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Efficient expression of a novel thermophilic fungal β-mannosidase from *Lichtheimia ramosa* with broad-range pH stability and its synergistic hydrolysis of locust bean gum

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β-Mannosidase (EC 3.2.1.25) is an exoglycosidase specific for the hydrolysis of terminal β-1,4-glycosidic linkage in mannan which can be applied in the food manufacture, animal feed, bioethanol making and coffee extraction industries. A novel β-mannosidase gene (*Lrman5A*) from *Lichtheimia ramosa* was synthesized and recombinantly expressed in *Pichia pastoris* X33. Lrman5A encodes 444 amino acids with a calculated molecular mass of 51.0 kDa which shares the highest identity (73%) with the β-mannosidase from *Rhizomucor miehei*. Purified recombinant Lrman5A showed the maximal activity at pH 6.0 and 65°C, had broad-range pH stability (retaining >65% activity after incubation at pH 3.0–8.5 at 37°C for 24 h), and was highly thermostable (retaining >80% activity after incubation at 65°C for 10 min). The specific activity, and K_m of Lrman5A was 17.5 U/mg and 1.377 mM, respectively. Lrman5A and GH5 β-mannanase displayed significant synergistic effects on the degradation of locust bean gum (LBG) and released more mannose (up to 2.89 folds) by simultaneous or sequential addition. Due to its hydrolytic properties, Lrman5A may have potential applications in the area of bioenergy, feed and food processing.

[**Key words:** *Lichtheimia ramosa*; β-Mannosidase; β-Mannanase; Recombinant expression; Synergistic hydrolysis; Locust bean gum]

Mannan is widely distributed in cell wall of plants, and it is the main component of hemicellulose. Mannan could be classified into four types: linear mannan, glucomannan, galactomannan and galactoglucomannan (1). The backbone of mannan molecule links mannose or combination of glucose and mannose residues by β-1,4-glycosidic bonds, which can be substituted with the α-1,6-linked galactose residues in the side chain (2). Due to the complicate structure of mannan, its hydrolysis requires a series of enzymes, including β-mannanase (EC 3.2.1.78), β-mannosidase (EC 3.2.1.25), α-galactosidase (EC3.2.1.23), and acetyl mannan esterase (EC 3.1.1.6) (3). Among these enzymes, β-mannanase randomly cuts the β-1,4 glycosidic bonds of the backbone to form the mannooligosaccharides. The α-galactosidase and acetyl mannan esterase are combined together to hydrolyze the side-chain substituents in galactomannan and galactoglucomannan. Finally, β-mannosidase hydrolyzes the mannooligosaccharides and release mannose monomers.

β-Mannosidase was the key enzyme in the transformation of mannan to mannose which could be applied in the food manufacture, animal feed, bioethanol making, coffee extraction, and paper and pulp industries (4). Up to now, many β-mannosidases have been purified and characterized from a variety of microorganisms including bacteria (5–9), fungi (10–16) and archaea (17,18). Among them, many β-mannosidases have been cloned and expressed heterologously to obtain high yields for industrial applications. To date, β-mannosidases were mainly expressed in *Escherichia coli* (5,6,17), and only *Aspergillus niger* β-mannosidase Man2 was expressed in *Pichia pastoris* (11). However, the low yield of recombinant β-mannosidase and their inefficient secretion in *E. coli* hinders its industrial applications.

Lichtheimia ramosa, a filamentous fungus of the *Mucorales*, has been reported to produce several enzymes including α-amylase (19), β-glucosidases (20,21), xylanases (22) and carboxymethylcellulase (23). In this study, we found a new thermophilic fungi β-mannosidase with broad-range pH stability from *L. ramosa*, and achieved high-efficiency expression of Lrman5A in *P. pastoris*. Enzymatic properties of the recombinant enzyme were investigated. Its synergistic effect with β-mannanase in the hydrolysis of locust bean gum (LBG) was also evaluated.

MATERIALS AND METHODS

Strains, media, vectors and chemicals *P. pastoris* strain X33 and the expression vector pPICzαA were purchased from Invitrogen (San Diego, CA, USA). *E. coli* DH5α (TransGen, Beijing, China) was used for plasmid transformation and amplification. The substrates *p*-nitrophenyl-β-D-mannopyranoside (pNPM), oligomannose and LBG were purchased from Sigma (St. Louis, MO, USA). Yeast extract

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peptone dextrose (YPD) medium, YPD medium with sorbitol (YPDS), buffered glycerol complex (BMGY) medium, buffered methanol complex (BMMY) medium were prepared according to the manual of *Pichia* Expression Kit (version F, Invitrogen). Fermentation basal salts (BSM) medium and PTM_1 trace salts were prepared according to the *Pichia* Fermentation Process Guidelines (24).

Gene annotation of Lrman5A and homology modeling BlastP analysis of *Rhizomucor miehei* mannosidase RmMan5B (GenBank number: AGV01048.1) (16) illustrated that a hypothetical protein (named Lrman5A) with glycoside hydrolase functional domain (GenBank number: CDS12333.1) from *L. ramosa* showed 73% sequence identity with the amino acid sequence of RmMan5B. Signal peptide was predicted by SignalP 4.1 server (25). The molecular mass of the protein was calculated by Compute pI/MW tool (https://web.expasy.org/compute_pi). Multiple sequence alignment of mannosidase was carried out by ClustalX2. Putative glycosylation site was predicted using a web-based tool NetNglyc1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/). The protein three-dimensional structure of Lrman5A was constructed via the SWISS-MODEL server (26) (https://www.swissmodel.expasy.org/).

Gene synthesis and construction of recombinant expression cassette plasmids The sequence of Lrman5A was subjected to codon optimization using software gene designer 2.0 (27). Mature coding sequence without signal peptide was synthesized by Generay (Generay, Shanghai, China) and inserted into cloning vector pUC57 (pUC57-Lrman5A). The coding sequence of mature enzyme was amplified from pUC57-Lrman5A using two primers (Lrman5A-F: 5′-atagaattccaaccattggattctagaaagg-3′ and Lrman5A-R: 5′-atagcggccgcttaatgttccaacttcttcaat-3′) by PCR amplification. Restriction sites *Eco*RI and *Not*I were added to the forward and reverse primers, respectively. PCR amplification was performed as follows: one cycle at 95°C for 3 min, 30 cycles at 98°C for 10 s, 58°C for 15 s, 72°C for 1.5 min. PCR product of Lrman5A and the plasmid pPICzαA were digested by *Eco*RI and *Not*I. The purified Lrman5A fragment was inserted into the vector pPICzαA, obtaining the expression cassette plasmid pPICZαA-Lrman5A. Then, pPICZαA-Lrman5A was transformed into *E. coli* DH5α competent cells. Transformants were selected on LB plates with zeocin (25 μg/mL). Plasmid construction was verified by double digestion and sequencing.

Transformation of *P. pastoris* and screening of transformants The expression plasmid pPICZαA-Lrman5A was linearized with *Sac*I and transformed into *P. pastoris* strain X33 by electroporation using a MicroPulse (Bio-Rad, CA, USA). Yeast transformants were cultured on YPDS agar plates with zeocin (100 μg/mL) and incubated at 30°C for 3 days. Ten macrocolonies were selected and cultivated in 50 mL BMGY medium in 250 mL shake flask at 28°C, 250 rpm, for 24 h. Cells were collected by centrifugation at 3000 ×*g* for 5 min at 4°C, resuspended in 50 mL BMMY and cultivated under the same conditions. Methanol was added to a final concentration of 0.5% to maintain induction. After 72 h cultivation with methanol, the culture supernatant was collected by centrifugation for protein purification.

Purification and SDS-PAGE analysis of recombinant Lrman5A The fermentation supernatant was obtained by centrifugation (5000 ×*g*, 10 min), and purified by the 0.22 μm filter. The supernatant was diluted with a two volume of Na_2HPO_4-citric acid buffer (pH 6.0), and used as the crude enzyme. The crude enzyme was concentrated by ultrafiltration using cellulose membranes with a 30 kDa cut-off (Millipore, Burlington, MA, USA). The concentrated recombinant enzyme was finally purified by gel filtration in Sephadex G-100 column (1.0 × 30 cm, 50 mM Na_2HPO_4-citric acid buffer, pH 6.0, GE Healthcare Life Sciences, Beijing, China) using AKTA fast protein liquid chromatography (FPLC) system (GE Healthcare, Uppsala, Sweden), according to the manufacturer's instruction. SDS-PAGE was carried out on a 12% separation gel and stained with Coomassie Blue (28). Protein concentration was measured using the Bradford assay (Bio-Rad) with bovine serum albumin as the standard (29).

β-Mannosidase and β-mannanase activity assay The β-mannosidase activity was measured according to the method of Béki et al. (5) with simple modifications. The activity was assayed by adding 100 μL of 15 mM pNPM in 100 mM Na_2HPO_4-citric acid buffer (pH 5.5) and 100 μL appropriate dilute enzyme solution in a 0.5 mL of 100 mM Na_2HPO_4-citric acid buffer (pH 5.5) at 50°C for 10 min. The reaction was terminated by the addition of 2 mL of 1 M sodium carbonate solution, and the release of *p*-nitrophenol (pNP) was monitored at 405 nm. Blank solution contained the same components except for the enzyme solution. All assays were performed in triplicates. One unit of β-mannosidase activity is defined as the amount of enzyme which liberates 1 μmol of pNP per min under the assay condition.

The β-mannanase activity was measured by the 3,5-dinitrosalicylic acid (DNS) method with some modifications (30). The reaction was started by mixing 0.2 mL of appropriately diluted enzyme sample with 1.8 mL of 20 mg/mL locust bean gum in 100 mM Na_2HPO_4-citric acid buffer (pH 5.5). After 10 min incubation at 50°C, the reaction was stopped by the addition of 3 mL DNS reagent. All assays were performed in triplicates. One unit of β-mannanase activity is defined as the amount of enzyme which liberates 1 μmol of mannose per min under the assay condition.

Biochemical characterization of the recombinant enzyme The optimal pH of purified recombinant Lrman5A was measured in 100 mM Na_2HPO_4-citric acid buffer with various pH from 3.0 to 8.0 at 50°C for 10 min. To determine the pH stability of the purified recombinant Lrman5A, the enzyme was incubated in Na_2HPO_4-citric acid buffer at 37°C for 24 h, and residual activity was measured by standard assay.

The optimal temperature was determined at range of 35–75°C under the optimal pH condition for 10 min. To determine the thermostability of the purified recombinant Lrman5A, the enzyme was incubated at 30–70°C for 10 min in 100 mM Na_2HPO_4-citric acid buffer (pH 5.5), and then cooled on ice for 30 min. The residual activities were measured.

To investigate the effects of metal ions and chemical reagents on the enzyme activity of Lrman5A, various salts with different metal ions (KCl, $MnCl_2$, $ZnSO_4$, NaCl, $CuSO_4$, $CoCl_2$, $FeSO_4$, $FeCl_3$ and $CaCl_2$) and chemical reagents (EDTA, SDS, DTT, DMSO) were added individually into the reaction system with a final concentration of 10 mM, except for DMSO and DTT (with a final concentration of 10%). The residual enzyme activity was determined under the standard conditions. The blank experiment was the original reaction system without any additive.

Enzyme kinetic parameters To determine the kinetic parameters (K_m, V_{max}) of Lrman5A, the activity was measured with various concentration of substrates (from 0.16 mM to 6.4 mM). Other reaction conditions were the same with the standard enzymic activity assay method. Substrate-velocity curve and Lineweaver–Burk plot of Lrman5A were carried out by GraphPad Prism 5.0.

High cell density fermentation High cell density fermentation was performed in a 5 L fermentor according to *Pichia* Fermentation Process Guidelines of Invitrogen (24). Activation and growth of the recombination *P. pastoris* were performed on YPD medium. After the cell density reached an OD_{600} of more than 10.0 at 28°C in YPD medium, then the seed culture was transferred to the 5 L fermentor (Biotech-7BG, Baoxing, Shanghai, China) containing 2 L BSM medium with 8 mL PTM_1 solution at a 10% inoculation amount. The initial culture medium was adjusted to pH 5.5 by adding ammonium hydroxide. The fermentation was proceeded at 28°C with the dissolved oxygen (DO) maintained at above 20% of air saturation, which was coupled with the nutrient feed. The nutrient consisting of 50% glycerol and 1.2% PTM_1 solution was pumped into the fermentor to keep oxygen homeostasis. When the fermentation process was at the induction phase, pure methanol mixed with 1.2% PTM_1 solution was flowed at the appropriate flow rate for the oxygen homeostasis. During the fermentation, the broth was sampled at intervals for protein concentration assay, optical density measurements, and enzyme activity assay.

Synergistic hydrolysis of mannans by β-mannanase and β-mannosidase from *L. ramosa* The β-mannosidase Lrman5A was recombinantly expressed in this research, and the β-mannanase (*L. ramosa*) was purchased from Asiapac (Dongguan, China). The Synergistic hydrolysis of LBG by Lrman5A and β-mannanase was tested as the method described by Gübitz et al. (12) with some modifications. The reaction system (2 mL) contained 50 mg LBG, 0.25 U Lrman5A (or 100 U β-mannanase), and 50 mM Na_2HPO_4-citric acid buffer (pH 5.5). The reaction system with both β-mannanase and β-mannosidase, was incubated at 50°C for 3 h in 50 mM Na_2HPO_4-citric acid buffers (pH 5.5) and then boiled for 10 min. For sequential reactions, after incubation with the first enzyme at 50°C for 3 h, the first enzyme was inactivated by boiling with 10 min. Then, the other enzyme was added, and the reaction system was incubated at 50°C for 3 h. The reducing sugar released in the reaction system was detected via the DNS method, with mannose as the standard. The amount of released mannose was measured by HPLC, with the following conditions: Alltima Amino column (4.6 × 250 mm, 5 μm); mobile phase of acetonitrile:water (80:20); the flow rate was 1 mL/min; column temperature is 40°C. All assays were carried out in triplicates, and differences between different enzyme combination were evaluated using One-way analysis of variance by GraphPad Prism 5.

RESULTS

Gene annotation of β-mannosidase Lrman5A In order to obtain new β-mannosidases, we performed BLAST search of the reported β-mannosidases and found a putative protein from *L. ramosa*. The amino acid sequence of the putative protein shared 73%, 69%, and 32% identity with β-mannosidases from *R. miehei* (AGV01048) (16), *Absidia corymbifera* (AMB61311) (15), and *Cellvibrio mixtus* NCIMB 8633 (AAS19695.1) (31), respectively (Fig. 1A). Due to the high amino acid identity with those β-mannosidases, we assumed that it was a new β-mannosidase, termed as Lrman5A. To further annotate Lrman5A, we performed bioinformatics analysis. The Lrman5A gene consists of 1335 bp, encoding 444 amino acids and a signal peptide of 19 amino acids was predicted by SignalP4.1 program. The molecular mass of deduced Lrman5A is 51.0 kDa and its isoelectric point (pI) is 6.03. Homology modeling of Lrman5A based on the *R. miehei* Man5B (4lyp.1.A) structure was achieved using SWISS-MODEL server (26) (Fig. 1B). The model contains a classical $(\alpha/\beta)_8$-TIM barrel fold, suggesting that this enzyme belongs to GH5 family. Multiple

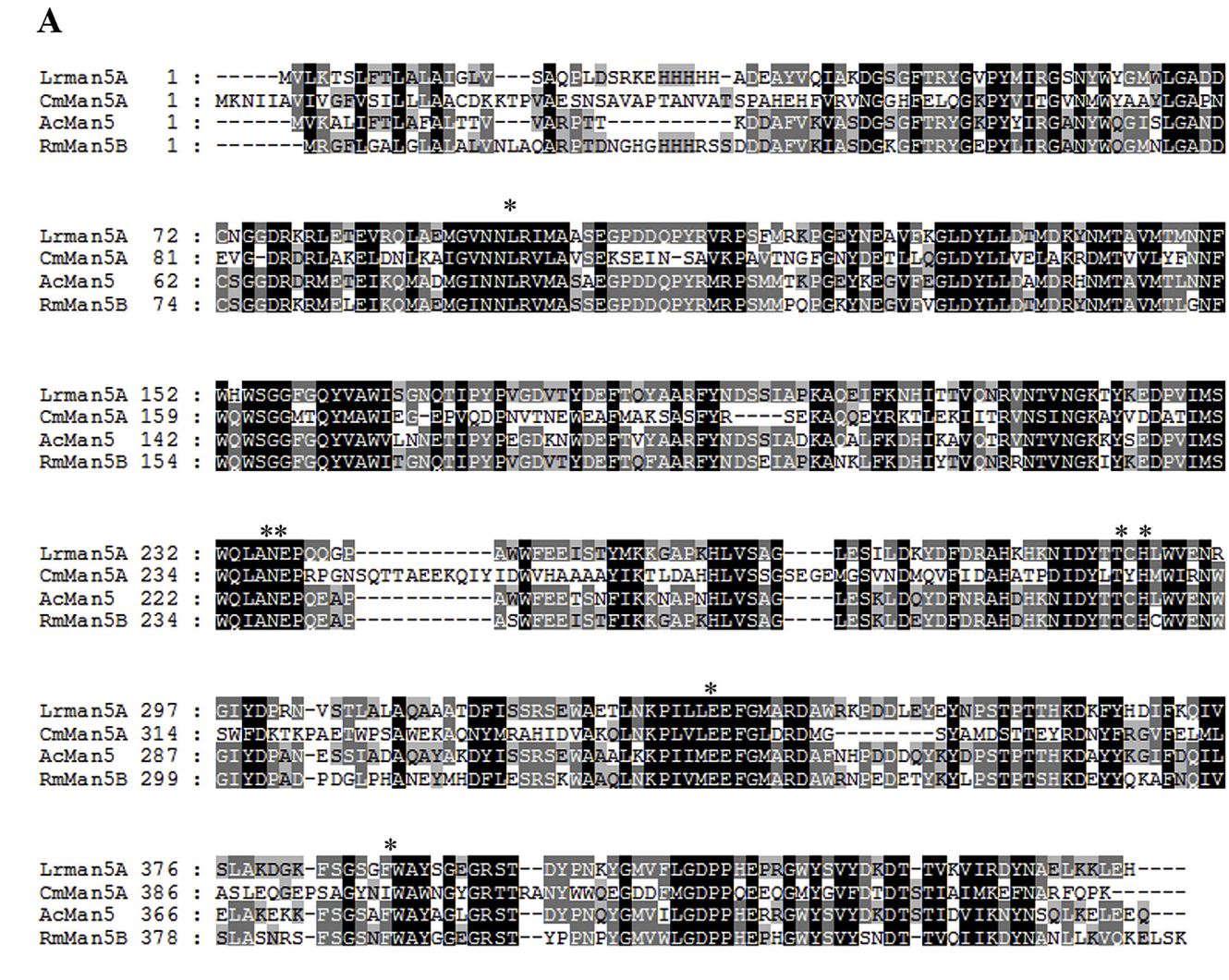

FIG. 1. Sequence and structure analysis of Lrman5A. (A) Multiple alignment of the amino acid sequences of Lrman5A with other identified GH family 5 β-mannosidases. RmMan5B from *R. miehei*, AcMan5A from *A. corymbifera*, CmMan5 from *C. mixtus*. Identical and similar amino acids are indicated by black and grey shades. The conserved catalytic residues are indicated by asterisk. (B) Predicted structure of Lrman5A by swiss-model.

sequence alignment and homology modeling analysis indicated that E237 and E336 are putative catalytic residues and seven strictly conserved residues of GH5 (Arg96, Asn236, Glu237, His290, Trp292, Glu336 and Trp389) forms a slot-like pocket which is located on connecting loops at the C-terminal end of the β-sheets of the $(\alpha/\beta)_8$-TIM barrel which might be related to substrate binding.

In order to assess the reliability of the structure and its suitability for the structural analysis, a model evaluation was implemented by the Structure Assessment tools of SWISS-MODEL server. Ramachandran plot (Fig. 1C) showed that 97.3% (395/406) of all residues were located in favored regions and 100.0% (406/406) of all residues were located in allowed regions, indicating that there was no residue (<2%) located in disallowed regions and the model is reasonable and reliable (32). The QMEAN4 value of the Lrman5A structure was 0.63, and the score is clearly within the expected quality range. It has an average score deviation of less than 1.0 from sized high-quality proteins from the reference dataset (33), therefore the homologous structure model of Lrman5A is reasonable.

Expression and purification of recombinant β-mannosidase Lrman5A To obtain and characterize the putative β-mannosidase Lrman5A, it was subjected to codon usage optimization. The optimized gene Lrman5A-opt without signal peptide was synthesized and cloned into the pPICZαA vector to construct the recombinant expression cassette (Fig. 2A) and then transformed into *P. pastoris* X33. Transformants were verified and cultivated in flasks and 5 L fermentor for 5 days, using methanol as the inducing carbon source. We assayed the activity of the culture supernatant with pNPM as substrate, the maximum activity reached to 6.1 U/mL and 27.9 U/mL, respectively. LBG and oligomannose were hydrolyzed into mannose by the culture supernatant which was assayed by HPLC (Fig. 2C). The recombinant Lrman5A was purified by 2.7-fold using ultrafiltration and gel chromatography using Sephadex G-100, with a recovery yield of 68.5%. The specific activity of the enzyme increased to 17.5 U/mg from 6.5 U/mg. SDS-PAGE illustrated that the molecular mass of the purified recombinant Lrman5A was 60 kDa (Fig. 2B), which was bigger than the theoretical value (51 kDa). The recombinant Lrman5A was predicted to own 5 potential *N*-glycosylation sites within the conserved Asn-Xaa-Ser/Thr sequence using the NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/), suggesting that it might be *N*-glycosylated when expressed in *P. pastoris*.

Enzymatic characteristics of recombinant Lrman5A To determine the enzymatic characteristics of recombinant Lrman5A, enzymatic activity of Lrman5A was measured at various temperatures and pH values. The optimal pH of the recombinant Lrman5A was 5.5, and it retains more than 80% activity at pH 4.5–6.0 (Fig. 3A). Lrman5A was stable at pH 3.0–8.5 and retained more than 60% of its original activity after incubation for 24 h at room temperature (Fig. 3B). Lrman5A exhibited its maximum activity at 65°C, retained more than 80% of its highest activity between 60°C and 70°C (Fig. 3C). It was fairly stable up to 60°C in 50 mM Na_2HPO_4-citric acid buffer (pH 5.5), maintaining its initial activity

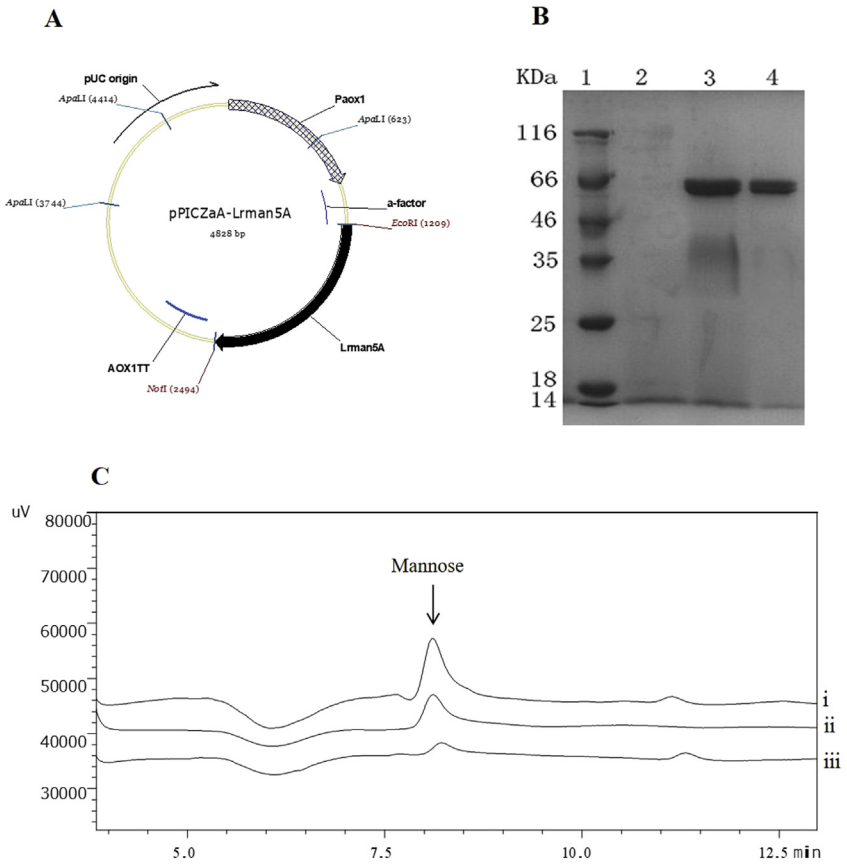

FIG. 2. Expression analysis of Lrman5A. (A) The Plasmid (pPICZαA-Lrman5A) with Lrman5A expression cassette. Paox1, alcohol oxidase 1 promoter region induced by methanol. Lrman5A, the optimized gene encoding β-mannosidase from *L. ramosa*. Zeo, zeocin resistance gene. AOX1 TT, native transcription termination. The restriction sites indicated are *Eco*RI and *Not*I. (B) SDS-PAGE analysis of recombinant Lrman5A expressed in *P. pastoris* X33. Lane 1, molecular mass markers; lane 2, culture supernatant of *P. pastoris* X33 with transformed pPICZαA plasmid; lane 3, culture supernatant of *P. pastoris* X33 with transformed pPICZαA-Lrman5A plasmid; lane 4, purified recombinant Lrman5A. (C) Product analysis of LRman5A hydrolyzed LBG and oligomannose. (i) The hydrolysis products released from oligomannose by Lrman5A. (ii) Mannose standard. (iii) The hydrolysis products released from LBG by Lrman5A.

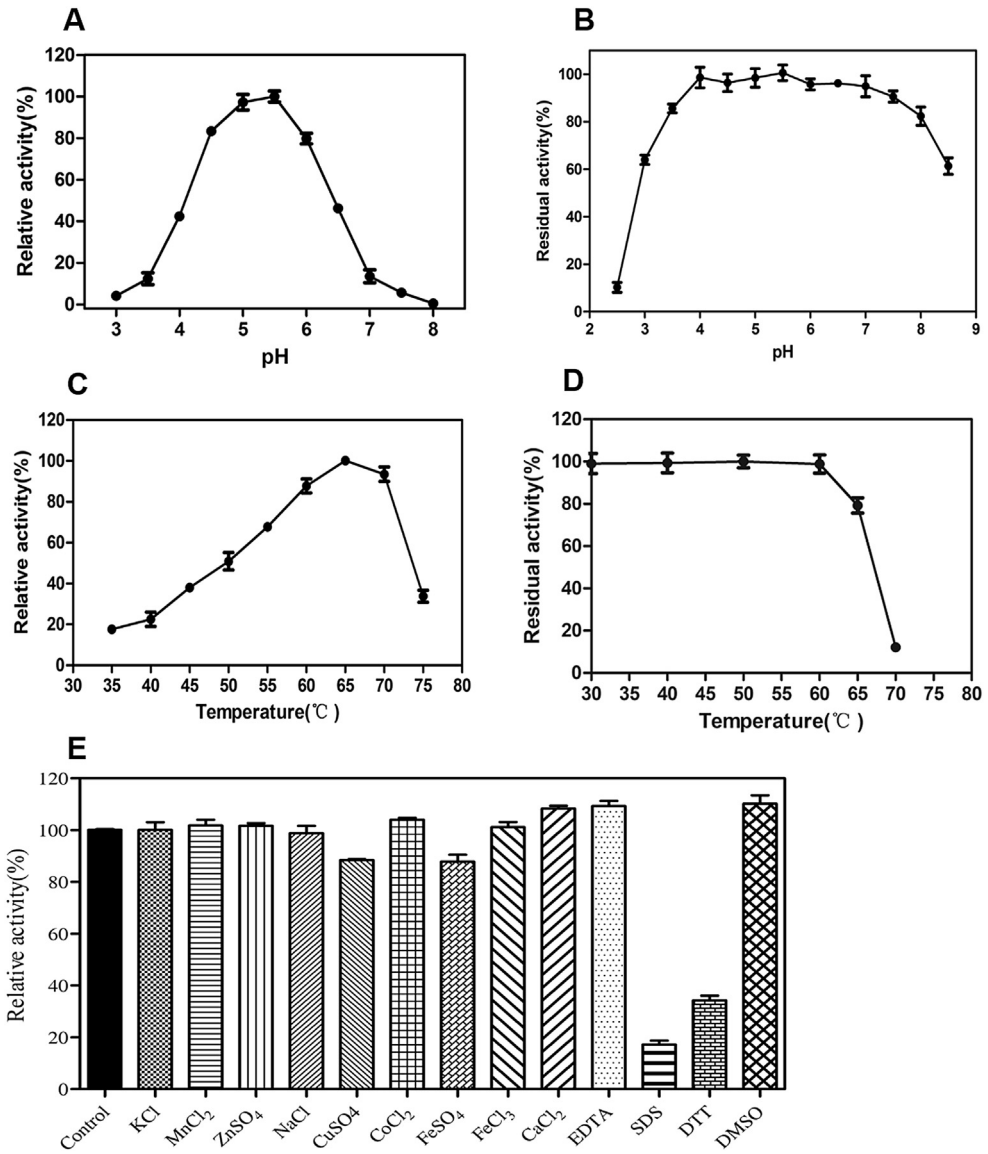

FIG. 3. (A) Optimal pH stability, (B) pH stability, (C) optimal temperature, (D) thermostability and (E) metal ions and chemical reagents effects of the recombined Lrman5A from *L. ramosa*. The influence of pH on enzyme activity was determined at 55°C using 50 mM of Na_2HPO_4-citric acid buffer with pH ranging from 3.0 to 8.0. For pH stability, the residual activities were measured after incubating the enzyme at 37°C for 24 h over various pH ranges. The optimal temperature was evaluated by measuring the enzyme activity at various temperatures (35–75°C) in 50 mM Na_2HPO_4-citric acid buffer (pH 5.5). For determination of thermostability, the residual activities of Lrman5A were measured after incubating at different temperatures (30–70°C) for 10 min. Metal ions, SDS, EDTA were added to the standard assay in 10 mM final concentration, DTT and DSMO were added in 10% final concentration. The activity was determined under standard assay condition.

at 60°C for 10 min (Fig. 3D). Moreover, more than 75% of the enzyme activity was retained after incubation at 65°C for 10 min.

The effect of various metal ions and chemical reagents on the activity of Lrman5A were investigated (Fig. 3E). Compared with the activity without metal ions, Co^{2+} and Ca^{2+} improved the mannosidase activity to 104.1% and 108.3%, respectively. Cu^{2+} and Fe^{2+} showed obvious inhibition by 12% decrease, and the other ions tested in this study showed little effect on the activity of Lrman5A. In addition, the activity was seriously inhibited by SDS and DTT, with 18.2% and 34.2% decrease, respectively. On the contrary, EDTA and DMSO increased the enzyme activity by 9.4% and 10.1%, respectively.

In order to evaluated the catalytic ability of Lrman5A for different substrates, we used pNPM, pNPG, pNPX and LBG as substrates to assay the enzyme activity. Lrman5A were active on pNPM and LBG with 17.5 U/mg β-mannosidase activity and 0.3 U/mg β-mannanase activity, respectively, while it was inactive on pNPG and pNPX. The K_m and V_{max} values for the recombinant β-mannosidase Lrman5A towards pNPM were determined to be 1.377 mM and 23.15 μmol min^{-1} mg^{-1} (Fig. 4). The enzyme turnover number (k_{cat}) was calculated to be 19.68 s^{-1} which resulted in a catalytic efficiency (k_{cat}/K_m) of 14.29 mM^{-1} s^{-1}.

Degradation of LBG by the synergistic hydrolysis of Lrman5A and β-mannanase In order to evaluate the synergistic effect of β-mannanase and β-mannosidase from *L. ramosa*, LBG was simultaneous and sequential hydrolyzed by Lrman5A and β-mannanase (Table 1 and Fig. 5). Both of simultaneous reaction and sequential reaction (β-mannanase→Lrman5A) showed very significant synergistic effect on the degradation of LBG (p value <0.001), resulting in the release of mannose increased to 2.89-fold and 2.72-fold, compared with the sum of mannose released by hydrolysis of single Lrman5A and β-mannanase. However, this synergistic effect is not significant in the release of reducing sugar.

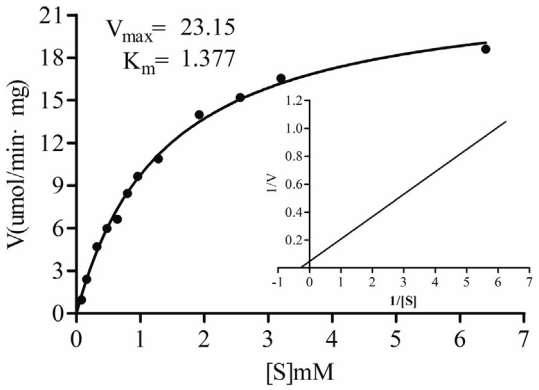

FIG. 4. Substrate-velocity curve and Lineweaver–Burk plot of Lrman5A was drawn by GraphPad Prism 5.0. The activity was measured using different concentrations (0.08 mM-6.4 mM) of pNPM as substrate under standard assay condition.

TABLE 1. Simultaneous or sequential hydrolysis of LBG by Lrman5A and β-mannanase.

Enzyme added		Amount of mannose released/mg[a]	Amount of reducing sugar released/mg[a]
First reaction	Second reaction		
β-Mannanase	None	3.54 ± 0.13	16.13 ± 0.41
Lrman5A	None	0.94 ± 0.09	3.74 ± 0.17
Lrman5A+ β-mannanase	None	12.97 ± 0.23	18.66 ± 0.53
β-Mannanase	Lrman5A	12.19 ± 0.35	19.86 ± 0.49
Lrman5A	β-Mannanase	4.14 ± 0.07	16.02 ± 0.38
None	None	0.00	0.08 ± 0.04

[a] Values represent means ± SD (n = 3).

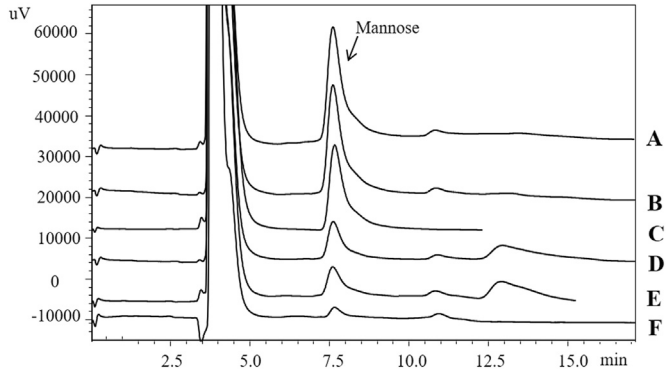

FIG. 5. HPLC profiles of the degradation of LBG by Lrman5A and endo-β-mannanase. Line: A, the hydrolysis products released by simultaneous action of Lrman5A and endo-β-mannanase. Line B, the hydrolysis products released by sequential interactions of endo-β-mannanase followed by Lrman5A. Line C, mannose standard. Line D, the hydrolysis products released by sequential interactions of Lrman5A followed by endo-β-mannanase. Line E, the hydrolysis products released by endo-β-mannanase. Line F, the hydrolysis products released by Lrman5A.

Simultaneous reaction and sequential reactions (Lrman5A→β-mannanase) had anti-synergistic effect on releasing of reducing sugar, and the amounts of reducing sugar were decreased by 6% and 19%, respectively.

DISCUSSION

So far, a number of β-mannosidases from plants, animals and microorganisms have been identified (3,4), but there is no report of β-mannosidases from *L. ramosa*. In this study, we obtained a new β-mannosidase gene (Lrman5A) from *L. ramosa* by bioinformatic analysis and Lrman5A was highly expressed for the first time in *P. pastoris*. The yield of recombinant enzyme was up to 4.3 g/L, and enzyme activity was 27.9 U/mL, which was much higher than the recombinant *A. niger* β-mannosidase Man2 in *P. pastoris* (0.052 g/L and 0.176 U/mL) (11). The β-mannosidase Lrman5A consists of 444 amino acids with a classical (α/β)8-TIM barrel fold of GH5 family, and it owned the high sequence identity with the reported GH5 β-mannosidases such as *R. miehei* (73%) and *A. corymbifera* (69%). The molecular weight of the recombinant Lrman5A (60 kDa) was higher than the theoretical calculating value (51 kDa), and is comparable to the previous GH5 family β-mannosidases from *A. corymbifera* (51.9 kDa) (15) and *C. mixtus* (53 kDa) (31). However, its molecular weight is smaller than all of the reported GH2 family β-mannosidases which are usually larger than 90 kDa (BRENDA Enzyme Database: https://www.brenda-enzymes.info), such as *A. niger* (130 kDa) (11) and *Thermobifida fusca* (94 kDa) (5).

Lrman5A was maximally active at pH 5.5 and was stable in the pH range of 3.0–8.5 (Fig. 5A and B). The optimal pH of Lrman5A (pH 5.5) is in accordance with other β-mannosidases from most fungi which exhibit optimal activity within the acidic pH range of 4.0–6.0 (10,11,16,34). However, Lrman5A with broad-range pH stability was much more stable across a wider pH range (pH 3.0–8.5) than other fungal counterparts from *A. niger* (pH 4.0–6.0) (35), *Aspergillus awamori* (pH 4.0–7.0) (14), *Thermoascus aurantiacus* (pH 3.0–7.0) (36) and *Thermotoga thermarum* (pH 5.0–8.5) (7). Lrman5A exhibited excellent thermostability. It displayed its maximum activity at 65°C and was highly stable at 65°C or below. This is superior to most of the previously reported mannosidases. For example, the mannosidases of *Myceliophthora thermophila*, *Streptomyces* sp. and *Cellulomonas fimi* own the highest activity at 40°C, 50°C and 55°C, respectively, and all of them are unstable when temperatue is higher than 50°C (6,8,34). Due to the broad-range pH stability and superior thermostability, Lrman5A may serve as a good candidate for its application in fundamental research and various industrial fields. For example, Lrman5A can be used in paper pulp bleaching at high-heat and variable pH condition (varies from acidic to alkaline pH) with together β-mananase (37,38). Lrman5A can be used in degradation of mannans in feed at animal digestive tract (pH 2.5–7.0) (39), to reduce the anti-nutritional factors of feed and improve the utilization of feed. The enzyme activity of Lrman5A is remarkably activated by Ca^{2+} and EDTA and is largely inhibited by SDS, DTT, Cu^{2+} and Fe^{2+}, which is consistent with previous reports (5,15,17). However, the enzyme activity is greatly activated by DMSO, which is different from Samra's report on human β-mannosidase whose activity was completely inhibited by 5 mM DMSO (40). The K_m values for the β-mannosidases towards pNPM were reported from 0.12 mM to 11 mM (BRENDA Enzyme Database: https://www.brenda-enzymes.info/). Compared with the reported β-mannosidases, the K_m value (1.377 mM) of Lrman5B is relatively low, only higher than *Trichoderma reesei* (0.12 mM) (10), *T. fusca* (0.18 mM) (5), *Thermotoga maritima* (0.49 mM) (9) and *T. aurantiacus* (1.1 mM) (36). However, this does not indicate that Lrman5A has lower catalytic efficiency for pNPM than the above mentioned β-mannosidases. Lrman5A has higher catalytic efficiency due to higher conversion number. For instance, the k_{cat} of Lrman5A (19.68 s^{-1}) is higher than the value for the β-mannosidases from *T. maritima* (8.43 s^{-1}), and k_{cat}/K_m (14.29 mM^{-1} s^{-1}) is comparable to the value for the β-mannosidases from *T. maritima* (17.22 mM^{-1} s^{-1}) (9). Thus, the enzyme could perform specific cleavage in β-mannoside linkage and own high catalytic efficiency compared to β-mannosidases from other sources.

Complete degradation of a structurally complex mannan is accomplished by synergistic action of several mannan-degrading

enzymes including β-mannanase and β-mannosidase. Synergy between two main-chain-cleaving enzymes (β-mannanase and β-mannosidase) for mannans degradation have been reported (3). For example, simultaneous or sequential incubation of Man2S27 and Man5S27 from *Streptomyces* sp. S27 had no synergistic or additive effect on LBG hydrolysis. Meanwhile, sequential interaction of Man5S27 and Man2S27 caused a significant 2.6-fold sugar release (8). In this study, simultaneous hydrolysis results in the release of less reducing sugar than the sum of reducing sugars produced by hydrolysis single enzyme. This phenomenon is similar to β-mannosidase from *Sclerotium rolfsii* (12) and *Streptomyces* sp. S27. The reason for this phenomenon is unclear, probably because both enzymes belongs to the GH5 family and they may have similar substrate binding sites. This anti-synergy phenomenon was observed in studies where two β-mannanases might be due to the competition for the same substrate binding sites (12,41). However, simultaneous reaction and sequential reaction (β-mannanase → Lrman5A) showed a strong synergistic effect on the release of monomeric mannose, which greatly improved the total amount of released mannose. The reason might be that small molecule products of β-mannanase, such as mannooligosaccharides with non-reducing ends were more prone to be hydrolyzed by β-mannosidase. It is well known that mannose is fermentable sugar which can be used in the production of biofuels and value-added chemicals. Therefore, Lrman5A there might be a potential application of Lrman5A in bioenergy production and food processing, due to its excellent characteristics of releasing mannose.

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