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Truncation of a mannanase from *Trichoderma harzianum* improves its enzymatic properties and expression efficiency in *Trichoderma reesei*

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Abstract To obtain high expression efficiency of a mannanase gene, *ThMan5A,* cloned from *Trichoderma harzianum* MGQ2, both the full-length gene and a truncated gene (*ThMan5A*△CBM) that contains only the catalytic domain, were expressed in *Trichoderma reesei* QM9414 using the strong constitutive promoter of the gene encoding pyruvate decarboxylase (*pdc*), and purified to homogeneity, respectively. We found that truncation of the gene improved its expression efficiency as well as the enzymatic properties of the encoded protein. The recombinant strain expressing *ThMan5A*△CBM produced 2,460 ± 45.1 U/ml of mannanase activity in the culture supernatant; 2.3-fold higher than when expressing the full-length *ThMan5A* gene. In addition, the truncated mannanase had superior thermostability compared with the full-length enzyme and retained 100 % of its activity after incubation at 60 °C for 48 h. Our results clearly show that the truncated ThMan5A enzyme exhibited improved characteristics both in expression efficiency and in its thermal stability. These characteristics suggest that ThMan5A△CBM has potential applications in the food, feed, paper, and pulp industries.

Keywords Mannanase · *Trichoderma harzianum* · *Trichoderma reesei* · Carbohydrate-binding module · Thermostability

Introduction

Mannan is a major hemi-cellulose that is present in the cell walls and in the seeds of plants [4, 20, 32]. Endo β-1, 4-mannanases (β-mannanases, EC 3.2.1.78) can catalyze the hydrolysis of structurally different mannans. Based on sequence similarities, these enzymes are mainly grouped into glycoside hydrolase families 5 and 26 [3]. Mannanases are ubiquitous in nature and a vast variety of bacteria, actinomycetes, yeasts, and filamentous fungi are known to be mannan degraders [28, 34, 37]. There are many applications for mannanases in industrial processes. Mannanases are used mainly for improving the quality of food and feed as supplements. In the paper and pulp industry, mannanases could be used as an enzymatic pretreatment to substitute for the ultrahot alkaline extraction stage within a pulp-bleaching sequence [5], thereby offering the possibility of a significant reduction in environmental pollution. Mannanases with substrate specificities for galactomannan constituents have significant advantages in enzymatic bleaching of softwood pulps [7]. Moreover, because pulping is generally performed at elevated temperatures, thermophilic mannanases could offer significant advantages because of their higher intrinsic stability and catalytic efficiencies

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[17]. Mannanases also have applications in textiles and cellulosic fiber, hydrolysis of coffee extract, the detergent industry, oil drilling, and in bioactive oligosaccharides production [6, 9].

Here, we report a mannanase from *Trichoderma harzianum* MGQ2, a strain that was isolated from mangrove soil, with significant mannanase activity. A β-mannanase gene from this strain was cloned and the full-length mannanase *ThMan5A* was expressed in *T. reesei* using a constitutive strong promoter *pdc* reported in our previous study [11]. To obtain high expression levels, the truncated mannanase *ThMan5A△CBM* was expressed under the control of the same promoter. This is the first report of the cloning of a mannanase gene and the characterization of a truncated enzyme from *T. harzianum*. The recombinant truncated mannanase has some superior properties: high extracellular enzyme activity, specific activity, proportion of the total amount of the extracellular enzyme, thermal stability, and pH stability.

Materials and methods

Microorganism isolation and identification

The soil samples collected from the Mangrove Nature Reserve (located 114°03′ E,22°32′ N, in Shenzhen, Guangdong Province, China) were diluted in sterile dilution solution (0.9 % saline). Aliquots were spread on modified Martin Agar medium containing 1 % konjac powder as the sole carbon source, and the plates were incubated at 28 °C for 48 h. One strain, MGQ2, with significant mannanase activity, was selected for further study. Identification of the isolated strain was determined using the Microlog system software, release 6.0. Differences in the metabolic rates of MGQ2 in different carbon sources were analyzed using a microbial automatic analyzer (Biolog, Hayward, CA, USA). For sequence analysis, the ITS1-5.8 S-ITS2 sequence of the rDNA region of the fungus was amplified by polymerase chain reaction (PCR) using the primer sets of White et al. [43]: pITS1 5′-TCCGTAGGTGAACCTGC CG-3′ and pITS4 5′-TCCTCCGCTTATTGATATGC-3′. The 640 bp amplicon that was obtained was cloned and sequenced.

Strains, plasmids, and cultivation conditions

Escherichia coli (*E. coli*) Top10F' (Invitrogen, San Diego, CA, USA) was used for plasmid construction and maintenance. *T. reesei* QM9414 (ATCC 26921) was used as the parental strain throughout the study. The *E. coli* strain was cultivated in LB medium in which ampicillin (100 µg/ml; Invitrogen) was supplemented when necessary. The *T.*

reesei and *T. harzianum* strains were maintained on potato dextrose agar (PDA), and for liquid cultivation, they were grown in a Mandels' medium that contained 2 % glucose [19]. *T. harzianum* produced mannanase with 5 g/l konjac powder as the inducer.

The recombinant *T. reesei* strains were placed on PDA agar supplemented with hygromycin B (100 µg/ml), and for recombinant mannanase production, the strains were cultivated in a modified Mandels' medium supplemented with 7 % glucose, 5 % soybean powder, and 1 % peptone. *E. coli* was routinely cultured at 37 °C, whereas *T. reesei* and *T. harzianum* strains were cultured at 28 °C. Plasmid pUC19 was used for the construction of *TrMan5A* expression cassettes. Plasmid pAN7-1, which contained the hygromycin B-resistant cassette, was used as an assisting plasmid for the transformation of *T. reesei* [29].

Chemicals and reagents

PrimeSTAR HS DNA polymerase, TaKaRa LA Taq, plasmid pUC19 and all the restriction enzymes used in this study were purchased from Takara Bio Inc. (Japan). The substrates, sodium carboxymethyl cellulose (CMC-Na), Avicel, and the xylans from birchwood, beechwood, and locust bean gum (LBG), were purchased from Sigma-Aldrich, Brondby, Denmark. Mannan and konjac powder were purchased from Jiang Lai Biotechnology Co., Ltd (Shanghai, China). All other chemicals were of analytical grade.

Cloning of *ThMan5A*

Total cellular DNA from *T. harzianum* MGQ2 was isolated with a Wizard Genomic DNA Purification Kit (Promega, Fitchburg, WI, USA) and used as the template for the PCR amplifications. Degenerate primers (D1, D2) were designed for PCR amplification based on the conserved regions of the genes that encode the GH5 mannanases in *Trichoderma* species. Then, a thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) [13] was performed to amplify the whole sequence of *ThMan5A* using four arbitrary degenerate primers from the Genome Walking Kit (Takara) and six specific primers (three UT-primers for the upstream and three DT-primers for the downstream amplifications). The complete DNA of *ThMan5A* was amplified using primers C1 and C2 based on the putative coding region. The primers used in this study are listed in Table 1.

Construction of expression cassettes

The promoter (P*pdc*, 1,344-bp upstream fragment starting from the start codon of *pdc*) and the terminator of the *pdc*

Table 1 Primers used for cloning of *ThMan5A* and the promoter, terminator of the PDC gene

Primer name	Sequences[a]
D1	5′ TGGGGYTTYAAYGAYGTSA 3′
D2	5′ AYCCAKCCRTTGCCCCA 3′
UT1	5′ CGTTAGCCAGCTCCCACGCAAAGATC 3′
UT2	5′ CGTGTTAGTGAACCAGGTGGTCGCA 3′
UT3	5′ CTTGAGGTTATGCTGCTCGGCGGACT 3′
DT1	5′ GCGATCTTTGCGTGGGAGCTGGCTAA 3′
DT2	5′ TGACGCTGGGAGATGAGGGACTTGGT 3′
DT3	5′ GACTTTGGCACCTTTCACCTGTATCC 3′
C1	5′ GG<u>ACTAGT</u>ATGATGATGCTCTCAAAGA3′
C2	5′ TCATGCAGACAGGCATTGCGAGT 3′
P*pdc*-F	CTAG<u>TCTAGA</u>CATCTTTCGAGGACGGACG
P*pdc*-R	CCG<u>GAATTC</u>GG<u><u>ACTAGT</u></u>GATTGTGCTGTAGCTG CGCT
T*pdc*-F	<u><u>CACCACCATCACCATCACTGA</u></u>CCCGGCATGAA GTCTGACC
T*pdc*-R	CCG<u>GAATTC</u>TGGACGCCTCGATGTCTTC

[a] Restriction sites are underlined; overlapping regions in P*pdc*-R and T*pdc*-F are double underlined

gene (T*pdc*, 1,030-bp downstream fragment starting from the stop codon of *pdc*) were amplified using the forward (F) and reverse (R) primers P*pdc*-F, P*pdc*-R, and T*pdc*-F, T*pdc*-R, (listed in Table 1), and using the genomic DNA of *T. reesei* QM9414 as the template.

Exons 1, 2, and 3 of the *ThMan5A* gene and the 6×histidine (His) tag were fused by overlapping extension PCR. P*pdc* and the *ThMan5A* gene with the 6×His tag were fused by *Spe*I. The P*pdc*-*ThMan5A* and T*pdc* were fused by overlapping extension PCR. The fusion product was amplified by PCR to regions upstream and downstream of the *Xbal*I and *Eco*R sites, respectively, and cloned into a pUC19 vector to generate the recombinant plasmid pUC19-P*pdc*-*ThMan5A*-T*pdc*. The regions encoding the linker (T374 to Q407) and CBM1 (C408 to A437) were deleted from the *ThMan5A* gene to construct the recombinant plasmid pUC19-P*pdc*-*ThMan5A*△CBM-T*pdc*.

Protoplast preparation and transformation of *T. reesei*

Protoplast preparation and transformation of *T. reesei* were performed using the polyethylene glycol method as described in Penttilä et al. [25].

Southern-blot hybridization

The chromosomal DNA was extracted and purified by the phenol/chloroform method. The DNA was digested with *Sac*I and *Bam*HI, fractionated on 0.7 % (w/v) agarose gels before being transferred to nylon membranes (Roche, Basel, Switzerland). High-stringency probing was carried out at 50 °C overnight using digoxigenin (DIG)-labeled DNA probes, which were produced by amplifying a 497-bp fragment of *pdc* and the *ThMan5A* gene with the primers Probe-F (5′-GTGTTGGCTCACGTCTCCAAT-3′) and Probe-R (5′-GATGTGGCTAAGGTCGAGT-3′) and labeled with digoxigenin DNA labeling mix (Roche). Chromogenic signal detection was performed using the detection system from Roche Molecular Biochemicals. NBT/BCIP was used as the chromogenic substrate.

Purification of ThMan5A and the truncated enzyme

The methods of transformants cultivation and protein preparation referenced Wang et al. [40].

Electrophoresis

Purified preparations of the enzyme samples were analyzed for homogeneity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12.5 % polyacrylamide gel as described by Laemmli et al. [10]. Protein concentration was determined by the Bradford method [1] using bovine serum albumin as the standard. The bands were visualized by staining with Coomassie Brilliant Blue G250.

Enzyme assays

For recombinant mannanase production, about 10^5 spores of the recombinant *T. reesei* strains were inoculated into 30 ml of Mandels' medium and maintained at 28 °C and 250 rpm/min for 48 h. Then, 1.5 ml of the supernatants was transferred into 30 ml of a modified Mandels' medium, and maintained at 28 °C and 250 rpm/min for about 168 h. The modified minimal medium contained 7 % glucose, 1 % peptone, and 5 % soybean powder.

Mannanase activity was assayed as described by Stålbrand et al. [35] with minor modifications. For a substrate, 0.5 % LBG (W/V) in 0.2 M sodium pyrophosphate (Na_2HPO_4)-citric acid buffer at pH 5.5 was used. Appropriate dilutions of the recombinant protein (or culture supernatant) in 0.2 M sodium citrate buffer (pH 5.5) was used as the enzyme source. The assay mixture was incubated at 60 °C for 5 min. The amount of released sugar was determined by the dinitrosalicyclic acid (DNS) method described by Miller [21]. Mannanase activity was calculated from the calibration curve constructed using mannose as the standard. One unit of enzyme activity was defined as the quantity of enzyme required to liberate 1 μmol of mannose equivalent per minute at 60 °C, and specific activity was defined as units per milligram protein. Protein concentrations were

measured using a method described by Sedmak and Grossberg [33].

Biochemical characterization of the purified recombinant enzymes

Assays at different pH values were performed at 60 °C over a pH range of 3.0–8.0. Two different buffers were used: Na_2HPO_4-citric acid buffer (0.2 M) was used for pHs 2.2–8, and Gly-NaOH buffer (0.05 M) was used for pHs 8–10. The pH stability of recombinant enzymes was determined under standard conditions after pre-incubation of the enzyme solution in the buffers without substrate at 30 °C for 24 h. The optimal temperature for the mannanase enzyme was obtained by assaying the enzyme activity at temperatures ranging from 30–90 °C. The thermostability of the recombinant enzymes were determined under standard conditions (pH 5.5, 60 °C, 5 min) after pre-incubation of the enzyme solution at 50 and 60 °C for various periods (24, 48, 72, 96, 120 h) without substrate. The enzymes were incubated with 10 mM solution of K^+, Na^+, Ca^{2+}, Li^+, Fe^{2+}, Cu^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+}, or with ethylenediamine tetraacetic acid (EDTA), β-mercaptoethanol and/or SDS at 37 °C for 1 h. The system without any chemicals was treated as the control. Residual activity was measured under standard conditions. Each experiment was performed in triplicate.

Substrate specificity analysis and kinetic parameters

Substrate specificity was determined by incubating the samples in the presence of various polysaccharide substrates for 5 min. Controls contained no enzyme. Solutions containing 0.5 % (w/v) concentrations of LBG, konjac powder, and chitosan in 0.2 M Na_2HPO_4-citric acid buffer at pH 5.5, were individually incubated at 60 °C for 5 min. Xylanase, CMCase, and filter paper hydrolase activities were measured in the presence of 1 % xylan, 1 % CMC-Na, and 20 mg of filter paper in 200 µl of 50 mmol/l sodium citrate buffer at pH 6.0. One unit of enzyme was defined as the amount required for the generation of 1 µmol/min of reducing sugars under the assay conditions. Mannose was used as the standard for calculating the activity against LBG and konjac powder, glucose was used as the standard for activity against CMC-Na, Avicel and chitosan, and xylose was used as the standard for activity against the birchwood and beechwood xylans. The K_m and V_{max} values of the purified recombinant enzymes were determined using 1–20 mg/ml of LBG or konjac powder. The data were plotted according to the Lineweaver and Burk [12] method. Each experiment was repeated three times.

Results

Microorganism identification

Strain *T. harzianum* MGQ2 was isolated from a soil sample collected from the mangrove soil. The amplified internal transcribed spacer (ITS) rDNA gene sequence (GenBank: KC342029) was compared to other ITS sequences in the GenBank database. The results indicated that the strain belonged to the genus *Trichoderma*. The metabolic rates of the strain cultured in different carbon sources for 48 and 72 h were almost the same as those of *T. harzianum*. Therefore, strain MGQ2 was identified as *T. harzianum*.

Cloning and analysis of *ThMan5A*

The full-length DNA of *ThMan5A* was 1,438 bp long (GenBank: KC342042). The coding region of the gene is separated by two introns and encodes a protein of 437 amino acids. On the basis of amino acid sequence similarity, this enzyme was assigned to family 5 of the glycosyl hydrolase (GH5) classification system. *ThMan5A* is the first mannanase gene to be cloned from *T. harzianum*. The deduced amino acid sequence of *ThMan5A* showed high homology with sequences from *Hypocrea rufa* (GenBank: AFP95336.1) (99 % identity), *T. longibrachiatum* (GenBank: ADN93457.1) (98 % identity), and *T. reesei* (GenBank accession no. AAA34208.1) (identity 94 %), and lower homology with sequences from *T. virens* Gv29-8 (GenBank: EHK24618.1) (80 % identity), and *T. atroviride* (GenBank: EHK45464.1) (69 % identity). The N-terminal region of the ThMan5A sequence contained a signal peptide sequence of 27 amino acid residues, indicating that it is an extracellular enzyme. The deduced ThMan5A sequence also contained a GH5 catalytic domain, a carbohydrate-binding module (CBM) that belongs to family 1 (CBM1), and a Ser/Thr-rich linker region.

Expression and purification of the recombinant ThMan5A and the truncated enzyme in *T. reesei*

Trichoderma reesei QM 9414 was used as the host strain for the heterologous expression of the *ThMan5A* gene from *T. harzianum*. To increase the expression efficiency of *ThMan5A* and the production of the enzyme, the truncated mannanase gene *ThMan5A*△CBM was expressed under the control of the same promoter. About 20 % of the transformants that were resistant to hygromycin B contained the co-transformed expression cassette. The transformants that exhibited the highest mannanase productivity for each expression cassette were designated as *T. reesei* M and *T. reesei* M△CBM, respectively. The expression cassettes in the transformants were PCR amplified, with the genomic

DNA as the template, and the PCR-amplified products were sequenced and shown to be correctly constructed. Southern-blot analysis showed that the two transformants both had a single-copy insert of the expression cassette. The expression cassettes might be inserted randomly into the genome of *T. reesei* because the endogenous *pdc* cassettes remained intact in the transformants.

The *T. reesei* M and *T. reesei* M△CBM transformants were cultivated in shake flasks. The recombinant ThMan5A and the truncated enzyme were purified by immobilized metal ion affinity chromatography (IMAC) according to the 6×His tag in the C terminal of the protein sequence. The recombinant enzymes were purified to apparent homogeneity as described elsewhere [38]. The fermentation supernatant of *T. reesei* M and *T. reesei* M△CBM showed thick bands on SDS-PAGE at approximately 53 kDa and 37 kDa, respectively. This result is consistent with the molecular weight of the purified ThMan5A and the truncated enzyme (Fig. 1). The molecular weight of recombinant ThMan5A (53 kDa) is slightly higher than that the molecular weight deduced from the protein sequence (48 kDa). This difference may indicate overglycosylation of the ThMan5A enzyme expressed in *T. reesei*.

When the transformants were cultured without an inducer, the mannanase activities reached the maximum

Fig. 1 SDS-PAGE analysis of ThMan5A and ThMan5A△CBM. *M* marker; *lane 1* culture filtrate QM9414; *lanes 2, 3* culture filtrate from positive transformant of recombinant ThMan5A and ThMan5A△CBM, respectively; *lanes 4, 5* purified recombinant ThMan5A and ThMan5A△CBM

Table 2 Extracellular mannanase activities of transformants and *Trichoderma harzianum*

Transformants	Mannanase activity (U/ml) (7th day)	*Trichoderma harzianum* MGQ2 (U/ml) (4th day)	Improved multiples
T. reesei M	1,063 ± 14.8	3.27 ± 0.13	325
T. reesei M△CBM	2,460 ± 45.1	3.27 ± 0.13	752

after 7 days, while when *T. harzianum* was cultured with konjac powder as the inducer, 4 days were required for the mannanase activity to reach the maximum (3.27 ± 0.13 U/ml). The extracellular mannanase activities towards LBG in the fermentation liquid of the *T. reesei* M and *T. reesei* M△CBM transformants were 325- and 752-fold higher, respectively, compared to the extracellular mannanase activity for the wild strain (Table 2).

Effect of temperature and pH on enzyme activity and stability

The optimal temperatures of the purified ThMan5A and ThMan5A△CBM mannanases were 70 and 80 °C, respectively (Fig. 2a). The two mannanases were stable after incubation at 50 °C for 120 h. The ThMan5A enzyme retained 45 % of its activity after incubation at 60 °C for 72 h, while the truncated enzyme from ThMan5A△CBM was stable (retained 100 % activity) after incubation at 60 °C for 72 h (Fig. 2b). The maximum activities of the ThMan5A and ThMan5A△CBM enzymes were observed at pH 6.0 and pH 5.5, respectively (Fig. 2c). After being pre-treated in buffers ranging from pHs 2.2–10.0 for 24 h at 30 °C, both the mannanases were found to be stable between pHs 3.0–9.0, showing that the two enzymes were acid and alkali tolerant. ThMan5A exhibited over 69 % of its activity at pH 3.0 and over 78 % of its activity at pH 9.0; the truncated enzyme exhibited over 86 % of its activity at pH 3.0 and over 78 % of its activity at pH 9.0 (Fig. 2d).

Additional effect of chemicals on enzyme activity

The mannanase activity of purified ThMan5A and ThMan5A△CBM in the presence of 10 mM of different metal ions or chemical reagents is shown in Table 3. The activity was partial inhibited by K^+, Na^+, Ca^{2+}, Fe^{2+}, Cu^{2+}, Mg^{2+}, Zn^{2+}, EDTA, β-mercaptoethanol and strongly inhibited by Mn^{2+}. The presence of SDS obviously promoted the activity of ThMan5A and partial inhibit activity of ThMan5A△CBM.

Substrate specificity and kinetic parameters

The hydrolytic activities of purified recombinant ThMan5A and ThMan5A△CBM towards various substrates were assayed. Both recombinant enzymes exhibited high activity towards LBG and konjac powder. The highest specific activity of ThMan5A was to LBG, up to 864 ± 7.9 U/mg of protein. However, the specific activities of ThMan5A△CBM to LBG and konjac powder were $1,544 \pm 17.7$ and $1,159 \pm 8.8$ U/mg of protein, respectively; 1.8 and 1.6 times higher that of ThMan5A to LBG and konjac powder, respectively. The specific activities of
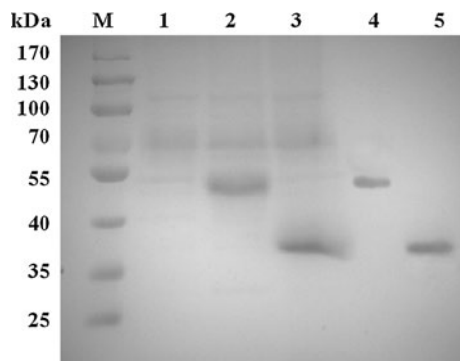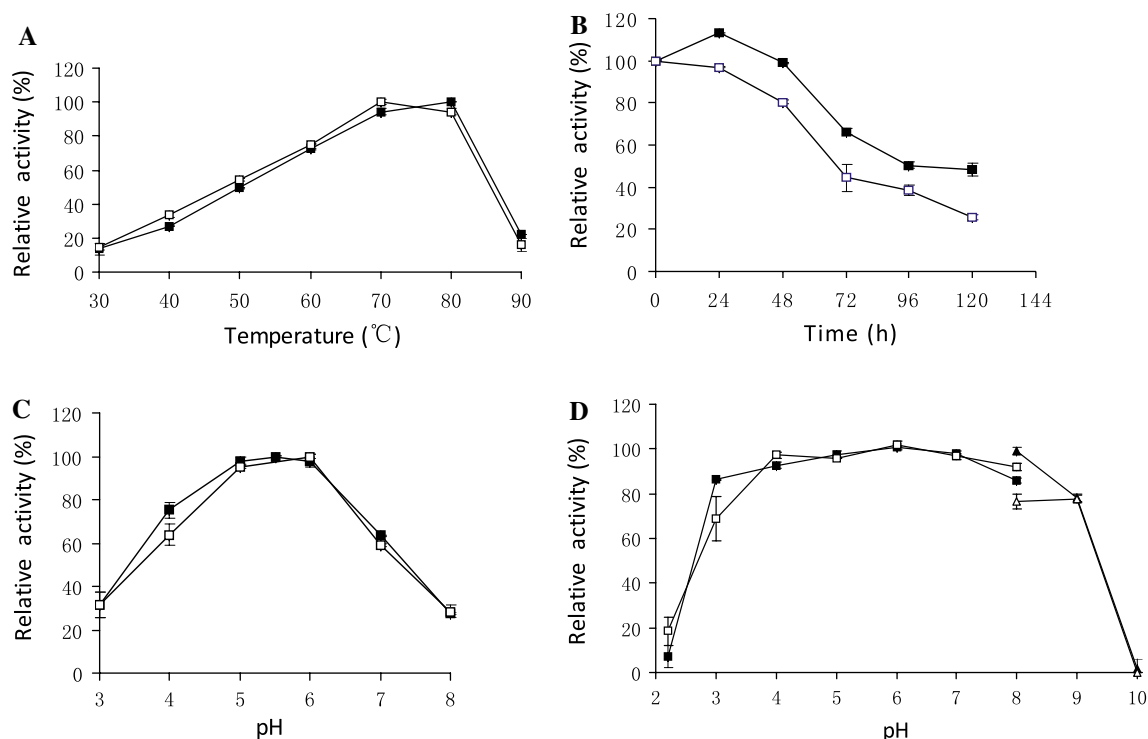
Fig. 2 Properties of the purified recombinant ThMan5A and ThMan5A△CBM. **a** Effect of temperature on mannanase activity of ThMan5A (*open squares*) and ThMan5A△CBM (*filled squares*). The assay was performed at temperatures that ranged from 30 to 90 °C. **b** Temperature stability of ThMan5A and ThMan5A△CBM. The enzyme was pre-incubated at 60 °C in 50 mM sodium pyrophosphate (Na_2HPO_4)-citric acid buffer (pH 5.0) without substrate. **c** Effect of pH on activity of purified enzymes. **d** The pH stability of purified ThMan5A and the truncated enzyme. For optimum pH, assays were performed at 60 °C over a pH range of 3.0–8.0. For pH stability, the following buffers were used: 0.2 M Na_2HPO_4-citric acid buffer (pH 2.2–8). 0.05 M Gly-NaOH buffer (pH 8–10). The enzyme was incubated at 30 °C for 24 h in buffers of pH 2.2–10.0, then the activity was measured under standard conditions (*open diamonds*). *Open squares* ThMan5A, *filled squares* ThMan5A△CBM

Table 3 Effect of metal ions and chemical reagents on the activity of recombinant ThMan5A and truncated mutant

Chemical (10 mM)	Relative activity (%)	
	ThMan5A	ThMan5A△CBM
Control	100 ± 1.6	100 ± 2.8
K^+	99.2 ± 3.0	88.8 ± 0.8
Na^+	96.3 ± 1.0	99.5 ± 0.5
Ca^{2+}	84.6 ± 0.8	83.8 ± 1.3
Fe^{2+}	82.9 ± 1.6	82.6 ± 1.8
Cu^{2+}	85.4 ± 0.1	96.5 ± 0.1
Mn^{2+}	17.5 ± 0.1	18.3 ± 0.1
Mg^{2+}	86.9 ± 1.9	90.3 ± 2.1
Zn^{2+}	92.7 ± 0.1	80.1 ± 3.5
EDTA	91.9 ± 3.8	87.2 ± 0.9
SDS	113.6 ± 0.1	85.3 ± 1.1
β-Mercaptoethanol (1 %)	76.8 ± 0.5	71.9 ± 3.3

the two enzymes towards Avicel were different; the specific activity of ThMan5A (13.2 ± 0.2 U/mg) was 5.7 times higher than that of ThMan5A△CBM (2.3 ± 0.1 U/mg), which are related with the binding ability of CBM1 to Avicel [8, 26]. Neither ThMan5A nor ThMan5A△CBM had any activity towards filter paper, birchwood xylan, beechwood xylan, chitosan, and CMC-Na.

The calculated K_m of ThMan5A to both LBG and konjac powder was 1.0 mg/ml; V_{max} was 1,429 and 1,666 µmol/mg/min to LBG and konjac powder, respectively. The calculated K_m of ThMan5A△CBM to LBG and konjac powder was 4.0 and 1.7 mg/ml, respectively; V_{max} was 5,000 and 3,333 µmol/mg/min, to LBG and konjac powder, respectively (Table 4). Thus, the V_{max} for ThMan5A△CBM was 3.5 and 2.0 times higher that the V_{max} for ThMan5A for the two substrates, respectively.

Discussion

Mangrove fungi play an important role in the decomposition of lingo-cellulose materials in tropical and subtropical coastal ecosystems. The activities of lingo-cellulolytic enzymes such as cellulases, laccases, and peroxidases in the mangrove fungi have been reported previously [27].

Table 4 Specific activity and kinetic parameters of the purified recombinant enzymes

Purified recombinant enzymes	Substrate (locust bean gum)			Substrate (konjac powder)		
	Specific activity (IU/mg)	K_m (mg/ml)	V_{max} (μmol/mg/min)	Specific activity (IU/mg)	K_m (mg/ml)	V_{max} (μmol/mg/min)
ThMan5A	864 ± 7.9	1	1,429	738 ± 4.0	1	1,666
ThMan5A△CBM	1,544 ± 17.7	4	5,000	1,159 ± 8.8	1.67	3,333

However, little attention has been given to mannanases and no mannanase genes from mangrove fungi have been cloned and expressed until now. In the present study, the *T. harzianum* MGQ2 strain that was isolated from mangrove soil was found to have significant mannanase activity. This is the first report on the cloning and characterization of a mannanase gene from *T. harzianum*.

Trichoderma reesei is an industrially important fungus that is used in the production of enzymes with hydrolytic activity against the polysaccharides that are abundant in woody materials [35]. *T. reesei* is an attractive host for the expression of homologous and heterologous proteins because of its ability to secrete large amounts of hydrolytic enzymes [22, 39]. In recent years, a large number of foreign genes have been expressed in *T. reesei*, especially genes from filamentous fungi [16, 31, 44]. In the present study, the *ThMan5A* from *T. harzianum* was expressed in *T. reesei* QM9414 using the *pdc* promoter, a constitutive strong promoter reported in our previous study [11]. In the present study, both the mannanase and truncated mannanase genes were successfully expressed in *T. reesei* QM9414. The recombinant full-length and the truncated enzymes accounted for approximately 83.6 and 91.7 % of the total protein secreted by *T. reesei*, respectively. The production of the recombinant full-length and the truncated enzymes was 1.2 and 1.6 g/l, respectively. These results indicated that *T. reesei* is a suitable host for the *Trichoderma*-derived enzyme ThMan5A.

Interestingly, the *T. reesei* M△CBM transformant that expressed the truncated gene produced 2.3-fold more mannanase activity in the culture supernatant than the *T. reesei* M transformant that expressed the full-length gene (Table 2). We inferred that the increase of truncated enzyme production is related with the increases in the mRNA signals in the transformant. Quite unexpectedly, we found that the thermostability of the truncated ThMan5A△CBM enzyme was higher compared with ThMan5A. A similar result was reported for a truncated *N. flexuosa* Xyn11A, which had enhanced thermostability compared with the full-length enzyme [24]. However, this is the first report of a truncated mannanase with enhanced thermostability, acidic tolerance, and production. It has been reported previously by Pham et al. [26] that the fused mannanase-CBM enzyme from *Aspergillus aculeatus* was more thermostable

than the wild type, which is contrary to our results. So far, based on data from literature [18, 23, 24, 26, 30, 36], adding or truncating CBMs could affect the enzyme stability profile, but it is difficult to predict their effect on enzyme characteristics. The mechanism of improving thermal stability of enzymes by removing or fusing a CBM is unclear.

The CBM1 domain has been found in other mannanases [8, 26] and in other types of hemicellulose degrading enzymes [24, 40]. Previous studies indicate that the CBM domains of modular mannanases are nonessential for the hydrolysis of soluble mannan. For example, Man5A and Man5A△CBM from *T. reesei* exhibited similar hydrolytic activity towards mannopentaose and soluble LBG galactomannan [8]. In the present study, the truncated mannanase had higher specific activity to LBG and konjac powder than the full-length enzyme. However, the full-length enzyme had higher activity to insoluble substrates than the truncated enzyme. The activity of ThMan5A to Avicel was 5.7-fold higher compared with that of the ThMan5A△CBM. Our results are consistent with the results from previous studies [8]. ThMan5A and ThMan5A△CBM can usefully be applied for different substrates.

In the present study, mannanase activity was assayed in the presence of metal ions and chemicals. We found that mannanase activity was negatively affected by divalent cations such as Ca^{2+}, Fe^{2+}, Cu^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+} and by the antioxidant, β-mercaptoethanol, whereas in a previous study it was found that divalent metal ions such as Mn^{2+}, Zn^{2+}, Ca^{2+}, Fe^{2+} and the antioxidant β-mercaptoethanol significantly enhanced the enzyme activity of Man5S27 (which, like ThMan5A, contains a GH5 domain) from *Streptomyces* sp. S27 [34]. In the present study, the denaturant SDS enhanced the activity of ThMan5A and partially inhibited the activity of ThMan5A△CBM although 85.3 % of its original activity remained. These results suggested that the CBM and the linker region of ThMan5A play an important role in resistance to SDS denaturation. The catalytic region of ThMan5A may have some anti-SDS role because the activity of mannanases from other sources was almost completely inhibited by SDS; for example, the activities of mannanases from *Bacillus subtilis* G1 [38], *Bispora* sp. MEY-1 [15], *Penicillium freii* F63 [42], *Humicola insolens* Y1 [14], *Pantoea agglomerans* A021 [41], and *Penicillium pinophilum* C1 [2] were strongly inhibited

by SDS. Together, these results suggest that mannanases from different sources may have different catalytic mechanisms even though some of the enzymes belong to the same glycoside hydrolase family.

In conclusion, we successfully cloned the mannanase gene from *T. harzianum* in *T. reesei* and produced a truncated *T. harzianum* Man5A protein devoid of the CBM and linker region. The enzyme product of the truncated mannanase gene was enhanced 1.3-fold, the enzyme activity of the truncated mannanase in the culture supernatant was enhanced 2.3-fold, and the thermal stability at 60 °C was obviously increased compared with that of the full-length ThMan5A. Thus, we have shown that the truncated enzyme exhibited superior characteristics over the full-length enzyme both at production and for its thermal stability. These characteristics make the truncated mannanase a promising enzyme for a variety of industrial uses.

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