RESEARCH ARTICLE

Biochemical characterization of a novel thermophilic α -galactosidase from *Talaromyces leycettanus* JCM12802 with significant transglycosylation activity

Caihong Wang^{1,§}, Huimin Wang^{1,§}, Rui Ma^{1,2}, Pengjun Shi¹, Canfang Niu¹, Huiying Luo¹, Peilong Yang^{1,3}, and Bin Yao^{1,*}

Key Laboratory for Feed Biotechnology of the Ministry of Agriculture, Feed Research Institute, Chinese Academy of Agricultural Sciences, Beijing 100081, People's Republic of China,¹ Biotechnology Institute, Chinese Academy of Agricultural Sciences, Beijing 100081, People's Republic of China,² and CAAS-ICRAF Joint Laboratory on Agroforestry and Sustainable Animal Husbandry, Beijing 100193, People's Republic of China³

Running title: A thermophilic α -galactosidase from *T. leycettanus*

* Corresponding author at: Key Laboratory for Feed Biotechnology of the Ministry of Agriculture, Feed Research Institute, Chinese Academy of Agricultural Sciences, No. 12 Zhongguancun South Street, Beijing 100081, P. R. China. Tel.: +86 10 82106053; fax: +86 10 82106054.

E-mail addresses: binyao@caas.cn; yaobin@caas.cn.

[§]These authors contributed equally to this work.

ABSTRACT

Thermophilic α -galactosidases have great potentials in biotechnological and medicinal applications due to their high-temperature activity and specific stability. In this study, a novel α -galactosidase gene of glycoside hydrolase family 27 (*aga27A*) was cloned from *Talaromyces leycettanus* JCM12802 and successfully expressed in *Pichia pastoris* GS115. Purified recombinant Aga27A (rAga27A) was thermophilic and thermotolerant, exhibiting the maximum activity at 70°C and retaining stability at 65°C. Like most fungal α -galactosidases, rAga27A had an acidic pH optimum (pH 4.0) but retained stability over a boarder pH range (3.0–11.0) at 70°C. Moreover, the enzyme exhibited strong resistance to most metal ions and chemicals tested (except for Ag⁺ and SDS) and great tolerance to galactose (19 mM). The preferable transglycosylation capacity of rAga27A with various substrates further widens its application spectrum. Thus rAga27A with excellent enzymatic properties will be ideal for applications in various industries, especially for the synthesis of galactooligosaccharides.

[Keywords: *Talaromyces leycettanus* JCM12802; α -Galactosidase; Thermophilic; Transglycosylation; Galactose tolerance]

INTRODUCTION

α -Galactosidases (α -D-galactoside galactohydrolase; EC3.2.1.22) catalyze the removal of α -linked terminal nonreducing galactose residues from different substrates (1). Besides the hydrolysis activity, some α -galactosidases have transglycosylation activity at high substrate concentrations to produce α -galactooligosaccharides as well. So far, about 600 α -galactosidase sequences from microorganisms, plants and animals have been released and 133 have been characterized in the carbohydrate active enzyme (CAZy) database (www.cazy.org, by April 9, 2015). Based on the sequence similarities, α -galactosidases are classified into glycoside hydrolase (GH) families 4, 27, 36, 57, 97 and 110 (2), and most fungal α -galactosidases belong to GH27. The resolved crystal structures of three GH27 α -galactosidases from *Saccharomyces cerevisiae*, *Trichoderma reesei* and *Umbelopsis (Mortierella) vinacea* share a $(\beta/\alpha)_8$ barrel fold and a retaining reaction mechanism (3–5).

α -Galactosidases are of great interest for various industrial applications. In the sugar-making industry, it is used to improve the crystallization of sucrose (6); in the pulp and paper industry, it aims to enhance the bleaching of pulp (7); in the food and feed industries, it is added to eliminate the α -D-galactosides (mainly raffinose and stachyose), the main anti-nutritional factor, in legume seeds, and to improve digestibility alone or in combination with other enzymes (8); in the medicinal field, it is used for Fabry disease treatment (9) and synthesis of galactooligosaccharide (10).

Thermophilic α -galactosidases are of great interest due to their high activity and

specific stability under high temperatures. Thermophilic fungi are the main microbial source of thermophilic/thermotolerant α -galactosidases. In our previous study, thermophilic *Talaromyces leycettanus* strain JCM12802 has been identified as an excellent CAZy producer, from which a gene encoding a thermophilic GH5 mannanase has been cloned and functionally characterized (11). To meet the industrial requirements, we cloned a novel α -galactosidase gene (*aga27A*) from this strain, and produced the gene product in *Pichia pastoris*. Biochemical characterization of the recombinant enzyme revealed its superior properties like adaptability and stability at high temperatures and over a broad pH range, strong resistance to a panel of metal ions and galactose, and significant transglycosylation capacity. All these excellent characteristics make it a good candidate for applications in various industries.

MATERIALS AND METHODS

Strains, media, vectors, and chemicals *T. leycettanus* JCM12802 was purchased from Japan Collection of Microorganisms RIKEN BioResource Center, Tsukuba, Japan. *Escherichia coli* Trans1-T1 and the pEASY-T₃ vector from TransGen (Beijing, China) were used for gene cloning. *P. pastoris* GS115 and pPIC9 from Invitrogen (Carlsbad, CA, USA) were used for heterologous gene expression. The media for gene expression were prepared according to the manual of the *Pichia* Expression kit (Invitrogen).

The DNA isolation kit and DNA polymerase *pfu* were purchased from Tiangen (Beijing, China). The Genome Walking kit and LA Taq DNA polymerase were obtained from TaKaRa (Tsu, Japan). The DNA purification kit was purchased from OMEGA (Cowpens, SC, USA). The SV Total RNA Isolation kit and Reverse Transcription kit were purchased from Promega (Madison, WI, USA) and TOYOBO (Osaka, Japan), respectively. T4 DNA ligase and endo- β -*N*-acetylglucosaminidase H (Endo H) were supplied by New England Biolabs (Ipswich, MA, USA). *p*-Nitrophenyl- β -D-galactopyranoside (*p*NPGal), galactose, melibiose, raffinose, and stachyose were purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade.

Cloning and sequence analysis of the α -galactosidase gene *aga27A* The genomic DNA of *T. leycettanus* JCM12802 was extracted and purified with the commercial kits. A degenerate primer set GH27F and GH27R (Table 1) specific for fungal GH27 α -galactosidases was designed and used to clone the core region of *T. leycettanus* α -galactosidase gene. The resulting PCR products (377 bp) were purified, ligated with vector pEASY-T₃, and then transformed into *E. coli* Trans1-T1 for sequencing. To obtain the 5' and 3' flanking regions of the core region, thermal asymmetric interlaced (TAIL)-PCR (12) was conducted with the TaKaRa genome walking kit and four nested insertion-specific primers (Table 1). The flanking regions were sequenced, and assembled with the known sequence to give the full-length gene (*aga27A*).

To obtain the cDNA sequence of *aga27A*, *T. leycettanus* JCM12802 was grown at 45°C for 3–5 days in soybean meal medium as described previously (13). Mycelia were collected and immediately ground into a fine powder in liquid nitrogen. Total RNA was extracted from mycelia using the SV Total RNA Isolation System, and the cDNA was synthesized with the TOYOBO reverse transcription kit. The full-length cDNA of *aga27A* was amplified using the specific primers Aga27AF and Aga27AR (Table 1) with an annealing temperature of 60°C. The resulting PCR products were purified, ligated with vector pEASY-T3, and then transformed into *E. coli* Trans-T1 for sequencing.

The sequence assembly was performed using the Vector NTI Advance 10.0 software (Invitrogen). Nucleotide and amino acid sequences were aligned using the BLASTx and BLASTp programs (<http://www.ncbi.nlm.nih.gov/BLAST/>), respectively. Introns, exons, and transcription initiation sites were predicted using the online software GENSCAN (<http://genes.mit.edu/GENSCAN.html>). The potential signal peptide sequence and *N*-glycosylation sites of *aga27A* were predicted online (<http://www.cbs.dtu.dk/services/SignalP/> and <http://www.cbs.dtu.dk/services/NetNGlyc/>, respectively). Multiple sequence alignment of the deduced amino acid sequence of Aga27A and other α -galactosidases from *Neosartorya fischeri* (KF640698 and KJ739690), *Lachancea thermotolerans* (BAE93466), *Saccharomyces cerevisiae* (3LRK_A), *Lachancea cidri* (Q99172.1), and *Trichoderma reesei* (1SZN_A) were conducted using ClustalW (www.genome.jp/tools/clustalw/). Homology modeling was conducted using the

Accelrys Discovery Studio software DS 2.5 (San Diego, CA, USA) with the GH27 α -galactosidases from *M. vinacea* (3A5V_A; 53% identity) (4) and *S. cerevisiae* (3LRK_A; 48% identity) (5) as the template. Further phylogenetic analysis of Aga27A and other counterparts from bacteria, fungi and plants was performed using the Neighbor-joining method with MEGA 4.0 (www.megasoftware.net).

Expression and purification of recombinant Aga27A The gene fragment coding for the mature Aga27A without the putative signal peptide-coding sequence was amplified by expression primers rAga27AF and rAga27AR (Table 1). The PCR product was digested with *EcoRI* and *NotI* and was ligated into the pPIC9 vector. The recombinant plasmid pPIC9-aga27A was linearized using *BglII* and transformed into *P. pastoris* GS115 competent cells by electroporation according to the manufacturer's instructions (Invitrogen). Gene expression and induction was conducted as described previously (14). After centrifugation at 12,000 $\times g$, 4°C for 10 min, the culture supernatants were collected and subject to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and α -galactosidase activity analysis as described below. The transformant exhibiting the highest activity was selected for large-scale fermentation in a 1-l Erlenmeyer flask as described (15).

The culture supernatants from 1-l flasks were centrifuged at 12,000 $\times g$, 4°C for 10 min and ultrafiltrated through a 10-kDa cut-off membrane (Vivascience, Hannover, Germany). The crude enzyme was dialyzed against 20 mM Na₂HPO₄-citric acid buffer (pH 7.0), and loaded onto a HiTrap Q Sepharose XL FPLC column (GE

Healthcare, Uppsala, Sweden) that was equilibrated with the same buffer. A linear gradient of NaCl (0–1.0 M) was used to elute the proteins at a flow rate of 2.0 ml/min. Fractions exhibiting α -galactosidase activity were pooled and subjected to SDS-PAGE analysis. The protein concentration was measured by a protein assay kit (Bio-Rad, Hercules, CA) using bovine serum albumin as the standard (16). To remove any *N*-glycosylation, the purified protein (1 μ g) was treated with 30 U of Endo H for 1 h at 37°C following the supplier's instructions (New England Biolabs). The deglycosylated protein was also analyzed on SDS-PAGE gel.

Enzyme activity assay α -Galactosidase activity was determined by using *p*NPGal as the substrate (17). Appropriately diluted enzyme (100 μ l), *p*NPGal (125 μ l of 4 mM substrate) and 275 μ l of McIlvaine buffer (200 mM Na₂HPO₄, 100 mM citric acid, pH 4.0) were incubated at 70°C for 5 min. The reaction was terminated by addition of 1.5 ml of 1 M Na₂CO₃, and the absorbance at 405 nm was measured spectrophotometrically when the reaction systems cooled down to room temperature. One unit of α -galactosidase activity was defined as the amount of enzyme that released one μ mol of *p*-nitrophenol (*p*NP) per min at pH 4.0 and 70°C. All assays were performed in triplicate.

Characterization of the purified recombinant Aga27A (rAga27A) The pH-activity profile of purified recombinant Aga27A (rAga27A) was determined by measuring its activity in different buffers of pH 2.0–8.0 at 70°C for 5 min. The pH

stability of rAga27A was assessed by measuring the residual activities under standard conditions (pH 4.0, 70°C, and 5 min) after 1-h incubation in buffers of pH 2.0–12.0 at 37°C. The buffers used were 100 mM glycine-HCl for pH 2.0–3.0, McIlvaine buffer for pH 3.0–8.0, 100 mM Tris-HCl for pH 8.0–9.0, and 100 mM glycine-NaOH for pH 9.0–12.0. The temperature-activity profile was determined at pH 4.0 and 30 to 90°C for 5 min. For thermal stability assay, rAga27A was incubated at pH 4.0 and 60°C, 65°C or 70°C for 2, 5, 10, 20, 30 or 60 min without substrate, and the residual activities were measured under standard conditions as described above, respectively.

The effects of metal ions and chemical reagents on rAga27A activity were examined by monitoring the residual enzymatic activities under standard conditions in the presence of 5 mM of NaCl, KCl, CaCl₂, LiCl, CoCl₂, CrCl₃, NiSO₄, CuSO₄, MgSO₄, FeCl₃, MnSO₄, ZnSO₄, Pb(CH₃COO)₂, AgNO₃, SDS, β -mercaptoethanol or EDTA. The reactions without any additive were used as a control.

Substrate specificity and kinetic parameters The substrate specificity of rAga27A was determined in the presence of *p*NPGal, melibiose, raffinose and stachyose, respectively. Among them, melibiose is a reducing disaccharide of galactose and glucose linked by an α -1,6 linkage; raffinose is a trisaccharide composed of galactose, glucose, and fructose; and stachyose is a tetrasaccharide consisting of two α -D-galactose units, one α -D-glucose unit and one β -D-fructose unit. The amounts of *p*NP released from *p*NPGal were determined as above. The α -galactosidase activity of rAga27A with melibiose as the substrate was determined

using the glucose oxidase-peroxidase method with a commercial kit (Biosino, Beijing, China) according to the manufacturer's instructions. The hydrolysis activities towards raffinose and stachyose were determined by measuring the reducing sugars released using the 3,5-dinitrosalicylic acid (DNS) method (18).

The K_m and V_{max} values for the purified rAga27A were determined at 70°C in McIlvaine buffer (pH 4.0) containing 0.01–2 mM *p*NPGal as the substrate. The experiments were carried out three times, and each experiment included three replicates. The data were calculated using the nonlinear regression computer program GraFit (Erithacus Software, Horley, UK) and plotted according to the Lineweaver-Burk method.

Transglycosylation activity assay Purified rAga27A (2 U) was incubated with 25 mM of *p*NPGal (donor) and 250 mM of galactose or melibiose (acceptor) in McIlvaine buffer (pH 4.0) at 45°C in a water bath with continuous agitation at 100 rpm for 5 h, 12 h and 24 h, respectively. Reactions were stopped by 5 min boiling. After cooling down to room temperature and centrifugation at 12,000 $\times g$, 4°C for 10 min, the compositions of transglycosylation products in the supernatants were analyzed by the thin-layer chromatography (TLC) plate (Silica gel 60F₂₅₄, Merck, Darmstadt, Germany). 1-Butanol, acetic acid and water at the ratio of 2:1:1 (vol/vol/vol) was used as the solvent. The plate was air dried and soaked in ethanol containing 5% of concentrated H₂SO₄. The plate was then dried and placed in an oven at 110°C for 5 min. Galactose and melibiose were used as the standards.

The transglycosylation activity of rAga27A was also determined with melibiose as both the donor and the acceptor. The reaction systems containing 1 U of rAga27A and 100 mM of melibiose were incubated under the same conditions as described above for 10 min, 30 min, 1 h, 5 h, 12 h and 24 h, respectively. The reactions were stopped by 5 min boiling. The products were also analyzed by using TLC.

Tolerance to galactose inhibition The inhibitory effect of galactose on rAga27A activity was determined by fitting to the Dixon plot (19). Aliquots of enzyme solution (100 μ l) were added to 275 μ l of McIlvaine buffer (pH 4.0) containing 2–100 mM galactose and 125 μ l of 3 or 4 mM *p*NPGal. The residual enzyme activities were measured at 70°C, pH 4.0 for 5 min. The K_i value was calculated by plotting the reciprocal of the reaction velocity and inhibitor concentration at each *p*NPGal concentration.

Nucleotide sequence accession number The nucleotide sequence for the *T. leycettanus* JCM12802 GH27 α -galactosidase gene (*aga27A*) was deposited in the GenBank database under accession number KP269074.

RESULTS AND DISCUSSION

Cloning and sequence analysis of the α -galactosidase gene *aga27A* The full-length *aga27A*, 1,711 bp in length, contains three introns (90 bp, 95 bp, and 68 bp,

respectively). The 1,458 bp-cDNA of *aga27A* encodes a polypeptide of 485 amino acids that consists of a typical hydrophobic signal sequence at residues 1–18 and a catalytic domain of GH27 (residues 19–485). The molecular mass and *pI* value were estimated to be 52.5 kDa and 5.2, respectively. The deduced amino acid sequence of *aga27A* showed the highest identity of 80% with the hypothetical α -galactosidase of GH27 from *Neosartorya fischeri* NRRL 181 (XM_001257444.1). To our best knowledge, it is the first α -galactosidase from *T. leycettanus*. The putative three-dimensional structure of Aga27A shows the $(\beta/\alpha)_8$ -barrel fold typical of GH27 members. Two putative catalytic aspartate residues (D149 and D209) located in the central cavity region, eight key residues involved in substrate binding (W34, D72, D73, C121, K147, C186, W188 and W204), and two protein motifs conserved among GH27 members, DD(G/C)W and YLKDYDNC, were also identified in deduced Aga27A (3, 4, 14).

Multiple sequence alignment (Fig. S1) and structural comparison (Fig. S2) of Aga27A and other homologs revealed significant differences in the loop regions. The loops (L1) in α -galactosidases of *N. fischeri* (KF640698) and *T. reesei* (1SZN_A) are distinctive, whereas the loops (L2 and L3) in Aga27A and α -galactosidases of *N. fischeri* (KJ739690), *L. thermotolerans* (BAE93466), *L. cidri* (Q99172.1) and *S. cerevisiae* (3LRK_A) are relatively close and form a smaller tunnel. Further phylogenetic analysis (Fig. S3) indicated that Aga27A is more closely related to the GH27 counterparts from *N. fischeri*, *L. thermotolerans*, *L. cidri* and *S. cerevisiae* but far from those from other fungi, bacteria and plants.

Expression and purification of rAga27A The gene fragment coding for mature Aga27A without the putative signal peptide was successfully expressed in *P. pastoris* with methanol induction. The recombinant enzyme was purified to electrophoretic homogeneity by anion exchange chromatography. SDS-PAGE (Fig. 1) revealed a single band with an apparent molecular mass of ~72 kDa, which is higher than its theoretical molecular mass (52.5 kDa). Glycosylation is one of the most prevalent and well-studied post-translational modifications in eukaryotes, which occurs in the secretory pathway (20). Sequence analysis with online tools indicated that deduced Aga27A has seven potential *N*-glycosylation sites. During heterologous expression in *P. pastoris*, rAga27A is likely to be *N*-glycosylated and showed an increased molecular weight. After Endo H treatment to remove any *N*-glycosylation, purified Aga27A migrated as a single band of ~55 kDa on the gel.

Enzyme properties of purified rAga27A As most fungal α -galactosidases have an acidic pH optimum (pH 3.0–5.0) (Table 2), purified rAga27A showed maximum activity at pH 4.0 and retained more than 60% of the activity at pH 3.0–5.0 (Fig. 2A). The enzyme displayed remarkable tolerance to acidic and alkaline pHs, retaining more than 80% of the initial activity over a broad pH range from 3.0 to 11.0 and more than 50% activity even at pH 2.0 (Fig. 2B). rAga27A showed excellent stability over a pH range of 3.0 to 11.0, which is broader than the α -galactosidases from *Thermomyces lanuginosus* (21), *Penicillium canescens* (22), *Bispora* sp. MEY-1 (23)

and *Debaryomyces hansenii* UFV-1 (24), but is similar to that of *N. fischeri* P1 α -galactosidase (14). *T. leycettanus* JCM12802 is a thermophilic strain that produces high-temperature active extracellular enzymes. As a result, rAga27A had a temperature optimum of 70°C (Fig. 2C) as most thermophilic α -galactosidases did (21, 25–28), but showed better thermostability at high temperatures than most fungal counterparts (Table 2). The enzyme was stable at 65°C (Fig. 2D), retaining more than 60% of the initial activity after 60 min incubation at pH 4.0, but lost activity rapidly and completely at 70°C within 20 min. rAga27A is thus favorable for the increased reaction rates at higher temperatures and lower risk of contamination by mesophilic organisms in industrial applications (29). The effect of different metal ions or chemical reagents on the α -galactosidase activity of rAga27A was shown in Table 3. The enzyme was intensely resistant to most chemicals tested except for SDS and Ag⁺. The excellent adaptability and stability of rAga27A over a broad pH range and at high temperatures and strong resistance against metal ions and chemicals may widen its application spectrum in various industries.

Substrate specificity and kinetic parameters rAga27A exhibited the highest activity of 43 U/mg to *p*NPGal (defined as 100%), followed by stachyose (23%), raffinose (9.3%) and melibiose (6.4%) (Table S1). It is common that α -galactosidases exhibit higher activities against synthetic substrates (i.e. *p*NPGal) than natural substrates (melibiose, raffinose and stachyose) (17, 31, 32).

Using *p*NPGal as the substrate, the K_m , V_{max} and k_{cat} values of rAga27A were

determined to be 1.32 mM, 389.8 $\mu\text{mol}^{-1} \text{min}^{-1} \text{mg}^{-1}$, and 341.0 s^{-1} , respectively (Fig. S4). The catalytic efficiency ($k_{\text{cat}}/K_{\text{m}}$) of rAga27A was 258.4 $\text{s}^{-1} \text{mM}^{-1}$, which is within the range of all characterized fungal counterparts (14.6–4730 $\text{s}^{-1} \text{mM}^{-1}$, Table 2).

Transglycosylation activity of Aga27A TLC analysis indicated that rAga27A had significant transglycosylation capacity. When using *p*NPGal as the donor and galactose as the acceptor, a disaccharide (galactosyl galactose) and a trisaccharide (galactosyl melibiose) were generated (Fig. 3A). When using melibiose as the acceptor, transglycosylation products of higher polymerization were produced, including trisaccharide (galactosyl melibiose), tetrasccharide (galactosyl trisaccharide) and pentasaccharide (galactosyl tetrasccharide) (Fig. 3B). When using melibiose as the donor and the acceptor, melibiose was firstly degraded into galactose, and a trisaccharide was then formed since the generated galactose was transferred to melibiose (Fig. 3C). Along with extended duration, the trisaccharide was gradually degraded, and galactose and melibiose were generated again. GH27 α -galactosidases catalyze the hydrolysis via a double-displacement mechanism, resulting in net retention of the stereochemistry at the anomeric centre (1). So the results indicated that rAga27A has both hydrolysis and transglycosylation activities against various substrates. α -Galactosidases have been reported to form α -galactooligosaccharides at high substrate concentrations by catalyzing the transfer of a galactosyl moiety to an acceptor (33, 34). Now galactooligosaccharides have been used as nondigestible,

carbohydrate-based food ingredients in human and animal nutrition (35). Thus rAga27A with the transglycosylating capacity is promising for producing oligosaccharides (36), and could greatly reduce the cost for industrial production.

Galactose tolerance Galactose is one of the products of α -galactosidase-catalyzed hydrolysis of the raffinose family of sugars. It is well known that galactose inhibits α -galactosidase. High tolerance towards galactose accumulation is a requirement of fungal α -galactosidase for industrial applications. Except the peanut α -galactosidase from *Arachis hypogaea* (37) that exhibited higher galactose tolerance with a K_i value of 189 mM, rAga27A was more tolerant to galactose (K_i 19 mM) than any other α -galactosidases, such as the α -galactosidases from *D. hansenii* UFV-1 (K_i 0.7 mM) (24) and *Talaromyces flavus* CCF2686 (K_i 0.38 mM) (38). These results agree with the inhibition patterns for structural analogues which have been reported. For example, Suzuki *et al.* (1970) reported that pNPGal, melibiose, raffinose, galactose, and other analogues inhibited the activity of *M. vinacera* α -galactosidase (39). The results indicated that galactose is a competitive inhibitor of rAga27A.

In this study we successfully cloned and heterologously expressed a novel high-temperature active α -galactosidase encoding gene, *aga27A*, from thermophilic *T. leycettanus* JCM12802. In comparison to other fungal counterparts, Aga27A exhibited both hydrolysis and transglycosylation activities, retained active and stable over a broader pH range and at high temperature, and was strongly resistant to most

metal ions, chemicals and galactose. These excellent properties reveal the great potential of rAga27A for applications in many industrial fields, especially the food, feed and medicine industries.

ACKNOWLEDGEMENTS

This research was supported by the National Science Foundation for Distinguished Young Scholars of China (31225026), the National Natural Science Foundation of China (31402110), the National High Technology Research and Development Program of China (2012AA022208), the 948 program of the Ministry of Agriculture of China (2014-S1).

References

1. **Zechel, D. L. and Withers, S. G.:** Glycosidase mechanisms: anatomy of a finely tuned catalyst, *Acc. Chem. Res.*, **33**, 11–18 (2000).
2. **Cantarel, B. L., Coutinho, P. M., Rancurel, C., Bernard, T., Lombard, V., and Henrissat, B.:** The Carbohydrate-Active EnZymes database (CAZy): an expert resource for glycogenomics, *Nucleic Acids Res.*, **37**, D233–D238 (2009).
3. **Golubev, A. M., Nagem, R. A., Brandao Neto, J. R., Neustroev, K. N., Eneyskaya, E. V., Kulinskaya, A. A., Shabalin, K. A., Savel'ev, A. N., and Polikarpov, I.:** Crystal structure of α -galactosidase from *Trichoderma reesei* and its complex with galactose: implications for catalytic mechanism, *J. Mol. Biol.*, **339**, 413–422 (2004).

-
- 370 4. **Fujimoto, Z., Kaneko, S., Kim, W. D., Park, G. G., Momma, M., and**
371 **Kobayashi, H.:** The tetramer structure of the glycoside hydrolase family 27
372 α -galactosidase I from *Umbelopsis vinacea*, Biosci. Biotechnol. Biochem., **73**,
373 2360–2364 (2009).
- 374 5. **Fernández-Leiro, R., Pereira-Rodríguez, Á., Cerdán, M. E., Becerra, M.,**
375 **and Sanz-Aparicio, J.:** Structural analysis of *Saccharomyces cerevisiae*
376 α -galactosidase and its complexes with natural substrates reveals new insights
377 into substrate specificity of GH27 glycosidases, J. Biol. Chem., **285**,
378 28020–28033 (2010).
- 379 6. **Linden, J. C.:** Immobilized α -D-galactosidase in the sugar beet industry, Enzyme
380 Microb. Tech., **4**, 130–136 (1982).
- 381 7. **Clarke, J. H., Davidson, K., Rixon, J. E., Halstead, J. R., Fransen, M. P.,**
382 **Gilbert, H. J., and Hazlewood, G. P.:** A comparison of enzyme-aided bleaching
383 of softwood paper pulp using combinations of xylanase, mannanase and
384 α -galactosidase, Appl. Microbiol. Biotechnol., **53**, 661–667 (2000).
- 385 8. **Ghazi, S., Rooke, J. A., and Galbraith, H.:** Improvement of the nutritive value
386 of soybean meal by protease and α -galactosidase treatment in broiler cockerels
387 and broiler chicks, Brit. Poult. Sci., **44**, 410–418 (2003).
- 388 9. **Tsuboi, K.:** Enzyme replacement therapy in patients with Fabry's disease, J. Int.
389 Med. Res., **35**, 574–581 (2007).
- 390 10. **Rodriguez-Colinas, B., Poveda, A., Jimenez-Barbero, J., Ballesteros, A. O.,**
391 **and Plou, F. J.:** Galacto-oligosaccharide synthesis from lactose solution or skim

- 392 milk using the β -galactosidase from *Bacillus circulans*, J. Agric. Food. Chem., **60**,
393 6391–6398 (2012).
- 394 11. **Wang, C., Luo, H., Niu, C., Shi, P., Huang, H., Meng, K., Bai, Y., Wang, K.,**
395 **Hua, H., and Yao, B.:** Biochemical characterization of a thermophilic
396 β -mannanase from *Talaromyces leycettanus* JCM12802 with high specific
397 activity, Appl. Microbiol. Biotechnol., **99**, 1217–1228 (2015).
- 398 12. **Liu, Y. G. and Whittier, R. F.:** Thermal asymmetric interlaced PCR:
399 automatable amplification and sequencing of insert end fragments from P1 and
400 YAC clones for chromosome walking, Genomics, **25**, 674–681 (1995).
- 401 13. **Wang, H., Ma, R., Shi, P., Xue, X., Luo, H., Huang, H., Bai, Y., Yang, P., and**
402 **Yao, B.:** A new α -galactosidase from thermoacidophilic *Alicyclobacillus* sp. A4
403 with wide acceptor specificity for transglycosylation, Appl. Biochem. Biotechnol.,
404 **174**, 328–338 (2014).
- 405 14. **Wang, H., Shi, P., Luo, H., Huang, H., Yang, P., and Yao, B.:** A thermophilic
406 α -galactosidase from *Neosartorya fischeri* P1 with high specific activity, broad
407 substrate specificity and significant hydrolysis ability of soymilk, Bioresour.
408 Technol., **153**, 361–364 (2014).
- 409 15. **Luo, H., Wang, Y., Wang, H., Yang, J., Yang, Y., Huang, H., Yang, P., Bai, Y.,**
410 **Shi, P., Fan, Y., and Yao, B.:** A novel highly acidic β -mannanase from the
411 acidophilic fungus *Bispora* sp. MEY-1: gene cloning and overexpression in
412 *Pichia pastoris*, Appl. Microbiol. Biotechnol., **82**, 453–461 (2009).
- 413 16. **Bradford, M. M.:** A rapid and sensitive method for the quantitation of

- 414 microgram quantities of protein utilizing the principle of protein-dye binding,
415 Anal. Biochem., **72**, 248–254 (1976).
- 416 17. **Cao, Y., Wang, Y., Luo, H., Shi, P., Meng, K., Zhou, Z., Zhang, Z., and Yao,**
417 **B.:** Molecular cloning and expression of a novel protease-resistant GH-36
418 α -galactosidase from *Rhizopus* sp. F78 ACCC 30795, J. Microbiol. Biotechnol.,
419 **19**, 1295–1300 (2009).
- 420 18. **Miller, G. L.:** Use of dinitrosalicylic acid reagent for determination of reducing
421 sugar, Anal. Chem., **31**, 426–428 (1959).
- 422 19. **Uchima, C. A., Tokuda, G., Watanabe, H., Kitamoto, K., and Arioka, M.:**
423 Heterologous expression in *Pichia pastoris* and characterization of an
424 endogenous thermostable and high-glucose-tolerant β -glucosidase from the
425 termite *Nasutitermes takasagoensis*, Appl. Environ. Microbiol., **78**, 4288–4293
426 (2012).
- 427 20. **Helenius, A. and Aebi, M.:** Roles of *N*-linked glycans in the endoplasmic
428 reticulum, Annu. Rev. Biochem., **73**, 1019–1049 (2004).
- 429 21. **Puchart, V., Vršanská, M., Bhat, M. K., and Biely, P.:** Purification and
430 characterization of α -galactosidase from a thermophilic fungus *Thermomyces*
431 *lanuginosus*, BBA. Gen. Sub., **1524**, 27–37 (2000).
- 432 22. **Sinitsyna, O., Fedorova, E., Vakar, I., Kondratieva, E., Rozhkova, A.,**
433 **Sokolova, L., Bubnova, T., Okunev, O., Chulkin, A., and Vinetsky, Y. P.:**
434 Isolation and characterization of extracellular α -galactosidases from *Penicillium*
435 *canescens*, Biochemistry (Mosc), **73**, 97–106 (2008).

-
- 436 23. **Wang, H., Luo, H., Li, J., Bai, Y., Huang, H., Shi, P., Fan, Y., and Yao, B.:** An
437 α -galactosidase from an acidophilic *Bispora* sp. MEY-1 strain acts synergistically
438 with β -mannanase, *Bioresour. Technol.*, **101**, 8376–8382 (2010).
- 439 24. **Viana, P. A., de Rezende, S. T., Passos, F. M. L., Oliveira, J. S., Teixeira, K.**
440 **N., Santos, A. M., Bemquerer, M. P., Rosa, J. C., Santoro, M. M., and**
441 **Guimarães, V. M.:** *Debaryomyces hansenii* UFV-1 intracellular α -galactosidase
442 characterization and comparative studies with the extracellular enzyme, *J. Agric.*
443 *Food. Chem.*, **57**, 2515–2522 (2009).
- 444 25. **Simila, J., Gernig, A., Murray, P., Fernandes, S., and Tuohy, M. G.:** Cloning
445 and expression of a thermostable α -galactosidase from the thermophilic fungus
446 *Talaromyces emersonii* in the methylotrophic yeast *Pichia pastoris*, *J. Microbiol.*
447 *Biotechnol.*, **20**, 1653–1663 (2010).
- 448 26. **Cavazzoni, V., Adami, A., and Craveri, R.:** α -Galactosidase from the yeast
449 *Candida javanica*, *Appl. Microbiol. Biotechnol.*, **26**, 555–559 (1987).
- 450 27. **Shankar, S., Kumar, S. P., and Mulimani, V.:** Calcium alginate entrapped
451 preparation of α -galactosidase: Its stability and application in hydrolysis of
452 soymilk galactooligosaccharides, *J. Ind. Microbiol. Biotechnol.*, **38**, 1399–1405
453 (2011).
- 454 28. **Wang, H., Ma, R., Shi, P., Huang, H., Yang, P., Wang, Y., Fan, Y., and Yao, B.:**
455 Insights into the substrate specificity and synergy with mannanase of family 27
456 α -galactosidases from *Neosartorya fischeri* P1, *Appl. Microbiol. Biotechnol.*,
457 10.1007/s00253-014-6269-3 (available online 10 December 2014).

-
- 458 29. **Turner, P., Mamo, G., and Karlsson, E. N.:** Potential and utilization of
459 thermophiles and thermostable enzymes in biorefining. *Microb. Cell Fact.* **6**,
460 1–23 (2007).
- 461 30. **Fernández-Leiro, R., Pereira-Rodríguez, A., Esperanza, M., Becerra, M.,**
462 **and Sanz-Aparicio, J.:** Structural analysis of *saccharomyces*
463 *cerevisiae* α -galactosidase and its complexes with natural substrates reveals new
464 insights into substrate specificity of GH27 glycosidases. *J. Biol. Chem.*, **285**,
465 28020–28033 (2010).
- 466 31. **Cervera-Tison, M., Tailford, L., Fuell, C., Bruel, L., Sulzenbacher, G.,**
467 **Henrissat, B., Berrin, J. G., Fons, M., Giardina, T., and Juge, N.:** Functional
468 analysis of family GH36 α -galactosidases from *Ruminococcus gnavus* E1:
469 insights into the metabolism of a plant oligosaccharide by a human gut symbiont,
470 *Appl. Environ. Microbiol.*, **78**, 7720–7732 (2012).
- 471 32. **Katrolia, P., Jia, H., Yan, Q., Song, S., Jiang, Z., and Xu, H.:** Characterization
472 of a protease-resistant α -galactosidase from the thermophilic fungus *Rhizomucor*
473 *miehei* and its application in removal of raffinose family oligosaccharides,
474 *Bioresour. Technol.*, **110**, 578–586 (2012).
- 475 33. **Goulas, T., Goulas, A., Tzortzis, G., and Gibson, G. R.:** A novel
476 α -galactosidase from *Bifidobacterium bifidum* with transgalactosylating
477 properties: gene molecular cloning and heterologous expression, *Appl. Microbiol.*
478 *Biotechnol.*, **82**, 471–477 (2009).
- 479 34. **Zhao, H., Lu, L., Xiao, M., Wang, Q., Lu, Y., Liu, C., Wang, P., Kumagai, H.,**

and Yamamoto, K.: Cloning and characterization of a novel α -galactosidase from *Bifidobacterium breve* 203 capable of synthesizing gal- α -1,4 linkage, FEMS Microbiol. Lett., **285**, 278–283 (2008).

35. **Intanon, M., Arreola, S. L., Pham, N. H., Kneifel, W., Haltrich, D., and Nguyen, T. H.:** Nature and biosynthesis of galacto-oligosaccharides related to oligosaccharides in human breast milk, FEMS Microbiol. Lett., **353**, 89–97 (2014).

36. **Wang, L. X.:** Chemoenzymatic synthesis of glycopeptides and glycoproteins through endoglycosidase-catalyzed transglycosylation, Carbohydr. Res., **343**, 1509–1522 (2008).

37. **Bryant, R. J. and Rao, D. R.:** Purification and characterization of α -galactosidase from peanuts 1, J. Food Biochem., **25**, 139–156 (2001).

38. **Simerská, P., Monti, D., Čechová, I., Pelantová, H., Macková, M., Bezouška, K., Riva, S., and Křen, V.:** Induction and characterization of an unusual α -D-galactosidase from *Talaromyces flavus*, J. Biotechnol., **128**, 61–71 (2007).

39. **Suzuki, H., Li, S. C., and Li, Y. T.:** α -Galactosidase from *Mortierella vinacea* crystallization and properties, J. Biol. Chem., **245**, 781–786 (1970).

Figure legends

FIG. 1. SDS-PAGE (12%) analysis of the purified rAga27A. Lanes: M, the protein molecular weight markers; 1, the purified rAga27A; 2, the deglycosylated rAga27A with Endo H treatment.

FIG. 2. Characterization of the purified rAga27A. (A) The effect of pH on enzyme activity. The activity assay was performed at 70°C in buffers of pH 2.0–8.0 for 5 min. (B) pH stability. After pre-incubating the enzyme at 37°C for 1 h at pH 2.0–12.0, the residual activities were measured in McIlvaine buffer (pH 4.0) at 70°C for 5 min. (C) The effect of temperature on enzyme activity measured in McIlvaine buffer (pH 4.0) for 5 min. (D) Thermostability. The enzyme was pre-incubated at 60°C, 65°C or 70°C in McIlvaine buffer (pH 4.0). Aliquots were removed at specific time points for measurement of residual activities at 70°C and pH 4.0 for 5 min. Each value in the panel represents the mean $\pm$ SD ($n = 3$).

FIG. 3. TLC analysis of the transglycosylation activity of rAga127A with different donors and acceptors. M shows the oligosaccharides standards (galactose and melibiose). (A) Transglycosylation with *p*NPGal as the donor and galactose as the acceptor. 1 shows the *p*NPGal; 2 shows the galactose; 3–5 show the products after incubation for 5 h, 12 h and 24 h, respectively. (B) Transglycosylation with *p*NPGal as the donor and melibiose as the acceptor. 1 shows the *p*NPGal; 2 shows the

521 melibiose; 3–5 show the products after incubation for 5 h, 12 h, 24 h, respectively. (C)
522 Transglycosylation with melibiose as the donor and acceptor. 1 shows the melibiose;
523 2–8 show the products after incubation for 10 min, 30 min, 1 h, 5 h, 12 h, 24 h and 36
524 h, respectively.

525

Figure 1

Figure 2

Figure 3

536

537

^a The restriction sites are underlined.

538

Table 2. Enzymatic property comparison of rAga27A with other thermophilic fungal α -galactosidases of GH27 ^a.

Organism	Optimal pH/pH stability	Optimum temperature/ thermostability	K_m (mM)	V_{max} ($\mu\text{mol min}^{-1}$ mg^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{s}^{-1} \text{mM}^{-1}$)	References
<i>T. leycettanus</i> JCM12802	pH4.0/pH 3.0–11.0	70 °C/65 °C for 1 h	1.32	389.8	341.0	258.4	This study
<i>N. fischeri</i> P1	pH4.0/pH 3.0–11.0	75 °C/70 °C for 1 h	2.84	1850	1621	570.8	(28)
<i>Talaromyces emersonii</i>	pH4.5/-	70 °C/50 °C for 10 d	0.29	240.3	200.3	690.5	(25)
<i>Candida javanica</i>	pH4.0/-	70 °C/70 °C for 15 min	11	100	-	-	(26)
<i>Thermomyces lanuginosus</i>	pH4.5–5.0/pH 3.0–7.5	65–70 °C/60 °C for 6 h	0.50	52.4	49.8	99.6	(21)
<i>Aspergillus terreus</i>	pH5.0/-	65 °C/65 °C for 30 min	0.11	7.2	-	-	(27)
<i>N. fischeri</i> P1	pH4.5/pH 2.0–12.0	60 °C/60 °C for 10 min	0.8	449.5	368.6	460.8	(14)

<i>Penicillium canescens</i>	pH4.0–5.0/pH 3.0–6.0	55 °C/40–50 °C for 3 h	0.48	6.89	7.0	14.6	(22)
------------------------------	----------------------	------------------------	------	------	-----	------	------

539 ^a The enzyme activities and kinetic values were determined by using *p*PNGal as the substrate.

Table 3. Effects of metal ions and chemical reagents on the activities of purified
rAga27A.

Chemicals	Relative activity (%) ^a	Chemicals	Relative activity (%)
None	100.0 ± 0.3	Cr ³⁺	101.1 ± 3.5
Pb ²⁺	106.4 ± 2.3	Cu ²⁺	101.1 ± 0.4
Fe ³⁺	106.2 ± 1.8	Ca ²⁺	98.6 ± 1.4
Na ⁺	106.1 ± 1.6	Ni ²⁺	98.5 ± 3.0
K ⁺	105.9 ± 1.7	Co ²⁺	95.6 ± 2.5
Zn ²⁺	105.2 ± 1.2	Ag ⁺	0.2 ± 0.0
Li ⁺	104.2 ± 0.3	EDTA	103.9 ± 0.3
Mg ²⁺	103.3 ± 2.6	β-Mercaptoethanol	98.6 ± 3.4
Mn ²⁺	101.2 ± 2.4	SDS	0.0 ± 0.0

^a Values represent the mean ± SD (*n* = 3) relative to the control samples without
any addition.