.

In conclusion, a novel mannanase gene *man26B*, cloned from *Paenibacillus* sp. BME-14, was found to encode an acidic mannanase that may be useful in the feed industry. Moreover, Man26Bcd, which was produced by cleaving the CBM6 from Man26B, exhibited high thermal stability, indicating that this enzyme may also have industrial applications. Accordingly, additional studies are being conducted to elucidate the relationship between the CBM and the thermal stability of the mannanase. The results of the present study and the ongoing studies will be of value in the engineering of mannanase and other enzymes.

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References

1. Araki, R., S. Karita, A. Tanaka, T. Kimura, and K. Sakka. 2006. Effect of family 22 carbohydrate-binding module on the thermostability of Xyn10B catalytic module from *Clostridium stercorarium*. *Biosci. Biotechnol. Biochem.* **70:** 3039–3041.
2. Bolam, D. N., N. Hughes, R. Virden, J. H. Lakey, G. P. Hazlewood, B. Henrissat, K. L. Braithwaite, and H. J. Gilbert. 1996. Mannanase A from *Pseudomonas fluorescens* ssp. *cellulosa* is a retaining glycosyl hydrolase in which E212 and E320 are the putative catalytic residues. *Biochemistry* **35:** 16195–16204.
3. Braithwaite, K. L., G. W. Black, G. P. Hazlewood, B. R. Ali, and H. J. Gilbert. 1995. A non-modular endo-beta-1,4-mannanase from *Pseudomonas fluorescens* subspecies *cellulosa*. *Biochem. J.* **305:** 1005–1010.
4. Cho, K. M., S. Y. Hong, S. M. Lee, Y. H. Kim, G. G. Kahng, H. Kim, and H. D. Yun. 2006. A *cel44C–man26A* gene of endophytic *Paenibacillus polymyxa* GS01 has multi-glycosyl hydrolases in two catalytic domains. *Appl. Microbiol. Biotechnol.* **73:** 618–630.
5. Filichkin, S. A., J. M. Leonard, A. Monteros, P. P. Liu, and H. Nonogaki. 2004. A novel endo-beta-mannanase gene in tomato LeMAN5 is associated with anther and pollen development. *Plant Physiol.* **134:** 1080–1087.
6. Fu, X., P. Liu, L. Lin, Y. Hong, X. Huang, X. Meng, and Z. Liu. 2009. A novel endoglucanase (Cel9P) from a marine bacterium *Paenibacillus* sp. BME-14. *Appl. Biochem. Biotechnol.* doi: 10.1007/s12010-009-8648-2.

7. Gibbs, M. D., A. U. Elinder, R. A. Reeves, and P. L. Bergquist. 1996. Sequencing, cloning and expression of a beta-1,4-mannanase gene, *manA*, from the extremely thermophilic anaerobic bacterium, *Caldicellulosiruptor* Rt8B.4. *FEMS Microbiol. Lett.* **141:** 37–43.
8. Gibbs, M. D., R. A. Reeves, A. Sunna, and P. L. Bergquist. 1999. Sequencing and expression of a beta-mannanase gene from the extreme thermophile *Dictyoglomus thermophilum* Rt46B.1, and characteristics of the recombinant enzyme. *Curr. Microbiol.* **39:** 351–357.
9. Halstead, J. R., P. E. Vercoe, H. J. Gilbert, K. Davidson, and G. P. Hazlewood. 1999. A family 26 mannanase produced by *Clostridium thermocellum* as a component of the cellulosome contains a domain which is conserved in mannanases from anaerobic fungi. *Microbiology* **145:** 3101–3108.
10. Handford, M. G., T. C. Baldwin, F. Goubet, T. A. Prime, J. Miles, X. Yu, and P. Dupree. 2003. Localisation and characterisation of cell wall mannan polysaccharides in *Arabidopsis thaliana*. *Planta* **218:** 27–36.
11. Henrissat, B. 1991. A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem. J.* **280:** 309–316.
12. Kansoh, A. and Z. Nagieb. 2004. Xylanase and mannanase enzymes from *Streptomyces galbus* NR and their use in biobleaching of softwood kraft pulp. *Antonie Van Leeuwenhoek* **85:** 103–114.
13. Kurokawa, J., E. Hemjinda, T. Arai, S. Karita, T. Kimura, K. Sakka, and K. Ohmiya. 2001. Sequence of the *Clostridium thermocellum* mannanase gene *man26B* and characterization of the translated product. *Biosci. Biotechnol. Biochem.* **65:** 548–554.
14. Laemmli, U. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685.
15. Lusterio, D., F. Suizo, N. Labunos, M. Valledor, S. Ueda, S. Kawai, *et al.* 1992. Alkali-resistant, alkaline endo-1,4-β-glucanase produced by *Bacillus* sp. PKM-5430. *Biosci. Biotechnol. Biochem.* **56:** 1671–1672.
16. Marga, F., C. Ghakis, C. Dupont, R. Morosoli, and D. Kluepfel. 1996. Improved production of mannanase by *Streptomyces lividans*. *Appl. Environ. Microbiol.* **62:** 4656–4658.
17. Miller, G. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. chem.* **31:** 426–428.
18. Politz, O., M. Krah, K. K. Thomsen, and R. Borriss. 2000. A highly thermostable endo-1,4-beta-mannanase from the marine bacterium *Rhodothermus marinus*. *Appl. Microbiol. Biotechnol.* **53:** 715–721.
19. Stoll, D., A. Boraston, H. Stalbrand, B. W. McLean, D. G. Kilburn, and R. A. Warren. 2000. Mannanase Man26A from *Cellulomonas fimi* has a mannan-binding module. *FEMS Microbiol. Lett.* **183:** 265–269.
20. Wang, Y., H. Yuan, J. Wang, and Z. Yu. 2009. Truncation of the cellulose binding domain improved thermal stability of endo-β-1,4-glucanase from *Bacillus subtilis* JA18. *Bioresource Technol.* **100**: 345–349.
21. Xu, B., D. Sellos, and J. C. Janson. 2002. Cloning and expression in *Pichia pastoris* of a blue mussel (*Mytilus edulis*) beta-mannanase gene. *Eur. J. Biochem.* **269:** 1753–1760.