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Purification, Cloning, Functional Expression, Structure, and Characterization of a Thermostable β-Mannanase from *Talaromyces trachyspermus* B168 and Its Efficiency in Production of Mannooligosaccharides from Coffee Wastes

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Abstract: Highly thermostable β-mannanase, belonging to glycoside hydrolase family 5 subfamily 7, was purified from the culture supernatant of *Talaromyces trachyspermus* B168 and the cDNA of its transcript was cloned. The recombinant enzyme showed maximal activity at pH 4.5 and 85 °C. It retained more than 90 % of its activity below 60 °C. Obtaining the crystal structure of the enzyme helped us to understand the mechanism of its thermostability. An antiparallel β-sheet, salt-bridges, hydrophobic packing, proline residues in the loops, and loop shortening are considered to be related to the thermostability of the enzyme. The enzyme hydrolyzed mannans such as locust bean gum, carob galactomannan, guar gum, konjac glucomannan, and ivory nut mannan. It hydrolyzed 50.7 % of the total mannans from coffee waste, producing mannooligosaccharides. The enzyme has the highest optimum temperature among the known fungal β-mannanases and has potential for use in industrial applications.

Key words: glycoside hydrolase family 5, β-mannanase, *Talaromyces trachyspermus*, thermophilic fungus, coffee waste

INTRODUCTION

Mannan is an important component of the hemicellulose family of compounds. This family can be divided into four subfamilies: linear mannans, glucomannans, galactomannans, and galactoglucomanans.[1)2)] Each of these polysaccharides presents a β-1,4-linked backbone containing mannose or a combination of glucose and mannose residues.[3)4)] In addition, the mannan backbone can be substituted with side chains of α-1,6-linked galactose residues.

β-Mannanase (EC 3.2.1.78) hydrolyzes β-1,4-glycosidic links within the mannan backbone to yield short-chain mannooligosaccharides of varying length. Based on the amino acid sequences of the catalytic domains, β-mannanases have been classified into four glycoside hydrolase (GH) families: 5, 26, 113, and 134.[5)] Thermostable enzymes have significant advantages in bioprocessing, such as prolonged storage (at room temperature), high tolerance to organic solvents,[6)] reduced risk of contamination, as well as low activity losses during processing (below the T_m of the enzyme). These properties are maintained even at the elevated temperatures often used in raw material pretreatments. In biorefining, renewable resources such as agricultural crops or wood are utilized for extraction of intermediates or for direct bioconversion into chemicals, commodities, and fuels.[7)8)] Thermostable enzymes have an obvious advantage as catalysts in these processes; high temperatures often im-

prove enzyme penetration and cell-wall disruption in the raw materials.[9] To date, thermostable β-mannanases have been isolated from *Bacillus* spp*., Bacillus nealsonii, Caldibacillus cellulovorans, Caldicellulosiruptor saccharolyticus, Dictyoglomus thermophilum, Rhodothermus marinus, Thermomonospora fusca, Thermotoga* spp*., Aspergillus* spp*., A. nidulans, Neosartorya fischeri, Talaromyces leycettanus,* and *Trichoderma reesei*.[10][11][12][13][14][15][16] Even the both thermostable enzymes from bacteria and fungi have been studied, in comparison to bacteria, fungi secrete considerably higher amounts of proteins; they have a significant advantage over bacteria as enzyme source for many applications.

In this study, we focused on a thermostable β-mannanase from a thermophilic fungus *Talaromyces trachyspermus* B168 (TtMan5A), and describe the purification, cDNA cloning and its efficient expression in *Pichia pastoris*, the solution of crystal structure, and characterization of the enzyme. The efficiency of the enzyme in production of mannooligosaccharides from agro-industrial residues is also presented.

MATERIALS AND METHODS

Strains. *T. trachyspermus* B168 was isolated from *Picrasma quassioides* roots from Kodaira, Tokyo, Japan, and was identified by the Tokyo Metropolitan Medicinal Botanical Gardens and deposited as strain RD000972. *Escherichia coli* DH5α (Takara Bio, Inc., Otsu, Japan) and *P. pastoris* KM71H (Invitrogen Corporation, Carlsbad, CA, USA) were used as the cloning and expression host, respectively.

Purification of β-mannanase from T. trachyspermus. *T. trachyspermus* B168 was grown in 3 L of mannan medium (MM) containing 2 g/L locust bean gum (Sigma Chemical Co., St. Louis, MO, USA), 1 g/L Bacto Peptone, 1 g/L yeast extract, 5 g/L potassium dihydrogen phosphate, and 0.5 g/L magnesium sulfate at 25 °C for 6 days at 150 rpm. The culture filtrate containing 4,610 units of mannanase activity was concentrated by ultrafiltration using a 10-kDa polyethersulfone ultrafiltration membrane (Biomax; Merck Millipore Co., Billerica, MA, USA) and dialyzed against 50 mM HEPES buffer (pH 8.0). After centrifugation, the supernatant was applied to a Q-Sepharose Fast Flow (GE Healthcare UK Ltd., Buckinghamshire, UK) 2.5 × 20 cm column equilibrated with 50 mM HEPES buffer (pH 8.0). β-Mannanase activity was eluted with a linear gradient of 0–1 M sodium chloride. The active fractions were combined, concentrated using an Ultracel-10K (Merck Millipore Co.), and loaded onto a Sephacyl S-200 (GE Healthcare) 2.5 × 120 cm column equilibrated with 20 mM Tris-HCl buffer (pH 7.0) containing 0.2 M sodium chloride. The eluted proteins were pooled and concentrated. Total 77 mg of protein with 2,770 units of mannanase activity was recovered. The final preparation was used as purified native TtMan5A for enzyme assay and crystallization.

Enzyme assay and protein determination. β-Mannanase activity was determined by measuring the liberated reducing sugars as mannose equivalents using locust bean gum as the substrate, as previously described.[17] One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of mannose per minute from the substrate at 85 °C in McIlvaine buffer (0.2 M dipotassium hydrogen phosphate and 0.1 M citric acid, pH 4.5). The effects of pH and temperature on enzyme activity and stability were investigated as previously described.[18]

The protein concentration was determined by measuring absorbance at 280 nm, assuming that protein concentration of 1 mg/mL gives A_{280} of 1.0. The purity of the proteins was assessed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE),[19] on 12 % (w/v) polyacrylamide gels stained with Coomassie Brilliant Blue R-250. N-terminal amino acid sequence of mature TtMan5A was determined using a protein sequencer (Procise 491 cLC, Applied Biosystems, Inc., Foster, CA ,USA).

Crystallography. Crystallization was performed at 20 °C for 2 weeks, using the sitting-drop vapor-diffusion method. Crystals were obtained by mixing 0.3 μL of protein solution, comprising 150 mg/mL of native TtMan5A and 20 mM Tris-HCl buffer (pH 7.0), and 0.3 μL of reservoir solution consisting of 2.0 M ammonium phosphate and 0.1 M Tris-HCl buffer (pH 8.5). Diffraction experiments for native crystals were conducted at beamline BL5A at the Photon Factory, High Energy Accelerator Research Organization, Tsukuba, Japan. Crystals were flash-cooled at 95 K in a stream of nitrogen gas. Diffraction data were collected at a wavelength of 1.0 Å. Data were processed using the HKL-2000 program.[20] Molecular replacement was performed using the MOLREP program.[21] Manual model building and molecular refinement were performed using the Coot and Refmac5 programs.[22][23] Data collection and refinement statistics are provided in Table 1. Molecular graphic images were prepared using the PyMol program (DeLano Scientific LLC, Palo Alto, CA, USA).

Cloning of the β-mannanase cDNA from T. trachyspermus. Total RNA was extracted from *T. trachyspermus* grown in MM at 25 °C for 6 days at 150 rpm and purified using the RNeasy Mini kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. The first-strand cDNA from the total RNA was synthesized using the ReverTra Dash kit (Toyobo Co., Ltd., Osaka, Japan). A partial cDNA fragment of the *T. trachyspermus* β-mannanase gene was amplified by polymerase chain reaction (PCR) using degenerated primers (forward; 5′-GAYACNTTYCCNGGNACNAAY-3′ and reverse; 5′-TTNRCNAGYTCCCANGCRAA-3′) designed on the basis of the N-terminal amino acid sequence of the mature TtMan5A and highly conserved amino acid sequences in GH5 β-mannanases; the CODEHOP program was employed.[24] The amplified DNA fragment was subcloned into the pGEM-T Easy vector (Promega Corporation, Madison, WI, USA) and sequenced using the ABI PRISM 310 genetic analyzer (Life Technologies Co., Carlsbad, CA,USA). To obtain the full-length sequences of the β-mannanase gene, 5′ and 3′ rapid amplification of cDNA ends (RACE) was performed using the FirstChoice RLM-RACE Kit (Ambion, Austin, TX, USA) and the 3′-Full RACE Core Set (Takara Bio, Inc.) in accordance with the manufacturers' instructions.

Table 1. Data collection and structure refinement statistics of the TtMan5A.

Data	TtMan5A
Data collection	
Space group	$P2_12_12_1$
	$a = 58.4$
Unit-cell (Å)	$b = 76.0$
	$c = 79.7$
Resolution (Å)	50.0–1.60
	(1.63–1.60)
Total reflections	330,230
Unique reflections	47,615
Completeness (%)	99.2 (99.2)
R_{merge} (%)	7.4 (9.1)
$I/\sigma(I)$	30.9 (13.5)
Redundancy	7.0 (6.8)
Structure refinement	
Resolution (Å)	19.28–1.60
R/R_{free} (%)	11.4/13.8
Number of reflections	44,734
Number of atoms	3,128
RMSD	
Bond lengths (Å)	0.026
Bond angles (°)	2.453

Values in parentheses are for the highest-resolution shell. RMSD, root mean square deviation.

Expression and purification of recombinant β-mannanase. The DNA fragment encoding the mature protein was amplified by PCR using specific primers (forward; 5′-GAATTCGATACGTTTCCGGGGACGAATGGACT-3′ and reverse; 5′-TCTAGATCAAATCGACGCAACGC-GATCGGTA-3′). The amplified DNA fragment was digested with *Eco*RI and *Xba*I (underlined) and ligated into the corresponding site of the pPICZαA (Life Technologies Co.). The plasmid was transformed into *P. pastoris* KM71H and the transformants were selected in accordance with the manufacturers' instructions. Expression of recombinant protein was performed as previously described.[18] The recombinant TtMan5A was purified in a manner similar to that of the native TtMan5A.

Substrate specificity. The substrate specificity of the recombinant TtMan5A was examined using polysaccharides as the substrates, by measuring the liberated reducing sugars as described above, with the exception that the reactions were performed at 50 °C. Carob galactomannan was purchased from Megazyme International (Wicklow, Ireland) and Avicel from Merck KGaA (Darmstadt, Germany). Other substrates used in this study were purchased from Megazyme International, Merck KGaA, and Sigma Chemical Co. as previously described.[17)25]

Enzymatic hydrolysis of coffee waste. Coffee waste was obtained by grinding commercial coffee beans in a hand mill and mixing them with boiled water, followed by filtration. The residues were collected and dried. A portion of the material was further ground to a powder at 1,200 rpm for 1 min using Auto-mill TK-AM5 (Tokken Inc., Chiba, Japan).

Recombinant TtMan5A (0.4 mg) was incubated with the dried coffee waste (15 mg) in McIlvaine buffer (pH 4.5) at 50 °C for up to 69 h in a final reaction volume of 1 mL. The reaction mixture was filtrated using Amicon Ultra-cel-10 (Merck Millipore Co.). Saccharification ability of TtMan5A was evaluated by measuring the reducing sugars of the flow-through fractions and by analyzing the sugar composition of the residues. The hydrolysis products were analyzed using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (DX-500; Dionex Corp., Sunnyvale, CA, USA). β-1,4-Mannooligosaccharides and galacto-mannooligosaccharides, such as 6^1-mono-α-D-galactopyranosyl-β-1,4-mannobiose (GM2) and 6^1-mono-α-D-galactopyranosyl-β-1,4-mannotriose (GM3), from Megazyme International were used as standards. Enzyme-treated and -untreated residues were washed three times with distilled water and freeze-dried. Sugar composition of the residues was analyzed using trifluoroacetic acid (TFA) hydrolysis and HPAEC-PAD as previously described.[26)27]

RESULTS AND DISCUSSION

Purification of native TtMan5A.
When *T. trachyspermus* B168 was grown in a liquid medium containing wheat bran or cedar powder, the culture supernatant showed significant β-mannanase activity (data not shown). For the isolation of enzymes with β-mannanase activity, the strain was grown in a liquid medium containing 2 mg/mL locust bean gum. A 41-kDa protein, *T. trachyspermus* β-mannanase (TtMan5A), was purified from the culture supernatant using ion exchange and size-exclusion chromatography (Fig. 1, lane 2). With locust bean gum as the substrate, the enzyme achieved its maximal activity at 85 °C (Fig. 2b, filled symbols, solid lines). Activity was highly thermostable with >90 % activity being retained below 60 °C when the enzyme was incubated at pH 4.5 for 1 h (Fig. 2d, filled symbols, solid lines). Several β-mannanases have the optimum temperature at around 85 °C. Enzymes from *C. cellulovorans* (85 °C), *R. marinus* (85 °C), *T. neapolitana* (92 °C),[28] and *T. maritima* (90 °C) are known but all these enzymes are produced by bacteria.[10] Among fungal β-mannanases, the enzymes from *A. awamori*,[11] *A. nidulans*,[14] and *A. niger*[29)30] and *N. fischeri*[15] have a high optimum temperature for activity at 80 °C. TtMan5A has the highest optimum temperature among the known fungal β-mannanases with Man5A1 and Man5A2 from *T. leycettanus*.[16]

Edman sequencing analysis of purified TtMan5A yielded an amino acid sequence of its N-terminal, DTFPGTNGLDFTIDGTAGYFAGSNAYW. BLASTP search performed using the 27-amino acid sequence revealed high similarity to the N-terminal sequences of GH5 β-mannanases; therefore, we assumed that TtMan5A may belong to the GH5 family.

Cloning, expression, and purification of recombinant TtMan5A.
The full-length cDNA encoding TtMan5A was cloned as

described in the Materials and Methods. The cDNA sequence contained an open reading frame (1,128 bp) encoding a 375-amino acid protein (Fig. 3). The N-terminus
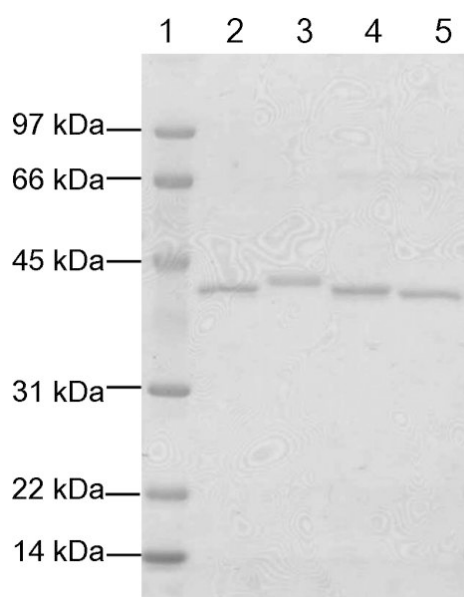

Fig. 1. SDS-PAGE analysis of native and recombinant TtMan5A.
Lane 1, molecular mass markers (1 μg of each); lane 2, native TtMan5A (1 μg); lane 3, recombinant TtMan5A (1 μg); lane 4, endoglycosidase H-treated native TtMan5A (1 μg); and lane 5, endoglycosidase H-treated recombinant TtMan5A (1 μg).

(amino acids 1–33) was a putative signal sequence. The deduced amino acid sequence corresponding to the mature region of TtMan5A (amino acids 34–375) resembled the sequences of the following proteins: putative β-mannanase from *Penicillium digitatum* Pd1 (EKV11756, 74 % identity, 86 % similarity), putative β-mannanase from *Penicillium chrysogenum* Wisconsin 54-1255 (CAP96302, 74 % identity, 85 % similarity), β-mannanase from *A. niger* (ACJ06979, 70 % identity, 83 % similarity), β-mannanase from *A. niger* (CAK96471, 71 % identity, 84 % similarity), and β-mannanase from *A. usamii* YL-01-78 (ADZ99027, 71 % identity, 83 % similarity). These results suggest that TtMan5A is a member of subfamily GH5_7.[31] To examine the function of TtMan5A in detail, we constructed the recombinant protein. The mature region of TtMan5A was successfully expressed in *P. pastoris* and the recombinant protein was purified (Fig. 1, lane 3). TtMan5A has two potential *N*-glycosylation sites (Fig. 3, double-underlined), so that it seems to be expressed as a glycoprotein. After endoglycosidase H treatment, TtMan5A appeared as a smaller band of approximately 39 kDa on SDS-PAGE (Fig. 1, lane 5), which is similar to the expected size of 37 kDa. In addition, native TtMan5A was treated with endoglycosidase H, but the glycosylation appeared to be resistant to this enzyme (Fig. 1, lane 4).
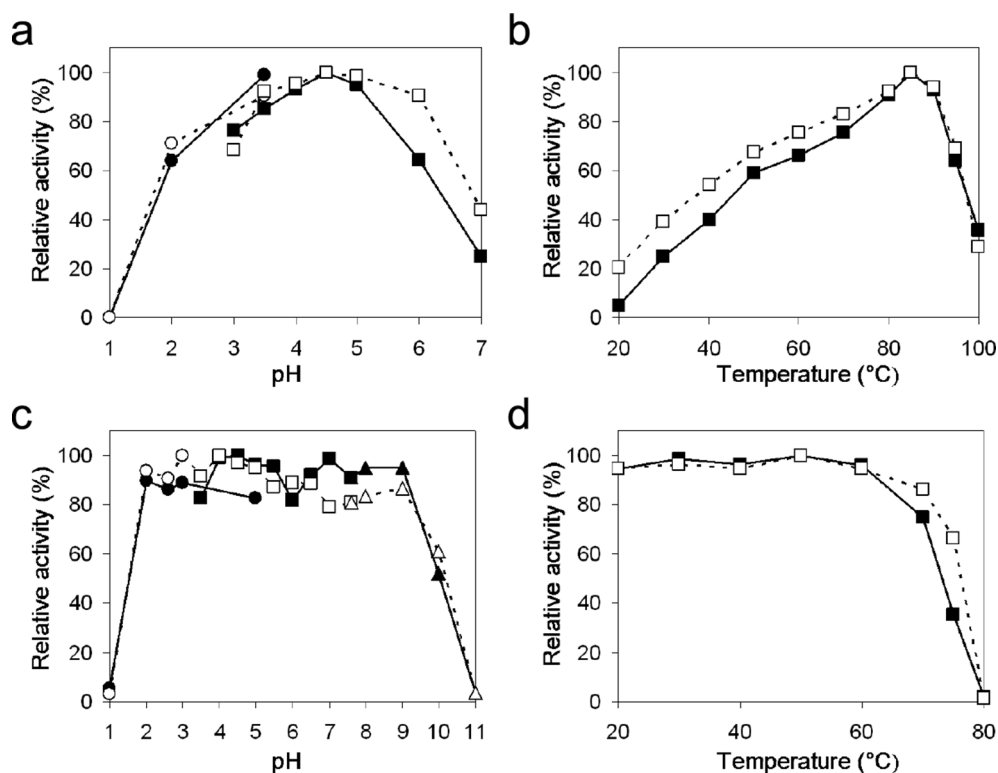

Fig. 2. Effect of pH and temperature on the activity [a and b] and stability [c and d] of native (filled symbols, solid lines) and recombinant (open symbols, dashed lines) TtMan5A.
Symbols: circle, glycine–HCl buffer; and square, McIlvaine buffer; triangle, Atkins-Pantin buffer. In order to determine the effect of pH on enzyme stability, the enzymes were preincubated at various pH values (glycine–HCl buffer (pH 1.0–3.5), McIlvaine buffer (pH 4.0–7.5), and Atkins-Pantin buffer (pH 8.0–11.0)) in the absence of substrate at 30 °C for 1 h, and the residual activity was then assayed using the standard method (15 min reaction at pH 4.5 and 85 °C). To determine the thermostability of the mannanases, the purified enzymes were incubated at different temperatures (20–80 °C) at pH 4.5 in the absence of substrate. After a 1 h incubation, residual mannanase activities were determined as described above.
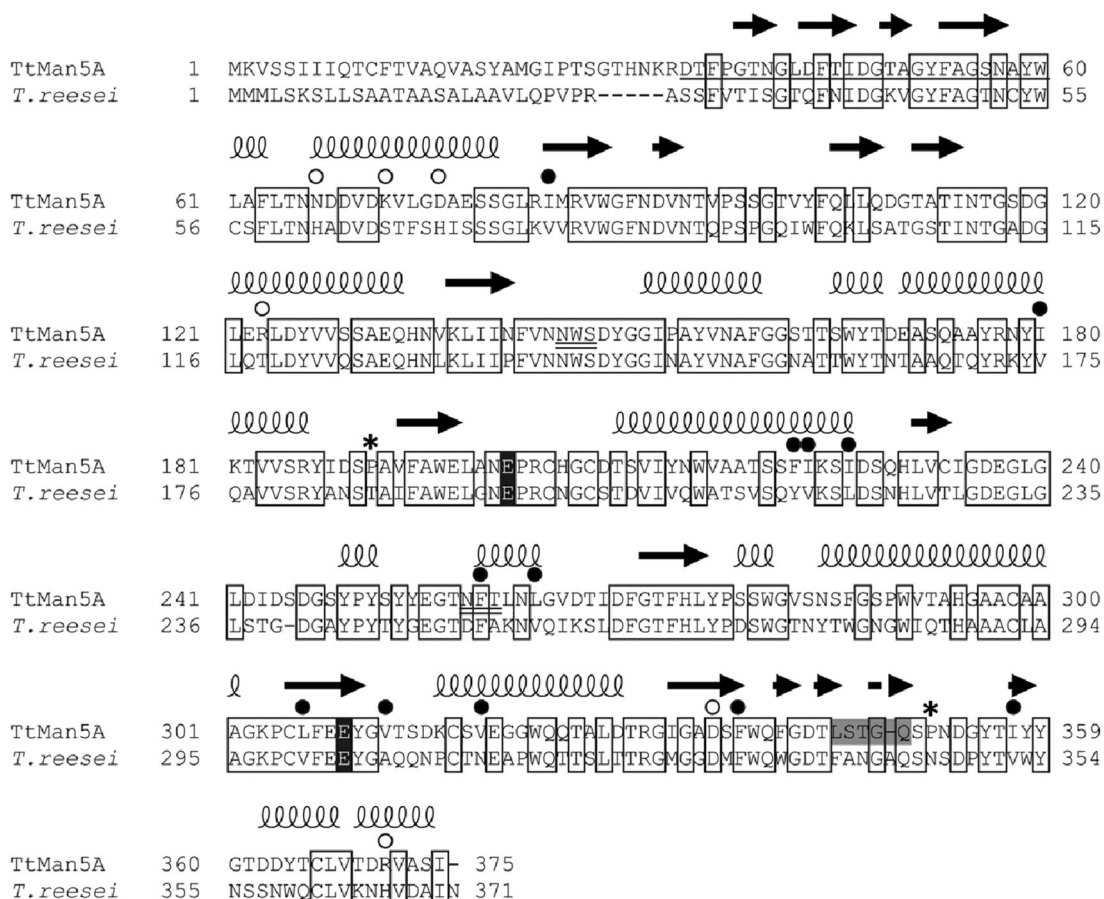
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TtMan5A    1    MKVSSIIIQTCFTVAQVASYAMGIPTSGTHNKRDTFPGTNGLDFTIDGTAGYFAGSNAYW   60
T.reesei   1    MMMLSKSLLSAATAASALAAVLQPVPR-----ASSFVTISGTQFNIDGKVGYFAGTNCYW   55

TtMan5A    61   LAFLTNNDDVDKVLGDAESSGLRIMRVWGFNDVNTVPSSGTVYFQLIQDGTATINTGSDG   120
T.reesei   56   CSFLTNHADVDSTFSHISSSGLKVVRVWGFNDVNTQPSPGQIWFQRLSATGSTINTGADG   115

TtMan5A    121  LERLDYVVSSAEQHNVKLIINFVNNWSDYGGIPAYVNAFGGSITSWYTDEASQAAYRNYI   180
T.reesei   116  LQTLDYVVQSAEQHNLKLIIPFVNNWSDYGGINAYVNAFGGNATTWYTNTAAQTQYRKYV   175

TtMan5A    181  KTVVSRYIDSPAMFAWELANEPRQHGCDTSVIYNWVAATSSFIKSIDSQHLVCIGDEGLG   240
T.reesei   176  QAVVSRYANSTAIFAWELGNEPRQNGCSTDVIVQWATSVSQYVKSLDSNHLVTLGDEGLG   235

TtMan5A    241  LDIDSDGSYPYSYMEGTNFTLNLGVDTIDFGTFHLYPSSWGVSNSHGSPWVTAHGAACAA   300
T.reesei   236  LSTG-DGAYPYTYGEGTDFAKNVQIKSLDFGTFHLYPDSWGTNYTWGNGWIQTHAAACLA   294

TtMan5A    301  AGKPCLFEEYGVTSDKCSVEGGWQQTALDTRGIGADSFWQFGDTLSTGQSPNDGYTIYY   359
T.reesei   295  AGKPCVFEEYGAQQNFCTNEAPWQTTSLITRGMGGDMFWQWGDTFANGAQSNSDPYTVWY   354

TtMan5A    360  GTDDYTCLVTDRVASI-   375
T.reesei   355  NSSNWQCLVKNHVDAIN   371
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Fig. 3.   Alignment of TtMan5A and *T. reesei* β-mannanase amino acid sequences.

   We show the alignment of deduced protein sequences of TtMan5A (present study) and the catalytic domain of *T. reesei* β-mannanase (Gen-Bank accession no. AAA34208). The identified amino acid residues are enclosed in boxes. Secondary structures for TtMan5A are shown. α-Helices are displayed as squiggles; β-strands are rendered as arrows. The N-terminal amino acid sequence determined is underlined. Two catalytic residues are indicated by white letters in black boxes. Possible *N*-glycosylation sites are double underlined. Structural features of TtMan5A responsible for thermostability are shown in Fig. 4 (c). Residues involved in the salt-bridge formation and increase of hydrophobic packing are indicated by ○ and ●, respectively. Proline residues in loops are indicated by asterisks. Loop shortening is presented on a gray background. Sequence alignment was performed using ClustalW followed by ESPript.[40]

Crystal structure of TtMan5A.

   To understand the reason for thermostability of TtMan5A, we crystallized the native enzyme and solved its three-dimensional structure (Fig. 4). Structure refinement statistics are summarized in Table 1. The crystal structure of TtMan5A was determined by molecular replacement at a resolution of 1.6 Å using the structure of *T. reesei* β-mannanase (PDB code 1QNO) as a search model. The structure was refined to R/R_{free} factors of 12.7/15.7 %. The final model included one TtMan5A molecule in an asymmetric unit, as well as the surrounding water molecules, five glycerols, one Tris, and one *N*-acetylglucosamine. The TtMan5A molecule was composed of a single polypeptide chain of 342 amino acids (34–375), but the N-terminal residue Asp34 could not be identified because of the lack of electron density.

   TtMan5A shows the classical TIM (β/α)$_8$-barrel architecture typical of the GH5 proteins of the clan GH-A (Fig. 4a). TtMan5A contains three disulfide bonds among Cys204–Cys207, Cys298–Cys305, and Cys317–Cys366. The amino acid sequence of TtMan5A includes two potential *N*-glycosylation sites (Fig. 3, double-underlined). Electron density

of one GlcNAc residue can be observed at one of the two potential *N*-glycosylation sites, Asn258. In the Dali structural similarity search, TtMan5A presents the highest similarity to *T. reesei* β-mannanase. The *T. reesei* enzyme belongs to the subfamily GH5_7 and has a domain belonging to carbohydrate-binding module family 1, which binds to cellulose at the C-terminus. In contrast, TtMan5A lacks such a carbohydrate-binding domain. On the basis of the crystal structure, the catalytic acid/base and the catalytic nucleophile of TtMan5A were assigned to Glu201 and Glu309, respectively. One Tris and one glycerol molecule are bound to subsite −1 and +1 of TtMan5A. The interactions and the active site structure are similar to that of the *T. reesei* enzyme complexed with Tris and glycerol.[32]

   The optimum temperature for activity of TtMan5A is 85 °C, whereas that for *T. reesei* enzyme is 70 °C.[12] To elucidate the reason for the thermostability of TtMan5A, we compared the structures of TtMan5A with that of the *T. reesei* enzyme. The protein structures superimposed almost perfectly, except for three differences in the secondary structures (Fig. 4b). A loop in the *T. reesei* enzyme was replaced in TtMan5A using an antiparallel β-sheet composed
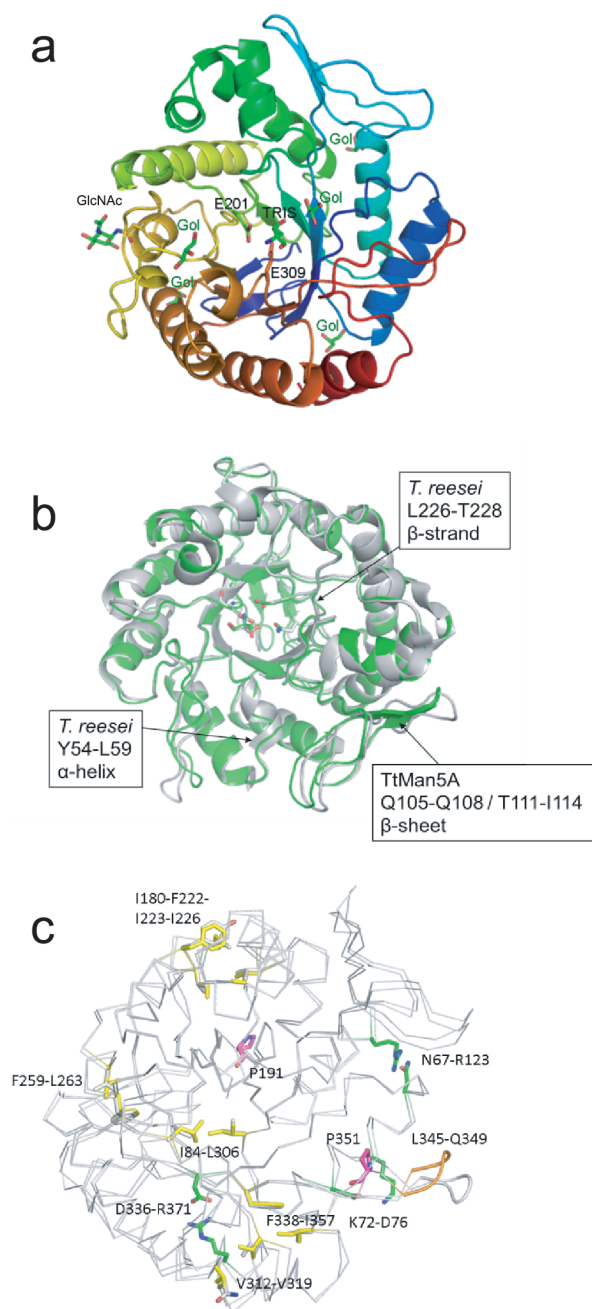
Fig. 4. Crystal structure of TtMan5A.
(a) Overall structure of TtMan5A. Catalytic residues, bound Tris and glycerols (Gol), and one GlcNAc residue at Asn258 are indicated as sticks. (b) Superimposition between TtMan5A (green) and *T. reesei* β-mannanase (gray, PDB code 1QNO). Differences between secondary structures of TtMan5A and *T. reesei* β-mannanase are shown. (c) Structural features responsible for thermostability of TtMan5A. Salt-bridge formation (green), increase of hydrophobic packing (yellow), presence of proline residues in loops (violet), and shortened loop (orange) were observed.

of the two β-strands, Gln105–Gln108 and Thr111–Ile114. In general, the antiparallel β-sheet is significantly stable because of the well-aligned H-bonds. The antiparallel β-sheet of TtMan5A is exposed on the surface of the protein; the compact structure of TtMan5A is stabilized by the antiparallel β-sheet. There are several structural features of TtMan5A responsible for thermostability.[33] As shown in

Table 2. Substrate specificity of TtMan5A.

Substrate	Specific activity (units/mg)	Relative activity (%)
Carob galactomannan	17.9 ± 0.7	100
Locust bean gum (galactomannan)	12.6 ± 1.2	73
Konjac glucomannan	13.1 ± 1.3	70
Ivory nut mannan (crystalline mannan)	2.7 ± 0.6	15
Guar gum (galactomannan)	1.0 ± 0.1	6
Carboxylmethyl cellulose	n.d.	0
Avicel (crystalline cellulose)	n.d.	0
Barley β-glucan (β-1,3-β-1,4-glucan)	n.d.	0
Lichenan (β-1,3-β-1,4-glucan)	n.d.	0
Laminarin (β-1,3-β-1,6-glucan)	n.d.	0
Curdlan (β-1,3-glucan)	n.d.	0
Pustulan (β-1,6-glucan)	n.d.	0
Tamarind xyloglucan	n.d.	0
Birch wood xylan	n.d.	0
Oat spelt xylan	n.d.	0
Chitosan	n.d.	0

TtMan5A was incubated 5 mg/mL substrate in McIlvaine buffer (pH 4.5) at 50 °C. n.d., not detected.

Fig. 4c, three salt-bridge formations (Fig. 3, indicated by ○), five hydrophobic packings (Fig. 3, indicated by ●), proline residues in two loops (Fig. 3, asterisks), and a shortening of one loop (Fig. 3, gray background) were observed in TtMan5A but not in the *T. reesei* enzyme.

Properties and substrate specificity of TtMan5A.

Enzymatic properties of recombinant TtMan5A were examined in a similar manner to those of the native enzyme (Fig. 2, open symbols, dashed lines). The enzyme achieved maximal activity at 85 °C, at pH 4.5 (Fig. 2a and b). It was stable over a broad pH range, i.e., from pH 2.0 to 9.0 (Fig. 2c). It retained more than 90 % of its activity below 60 °C (Fig. 2d). Under the optimal conditions, the specific activities of the native and the recombinant enzyme were 35.9 and 20.9 units/mg, respectively. The larger glycosylation of the recombinant enzyme probably affects the activity. Although the specific activity of the recombinant enzyme was lower than that of the native enzyme, the enzymatic properties of both enzymes were similar, as shown in Fig. 2.

Subsequently, the substrate specificity of recombinant TtMan5A was examined (Table 2). When the reaction was performed at 50 °C and pH 4.5 using polysaccharides listed in Table 2 as substrates, the recombinant enzyme hydrolyzed only mannans such as galactomannan, glucomannan, and crystalline mannan. The enzyme showed high activity with carob galactomannan (100 %) and locust bean gum (73 %), but it presented low activity against guar gum (6 %). The Gal/Man proportions of carob galactomannan and locust bean gum are 1:3.8–1:4, whereas the ratio for guar gum is 1:2.[34] Guar gum contains approximately twice as many α-D-Gal stubs as carob galactomannan and locust bean gum. Therefore, guar gum is more difficult to hydro-

Table 3. Sugar composition of the coffee wastes.

Sample	Rha	Ara	Xyl	Man	Gal	Glc	UA
TtMan5A-untreated	1.0 ± 0.0	3.5 ± 0.3	0.0 ± 0.0	47.7 ± 6.3	27.5 ± 4.0	2.4 ± 0.4	0.5 ± 0.7
TtMan5A-treated	1.0 ± 0.0	4.1 ± 0.2	0.0 ± 0.0	24.2 ± 1.1	18.2 ± 0.9	1.8 ± 0.1	1.0 ± 0.4

Values are listed by sugar: rhamnose molar ratio. Values represent the average and the standard deviation of triplicate experiments. Rha, rhamnose; Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose; UA, uronic acid.
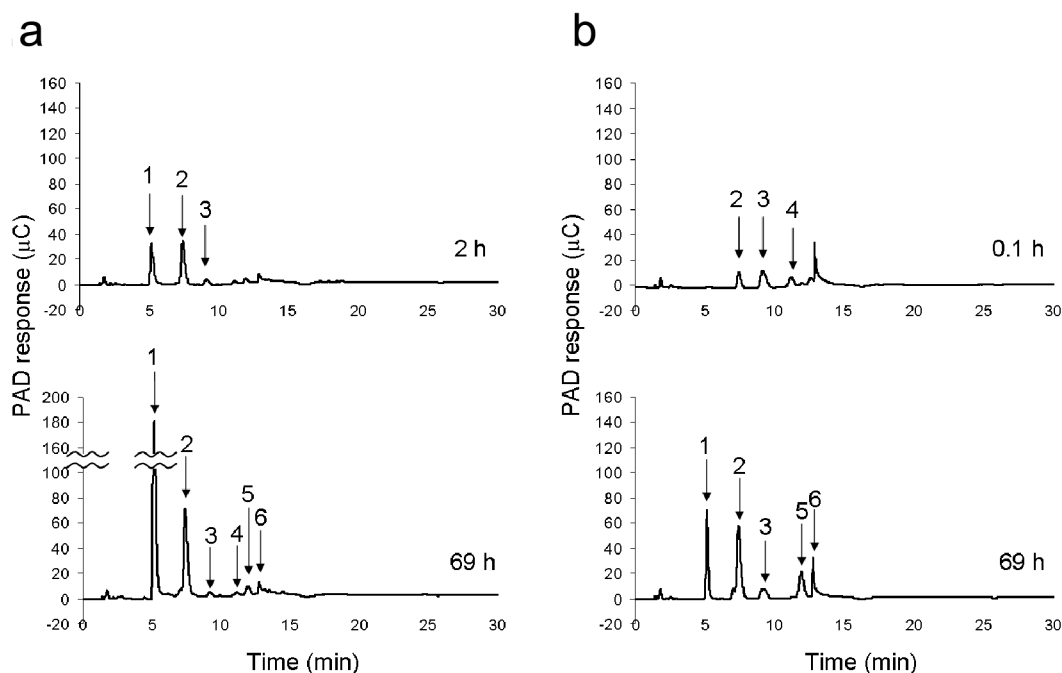
Fig. 5.   Hydrolysis products of coffee wastes (a) and locust bean gum (b) generated by TtMan5A.

Recombinant enzyme was incubated with 15 mg/mL coffee waste or 5 mg/mL locust bean gum in McIlvaine buffer (pH 4.5) at 50 C; the samples were subjected to HPAEC-PAD analysis. Peak 1, mannose (M1); peak 2, β-1,4-mannobiose (M2); peak 3, β-1,4-mannotriose (M3); peak 4, β-1,4-mannotetraose (M4); peak 5, 6¹-mono-α-D-galactopyranosyl-β-1,4-mannobiose (GM2); and peak 6, 6¹-mono-α-D-galactopyranosyl-β-1,4-mannotriose (GM3).

lyze. TtMan5A can digest crystalline mannan (15 %). Ivory nut mannan contains no α-D-Gal stubs, but a steric hindrance can be caused by the crystalline structure of the substrate.

Efficiency of TtMan5A in production of mannooligosaccharides from coffee waste.

With the industrial application of TtMan5A in mind, we evaluated the efficiency of the enzyme in the production of mannooligosaccharides from coffee waste. The polysaccharides in coffee bean consist of three major types: mannans or galactomannans, arabinogalactan-proteins, and cellulose.[35] In addition, there are small amounts of pectic polysaccharides and xyloglucan. The molecular weight and sugar composition of the coffee bean polysaccharide content are changed during roasting.[36] Sugar composition of the coffee waste used in this study was analyzed using TFA hydrolysis and HPAEC-PAD (Table 3, untreated) and determined as Ara:Gal:Man:Glc = 3.5:27.5:47.7:2.4 (molar ratio, Table 3). Mussatto *et al.* have reported that the spent coffee grounds consist of cellulose and hemicellulose (as arabinans, galactans, and mannans).[37] The sugar composition of hemicellulose is in good agreement with our experimental data because only hemicellulose is hydrolyzed and

crystalline cellulose is left behind under the employed hydrolysis condition.[25]

The products of the hydrolysis of coffee waste by TtMan5A were analyzed using HPAEC-PAD (Fig. 5a). TtMan5A generated mainly mannose (M1, peak 1) and β-1,4-mannobiose (M2, peak 2) at the initial stages of the reaction (Fig. 5a, upper panel). β-1,4-Mannotriose (M3, peak 3), β-1,4-mannotetraose (M4, peak 4), GM2 (peak 5), and GM3 (peak 6) were represented by small peaks. As the reaction progressed, the amounts of M1 and M2 significantly increased (Fig. 5a, lower panel). In addition, the hydrolysis products of locust bean gum were analyzed using HPAEC-PAD (Fig. 5b). M2, M3, and M4 (but not M1) were generated by the enzyme when the amounts of reducing sugars obtained from locust bean gum were nearly equal to those obtained from the coffee waste during the 2-h reaction (Fig. 5b, upper panel). As the reaction progressed, the amounts of M1 and M2 increased (Fig.5b, lower panel). This indicated the progress of the hydrolysis of oligosaccharides with the release of the final products such as M1 and M2.

Coffee wastes used in this study contained Man and Gal in the ratio of 1.7:1 (Table 3). In general, coffee bean mannans contain high amounts of Man, ranging from Man/Gal

ratio of 7:1 to 40:1.[35] The residue of coffee wastes after TtMan5A treatment contained Man and Gal in a ratio of 1.3:1 (Table 3). There was no difference between the Man/Gal ratio of coffee waste before and after treatment with TtMan5A. The enzyme did not hydrolyze galactans, suggesting that galactans were solubilized by the hydrolysis of mannans. This is also apparent from the data in Table 3 (TtMan5A-treated). Only the contents of Man and Gal individually were reduced by the TtMan5A treatment. The saccharification yield of mannans estimated using the amounts of Man by TtMan5A was 50.7 %.

There are several studies reporting the hydrolysis of coffee mannans. Most these studies analyzed mannans in liquid coffee extracts, although the coffee wastes left after extraction are extremely rich in mannans. Mannooligosaccharides are prepared from spent coffee grounds using hydrothermal hydrolysis. Hydrothermal treatment increases the amount of monosaccharides in the solution and promotes further reactions such as rearrangements and fragmentation of monosaccharides.[38] Enzymatic hydrolysis has an advantage over hydrothermal hydrolysis because it avoids excessive decomposition of sugars. Most recently, Jooste *et al*. reported enzymatic hydrolysis of the spent coffee grounds using recombinant β-mannanase Man1 from *A. niger*.[30] In that study, the enzymatic hydrolysis yield of the coffee wastes reached 17 %. Although the amounts of enzyme were different, our method was more efficient, reaching considerably higher yields. The results demonstrate that TtMan5A hydrolyzes the mannans of the coffee wastes with high efficiency, producing mannooligosaccharides.

In addition to their application in the extraction and hydrolysis of coffee mannans, thermostable β-mannanases are expected to be widely used. These enzymes may help in improving the quality of food and animal feed, aiding in enzymatic bleaching of softwood pulps in the paper and pulp industries, and enhancing the flow of oil or gas in drilling operations.[10] The highly thermostable enzyme TtMan5A would be particularly suitable in the oil and gas well stimulation.[39] No fungal β-mannanases have been used for this application because of the extreme temperatures in the wells (> 80 °C). TtMan5A has the optimum temperature at 85 °C; therefore, the enzyme is an excellent potential candidate for such applications.

In conclusion, highly thermostable β-mannanase TtMan5A was purified from *T. trachyspermus*. It has the highest optimum temperature among the reported fungal β-mannanases. We determined the crystal structure of the native enzyme and clarified the mechanism of the thermostability of TtMan5A. We obtained and characterized the recombinant enzyme and evaluated the efficiency of the enzyme in production of mannooligosaccharides from coffee wastes. TtMan5A approximately provided a three times higher hydrolysis yield of the coffee wastes than the *A. niger* enzyme. TtMan5A is highly thermostable and acts over a broad pH range, suggesting that it is an excellent potential candidate for industrial applications.

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