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Gene Cloning, DNA Sequencing, and Expression of Thermostable β-Mannanase from *Bacillus stearothermophilus*

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A DNA genomic library constructed from *Bacillus stearothermophilus*, a gram-positive, facultative thermophilic aerobe that secretes a thermostable β-mannanase, was screened for mannan hydrolytic activity. Recombinant β-mannanase activity was detected on the basis of the clearing of halos around *Escherichia coli* colonies grown on a dye-labelled substrate, Remazol brilliant blue-locust bean gum. The nucleotide sequence of the mannanase gene, *manF*, corresponded to an open reading frame of 2,085 bp that codes for a 32-amino-acid signal peptide and a mature protein with a molecular mass of 76,089 Da. From sequence analysis, ManF belongs to glycosyl hydrolase family 5 and exhibits higher similarity to eukaryotic than to bacterial mannanases. The *manF* coding sequence was subcloned into the pH6EX3 expression plasmid and expressed in *E. coli* as a recombinant fusion protein containing a hexahistidine N-terminal sequence. The fusion protein has thermostability similar to the native enzyme and was purified by Ni^{2+} affinity chromatography. The values for the kinetic parameters V_{max} and K_m were 384 U/mg and 2.4 mg/ml, respectively, for the recombinant mannanase and were comparable to those of the native enzyme.

Hemicelluloses, the second most abundant polysaccharide in nature, are found in plant cell walls as linkers between lignin and cellulose constituents. In wood fibers, hemicelluloses are known to affect fiber swelling and flexibility (44). Mannan exists in nature in two forms: as acetylated galactoglucomannan, a principal component of hemicellulose found in softwoods, which has a heterogeneous backbone of β-1,4-linked mannose and glucose residues; and galactomannan, which is found in seeds of leguminous plants and beans of carob trees and is composed of a homogeneous backbone of β-1,4-linked mannose residues. Mannose residues often possess α-1,6-galactose as side groups and may be acetylated at the O-2 and O-3 positions. The β-1,4-glycosidic linkages in the main chain of β-linked mannan are hydrolyzed by β-1,4-mannanases, yielding small oligomannoside products (15).

Use of hemicellulases has been shown to promote pulp bleaching in the manufacture of kraft pulp (48, 49). Although a considerable number of studies have focused on the use of xylanase for bleaching, usage of mannanases has not been addressed until recently. Previous studies concentrated on the isolation and characterization of β-mannanases in bacteria, fungi, and plants (2, 15, 29, 55). Until 1993, only three nucleotide sequences of the β-mannanase gene were known (1, 3, 19, 26). However, after studies clearly demonstrated the usefulness of mannanases in enhancing kraft pulp bleachability following oxygen delignification (10–12, 14, 40, 45, 50), an increasing number of β-mannanase sequences were reported in fungi (13, 16, 32, 43, 53), bacteria (9, 18, 20, 31, 33, 38, 39, 47), and plants (8). These enzymes have been shown to belong to either glycosyl hydrolase family 5 or 26 (23). Interestingly, in family 5, fungal mannanases from *Trichoderma reesei* (43) and *Agaricus bisporus* (53) as well as two bacterial mannanases from *Caldocellum saccharolyticum* (19, 33) possess, in addition to the catalytic core, a cellulose-binding domain (CBD) linked to the catalytic core by proline-threonine- and serine-rich linkers.

Bacillus stearothermophilus, a thermophilic bacterium, is an important producer of hemicellulases, including α-L-arabinofuranosidase (21), β-xylosidase (34), xylanase (24, 34), α-galactosidase, and β-mannanase (46). The purification and characterization of a thermostable and alkalitolerant β-mannanase from *B. stearothermophilus* have been reported previously (46). A DNA genomic library was constructed from *B. stearothermophilus* and probed for mannanase activity. The DNA coding sequence for the β-mannanase gene, *manF*, was cloned, overexpressed in *Escherichia coli*, and characterized.

MATERIALS AND METHODS

Bacterial strains and cloning vectors. Chromosomal DNA was extracted from *B. stearothermophilus* obtained from the American Type Culture Collection (ATCC; catalog no. 12016). The bacterial strain was grown in ATCC 266 medium at 55°C. *E. coli* strains DH5αMCR and BL21 were used as hosts for recombinant plasmids. Plasmid pUC18 was used as a cloning and sequencing vector, whereas pH6EX3 (7) was used for expression.

Determination of the N-terminal protein sequence. The β-mannanases of *B. stearothermophilus* were purified as described by Talbot and Sygusch (46). The NH_2-terminal amino acid sequence was determined by C. Lazure (Institut de Recherche Clinique de Montréal) with a 470A Applied Biosystems gas-phase sequenator.

Isolation and cloning of DNA. Genomic DNA from *B. stearothermophilus* was isolated as described by Yang et al. (54). DNA was partially digested with *Eco*RI and fractionated on a linear 10-to-40% sucrose density gradient. Fractions containing DNA fragments ranging in size from 2 to 10 kb were pooled and ligated into the dephosphorylated *Eco*RI site of pUC18. The plasmid DNA was transformed into the hypercompetent *E. coli* DH5αMCR strain. Transformed cells were grown on 2× YT (41) agar plates supplemented with ampicillin at 37°C for 18 h. Replicates were made with Hybond nylon membranes (Amersham) and overlaid onto a plate containing M9 minimal medium containing 0.2% (wt/vol) mannitol instead of glucose, 0.5% (wt/vol) Remazol brilliant blue-locust bean gum (RBB-LBG), and ampicillin at a final concentration of 60 μg/ml. Transformants were grown for 18 h at 37°C and then incubated at 55°C for an additional 24 h. Detection of mannanase activity was indicated by the presence of clearing halos around transformed colonies. Approximately 10,000 transformants were screened for mannanase activity.

DNA analysis. Restriction enzymes were purchased from Pharmacia Biotech and were used as specified. Restriction maps were constructed from single and

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multiple restriction enzyme digests of plasmids expressing mannanase activity. Restriction fragments were subcloned into pUC18 and transformed in *E. coli* DH5αMCR. Cloning procedures and transformations were performed as described by Sambrook et al. (41). Clones were sequenced by the dideoxynucleotide chain termination method of Sanger et al. (42) using Redivue [α-^{35}S]dATP (Amersham) and a T7 sequencing kit (Pharmacia Biotech) or *Bst* DNA polymerase (Bio-Rad) (30).

Localization of mannanase activity in *E. coli*. The extracellular, periplasmic, and intracellular fractions of *E. coli* were prepared as described by Nilsson et al. (35) for the 7.6-, 10.7-, and 16.9-kb insert clones. Fractions were dialyzed against 50 mM KH_2PO_4 (pH 6.5), and enzyme activity was measured.

Construction of fusion protein expression plasmid. For recombinant protein overexpression, a mannanase fragment was generated by PCR amplification from the 16.9-kb clone. The twofold strategy made use of (i) a 49-mer PCR primer sequence that hybridized to the mature protein N-terminus coding sequence and which contained a 5′ *Bam*HI restriction site as well as a recognition site for protease, factor Xa (ATCGAGGGTAGG); and (ii) a 42-mer PCR primer complementary to the C-terminus coding sequence that included a 3′ *Xma*I restriction site. The PCR was performed in Expand High Fidelity buffer (Boehringer Mannheim) containing 1.5 mM $MgCl_2$, 10 ng of DNA, 20 pmol of primers, 200 μM deoxynucleoside triphosphates, and 2.6 U of Expand High Fidelity PCR DNA polymerase (Boehringer Mannheim) in a final reaction volume of 50 μl. The DNA to be amplified was initially denatured at 95°C for 3 min, and the reaction was allowed to proceed through 30 cycles at a programmed temperature profile of 40 s at 95°C, 40 s at 55°C, and 2 min 20 s at 72°C. The resultant PCR fragment was cloned into a hexa-His-Tag vector (pH6EX3), yielding plasmid pH6MAN. The pH6EX3 vector codes for an N-terminus hexahistidine sequence suitable for affinity purification and whose expression is controlled by a strong *tac* promoter.

Production of β-mannanase activity in *E. coli*. Plasmid pH6MAN was transformed into *E. coli* BL21, and positive transformants were screened for mannanase activity and were verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for mannanase production. Correct insertion of the mannanase-coding DNA fragment was verified by DNA sequencing of the region about each cloning site. *E. coli* BL21 cells transformed with pH6MAN were grown in 2 liters of 2× YT medium at 37°C containing 100 μg of ampicillin per ml until an optical density of 0.4 to 0.7 at 600 nm was attained. Mannanase activity was induced by isopropyl-β-D-thiogalactopyranoside (IPTG) addition to a final concentration of 0.3 mM and incubated for 5 h at 37°C.

Purification of recombinant His-tagged β-mannanase. Induced cultures were centrifuged, and bacterial pellets were lysed by alumina grinding (2 g/g of bacteria) in a cold mortar in the presence of DNase I and RNase A (0.1 mg/g of bacteria). The lysate was resuspended in 15 ml of lysis buffer (5 mM Tris-HCl [pH 7.4], 10 mM $MgCl_2$, 5% glycerol, 5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) and ultracentrifuged. The supernatant was dialyzed against a binding buffer (50 mM NaH_2PO_4 [pH 8.0], 300 mM NaCl) and applied to an affinity Ni-nitrilotriacetic acid resin (Qiagen) packed into a fast protein liquid chromatography column (Pharmacia Biotech) previously equilibrated with binding buffer. The column was rinsed with a washing buffer (50 mM NaH_2PO_4 [pH 6.0], 1 M NaCl, 20% glycerol, 20 mM β-mercaptoethanol), and recombinant fusion protein was eluted against a decreasing linear pH gradient (pH 6 → 4) of the same buffer. Active fractions were pooled and dialyzed against a 95% saturated $(NH_4)_2SO_4$ solution. The precipitated protein was stored at 4°C.

Protein assay. The protein concentration was determined with bicinchonic acid protein assay reagent purchased from Pierce Chemical Co. (Rockford, Ill.).

Enzyme assay. The β-mannanase activity was assayed by the 3,5-dinitrosalicylic acid method (6) with LBG as a substrate, as described previously (46).

SDS-PAGE. The relative molecular mass of the purified His-tagged-β-mannanase was determined by SDS-PAGE (8% polyacrylamide) (25). Migration of protein bands was detected by Coomassie brilliant blue staining and compared against molecular weight standards purchased from New England Biolabs (Protein Marker Broad Range set).

Enzymatic activity. Michaelis-Menten kinetic parameters K_m and V_{max} for the purified recombinant mannanase were determined at 70°C in 50 mM KH_2PO_4 buffer (pH 6.5) from double reciprocal plots. The substrate concentrations utilized ranged from 1 to 50 mg of LBG/ml, and each activity assay was performed for 20 min or less.

Nucleotide sequence accession number. The sequence reported in this paper has been deposited in the GenBank database under accession no. AF038547.

RESULTS AND DISCUSSION

Cloning of the mannanase gene. Approximately 10,000 *E. coli* transformants from a *B. stearothermophilus* genomic library were screened for mannanase activity. Detection of positive clones was based on clearing of halo formation around transformed colonies that grew on dye-labelled substrate. Eight positive clones were isolated and further characterized. For all clones, ~75% of total mannanase activity was found in the periplasm, and the remaining activity was found in the intracellular fraction. Three clones had genomic DNA inserts of 7.6 kb, four others contained inserts of 10.7 kb, and one possessed an insert of 16.9 kb. *Eco*RI digestions of all inserts showed common fragments of 1.2 and 6.4 kb. Single and multiple digestions with *Bam*HI, *Hin*dIII, and *Eco*RI confirmed the order of the *Eco*RI fragments in all clones. A clone containing a 7.6-kb genomic insert was used for further study. For DNA sequencing, a detailed restriction map was first constructed, and appropriate restriction fragments were subcloned.

Nucleotide sequence analysis. Sequencing by primer walking of a 1.7-kb region encompassing the 1.2-kb fragment of the 7.6-kb insert revealed an open reading frame (ORF) sequence. The corresponding amino acid sequence of this region was consistent with a protein expressing mannanase activity; however, the gene sequence did not reveal a stop codon and abutted the *Eco*RI cloning site of the pUC18 vector. Nucleotide sequencing was continued with a plasmid containing the 16.9-kb DNA insert, which extends beyond the *Eco*RI cloning site of the 7.6-kb fragment. This strategy allowed the determination of the remaining nucleotide sequence. A single ORF corresponding to an initiation ATG codon beginning at position 351 of the determined sequence and ending with a TAA codon at position 2433 was found. The nucleotide sequence of the β-mannanase gene, designated *manF*, coded for a 694-amino-acid protein. Residues 33 to 47 of the translated sequence are in agreement with the N-terminal amino acid sequence of purified β-mannanase from *B. stearothermophilus* (KTKREPATPTKDNEF). The N-terminal 29-amino-acid sequence is characteristic of a bacterial signal peptide sequence (51) containing a relatively short basic region, followed by a hydrophobic region and having a large polar residue (Q) located 4 to 8 residues before the cleavage site. Amino acids 30 to 32 (V-H-A) from the deduced sequence represent a signal peptidase recognition site (36). The presence of a signal peptide is consistent with the fact that the native protein is secreted by *B. stearothermophilus* (46). The calculated molecular mass of the mature protein was 76,089 Da and agrees well with the M_r of ~73 kDa previously determined for the purified native mannanase (46).

A nucleotide sequence, 5′ GGAGGAG 3′, consistent with a putative ribosome-binding site, is located within a few bases upstream of the ATG start codon. The nucleotide sequence from position 175 to position 201, (TTGACA-15 bp-TATAAA) corresponds to the expected −35 and −10 sequences of a putative promoter. A sequence that could act as transcription terminator was found 10 nucleotides downstream from the termination codon of the ORF. This structure was composed of a 20-bp inverted repeat sequence followed by a series of T nucleotides (37).

Amino acid sequence similarity. The deduced β-mannanase amino acid sequence from *B. stearothermophilus* was compared with those of other mannanases. Similarity to the ManF sequence was essentially confined to its N-terminal region (amino acids 33 to 417), which exhibited 33, 32, 36, and 26% identity to β-mannanases from *Aspergillus aculeatus* (13), *Trichoderma reesei* (43), *Agaricus bisporus* (53), and germinated tomato seeds (*Lycopersicon esculentum*) (8), respectively. The sequence alignment is consistent with conservation of seven amino acid residues in *B. stearothermophilus* β-mannanase (Arg-96, His-182, Asn-227, Glu-228, His-306, Tyr-308, and Glu-345) that are characteristic of glycosyl hydrolase family 5 (4, 5, 22, 27, 52).

β-Mannanase sequences are classified, based on amino acid sequence similarity and hydrophobic cluster analysis (HCA), into two distinct families (23), glycosyl hydrolase families 5 and

TABLE 1. Bacterial and fungal mannanase sequences reported to date[a]

Enzyme class produced by organism[b]	Protein	Mannanase domain	Additional domain function	Reference
Family 26				
Bacillus sp. strain AM-001	ManA	N terminal	Unknown	1
	ManB	Entire		1
Prevotella ruminicola $B_1$4	Mannanase	Entire		18
Bacillus subtilis NM-39	Mannanase	Entire		31
Pseudomonas fluorescens	ManA	Entire		9
Piromyces sp.	ManA	N terminal	Protein docking domain	16
	ManB	N terminal	Protein docking domain	32
	ManC	N terminal	Protein docking domain	32
Caldocellum saccharolyticum Rt8B.4	ManA	C terminal	Unknown	20
Dictyoglomus thermophilun Rt46B.1	ManA	Entire		39
Rhodothermus marinus	Mana	?	?	38
Family 5				
Prokaryotic				
Caldocellum saccharolyticum	ManA	N terminal	CBDs and endoglucanase	19
Streptomyces lividans 66	ManA	Entire		3
Caldocellum saccharolyticum	ManB in CelC	C terminal	CBDs and endoglucanase	33
Vibrio sp.	ManA	Entire		47
Eukaryotic				
Aspergillus aculeatus	Man1	Entire		13
Trichoderma reesei	Man1	N terminal	CBD	43
Agaricus bisporus	Cel4	C terminal	CBD	53
Lycopersicon esculentum (tomato)	Mannanase	Entire		8
Bacillus stearothermophilus[c]	ManF	N terminal	Unknown	This study

[a] The position of the catalytic core with respect to the protein sequence is indicated. In the case of additional domains, their functions are indicated if known.
[b] Mannanase classes having amino acid sequence similarities less than 25% have been separated by spaces.
[c] Note that *B. stearothermophilus* ManF has been classified with the eukaryotic family 5 proteins because of the unusually high similarity between its N-terminal domain and those of the eukaryotic mannanases (see text for details).

26, as shown in Table 1. Family 5 includes a collection of highly divergent sequences, comprising not only endoglucanases (23, 52), but also bacterial (3, 19, 33, 47), fungal (13, 43, 53), and plant (8) β-mannanases. Mannan hydrolases in family 5, shown in Table 1, can be further divided into prokaryote and eukaryote classes by sequence analysis. Within the classes, there is at least 59% similarity among prokaryotic enzymes and 26 to 58% identity among eukaryotic enzymes, the low sequence similarity between the classes being attributed to evolution from unrelated ancestral proteins (8). The high similarity between the N-terminal domain of ManF (residues 33 to 417), a bacterial mannanase, and that of eukaryotic mannanases is novel. The absence of sequence similarity of ManF to other bacterial mannanases suggests that they may have evolved from different ancestors.

Sequence alignment of ManF indicates two distinct polypeptide folds, an N-terminal catalytic core and a C-terminal domain. The function of the N-terminal domain is consistent with the presence of the seven invariant amino acid residues (27, 52). In contradistinction, the last 277 amino acids of ManF display no detectable homology with any related family 5 enzyme. That the truncated ManF sequence, missing 136 amino acids from its C terminus because of an internal restriction site, also codes for β-mannanase activity suggests that this portion of the C-terminal domain is not essential for mannanase activity. Deletion of the C-terminal domain in two cellulose-hydrolyzing enzymes, a cellulase from *Bacillus* sp. and *Bacillus subtilis* endoglucanase, also showed that this region was not required for enzymatic activity (17, 28). The last 122 C-terminal amino acids of ManF have 49% similarity to the N-terminal domain of the extreme thermophile *C. saccharolyticum* Rt8B.4 ManA (20), which belongs to glycosyl hydrolase family 26 and is shown in Table 1. The catalytic domain of ManA is located in the C-terminal region, whereas its N-terminal region does not have any reported function.

Purification of the recombinant β-mannanase. For overexpression in *E. coli* as well as to facilitate subsequent purification, the *manF* gene sequence coding for the mature β-mannanase was subcloned into the expression vector pH6EX3 (7). From the SDS-PAGE shown in Fig. 1, a single affinity chromatography purification step rendered the overexpressed protein ~90% pure, with a yield of ~50 mg of β-mannanase from a 2-liter *E. coli* culture. This level of expression is comparable to previously reported yields obtained with this expression system (7). The apparent M_r of the fusion protein, which included a 2,677-Da fusion tag ($MSPIH_6LVPRGSIEGR$), was estimated to be 74 kDa by SDS-PAGE and is consistent with the M_r reported for native β-mannanase (46).

Kinetic characterization of the recombinant β-mannanase was determined at 70°C and pH 6.5, as previously reported (46). The double reciprocal plot of the kinetic data was consistent with enzymatic inhibition at substrate concentrations exceeding 10 mg/ml. Such substrate inhibition was also observed with the native enzyme and may in part be due to the viscosity of the enzyme substrate at high concentration (46). Kinetic constants were determined by linear regression of the double reciprocal plot for substrate concentrations between 1 and 10 mg/ml. The kinetic parameters obtained correspond to a V_{max} of 384 U/mg and a K_m of 2.4 mg/ml for the recombinant mannanase. These values are comparable to those reported for the native enzyme (V_{max} = 455 U/mg, and K_m = 1.5 mg/ml). The results indicate that the recombinant β-mannanase pos-

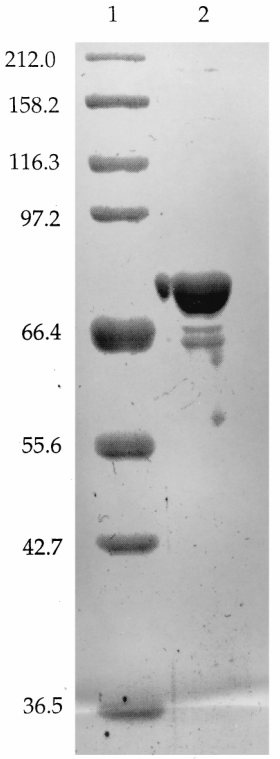

FIG. 1. SDS-PAGE (8% [wt/vol] polyacrylamide) of *B. stearothermophilus* β-mannanase purified from *E. coli*. Lane 1 contains the protein markers myosin (212.0 kDa), maltose binding protein–β-galactosidase (158.2 kDa), β-galactosidase (116.3 kDa), phosphorylase *b* (97.2 kDa), serum albumin (66.4 kDa), glutamine dehydrogenase (55.6 kDa), maltose binding protein (42.7 kDa), and lactate dehydrogenase M (36.5 kDa). Lane 2 contains *B. stearothermophilus* β-mannanase purified from *E. coli*. The lane was loaded with 2 μg of protein, and the gel was stained with Coomassie brilliant blue R.

sesses kinetic properties identical to those of the native one, even though different expression systems were employed and the recombinant β-mannanase included a 20-amino-acid fusion tag.

Expression of recombinant mannanase represents at least 10% of the soluble protein in the *E. coli* lysate (data not shown). This high level of expression together with its thermostability makes usage of the recombinant mannanase in biobleaching commercially possible.

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