J. Microbiol. Biotechnol. (2004), **14**(6), 1295–1302

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Cloning of a *Bacillus subtilis* WL-7 Mannanase Gene and Characterization of the Gene Product

KWEUN, MIN A[1], MI-SUNG LEE[1], JOON HO CHOI[3], KI HAENG CHO[4], AND KI-HONG YOON[1,2]*

[1]School of Food Science and Biotechnology, [2]Bioresouce and Application Research Center, Woosong University, Daejeon 300-718, Korea
[3]R&D Center, KoBioTech Co., Incheon, [4]R&D Center, CTCBIO Inc., Seoul, Korea

Received: July 5, 2004
Accepted: August 23, 2004

Abstract A gene encoding the mannanase of *Bacillus subtilis* WL-7, which had been isolated from Korean soybean paste, was cloned into *Escherichia coli*, and the gene product was purified from the culture filtrate of the recombinant *E. coli*. This mannanase gene, designated *manA*, consisted of 1,086 nucleotides, encoding a polypeptide of 362 amino acid residues. The deduced amino acid sequence was highly homologous to those of mannanases belonging to the glycosyl hydrolase family 26. The molecular mass of the purified mannanase was 38 kDa as estimated by SDS-PAGE. The enzyme had a pH optimum at 6.0 and a temperature optimum at 55°C. The enzyme was active on locust bean gum, konjak, guar gum, and lichenan, while it did not exhibit activity towards yeast mannan, laminarin, carboxymethylcellulose, β-glucan, xylan, and *para*-nitrophenyl-β-mannopyranoside.

Key words: *Bacillus subtilis* WL-7, mannanase, gene, purification, recombinant *Escherichia coli*

Mannan-type polysaccharides are some of the major constituents of the hemicellulose fraction in both hardwoods and softwoods as well as in the endosperm of many leguminous seeds and in some mature seeds of non-leguminous plants. β-Mannans, found in non-leguminous plants including ivory nut, are unsubstituted linear polysaccharides, having a backbone composed of β-1,4-linked mannose. Glucomannans, found in konjak, are polysaccharides having a backbone more or less regularly alternating β-1,4-linked mannose and glucose. Galactomannans and galactoglucomannans are β-mannans and glucomannans having α-galactose linked to *O*-6 of mannose residues, respectively. They can also be acetylated to various degree on *O*-2 and *O*-3 of the mannose residues [34]. Galactomannans are found in large quantities in seeds of leguminous plants, and acetylated galactoglucomannans are main constituents of softwood.

Mannanases, which catalyze the random hydrolysis of the β-D-1,4-mannopyranosyl linkages within the backbone of various mannan-based polysaccharides, are enzymes useful in food, feed, paper, and laundry industries. Although the mannanases are widely distributed in microorganism, higher plants, and animals, much attention has been focused on the microbial mannanases for their industrial applications. So far, numerous mannanases and their genes have been identified from bacteria and fungi. Bacterial mannanase genes were obtained and characterized from many strains of genus, including *Bacillus* [19], *Clostridium* [24], *Thermotoga* [32], *Cellulomonas* [39], *Cellvibrio* [15], *Caldicellulosiruptor* [11], *Rhodothermus* [33], *Thermoanaerobacterium* [7], *Dictyoglomus* [12], *Caldibacillus* [41], *Streptomyces* [5], and *Caldocellulosiruptor* [29]. Fungal mannanase genes were mostly reported in strains of *Trichoderma* [14], *Aspergillus* [8], and *Piromyces* [10]. On the basis of amino acid similarity, these mannanases have been shown to belong to either glycosyl hydrolase (GH) family 5 or 26. Most GH26 mannanases are prokaryotic in origin, whereas GH5 mannanases are derived from bacteria and fungi.

Soybean meal is one of the major components in feeds for swine, chikens, dogs, and fish. Galactomannans of leguminous seeds are regarded as antinutritional factors, because monogastric animals are unable to digest them rapidly enough to obtain the edible sugars. In addition to phytase [23] and xylanase [16, 20], mannanase has been used as a feed additive enzyme to increase nutritional value of soybean meal [22]. For the applications of mannanase in the fields of food processing and animal feed additive, we have previously isolated a mannanase-producing bacterium, *Bacillus subtilis* WL-7, from Korean soybean paste [25]. The isolate produced significant amounts

*Corresponding author
Phone: 82-42-630-9742; Fax: 82-42-636-2676;
E-mail: ykh@lion.woosong.ac.kr

of extracellular mannanase in the presence of inducers, such as locust bean gum and konjak. In this work, the *B. subtilis* WL-7 mannanase gene was cloned into *Escherichia coli* and the enzyme was purified from the recombinant *E. coli*.

MATERIALS AND METHODS

Chemicals and Enzymes

Restriction endonucleases, protease, and RNase were obtained from Boehringer Mannheim (Manheim, Germany), and T4 DNA ligase was from Solgent Co. (Deajeon, Korea), and they were used as recommended by the manufacturers. Locust bean gum (LBG), guar gum, mannan, lichenan, barley β-glucan, laminarin, oat spelt xylan, and birchwood xylan were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and mannooligosaccharides and arabinoxylan were from Megazyme (Wicklow, Ireland).

Bacterial Strains, Plasmids, and Media

B. subtilis WL-7 was used as the source of the gene coding for mannanase, *E. coli* XL-1 blue (*supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*46 *relA*1 *thi lac*$^-$ F'[*proAB*$^+$ *lacI*^{q} *lacZ* M15 Tn10(*tet*^{r})]) as host for recombinant plasmids, recombinant *E. coli* (pWL7M) for producing the mannanase of *B. subtilis* WL-7, and plasmid pUC19 for all cloning and sequencing experiments. *E. coli* and *B. subtilis* were cultured at 37°C in LB broth (10 g of tryptone, 5 g of yeast extract, 10 g of NaCl per liter, pH 7.0). Ampicillin (50 μg/ml) was used for the selection of transformants of *E. coli*.

DNA Manipulation and Construction of a *B. subtilis* WL-7 Genomic Library

The standard procedures of Sambrook *et al.* [37] were used for DNA manipulation. The chromosomal DNA was isolated from *B. subtilis* WL-7 cells grown exponentially in LB medium according to the preparative method described by Rodriquez and Tait [35]. The chromosomal DNA was partially digested with *Sau*3AI, and DNA fragments ranging from 2 to 10 kb were isolated from agarose gel. The *Sau*3AI-generated chromosomal DNA fragments were introduced into the dephosphorylated *Bam*HI site of pUC19, and the ligation mixture was transformed into *E. coli* XL-1 blue by the electroporation method.

DNA Sequencing and Computer Analysis

Restriction endonuclease-generated DNA fragments of the *B. subtilis* DNA were subcloned into pUC19. The nucleotide sequences of the fragments were determined with a DNA sequencer (ABI Prism 377, Perkin Elmer Co., Foster, CA, U.S.A.). The DNA and protein sequences were analyzed, using the DNASIS (Hitachi Software Engineering, Japan) program.

Mannanase Purification

The recombinant *E. coli* XL-1 blue carrying pWL7M was grown overnight at 37°C in 400 ml of LB medium containing ampicillin. The culture filtrate was concentrated by ultrafiltration (PM10), followed by fractionation with ammonium sulfate (30–70% saturation). The precipitate was collected and resuspended in 4 ml of 20 mM Tris-HCl buffer (pH 8.0). After dialyzing against the same buffer, the crude enzyme suspension was applied to a DEAE-Sepharose column (2.5×15 cm) which had been equilibrated with 20 mM Tris-HCl, pH 8.0. After washing with the same buffer, bound proteins were eluted with a linear NaCl gradient (0 to 0.4 M) at a flow rate of 1 ml/min. The active fractions were concentrated by ultrafiltration and dialyzed against 20 mM Tris buffer, pH 8.0. The dialyzed sample was loaded onto a Q-Sepharose column (1.5×10 cm), and the proteins were then eluted with a linear NaCl gradient (0 to 0.3 M). The active fractions were pooled and concentrated by ultrafiltration. Proteins were analyzed by SDS-PAGE.

Enzyme and Protein Assays

The mannanase activity was determined by measuring the amount of reducing sugars liberated during the hydrolysis of LBG by the dinitrosalicylic acid method [28]. The standard assay reaction mixture consisted of 0.5% (w/v) of polysaccharide substrates supplemented with 50 mM sodium citrate buffer (pH 6.0) and enzyme to make a final volume of 0.3 ml. The reaction mixture was incubated at 50°C for 15 min. One unit of the enzyme activity was defined as the amount of enzyme to produce 1 μmol of reducing sugar per min. Protein concentrations were determined by the Bradford method [6] with bovine serum albumin as a standard.

Effects of pH and Temperature on Enzyme Activity

The effect of pH on the reaction rate was determined by measuring mannanase activity at different pH values under standard assay conditions, using 50 mM sodium citrate (pH 4.0 to 6.0), sodium phosphate (pH 6.0 to 8.0), and KCl-borate (pH 8.0 to 9.0) buffers. The activity of the purified enzyme was also assayed at temperatures ranging from 35°C to 70°C under standard assay condition. After the enzyme suspension was allowed to stand at 4°C for 1 h at various pH values (pH 4.0 to 9.0) without the substrate, the remaining activity was measured. The thermostability of mannanase was also examined by measuring the remaining activity after preincubating at various temperatures (35 to 70°C) without substrate.

N-Terminal Sequencing

The N-terminal amino acid sequence of the purified enzyme was determined by automatic Edman degradation, using a Procise 491 protein sequencer (Applied Biosystems, U.S.A.), at Korea Basic Science Institute Seoul Branch.

RESULTS AND DISCUSSION

Cloning and Nucleotide Sequence of a *B. subtilis* WL-7 Mannanase

The genomic library of *B. subtilis* WL-7 was constructed in *E. coli* XL-1 Blue, using pUC19 as a cloning vector. Approximately 3,000 transformants were transferred into LB agar plates containing ampicillin. After overnight incubation, these plates were overlayed with soft agar (0.7%) containing 0.2% LBG and 80 ppm trypan blue for screening *E. coli* clone with mannanase activity. After incubation for 2 h at 50°C, a single *E. coli* clone capable of hydrolyzing LBG was obtained by scoring the clear zone around the colony. To investigate secretion of the mannanase produced by the *E. coli* clone containing the *B. subtilis* WL-7 mannanase gene, mannanase activity was measured, using LBG as a substrate, with the culture filtrate and cell-free extract of the *E. coli* clone grown in LB broth for 12 h. Mannanase activity was equally detected in both the culture filtrate and cell-free extract, indicating that about 50% of the WL-7 mannanase was secreted by the recombinant *E. coli* cell. It was reported that 58% of mannanase were localized in the extracellular and periplasmic fractions of recombinant *E. coli* cell, harboring *Bacillus* sp. AM-011 mannanase gene [2]. The recombinant plasmid, named pWL7M, was isolated from the *E. coli* clone and analyzed with various restriction enzymes. As shown in Fig. 1, the mannanase gene, designated as *manA*, was found in the 2.0-kb insert DNA fragment on plasmid pWL7M.

The complete 2,087-bp sequence of the insert was determined, and the deduced amino acid sequence of mannanase yielded an open reading frame of 1,086 nucleotides coding for a protein of 362 amino acids with an estimated molecular mass of 40,871 Da (Fig. 2). The *manA* gene is preceded and followed by two *orf*s (nt positions 1–238 for *orf*1 and 1,404–2,087 for *orf*2) encoding the carboxy termini of truncated proteins showing 96% homology to a 78 amino-acid stretch of phosphomannose isomerase of *B. subtilis* 168 (GenBank accession number Z99107) and 81.7% homology to a 227 amino-acid stretch of catalase from *B. anthracis* Ames (accession number NP_845456), respectively (data not shown). A putative start codon, TTG, of *manA* is located at nucleotide position 257, which is separated by 18 nucleotides after the termination codon of the truncated phosphomannose isomerase (*pmi*) gene, whereas the termination codon, TGA, is at position 1,343. According to a putative ribosome binding site (GGGGGAG) which was followed at a spacing of 6 bp by the codon, the putative initiation codon was expected. Sadaie *et al.* [36] reported that the start codon of a *B. subtilis* 168 mannanase gene is TTG, while the start codon of mannanase genes was predicted to be ATG from *Bacillus* sp. 5H [19], *B. subtilis* NM-39 [26], and *B. subtilis* HB002 (accession number AF324506). The start codon of *B. subtilis* HB002 gene corresponded to nucleotide position 242 of *B. subtilis* WL-7 *manA* gene, but the nucleotide sequence for putative RBS was not found upstream from nucleotide position 242 of the *manA* gene. The initiation codon of a *B. stearothermophilus* No. 236 β-xylosidase gene was also known to be TTG [21]. Possible promoter sequences, TTGCCT for the –35 region and TTCAATA for the –10 region with a 17-bp spacing between them, were found within the truncated *pmi* gene. A possible transcriptional terminator consisting of a 15-bp

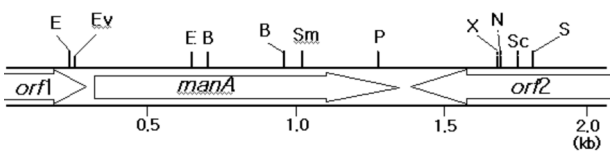

Fig. 1. Restriction endonuclease map of the pWL7M insert DNA.
Below the restriction map, the *manA* indicates a structural gene of mannanase. The *orf*1 and *orf*2 correspond to putative truncated genes, encoding phosphomannose isomerase and catalase, respectively. The arrows indicate the directions of transcription. Restriction site abbreviations are as follows: B, *Bcl*I; E, *Eco*RI; Ev, *Eco*RV; N, *Nsi*I; P, *Pvu*II; S, *Sal*I; Sc, *Sac*I; Sm, *Sma*I; X, *Xho*I.

Fig. 2. Nucleotide sequence and deduced amino acid sequence of the mannanase gene.
The amino acid deduced from the open reading frame is shown with the one-letter code below the nucleotide sequence. Putative ribosome binding site (SD) and promoter sequence (–35 and –10) are underlined. Amino acids corresponding to the signal sequence are italicized. Underlined amino acids indicate the N-terminus of mature protein purified from culture filtrate of recombinant *E. coli* carrying a *B. subtilis* WL-7 mannanase gene. Palindrome sequence that may act as a transcription terminator is indicated by arrows facing each other. The numbers at the end of each line correspond to the nucleotide positions. The nucleotide sequence has been deposited with GenBank under accession number AY601725.

palindrome was found downstream of the TGA termination codon. This palindromic sequence was also followed by a TAA termination codon of the putative truncated catalase gene, suggesting that transcriptional terminations of both *manA* and catalase gene occur at the same palindromic sequence in reverse direction with each other.

The codon utilization pattern for the *manA* gene resembled that of the highly expressed *B. subtilis* gene in amino acid residues such as Leu, Pro, Gln, and Phe. But, for Tyr, Ile, Asn, Asp, and Gly, it resembled that of weakly expressed gene [13]. In addition, the *manA* had a total 42.5% G/C content while the G/C content was 39.9% at the third base of the codon. The *manA* did not show any preference for G or C residues at the third base of the codons, similar to the weakly expressed *E. coli* genes [38].

Comparison of *B. subtilis* Mannanase with Others

When the deduced amino acid sequence of a WL-7 mannanase was compared with the sequences of other mannanases in the NCBI database, using the BLAST search program [3], the present mannanase showed high homology with those of mannanases from *Bacillus* strains belonging to glycosyl hydrolase family 26; *B. subtilis* 168 (98% identity), *B. subtilis* HB002 (identity: 96%), *Bacillus* sp. 5H (identity: 95%), and *B. subtilis* NM-39 (identity: 66%). Amino acid sequences of the mannanase from the above *Bacillus* species are aligned in Fig. 3. These mannanases had a catalytic module, while many GH26 mannanases from *Clostridium thermocellum* [24], *Caldicellulosiruptor* sp. Rt8.B4 [11], *Cellulomonas fimi* [39], and *Caldibacillus cellulovorans* [41] were composed of at least two modules.

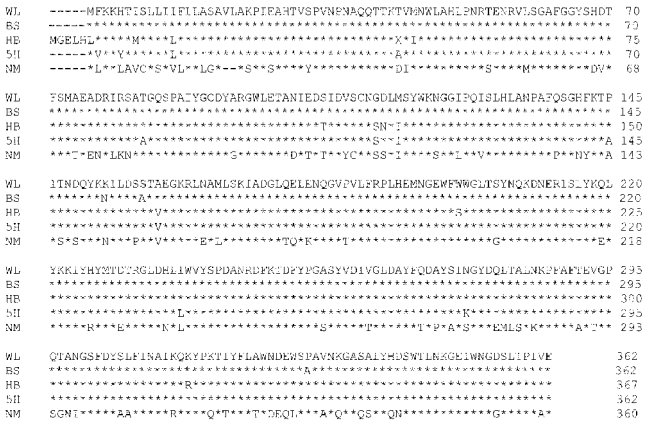

Fig. 3. Comparison of the *B. subtilis* WL-7 mannanase with others.

The amino acid sequences of five mannanases from *B. subtilis* WL-7 (WL), *B. subtilis* 168 (BS), *B. subtilis* HB002 (HB), *Bacillus* sp. 5H (5H), and *B. subtilis* NM-39 (NM) are given in the one-letter code and have been aligned by introducing gaps (hypens) to maximize similarities. Residues identical to amino acid sequence of the WL-7 mannanase are indicated by asterisks in other sequences. Numbers at the end of each line correspond to the amino acid position in the protein.

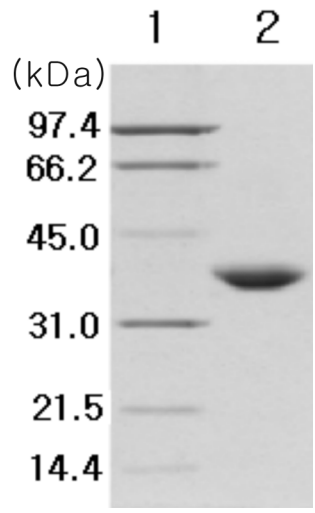

Fig. 4. SDS-PAGE of the mannanase purified from recombinant *E. coli*.

Lane 1, the molecular weight markers; lane 2, the purified enzyme. Molecular size is shown in kilodaltons to the left side of the gel.

The 26 amino acid stretch in the N-terminus of the predicted amino acid sequence has properties similar to typical signal peptides in *Bacillus* sp. consisting of positively charged amino acids followed by a hydrophobic amino acid stretch. When the N-terminal region of WL-7 mannanase was compared with the cleavage sites of other signal peptides, the amino acid sequence (I-E-A) for cleavage site was not identical to a typical signal peptidase processing site (A-X-A) [45]. In the present study, the N-terminal amino acid sequence of mannanase purified from culture filtrate of recombinant *E. coli* (pWL7M) was determined to be HTVSPVNPNA, which is identical to that of the mannanase purified from *Bacillus* sp. 5H [19]. This corresponded to the amino acid sequence from positions 27 to 36, suggesting that this protein was secreted by both the recombinant *E. coli* and *B. subtilis* WL-7 with the same leader peptide processing site in the premature mannanase. Thus, the presently purified mannanase was estimated to have a molecular mass of 38 kDa, which matches closely to the one determined by SDS-PAGE, as shown in Fig. 4. Mannanase produced by recombinant *E. coli*, carrying a *C. fimi* mannanase gene, was reported to be longer at the N-terminus than that from *C. fimi* [40].

Physicochemical Characterization of the Purified Mannanase

From the culture filtrate of *E. coli* carrying pWL7M, the mannanase was purified 27-fold with specific activity of 10,080 U/mg and 21.6% yield. As shown in Fig. 4, SDS-PAGE showed a single band corresponding to molecular mass of 38 kDa, which agrees with that of mature mannanase as predicted by the nucleotide sequence of

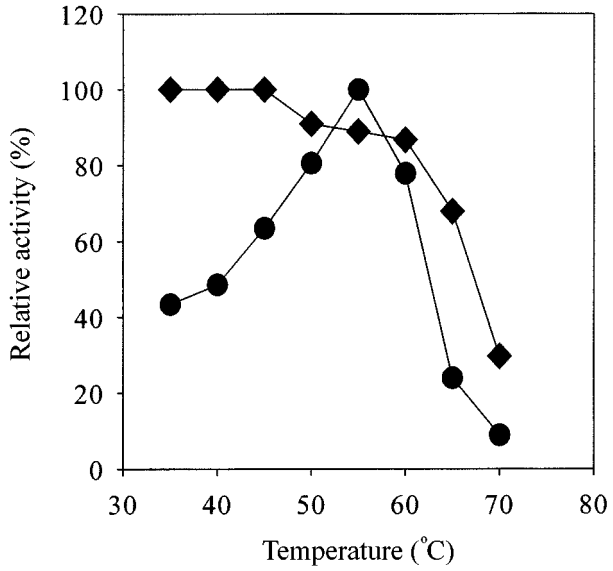

Fig. 5. Temperature optimum and thermostability of the purified mannanase.
The enzyme reaction was performed at various temperatures (●) for 15 min in 50 mM sodium citrate buffer (pH 6.0). Thermostability (◆) was determined by measuring the residual activities after pre-incubation for 1 h at different temperatures.

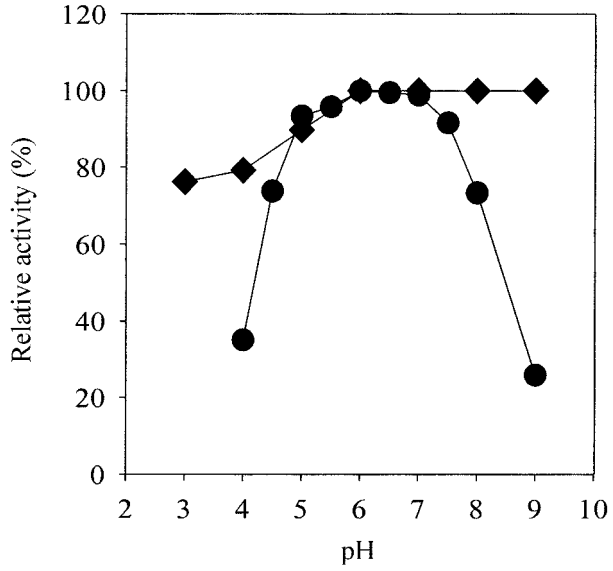

Fig. 6. pH optimum and pH stability of the purified mannanase.
Buffers (50 mM) used were as follows: sodium citrate (pH 3–6), sodium phosphate (pH 6–8), and KCl-borate (pH 8–9). Mannanase activity (●) was assayed at various pHs at 50°C. pH stability (◆) was determined by measuring the residual activities after pre-incubations for 1 h in different pHs at 4°C.

manA in Fig. 1. Its molecular mass was similar to those of mature mannanases from *B. subtilis* (38 kDa) [27], *B. pumilus* (38 kDa) [4], *Aspergillus niger* (40 kDa) [1], *C. cellulovorans* (38 kDa) [44], or *Dictyoglomus thermophilum* (40 kDa) [12]. Most of other mannanases have molecular mass higher than that of *B. subtilis* WL-7, including mannanases from *Vibrio* sp. (49 kDa) [43], *T. neapolitana* (65 kDa) [9], *B. stearothermophilus* (73 kDa) [42], and *Enterococcus casseliflavus* (142 kDa and 137 kDa) [31]. In particular, mannanase of *Pseudomonas* sp. PT-5 has been reported to have a 34 kDa molecular mass [46].

The purified mannanase was optimally active on LBG at 55°C with greater than 50% activity at temperature between 45°C and 60°C (Fig. 5). Thermostability of the enzyme was determined by measuring remaining activity after preincubation in citrate buffer (50 mM, pH 6.0) for 1 h at various temperatures. The enzyme was stable up to 45°C, and its stability rapidly decreased at above 60°C. The mannanase exhibited optimal activity in the pH range of 5.0–7.5 with the highest activity at pH 6.0 (Fig. 6). At least 80% of the activity were maintained at pH 4.5 to 9.0, and no residual activity was detected at pH 3.0. In a previous report, the mannanase activity in the culture supernatant of *B. subtilis* WL-7 was detected maximally also under the reaction condition of 55°C and pH 6.0 [25].

The effects of various reagents, including metal salts, EDTA, dithiothreitol, and SDS, on the mannanase activity, was investigated. As shown Table 1, the activity of the purified mannanase was not enhanced by any of the reagents tested. However, 5 mM SDS, EDTA, or Mn^{2+} were inhibitory. Many mannanases of other strains, such as *Pseudomonas* sp. PT-5 [46], *Streptomyces galbus* [17], *D. thermophilum* Rt46B.1 [12], and *Vibrio* sp. MA-138 [43], were inhibited by Cu^{2+}, but the WL-7 mannanase was not inhibited by Cu^{2+}. Mannanases of both *E. caseeliflavus* [31] and *Streptomyces ipomoea* [30] were activated by Mn^{2+}, whereas the enzyme of *S. galbus* was inhibited by the metal ion, similar to the WL-7 mannanase.

Substrate Specificity

The purified mannanase was assayed with various substrates to investigate its substrate specificity. When

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

Effector (5 mM)	Relative activity (%)
None	100.0
NaCl	105.2
KCl	100.0
$MgCl_2$	93.0
$MnCl_2$	76.5
$CaCl_2$	97.8
$CuCl_2$	100.9
$FeCl_2$	101.8
EDTA	88.8
SDS	67.7
Dithiothreitol	107.6

Table 2. Substrate specificity of the purified mannanase.

Substrates	Specific activity (U/mg protein)
Locust bean gum	10,082.0
Konjak	7,080.0
Guar gum	1,295.0
Lichenan	7.6
Oat spelt xylan	ND
Birchwood xylan	ND
Arabinoxylan	ND
Carboxymethylcellulose	ND
Laminarin	ND
Barley β-glucan	ND
pNP-β-mannoside	ND
pNP-β-cellobioside	ND
pNP-β-galactoside	ND
pNP-β-glucoside	ND
pNP-β-xyloside	ND

ND, not detected.

polysaccharides were used as substrates, the activity was determined by the release of reducing sugars, and the synthetic substrate derivatives were assayed by the release of *para*-nitrophenol (pNP). As shown in Table 2, no detectable reducing sugars were released from xylans, barley β-glucan, carboxymethyl cellulose, and laminarin. The largest amount of reducing sugars were liberated when galactomannan LBG (mannose/galactose ratio, 4:1) was used as the substrate. In the presence of konjak glucomannan (mannose/glucose ratio, 1.5:1), approximately 70% of the maximum activity was observed, while replacement of LBG by the galactomannan guar gum (mannose/galactose ratio, 2:1) resulted in a reduction of 90% activity. The lower activity towards guar gum than LBG supports the hyphothesis that the enzyme activity is limited by a number of branched α-galactose residues. Furthermore, no detectable reducing sugars were released with *Saccharomyces cerevisiae* mannan (with α-1,6, α-1,2, and α-1,3 mannosidic linkages). It was also reported that mannanase A of *Caldibacillus cellulovorans* could hydrolyze LBG, but not *Saccharomyces cerevisiae* mannan [41], and that mannanases from *C. cellulovorans* and *Clostridium tertium* [18] could comparably hydrolyze galactomannan guar gum to LBG, but konjak poorly, indicating that substrate specificity of the WL-7 mannanase was different from them.

The WL-7 mannanase was not active on synthetic substrate derivatives such as pNP-β-mannoside, pNP-β-cellobioside, pNP-β-galactoside, pNP-β-glucoside, and pNP-β-xyloside, indicating that the mannanase could not hydrolyze the β-1,4-cellulosic linkages and had no β-mannosidase activity. On the other hand, this enzyme could hydrolyze a small amount of lichenan which has much higher proportion of 1,3- to 1,4-β linkage than barley β-glucan, while it could not hydrolyze barley β-glucan. Thus, the GH26 mannanases appear to have very strict substrate specificity, hydrolyzing mannan and glucomannan but not other β-glycans such as barley β-glucans or soluble derivatives of cellulose.

REFERENCES

1. Ademark, P., A. Varga, J. Medve, V. Harjunpaa, T. Drakenberg, F. Tjerneld, and H. Stalbrand. 1998. Softwood hemicellulose-degrading enzymes from *Aspergillus niger*: Purification and properties of a β-mannanase. *J. Biotechnol*. **63:** 199–210.
2. Akino, T., C. Kato, and K. Horikoshi. 1989. The cloned β-mannanase gene from alkalophilic *Bacillus* sp. AM-001 produced two β-mannanases in *Escherichia coli*. *Arch. Microbiol*. **152:** 10–15.
3. Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. Basic local alignment search tool. *J. Mol. Biol*. **215:** 403–410.
4. Araujo, J. and O. P. Ward. 1990. Production, purification and properties of the mannanase components of *Bacillus pumilus*. *Appl. Environ. Microbiol*. **56:** 1954–1956.
5. Arcand, N., D. Kluepfel, F. W. Paradis, R. Morosoli, and F. Shareck. 1993. β-Mannanase of *Streptomyces lividans* 66: Cloning and DNA sequence of the *manA* gene and characterization of the enzyme. *Biochem. J*. **290:** 857–863.
6. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.
7. Cann, I. K., S. Kocherginskaya, M. R. King, B. A. White, and R. I. Mackie. 1999. Molecular cloning, sequencing, and expression of a novel multidomain mannanase gene from *Thermoanaerobacterium polysaccharolyticum*. *J. Bacteriol*. **181:** 1643–1651.
8. Christgau, S., S. Kauppinen, J. Vind, L.-V. Koffod, and H. Dalboge. 1994. Expression cloning, purification and characterization of a β-1,4-mannanase from *Aspergillus aculeatus*. *Biochem. Mol. Biol. Int*. **33:** 917–925.
9. Duffaud G. D., C. M. McCutchen, P. Leduc, K. N. Parker, and R. M. Kelly. 1997. Purification and characterization of extremely thermostable β-mannanase, β-mannosidase, and α-galactosidase from the hyperthermophilic eubacterium *Thermotoga neapolitana* 5068. *Appl. Environ. Microbiol*. **63:** 169–177.
10. Fanutti, C., T. Ponyi, G. W. Black, G. P. Hazlewood, and H. J. Gilbert. 1995. The conserved noncatalytic 40-residue sequence in cellulases and hemicellulases from anaerobic fungi functions as a protein docking domain. *J. Biol. Chem*. **270:** 29314–29322.
11. Gibbs, M. D., A. U. Elinder, R. A. Reeves, and P. L. Bergquist. 1996. Sequencing, cloning and expression of a β-1,4-mannanase gene, *man*A, from the extremely thermophilic anaerobic bacterium, *Caldicellulosiruptor* Rt8B.4. *FEMS Microbiol. Lett*. **141:** 37–43.

12. Gibbs, M. D., R. A. Reeves, A. Sunna, and P. L. Bergquist. 1999. Sequencing and expression of a β-mannanase gene from the extreme thermophile *Dictyoglomus thermophilum* Rt46B.1, and characteristics of the recombinant enzyme. *Curr. Microbiol*. **39:** 351–357.
13. Grosjean, H. and W. Fiers. 1982. Preferential codon usage in genes: The optimal codon-anticodon interaction energyand the selective codon usage in efficiently expressed genes. *Gene* **18:** 199–209.
14. Hagglund, P., T. Eriksson, A. Collen, W. Nerinckx, M. Claeyssens, and H. Stalbrand. 2003. A cellulose-binding module of the *Trichoderma reesei* β-mannanase Man5A increases the mannan-hydrolysis of complex substrates. *J. Biotechnol*. **101:** 37–48.
15. Hogg, D., G. Pell, P. Dupree, F. Goubet, S. M. Martin-Orue, S. Armand, and H. J. Gilbert. 2003. The modular architecture of *Cellvibrio japonicus* mannanases in glycoside hydrolase families 5 and 26 points to differences in their role in mannan degradation. *Biochem. J*. **371:** 1027–1043.
16. Johnvesly, B., S. Virupakshi, G. N. Patil, Ramalingam, and G. R. Naik. 2002. Cellulase-free thermostable alkaline xylanase from thermophilic and alkalophilic *Bacillus* sp. JB-99. *J. Microbiol. Biotechnol*. **12:** 153–156.
17. Kansoh, A. L. and Z. A. 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.
18. Kataoka, N. and Y. Tokiwa. 1998. Isolation and characterization of an active mannanase-producing anaerobic bacterium, *Clostridium tertium* KT-5A, from lotus soil. *J. Appl. Microbiol.* **84:** 357–367.
19. Khanongnuch, C., T. Ooi, and S. Kinoshita. 1999. Cloning and nucleotide sequence of β-mannanse and cellulase genes from *Bacillus* sp. 5H. *Wor. J. Microbiol. Biotechnol*. **15:** 249–258.
20. Kim, K. C., S.-S. Yoo, Y.-A. Oh, and S.-J. Kim. 2003. Isolation and characteristics of *Trichoderma harzianum* FJ1 producing cellulases and xylanase. *J. Microbiol. Biotechnol*. **13:** 1–8.
21. Kim, M. D., K.-N. Kim, and Y. J. Choi. 2003. Enhanced expression of β-xylosidase of *Bacillus stearothermophilus* No. 236 by change of translational initiation codon in *Escherichia coli* and *Bacillus subtilis*. *J. Microbiol. Biotechnol*. **13:** 584–590.
22. Kim, S. W., D. A. Knabe, K. J. Hong, and R. A. Easter. 2003. Use of carbohydrases in corn-soybean meal-based nursery diets. *J. Anim. Sci*. **81:** 2496–2504.
23. Kim, Y.-H., M.-N. Gwon, S.-Y. Yang, T.-K. Park, C.-G. Kim, C.-W. Kim, and M.-D. Song. 2002. Isolation of phytase-producing *Pseudomonas* sp. and optimization of its phytase production. *J. Microbiol. Biotechnol*. **12:** 279–285.
24. 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.
25. Kweun, M. A., H. S. Kim, M.-S. Lee, J. H. Choi, and K.-H. Yoon. 2003. Mannanase production by a soybean isolate, *Bacillus subtilis* WL-7. *Kor. J. Microbiol. Biotechnol*. **31:** 277–283.
26. Mendoza, N. S., M. Arai, K. Sugimoto, M. Ueda, T. Kawaguchi, and L. M. Joson. 1995. Cloning and sequencing of β-mannanase gene from *Bacillus subtilis* NM-39. *Biochim. Biophys. Acta* **1243:** 552–554.
27. Mendoza, N. S., M. Arai, T. Kawaguchi, T. Yoshida, and L. M. Joson. 1994. Purification and properties of mannanase from *Bacillus subtilis*. *Wor. J. Microbiol. Biotechnol*. **10:** 551–556.
28. Miller, M. L., R. Blum, W. E. Glennon, and A. L. Burton. 1960. Measurement of carboxymethylcellulase activity. *Anal. Biochem*. **2:** 127–132.
29. Morris, D. D., R. A. Reeves, M. D. Gibbs, D. J. Saul, and P. L. Bergquist. 1995. Correction of the β-mannanase domain of the *celC* pseudogene from *Caldocellulosiruptor saccharolyticus* and activity of the gene product on kraft pulp. *Appl. Environ. Microbiol*. **61:** 2262–2269.
30. Montiel, M. D., M. Hernandez, J. Rodriguez, and M. E. Arias. 2002. Evaluation of an endo-β-mannanase produced by *Streptomyces ipomoea* CECT 3341 for the biobleaching of pine kraft pulps. *Appl. Microbiol. Biotechnol*. **58:** 67–72.
31. Oda, Y., T. Komaki, and K. Tonomura. 1993. Purification and properties of extracellular β-mannanases produced by *Enterococcus casseliflavus* FL2121 isolated from decayed konjac. *J. Ferment. Bioeng*. **76:** 14–18.
32. Parker, K. N., S. R. Chhabra, D. Lam, W. Callen, G. D. Duffaud, M. A. Snead, J. M. Short, E. J. Mathur, and R. M. Kelly. 2001. Galactomannanases Man2 and Man5 from *Thermotoga* species: growth physiology on galactomannans, gene sequence analysis, and biochemical properties of recombinant enzymes. *Biotechnol. Bioeng*. **75:** 322–333.
33. Politz, O., M. Krah, K. K. Thomsen, and R. Borriss. 2000. A highly thermostable endo-(1,4)-β-mannanase from the marine bacterium *Rhodothermus marinus*. *Appl. Microbiol. Biotechnol*. **53:** 715–721.
34. Puls, J. and J. Schuseil. 1996. Chemistry of hemicelluloses: Relationship between hemicellulose structure and enzymes required for hydrolysis, pp. 1–28. *In* M. P. Coughlan and G. P. Hazlewood (eds.). *Hemicelluloses and Hemicellulases*. Portland Press, London, England.
35. Rodriquez, R. L. and R. C. Tait. 1983. *Recombinant DNA Techniques - An Introduction*, pp. 45–46. Addison-Wesley Pub. Co., Reading, MA, U.S.A.
36. Sadaie, Y., K. Yata, M. Fujita, H. Sagai, M. Itaya, Y. Kosahara, and N. Ogasawara. 1997. Nucleotide sequence and analysis of the *phoB-rrnE-groESL* region of the *Bacillus subtilis* chromosome. *Microbiology* **143:** 1861–1866.
37. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning. A Laboratory Mannual*. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.
38. Sharp, P. M., D. G. Higgins, D. C. Shields, and K. M. Devine. 1990. Protein-coding genes: DNA sequence database and codon usage, pp. 557–569. *In* C. R. Harwood and S. M. Cutting (eds.). *Molecular Biological Methods for Bacillus*. John Wiley & Sons, Chichester, England.
39. Stoll, D., A. Boraston, H. Stalbrand, B. W. McLean, D. G. Kilburn, and R. A. J. Warren. 2000. Mannanase Man26A

from *Cellulomonas fimi* has a mannan-binding module. *FEMS Microbiol. Lett.* **183:** 265–269.
40. Stoll, D., H. Stalbrand, and R. A. Warren. 1999. Mannan-degrading enzymes from *Cellulomonas fimi*. *Appl. Environ. Microbiol.* **65:** 2598–2605.
41. Sunna, A., M. D. Gibbs, C. W. J. Chin, P. J. Nelson, and P. L. Bergquist. 1999. A gene encoding a novel multidomain β-1,4-mannanase from *Caldibacillus cellulovorans* and action of the recombinant enzyme on kraft pulp. *Appl. Environ. Microbiol*. **66:** 664–670.
42. Talbot, G. and J. Sygusch. 1990. Purification and characterization of thermostable β-mannanase and α-galactosidase from *Bacillus stearothermophilus*. *Appl. Environ. Microbiol*. **56:** 3505–3510.
43. Tamaru, Y., T. Araki, H. Amagoi, H. Mori, and T. Morishita. 1995. Purification and characterization of an extracellular β-1,4-mannanase from a marine bacterium, *Vibrio* sp. strain MA-138. *Appl. Environ. Microbiol*. **61:** 4454–4458.
44. Tamaru, Y. and R. H. Doi. 2000. The *engL* gene cluster of *Clostridium cellulovorans* contains a gene for cellulosomal ManA. *J. Bacteriol*. **182:** 244–247.
45. Valentine, P. J. and A. A. Salyers. 1992. Analysis of proteins associated with growth of *Bacteroides ovatus* on the branched galactomannan guar gum. *Appl. Environ. Microbiol*. **58:** 1534–1540.
46. Yamaura, L., T. Matsumoto, M. Funatsu, and Y. Funatsu. 1990. Purification and some properties of endo-1,4-β-D-mannanase from *Pseudomonas* sp. PT-5. *Agric. Biol. Chem*. **54:** 2425–2427.