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ARTICLE

An Acidophilic and Acid-Stable β-Mannanase from *Phialophora* sp. P13 with High Mannan Hydrolysis Activity under Simulated Gastric Conditions

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A β-mannanase gene, *man5AP13*, was cloned from *Phialophora* sp. P13 and expressed in *Pichia pastoris*. The deduced amino acid sequence of the mature enzyme, MAN5AP13, had highest identity (53%) with the glycoside hydrolase family 5 β-mannanase from *Bispora* sp. MEY-1. The purified recombinant β-mannanase was acidophilic and acid stable, exhibiting maximal activity at pH 1.5 and retaining >60% of the initial activity over the pH range 1.5−7.0. The optimum temperature was 60 °C. The specific activity, K_m and V_{max} for locust bean gum substrate were 851 U/mg, 2.5 mg/mL, and 1667.7 U/min·mg, respectively. The enzyme had excellent activity and stability under simulated gastric conditions, and the released reducing sugar of locust bean gum was significantly enhanced by one-fold in simulated gastric fluid containing pepsin in contrast to that without pepsin. All these properties make MAN5AP13 a potential additive for use in the food and feed industries.

KEYWORDS: ***Phialophora* sp.; acidophilic and acid-stable enzyme; β-mannanase; *Pichia pastoris***

INTRODUCTION

Hemicelluloses, a group of complex polysaccharides, are the main constituents of plant cell walls. Based on their backbone composition, hemicelluloses can be classified as xylans, mannans, arabinogalactans or arabinans (*1*). Among them, mannans and heteromannans are the second most abundant hemicellulosic polysaccharides in nature (*2*, *3*), and their hydrolysis is catalyzed by β-mannanases (*endo*-1,4-β-mannanase; EC 3.2.1.78). To date, most mannanases have been classified into glucoside hydrolase (GH) families 5 and 26 based on amino acid sequence (http://www.cazy.org/) (*4*). A new family, GH 113, also contains some mannanases (*5*). According to their three-dimensional structures, β-mannanases in these three families all belong to GH clan-A (*5*, *6*).

Many mannanase genes have been cloned from bacteria, fungi and plants (*7*, *8*). Some mannanases retain their activity under extreme conditions, such as the β-mannanases from *Bispora* sp. MEY-1 (*9*), *Trichoderma harzianum* T4 (*10*), and *Aspergillus sulphureus* (*11*), which have extremely acidic pH optima, and the mannanases from *Bacillus* sp. JAMB-750 (*12*) and N16-5 (*13*), which have extremely alkaline pH optima. As such, these mannanases have attracted much attention owing to their potential use in diverse biotechnological processes.

Here we report the cloning of a mannanase gene from the acidophilic fungal strain *Phialophora* sp. P13 and expression of the gene in *Pichia pastoris*. The recombinant mannanase, denoted MAN5AP13, showed maximum activity at pH 1.5, had strong resistance to proteolysis by pepsin or trypsin, and remained stable under simulated gastric conditions. These properties suggest its potential application in the food and feed industries. To our knowledge, this is the first report on the cloning and expression of a β-mannanase gene from the genus *Phialophora*.

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MATERIALS AND METHODS

Microorganism Isolation. Strain 13 was isolated from the acidic wastewater of a tin mine in Yunnan Province, China. Mud and water samples were mixed thoroughly at a ratio of 1:1 (w/w) and spread onto potato dextrose agar plates (contained: 20 g/L of potato, 20 g/L sucrose, and 17 g/L agar, pH 3.0) containing 50 mg/L ampicillin. Colonies were subcultured on potato dextrose agar plates to obtain purity. The taxon of strain P13 was identified based on morphologic characteristics and on internal transcribed spacer (ITS) sequence in rDNA.

Strains, Media, Vectors, and Chemicals. *Escherichia coli* Trans1-T1 (TransGen, Beijing, China) was cultivated at 37 °C in Luria−Bertani medium supplemented with 100 μg/mL ampicillin for positive transformant selection. *P. pastoris* GS115 (Invitrogen, Carlsberg, CA) was cultivated at 30 °C in yeast extract peptone dextrose medium.

The medium for induction of β-mannanase was composed of 30 g/L wheat bran, 30 g/L corn cob, 30 g/L soybean meal, 5 g/L konjac flour, 5 g/L $(NH_4)_2SO_4$, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.5 g/L KCl, 0.2 g/L $CaCl_2$ and 0.01 g/L $FeSO_4 \cdot 7H_2O$, pH 3.0. Buffered glycerol-complex medium, buffered methanol-complex medium, minimal dextrose medium, and minimal methanol medium were prepared according the manual of the *Pichia* expression kit (Invitrogen).

The plasmids pGEM-T Easy (Promega, Madison, WI) and pPIC9 (Invitrogen) were used for gene cloning and expression, respectively. Locust bean gum (LBG) was purchased from Sigma (St. Louis, MO). The DNA purification kit, *LA Taq* DNA polymerase, and restriction endonucleases were purchased from TaKaRa (Kyoto, Japan), and T4 DNA ligase was from New England Biolabs (Hitchin, U.K.). Trans1-T1 phage resistant chemically competent cells were supplied by TransGen. All other chemicals are of analytical grade and commercially available.

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Table 1. Primers Used in This Study

primer name	sequence (5′→3′)[a]	size (bp)
M P1	AAYAAYTGGGAYGAYTWYGGNGG	23
M P2	GGYTCRYYNSCNARYTCCCANGC	23
spF1	CAACACCTACTGCGCCGTGTACGG	24
spF2	GCAATGCTACCACTTTCTACACTAGCGCG	29
spF3	CCGCAGGAACTATATCCAGTTTATCGTCAACC	32
spR1	GGTTGACGATAAACTGGATATAGTTCCTGCGG	32
spR2	GTTCCTGCGGAGAATCGTTAGCTCTGAC	28
spR3	CCGTACACGGCGCAGTAGTGTTG	23
Prt-F	GCAATGGTGTCCTCTACTACTCTTGT	26
Prt-R	GTTAAGGTACTACAGGCTTCGCGT	24
PpicF	GGGGAATTCGTGTCATGGGGCTTTGGCCAGGG	32
PpicR	GGGGCGGCCGCTTAAGGTACTACAGGCTTCGCGTCCATAG	40

[a]Y = C/T, W = A/T, R = A/G, S = C/G, N = A/T/G/C; restriction sites incorporated into primers are underlined.

Cloning of the Full-Length Chromosomal β-Mannanase Gene. The core region of the β-mannanase gene from strain P13 was amplified by a degenerate primer set (M P1 and M P2) specific for GH 5 β-mannanases designed by Luo et al. (*9*). The genomic DNA of strain P13 was extracted and used as a template for PCR amplification. PCR reactions were performed as follows: 4 min at 95 °C, 10 cycles of 95 °C for 30 s, 50 to 45 °C for 30 s (decreasing 0.5 °C after each cycle), and 72 °C for 1 min, followed by 30 cycles of 95 °C for 30 s, 45 °C for 30 s, and 72 °C for 1 min, and a final extension at 72 °C for 8 min. The PCR products were purified and ligated into the pGEM-T Easy vector for sequencing. The sequence of the core region fragment was analyzed by BLAST (http://www.ncbi.nlm.nih.gov/BLAST). The 5′ and 3′ flanking fragments of the core region were amplified using thermal asymmetric interlaced (TAIL)-PCR (*14*) with three forward special primers (spF 1−3) and three reverse special primers (spR 1−3) (**Table 1**).

Total RNA Isolation and Cloning of a Full-Length Complementary DNA of the β-Mannanase Gene. Strain P13 was grown in 100 mL of inducing medium at 28 °C for 6 days with constant agitation of 200 rpm. Mycelia were collected, and the total RNA was extracted and purified using the RNeasy Plant Mini kit (QIAGEN, Hilden, Germany) according to the instructions of manufacturer. Reverse transcription was performed using the ReverTra Ace-α-TM kit (TOYOBO, Osaka, Japan). The full-length cDNA of the β-mannanase gene (*man5AP13*) was obtained from total RNA by RT-PCR with pfu DNA polymerase (TIANGEN, Beijing, China) using the primers Prt-F and Prt-R (see **Table 1**) designed based on the chromosomal DNA sequence.

Sequence Analysis. The exon-intron structure of the full-length mannanase gene was predicted using GENESCAN (http://genes.mit.edu/GENSCAN.html) and FGENESH (http://www.softberry.com/berry.phtml/). The open-reading frame (ORF) finder tool (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) was used to identify the ORF in the cDNA sequence. The signal peptide of the β-mannanase from strain P13 was predicted using SignalP (http://www.cbs.dtu.dk/services/SignalP/). The molecular mass of the mature peptide was calculated using Vector NTI 7.0 software. The search for protein families, domains, and active sites was performed using the InterProScan Web site (http://www.ebi.ac.uk/InterProScan). The three-dimensional structure and charge distribution on the surface of MAN5AP13 was predicted using Swiss-pdb viewer (http://www.expasy.ch/spdbv) and Discovery Studio 2.5 MODELER (Accelrys, San Diego, CA), respectively, with the β-mannanase from *Trichoderma reesei* (pdb number 1QNO) (*15*) as template.

Expression of the β-Mannanase Gene *man5AP13* in *P. pastoris*. The gene fragment containing *man5AP13* without the signal peptide coding sequence was amplified by PCR using primers PpicF and PpicR (**Table 1**). The PCR product was digested with *Eco*RI and *Not*I and ligated into pPIC9 under the control of the alcohol oxidase promoter. The resulting construct, pPIC9-*man5AP13*, was confirmed by restriction digestion and DNA sequencing. The correct expression vector was linearized using *Bgl*II and then transformed into *P. pastoris* GS115 competent cells by electroporation. The transformed cells were cultured on minimal dextrose plates at 30 °C for 2−3 days until colonies formed. His^+ transformants were then transferred to minimal methanol and minimal dextrose plates and grown for 2−3 days at 30 °C to screen positive clones. Colonies that appeared on plates were transferred into 3 mL of buffered glycerol-complex medium and grown at 30 °C in a shaking incubator for about 2 days. The cells were pelleted by centrifugation and resuspended in 1 mL of buffered methanol-complex medium containing 0.5% methanol for induction at 20 °C. The culture supernatant was collected by centrifugation and subjected to β-mannanase activity assay. The transformant exhibiting the highest enzymatic activity was selected for fermentation in a 1 L flask. The scale-up fermentation protocol was the same as described above, except that 100% methanol was added to a final concentration of 0.5% every 24 h to maintain induction.

Purification and Deglycosylation of Recombinant MAN5AP13. The induced culture was centrifuged at 12000*g* for 10 min at 4 °C to remove cell debris. The culture supernatant was concentrated by a hollow fiber (cutoff 6 kDa; Motianmo, Tianjin, China) and subjected to ammonium sulfate fractionation (20−60%). The fraction containing the enzymatic activity was dissolved in 7 mL of 20 mM citric acid−Na_2HPO_4 buffer (pH 7.0) and dialyzed against the same buffer overnight. The clear supernatant was loaded onto a HiTrap Q Sepharose XL 5 mL FPLC column (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with the same citric acid−Na_2HPO_4 buffer. A linear gradient of NaCl (0−1.0 M) was used to elute the proteins. Fractions having β-mannanase activity were pooled and concentrated by ultrafiltration at 4000*g* for 20 min at 4 °C using an Amicon Ultra Centrifugal Filter Device PL-10 (Millipore, Chicago, IL).

Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was carried out with a 5% stacking gel and a 12% separation gel as described by Laemmli (*16*). The concentration of the recombinant enzyme was determined by Bradford assay using bovine serum albumin as the standard (*17*). A sample of purified recombinant MAN5AP13 (5 μg) was subjected to deglycosylation using 250 U of *endo*-β-*N*-acetylglucosaminidase H (Endo H) for 2 h at 37 °C following the manufacturer's instructions (New England Biolabs) and then analyzed by SDS−PAGE.

Enzyme Activity Assay. The β-mannanase activity of MAN5AP13 was determined using the 3,5-dinitrosalicylic acid (DNS) method (*18*) by measuring the amount of reducing sugar released. The reaction system was composed of 0.1 mL of appropriately diluted enzyme and 0.9 mL of 0.1 M glycine-HCl buffer (pH 1.5) containing 0.5% (w/v) LBG. After incubation at 37 °C for 10 min, the reaction was stopped by the addition of 1.5 mL of DNS reagent, boiled in a water bath for 10 min, and cooled to room temperature. The absorbance at 540 nm was measured. One unit of β-mannanase activity was defined to be the amount of enzyme required to release 1 μmol of reducing sugar from the substrate per minute under the assay conditions.

Biochemical Characterization of Purified Recombinant MAN5AP13. The optimum pH of purified recombinant MAN5AP13 for β-mannanase activity was determined at 37 °C for 10 min in the following buffers: 0.1 M glycine-HCl (pH 1.0−3.0), 0.1 M citric acid-Na_2HPO_4 (pH 3.0−8.0), 0.1 M Tris-HCl (pH 8.0−9.0), and 0.1 M glycine-NaOH (pH 9.0−11.0). To estimate the pH stability, the enzyme was preincubated in the buffers described above without substrate at 37 °C for 2 h, and the residual activity was assayed at optimal pH and temperature for 10 min (standard conditions).

The optimal temperature for β-mannanase activity was determined at various temperatures from 20 to 75 °C and pH 1.5 for 10 min. Thermal stability of the purified recombinant enzyme was determined by measuring the residual enzyme activity under standard conditions after incubation of the enzyme at 50, 55, or 60 °C for 2, 5, 10, 20, 30, 60, or 120 min.

The resistance of purified recombinant MAN5AP13 against proteolysis by pepsin or trypsin was determined by incubating the enzyme with pepsin in 0.1 M glycine-HCl (pH 2.0) or trypsin in 0.1 M Tris-HCl (pH 7.0) at 37 °C for different periods at a ratio of MAN5AP13:protease (w/w) of 1:0.1, and measuring the residual enzyme activity under standard conditions following protease treatment.

To determine the effect of metal ions and chemical reagents on the enzyme activity of purified recombinant MAN5AP13, 1 or 10 mM of various metal ions (NaCl, KCl, $CaCl_2$, LiCl, $CoCl_2$, $CrCl_3$, $NiSO_4$, $CuSO_4$, $MgSO_4$, $FeCl_3$, $MnSO_4$, $ZnSO_4$, $Pb(CH_3COO)_2$, $AgNO_3$, or $HgCl_2$) or reagents (SDS, β-mercaptoethanol or EDTA) was added in the assay system, and the residual enzyme activity was determined. The control was the standard system without any additive.

Substrate Specificity and Kinetic Parameters. The substrate specificity of MAN5AP13 was determined by measuring the enzyme activity in

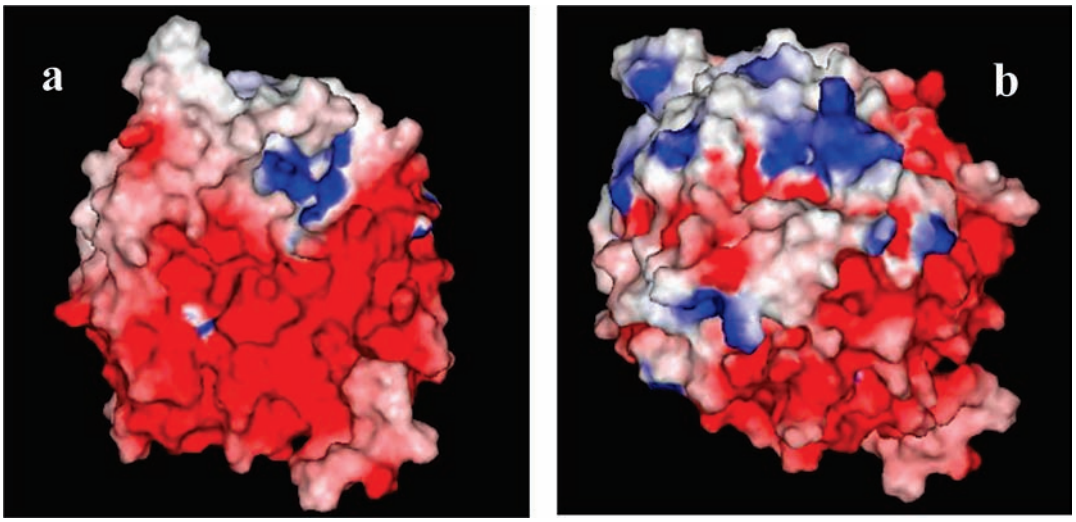

Figure 1. Charge distribution on the surface of a three-dimensional model of MAN5AP13 predicted using Swiss-pdb viewer (http://www.expasy.ch/spdbv) with the β-mannanase from *Trichoderma reesei* (44% identity; pdb number 1QNO) (*15*) as template and calculated with Discovery Studio 2.5 MODELER at pH 7.0. Positive charges are depicted in blue and negative charges in red. (**a**) The surface showing the substrate cleft with the active site. (**b**) The opposite-side surface.

0.1 M glycine-HCl containing 0.5% of LBG, guar gum, konjac flour, carboxymethyl cellulose (sodium), or birchwood xylan at 60 °C and pH 1.5 for 10 min. The amount of reducing sugars produced was estimated using the DNS method described above.

Enzyme kinetic assays were performed in 0.1 M glycine-HCl buffer (pH 1.5) containing 0.25−10 mg/mL substrates at 60 °C for 10 min. The K_m and V_{max} values were determined based on Lineweaver−Burk plotting (*19*). The experiments were carried out three times, and each experiment included three replicates.

Analysis of Hydrolysis Products. The reaction system containing 100 U purified MAN5AP13 and 800 μg of LBG, konjac flour, or guar gum in 800 μL of 0.1 M glycine-HCl (pH 1.5) was incubated at 50 °C for 16 h. The enzyme was removed from the reaction system using the Nanosep centrifugal 3 K device (Pall, Port Washington, NY). The remaining products were analyzed using high-performance anion exchange chromatography with a model 2500 system from Dionex (Sunnyvale, CA) (*20*). Mannose, mannobiose, mannotriose, mannotetraose, and manno pentaose were used as standards.

Enzyme Stability and Hydrolysis Capacity in Simulated Gastric Fluid. The stability of the purified recombinant MAN5AP13 in simulated gastric fluid (SGF; 0.2 M glycine-HCl or 0.2 M sodium acetate/acetic acid containing 2.0 mg/mL NaCl and 3.2 mg/mL pepsin) (*21*, *22*) was determined by incubating the enzyme (20 U) in 50 mL of SGF at 37 °C for 60 min at the following pHs: 1.5, 2.0, 2.8, 3.8, 4.6, or 5.5 (pH was adjusted with HCl or sodium acetate). The residual enzyme activity was determined at pH 1.5, 37 °C for 10 min. The buffer (0.2 M glycine-HCl or 0.2 M sodium acetate/acetic acid) and SGF without pepsin (N-SGF) were used as controls.

The hydrolysis capacity of recombinant MAN5AP13 to LBG was determined in SGF with a pH gradient model (*23*) with little modification. Reaction system of 0.5% substrate (w/v) and 0.1% purified enzyme (1 U/mL; v/v) in 30 mL SGF was incubated at 37 °C with constant agitation at 220 rpm as follows: pH 2.0, 20 min; pH 5.5, 10 min; pH 4.6, 10 min; pH 3.8, 10 min; pH 2.8, 20 min; and pH 2.0, 40 min. At the beginning of each pH step, 0.53 mg/mL pepsin was added to the reaction system (*21*). The entire experiment was carried out in a rotary incubator. SGF without pepsin was used as a control.

Nucleotide Sequence Accession Numbers. Nucleotide sequences for the *Phialophora* sp. P13 ITS region and the β-mannanase gene (*man5AP13*) were deposited in GenBank under accession numbers GU082376 and GU082377, respectively.

RESULTS

Microorganism Isolation and Identification. Colony of strain P13 was woolly to velvety. The hyphae were branched, and yellow or green. Conidia were unicellular, hyaline or brown, oval in shape, and accumulated at the apexes of the phialides. The full-length ITS sequence (582 bp) had the highest nucleotide identity (97%) with that from *Phialophora* sp. WRCF-AW14 (GenBank accession no. AY618682.1). Based on morphology and ITS sequence analysis, strain P13 was identified to belong to the genus *Phialophora* and deposited in the China General Microbiological Culture Collection Center under CGMCC3329. *Phialophora* sp. P13 grew optimally at pH 3.0, and the mannanase activity in the inducing medium was 1.77 U/mL.

Cloning and Sequence Analysis of the β-Mannanase Gene, *man5AP13*, from *Phialophora* sp. P13. A partial β-mannanase gene fragment from *Phialophora* sp. P13, 232 bp, was amplified by PCR with degenerate primers M P1 and M P2 (*4*) and cloned into pGEM-T Easy for sequencing. The 5′ and 3′ flanking regions of the core region were amplified by TAIL-PCR using the nested internal primers designed based on the core region. The flanking and core regions were assembled with vector NTI 7.0 and resulted in a DNA fragment of 2,510 bp. Based on the analysis of GENESCAN and FGENESH, a complete chromosomal gene consisting of 1,471 bp was identified.

The full-length cDNA sequence for the β-mannanase was obtained via reverse transcription-PCR using the specific primers Prt-F and Prt-R and contained a 1,260-bp ORF. Four introns interrupted the β-mannanase coding sequence. The mature protein was 400 residues with a calculated molecular mass of 44.26 kDa. SignalP and sequence analysis indicated the presence of an N-terminal signal peptide at residues 1−20 and a GH 5 catalytic domain. No cellulose binding domain or Ser/Thr/Pro-rich peptide linker was found in MAN5AP13. A BlastP search in the NCBI database showed that the deduced amino acid sequence of MAN5AP13 was most similar to the β-mannanases from *Bispora* sp. MEY-1 (53% identity) and *Emericella nidulans* (51% identity). Using the β-mannanase from *T. reesei* (44% identity) (*15*) as template, the three-dimensional structure of MAN5AP13 was predicted. Two strictly conserved catalytic residues (Glu194 and Glu312) and five active-site residues (Arg75, Asn193, His277, Tyr279 and Trp356) conserved in all GH 5 β-mannanases were identified. Most acidic residues were located at the surface of the predicted structure (**Figure 1**). A surface charge analysis of modeled MAN5AP13 at pH 7.0 using Discovery Studio 2.5 MODELER predicted that the protein

surface was mostly negatively charged, especially near the active site.

Expression of man5AP13 in *P. pastoris*. The gene *man5AP13* encoding the mature protein without the signal peptide was cloned into pPIC9 to obtain the expression vector pPIC9-*man5-AP13*. Then the vector was transformed into *P. pastoris* GS115 competent cells. Positive transformants were screened based on β-mannanase activity. The transformant with highest β-mannanase activity (11.8 U/mL) was obtained from shaker flask culture after induction with methanol for 60 h at 20 °C and 200 rpm. The recombinant MAN5AP13 migrated as a band of approximately 60 kDa on SDS−PAGE, which is greater than the calculated molecular mass (44.26 kDa). Based on analysis of the deduced amino acid sequence, four potential N-glycosylation sites (Asn-X-Ser/Thr) were identified (http://www.expasy.ch) (*24*). Thus, the higher apparent molecular mass of recombinant MAN5AP13 might be attributable to glycosylation.

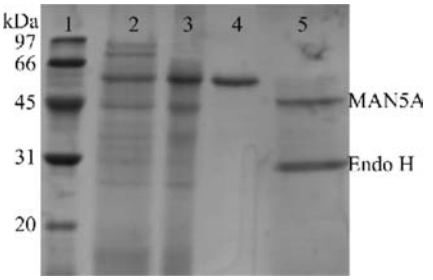

Figure 2. SDS−PAGE analysis of the recombinant β-mannanase (MAN5AP13) from *Phialophora* sp. P13. Lane 1, molecular mass markers; lane 2, culture supernatant of the induced transformant; lane 3, crude enzyme after $(NH_4)_2SO_4$ precipitation; lane 4, purified recombinant MAN5AP13 after anion exchange chromatography; lane 5, purified recombinant enzyme after deglycosylation (a band at ~30 kDa corresponding to Endo H is also indicated).

Purification and Deglycosylation of MAN5AP13. Recombinant MAN5AP13 was purified to electrophoretic homogeneity by ammonium sulfate precipitation and anion-exchange chromatography (**Figure 2**). The specific activity of purified recombinant MAN5AP13 was 851 U/mg. After treatment with Endo H, a protein band of about 45 kDa was observed on SDS−PAGE. Excluding the surplus apparent mass (0.95 kDa) derived from the expression vector pPIC9, the deglycosylated MAN5AP13 showed a similar molecular weight to the calculated value (44.26 kDa).

Properties of Purified Recombinant MAN5AP13. The optimum pH of MAN5AP13 for maximum activity was pH 1.5 at 37 °C, and more than 50% of the maximum activity was retained when the enzyme was assayed at various pHs ranging from 1.0 to 6.0 (**Figure 3a**). The enzyme was stable at acidic pHs, retaining more than 80% of the initial activity after incubation at pH 1.5−7.0, 37 °C for 2 h (**Figure 3b**).

The apparent temperature for maximum enzyme activity was 60 °C at pH 1.5, and more than 70% of the maximum activity was retained when assayed between 50 and 70 °C (**Figure 3c**). The thermostability of MAN5AP13 was determined at 50, 55, or 60 °C. The recombinant enzyme retained 83% and 97% of maximum activity at 55 and 50 °C for 2 h, respectively, and retained 37% of maximum activity at 60 °C for 20 min (**Figure 3d**). Recombinant MAN5AP13 exhibited excellent resistance to proteolysis by pepsin (95% activity retained after incubation with this protease for 1 h) or trypsin (90% activity retained) (data not shown).

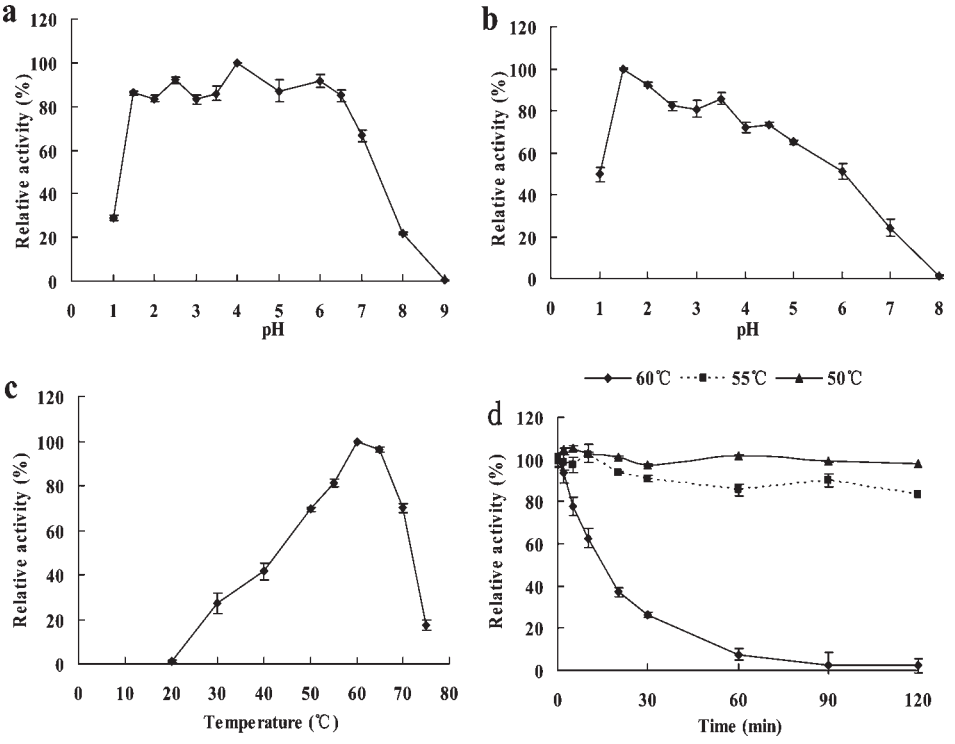

Figure 3. Characterization of purified recombinant MAN5AP13. (**a**) Effect of pH on β-mannanase activity. The activity assay was performed at 37 °C in buffers of pH 1.0−8.0 for 10 min. (**b**) Effect of pH on the stability of MAN5AP13. The enzyme was preincubated at 37 °C for 2 h in buffers of pH 1.0−9.0, and the remaining activity was determined in 0.1 M glycine-HCl buffer (pH 1.5) at 60 °C. (**c**) Effect of temperature on MAN5AP13 activity measured in 0.1 M glycine-HCl (pH 1.5) for 10 min at different temperatures. (**d**) Thermostability of purified recombinant MAN5AP13. The enzyme was preincubated at 60 °C (diamond), 55 °C (square), or 50 °C (triangle) in 0.1 M glycine-HCl (pH 1.5). Aliquots were removed at different time points for the measurement of residual activity at 60 °C. Each value in the panel represents the mean of triplicates plus standard deviation.

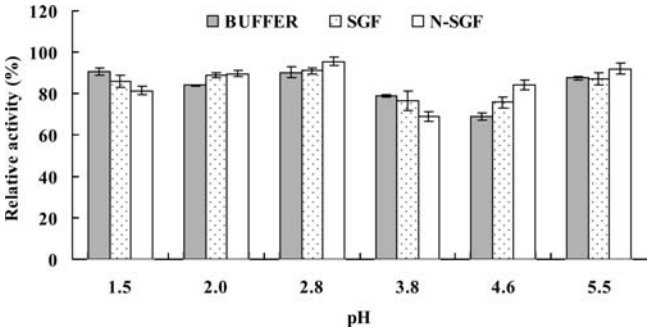

Figure 4. Stability of purified recombinant MAN5AP13 in simulated gastric fluid (SGF). The residual activity was determined after incubating MAN5AP13 at pH 1.5, 2.0, 2.8, 3.8, 4.6, or 5.5 for 60 min in either buffer (0.2 M glycine-HCl with pH 1.5, 2.0, 2.8, or 0.2 M sodium acetate−acetate acid with pH 3.8, 4.6, or 5.5), SGF without pepsin (N-SGF), or SGF. Each experiment was repeated three times.

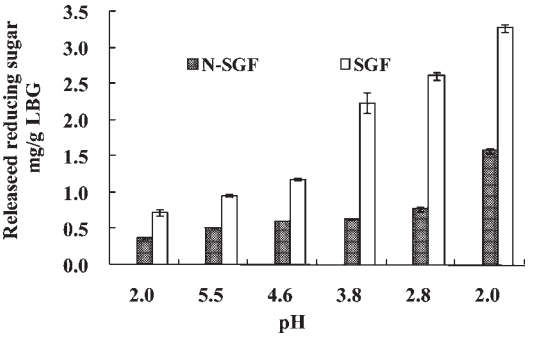

Figure 5. Effect of a pH gradient on the activity of purified recombinant MAN5AP13 in SGF. The amount of released reducing sugars was quantified after incubation of each test MAN5AP13 sample at various pHs, in succession, for the indicated times: pH 2.0 for 20 min, pH 5.5 for 10 min, pH 4.6 for 10 min, pH 3.8 for 10 min, pH 2.8 for 20 min, and pH 2.0 for 40 min. The experiment was repeated three times. The activity of purified recombinant MAN5AP13 in SGF without pepsin (N-SGF) was used as a control. LBG: locust bean gum.

The effect of metal ions and other chemical reagents on MAN5AP13 activity was determined. The activity increased by 25% and 16% in the presence of 1 mM Co^{2+} and β-mercaptoethanol, respectively (data not shown). Partial inhibition was observed in the presence of some metal ions and chemicals, including 1 mM Cr^{3+}, Ni^{+}, Fe^{2+}, Zn^{2+}, Pb^{2+}, Ag^{+} and EDTA, and SDS (1 mM) strongly inhibited the activity. Other chemicals had no significant effect on activity. At a concentration of 10 mM, all chemicals except for Co^{2+} and β-mercaptoethanol inhibited the activity either to a greater or lesser extent compared with that measured at 1 mM (data not shown).

Substrate Specificity and Kinetic Parameters. Purified recombinant MAN5AP13 had the highest activity for LBG (100%), a little lower for konjac flour (79%), and relatively weak activity for guar gum (48%). No activity was detected when carboxymethyl cellulose (sodium) or birchwood xylan was used as the substrate. The kinetic parameters for LBG and konjac flour were determined. The K_m and V_{max} values were 2.5 mg/mL and 1,668 U/min·mg, respectively, for LBG, and 1.3 mg/mL and 1,000 U/min·mg, respectively, for konjac flour.

Enzyme Stability and Hydrolysis Ability of MAN5AP13 in SGF. Recombinant MAN5AP13 retained more than 70% activity after incubation at 37 °C for 60 min at various pH values in buffer, SGF without pepsin (N-SGF), or SGF containing 3.2 mg/mL pepsin (**Figure 4**). The stomach digestion phase for LBG hydrolysis was simulated *in vitro*. The resultant products accumulated gradually, and the cumulative reducing sugar was 3.3 mg/g LBG and 1.6 mg/g LBG in SGF and N-SGF, respectively (**Figure 5**).

Analysis of Hydrolysis Products. The hydrolysis products of LBG, konjac flour, and guar gum by MAN5AP13 were analyzed by high-performance anion exchange chromatography. The main hydrolysis products from LBG were 7.1% mannose, 21.5% mannobiose, 1.6% mannotriose, 5.0% mannopentaose, and 64.9% other mannanoligosaccharides. The mass composition of the hydrolysis products from konjac flour was 16.4% mannose, 18.1% mannobiose, 2.2% mannotriose, and 63.3% other mannanoligosaccharides. The constituents of hydrolysis products from guar gum were 5.6% mannose, 11.9% mannobiose, 2.9% mannotetraose, and 79.6% other mannanoligosaccharides.

DISCUSSION

Extremophiles can survive in harsh conditions, and these organisms constitute sources for the isolation of enzymes with extremophilic properties that may have important applications in various biotechnological processes. For example, the β-mannanase from acidophilic *Bispora* sp. MEY-1 has a pH optimum of 1.5 (*9*), the alkaline protease from alkalophilic *Bacillus* sp. KSM-K16 has a pH optimum of 11.0 (*25*), and the thermophilic α-galactosidase from hyperthermophilic *Thermotoga neapolitana* 5068 has a temperature optimum of 100−105 °C (*26*). In our present study, a *Phialophora* strain was isolated from the acidic wastewater of a tin mine and showed optimal growth at pH 3.0. Activities of several glucoside hydrolases were detected in the culture medium of this strain, and one β-mannanase gene was cloned and expressed in a heterologous system. To our knowledge, this is the first report of the cloning and heteroexpression of a β-mannanase gene from *Phialophora* sp. in *P. pastoris*.

Recombinant MAN5AP13 from *Phialophora* sp. P13 showed relatively low amino acid sequence identity (53%) with the homologue MAN5A from *Bispora* sp. MEY-1 (*9*), a GH 5 β-mannanase, indicating that *man5AP13* might be a unique β-mannanase gene. Besides sequence similarity, MAN5AP13 shares certain similarities in microbial source and enzymatic properties with the mannanase from *Bispora* sp. MEY-1. The genes encoding both of these enzymes were cloned from fungi growing in extreme environments (*Bispora* sp. MEY-1 from uranium mine wastewater, and *Phialophora* sp. P13 from tin mine wastewater), and both of these organisms grow optimally at pH 3.0. Both enzymes exhibited optimum activity at pH 1.5 and at 60−65 °C, remained stable under acidic conditions, and were resistant to proteolysis by pepsin or trypsin. Certain enzymatic properties of MAN5AP13 are superior to those of the *Bispora* sp. β-mannanase, however. For example, compared with *Bispora* sp. β-mannanase, purified MAN5AP13 had relatively higher activity for hydrolysis of konjac flour (79.0% vs 69.8%) and guar gum (48.0% vs 14.8%), suggesting that MAN5AP13 has greater capacity to hydrolyze mannan-containing substrates. On the other hand, the hydrolysis products of both enzymes varied substantially; the main products of LBG and konjac flour identified for the *Bispora* sp. β-mannanase were mannose and mannotriose, whereas those for MAN5AP13 were mannose and mannobiose. These results indicate that these enzymes cleave different glycosidic linkages.

MAN5AP13 from *Phialophora* sp. P13 had an acidophilic pH optimum and excellent stability over a broad range of acidic pH. The mechanism underlying the acidophilic properties of MAN5AP13 might be ascribed to the pK_a values of its catalytic residues (*27*), which are 5.8 (Glu194) and −5.1 (Glu312) based on calculations according to the site http://biophysics.cs.vt.edu/H++/uploadpdb.php. The ratio of acidic to basic residues might

be another reason (*28*); MAN5AP13 has more acidic residues, and the ratio of acidic to basic residues is 36:25. Furthermore, at extreme acidic pH, electrostatic repulsion between enzyme and substrate is low, facilitating enzyme−substrate interactions (*29*). The low p*I* (∼4.5) for MAN5AP13 and excess of acidic residues close to the catalytic site and on the surface might explain the stability of MAN5AP13 over a broad range of acidic pH (*30*). On one hand, at low pH the negatively charged groups of acidic residues are protonated and their electrostatic repulsion is weak, thereby stabilizing the enzyme structure (*28*, *29*). On the other hand, modeling of the surface charges of MAN5AP13 at pH 7.0 also predicted a mostly negatively charged surface (**Figure 1**), which may contribute to the acid stability of MAN5AP13. Because the sequence identity of MAN5AP13 to known proteins is low and modeling to homologous enzymes gives only partial information, the true attributes that underlie the observed acidophilic and acid-stable properties of MAN5AP13 remain unclear. Crystallization and subsequent structure analysis of the enzyme and its site-specific mutants might provide such answers.

The hydrolytic activities of recombinant MAN5AP13 in simulated gastric fluid containing pepsin (SGF) and without pepsin (N-SGF) were compared, and the released reducing sugar under SGF condition was about 2-fold as much as that in N-SGF; this phenomenon was analogous to the cooperation of trypsin and α-galactosidase, in which, the hydrolytic activity of α-galactosidase was promoted by 37% in the presence of trypsin (*30*). However, further studies will be required to identify this mechanism.

In summary, MAN5AP13 from *Phialophora* sp. P13 is optimally active at extreme acidic pH, has good pH stability in acidic condition and resistance to proteases, exhibits excellent stability and activity under simulated gastric conditions, and preferentially hydrolyzes mannan-containing substrates. All these properties suggest that MAN5AP13 has great potential to improve food and feed quality and increase nutrient uptake.

ABBREVIATIONS USED

GH, glycoside hydrolase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DNS, 3,5-dinitrosalicylic acid; SGF, simulated gastric fluid.

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