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A Novel β-mannanase with High Specific Activity from *Bacillus circulans* CGMCC1554: Gene Cloning, Expression and Enzymatic Characterization

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Abstract A DNA fragment of 2,042 bp containing a novel β-mannanase gene, *man5A*, was identified from the genome of the mannan-degrading bacterium *Bacillus circulans* CGMCC1554. The open reading frame of *man5A* comprised 978 bp encoding a protein of 326 amino acids with a predicted molecular weight of 32 kDa. The amino acid sequence of the encoded mannanase, MAN5A, showed the highest identity (78.5%) to β-mannanases belonging to glycosyl hydrolases family 5. The gene *man5A* was efficiently expressed in *Escherichia coli* and *Pichia pastoris* with the highest activity of 541 U/ml in a 3-L fermenter. Recombinant MAN5A purified from *E. coli* had a high specific activity of 4,839 U/mg, which is much higher than that of enzymes that showed high sequence identity. The enzyme showed maximum activity at pH 7.6 and 60 °C and resistance to trypsin. After hydrolysis of LBG, oligomannosides accounted for 76% of the hydrolysis products. All these properties collectively make MAN5A a better candidate than current mannanases for use in the food and feed industry.

Keywords β-Mannanase · *Bacillus circulans* · Expression · Characterization · High specific activity

Introduction

Acetylated galactoglucomannans and other types of heteromannans that are the major hemicelluloses in softwoods and hardwoods consist of a backbone of β-1,4-linked mannose

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and glucose residues with α-1,6-linked galactosyl side groups [1, 2]. β-Mannanases (endo-1,4-β-d-mannanase, EC 3.2.1.78) are endohydrolases that catalyze the random hydrolysis of the β-d-1,4-mannopyranosyl linkages within the main chain of mannans and various polysaccharides mainly consisting of mannose to yield small oligomannoside products [3]. β-Mannanases are very useful in the feed and foodstuff industries [4, 5]. The enzymes are also used in the pulp and paper industry to improve the bleaching of pulp by facilitating the release of lignin from paper pulp [6, 7].

Several β-mannanase genes from bacteria, fungi, and animals have been isolated and expressed in heterologous systems [8–13]. Based on the amino acid sequence similarity, these β-mannanases have been grouped into glycosyl hydrolases (GH) families 5 and 26 [14, 15] and belong to the 3D structure group $(\beta/\alpha)_8$ fold catalytic module characteristic of clan Glycoside hydrolase-A [16]. β-mannanases from different sources displayed different properties, such as optimal pH and temperature, pH and thermal stability, etc. [17]. In an attempt to meet the ever increasing demands for their application in industries and reduce the cost as well, novel β-mannanases with outstanding enzymatic properties, especially high specific activity, are required.

Cultures of mannan-degrading *Bacillus circulans* CGMCC1554 can produce β-mannanase activity of 5.32 U/ml in shaker flask (unpublished data). Here, we report the cloning of the *B. circulans* CGMCC1554 β-mannanase gene, *man5A,* and its efficient expression in *Escherichia coli* and *Pichia pastoris.* The purified enzyme showed a high specific activity and could be an alternative for β-mannanases currently used in industrial processes.

Materials and Methods

Materials

The strains *E. coli* JM109 and *E. coli* BL21 (DE3), and plasmids pGEM-T Easy and pET-22b(+), were purchased from Novagen (USA). The *P. pastoris* expression system containing host strain *P. pastoris* GS115 (His^+ Mut^s) and plasmid vector pPIC9 was obtained from Invitrogen (USA). T4 DNA ligase was purchased from Gibco (USA). Taq DNA polymerase and restriction enzymes were from Takara (Japan). Locust bean gum (LBG), guar gum, and bovine serum albumin were purchased from Sigma (USA). Other chemicals used were of reagent grade and available commercially.

Medium and Culture Conditions

B. circulans CGMCC1554 was cultured in medium consisting of 1% (*w*/*v*) tryptone, 0.3% beef extract, and 0.5% NaCl (pH 7.0) on an orbital shaker at 37 °C, 240 rpm for 2 days. To induce β-mannanase production, inoculums (1%, *v*/*v*) of the cell suspension were grown in medium containing 2% LBG (*w*/*v*), 2% yeast extract, 0.3% NH_4Cl, 0.03% KH_2PO_4, 0.3% $CaCl_2$, 0.06% $MgCl_2{\cdot}6H_2O$, and 0.35% Na_2CO_3 (pH 7.0) at 30 °C, 240 rpm for 3 days. *E. coli* JM109 and *E. coli* BL21(DE3) were grown at 37 °C in Luria–Bertani medium containing 0.1 mg/ml ampicillin.

Enzyme Activity Assays

β-Mannanase activity was estimated by the Somogyi [18] and dinitrosalisylic (DNS) method as described [19, 20]. The standard reaction system contained 0.4 ml 0.3% (*w*/*v*)

LBG in Tricine-NaOH buffer (pH 7.6) and 0.1 ml diluted properly enzyme. The reaction was incubated at 60 °C for 10 min. One unit of mannanase activity was defined as the amount of enzyme required to liberate 1 μmol/min of mannose at a given assay temperature.

Cloning of the *man5A* Gene

Genomic DNA of *B. circulans* CGMCC1554 was extracted as described by Wang et al. [21] and used as template for degenerate polymerase chain reaction (PCR). Primers S1, 5′-AAGTHCATGAYGCYACRGG-3′, and S2, 5′-CCWGCATAYTCRTACATATGG-3′ (H stands for A, C, or T; Y stands for C or T; R stands for A or G; W stands for A or T), were designed based on the conserved sequences of β-mannanases from *Bacillus*. The PCR amplifications were conducted with 30 cycles of 94 °C for 40 s, 50 °C for 30 s, and 72 °C for 40 s after the initial denaturation at 95 °C for 3 min. The PCR product was cloned into the pGEM-T Easy vector for sequencing.

Inverse PCR was used to amplify the upstream and downstream sequences. Two additional primers, C1, 5′-ACCCCAGCCTGCTGCATCTACAATCAAGG-3′, and C2, 5′-CTCAATCGATCGTCGATTACGGACAAAGCG-3′, were designed on the basis of the sequence obtained above. The amplification program of inverse PCR was carried out as in Li et al. [19]. The nucleotide sequences were analyzed by NCBI ORF Finder tool (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The presence of signal peptide in the deduced amino acid sequences was predicted using SignalP (http://www.cbs.dtu.dk/services/SignalP/). Phylogenetic analyses were conducted using CLUSTAL W (http://www.ebi.ac.uk/clustalw/).

Expression of *man5A* in *E. coli*

Primers E1 (5′-CGCCATGGCAGCTACAGGTTTTTAC-3′, *Nco*I restriction site underlined) and E2 (5′-ATCTCGAGGCGACCTTCGATAAAGATTCCCGCTTTTTGGG-3′, *Xho*I restriction site underlined and factor Xa recognition site shaded) were designed based on the complete sequence of *man5A* without the signal peptide. The open reading frame was amplified by PCR using genomic DNA of *B. circulans* CGMCC1554 as template. PCR was performed with 32 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 80 s. The DNA fragment was digested and ligated into vector pET22b(+), which encoded a C-terminal His_6-tag sequence. The recombinant plasmid was transformed into *E. coli* BL21 (DE3), and positive transformants were cultured in a 1-L flask with 100 ml Luria–Bertani medium containing 0.1 mg/ml ampicillin at 37 °C with shaking to an optical density of OD_{600}=0.5. After induction with isopropyl-β-d-1-thiogalactopyranoside to a final concentration of 1 mM and incubation at 18 °C for 12 h, the culture was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and enzyme activity assay.

Expression of *man5A* in *P. pastoris*

The gene fragment encoding the mature protein of MAN5A was amplified by PCR using genomic DNA of *B. circulans* CGMCC1554 as template and primers E3 5′-GCTACGTAG CAGCTACAGGTTTTTACG-3′ (*SnaB*I restriction site underlined), and E4 5′-ATGCGGCCGCATAGCTTTAAAAGATTCC-3′ (*Not*I restriction site underlined). The amplified fragment was inserted into pPIC9 digested by the same restriction enzymes to generate a recombinant plasmid pPIC9-*man5A*, in which the gene was in frame with alpha

factor signal sequence in the vector. Transformation and expression of the gene was carried out as in Yang et al. [22]. The fermentation was carried out in a 3-L fermenter (Bioengineering, Switzerland) at 20 °C and pH 4.5 with an agitation rate of 1,000 rpm.

Purification and Characterization of the Recombinant MAN5A

The periplasmic MAN5A was extracted from *E. coli* as described [19]. The properties of MAN5A, including the optimum pH and temperature, thermostability, stability under different pH values, and effects of various chemicals on the activity and resistance of the activity to pepsin and trypsin, were tested according to the methods of Li et al. [19].

Analysis of the Hydrolysis Products

To analyze the hydrolysis products, 0.6% (*w*/*v*) LBG was hydrolyzed by the purified MAN5A at 60 °C for 6 h, when the substrate LBG was completely hydrolyzed. The hydrolysis products were assayed by high-performance anion exchange chromatography (HPAEC) with a Dionex model 2500 HPAEC system (Dionex, USA) [22].

Results

Cloning and Sequence Analysis of *man5A* from *B. circulans* CGMCC1554

To isolate the β-mannanase gene from *B. circulans* CGMCC1554 using degenerate PCR, we identified two conserved regions in the nucleotide sequences encoding β-mannanases from *Bacillus* spp., and a set of degenerate primers, S1 and S2, were designed based on the sequence alignment. The expected conserved fragment of 330 bp was amplified. The upstream (1.5 kb) and downstream (668 bp) sequences were then obtained using inverse PCR. As a result, a fragment of 2,042 bp containing the complete gene and the flanking region was assembled and registered in GenBank under accession no. AY913796.

An open reading frame of 978 bp was found, encoding a protein of 326 amino acid residues with a predicted signal peptide of 30 residues at its *N* terminus. The predicted molecular weight of the mature protein was 32 kDa. The amino acid sequence encoded by *man5A* showed the highest identity with the β-mannanase from *B. circulans* B48 (78.5%) [19] and 69.8% and 56.8% identities with the catalytic domains of *B. circulans* K-1 β-mannanase [23] and *Bacillus clausii* KSM-K16 β-mannanase [24], respectively, both of which belong to GH family 5 [14] (Fig. 1). The amino acid sequence of *man5A* contains the three conserved residues (Asn-125, Glu-126, and Glu-221) at active sites that are typical of all GH family 5 members [25–29]. Based on these results, we hypothesized that *man5A* encoded a new GH family 5 β-mannanase.

Expression of *man5A* in *E. coli* and *P. pastoris*

After transformation and induction, distinct β-mannanase activity was detected in the cell lysate and periplasm extract of *E. coli* BL21 (DE3) harboring pET22-*man5A*, whereas no activity was detected in the strain transformed with the empty vector pET22b(+). The β-mannanase activity was 17.21 U/ml in the cell lysate and 9.82 U/ml in the periplasm after induction for 4 h. The apparent molecular weight of the expressed MAN5A protein was about 32 kDa by SDS-PAGE (Fig. 2a).

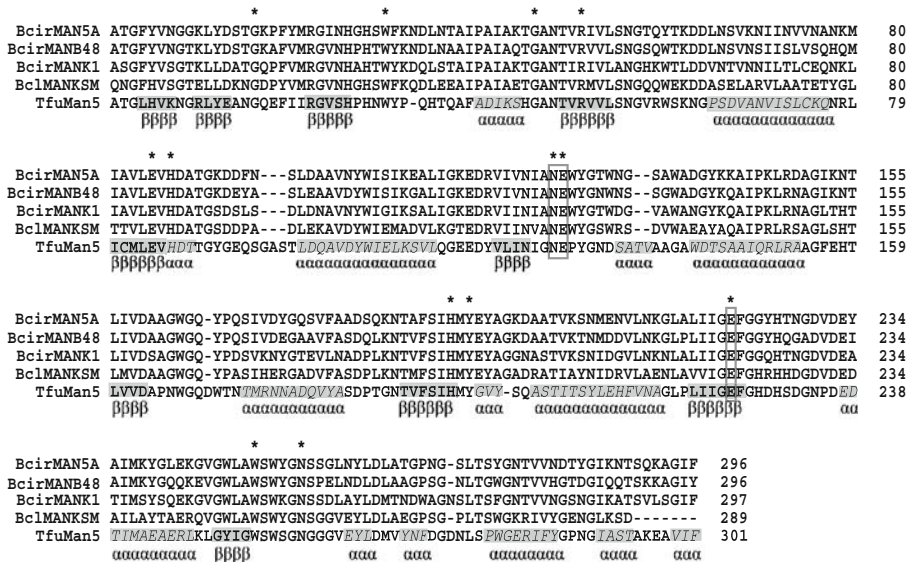

Fig. 1 Structure-based sequence alignment of MAN5A and related amino acid sequences of mannanases with known structures. The alignment was carried out by ClustalX. The catalytic residues are *boxed*, and conserved residues are marked with *asterisks* (*). The residues in *bold in grey boxes* indicate beta strands; the residues in *italics in grey boxes* indicate alpha helices. The structure elements were analyzed with the sequence of β-mannanase from *Thermomonospora fusca* (PDB ID 1bqc) using the DALI program (http://ekhidna.biocenter.helsinki.fi/dali_server/)

We also attempted to express the β-mannanase gene *man5A* with high yield in *P. pastoris*. A DNA fragment lacking the sequence encoding the native signal peptide was inserted into pPIC9 to generate an in-frame fusion to the yeast alpha factor signal sequence and transformed into *P. pastoris* strain GS115. The β-mannanase activity of clone GS115/pPIC9-*man5A*-3 was the highest, reaching 1.96 U/ml in the supernatant of a shaker flask culture. A 3-L fermenter culture of this clone was induced with methanol for 108 h, and we found that the β-mannanase activity in the culture supernatant and cell lysate continuously increased up to 14.31 and 254.39 U/ml using Somogyi-Nelson method, which corresponding to 30.4 and 541 U/ml using the DNS method, respectively, suggesting that the protein was mainly expressed intracellularly. The molecular weight of the intracellular and secreted recombinant MAN5A was about 32 kDa determined by SDS-PAGE, corresponding to the calculated value.

Purification and Characterization of the Recombinant MAN5A

The *E. coli* periplasm extract was subjected to ammonium sulfate precipitation and nickel affinity chromatography, and this procedure yielded a purified his_6-tagged MAN5a that was homogeneous as assessed by its migration as a single band on SDS-PAGE (Fig. 2b). After cleavage of the histidine tag with factor Xa, the apparent molecular weight of the purified recombinant MAN5A was estimated to be 32 kDa, slightly smaller than the non-cleaved protein and in agreement with the calculated value (Fig. 2b). The specific β-mannanase activity of the purified MAN5A was 2277 U/mg after 10.8-fold purification with a 49% yield. When analyzed by the DNS method, the specific activity of MAN5A was 4,839 U/mg. The cleaved enzyme was used for further enzyme characterization.

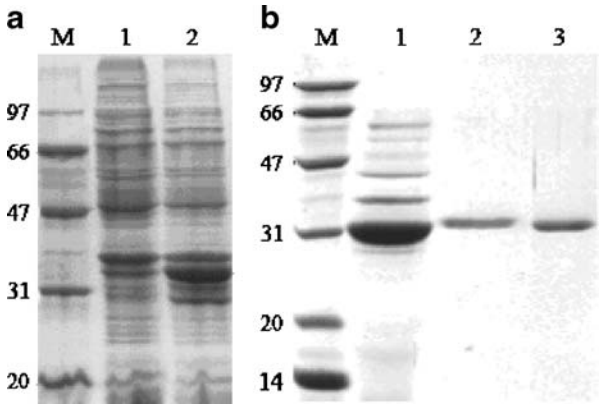

Fig. 2 SDS-PAGE analysis of the expression and purification of recombinant MAN5A from *E. coli* BL21 (DE3). **a** SDS-PAGE analysis of recombinant MAN5A expressed in *E. coli. Lane M* standard protein molecular weight markers (kDa); *lane 1* periplasm extract of uninduced transformant harboring the empty pET-22b(+) vector; *lane 2* periplasm extract of induced transformant harboring pET22-*man5A*. **b** SDS-PAGE analysis of recombinant MAN5A purified using nickel chromatography. *Lane M* standard protein molecular weight markers; *lane 1* periplasmic protein extract from induced transformant harboring pET22-*man5A*; *lane 2* purified recombinant MAN5A after nickel chelate chromatography; *lane 3* purified recombinant MAN5A after cleavage by factor Xa to remove the his_6 tag

The β-mannanase activity of MAN5A at various pH values (2.2–10.0) was measured in the following buffers: citric acid-Na_2HPO_4 (pH 2.2–7.5), Tricine-NaOH (pH 7.5–9.0), and glycine-NaOH (pH 9.0–11.0). The optimum pH for MAN5A activity was 7.6, and more than 75% of its maximal activity was retained in the pH range from 7.0 to 8.0 (Fig. 3a). The pH stability of MAN5A was determined by incubating the enzyme in buffers of different pH values at 37 °C for 60 min and measuring the residual activity in the standard assay. The results indicated that MAN5A has considerable stability, retaining more than 75% of the maximum β-mannanase activity at pH 6.0 to 10.0 and 27% at pH 11.0 (Fig. 3b).

The enzyme had an optimal temperature of 60 °C (Fig. 3c). The thermal stability of MAN5A was also determined by incubating the enzyme at 50 or 60 °C for various times and measuring the residual activity. MAN5A retained 90% of its activity at 50 °C for 60 min but lost all activity after treatment of 60 °C for 20 min (Fig. 3d).

As shown in Table 1, MAN5A was strongly inhibited by Hg^{2+}, Ag^{+}, and SDS and was partially inhibited by Mn^{2+}, Zn^{2+}, and ethylene diamine tetraacetic acid. Other metal ions and chemicals had no obvious effect on MAN5A activity. After treatment with 80 U/ml trypsin for 30 min at 37 °C, the purified MAN5A retained 90% of the maximum activity but lost all activity when pretreated with pepsin for 30 min at 37 °C before β-mannanase activity was assayed. Therefore, MAN5A was resistant to trypsin but not pepsin, suggesting that MAN5A was selectively sensitive to proteolytic enzymes.

Analysis of Hydrolysis Products of MAN5A Digestion

After hydrolysis of a standard substrate LBG, the enzyme protein was removed from the reaction mixture by precipitation, followed by use of 10-kDa cutoff ultrafiltration tubes. The hydrolysis products were analyzed by HPAEC using oligosaccharide standards. The results indicated (Fig. 4) that oligomannosides were the main products, accounting for 76%

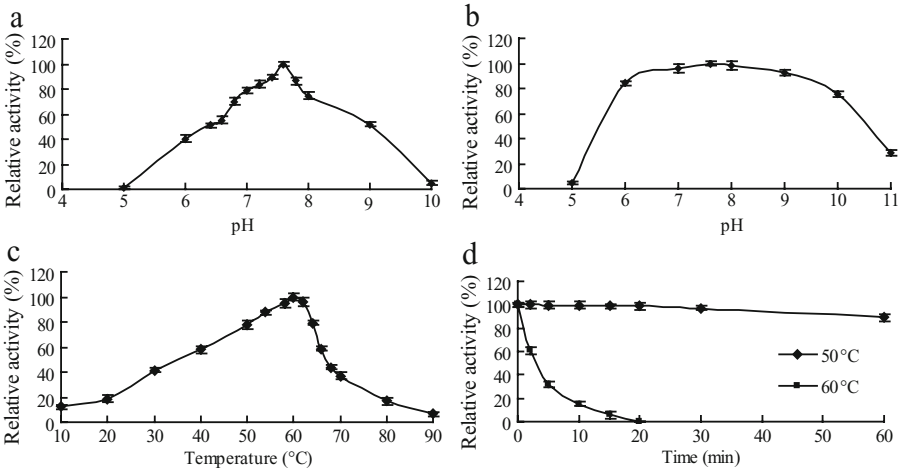

Fig. 3 Characterization of the purified recombinant MAN5A. **a** Effects of pH on the activity of recombinant MAN5A. The β-mannanase activity assay was conducted at 60 °C for 10 min in buffers of pH 2.2–10.0. The maximum activity at pH 7.6 was defined as 100%. **b** Effects of pH on stability of recombinant MAN5A. β-Mannanase activity was measured at 60 °C after incubation at 37 °C for 30 min in buffers of pH 2.2–10.0. The activity of untreated recombinant MAN5A was defined as 100%. **c** Effects of temperature on β-mannanase activity of recombinant MAN5A. The β-mannanase activity was measured in Tricine-NaOH buffer (pH 7.6) at 10–90 °C for 10 min. The maximum activity at 60 °C was defined as 100%. **d** Thermostability of the recombinant MAN5A. The enzyme was preincubated at 50 or 60 °C in Tricine-NaOH buffer (pH 7.6) for 30 min, and then was subjected to the activity assay at 60 °C for 10 min. The activity of untreated recombinant MAN5A was defined as 100%

of the total products. Mannobiose, mannotriose, mannotetraose, and mannopentaose were 25.2%, 15.0%, 23.6%, and 12.1%, respectively.

Discussion

In this study, a β-mannanase gene was cloned from *B. circulans* CGMCC1554. Sequence analysis demonstrated that the mature MAN5A protein has the highest sequence identity with *B. circulans* B48 β-mannanase (78.5%) [19] and has 69.8% and 56.8% sequence

Table 1 Effects of various chemicals on purified recombinant MAN5A activity.

Chemical (1 mM)	Relative activity (%)	Chemical (1 mM)	Relative activity (%)
CK	100	Cu^{2+}	86
Ca^{2+}	110	Ni^{2+}	80
Na^{+}	101	Zn^{2+}	35
K^{+}	100	Mn^{2+}	31
Mg^{2+}	100	Hg^{2+}	8
Co^{2+}	98	Ag^{+}	2
Li^{2+}	95	EDTA	27
Fe^{3+}	91	SDS	ND
Al^{3+}	86		

CK no chemicals added in the reaction system, *ND* not detected

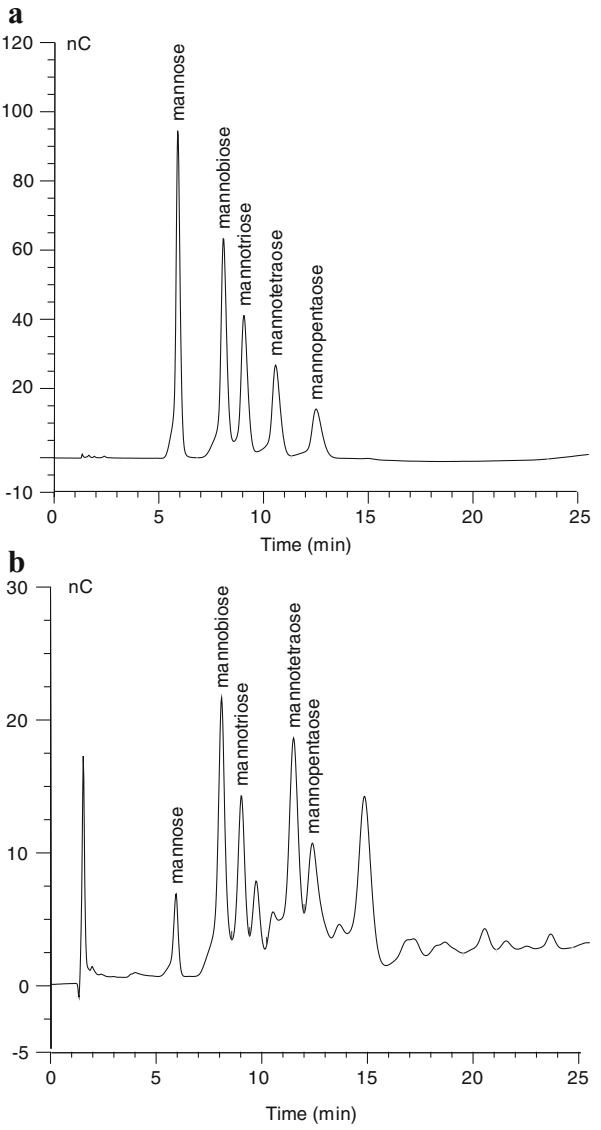

Fig. 4 HPAEC analysis of LBG hydrolysis products after digestion with recombinant MAN5A. **a** Standard of oligomannosides, including mannose, mannobiose, mannotriose, mannotetraose, and mannopentaose. **b** LBG hydrolysis products of MAN5A digestion

identity with the catalytic domains of *B. circulans* K-1 β-mannanase [23] and *B. clausii* KSM-K16 β-mannanase, respectively [24]. These three β-mannanases have all been identified as GH family 5 members. Structure-based alignment of MAN5A and the β-mannanase from *Thermomonospora fusca* (PDB ID 1bqc) [30], which had 41.4% identity with MAN5A, showed a similar structure for the two β-mannanases. MAN5A contained the three highly conserved active-site residues, Asn-125, Glu-126, and Glu-221, and the strictly conserved residues, Gly-16, Trp-30, Gly-46, Arg-51, Glu-85, His-87, His-191, Tyr-193, Trp-250, and Asn-255, which are typical of all GH family 5 enzymes [25–30]. Conservation of these residues strongly suggests that MAN5A is a new GH family 5 β-mannanase.

β-mannanases from different sources showed different properties. In this study, the characteristics of MAN5A, including optimal pH and temperature, thermal stability, proteinase and ion sensitivity, etc., were similar with MANB48 from *B. circulans* B48 [19], both of which shared the highest identity. The relationship between enzyme properties and structure had been preliminarily studied. Based on the 3D structure and H1A-23A mutation, the thermal stability of mannanase BCman from *Bacillus subtilis* was affected by its disulfide bond and metal-bonding site (His1–Glu336–His23); however, the mechanisms were not determined in details [31]. The specific activity of the purified MAN5A was 4,839 U/mg, which was higher than that of MANB48 (481.55 U/mg) [19] and of the β-mannanase from *B. subtilis* KU-1 (407.7 U/mg) [32], and only lower than that of β-mannanase purified from *B. subtilis* WY34 (8302.4 U/mg) [33]. MAN5A contains the acidic residues Glu-110 and Asp-149, in contrast to Gly and Asn, respectively, in MANB48. The two acidic residues in MAN5A are close to the catalytic site and might serve as H^+ donors [30], thus, potentially improving the catalytic efficiency. The *B. subtilis* WY34 mannanase showed the highest specific activity reported to date. However, the amino acid and nucleotide sequence of the enzyme was not reported [33]. The role of these residues and the mechanism of the catalytic reaction require further study by protein engineering and structure determination.

β-Mannanases have been successfully expressed in various microorganisms, including bacteria, fungi, and yeasts [8–13]. The yeast *P. pastoris* expression system has been a favorite system for expressing heterologous proteins due to its many advantages [34]. To date, only β-mannanases from blue mussel (*Mytilus edulis*) [13], *Aspergillus sulphureus* [35], and *Agaricus bisporus* [36] have been expressed in *P. pastoris*, with respective activities of 41, 96, and 3.3 U/ml. The recombinant *P. pastoris* clone transformed with *man5A* showed a high activity of 254.39 U/ml during fermentation, which corresponds to 541 U/ml using the DNS method. The higher activity of the recombinant MAN5A might be due to the higher specific activity of the enzyme. However, the protein was expressed mainly intracellularly at a low concentration of target protein. The expression and enzyme activity could be further improved by optimizing the signal peptide and codon bias and by gene modification.

Most species of fishes do not have a stomach, and pepsin is directly secreted into gastrointestinal tract with neutral pH [8], thus, requiring the additive of enzyme having the properties such as neutral optimal pH and resistance to proteolysis. MAN5A was optimally active at pH 7.6 and highly resistant to trypsin, suggesting its great potential in fish feed industry. In the point of hydrolysis products, most products of LGB by MAN5A digestion were oligomannosides, implying the potential application of MAN5A in the food and feed industry [37].

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