Cloning and Functional Expression of an Acidophilic β-Mannanase Gene (Anman5A) from Aspergillus niger LW-1 in Pichia pastoris

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ABSTRACT: A cDNA fragment of the Anman5A, a gene that encodes an acidophilic β-mannanase of Aspergillus niger LW-1 (abbreviated as AnMan5A), was cloned and functionally expressed in Pichia pastoris. Homology alignment of amino acid sequences verified that the AnMan5A belongs to the glycoside hydrolase (GH) family 5. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) assay demonstrated that the recombinant AnMan5A (reAnMan5A), a N-glycosylated protein with an apparent molecular weight of 52.0 kDa, was secreted into the medium. The highest reAnMan5A activity expressed by one P. pastoris transformant, labeled as GSAnMan4-12, reached 29.0 units/mL. The purified reAnMan5A displayed the highest activity at pH 3.5 and 70 °C. It was stable at a pH range of 3.0–7.0 and at a temperature of 60 °C or below. Its activity was not significantly affected by an array of metal ions and ethylenediaminetetraacetic acid (EDTA). The K_m and V_{max} of the reAnMan5A, toward locust bean gum, were 1.10 mg/mL and 266.7 units/mg, respectively.

KEYWORDS: Aspergillus niger, Pichia pastoris, cloning, expression, β-mannanase

INTRODUCTION

β-1,4-D-Mannan mannohydrolase (EC 3.2.1.78), generally abbreviated as β-mannanase, can catalyze the random cleavage of internal β-1,4-D-mannosidic linkages of the mannan or heteromannan backbone.[1] To date, almost all known β-mannanases have been classified into glycoside hydrolase (GH) families 5, 26, and 113 based on their amino acid sequence alignment and hydrophobic cluster analysis (http://www.cazy.org/fam/acc_GH.html).[2] β-Mannanases have attracted much attention because of their potential applications in diverse industrial processes.[1,3] Similar other GHs, β-mannanases played important roles in simplifying the industrial processes and improving the quality of products while reducing the environmental pollution caused using the chemicals.[4]

During the past few decades, numerous β-mannanases have been purified and characterized from microorganisms, plants, and metazoans,[5] among which the filamentous fungi were considered to possess great potential for the industrial production of β-mannanases. Many studies have been conducted on exploiting novel β-mannanases with superior enzymatic properties, on increasing β-mannanase activities by mutating enzyme-producing strains and optimizing fermentation conditions, and on producing β-mannanases by fermentation on an industrial scale. However, commercialization and broad applications of β-mannanases were still hindered, owing to their low catalytic activities and expensive production costs.[3] To make β-mannanases be applied more efficiently and economically, more interests are being focused on improving their structures and catalytic properties with chemical or physical approaches and, recently, by means of genetic engineering. Many β-mannanase genes from filamentous fungi, such as Aspergillus niger CBS 513.88,[6] Aspergillus usamii YL-01-78,[7] Aspergillus sulphureus MAFIC001,[8] Aspergillus aculeatus MRC11624,[9] Biopora sp. MEY-1,[4] and Trichoderma reesei RutC30,[10] have been cloned, characterized, and modified, and some recombinant β-mannanases have been expressed in heterologous cells with high activities and superior properties.[4,9]

A. niger, recognized as a safe filamentous fungus, was one of the most common microorganisms used in the production of fermented foods, organic acids, and various hydrolases.[11] In our previous studies, the bimutation breeding and fermentation conditions optimizing of A. niger LW-1 for enhancing the β-mannanase activity were performed,[3,12] while its enzymatic properties were also assayed.[13] Here, we reported the cloning of a full-length cDNA of the Anman5A, a gene that encodes an acidophilic β-mannanase of A. niger LW-1, and functional expression of a cDNA fragment of the Anman5A in Pichia pastoris. In addition, enzymatic properties of the expressed reAnMan5A were also characterized.

MATERIALS AND METHODS

Strains, Vectors, and Culture Media. A. niger LW-1 was isolated from the soil in China as reported previously.[12] Here, the strain was used as the source for total RNA extraction. Escherichia coli JM109 and pUCm-T (Sangon, Shanghai, China) were used as a host–vector system for gene cloning and DNA sequencing. E. coli DH5α and pPICZαA (Invitrogen, San Diego, CA) were used for construction of the recombinant vector. A cDNA fragment of the Anman5A was extracellularly expressed in P. pastoris GS115 (Invitrogen, San Diego, CA). A. niger LW-1 was cultured at 30 °C in a liquid medium containing 5 g/L yeast extract, 10 g/L tryptone, 1 g/L KH₂PO₄, 1 g/L Na₂HPO₄, 0.5 g/L MgSO₄, 0.2 g/L CaCl₂, 1 g/L (NH₄)₂SO₄, 10 g/L dextrose, and 5 g/L locust bean gum (Sigma, St. Louis, MO), at pH

Table 1. Oligonucleotide Sequences of Primers for PCR Amplification

cloned fragments	primers	oligonucleotide sequences (5′−3′)[a]	size (bp)
	ManF1	GCTACTTYGCSGGVACSAAC	20
	ManF2	TCDACVATCAACACKGGNGC	20
3′-end cDNA fragment	dT-PR	GTTTTCCCAGTCACGAC-oligo dT	37
	PR	GTTTTCCCAGTCACGAC	17
	OP	CATGGCTACATGCTGACAGCCTA	23
5′-end cDNA fragment	IP	CGCGGATCCACAGCCTACTGATGATCAGTCGATG	34
	ManR1	ATTGTCGATTTGCCGTCCTG	20
	ManR2	GCAGTTGGTACCAGACTGTG	20
full-length cDNA of the *AnmanSA*	FManF	GTATCATAAACAAACCAAAGG	21
	FManR	TTAAGTTTTACTTGTTTGATTT	22
mature peptide cDNA	ManF	<u>GAATTC</u>TCCTTCGCCAGCACCTCC	24
	ManR	<u>GCGGCCGC</u>TTAAGCACTACCAATAGC	26

[a]Y, C/T; S, C/G; V, A/G/C; D, A/G/T; K, G/T; and N, A/T/G/C. The underlines of primers ManF and ManR represent the *Eco*RI and *Not*I sites, respectively.

6.0. *E. coli* JM109 and *E. coli* DH5α were grown at 37 °C in the Luria−Bertani medium.[14] *P. pastoris* GS115 was cultivated at 30 °C in a yeast extract peptone dextrose (YPD) medium. Zeocin-containing YPD, buffered glycerol-complex (BMGY), and buffered methanol-complex (BMMY) media were prepared as described in the manual of Multi-Copy Pichia Expression Kit (Invitrogen, San Diego, CA).

Total RNA Extraction. *A. niger* LW-1 was cultured at 30 °C in an above-mentioned mannan-containing medium on a rotary incubator with 220 rpm. After 36 h of cultivation, the mycelia were harvested through filtration and then thoroughly washed with sterile deionized water. The total RNA was extracted using RNA Extraction Kit (Sangon, Shanghai, China) according to the method reported previously.[15]

Primers for Polymerase Chain Reaction (PCR) Amplification. After aligning four fungal β-mannanase sequences from the GH family 5, *A. usamii* (ADZ99027), *A. sulphureus* (ABC59553), *A. aculeatus* (AAA67426), and *T. reesei* (AAA34208), we found the most conserved two peptide segments, GYFAGTNS(/C)YW and STINTGADGLQ, located in the N-terminal region. Therefore, two degenerate primers ManF1 and ManF2 were designed corresponding to GYFAGTN and STINTGA, respectively. Primers dT-PR and PR (original names, oligo dT-M13 primer M4 and M13 primer M4, respectively) were provided by RNA PCR Kit (TaKaRa, Dalian, China). Primers OP and IP (original names, 5′ RACE outer primer and 5′ RACE inner primer, respectively) were provided by 5′-Full RACE Kit (TaKaRa, Dalian, China). Primers FManF and FManR were designed to directly amplify a full-length cDNA sequence of the *AnmanSA*. A pair of specific primers ManF and ManR with *Eco*RI and *Not*I sites (underlined), respectively, was used for the cloning of a cDNA fragment encoding the AnMan5A. As listed in Table 1, all PCR primers (except those provided by kits) were synthesized by Sangon (Shanghai, China).

Cloning of a Full-Length cDNA. A 3′-end cDNA fragment of the *AnmanSA* was amplified by using RNA PCR Kit and nested PCR method.[15] The primer dT-PR was used for reverse transcription of the first-strand cDNA from the *A. niger* LW-1 total RNA. Using the resulting first-strand cDNA as the template, the first-round PCR was performed using primers ManF1 and PR as the following conditions: an initial denaturation at 94 °C for 2 min, 30 cycles at 94 °C for 30 s, 53 °C for 30 s, 72 °C for 75 s, and an extra elongation at 72 °C for 10 min. Then, the second-round PCR was carried out with the same conditions using primers ManF2 and PR for confirmation (nested PCR method). Next, a 5′-end cDNA fragment of the *AnmanSA*, starting from the transcription start site, was amplified using 5′-Full RACE Kit. The first-strand cDNA was used as template for the first-round PCR using primers OP and ManR1 and then subjected to the second-round PCR using primers IP and ManR2 for confirmation. Finally, a full-length cDNA of the *AnmanSA* could be either obtained by assembling above cloned 3′- and 5′-end cDNA fragments or directly amplified by conventional PCR using primers FManF and FManR.

Analysis of the AnMan5A Structure. The signal peptide of the preproAnMan5A was predicted using the SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/). The putative N-glycosylation sites of the AnMan5A were located using the NetNGlyc program 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/). Physicochemical properties were identified by the Protparam program (http://au.expasy.org/tools/protparam.html). Homology searches in GenBank were performed using the BLAST server. Multiple alignment of protein primary structures between the AnMan5A and the other four fungal β-mannanases from the GH family 5 was accomplished using the ClustalW program (http://www.ebi.ac.uk/ClustalW) and DNAMAN 6.0 software. The three-dimensional (3D) structure of the AnMan5A was modeled by the bitemplate-based method using both MODELER 9.9 (http://salilab.org/modeller/) and SALIGN (http://salilab.org/DBAli/?page=tools&action=f_salign) programs based on known protein crystal structures of the *T. reesei* β-mannanase (1QNO) and *Lycopersicon esculentum* β-mannanase (1RH9) from the GH family 5.

Construction of the Recombinant Vector. On the basis of the information of the cloned full-length cDNA sequence in this work (GenBank accession JN123356) and the determined sequence of N-terminal 15 amino acid residues (SFASTSGLQFTIDGE) of the native β-mannanase purified from the cultivated koji of *A. niger* LW-1,[3] a pair of specific primers ManF and ManR (see Table 1) was designed to amplify a cDNA fragment encoding the AnMan5A using the first-strand cDNA as the template with following conditions: an initial denaturation at 94 °C for 2 min, 30 cycles at 94 °C for 30 s, 56 °C for 30 s, 72 °C for 75 s, and an extra elongation at 72 °C for 10 min. After the target PCR product was purified using EZ-10 Spin Column DNA Gel Extraction Kit (BBI, Markham, Canada) and digested with *Eco*RI and *Not*I, the resulting cDNA fragment was inserted into pPICZαA vector digested with the same restriction enzymes, followed by transforming it into *E. coli* DH5α competent cells. The proper recombinant vector, named pPICZαA−*AnmanSA*, was confirmed by restriction enzyme analysis and DNA sequencing.

Transformation and Expression of a cDNA Gene. The pPICZαA−*AnmanSA* was linearized with *Sac*I and transformed into *P. pastoris* GS115 by electroporation on a Gene Pulser apparatus (Bio-Rad, Hercules, CA) according to the instructions of the manufacturer. The *P. pastoris* transformants were inoculated on YPD plates containing Zeocin at increasing concentrations of 100, 200, and 400 μg/mL for the screening of multiple copies of a cDNA gene. Using genomic DNA of the *P. pastoris* transformant as the template, extracted using Yeast Genomic DNA Extract Kit (Tianwei, Beijing, China), the integration of the cDNA gene into the yeast *P. pastoris* genome was confirmed by PCR analysis using 5′- and 3′-*AOX1* primers. The genomic DNA of *P. pastoris* GS115 with pPIZαA was used as the control. Expression of the cDNA gene in *P. pastoris* GS115 was performed according to the instructions of Multi-Copy Pichia Expression Kit (Invitrogen, San Diego, CA), with slight modification.[16] Each single colony of yeast transformants was inoculated into 30 mL

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1     GTATCATAAACAAACCAAAGGAAATCAATCAAACGGTCACAATGAAGCTTTCCAACGCCCTCCTCACCCTGGCTA
      FManF                                              M  K  L  S  N  A  L  L  L  T  L  A

76    GCCTGGCGCTGGCCAACGTCTCCACGGCTCTGCCCGAAAGCCTCCCCTGCACCGAGCACCAGCAGCAGTGCTGCCT
      S  L  A  L  A  N  V  S  T  A  L  P  K  A  S  P  A  P  S  T  S  S  S  A  A

151   CCACCTCCTTCGCCAGCACCTCCGGCCTCCAATTCACCATTGATGGCGAAACTGGCTACTTCGCCGGAACCAACA
                             ManF                                  ManF1
      S  T  S  F  A  S  T  S  G  L  Q  F  T  I  D  G  E  T  G  Y  F  A  G  T  N

226   GCTACTGGATCGGTTTCCTCACTGACAACGCGGACGTCGACCTCGTCATGGGCCACCTGAAGTCGTCCGGCCTCA
      S  Y  W  I  G  F  L  T  D  N  A  D  V  D  L  V  M  G  H  L  K  S  S  G  L

301   AGATCCTCCGCGTGTGGGGCTTCAACGATGTCACCTCGCAGCCCTCCTCCGGCACAGTCTGGTACCAACTGCACC
                                                          ManR2
      K  I  L  R  V  W  G  F  N  D  V  T  S  Q  P  S  S  G  T  V  W  Y  Q  L  H

376   AGGACGGCAAATCGACAATCAACACGGGTGCCGACGGTCTCCAGCGCCTCGACTACGTCGTCTCGTCTGCCGAAC
            ManR1                ManF2
      Q  D  G  K  S  T  I  N  T  G  A  D  G  L  Q  R  L  D  Y  V  V  S  S  A  E

451   AGCACGACATCAAACTCATCATCAACTTCGTCAACTACTGGACCGATTACGGTGGTATGTCTGCGTACGTGAGCG
      Q  H  D  I  K  L  I  I  N  F  V  N  Y  W  T  D  Y  G  G  M  S  A  Y  V  S

526   CGTATGGCGGATCCGGCGAGACGGATTTCTATACCAGTGATACCATGCAGAGTGCCTATCACACATATATCAAGA
      A  Y  G  G  S  G  E  T  D  F  Y  T  S  D  T  M  Q  S  A  Y  H  T  Y  I  K

601   CGGTCGTGGAGCGGTACAGTAACTCCTCGGCCGGTGTGTTGCGTGGGAGTTGGCGAATGAGCCGAGATGTCCGAGTT
      T  V  V  E  R  Y  S  N  S  S  A  V  F  A  W  E  L  A  N  E  P  R  C  P  S

676   GCGATACTTCTGTGTTGTATAACTGGATTGAGAAGACGAGTAAGTTTATTAAGGGGTTGGATGCGGATCGTATGG
      C  D  T  S  V  L  Y  N  W  I  E  K  T  S  K  F  I  K  G  L  D  A  D  R  M

751   TTTGTATTGGTGATGAGGGCTTCGGTCTCAACATCGACTCAGACGGCAGCTACCCTTATCAATTCTCCGAGGGCT
      V  C  I  G  D  E  G  F  G  L  N  I  D  S  D  G  S  Y  P  Y  Q  F  S  E  G

826   TGAACTTTACGATGAACCTCGGTATCGATACTATTGACTTTGGTACCCTCCACTTGTACCCTGATAGCTGGGGCA
      L  N  F  T  M  N  L  G  I  D  T  I  D  F  G  T  L  H  L  Y  P  D  S  W  G

901   CCTCCGACGACTGGGGCAACGGCTGGATCACCGCCCACGGCGCAGCCTGCAAAGCAGCCGGCAAGCCATGTCTCC
      T  S  D  D  W  G  N  G  W  I  T  A  H  G  A  A  C  K  A  A  G  K  P  C  L

976   TGCAGGAATACGGAGTCACCTCGAACCACTGCAGTGTGGAGGGCTCGTGTCAGAAGACAGCGCTCAGCACAACGG
      L  Q  E  Y  G  V  T  S  N  H  C  S  V  E  G  S  C  Q  K  T  A  L  S  T  T

1051  GCGTCGGCGCGGATCTGTTCTGGCAGTATGGTGATGATTTGAGTACCGGGAAGTCGCCGGATGATGGGAATACTA
      G  V  G  A  D  L  F  W  Q  Y  G  D  D  L  S  T  G  K  S  P  D  D  G  N  T

1126  TCTACTATGGGACTAGTGATTATCAGTGCCTGGTGACGGATCATGTTGCTGCTATTGGTAGTGCTTAAGGGGGAT
                                                             ManR
      I  Y  Y  G  T  S  D  Y  Q  C  L  V  T  D  H  V  A  A  I  G  S  A  *

1201  ACTGAGACTGAGATGTGGGGGAAAGGATGTGATTGTTGGTGATTTGAGAATGTATGTACAAAAGGGGGGAAGCGG

1276  TGAATGAGAGTTGTTCGTTGAGTTTTTGGTAAACAATTGGACAGTCAAACCATCAGCCAAGACTAGCTAAGATAC

1351  TGGGTATTTTGGTCACTGCTTTTCATAATAAAACATGCTATTTATAAATCAAACAAGTAAAACTTAA
                                          FManR
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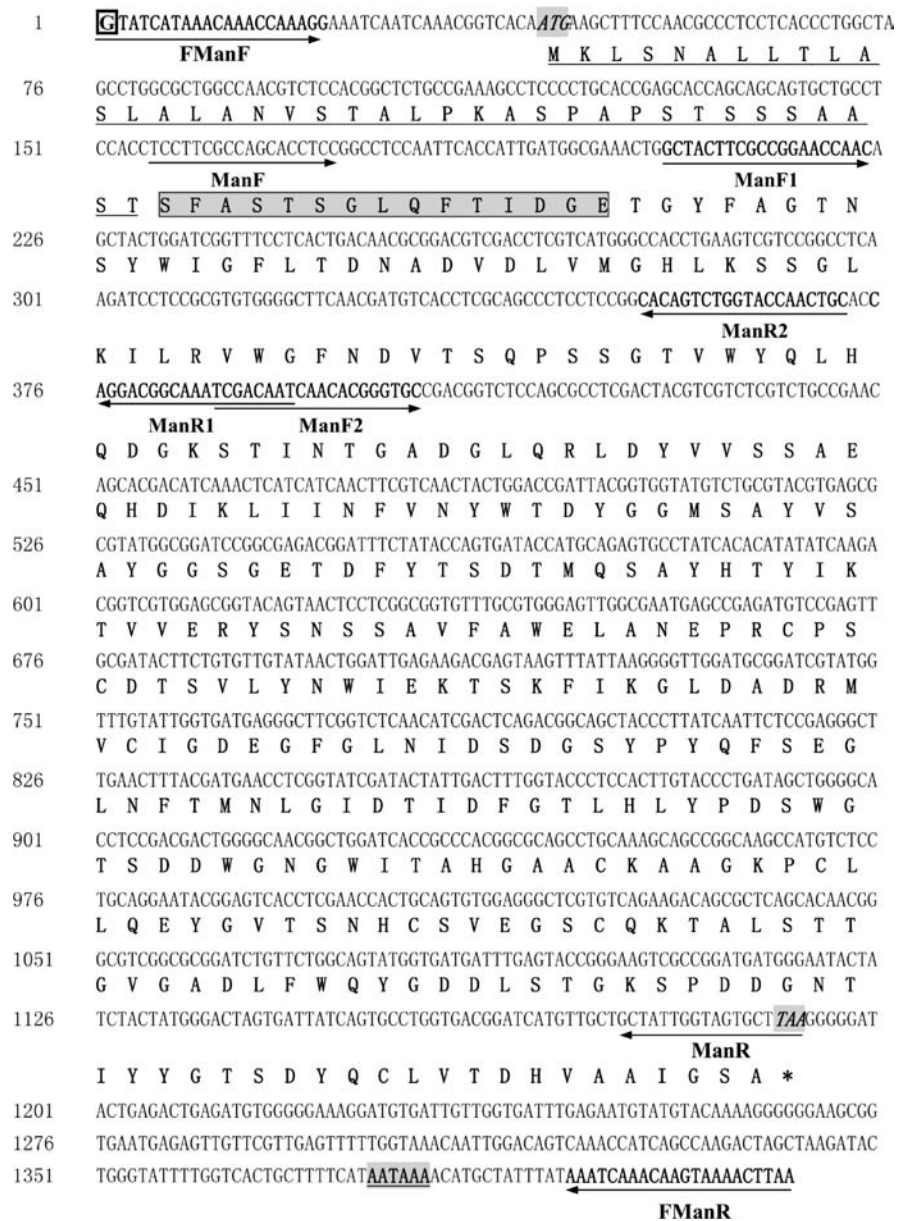
Figure 1. Nucleotide sequence of a full-length cDNA of the *Anman5A* from *A. niger* LW-1 and its deduced amino acid sequence of the AnMan5A. A signal peptide from Met[1] to Ala[21] and a propeptide from Leu[22] to Thr[38] are underlined. The determined N-terminal 15 amino acid sequence of the native AnMan5A is indicated in a gray box. The bold letter of G in the box indicates the transcription start site. The gray italic letters of ATG and TAG represent the initiation codon and stop codon, respectively. The putative polyadenylation signal, AATAAA, is shown as gray underlined letters. The bold arrows below the letters represent the primers for PCR amplification.

of BMGY medium in a 250 mL flask and cultured at 30 °C on a rotary incubator with 220 rpm until the OD_{600} reached 2−4. Then, the cells were harvested by centrifugation at 3000 rpm and resuspended in 30 mL of BMMY medium. The expression of the reAnMan5A was induced at 30 °C for 96 h by adding methanol to a final concentration of 1.0% (v/v) at 24 h intervals.

Purification of the Expressed reAnMan5A. After the *P. pastoris* transformant was induced by 1.0% methanol at 30 °C for 96 h, the cultured broth was centrifuged at 8000 rpm for 10 min to remove yeast cells. A total of 20 mL of resulting supernatant was fractionated by ammonium sulfate at concentrations ranging from 40 to 80% saturation, and the fraction containing the reAnMan5A activity was retained. The resulting precipitate was dissolved in 4.0 mL of 20 mM Na_2HPO_4–NaH_2PO_4 buffer (pH 6.5) and then dialyzed against the same buffer overnight. The dialyzed solution was concentrated to 1.0 mL by ultrafiltration at 8000 rpm for 20 min at 4 °C using a 10 kDa cutoff membrane (Millipore, Billerica, MA), then applied onto a Sephadex G-75 column (Amersham Pharmacia Biotech, Uppsala, Sweden; inner diameter of 1.6 × 80 cm), and followed by eluting with the same buffer at a flow rate of 0.4 mL/min. Aliquots of 2.0 mL eluent having the reAnMan5A activity were pooled, concentrated by ultrafiltration as above, and further characterized.

Enzyme Activity and Protein Assays. β-Mannanase activity was assayed using the 3,5-dinitrosalicylic acid (DNS) colorimetric method as described previously,[12] with slight modification. In brief, 100 μL of suitably diluted enzyme solution was incubated with 2.4 mL of 0.5% (w/v) locust bean gum, prepared with 50 mM Na_2HPO_4–citric acid buffer (pH 3.6), at 50 °C for 10 min. The reaction was terminated and color-developed by adding 2.5 mL of DNS reagent and boiling at 100 °C for 7 min. The absorbance of colored solution was measured at 540 nm using an ultraviolet−visible (UV−vis) spectrophotometer (Jinghua, Shanghai, China). A total of 1 unit of β-mannanase activity was defined as the amount of enzyme liberating 1 μmol of reducing sugar equivalent per minute under the assay conditions, using D-

mannose as the standard. The protein concentration was determined using the BCA-200 Protein Assay Kit (Pierce, Rockford, IL), using bovine serum albumin as the standard. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed on a 12.5% gel by the method from Laemmli,[17] and isolated proteins were visualized by straining with Coomassie Brilliant Blue R-250 (Sigma, St. Louis, MO).

Carbohydrate Content and Deglycosylation Assays. The carbohydrate content of the purified reAnMan5A expressed by the *P. pastoris* transformant was assayed by the phenol–sulfuric acid method,[18] using D-mannose as the standard. After the purified reAnMan5A was treated at 100 °C for 10 min and cooled to room temperature, an endoglycosidase H (New England Biolabs, Ipswich, MA), which can catalyze the release of N-linked oligosaccharides, was added to perform deglycosylation at 37 °C for 1.0 h. The manipulation followed the instructions of the manufacturer.

pH Optimum and Stability. The optimal pH of the purified reAnMan5A was assayed by the standard β-mannanase activity assay method as stated above, except 0.5% of substrate solutions, which were prepared with 20 mM Na_2HPO_4–citric acid buffer over the pH range of 2.5–7.5. To estimate the pH stability, aliquots of reAnMan5A solution were preincubated at 40 °C for 1.0 h in varied pH values (Na_2HPO_4–citric acid buffer, pH 2.5–7.5; Tris-HCl buffer, pH 8.0), and the residual reAnMan5A activities were assayed under standard conditions. The pH stability, in this work, was defined as a pH range, over which the residual reAnMan5A activities were more than 85% of the original activity.

Temperature Optimum and Stability. The optimal temperature of the reAnMan5A was determined at the optimal pH and at temperatures ranging from 40 to 80 °C. To evaluate thermostability, aliquots of reAnMan5A solution were mixed with Na_2HPO_4–citric acid buffer (pH 3.6) at a ratio of 1:1 (v/v) and then preincubated at various temperatures (40–75 °C) for 1.0 h. The residual reAnMan5A activities were assayed under standard conditions. Here, the thermostability was defined as a temperature, at or below which the residual reAnMan5A activities remained over 85% of the original activity.

Effects of Metal Ions and Ethylenediaminetetraacetic Acid (EDTA). To estimate the tolerance of the reAnMan5A to metal ions and EDTA, aliquots of reAnMan5A solution were preincubated with various metal ions and EDTA at a final concentration of 5.0 mM in 20 mM Na_2HPO_4–citric acid buffer (pH 3.6) at 40 °C for 1.0 h. The residual reAnMan5A activities were assayed under standard conditions. The reAnMan5A solution without any additive was used as the control.

Substrate Specificity and Kinetic Parameters. The substrate specificity of the reAnMan5A was determined by measuring the enzyme activities at 50 °C for 10 min toward 0.5% (w/v) of locust bean gum, konjac flour, guar gum, sodium carboxymethyl cellulose, or birchwood xylan (Sigma, St. Louis, MO), which was prepared with 50 mM Na_2HPO_4–citric acid buffer (pH 3.6). The amount of reducing sugars produced was assayed using the DNS colorimetric method as described above.

Hydrolyzing reaction rates (units/mg) of the reAnMan5A were assayed under standard conditions (pH 3.6 and 50 °C for 10 min), except the substrate (locust bean gum) concentrations ranging from 1.0 to 10 mg/mL. The hydrolyzing reaction rate versus the substrate concentration was plotted to verify whether the hydrolyzing mode of the reAnMan5A conforms to the Michaelis–Menten equation. Kinetic parameters, K_m and V_{max}, were graphically determined from Line-weaver–Burk plotting.

RESULTS AND DISCUSSION

Cloning of a Full-Length cDNA of the *Anman5A*. Analytical results of the total RNA extracted from *A. niger* LW-1 showed that the ratio of OD_{260}/OD_{280} was 1.96 and that the 18S rRNA and 28S rRNA bands, characterized as eukaryotes, on formaldehyde denatured agarose gel electrophoresis were specific (data not shown), indicating that the total RNA has high purity and is not decomposed.

Using the reverse-transcribed first-strand cDNA as the template, one approximate 1.2 kb clear band and several faint bands were amplified by the first-round PCR amplification using primers ManF1 and PR. On the basis of the principle of the nested PCR technique, each band was agarose-gel-purified and subjected to the second-round PCR using primers ManF2 and PR for confirmation. As a result, an about 1.0 kb specific band was amplified only using the 1.2 kb band as the template. The DNA sequencing result verified that the cloned 3′-end cDNA fragment is exactly 1213 bp in length (except the complementary sequence of dT-PR). An approximate 450 bp band of a 5′-end cDNA fragment was first amplified using primers OP and ManR1 as a major PCR product and subjected to the second-round PCR using primers IP and ManR2 for confirmation. The DNA sequencing result showed that the first-round major PCR product is exactly 394 bp in length (except primers OP and IP), containing a 190 bp sequence identical to that between ManF1 and ManR1 and a new 204 bp sequence, in which a transcription start site (G) and cDNA fragment encoding a 21 amino acid signal peptide, a 17 amino acid propeptide, and a determined N-terminal 15 amino acid residue (SFASTSGLQFTIDGE) of the native β-mannanase were recognized (Figure 1).

Using the first-strand cDNA as the template, an approximate 1.4 kb full-length cDNA sequence was directly amplified using primers FManF and FManR, which is entirely identical to that obtained by assembling cloned 3′- and 5′-end cDNA fragments. It is 1417 bp in length (except polyA), containing a 41 bp of the 5′-untranslated region (5′-UTR), a 1152 bp of the open reading frame (ORF) encoding a 383 amino acid preproAn-Man5A, and a 224 bp of the 3′-untranslated region (3′-UTR), in which the AATAAA segment as a putative polyadenylation signal was found at 183 bp downstream of the termination codon (see Figure 1). A full-length cDNA sequence of the *Anman5A*, along with its deduced AnMan5A sequence, reported in this paper, has been deposited in the GenBank database under accession number JN123356. To each amplified cDNA fragment, three independent clones were randomly picked out for DNA sequencing. The sequencing results were adopted when three inserted cDNA fragments were identical to one another, or else the experiment was redone.

Analysis of the Primary Structure. The SignalP 3.0 predicted an unambiguous signal peptide cleaving site between Ala^{21} and Leu^{22}, indicating that the AnMan5A is a secretory protein. With the information of the determined N-terminal 15 amino acid residues of the native β-mannanase purified from the cultivated koji of *A. niger* LW-1, it was probable that the preproAnMan5A of 383 amino acids was predicted to contain a 21 amino acid signal peptide from Met^1 to Ala^{21}, a 17 amino acid propeptide from Leu^{22} to Thr^{38}, and a 345 amino acid mature peptide (namely, AnMan5A) (Figure 1). Propeptides also exist in other β-mannanases or microbial enzymes.[19,20] A theoretical molecular weight of the AnMan5A is 37 539 Da that is in good agreement with the determined molecular weight (37.5 kDa) of the deglycosylated *A. usamii* AuMan5A,[7] and a calculated pI is 4.15 that is similar to that (pI 4.2) of the purified *A. usamii* AuMan5A determined by isoelectric focusing (IEF)–PAGE.[21] Homology alignment of amino acid sequences demonstrated that similarities of the AnMan5A of *A. niger* LW-1 (AEP84473) with five fungal β-mannanases of *A. niger* BK01 (ACJ06979), *A. usamii* (ADZ99027), *A. sulphureus* (ABC59553), *A. aculeatus* (AAA67426), and *T. reesei* (AAA34208) from the GH family 5 were 98.8, 98.6, 93.0,
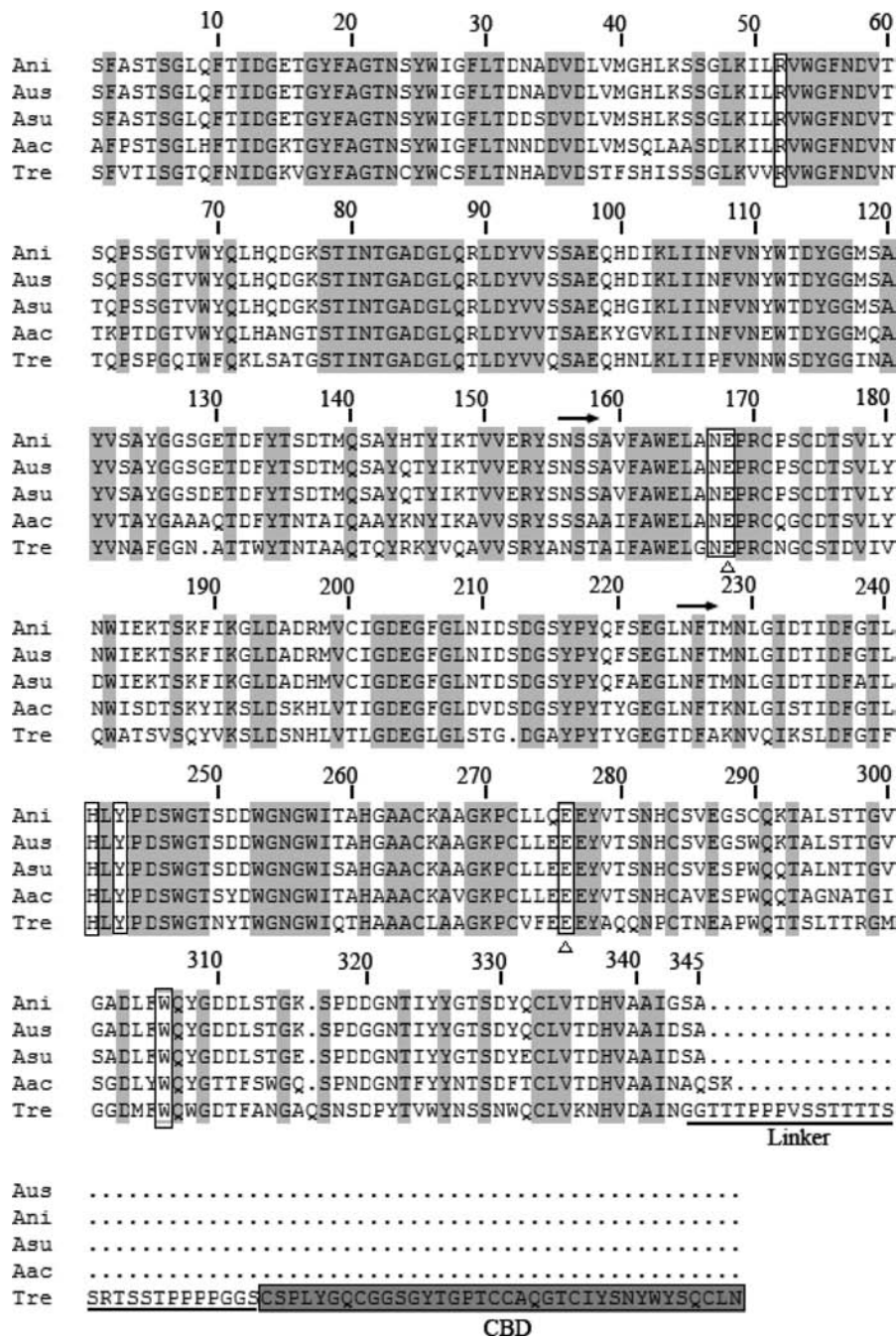
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              10        20        30        40        50        60
              |         |         |         |         |         |
Ani   SFASTSGLQFTIDGETGYFAGTNSYWIGFLTDNADVDLVMGHLKSSGLKILRVWGFNDVT
Aus   SFASTSGLQFTIDGETGYFAGTNSYWIGFLTDNADVDLVMGHLKSSGLKILRVWGFNDVT
Asu   SFASTSGLQFTIDGETGYFAGTNSYWIGFLTDDSDVDLVMSHLKSSGLKILRVWGFNDVT
Aac   AFPSTSGLHFTIDGKTGYFAGTNSYWIGFLTNNDDVDLVMSQLAASDLKILRVWGFNDVN
Tre   SFVTISGTQFNIDGKVGYFAGTNCYWCSFLTNHADVDSTFSHISSSGLKVVRVWGFNDVN

              70        80        90        100       110       120
              |         |         |         |         |         |
Ani   SQPSSGTVWYQLHQDGKSTINTGADGLQRLDYVVSSAEQHDIKLIINFVNYWTDYGGMSA
Aus   SQPSSGTVWYQLHQDGKSTINTGADGLQRLDYVVSSAEQHDIKLIINFVNYWTDYGGMSA
Asu   TQPSSGTVWYQLHQDGKSTINTGADGLQRLDYVVSSAEQHGIKLIINFVNYWTDYGGMSA
Aac   TKPTDGTVWYQLHANGTSTINTGADGLQRLDYVVTSAEKYGVKLIINFVNEWTDYGGMQA
Tre   TQPSPGQIWFQKLSATGSTINTGADGLQTLDYVVQSAEQHNLKLIIPFVNNWSDYGGINA

              130       140       150       160       170       180
              |         |         |         |         |         |
Ani   YVSAYGGSGETDFYTSDTMQSAYHTYIKTVVERYSNSSAVFAWELANEPRCPSCDTSVLY
Aus   YVSAYGGSGETDFYTSDTMQSAYQTYIKTVVERYSNSSAVFAWELANEPRCPSCDTSVLY
Asu   YVSAYGGSDETDFYTSDTMQSAYQTYIKTVVERYSNSSAVFAWELANEPRCPSCDTTVLY
Aac   YVTAYGAAAQTDFYTNTAIQAAYKNYIKAVVSRYSSSAAIFAWELANEPRCQGCDTSVLY
Tre   YVNAFGGN.ATTWYTNTAAQTCYRKYVQAVVSRYANSTAIFAWELGNEPRCNGCSTDVIV
                                                           △

              190       200       210       220       230       240
              |         |         |         |         |         |
Ani   NWIEKTSKFIKGLDADRMVCIGDEGFGLNIDSDGSYPYQFSEGLNFTMNLGIDTIDFGTL
Aus   NWIEKTSKFIKGLDADRMVCIGDEGFGLNIDSDGSYPYQFSEGLNFTMNLGIDTIDFGTL
Asu   DWIEKTSKFIKGLDADHMVCIGDEGFGLNTDSDGSYPYQFAEGLNFTMNLGIDTIDFATL
Aac   NWISDTSKYIKSLDSKHLVTIGDEGFGLDVDSDGSYPYTYGEGLNFTKNLGISTIDFGTL
Tre   QWATSVSQYVKSLDSNHLVTLGDEGLGLSTG.DGAYPYTYGEGTDFAKNVQIKSLDFGTF

              250       260       270       280       290       300
              |         |         |         |         |         |
Ani   HLYPDSWGTSDDWGNGWITAHGAACKAAGKPCLLQEEYVTSNHCSVEGSCQKTALSTTGV
Aus   HLYPDSWGTSDDWGNGWITAHGAACKAAGKPCLLEEEYVTSNHCSVEGSWQKTALSTTGV
Asu   HLYPDSWGTSDDWGNGWISAHGAACKAAGKPCLLEEEYVTSNHCSVESPWQQTALNTTGV
Aac   HLYPDSWGTSYDWGNGWITAHAAACKAVGKPCLLEEEYVTSNHCAVESPWQQTAGNATGI
Tre   HLYPDSWGTNYTWGNGWIQTHAAACLAAGKPCVFEEEYAQQNPCTNEAPWQTISLTTRGM
                                               △

              310       320       330       340       345
              |         |         |         |         |
Ani   GADLFWQYGDDLSTGK.SPDDGNTIYYGTSDYQCLVTDHVAAIGSA.............
Aus   GADLFWQYGDDLSTGK.SPDGGNTIYYGTSDYQCLVTDHVAAIDSA.............
Asu   SADLFWQYGDDLSTGE.SPDDGNTIYYGTSDYECLVTDHVAAIDSA.............
Aac   SGDLYWQYGTTFSWGQ.SPNDGNTFYYNTSDFTCLVTDHVAAINAQSK..........
Tre   GGDMFWQWGDTFANGAQSNSDPYTVWYNSSNWQCLVKNHVDAINGGTTTPPPVSSTTTTS
                                                            Linker

Aus   ...........................................
Ani   ...........................................
Asu   ...........................................
Aac   ...........................................
Tre   SRTSSTPPPPGGSCSPLYGQCGGSGYTGPTCCAQGTCIYSNYWYSQCLN
                       CBD
```

Figure 2. Multiple alignment of protein primary structures between the AnMan5A and the four representative fungal β-mannanases from GH family 5. Abbreviations: Ani, *A. niger* (AEP84473, in this work); Aus, *A. usamii* (ADZ99027); Asu, *A. sulphureus* (ABC59553); Aac, *A. aculeatus* (AAA67426); and Tre, *T. reesei* (AAA34208). The identical amino acid residues among five β-mannanases are marked in gray background. The seven functional amino acid residues are strictly conserved among the GH family 5 members: two triangles below the boxed letters indicate two catalytic residues (E^{168} and E^{276}), and five active site residues (R^{52}, N^{167}, H^{241}, Y^{243}, and W^{306}) are boxed. Two putative N-glycosylation sites are marked in bold arrows. In the *T. reesei* β-mannanase sequence, a Ser/Thr/Pro-rich peptide linker is underlined and a cellulose-binding domain (CBD) is located in a gray box.

73.9, and 56.3%, respectively, but with β-mannanases from the GH family 26 or 113 less than 20%. In addition, multiple homology alignment displayed that the AnMan5A sequence also contained seven functional amino acid residues (Figure 2) that were strictly conserved among the GH family 5 members:[8] two catalytic residues (acid/base, Glu^{168}; nucleophile, Glu^{276}), and five active site residues (Arg^{52}, Asn^{167}, His^{241}, Tyr^{243}, and Trp^{306}). These features verified that the *A. niger* LW-1 AnMan5A is a member of the GH family 5.

The GH family 5 β-mannanases may either only contain one catalytic domain (CD), such as *A. sulphureus* and *A. usamii* β-mannanases,[7] or, besides a CD, carry an additional cellulose-binding domain (CBD), which may locate at the C terminus, such as *T. reesei* β-mannanase,[10] or the N terminus, such as *Phanerochaete chrysosporium* β-mannanase.[22] A Ser/Thr/Pro-rich region (an O-glycosylated linker) in the *T. reesei* β-mannanase sequence links the CD with CBD. On the basis of amino acid sequence analysis, neither a CBD nor a Ser/Thr/

Pro-rich linker was observed in the AnMan5A sequence (Figure 2), suggesting that the AnMan5A has no ability to bind cellulose.

Analysis of the 3D Structure. On the basis of protein crystal structures of the *T. reesei* β-mannanase (1QNO) and *L. esculentum* β-mannanase (1RH9) from the GH family 5, we predicted the 3D structure of the AnMan5A using the bitemplate-based homology modeling method (Figure 3).

Figure 3. Three-dimensional structure of the AnMan5A predicted with the bitemplate-based homology modeling method using both MODELER 9.9 and SALIGN programs based on known protein crystal structures of the *T. reesei* β-mannanase (1QNO) and *L. esculentum* β-mannanase (1RH9). The 3D structure consists principally of the $(\alpha/\beta)_8$ TIM-barrel fold. Two catalytic residues (Glu[168] and Glu[276]) and five active site residues (Arg[52], Asn[167], His[241], Tyr[243], and Trp[306]) were strictly conserved among the GH family 5 members.

The 3D structure consists principally of the $(\alpha/\beta)_8$ TIM-barrel fold. The structure has been likened to a "salad bowl", with one face of the molecule having a large radius (approximately 45 Å) because of an elaborate loop architecture, while the opposite face, which consists of simple α/β turns, has a radius of approximately 30 Å. This is similar to the fold described for the GH family 10 enzymes, and both are members of the GH clan-A.[2] Indeed, these two families are quite closely related, and in addition to sharing a common fold, they have the same type of catalytic mechanism and share several common residues.[23,24] Two catalytic residues, Glu[168] and Glu[276], are located in the hydrophobic cleft of the AnMan5A, where the β-1,4-D-mannosidic linkages of the mannan or heteromannan backbone insert and are cleaved.

Screening and Expression of the Transformants. *P. pastoris* transformant that can resist a higher concentration of Zeocin might contain multiple copies of integration of the heterologous gene into the *P. pastoris* genome, which could potentially lead to a higher expression level of the heterologous protein as explained in the manual of Multi-Copy Pichia Expression Kit (Invitrogen, San Diego, CA). However, the expression level was not directly proportional to the concentration of Zeocin or the copy number of heterologous gene integration.[8] Because of these reasons, we randomly picked out 20 transformants resistant to 100, 200, and 400 μg/mL Zeocin labeled as *P. pastoris* GSAnMan1-1 to GSAnMan1-20, GSAnMan2-1 to GSAnMan2-20, and GSAnMan4-1 to GSAnMan4-20, respectively, for shake flask expression tests.

The *P. pastoris* GS115 transformed with pPICZαA vector (labeled as *P. pastoris* GSC) was used as a negative control. After 96 h of induction by adding 1.0% (v/v) methanol, the cultured supernatants of the transformants were harvested by centrifugation and used for reAnMan5A activity and protein assays. From these transformants tested, one *P. pastoris* transformant expressing the highest reAnMan5A activity (29.0 units/mL), labeled as GSAnMan4-12, was chosen and used for subsequent studies. No β-mannanase activity was detected in the cultured supernatant of the negative control (GSC) under the same expression conditions. The enzyme activities of β-mannanases from *A. sulphereus* MAFIC001,[8] *A. aculeatus* MRC11624,[9] *Bispora* sp. MEY-1,[4] and *T. reesei* RutC30[10] were 96.0, 7.38, 64.0, and 0.0132 units/mL, respectively. The enzyme activity of the reAnMan5A from *A. niger* LW-1 was lower than those of β-mannanases from *A. sulphereus* and *Bispora* sp. Therefore, it is necessary for us to increase the reAnMan5A activity by optimizing expression conditions of the GSAnMan4-12 or directed molecular modification in the future.

Verification of *P. pastoris* Transformants. To verify whether the cDNA fragment of the *Anman5A* was integrated into the *P. pastoris* GS115 genome, the genomic DNAs of three representative *P. pastoris* transformants, GSC, GSAnMan1-2, and GSAnMan4-12, were extracted as templates and analyzed by PCR amplification using 5'- and 3'-*AOX1* primers. A 2.2 kb complete alcohol oxidase 1 (*AOX1*) gene in *P. pastoris* GS115 and a 490 bp *AOX1* gene fragment on the pPICZαA vector were amplified by PCR from *P. pastoris* GSC (lane 1 of Figure
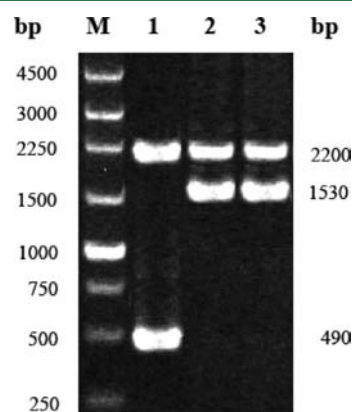

Figure 4. Verification of three representative *P. pastoris* transformants, GSC, GSAnMan1-2, and GSAnMan4-12, by PCR analysis using 5'- and 3'-*AOX1* primers. Lane M, DNA marker; lane 1, PCR products of the GSC genome; and lanes 2 and 3, PCR products of the GSAnMan1-2 and GSAnMan4-12 genomes, respectively.

4). However, a 2.2 kb complete *AOX1* gene and a 1.53 kb DNA fragment consisting of a 490 bp *AOX1* gene and a 1038 kb cDNA gene were amplified by PCR from *P. pastoris* GSAnMan1-2 or GSAnMan4-12 (lane 2 or 3 of Figure 4). These results demonstrated that, in positive transformants, the cDNA gene encoding the AnMan5A had been integrated into the *P. pastoris* GS115 genome.

Purification of the reAnMan5A. It was reported that the purity of the recombinant *A. sulphureus* β-mannanase expressed in *P. pastoris* X-33 was 97%.[8] In our work, the purity of the reAnMan5A in the cultured supernatant of *P. pastoris* GSAnMan4-7 was more than 85% (lane 3 of Figure 5),
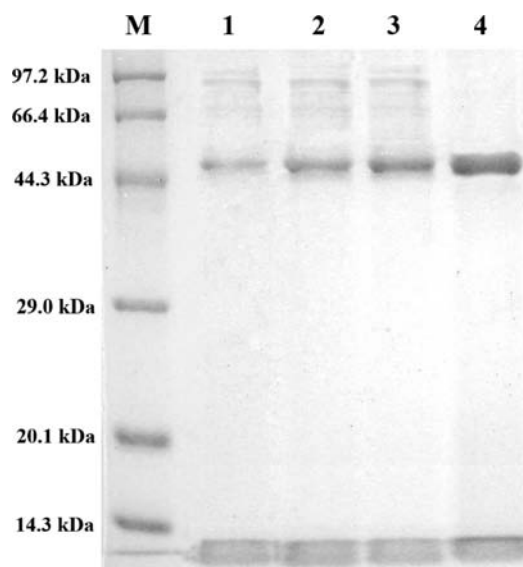
Figure 5. SDS−PAGE analysis of the reAnMan5A secreted by the *P. pastoris* GSAnMan4-12. Lane M, protein marker; lane 1, cultured supernatant of the GSAnMan4-12 (induction period of 72 h); lane 2, cultured supernatant of the GSAnMan4-12 (induction period of 84 h); lane 3, cultured supernatant of the GSAnMan4-12 (induction period of 96 h); and lane 4, purified reAnMan5A of the GSAnMan4-12 (induction period of 96 h).

which will greatly facilitate the purification of the reAnMan5A and decrease the industrial production and application costs. Hence, the expressed reAnMan5A could be purified to homogeneity by a simple combination of the ammonium sulfate fraction, ultrafiltration, and Sephadex G-75 gel filtration. The specific enzyme activity of the purified reAnMan5A, toward locust bean gum at pH 3.6 and 50 °C for 10 min, was 211.8 units/mg, which was similar to those of the purified native β-mannanases from *A. niger* WM-20[25] and *A. niger* ATCC-46890[19] but lower than that of the recombinant β-mannanase from *A. niger* BK01.[26]

SDS−PAGE analysis of the purified reAnMan5A showed one single protein band with an apparent molecular weight of about 52.0 kDa (lane 4 of Figure 5), which is much larger than the theoretical molecular weight of the AnMan5A (37 539 Da). *P. pastoris* enables some post-translational modifications, including the assembly of disulfide bond, the exclusion of the signal peptide, the glycosylation of mature peptide, etc. To verify whether the difference between the apparent molecular weight and theoretical molecular weight is due to N-glycosylation, deglycosylation analysis was carried out. After the reAnMan5A was catalyzed with endoglycosidase H to remove carbohydrate moieties, a clear protein band of about 38.0 kDa and several faint bands located between 38.0 and 52.0 kDa were observed on SDS−PAGE (data not shown). Furthermore, the carbohydrate content of the purified reAnMan5A was determined to be 27.3% by the phenol−sulfuric acid method. These analytical results strongly verified that the reAnMan5A is a N-glycosylated protein, which is also in good agreement with the fact that there are two putative N-glycosylation sites, N^{156}−S−S^{158} and N^{225}−F−T^{227}, in the AnMan5A sequence deduced from its cDNA fragment of the *Anman5A* (Figure 2).

Enzymatic Properties of the reAnMan5A. The purified reAnMan5A exhibited higher enzyme activities over a pH range of 3.0−4.5, among which the highest specific enzyme activity

(100%) of 212.5 units/mg was at pH 3.5 (measured at 50 °C for 10 min). Preincubated at 40 °C for 1.0 h at varied pH values, the reAnMan5A displayed high stability at a wide pH range of 3.0−7.0, retaining more than 85% of the original activity (Figure 6a). The retained highest specific enzyme activity (100%) of 210.6 units/mg was at pH 4.5.
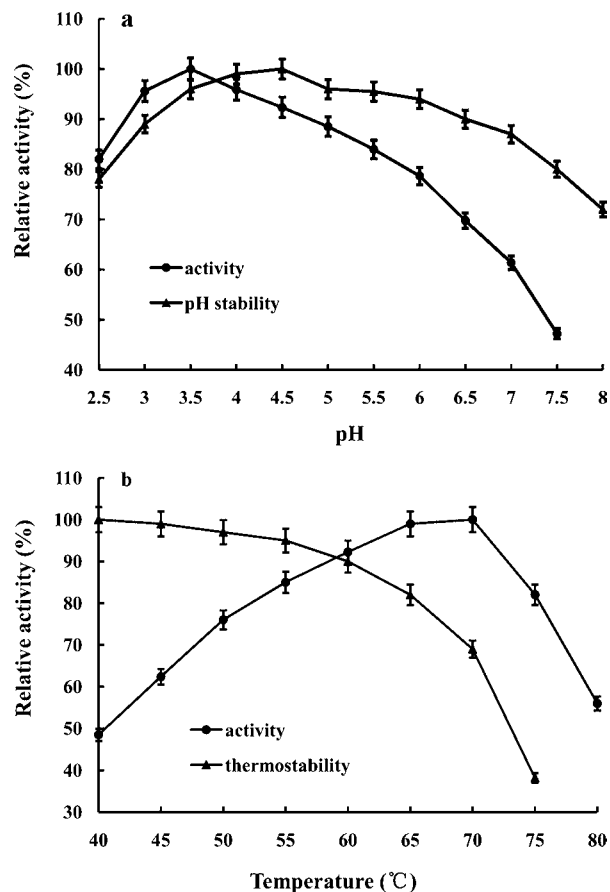

Figure 6. Effects of temperatures and pH values on the reAnMan5A activity. (a) pH optimum and stability. The optimal pH of the reAnMan5A was measured by the DNS method using Na_2HPO_4−citric acid buffer (pH 2.5−7.5), among which the highest specific enzyme activity (100%) of 212.5 units/mg was at pH 3.5. The pH stability of the reAnMan5A was determined by incubating in Na_2HPO_4−citric acid buffer (pH 2.5−7.5) and Tris-HCl buffer (pH 8.0) at 40 °C for 1.0 h, and then the residual enzyme activities were measured using the DNS method. The retained highest specific enzyme activity (100%) was 210.6 units/mg at pH 4.5. (b) Temperature optimum and stability. The optimal temperature of the reAnMan5A was measured using the DNS method, except the reaction temperatures ranging from 40 to 80 °C. The 100% specific enzyme activity of 285.4 units/mg was at 70 °C. The thermostability of the reAnMan5A was determined by incubating it at various temperatures (40−75 °C) for 1.0 h, and then the residual enzyme activities were measured using the DNS method. The reAnMan5A retained 100% specific enzyme activity of 206.8 units/mg at 40 °C.

The optimal temperature for the reAnMan5A activity was 70 °C (measured at pH 3.5 for 10 min), at which the highest specific enzyme activity (100%) was 285.4 units/mg. Preincubated at pH 4.0 for 1.0 h at various temperatures (40−75 °C), the reAnMan5A retained 100% specific enzyme activity of 206.8 units/mg at 40 °C, also retained over 85% of the original activity at a temperature of 60 °C or below,

declined over 60 °C, and only remained 38.2% of the original activity at 75 °C (Figure 6b).

Preincubated with various metal ions and EDTA at 40 °C for 1.0 h, the reAnMan5A activities were not significantly affected by Ca^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Al^{3+}, Fe^{2+}, Fe^{3+}, Zn^{2+}, Ba^{2+}, Pb^{2+}, Mn^{2+}, and EDTA but strongly inhibited by Ag^+ and Hg^{2+}, with only 39.2 and 32.5% of the original reAnMan5A activity, respectively. The enzymatic properties of the reAnMan5A described above are similar to those of the native β-mannanase purified from the cultivated koji of A. niger LW-1.[3]

Substrate Specificity and Kinetic Parameters. The purified reAnMan5A, toward locust bean gum at pH 3.6 and 50 °C for 10 min, had the highest β-mannanase activity of 211.8 units/mg (100%), followed by konjac flour (79.4%) and guar gum (41.1%). No activity was detected if sodium carboxymethyl cellulose or birchword xylan was used as the substrate. The K_m and V_{max} values of the purified reAnMan5A for locust bean gum were graphically determined from Lineweaver–Burk plotting to be 1.10 mg/mL and 266.7 units/mg, respectively.

The yeast P. pastoris expression system, as a eukaryotic expression system, has been a favorite system for expressing heterologous proteins, owing to its many advantages, such as protein processing, folding, post-translational modification, and secretion. In addition, P. pastoris can be grown and induced in inexpensive media, and the purity of the expressed recombinant protein was high. In this work, we cloned a full-length cDNA sequence and a cDNA fragment sequence of the Anman5A, performed its expression in P. pastoris, purified the reAnMan5A to electrophoretical homogeneity, and characterized its enzymatic properties. In addition, we predicted the 3D structure of the AnMan5A using the bitemplate-based homology modeling method. Considering the rapid accumulation of the primary and 3D structures of β-mannanases, we will further determine the 3D structure of the AnMan5A. In addition, our subsequent studies will focus on how to increase the reAnMan5A activity by optimizing expression conditions or directed molecular modification as well as how to realize the reAnMan5A production by fermentation on an industrial scale.

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■ REFERENCES

(1) van Zyl, W. H.; Rose, S. H.; Trollope, K.; Görgens, J. F. Fungal β-mannanases: Mannan hydrolysis, heterologous production and biotechnological applications. Proc. Biochem. 2010, 45, 1203–1213.

(2) Zhang, Y. L.; Ju, J. S.; Peng, H.; Gao, F.; Zhou, C.; Zeng, Y.; Xue, Y. F.; Li, Y.; Henrissat, B.; Gao, G. F.; Ma, Y. H. Biochemical and structural characterization of the intracellular mannanase AaManA of Alicyclobacillus acidocaldarius reveals a novel glycoside hydrolase family belonging to clan GH-A. J. Biol. Chem. 2008, 283, 31551–31558.

(3) Wu, M. C.; Tang, C. D.; Li, J. F.; Zhang, H. M.; Guo, J. Bimutation breeding of Aspergillus niger strain for enhancing β-mannanase production by solid-state fermentation. Carbohydr. Res. 2011, 346, 2149–2155.

(4) Luo, H. Y.; Wang, Y. R.; Wang, H.; Yang, J.; Yang, Y. H.; Huang, H. Q.; Yang, P. L.; Bai, Y. G.; Shi, P. J.; Fan, Y. L.; Yao, B. A novel highly acidic β-mannanase from the acidophilic fungus Bispora sp. MEY-1: Gene cloning and overexpression in Pichia pastoris. Appl. Microbiol. Biotechnol. 2009, 82, 453–461.

(5) Song, J. M.; Nam, K. W.; Kang, S. G.; Kim, C. G.; Kwon, S. T.; Lee, Y. H. Molecular cloning and characterization of a novel cold-active β-1,4-D-mannanase from the Antarctic springtail, Cryptopygus antarcticus. Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol. 2008, 151, 32–40.

(6) Zhao, W.; Zheng, J.; Zhou, H. B. A thermotolerant and cold-active mannan endo-1,4-β-mannosidase from Aspergillus niger CBS 513.88: Constitutive overexpression and high-density fermentation in Pichia pastoris. Bioresour. Technol. 2011, 102, 7538–7547.

(7) Tang, C. D.; Guo, J.; Wu, M. C.; Zhao, S. G.; Shi, H. L.; Li, J. F.; Wang, J. Q. Cloning and bioinformatics analysis of a novel acidophilic β-mannanase gene, AumanSA, from Aspergillus usamii YL-01-78. World J. Microbiol. Biotechnol. 2011, DOI: 10.1007/s11274-011-0775-6.

(8) Chen, X. L.; Cao, Y. H.; Ding, Y. H.; Lu, W. Q.; Li, D. F. Cloning, functional expression and characterization of Aspergillus sulphureus β-mannanase in Pichia pastoris. J. Biotechnol. 2007, 128, 452–461.

(9) Roth, R.; Moodley, V.; van Zyl, P. Heterologous expression and optimized production of an Aspergillus aculeatus endo-1,4-β-mannanase in Yarrowia lipolytica. Mol. Biotechnol. 2009, 43, 112–120.

(10) Stalbrand, H.; Saloheimo, A.; Vehmaanpera, J.; Henrissat, B.; Penttila, M. Cloning and expression in Saccharomyces cerevisiae of a Trichoderma reesei β-mannanase gene containing a cellulose binding domain. Appl. Environ. Microbiol. 1995, 61, 1090–1097.

(11) Bansal, N.; Tewari, R.; Gupta, J. K.; Soni, R.; Soni, S. K. A novel strain of Aspergillus niger producing a cocktail of hydrolytic depolymerising enzymes for the production of second generation biofuels. BioResources 2011, 6, 552–569.

(12) Li, J. F.; Wu, M. C.; Xia, W. S. Study on fermentation conditions of acidic β-mannanase from Aspergillus niger LW-1. J. Food Sci. Biotechnol. 2007, 26, 88–92.

(13) Li, J. F.; Wu, M. C.; Xia, W. S. Study on the koji plate fermentation process of the acidic β-mannanase from Aspergillus niger and its partial properties. Food Ferment. Ind. 2008, 34, 5–9.

(14) Sambrook, J.; Russell, D. W. Molecular Cloning: A Laboratory Manual, 3rd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 2001; pp 1595–1604.

(15) Wu, M. C.; Wang, J. Q.; Zhang, H. M.; Tang, C. D.; Gao, J. H.; Tan, Z. B. Cloning and sequence analysis of an acidophilic xylanase (XynI) gene from Aspergillus usamii E001. World J. Microbiol. Biotechnol. 2011, 27, 831–839.

(16) Li, J. F.; Tang, C. D.; Shi, H. L.; Wu, M. C. Cloning and optimized expression of a neutral endoglucanase gene (ncel5A) from Volvariella volvacea WX32 in Pichia pastoris. J. Biosci. Bioeng. 2011, 111, 537–540.

(17) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970, 227, 680–685.

(18) Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. Colorimetric method for determination of sugars and related substances. Anal. Chem. 1956, 28, 350–356.

(19) Ademark, P.; Varga, A.; Medve, J.; Harjunpaa, V.; Drakenberg, T.; Tjerneld, F.; Stalbrand, H. Softwood hemicellulose-degrading enzymes from Aspergillus niger: Purification and properties of a β-mannanase. J. Biotechnol. 1998, 63, 199–210.

(20) Wang, J. Q.; Zhang, H. M.; Wu, M. C.; Tang, C. D. Cloning and sequence analysis of a novel xylanase gene, Auxyn10A, from Aspergillus usamii. Biotechnol. Lett. 2011, 33, 1029–1038.

(21) Li, J. F.; Ma, L. P.; Wu, M. C.; Xia, W. S. Purification and some properties of an acidic β-mannanase from Aspergillus usamii. Food Ferment. Ind. 2006, 32, 5–9.

(22) Benech, R. O.; Li, X.; Patton, D.; Powlowski, J.; Storms, R.; Bourbonnais, R.; Paice, M.; Tsang, A. Recombinant expression,

characterization, and pulp prebleaching property of a *Phanerochaete chrysosporium endo-β*-1,4-mannanase. *Enzyme Microb. Technol.* **2007**, *41*, 740−747.

(23) Larsson, A. M.; Anderson, L.; Xu, B.; Munoz, I. G.; Uson, I.; Janson, J. C.; Stalbrand, H.; Stahlberg, J. Three-dimensional crystal structure and enzymic characterization of *β*-mannanase Man5A from blue mussel *Mytilus edulis*. *J. Mol. Biol.* **2006**, *357*, 1500−1510.

(24) Larson, S. B.; Day, J.; Barba de la Rosa, A. P.; Keen, N. T.; McPherson, A. First crystallographic structure of a xylanase from glycoside hydrolase family 5: Implications for catalysis. *Biochemistry* **2003**, *42*, 8411−8422.

(25) Zhu, J.; Li, J. F.; Wu, M. C. Studies on purification and some properties of the acidic *β*-mannanase from *Aspergillus niger*. *J. Agric. Univ. Hebei* **2007**, *30*, 55−59.

(26) Cuong, D. B.; Thu, D. T.; Berrin, J. G.; Haltrich, D.; Anh, T. K.; Sigoillot, J. C.; Yamabhai, M. Cloning, expression in *Pichia pastoris*, and characterization of a thermostable GH5 mannan *endo*-1,4-*β*-mannosidase from *Aspergillus niger* BK01. *Microb. Cell Fact.* **2009**, *8*, 59−70.