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Molecular Cloning and Biochemical Characterization of an Endo-β-mannanase Gene from Soybean for Soybean Meal Improvement

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ABSTRACT: Soybean meal is the most commonly used protein source in animal feeds. Among the undesirable attributes of soybean meal is the high level of β-mannan, which was determined to be detrimental to the growth performance of animals. β-Mannan is a type of hemicellulose in the plant cell wall and can be hydrolyzed by endo-β-mannanase. The goal of this study is to isolate and characterize an endo-β-mannanase gene from soybean that can be used for genetic improvement of soybean meal. From the sequenced soybean genome, 21 putative endo-β-mannanase genes were identified. On the basis of their relatedness to known functional plant endo-β-mannanases, four soybean endo-β-mannanase genes (*GmMAN1* to *GmMAN4*) were chosen for experimental analysis. *GmMAN1* and *GmMAN4* showed expression in the soybean tissue examined, and their cDNAs without the sequences for signal peptide were cloned and expressed in *Escherichia coli* to produce recombinant enzymes. Only GmMAN1 showed endo-β-mannanase hydrolase activity. Further gene expression analysis showed that *GmMAN1* is specifically expressed in cotyledons of seedlings, suggesting a role of GmMAN1 in degrading mannan-rich food reserves during soybean seedling establishment. Purified recombinant GmMAN1 exhibited an apparent K_m value of 34.9 mg/mL. The catalytic efficiency (k_{cat}/K_m) of GmMAN1 was determined to be 0.7 mL/(mg·s). GmMAN1 was also shown to be active in hydrolyzing the β-mannan-rich cell wall of soybean seeds.

KEYWORDS: cotyledon, recombinant enzyme, kinetics, gene expression, soybean seed, hydrolase

INTRODUCTION

Soybean meal, the product remaining after removal of most of the oil from soybean seeds, is a major source of protein in livestock and poultry feeds in most countries.[1] The quality of soybean meal is determined by a number of parameters, including protein content, amino acid composition, carbohydrate composition and digestibility, among which a high content of β-mannan was determined to be an undesirable character of soybean meal.[2] β-Mannan is one type of hemicellulosic polysaccharide found in the plant cell wall. It is composed of repeating units of mannose, with galactose, glucose, or both, often found attached to the β-mannan backbone.[3] The β-mannan content of soybean meal ranges from 1.0% to 1.5% for dehulled samples and from 1.3% to 2.1% for nondehulled samples.[1] The relatively high content of β-mannan in animal feeds appears to have a deleterious effect on animal performance. β-Mannan was shown to stimulate the innate immune system of animals, which causes an increase in proliferation of macrophages and monocytes, as well as increased cytokine production.[4] Although the stimulation of the immune system may provide benefits under certain conditions,[5] these physiological changes often lead to an increased severity of disease symptoms and a decrease in the efficiency of nutrient utilization.[6]

As a type of polysaccharide, β-mannan can be hydrolyzed by the action of a number of hydrolytic enzymes, with endo-β-mannanase (EC 3.2.1.78) being the most important. Endo-β-mannanase catalyzes the random hydrolysis of the β-mannosidic linkage in the mannan backbone. A number of studies have shown that the addition of exogenous endo-β-mannanase derived from microorganisms to diets containing soybean meal can improve the performance of poultry and swine.[7,8] Previous studies showed that the quality of soybean meal, such as the content of phytate, can be genetically improved.[9] These results suggest that the desirable soybean meal with a low content of β-mannan may be produced by overexpression of an endo-β-mannanase gene in soybean seeds through genetic engineering.

A successful genetic engineering program of soybean meal improvement for reducing β-mannan requires an endo-β-mannanase gene. No endo-β-mannanase gene has been reported from soybean, whereas genes encoding endo-β-mannanase have been isolated from a wide range of organisms, including plants, animals, and microorganisms.[10] For plants, endo-β-mannanase genes have been isolated from tomato (*Solanum lycopersicum*), coffee (*Coffea arabica*), and lettuce (*Lactuca sativa*).[11−13] Sequence comparison and phylogenetic analysis imply that plant endo-β-mannanase genes have a common evolutionary origin.[10] The biological function of plant endo-β-mannanase genes has been best characterized for their involvement in seed biology. For example, *LeMAN1*, the first endo-β-mannanase gene to be isolated from a plant, plays a role in postgermination reserve mobilization in tomato seeds.[11] In contrast, a germination-specific endo-β-mannanase gene from tomato, *LeMAN2*, was suggested to play a role in regulating germination by modulating the process of endosperm weakening.[14] In addition, endo-β-mannanase genes have been shown to be involved in fruit ripening[15] and pollen development.[16] While most of the known endo-β-mannanases were shown to possess hydrolase activity, a recent study suggested that some endo-β-mannanases may have transglycosylase activity.[17]

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Table 1. Primers Used for Expression Analysis of Selected Soybean Endo-β-mannanase Genes Using RT-PCR

gene name	forward primer (5′−3′)	reverse primer (5′−3′)
GmMAN1	ATGGGTTGGAATGGACGTCTCA	TTAATGCTTGTTCACTAAGCAATACTTG
GmMAN2	ATGGGGGACTCAGCTCGCATGTTAC	CTAAGCAATACTTGACATTTTGTGG
GmMAN3	ATGAAGGATATAGTCAATCCTCATCATCAC	TTATGCAAGACTTGACATCTTCTTAG
GmMAN4	ATGTTCCATGTCAGTGTGGAAGCAAGG	TCAGTTGTCAATGTATCTGCCTCCGTTTC
GmUBI-3	GGGTTTTAAGCTCGTTGTGT	TCCCCTCTAGCCAATTCAGA

Although any endo-β-mannanase gene can be used as a molecular tool for the genetic improvement of soybean meal quality, a gene from soybean will have an advantage for the creation of cisgenic plants, which are better perceived by the public.[18] The genome of soybean has recently been fully sequenced,[19] which provides a unique opportunity for the identification and isolation of endo-β-mannanase genes from soybean. In this study, an endo-β-mannanase gene (*GmMAN1*) that showed hydrolase activity with specific expression in cotyledons of germinated soybean seeds was selected from a family of 21 putative endo-β-mannanase genes and subjected to detailed biochemical characterization. Recombinant GmMAN1 is active in hydrolyzing the β-mannan-rich cell wall of soybean seeds, suggesting that *GmMAN1* can be used as a candidate gene for genetic improvement of soybean meal quality.

MATERIALS AND METHODS

Plant Material and Chemicals. Williams 82 soybeans,[20] the cultivar utilized for whole genome sequencing,[19] were used for gene cloning and expression analysis of soybean endo-β-mannanase genes. Germinating seed samples were collected from soybean seeds imbibed in water on germination paper at 22 °C at different time points. In a growth chamber, soybean seeds were planted in soil and seedlings were grown with a photoperiod set for 18 h at 25 °C in the light and 6 h at 23 °C in the dark. Soybean tissues for gene expression analysis, including root, epicotyls, hypocotyls, cotyledons, first foliage leaves, and leaf buds, were harvested from 14 day old seedlings. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Database Search and Sequence Analysis. The soybean genome sequence[19] was analyzed to identify endo-β-mannanase genes using the BLASTP program[21] with the protein sequence of the tomato endo-β-mannanase protein LeMAN1 (GenBank accession AAB87859) as a query. The *E*-value of ≤E−5 was set as the cutoff value for identifying significant protein matches. In phylogeny reconstruction, a multiple sequence alignment of selected endo-β-mannanase proteins was performed using ClustalW2[22] with default parameters. The neighbor-joining phylogenetic tree was constructed using the MEGA software (version 4.0).[23]

Gene Expression Analysis Using Real-Time Polymerase Chain Reaction. Total RNA was extracted from germinating seeds and other tissues using an RNeasy Plant Mini Kit (Qiagen, Valencia, CA) with DNA contamination removed using an on-column DNase treatment (Qiagen). After purification, total RNA (1.5 μg) was reverse-transcribed into first-strand cDNA in a 15 μL reaction volume using the first-strand cDNA Synthesis Kit (Amersham Biosciences, Piscataway, NJ) as previously described.[24,25] With the first strand cDNA as the template, real-time polymerase chain reaction (RT-PCR) was conducted using the gene-specific primers for analyzing the expression of *GmMAN1* to *GmMAN4*. The mixed cDNAs (1.0 μL) from various tissues were used as the template for the PCRs. In performing semiquantitative RT-PCR analysis for *GmMAN1*, soybean ubiqutin 3 gene (*GmUBI-3*, GenBank accession D28123) was used as an internal control. The primers used for gene expression analysis are listed in Table 1.

Initially, PCR analysis was performed with ubiquitin 3 specific primers using 0.1, 0.2, 0.5, and 1.0 μL of cDNA. The program used to amplify ubiquitin 3 was as follows: 94 °C for 2 min followed by 30 cycles at 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 45 s, with a final extension at 72 °C for 10 min. Amplified products were separated on 1.0% agarose gel. Gels were stained with ethidium bromide, visualized under UV light, and quantified using the Bio-Rad Quantity One software (Bio-Rad, Hercules, CA). Analysis showed that the amount of amplified products with the ubiquitin 3 specific primers increased linearly with increasing amounts of template cDNA. Therefore, 0.2 μL of cDNA was chosen as the optimal template concentration for PCR analysis with the *GmMAN1*-specific primers. The program used to amplify the *GmMAN1* fragment was as follows: 94 °C for 2 min followed by 35 cycles at 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 1 min 30 s, and a final extension at 72 °C for 10 min. All PCRs were replicated three times each with the first-strand cDNAs made from two independent RNA preparations.

cDNA Cloning for *GmMAN1* and *GmMAN4* and Protein Expression. Plant endo-β-mannanases are cell wall proteins and contain signal peptides. Previous studies showed that the removal of signal peptides is important for the activity of mature proteins.[14] We first analyzed the protein sequences of GmMAN1 and GmMAN4 using the SignalP program (http://www.cbs.dtu.dk/services/SignalP/), and their corresponding signal peptides were identified (first 28 and 18 amino acids for GmMAN1 and GmMAN4, respectively). To clone the truncated GmMAN1 and GmMAN4 without a signal peptide, 5′-ATGGGGGACTCAGCTCGCATATTG-3′ and 5′- ATGTTCCATGTCAGTGTGGAAGCAAGG-3′ were used as forward primers and 5′-TTAATGCTTGTTCACTAAGCAATACTTG-3′ and 5′-TCAGTTGTCAATGTATCTGCCTCCGTTTC-3′ were used as reverse primers for GmMAN1 and GmMAN4, respectively. PCR products were cloned into the pEXP5/CT-TOPO vector and fully sequenced. To express recombinant GmMAN1 and GmMAN4, the protein expression construct was transformed into the *Escherichia coli* strain BL21 (DE3) CodonPlus (Stratagene, La Jolla, CA). Protein expression was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) at a concentration of 500 μM for 18 h at 25 °C.

GmMAN1 was also expressed with a His-tag. GmMAN1 was first subcloned into the pET100/D-TOPO vector (Invitrogen, Carlsband, CA). *E. coli*-expressed GmMAN1 with a His-tag was purified from the *E. coli* cell lysate using Ni-NTA agarose following the manufacturer instructions (Invitrogen). Protein purity was verified by SDS−PAGE, and protein concentrations were determined by the Bradford assay.[26]

Endo-β-mannanase Hydrolase Activity Assay. Qualitative analysis of hydrolase activity of GmMAN1 and GmMAN4 was performed using a modified gel diffusion method.[27] The agarose 0.8% (w/v) plates containing 0.05% (w/v) locust bean galactomannan (Sigma) were solidified, and wells were formed on the plates by scoring with a 3 mm cork borer and removing the plug by suction. The extracts (2 μL) from purified recombinant protein solution were applied to the wells, and the plates were incubated at 28 °C for 20 h. After incubation, the agarose gel plates were stained by 0.5% (w/v) Congo red dye (Sigma). The hydrolyzed areas were visible as clear circles on a dark background.

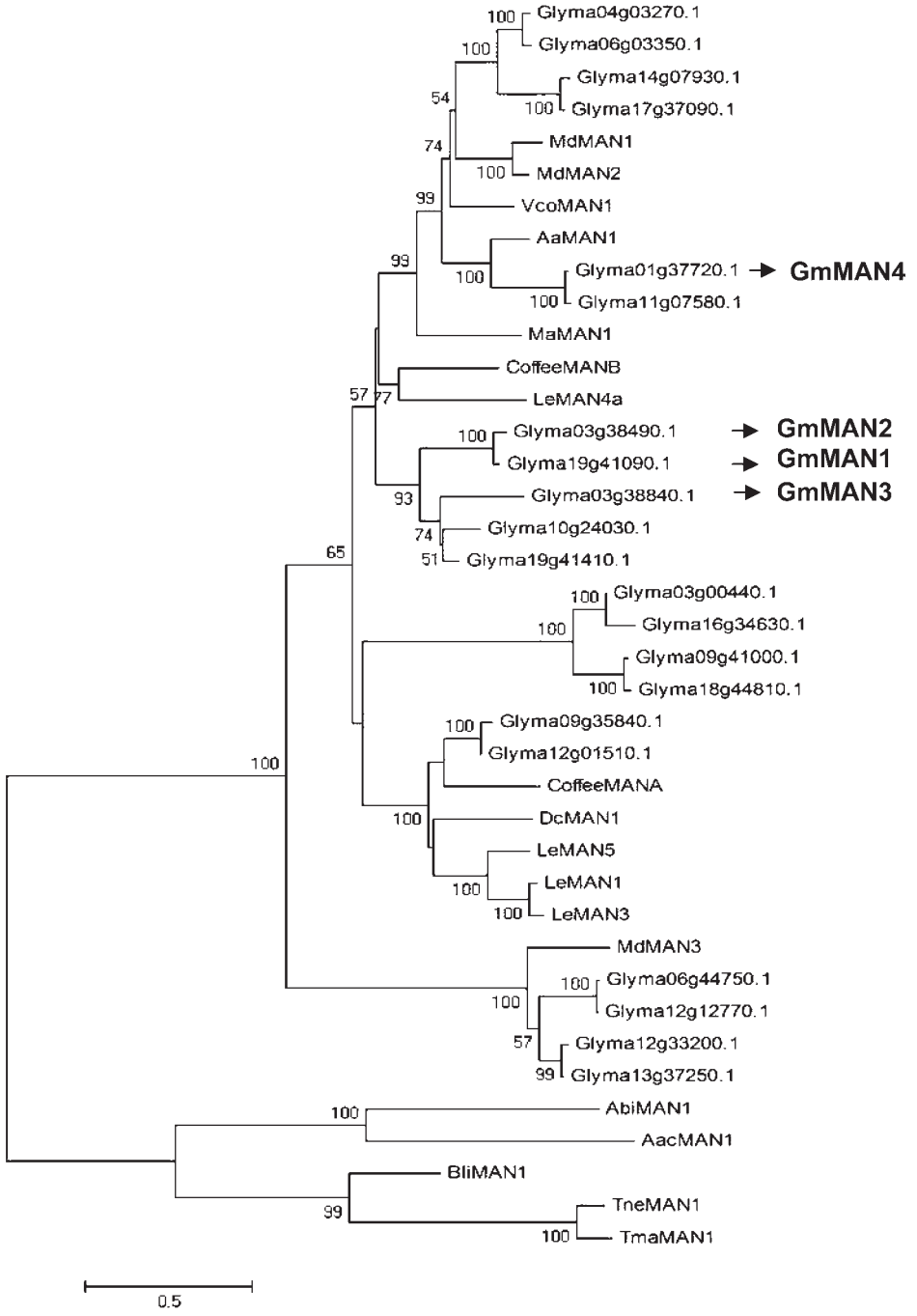

Figure 1. Phylogeny of 21 soybean endo-β-mannanase proteins, known endo-β-mannanases from other plants, and representative microbial endo-β-mannanases. All sequences that start with "Glyma" are soybean endo-β-mannanases. Known plant endo-β-mannanases include LeMAN1 (GenBank accession AAB87859), LeMAN3 (GenBank accession AAG14352), LeMAN4a (GenBank accession AAK97760), and LeMAN5 (GenBank accession AAG00315) from tomato, CoffeeMANA (GenBank accession CAC08208) and CoffeeMANB (GenBank accession CAC08442) from coffee (*Coffea arabica*), AaMAN1 (GenBank accession ACN78662) from *Actinidia arguta*, DcMAN1 (GenBank accession AAN34823) from carrot (*Daucus carota*), MdMAN1 (GenBank accession ACN78663), MdMAN2 (GenBank accession ACN78664), and MdMAN3 (GenBank accession ACN78665) from apple (*Malus domestica*), MaMAN1 (GenBank accession ABF69949) from *Musa acuminate*, and VcoMAN1 (GenBank accession ACN78666) from *Vaccinium corymbosum*. The microbial endo-β-mannanases include BliMAN1 (AAU23418.1) from *Bacillus licheniformis*, TneMAN1 (CAB56856.1) from *Thermotoga neapolitana*, TmaMAN1 (AAD36302.1) from *Thermotoga maritima*, AbiMAN1 (CAB76904.1) from *Agaricus bisporus*, and AacMAN1 (AAA67426.1) from *Aspergillus aculeatus*. Bootstrap values larger than 50% are shown in the phylogenetic tree. The four endo-β-mannanases studied in this paper, GmMAN1, GmMAN2, GmMAN3, and GmMAN4, are indicated.

The diameter of the hydrolyzed area is logarithmically related to the enzyme activity. The 1000-fold-diluted commercial endo-β-mannanase from *Aspergillus niger* (Megazyme, Bray, Ireland) was used as a positive control.

Figure 2. Expression analysis of four soybean *endo-β*-mannanase genes, *GmMAN1*, *GmMAN2*, *GmMAN3*, and *GmMAN4*, using RT-PCR. Soybean RNAs pooled from soybean tissues, including roots, epicotyls, hypocotyls, cotyledons, leaf buds, first foliage leaves, and imbibed soybean seeds, were reverse-transcribed to first-strand cDNA as the template for gene expression analysis.

The kinetic parameters of GmMAN1 were determined by measuring reducing sugars released after incubation with locust bean gum (LBG) in 0.1 M pH 5 McIlvaine buffer at 28 °C for 30 min according to a previously published method.[28] Absorbency was measured at 540 nm. The standard curve was obtained with D-mannose. One unit of endo-*β*-mannanase activity was defined as the amount of enzyme required to liberate 1 *μ*mol of mannose per minute at a given assay temperature. Increases in the reaction rate with increasing concentrations of LBG were evaluated through the endo-*β*-mannanase activity assay described above and were found to obey Michaelis−Menten kinetics. The appropriate enzyme concentration and incubation time were determined in time course assays. For determination of the catalytic parameters K_m and V_{max}, LBG served as the substrate and was used at concentrations of 1−10 mg/mL. The optimal pH of endo-*β*-mannanase activity was examined at pH 2.2−8.0 using 0.1 M McIlvaine buffer.

The effect of temperature on endo-*β*-mannanase activity was determined by incubating the purified enzyme with the substrate at temperatures ranging from 0 to 60 °C in 0.1 M McIlvaine buffer, pH 5.0. Thermal stability of the enzyme was determined by assaying for residual enzyme activity after incubation at various temperatures for 30 min in 0.1 M McIlvaine buffer, pH 5.0. The effect of various metal ions on endo-*β*-mannanase activity was determined by assaying for residual activity after adding metal ions to reactions in the form of chloride salts at 5 mM final concentration. Final values represent the average of three independent measurements.

Isolation of Cell Walls from Soybean Seeds. Williams 82 soybean seeds were ground and homogenized in 1 mL of distilled water and centrifuged at 10000*g* for 5 min. After the supernatant was decanted, the pellet was washed three times, each time with 1 M NaCl, 70% (v/v) ethanol, and chloroform−methanol (2:1), and then dried at room temperature. The dried cell wall material (20 mg) was suspended in 0.1 M McIlvaine buffer, pH 5, and subjected to enzyme digestion with the purified recombinant GmMAN1 protein (about 10 *μ*g) at 28 °C for 20 h. Reducing sugars released into the supernatant were assayed by the dinitrosalicylic acid (DNS) method using mannose as the standard.[28]

RESULTS

Identification of Endo-*β*-mannanase Genes in the Soybean Genome and Phylogenetic Analysis. A total of 21 genes encoding proteins that are significantly homologous to known plant endo-*β*-mannanases were identified from the soybean genome. These 21 genes are distributed in 14 chromosomes. The average length for these 21 mannanases is 422 amino acids, with the shortest being 365 amino acids and the longest being 471 amino acids.

To investigate the evolutionary relationships between the soybean mannanase genes and endo-*β*-mannanases of other origins, a phylogenetic tree of endo-*β*-mannanases was constructed (Figure 1). The 21 endo-*β*-mannanase genes in soybean

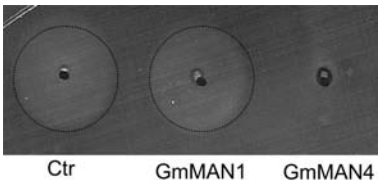

Figure 3. Hydrolase activity analysis of the crude protein extract of truncated *GmMAN1* and *GmMAN4* without signal peptide. Endo-*β*-mannanase from *Aspergillus niger* was used as a positive control (Ctr), and the crude protein extracts of truncated *GmMAN1* and *GmMAN4* were analyzed by the gel diffusion method. The size of the hydrolyzed area is indicated by a circle of broken line.

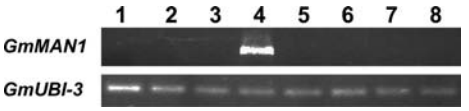

Figure 4. Expression of *GmMAN1* in various tissues of soybean. Semiquantitative RT-PCR was used to analyze *GmMAN1* expression. Seedling tissues, including roots (lane 1), epicotyls (lane 2), hypocotyls (lane 3), cotyledons (lane 4), first foliage leaves (lane 5), and leaf buds (lane 6), were collected from 14 day old soybean plants. Seed samples were collected at 36 h (lane 7) and 48 h (lane 8) after imbibition. Total RNA was extracted and used for RT-PCR analysis. PCR using primers for soybean ubiquitin 3 was used to judge the equality of concentration of cDNA templates in different samples.

are clustered into three major clades. On the basis of their gene structure and relatedness to functionally known plant endo-*β*-mannanases, four genes were selected for further experimental study. These include *Glyma19g41090.1* (*GmMAN1*), *Glyma 03g38490.1* (*GmMAN2*), *Glyma03g38840.1* (*GmMAN3*), and *Glyma01g37720.1* (*GmMAN4*).

Gene Expression Analysis of Four Soybean Endo-*β*-mannanase Genes. Soybean RNA was obtained from a pool of soybean tissues, including roots, epicotyls, hypocotyls, cotyledons, leaf buds, first foliage leaves, and imbibed soybean seeds. RNA was isolated and reverse-transcribed into first-strand cDNA, which was used as the template for PCR. Using specific primers, the amplified PCR products of soybean mannanase candidate genes were gained for *GmMAN1* and *GmMAN4* (Figure 2). Both of these amplified PCR products were cloned into a pEXP5/CT-TOPO vector and fully sequenced.

GmMAN1 and *GmMAN4* contain the opening reading frame sequences of 1233 and 1296 bp's, which encode proteins of 410 and 431 amino acids, respectively. GmMAN1 and GmMAN4 show moderate levels of sequence similarity to known plant endo-*β*-mannanases. For example, they are about 57% similar to LeMAN4a. Compared with LeMAN4a, whose three-dimensional (3-D) crystal structure has been solved,[29] the active sites of GmMAN1, including W97, N212, W295, W371, and I409, are strictly conserved.

Molecular Cloning of GmMAN1 and GmMAN4 and Their Biochemical Activities. The presence of a signal peptide in an endo-*β*-mannanase can interfere with its hydrolase activity.[14] Similarly, proteins expressed using full-length cDNAs of GmMAN1 and GmMAN4 did not show endo-*β*-mannanase activity. When the truncated forms of GmMAN1 and GmMAN4 without signal peptide were assayed, GmMAN1 showed endo-*β*-mannanase activity, but no endo-*β*-mannanase activity was detected for GmMAN4 (Figure 3).

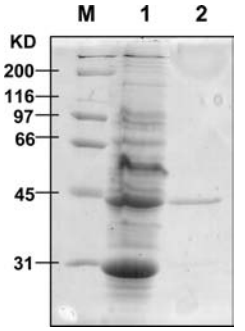

Figure 5. SDS−PAGE of purified recombinant GmMAN1 protein. His-tagged GmMAN1 expressed in *E. coli* was purified as described in the Materials and Methods. Lane M contained protein molecular weight markers. Lane 1 contained crude extract, and lane 2 contained about 2 μg of purified GmMAN1 protein. The gel was stained with Coomassie blue.

Expression Patterns of *GmMAN1*. To identify the specific tissues of soybean seedlings in which *GmMAN1* was expressed, total RNAs were isolated from 14 day old soybean seedling tissues, including roots, epicotyls, hypocotyls, cotyledons, first foliage leaves, and leaf buds. In addition, soybean seeds imbibed in water for 36 and 48 h were also collected. The cDNAs synthesized from individual RNAs were used for semiquantitative RT-PCR analysis. The expression of *GmMAN1* was only observed in the cotyledons of seedlings but not in other tissues examined (Figure 4).

Biochemical Properties of GmMAN1. Recombinant GmMAN1 with a His-tag expressed in *E. coli* was purified to electrophoretic homogeneity (Figure 5) and subjected to detailed biochemical characterization. When enzyme assays were performed in buffers of different pH values, GmMAN1 showed the highest level of catalytic activity at pH 5.0 (Figure 6A). The optimal temperature for GmMAN1 activity was 50 °C (Figure 6B). The enzyme was stable up to 37 °C, but about 80% of its activity was lost at 50 °C after 0.5 h of incubation (Figure 6C).

The effects of various metal ions on GmMAN1 activity were measured. While Zn^{2+}, Fe^{2+}, NH_4^+, K^+, and Na^+ had minimal effect on GmMAN1 activity, GmMAN1 activity was strongly inhibited by Mn^{2+} and moderately inhibited by Cu^{2+} and Ca^{2+} (Figure 6D).

The kinetic parameters of GmMAN1 were also determined. All assays were conducted at 28 °C in McIlvaine buffer, pH 5.0. The K_m of GmMAN1 was determined to be 34.86 ± 4.78 mg/mL, V_{max} was 33.80 ± 3.99 μmol/(min·mg), and K_{cat} was 25.2 s^{-1}. The catalytic efficiency of GmMAN1 (k_{cat}/K_m) was determined to be 0.72 mL/(mg·s).

Activity of Recombinant GmMAN1 on Isolated Cell Wall from Soybean Seeds. To test whether the recombinant GmMAN1 can hydrolyze the soybean cell wall, cell walls were isolated from soybean seeds. After 20 h of reaction, 10 μg of GmMAN1 recombinant enzyme hydrolyzed approximately 0.2 mg of the isolated soybean cell walls.

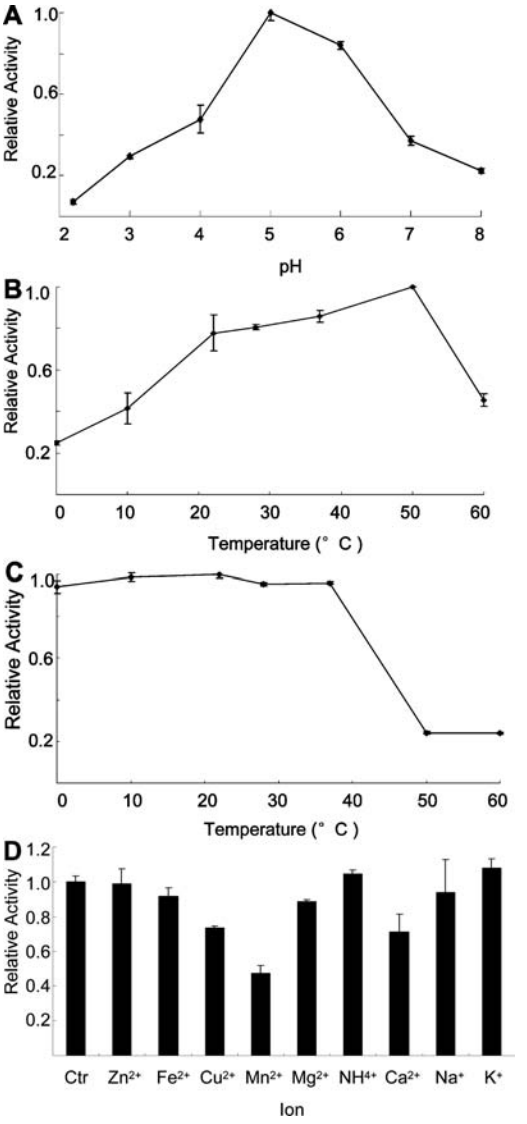

Figure 6. Biochemical properties of GmMAN1. (A) pH effect on GmMAN1 activity. The activity of the purified GmMAN1 was assayed from pH 2.2 to pH 8.0. The level of GmMAN1 activity in 0.1 M McIlvaine buffer, pH 5.0, was arbitrarily set at 1.0. (B) Optimal temperature of GmMAN1. The activity of purified GmMAN1 was assayed at temperatures ranging from 0 to 60 °C. The level of GmMAN1 activity at 50 °C was arbitrarily set at 1.0. (C) Temperature effect on the GmMAN1 stability. The thermostability of the purified GmMAN1 was determined by preincubating samples at temperatures ranging from 0 to 60 °C for 30 min and subsequently assaying the remaining activity. (D) Effects of metal ions on the activity of GmMAN1. Metal ions were added to reactions in the form of chloride salts at 5 mM final concentrations. The level of GmMAN1 activity without any metal ion added as the control (Ctr) was arbitrarily set at 1.0.

DISCUSSION

Hemicellulose β-mannan is rich in many seeds and has important biological functions. In tomato seeds, β-mannan is an important component of endosperm and plays a role in regulating seed germination.[14] In seeds of other plants, including soybean, β-mannan mainly serves as a food reserve to support seedling growth after germination.[30] Because there is essentially no endosperm in the mature soybean seeds, the high content of mannan is very likely localized in the cell walls of cotyledons. Despite its biological importance, the high content of β-mannan in soybean meal is undesirable.[2] The properties of soybean meal, including carbohydrate content and composition, can be modified genetically. Our long-term goal of this study is to improve soybean meal quality, attributed to β-mannan, through genetic

engineering. To this end, our first objective was to identify a useful soybean endo-β-mannanase gene.

Endo-β-mannanase genes belong to an ancient gene family, which is present in plants,[11−13] microorganisms,[31] and animals.[32] Phylogenetic analysis suggested that plant endo-β-mannanase genes share a common evolutionary origin.[10] Our research presents the first report, to our knowledge, on functional characterization of endo-β-mannanase genes from soybean. Despite the well-characterized functions of endo-β-mannanase in seed germination,[14] fruit ripening,[15] and pollen development,[16] the knowledge of endo-β-mannanases in plant biology is still limited. Because β-mannan is a component of the primary cell wall of many plants,[33,34] the modification of β-mannan via the action of endo-β-mannanase is likely involved in many biological processes. It is interesting that the soybean genome contains a relatively large family of endo-β-mannanase genes with 21 members (Figure 1), in contrast to *Arabidopsis*, rice, and poplar, which contain 8, 9, and 11 genes, respectively.[10] Therefore, soybean represents a useful model for studying the biochemical and biological functions of endo-β-mannanase genes. For instance, GmMAN4, which is 55% identical to GmMAN1 at the protein sequence level, did not show hydrolase activity (Figure 3). It will be interesting to determine whether GmMAN4 has transglycosylase activity and its specific biological role in soybean.

A number of endo-β-mannanse genes have been cloned from various plant species, including tomato, coffee, and lettuce.[11−13] However, at the biochemical level, most of these genes were only characterized to have hydrolase activity without detailed knowledge of their kinetics. Due to the many commercial uses of endo-β-mannanases, many microbial endo-β-mannanases have been fully characterized biochemically.[35,36] To our knowledge, GmMAN1 is among the first plant endo-β-mannanases to be fully characterized for its kinetics. The K_m of GmMAN1 is about 35 mg/mL on locust bean gum, which is significantly higher than those of microbial endo-β-mannanases. For instance, the K_m values for endo-β-mannanases from *Aspergillus tamarill* and *Streptomyces lividans* were reported to be 0.02 and 0.77 mg/mL, respectively.[37,38] While these differences may be due to the different mannan substrates used in the assays, they also suggest that GmMAN1 is less efficient than microbial endo-β-mannanases. Nonetheless, the detailed kinetic parameters of GmMAN1 presented in this study will be useful for a rational design of a genetic engineering strategy for reducing β-mannan in the soybean meal.

With an endo-β-mannanase gene at hand, our next objective will be to test the effect of overexpression of *GmMAN1* on the β-mannan content of soybean. This could be conducted using different strategies. One is to overexpress *GmMAN1* in developing seeds. Assuming that mannan is synthesized during seed development and maturation, the overexpression of *GmMAN1* in cotyledons will lead to immediate degradation of this hemicellulose. However, if this were to cause problems for seed development, an alternative strategy would be to accumulate the proteins in a different compartment, such as the endoplasmic reticulum, which would physically separate the enzyme from its substrate in intact seeds. Overproduced endo-β-mannanase can function in hydrolyzing β-mannan only after the soybean seeds have been milled, a strategy mimicking the addition of microbe-produced endo-β-mannanase to soybean meal. In this way, the potential negative impact of endo-β-mannanase overproduction on seed vigor and seedling establishment can be minimized.

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