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Gene Cloning and Enzymatic Characterization of an Alkali-Tolerant Endo-1,4-β-mannanase from *Rhizomucor miehei*

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ABSTRACT: An endo-1,4-β-mannanase gene (*RmMan5A*) was cloned from the thermophilic fungus *Rhizomucor miehei* for the first time and expressed in *Escherichia coli*. The gene had an open reading frame of 1330 bp encoding 378 amino acids and contained four introns. It displayed the highest amino acid sequence identity (42%) with the endo-1,4-β-mannanases from glycoside hydrolase family 5. The purified enzyme was a monomer of 43 kDa. RmMan5A displayed maximum activity at 55 °C and an optimal pH of 7.0. It was thermostable up to 55 °C and alkali-tolerant, displaying excellent stability over a broad pH range of 4.0−10.0, when incubated for 30 min without substrate. The enzyme displayed the highest specificity for locust bean gum (K_m = 3.78 mg mL^{-1}), followed by guar gum (K_m = 7.75 mg mL^{-1}) and konjac powder (K_m = 22.7 mg mL^{-1}). RmMan5A hydrolyzed locust bean gum and konjac powder yielding mannobiose, mannotriose, and a mixture of various mannose-linked oligosaccharides. It was confirmed to be a true endo-acting β-1,4-mannanase, which showed requirement of four mannose residues for hydrolysis, and was also capable of catalyzing transglycosylation reactions. These properties make RmMan5A highly useful in the food/feed, paper and pulp, and detergent industries.

KEYWORDS: *Alkali tolerance, cloning, characterization, endo-1,4-β-mannanase, Rhizomucor miehei*

INTRODUCTION

Mannans together with xylans are major constituents of the hemicellulose fraction.[1] β-1,4-Mannanase (β-1,4-D-mannan mannanohydrolase, EC 3.2.178) commonly known as β-mannanase, is an endo-acting hydrolase, which catalyzes the random hydrolysis of β-1,4-mannosidic linkages in the main chain of mannan polymers.[2] It releases linear and branched manno-oligosaccharides (MOS) of various lengths, which can be further hydrolyzed into monomers by β-mannosidase (EC 3.2.1.25) and α-galactosidase (EC 3.2.1.22).[3] Mannan-degrading enzymes have been classified into glycoside hydrolase (GH) families 5 or 26 on the basis of their amino acid sequence similarities.[4] Both enzyme families cleave glycosidic bonds by a double-displacement mechanism that leads to the retention of their anomeric configuration.[5,6] GH family 5 represents mannan-degrading enzymes from bacterial organisms and eukaryotic origin, while GH family 26 largely comprises β-mannanases from bacteria with the exception of a few anaerobic fungi.[7,8]

There has been growing interest over the years in the industrial potential of mannan-degrading enzymes, especially β-mannanase. This enzyme is widely applied in the food/feed industries, such as in poultry feed, to reduce the antinutritional factor of mannan polymers found in corn−soy-based feeds, for clarification of fruit juices and instant coffee, and extraction of coconut meats, and in the paper/pulp and detergent industries.[8−13] Mannan-rich agricultural crops, such as konjac, guar gum, and locust bean gum (LBG), are widely planted in Asian countries but have low commercial value.[14] Degradation of these mannan-rich agricultural residues by β-mannanases can produce MOS, which have several health benefits. The potential of β-mannanases to hydrolyze these cheap mannan-containing substrates makes them useful in conversion of cheap agricultural residues to products of commercial value.

As a result of their growing industrial potential, a large number of β-mannanase genes have been cloned from various microbial sources.[8,11] Thermostable and broad pH-tolerant enzymes are particularly suited for pulp and paper processing performed at elevated temperature and alkaline pH conditions. With the rapid expansion of genomic sequence data, a large number of β-mannanase genes have been putatively identified. Although many fungal β-mannanase genes have been cloned,[11,15,16] only a few genes from the thermophilic fungi have thus far been cloned and characterized.[16,17] Here, we report the gene cloning and enzymatic characterization of the first β-mannanase from the thermophilic fungus *Rhizomucor miehei*. Furthermore, the potential of the recombinant β-mannanase was also evaluated for possible application in the food/feed industries.

MATERIALS AND METHODS

Strains, Vectors, and Reagents. *Escherichia coli* JM109 (Strategene) and BL21 strains were used for propagation of the plasmids and as hosts for the expression of the β-mannanase gene, respectively. *R. miehei* CAU432 used in this investigation was deposited at the China General Microbiological Culture Collection Center under CGMCC 4967. pET-28a (+) vector was obtained from Novagen (Madison, WI). Restriction endonucleases and PrimeSTAR HS DNA polymerase (DR010A) were purchased from TaKaRa (Tokyo, Japan). T4 DNA ligase was from Biolabs (New England Biolabs, Ipswich, MA). Chelating Sepharose (Ni-IDA) resin matrix

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Table 1. Primers Used in This Study

primers	primer sequence (5′−3′)[a]	bases
ManDF	GAYTAYGGNGGNATGGA	17
ManDR	CANCKNGGYTCRTTIGC	17
Man5′GSP	ATCTCCCATCCAAAGATAGTCG	22
Man5′NGSP	CGATCATTTCGGTGTTCGTGTA	22
Man3′GSP	TGGATGGGAGATTGCCAATGAA	22
RmMan5F	CGCGGATCCGCTTCTTCGTTTGTCCAGACAAG	32
RmMan5R	CCGCTCGAGCTACTTCTTGGCCATGGCATCAGC	33

[a]N, A/G/C/T; R, A/G; Y, C/T; K, G/T; and I, hypoxanthine.

and Sephacryl S-100 resins were from GE Life Sciences (Pittsburgh, PA). LBG (G0753), guar gum (G4129), cellulose (S3504), birchwood xylan (X0502), and carboxymethylcellulose (CMC, sodium salt, low viscosity, C5678) were obtained from Sigma Chemical Company (St. Louis, MO). Avicel was from Merck, Ltd. (Darmstadt, Germany). All other chemicals were of analytical grade, unless stated otherwise.

Cloning of a β-Mannanase Gene from *R. miehei*. DNA manipulations were performed according to the recombinant DNA techniques as described by Sambrook and Russell.[18] To clone the β-mannanase gene (*RmMan5A*), degenerate primers ManDF and ManDR were designed based on the conserved sequences (DYGGMD and ANEPRC) of known fungal GH family 5 β-mannanases (Table 1).

Polymerase chain reaction (PCR) conditions were as follows: a hot start at 94 °C for 5 min, followed by the first five cycles at 94 °C for 30 s, 45 °C for 30 s, 72 °C for 1 min, 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min. The PCR product was purified, ligated into pMD18-T vector (TakaRa, Japan), and sequenced. Further, the full-length cDNA sequence of the β-mannanase was obtained by 5′ and 3′ rapid amplification of cDNA ends (RACE) using a SMART RACE cDNA amplification kit (Clontech, Palo Alto, CA). To amplify the 5′ end of the cDNA, RACE product was amplified with primer Man5′GSP and adapter primer UPM and then subjected to nested PCR using a nested gene-specific primer Man5′NGSP and adapter primer NUP. The PCR condition for RACE was 1 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 68 °C, and 1 min at 72 °C, and finally, 10 min at 72 °C. The primary PCR using primers Man3′GSP and UPM were followed by a nested PCR using nested gene-specific primer. The obtained PCR product was purified, cloned, and sequenced. The amplified PCR product of the DNA was purified and cloned into the pMD18-T vector and transformed into *E. coli* DH5α for sequencing. The 5′ and 3′ flanking sequences obtained by 5′ and 3′ RACE were assembled with that of the consensus region to form a whole-length cDNA sequence containing the open reading frame (ORF) of the β-mannanase gene. The nucleotide sequence has been submitted to the GenBank database and has been allocated the accession number JX255675.

Sequence assembly was performed using DNAMAN software (Lynnon Biosoft, Pointe-Claire, Quebec, Canada). BLAST analysis was performed at the NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple alignment analysis was performed by Clustal W2.0 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Structural analysis of deduced protein was carried out on the website of ExPASy. Signal peptide was analyzed by SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/). Putative catalytic residues were predicted using Motif Scan (http://myhits.isb-sib.ch/cgi-bin/motif_scan) and CAZY (http://www.cazy.org/).

Expression and Purification of RmMan5A. The coding region of *RmMan5A* without the signal peptide sequence was amplified by PCR from the cDNA of *R. miehei* CAU432 with the primers RmMan5F and RmMan5R (Table 1). *Bam*H I and *Xho* I sites (underlined) were added to the forward and reverse primers, respectively. The PCR product was cloned into the *Bam*H I/*Xho* I site of the pET28a (+) vector (Novagen) and transformed into *E. coli* BL21 for protein expression. *E. coli* BL21 cells harboring *RmMan5A* were inoculated at 37 °C in Luria-Bertani (LB) medium containing kanamycin (50 μg mL^{-1}) on a rotary shaker (200 rpm) until the OD_{600} reached about 0.6−0.8. The recombinant enzyme expression was induced by the addition of 1% lactose, and growth was continued further for 20 h at 35 °C. RmMan5A was purified using Ni-IDA resin. A total of 200 mL of *E. coli* culture was harvested by centrifugation and suspended in lysis buffer (50 mM sodium phosphate, 500 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride at pH 8.0), and the cells were disrupted by sonication. The lysate was clarified by centrifugation at 10000*g*, and the supernatant was applied to the Ni-IDA agarose resin column pre-equilibrated with buffer A (50 mM sodium phosphate, 500 mM NaCl, and 20 mM imidazole at pH 8.0) at a flow rate of 0.5 mL min^{-1}. The column was washed with 15 column volumes (CV) of buffer A followed by 5 CV of buffer B (50 mM sodium phosphate, 500 mM NaCl, and 50 mM imidazole at pH 8.0) and buffer C (50 mM sodium phosphate, 500 mM NaCl, and 75 mM imidazole at pH 8.0) at 1.0 mL min^{-1}. The bound protein was eluted with buffer D (50 mM sodium phosphate, 500 mM NaCl, and 200 mM imidazole at pH 8.0). The fractions showing β-mannanase activity were dialyzed against 50 mM sodium phosphate buffer (pH 6.5) and concentrated by ultrafiltration using a 10 000 Da molecular weight cutoff membrane.

Enzyme Activity Assay and Determination of the Protein Concentration. The β-mannanase activity was determined by the 3,5-dinitrosalicylic acid (DNS) method.[19] A total of 0.1 mL of enzyme in 50 mM sodium phosphate buffer (pH 7.0) was added to 0.9 mL of 0.5% LBG and incubated at 50 °C for 10 min. A total of 1 mL of DNS was added to the reaction mixture and boiled for 15 min. A total of 1 mL of 40% sodium−potassium tartarate was added, and the absorbance was measured at 540 nm. The amount of reducing sugars was calculated from a standard plot of mannose. A total of 1 unit of enzyme activity is defined as the amount of enzyme liberating 1 μmol of reducing sugars per minute under the conditions described. The protein content was determined by Lowry's method using bovine serum albumin as the standard.[20]

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE) and Molecular Mass Determination. The homogeneity and molecular mass of RmMan5A was analyzed on a 12.5% gel by SDS−PAGE as described by Laemmli.[21] The native molecular mass of the purified recombinant β-mannanase was determined using a Sephacryl S-100 gel filtration column (1 × 100 cm), which was equilibrated in 25 mM sodium phosphate buffer (pH 6.5) containing 150 mM NaCl at a flow rate of 0.3 mL min^{-1}. Standard proteins of known molecular masses (phosphorylase B rabbit muscle, 97.2 kDa; bovine serum albumin, 66 kDa; chicken egg white albumin, 44.3 kDa; α-chymotrypsinogen A from bovine pancreas, 25.6 kDa; and cytochrome *c*, 12.4 kDa) were loaded on the column and eluted under the same conditions for calculation of the molecular mass.

Biochemical Properties of RmMan5A. The optimal pH for β-mannanase activity and the pH stability were determined in 50 mM of different buffers within the pH range of 3.0−11.0 at 50 °C using 0.5% LBG as the substrate by the DNS method. The buffers used were McIlvaine (pH 3.0−7.5), sodium phosphate (pH 6.0−7.5), 2-(cyclohexylamino)ethanesulfonic acid (CHES) buffer (pH 8.0−10.0), and glycine-NaOH (pH 9.0−11.0). The effect of pH on β-mannanase stability was examined by incubating the purified enzyme in different buffers at 50 °C for 30 min. The residual activity was determined by the standard assay. The optimal temperature was determined by performing the standard assay at temperatures ranging

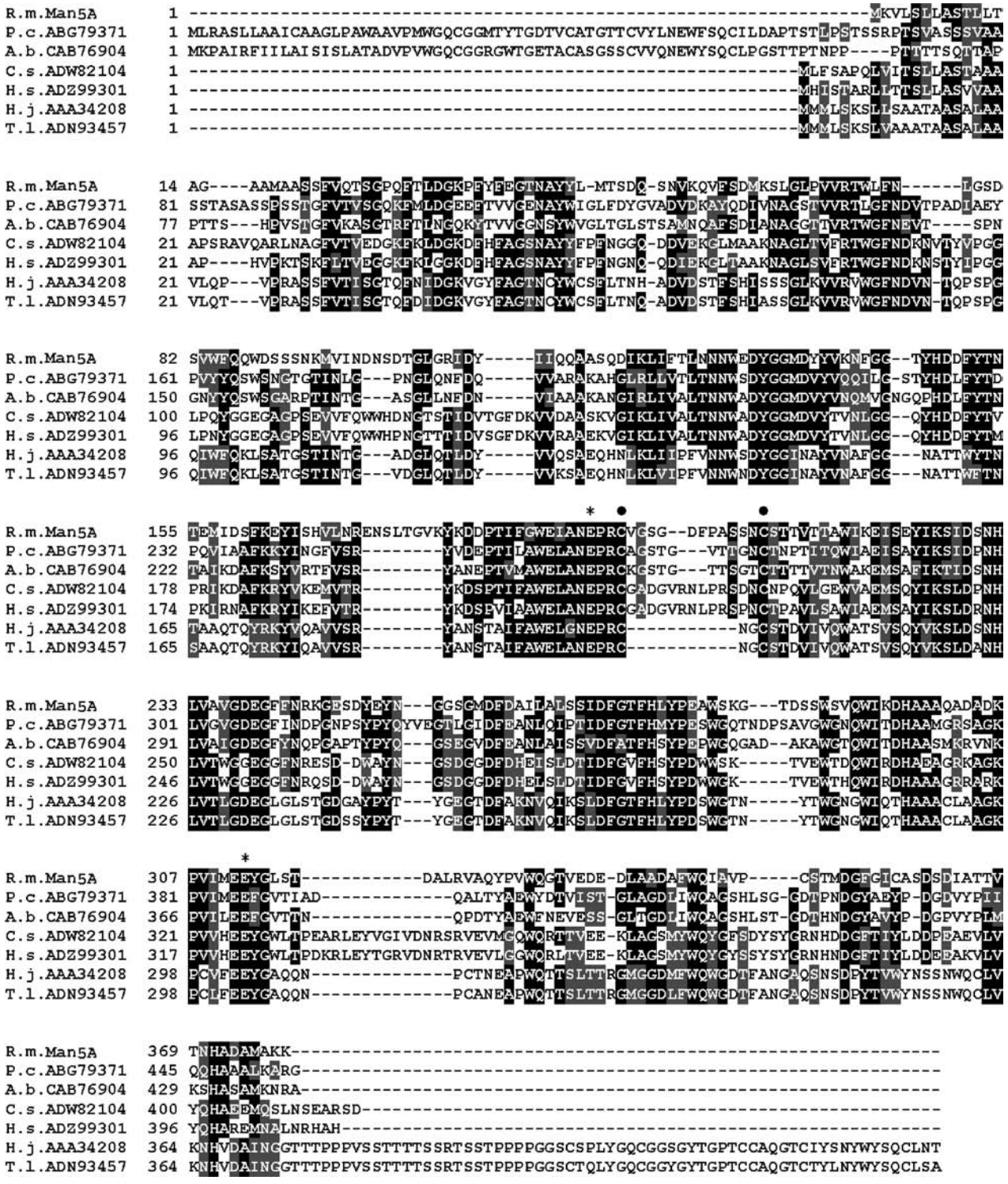

Figure 1. Comparison of amino acid sequence similarity of the *β*-mannanase from *R. miehei* with other fungal GH family 5 *β*-mannanases. Numbers on the left are the residue number of the first amino acid in each line. Sequences listed include those of *β*-mannanases from *P. chrysosporium* (*P.c.*ABG79371.1), Cel4a from *A. bisporus* (*A.b.*CAB76904.1), *Chaetomium* sp. CQ31 (*C.s.*ADW82104.1), *Humicola* sp. Y1 (*H.s.*ADZ99301.1), *H. jecorina* (*H.j.*AAA34208.1), and *T. longibrachiatum* (*T.l.*ADN93457.1). The single-letter amino acid code is used. Identical residues are shaded in black, and conserved residues are shaded in gray.

from 30 to 85 °C in 50 mM sodium phosphate buffer (pH 7.0). Thermal stability was measured by assessing the residual enzyme activity after incubation of the enzyme at different temperatures for 30 min. The influence of metal ions and various other agents on the activity of RmMan5A was determined. For this, the enzyme was incubated with 1 mM of the reagents at 50 °C in 50 mM sodium phosphate buffer (pH 7.0) for 30 min. The residual activity in the samples and the control (without reagents) was determined by the standard assay.

Substrate Specificity and Determination of Kinetic Parameters. The activity of the recombinant *β*-mannanase (RmMan5A) for 0.5% of various substrates, such as LBG, guar gum, konjac powder, birchwood xylan, CMC, starch, and Avicel, was measured at 50 °C in

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50 mM sodium phosphate buffer (pH 7.0) by the DNS method. Activity toward 5 mM *p*-nitrophenyl-*β*-D-mannopyranoside was measured by the rate of *p*-nitrophenol formed during hydrolysis from the substrates in 50 mM sodium phosphate buffer (pH 7.0) at 50 °C for 10 min and detected by spectrophotometry at 410 nm.

The Michaelis−Menten constants were determined for the substrates LBG, guar gum, and konjac powder in the concentration range of 2−7, 4−14, and 12−42 mg mL^{-1}, respectively, at 50 °C in 50 mM sodium phosphate buffer (pH 7.0) for 5 min using the DNS method. The kinetic parameters K_m and V_{max} were calculated using the software GraFit.[22]

Degradation of Mannan Polymers and Hydrolysis of MOS by RmMan5A. The potential of RmMan5A in degrading various mannan polysaccharides was analyzed as given below. A total of 5 units of purified RmMan5A was incubated with 1% of different mannan polysaccharides, such as LBG and konjac powder, at 40 °C in 50 mM sodium phosphate buffer (pH 7.0) for 24 h. Aliquots were withdrawn at different time points, boiled for 5 min, and analyzed by thin-layer chromatography (TLC). The reaction mixtures were spotted on a silica gel plate (Merck Silica Gel 60F 254, Germany) and developed twice in a solvent system containing *n*-propanol/ethanol/water (7:1:2, v/v/v). Saccharides were detected by heating in an oven after spraying the plates with a mixture of methanol/sulfuric acid (95:5, v/v). A mixture of MOS consisting of mannose (M1), mannobiose (*β*-1,4-mannobiose, M2), mannotriose (M3), mannotetraose (M4), and mannopentaose (M5) was used as the standard.

The ability of RmMan5A to hydrolyze various MOS, such as M2, M3, and M4, was studied. A total of 5 units of RmMan5A was incubated with 1% of different MOS in 50 mM sodium phosphate buffer (pH 7.0) at 40 °C for 24 h. Aliquots were withdrawn at different time points, boiled for 5 min, and analyzed by TLC. Transglycosylation of M4 was performed by incubating 5% M4 with 1 unit mL^{-1} purified recombinant *β*-mannanase in 50 mM sodium phosphate buffer (pH 7.0) at 40 °C for 8 h. The transglycosylation products were analyzed by TLC.

RESULTS

Cloning of a *β*-Mannanase Gene from *R. miehei* and Sequence Analysis. A partial *β*-1,4-mannanase gene from *R. miehei* was amplified by PCR using degenerate primers. The 5′ and 3′ RACE yielded 542 and 703 bp DNA fragments, respectively. The *β*-mannanase gene (*RmMan5A*) encodes 378 amino acids with an ORF comprising of 1330 bp with four introns. The translated protein has a predicted molecular mass of 41 859 Da. The N-terminal region contains a predicted signal peptide of 19 amino acids.

Amino acid homology alignment of the predicted RmMan5A with the homologous *β*-mannanases in GenBank was performed using a BLAST program. According to the homology search of the deduced amino acid sequence, RmMan5A showed low degree of homology with other fungal *β*-mannanases (Figure 1). It displayed the highest identity (42%) with the *β*-mannanase (Man5C) from *Phanerochaete chrysosporium* (accession number ABG79371.1)[12] and Cel4a from *Agaricus bisporus* (accession number CAB76904.1). The amino acid sequence identities with other fungal *β*-mannanases were 40% with *Chaetomium* sp. CQ31 (accession number ADW82104.1)[16] and *Humicola* sp. Y1 (accession number ADZ99301.1) and 39% with *Hypocrea jecorina* (accession number AAA34208.1) and *Trichoderma longibrachiatum* (accession number ADN93457.1). All of these fungal *β*-mannanases belong to GH family 5. This indicates that RmMan5A is a novel *β*-mannanase belonging to GH family 5.

Heterologous Expression and Purification of RmMan5A. The recombinant *β*-mannanase was expressed as a soluble, intracellular His-tagged enzyme with a specific activity of 1186 units mg^{-1}, which was purified to homogeneity using Ni-IDA chromatography (Figure 2). As shown in Table 2, the purified enzyme exhibited a high specific activity of 10 756 units mg^{-1}. The purified RmMan5A was found to be a monomer having a molecular mass of 42.6 kDa, which matches with that obtained by SDS−PAGE (44 kDa) and theoretical prediction analysis.

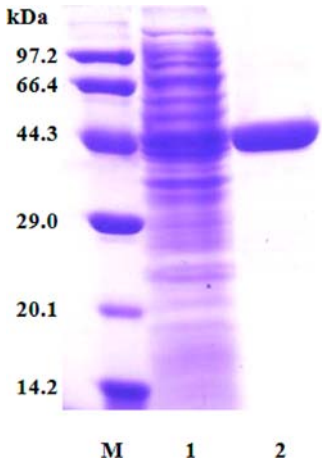

Figure 2. Analysis of purification of RmMan5A by SDS−PAGE. SDS−PAGE was performed on 12.5% gel. Lane M, low-molecular-mass protein marker; lane 1, crude lysate; and lane 2, purified RmMan5A.

Table 2. Summary of Purification of Recombinant *β*-Mannanase (RmMan5A) from 200 mL of *E. coli* Culture

purification steps	total activity (units)[a]	total protein (mg)[b]	specific activity (units mg^{-1})	purification factor (-fold)	yield (%)
crude extract	309897	261.3	1186	1.0	100
Ni-IDA column	234491	21.8	10756	9.1	75.6

[a]Activity was determined in 50 mM sodium phosphate buffer (pH 7.0) at 50 °C using LBG. [b]The protein was measured by the method from Lowry et al.,[20] using BSA as the standard.

Biochemical Properties of RmMan5A. The optimal pH for *β*-mannanase activity was pH 7.0 (Figure 3A). The enzyme displayed excellent stability over a broad pH range of 4.0−10.0, retaining more than 80% of its activity in this pH range (Figure 3B). Moreover, it retained more than 50% activity at pH 11.0, indicating that it is an alkali-tolerant *β*-mannanase. The optimal temperature of RmMan5A was 55 °C (Figure 3C). The enzyme was thermostable because it was fully active up to 55 °C for 30 min, at which it retained 90% of its activity (Figure 3D). The effect of various metal ions and other reagents on the *β*-mannanase activity was analyzed. Among the metal ions tested, Hg^{2+} and Ag^{+} completely inhibited the enzyme activity (Table 3). Ni^{2+} and Cu^{2+} were also found to inhibit the enzyme activity (Table 3). The enzyme activity was significantly enhanced by some metal ions, such as Mn^{2+} and Co^{2+}, whereas other metal ions did not have much effect. RmMan5A retained complete activity in the presence of a reducing agent as well as the detergent SDS.

Analysis of Substrate Specificity and Kinetic Parameters. The relative activity of RmMan5A on various galacto- and glucomannan substrates was determined because *β*-mannanases from different organisms are known to display

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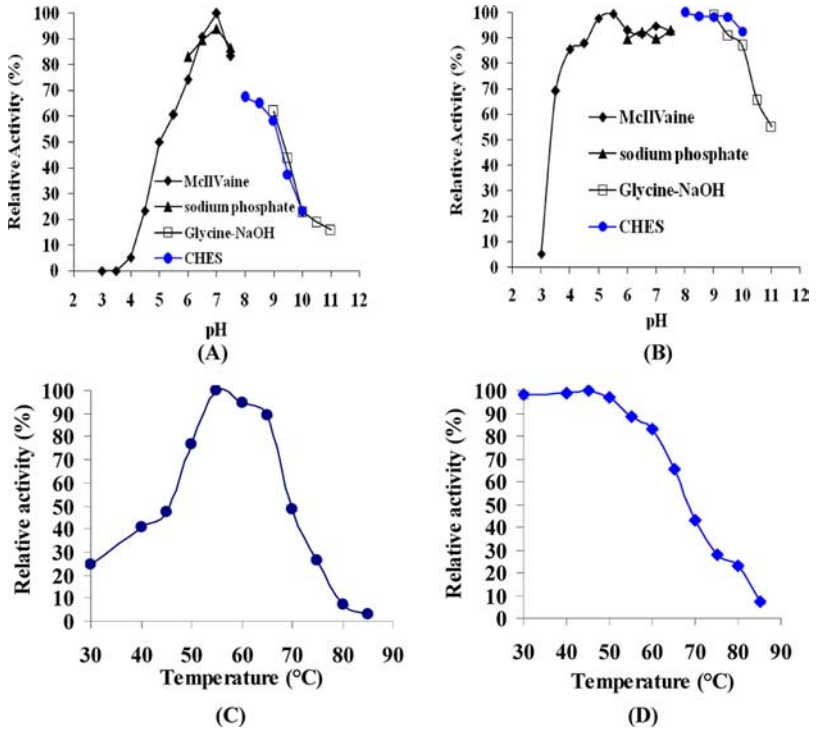

Figure 3. Determination of (A) optimal pH, (B) pH stability, (C) optimal temperature, and (D) thermostability of the purified RmMan5A. The influence of pH on β-mannanase activity was determined at 50 °C using 50 mM of different buffers. To determine pH stability, the remaining activity was measured after incubation for 30 min at 50 °C over various pH ranges. The temperature profile was measured at different temperatures in 50 mM sodium phosphate buffer (pH 7.0). For determination of thermostability, the residual activity of the treated enzyme was measured according to the standard assay after a 30 min of preincubation at different temperatures.

Table 3. Influence of Different Reagents on β-Mannanase Activity of RmMan5A

reagents	specific activity (units mg^{-1})[a]	relative activity (%)
control	10656	100.0
Cu^{2+}	6777	63.6 ± 0.9
Ca^{2+}	10249	96.2 ± 0.4
Ni^{2+}	8059	75.6 ± 0.4
Mg^{2+}	10387	97.5 ± 0.1
Zn^{2+}	9020	84.6 ± 0.1
Mn^{2+}	16194	152.0 ± 7.2
Co^{2+}	15772	148.0 ± 2.5
Na^{+}	10356	97.2 ± 0.05
EDTA	9327	87.5 ± 0.01
Ba^{2+}	9611	90.2 ± 0.3
Hg^{2+}	793	7.43 ± 1.0
Cr^{3+}	9673	90.8 ± 0.3
Ag^{+}	1983	18.6 ± 0.05
β-mercaptoethanol	11355	106.6 ± 0.9
SDS	11939	112.0 ± 0.01
DTT	8997	84.4 ± 0.4

[a]The residual enzyme activity was assayed after incubating with 1 mM of different reagents at 50 °C for 30 min.

great variation in their substrate specificities. RmMan5A displayed high activity for LBG galactomannan (10 756.3 units mg^{-1}, 100%), followed by konjac glucomannan (5317.6 units mg^{-1}, 49.4%) and guar gum galactomannan (1492.1 units mg^{-1}, 13.9%). The enzyme, however, did not show any detectable activity with *p*-nitrophenyl-β-D-mannopyranoside, CMC, starch, xylan, and Avicel. The Michaelis−Menten constants were determined for LBG, guar gum, and konjac powder. As shown in Table 4, RmMan5A exhibited higher affinity for galactomannans, such as LBG and guar gum, in comparison to glucomannan, such as konjac powder.

Table 4. Kinetic Parameters for the Purified RmMan5A[a]

substrate	V_{max} (μmol min^{-1} mg^{-1})	K_m (mg mL^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (mL mg^{-1} s^{-1})
LBG	21391.6 ± 538	3.8 ± 0.2	14.9	3.93
guar gum	6843.3 ± 267	7.8 ± 0.6	4.77	0.61
konjac powder	36936.2 ± 429	22.7 ± 0.6	25.8	1.14

[a]Enzymatic reactions were carried out for 5 min at 50 °C in 50 mM sodium phosphate buffer (pH 7.0).

Degradation of Mannan Polymers and Hydrolysis of MOS. The degradation of various mannan polymers, such as LBG and konjac powder, by the purified RmMan5A was performed. As shown in Figure 4, RmMan5A was able to degrade different types of mannan polymers, such as galacto- and glucomannans, releasing various galacto- and gluco-linked MOS. The hydrolysis of LBG yielded predominantly mannobiose (M2) and mannotriose (M3) as the main products (Figure 4A). The degradation of konjac powder resulted in the formation of a mixture of mannose-linked sugars (Figure 4B).

RmMan5A was incubated with different MOS, and the products were resolved by TLC (Figure 5). TLC analysis confirmed that RmMan5A is indeed an endo-β-mannanase, which cannot cleave the mannosidic linkage in M2 (Figure 5A). While the enzyme was unable to cleave M3 even after a prolonged incubation period of 24 h (Figure 5B), it efficiently hydrolyzed mannotetraose, forming M3 and M2 (Figure 5C). It

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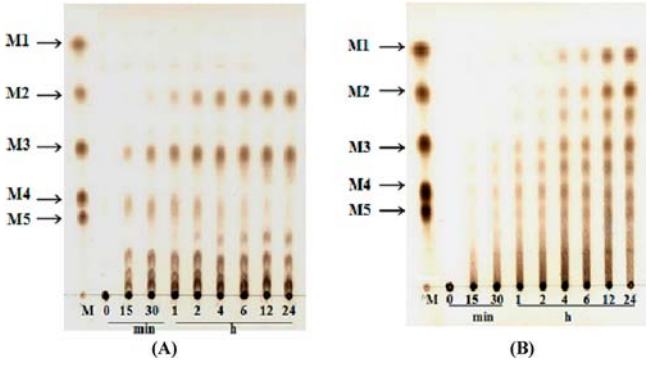

Figure 4. TLC analysis of the degradation of mannan polymers by RmMan5A. A total of 5 units mL^{-1} enzyme was incubated with different substrates, such as (A) 1% LBG or (B) 1% konjac mannan in 50 mM sodium phosphate buffer (pH 7.0) for 24 h at 40 °C. Incubation times (hours or minutes) are indicated. Lane M, standard MOS.

should be noted that, although M3 and M2 were detected during the hydrolysis of M4, an equal amount of M1 was not detected (Figure 5C), suggesting the occurrence of a transglycosylation reaction. When RmMan5A was incubated with a high concentration of M4, it was able to catalyze the transglycosylation reaction, resulting in the formation of MOS with a degree of polymerization (DP) > 5 (Figure 5D).

DISCUSSION

β-Mannanases from different fungi have been characterized for their potential applications in various industries.[11] In this study, the *β*-mannanase gene (*RmMan5A*) from the thermophilic fungus *R. miehei* was cloned. RmMan5A shared the highest identity (42%) with fungal *β*-mannanases belonging to GH family 5 (Figure 1). On the basis of their sequence similarity, microbial mannan-degrading enzymes are grouped into GH families 5 and 26. Fungal *β*-mannanases predominantly belong to GH family 5, with limited representation in GH family 26.[11] The acidic amino acids (aspartic or glutamic acid residues) were demonstrated to play the role of acid/base catalyst and nucleophile. On the basis of the sequence comparison between RmMan5A and other GH family 5 *β*-mannanases, E175 and E293 are predicted to be the catalytic acid/base and catalytic nucleophile, respectively, in RmMan5A.[7] RmMan5A was expressed intracellularly as soluble protein at high levels of 1305 mg L^{-1}, and the purified enzyme exhibited high specific activity of 10 756 units mg^{-1} (Table 2). Although extracellular enzymes are better suited for applications, RmMan5A was expressed intracellularly as a soluble enzyme and showed good

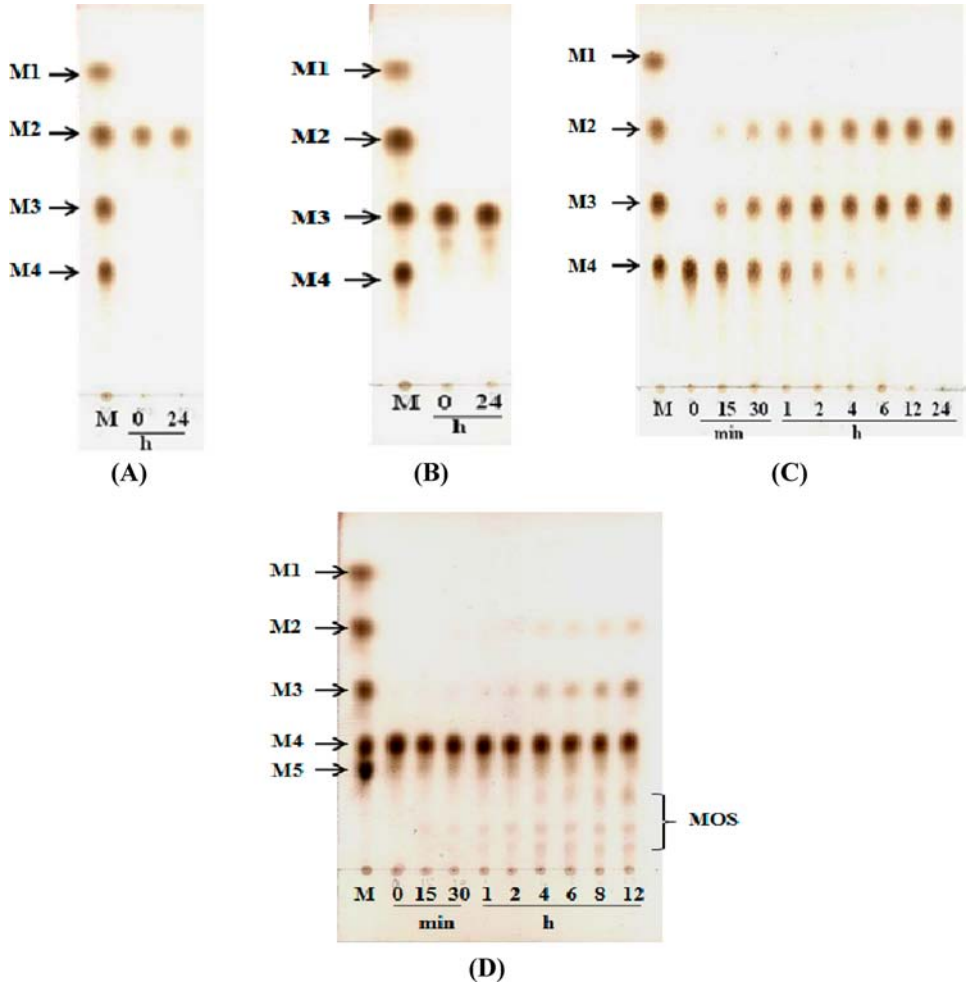

Figure 5. TLC analysis of hydrolysis of MOS by RmMan5A. For the hydrolysis reaction, 5 units mL^{-1} RmMan5A was incubated with 1% (A) mannobiose, (B) mannotriose, or (C) mannotetraose at 40 °C in 50 mM sodium phosphate buffer (pH 7). (D) Transglycosylation reaction was performed with 1 unit mL^{-1} enzyme and 5% mannotetraose. Incubation times (hours or minutes) are indicated. Lane M, standard MOS.

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activity and the expression levels and yield of the purified enzyme were high, making it economical for large-scale production.

The expression levels of RmMan5A was significantly higher than that of most reported β-mannanases, such as those from *T. longibrachiatum* (201 mg L^{-1}), *Aspergillus niger* BK01 (243 mg L^{-1}), and *Aspergillus sulphureus* (262 mg L^{-1}).[23−25]

The purified RmMan5A was a monomeric enzyme with a molecular mass of 42.6 kDa (Figure 2). The β-mannanases from GH family 5 have molecular masses in the range of 40−60 kDa and are usually monomeric in nature.[3,15,23] RmMan5A displayed much differences in terms of its pH optimum and pH stability compared to other fungal β-mannanases. The fungal β-mannanases characterized thus far exhibited optimal activity in the acidic pH range of 4.0−5.0,[12,16,23] while RmMan5A was maximally active at pH 7.0. RmMan5A was stable in the pH range from 4.0 to 10.0. The most remarkable feature of RmMan5A is its excellent tolerance to alkaline pH, because it retained >50% activity at pH 11.0 (Figure 3). Most of the fungal β-mannanases are stable in the acidic−neutral pH range and lose their activity at alkaline pH.[11] Thus far, only the fungal β-mannanases from *Humicola insolens* and *Chaetomium* sp. were found to be alkali-pH-tolerant.[16,26] However, they exhibited optimal β-mannanase activity in the acidic pH range (pH 5.0−5.5). The high alkali tolerance of RmMan5A provides obvious advantages for the application in the manufacture of kraft pulp and in the detergent industry, where high pH processes are applied. Until now, only bacterial β-mannanases exhibiting high stability at alkaline pH were found to be suitable for such industrial applications.[27] The optimal temperature of RmMan5A was 55 °C (Figure 3), similar to some other fungal β-mannanases belonging to GH family 5, which are optimally active in the range of 40−60 °C.[16,23,25] RmMan5A was thermostable up to 55 °C, which is also comparable to other GH family 5 β-mannanases.[12,17,23] The thermostable property of RmMan5A makes it useful in the food industry. The activity of RmMan5A was inhibited by some metal ions (Table 3). Metal ions, such as Ag^{+}, Hg^{2+}, Mn^{2+}, and Pb^{2+}, have been shown to strongly inhibit other β-mannanases.[16,23,25] Inhibition of RmMan5A activity by Hg^{2+} suggests that the enzyme contains an essential sulfhydryl group. Inhibition of β-mannanase activity has also been observed in the presence of certain metal ions, including Cu^{2+}, Ni^{2+}, and Zn^{2+}.[26] One notable feature of RmMan5A is its high activity in the presence of the detergent SDS, which makes it valuable in the detergent industries. SDS has been shown to inhibit the activity of many β-mananases.[15,26,28] Thus, RmMan5A displayed superior biochemical properties compared to other fungal β-mannanases belonging to GH family 5.

RmMan5A showed high specific activity with various mannan polymers, such as LBG, a galactomannan with a high mannose/galactose ratio of 4:1, guar gum, with mannose/galactose ratio of 2:1, and konjac, with a mannose/glucose ratio of 3:1. β-1,4-Mannanases act on and randomly hydrolyze β-1,4-linkages in diverse substrates, such as pure mannans, galactomannans, glucomannans, and galactoglucomannans.[3] The activity of β-mannanases for galacto- and galactoglucomannans is greatly dependent upon the extent and pattern of substitution of the mannan backbone. The high specific activity of RmMan5A toward these mannan polymers suggests its potential use in conversion of these widely available, cheap, mannan-rich, agricultural residues into products of commercial value. The absence of activity of RmMan5A toward cellulose suggests its possible use in the paper and pulp industries. The K_m of RmMan5A for LBG (3.78 mg mL^{-1}) was close to the K_m values obtained for some other fungal β-mannanases, such as from *P. chrysosporium* (K_m = 3.0 mg mL^{-1}) and *Chaetomium* sp. (K_m = 3.1 mg mL^{-1}); however, its V_{max} value was comparatively much higher.[12,16]

Hydrolysis of substituted or branched polysaccharides by β-1,4-mannanases is hindered by the degree and pattern of galactose substitution on the mannan backbone and the distribution of glucose within the main chain.[1] In most cases, an uninterrupted sequence of 3−5 unsubtituted mannose residues is required for cleavage to occur.[2] Most endo-1,4-β-mannanases cleave the main chain of mannans, yielding mainly mannotriose and mannobiose.[8,17] However, a few β-mannanases also liberate mannose from mannans.[29] A comparison of the hydrolysis behavior of RmMan5A to other GH family 5 β-mannanases indicates interesting facts. Mannobiose was the major product released by degradation of LBG by Man5D from *P. chrysosporium*, which is also the main hydrolysis product detected for RmMan5A along with mannotriose.[12] Contrary to this, low amounts of mannose, mannobiose, or mannotriose were formed by catalysis of LBG by Man5A from *H. insolens* Y1 and CsMan5A from *Chaetomium* sp.[16,26] This indicates that a great deal of variation in the substrate-binding mechanism of β-mannanases from the same family exists. Extensive hydrolysis of cheap and widely available LBG and konjac by RmMan5A resulted in a mixture of MOS containing various oligosaccharides that may have diverse prebiotic effects. Furthermore, because galactomannan cannot be efficiently used by monogastric animals and has been shown to have strong negative influences in monogastric animals, RmMan5A can find application in the feed industries.[9] Thus, RmMan5A could find potential application in the food/feed industries for the production of prebiotic MOS from cheap and widely available agricultural residues, such as konjac and LBG. Also, β-mannanases having substrate specificities for galactomannan constituents would make excellent candidates for use in enzymatic bleaching of softwood pulps.

The results in this study suggest that RmMan5A exhibits an action pattern with a demand of at least four mannose residues for effective cleavage. Apart from their ability to hydrolyze different mannans, some β-1,4-mannanases display transglycosylation activity.[5,17] Therefore, to confirm this, the transglycosylation property of RmMan5A was tested by incubation with high concentrations of M4. Product analysis by TLC confirmed that RmMan5A catalyzes the transglycosylation of M4, generating higher MOS. Usually, a DP of at least 4 is required for efficient hydrolysis, although some β-mannanases are also able to hydrolyze mannotriose.[5,16] The GH family 5 Man5D from *P. chrysosporium* required a chain length of three or more mannose residues for efficient catalysis and was also able to undergo transglycosylation reactions.[12]

In conclusion, the gene encoding a GH family 5 β-mannanase (*RmMan5A*) from the thermophilic fungus *R. miehei* was cloned and expressed in *E. coli*. The purified RmMan5A was optimally active at neutral pH and displayed many favorable properties, such as high stability at alkaline pH, high specific activity, and catalytic efficiency toward LBG. These properties make RmMan5A better suited for many industrial applications in the food, detergent, and paper/pulp industries compared to most other fungal β-mannanases.

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