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A protease-resistant α-galactosidase from *Pleurotus citrinopileatus* with broad substrate specificity and good hydrolytic activity on raffinose family oligosaccharides

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ABSTRACT

An acidic α-galactosidase designated as PCGI was isolated from the fruiting bodies of *Pleurotus citrinopileatus* with 264-fold purification and a specific activity of 7.92 units/mg. It was purified to homogeneity by ion exchange chromatography and gel filtration chromatography. PCGI is a heterodimeric protein consisting of a 33 kDa and a 27 kDa subunit in SDS-PAGE. The purified enzyme was identified by MALDI-TOF-MS. It belongs to the GH27 family. The optimum pH and temperature of the enzyme with pNPGal as substrate were 4.4 and 50 °C, respectively. Besides, it displayed remarkable resistance to acid protease, neutral protease, α-chymotrypsin, and trypsin. It was strongly inhibited by Cd^{2+}, Cu^{2+}, Hg^{2+}, Al^{3+}, Fe^{3+} and Ag^{+} ions. Diethypyrocarbonate (DEPC) doubled the activity of PCGI whereas *N*-bromosuccinimide (NBS) drastically decreased it. PCGI displayed wide substrate diversity with activity toward substrates such as stachyose, raffinose, melibiose. The Km values for hydrolysis of pNPGal, stachyose, raffinose, and melibiose were 0.2, 16.7, 18.9, and 6.3 mM, respectively. Galactose (Ki = 0.92 mM) and melibiose (Ki = 7.13 mM) competitively inhibited the enzymes. Futhermore, it completely degraded raffinose and sthachyose. These results suggest that PCGI has great potential for removal of the non-digestible and flatulence-causing oligosaccharides stachyose and raffinose from legumes.

1. Introduction

Pleurotus citrinopileatus, commonly called "golden oyster mushroom", is a popular edible mushroom because of its bright yellow color and unique flavor, and medicinal value. Polysaccharides or extracts from *P. citrinopileatus* have been found to have antitumor, immuno-enhancement, antihyperglycemic, antihyperlipidemic effects and antioxidant activities [1–4]. Moreover, a lectin purified from its fruiting bodies exhibit antitumor, mitogenic and HIV-1 reverse transcriptase inhibitory activities [5]. Recently, a novel nonlectin glycoprotein, PCP-3A isolated from the fruiting bodies of *P. citrinopileatus* was reported to inhibit the growth of hum an myeloid leukemic U937 cells by stimulating human MNC to secrete cytokines [6]. Up till now, studies on α-galactosidase from *P. citrinopileatus* have not been reported.

α-galactosidases (α-D-galactoside galactohydrolases; EC 3.2.1.22) catalyse the hydrolysis of α-1,6-linked α-galactoside residues in different substrates [7], thus they have potential applications in various industrial processes such as sugar production [8], pulp and paper [9], food and feed additives [10]. In addition to that, it is also used in medical treatment, such as blood group transformation, treatment of Fabry's disease, and xenotransplantation [11–14]. Despite its hydrolytic activity, α-galactosidases can also be applied to synthesis of α-galactosides by transglycosylation and reverse hydrolysis reactions [15].

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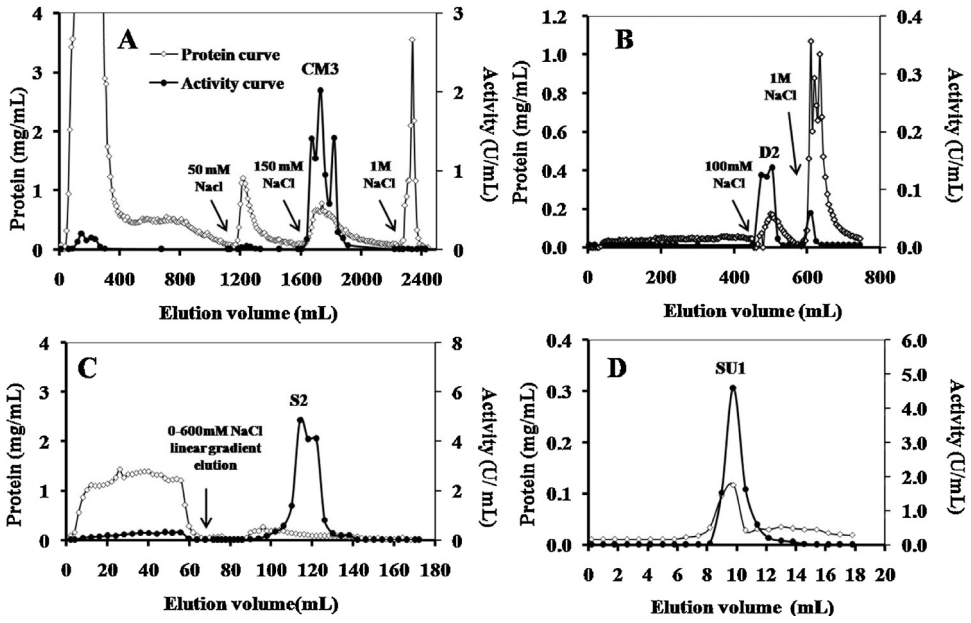

Fig. 1. Elution profile of PCGI on a (A) CM-cellulose column, (B) DEAE-cellulose column, (C) SP-sepharose column and (D) superdex 75HR 10/30 gel filtration.

α-D-Galactosides, mainly raffinose and stachyose, are identified as antinutritional factors in soybean and legume seeds because of indigestibility and induction of flatulence in monogastric animals. Therefore, α-galactosidases have been extensively used for degrading raffinose-family oligosaccharides(RFOs) in the food and feed industries [16], for instance used to improve the gelling properties of galactomannans to be used as food thickeners [17]. Furthermore, α-galactosidase has also been applied to the field of biotechnology for hydrolyzing raffinose in beet sugar syrups to facilitate the crystallization of sucrose [18].

In this study, we report the purification and enzymatic properties of an α-galactosidase from *P. citrinopileatus* (defined as PCGI). The ability of PCGI to hydrolyse various natural substrates, especially the RFOs, was also assessed in order to explore its potent applications in food and feed industries.

2. Materials and methods

2.1. Plant materials and chemicals

Fresh fruiting bodies of *P. citrinopileatus* were purchased from a local market in Beijing. DEAE-cellulose, CM-cellulose, and SP-Sepharose were obtained from Sigma Chemical Co., USA. Q-Sepharose, Superdex G-75HR 10/30 and AKTA Purifier were purchased from GE Healthcare, USA. The substrates 4-nitro-phenyl α-D-galactopyranoside (pNPGal), o-nitrophenyl α-D-galactoside (oNPαGal), 4-nitrophenyl β-D-glucuronide, locust bean gum, guar gum, melibiose, galactose, lactose, sucrose, glucose, xylose, fructose, stachyose and raffinose were purchased from Sigma Chemical Company (St. Louis, MO, USA). Acid protease, neutral protease, proteinase K, α-chymotrypsin, and trypsin proteases were purchased from Sigma Chemical Company. All of the other chemicals

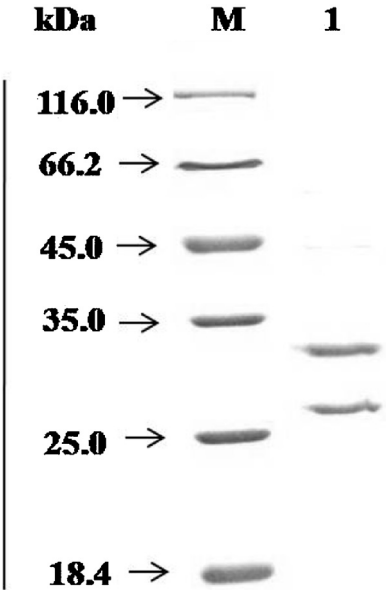

Fig. 2. SDS-PAGE analyses of PCGI. Lanes: M, molecular mass standards; 1, Superdex 75 fraction.

for buffers and other reagents used were of analytical grade or electrophoresis grade unless otherwise stated.

2.2. Enzyme activity assay

With minor modifications, α-galactosidase activity was assayed by determining the amount of p-nitrophenol (pNP) released from pNPGal [19]. The reaction mixture consisted of 20 μL diluted enzyme and 20 μL of 10 mM pNPGal (pH 4.6). Incubation was performed at 40 °C for 10 min. The reaction was ended with addition of 160 μL 0.5 M Na_2CO_3. The amount of p-nitrophenol released was determined spectrophotometrically at 405 nm. This procedure was defined as the standard assay. One unit of α-galactosidase activity was defined as the amount of enzyme that liberated 1 μmol p-nitrophenol per min at 40 °C and pH 4.6 under the assay conditions. The enzyme activity values presented are mean values of triplicate assays. Standard deviation values were always smaller than 10% of the mean value.

2.3. Protein estimation

Protein concentration was estimated according to the method of Lowry et al. [20] using bovine serum albumin (BSA) as standard. Fractions obtained during chromatographic purification were screened for protein by measurement of absorbance at 280 nm.

2.4. Isolation and purification of PCGI

P. citrinopileatus fruiting bodies (60 g) were homogenized in deionized water using a Waring blender. The homogenate was centrifuged at 10,000 × *g* for 10 min after overnight extraction at 4 °C. The supernatant was dialyzed in deionized water before applying to a column (20 cm × 20 cm) of CM-cellulose, which had previously been equilibrated with and was then eluted with 10 mM NaAc-HAc buffer (pH 4.6). After the unadsorbed proteins (collected as fraction CM1) had been eluted, elution was performed sequentially with an increasing gradient of 50 mM, 150 mM, 1 M NaCl prepared in equilibration buffer at the flow rate of 10 mL/min, and 10-mL fractions were collected. The active fractions, which were located in the 150 mM NaCl eluant (CM3), were pooled and dialyzed against deionized water.

After dialysis, fraction CM2 was subjected to ion exchange chromatography on a DEAE-cellulose column (10 cm × 20 cm), which had previously been equilibrated with 10 mM Tris-HCl buffer, pH 7.6. After the unadsorbed proteins (collected as fraction D1) had been eluted, the proteins were eluted in turn with the same buffer containing 100 mM NaCl, and then with buffer containing 1 M NaCl at a flow rate of 8 mL/min, and 5 mL fractions were collected. The active fraction (D2) was eluted with 100 mM NaCl, which were then pooled and dialyzed as described above.

Fraction D2 was injected into a column of SP-sepharose (1 cm × 10 cm) previously equilibrated with 10 mM NaAc-HAc buffer (pH 5.0). After the unadsorbed proteins (collected as fraction SP1) had been eluted, the column was further eluted with a linear 0–600 mM NaCl gradient in the same buffer. Fraction SP2 containing α-galactosidase activity was finally purified on a fast protein liquid chromatography (FPLC) on a Superdex 75HR 10/30 gel filtration (GE-Healthcare, USA), which was pre-equilibrated with 10 mM NaAc-HAc buffer (pH 4.6) containing 150 mM NaCl to prevent non-specific adsorption of proteins. The active fractions were pooled, dialyzed, freeze-dried and analyzed for purity by SDS-PAGE.

2.5. Native molecular mass

Native molecular mass of the purified PCGI was determined by a Superdex 75HR 10/30 gel filtration column as described above.

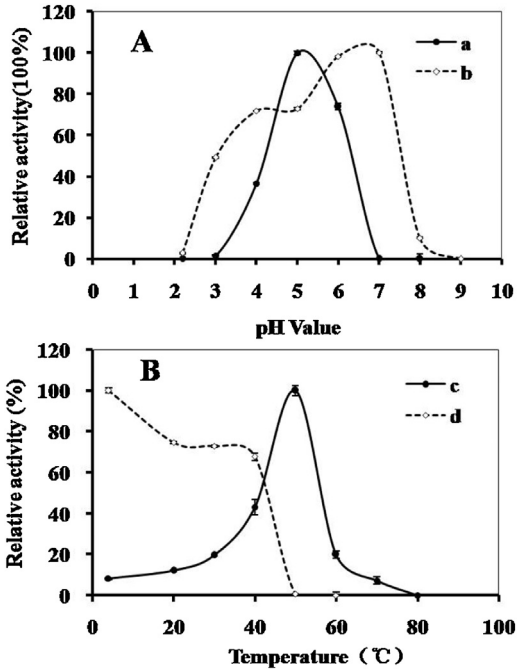

Fig. 3. pH and temperature profiles of PCGI. (A) Effects of pH on the activity (a) and stability (b) of PCGI (B) effects of temperature on the activity (c) and stability (d) of PCGI.

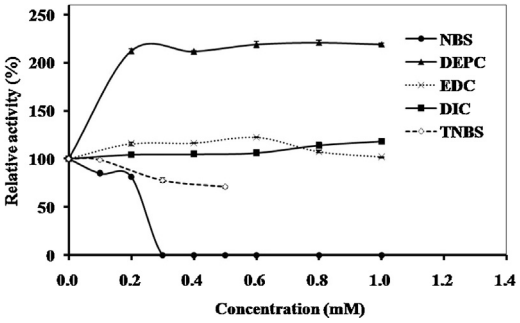

Fig. 4. Effect of chemical modification reagents on activity of PCGI.

Marker proteins with known molecular mass were used for column calibration. The proteins were eluted with the same buffer at a flow rate of 0.5 mL/min. Along with measurement of absorbance at 280 nm, the activity peak was checked by assaying the enzyme activity using pNPGal as substrate.

2.6. SDS-PAGE

Protein homogeneity of purified PCGI was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12.5% w/v polyacrylamide gel [21]. The proteins were stained with 0.25% Coomassie Brilliant Blue R-250, and molecular mass was determined by comparing the relative mobilities of appropriate protein markers. Medium molecular mass protein markers (Sigma Chemicals Co., USA) ranging from 14.4 to 116 kDa were used. The molecular mass protein standards used in gel filtration included bovine serum albumin (67 kDa), ovalbumin (43 kDa), ribonuclease A (13 kDa), aprotinin (6.5 kDa) and vitamin B (1.355 kDa).

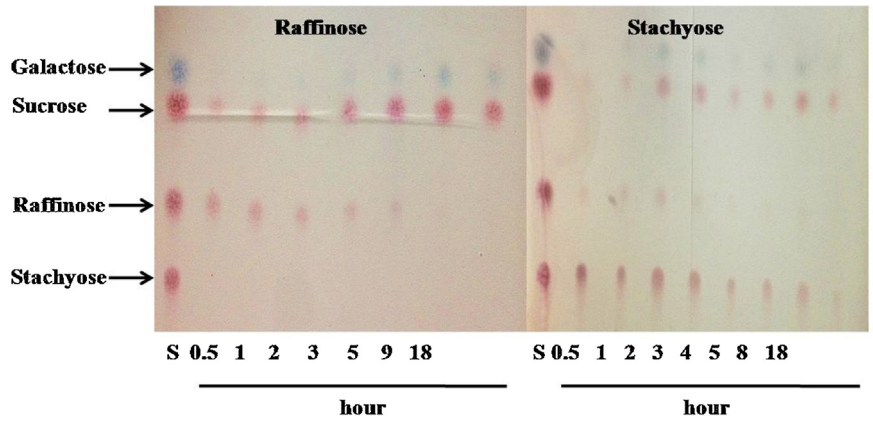

Fig. 5. TLC analysis of raffinose and stachyose hydrolyzed by PCGI. Lane S, a mixture of galactose, sucrose, raffinose, stachyose.

2.7. Analysis of amino acid sequence

There is no report for the amino acid sequence of PCGI in an available protein database. Therefore, the ratio of *m*/*z* values obtained from MALDI-TOF spectra corresponding to peptides of PCGI was matched with α-galactosidases from other sources using Mascot (www.matrixscience.com). The NCBI database was searched with fungi. Comparative analysis is based on parameters such as digestion with trypsin with 1 missed cleavage and the rest is by default. During our search, all of the peptide masses were assumed to be monoisotropic with a mass accuracy of ±0.2 Da.

2.8. Effects of pH and temperature on PCGI

The optimum pH of the purified enzyme was determined with pNPGal as the substrate in the pH range of 2.2–8.0 using 100 mM Na_2HPO_4-citric acid buffer. The pH stability was studied by incubating the purified enzyme in different buffers for 3 h at room temperature and then measuring the residual enzyme activity. The optimum temperature of the purified enzyme was determined at its optimum pH value with pNPGal as the substrate over the temperature range of 4–80 °C. Thermostability was investigated by measuring the enzyme activity remaining after incubation for 1 h at 4–60 °C.

2.9. Effects of various metal ions, chemical reagent, and chemical modification reagents on PCGI

The effects of various metal ions, chemical reagents, and chemical modification reagents on the purified enzyme were examined. The assay mixture consisted of appropriately diluted enzyme, 10 mM pNPGal in pH 4.6, 0.1 M NaAc-HAc buffer and different concentrations of metal ions, chemical reagents and chemical modification agents. The influence of metal ions (10, 5, 2.5, and 1.25 mM), chemical modification agents, and 2, 20, 200 mM EDTA, SDS, sodium acetate, $(NH4)_2SO_4$, NaCl, lactose, glucose, meliobiose, sucrose, xylose and fructose in sodium citrate buffer (pH 4.6) on PCGI was studied in a ratio of 1:1 at 40 °C for 60 min. The remaining activity was assayed using pNPGal and compared with the control (enzyme without the addition of reagents).

2.10. Protease treatments

To examine resistance to different proteases, purified PCGI (0.02 UmL^{-1}) was incubated at 37 °C for 1 h with 2 mg mL^{-1} and 20 mg mL^{-1} acid protease (pH 4.0), neutral protease (pH 7.0), trypsin (pH 7.0), α-chymotrypsin (pH 7.0), or proteinase K (pH 7.5). The residual enzyme activity was assayed in 50 mM citrate buffer (pH 4.6) by the standard method at 40 °C. The activity of the control sample (enzyme without protease) was regarded as 100%.

2.11. Substrate specificity

Enzymatic assays were performed with various synthetic, natural, and polymeric substrates. The synthetic substrates were the nitrophenyl derivatives, including 4-nitro-phenyl α-D-galactopyranoside (pNPGal), 2-nitrophenyl α-D-galactopyranoside (oNPGal) and 4-nitrophenyl β-D-glucuronide. The reaction mixture contained 20 μL of 5 mM nitrophenyl derivative in 0.1 M pH 4.6 NaAc-HAc buffer, and 20 μL diluted enzyme preparation. The reactions were performed at 40 °C for 10 min and terminated by adding 160 μL of 0.5 M Na_2CO_3. A pNP standard curve was plotted for each assay with the following concentrations: 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 μM/L pNP. For natural and polymeric substrates, the reaction mixture contained 40 μL of enzyme in solution 0.1 M NaAc-HAc buffer (pH 4.6), and 10 μL of natural and polymeric substrate, stachyose (50 mM), and raffinose (50 mM), or locust bean gum and guar gum solutions (1%). The activities were measured under standard assay conditions at 40 °C, 60 min. The temperature of incubation was chosen because it represents the temperature at which the enzyme exhibits long-term stability while maintaining a satisfactory level of activity. The substrate specificity for sucrose, raffinose, stachyose, locust bean gum and guar gum was determined by measuring the reducing sugar released using the 3,5-dinitrosalicylic acid reagent as described by Miller [22].

One unit of α-galactosidase activity was defined as the amount of enzyme that released 1 μmol of reducing sugar equivalent to galactose per minute under the assay conditions. A glucose standard curve was plotted for each assay with the following concentrations: 0, 83.33, 125, 166.67, 208.33, 250, 291.67, 333.33 and 416.67 μM/L glucose. The α-galactosidase activity with melibiose as substrate was determined as above and the glucose released was measured with a glucose-oxidase kit (Beijing BHKT clinical Reagent Co., Ltd.). One unit of enzyme activity is defined as the amount of enzyme that releases 1 μmol of glucose per minute at 40 °C. The data presented for all enzyme activity determinations are mean values SD of three measurements.

2.12. Determination of kinetic parameters

For all kinetic studies, dialyzed enzyme obtained after gel filtration chromatography was used. Kinetic experiments were performed at 40 °C. The Michaelis-Menten constant (Km) and Vmax for substrate hydrolysis were calculated by the Michaelis-Menten plot. The substrate concentrations expressed in mM were 0. 5, 1.0, 1.5,

2.0, 2.5, 3.0, 3.5, 4.0, 4.5, and 5.0 for pNP Gal; 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 for stachyose; 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 for raffinose.

2.13. Enzymatic hydrolysis of RFOs

A mixture of 400 μL of purified enzyme (containing 2.4 units/mL) and 100 μL of raffinose (50 mM) or 100 μL stachyose (50 mM) in 0.1 M NaAc-HAc buffer(pH 4.6), was incubated for various time periods at 40 °C. Aliquots (20 μL) of the solution were sampled from the reaction mixture during the hydrolysis process, boiled in a water bath for 5 min to stop the reaction, and centrifuged to remove denatured protein. The hydrolysates were analyzed qualitatively by thin-layer chromatography (TLC). About 3 μL of each of the hydrolysates was applied to silica gel G plates (10 cm × 10 cm) and developed by ascending chromatography using n-propanol/acetic acid/water (10:15:1; v/v/v) as the developing solvent system. The sugar spots were revealed by incubating the plates at 90 °C after immersing in chromogenic reagent (diphenylamine 1 g, aniline 1 mL, acetone 50 mL) when the silica gel plates were completely dried. The reduction of raffinose and stachyose was determined by measuring the increase of reducing sugars using the 3,5-dinitrosalicylic acid reagent as described by Miller [22].

3. Results and discussion

3.1. Purification of PCGI

PCGI was purified through a multistep purification procedure and the purification scheme of the PCGI is shown in Fig. 1. The crude extract was subjected to ion exchange chromatography on CM-cellulose resulting in the separation of one protein fraction CM3 with α-galactosidase activity (Fig. 1A). CM3 was used for the next step which was anion exchange chromatography on DEAE-cellulose, resulting in an active fractions D3 (Fig. 1B). The third step was cation-exchange chromatography on SP-Sepharose, and the activity resided in fraction S2 (Fig. 1C). After the abovementioned multi-stage purification, the specific activity against pNPGal was 24.07 Umg^{-1}. The final step was carried out by gel filtration chromatography on Superdex 75 and the resulting active fraction appeared as a single peak (Fig. 1D) designated as SU1. This step resulted in considerable enhancement of the specific activity, with a 274-fold purification of the enzyme and a specific enzyme activity of 39.59 Umg^{-1} toward pNPGal achieved (Table 1). It is worth noting that no invertase activity was detected in the final enzyme preparation. The purification fold of PCGI attained was higher than those α-galactosidases from *Tachigali multijuga* Benth. seeds (241.52) [23], *Aspergillus terreus* (E1, 73.15; E2, 60.26; E3, 50.87) [24], and *Debaryomyces hansenii* UFV-1 (16.70) [25].

3.2. Determination of PCGI molecular weight and amino acids sequence of inner peptide

As indicated as the result of the gel filtration chromatography (Fig. 1D), the size of PCGI was estimated to be 57 kDa. This was similar to the other α-galactosidases purified from *Aspergillus fumigatus*, and *Thermomyces lanuginosus* IMI 158749 which presented Mr values of 54.7 kDa [26], and 57 kDa [27], respectively. The molecular mass of the α-galactosidase was identified further by SDS-PAGE under reducing conditions (Fig. 2, lane 1). Two single bands were observed in the case of SDS-PAGE, with molecular mass corresponding to 33 kDa and 27 kDa respectively (Fig. 2, lanes 1). Thus, PCGI is a heterodimer in solution. It has been reported that *T. polysaccharolyticum* [28] and *T. maritima* Gal A [29], and *Escherichia coli* Mel A [30] were active as dimers.

High-quality MALDI-TOF peptide mass spectrum was obtained after tryptic digestion of PCGI. The amino acid sequences of inner peptide fragments with a molecular mass of 33 kDa and 27 kDa respectively were subjected to BLAST search against fungal α-galactosidase databases in NCBI. As for the 33-kDa subunit, seven important peptides which showed remarkable identity with other fungi α-galactosidases were obtained. Peptide **KFPDGISGVADKI** shared 100% identity with α-galactosidases from *Penicillium simplicissimum* (accession number O94221.1); Peptide **RVAALENGVARL** showed 100% query coverage and identity with glycoside hydrolase from *Auricularia delicata* TFB-10046 SS5 (accession number XP_007349296.1); Peptide **MKLLTVVGKI** harbored 100% query coverage and identity with α-galactosidase from *Talaromyces stipitatus* ATCC 10,500 (accession number XP_002485438.1); Peptide **RQAQLVRRF** shared 100% query coverage and identity with α-galactosidase from *Colletotrichum orbiculare* MAFF 240422 (accession number ENH87651.1); Peptide **KQAQLVRRY** shared 100% query coverage and identity with α-galactosidase from *Colletotrichum sublineola* (accession number KDN69325.1) and *Colletotrichum graminicola* (accession number M1.001 XP_008095900.1); Peptide **KEVIALNQDPLVKQ** shared 100% query coverage and identity with α-galactosidase from *C. sublineola* (accession number KDN69325.1). Peptide **RLVSVGDLRV** suggested 90% query coverage and 100% identity with α-galactosidase from *Streptomyces clavuligerus* (accession number WP_009996758.1). With respect to the 27-kDa subunit, we got only one peptide **KFPDGISGVADKI**, same as the 33-kDa subunit, which shared 100% identity with α-galactosidases from *Penicillium brasilianum* (accession number CEJ59911.1) and glycoside hydrolase family 27 protein from *Paxillus rubicundulus* Ve08.2h10 (accession number KIK91188.1);

On the basis of amino acid sequence similarity, α-galactosidases have been classified into glycoside hydrolase (GH) families 4, 27, 36, 57, 97 and 110 in CAZy database (http://www.cazy.org/). Many reported fungal α-galactosidases, for instance, *Bispora* sp.[31], *Clostridium josui* [32]. and *Neosartorya fischeri* [33] belong to GH family 27. We found that PCGI share higher identity with GH family 27 protein when we compared the amino acid sequence of its inner peptides above with other α-galactosidases. Moreover, the matched α-galactosidases introduced above all belong to the GH 27 family. So we proposed that PCGI is a novel member of GH family 27.

3.3. Biochemical properties of PCGI

As Fig. 3a indicated, PCGI achieved maximum activity at pH 5.0 with pNPGal in Na_2HPO_4-citric acid buffer and was stable over a range of pH 4.0–7.0 (Fig. 3b). When the pH was outside the range of 4.0 to 7.0, hardly any α-galactosidase activity could be detected. Moreover, incubation of α-galactosidsae in NaAc-HAc buffers with different pH as 3.8, 4.0, 4.2, 4.4, 4.6, 5.0 showed that its optimum pH is 4.4 (data did not show). This was in good agreement with the other α-galactosidase purified from other sources [34]. In general, the optimal pH of fungi α-galactosidases are within the acidic pH range (4.5–5.5) [33], which makes them valuable in several applications occurring at acidic pH [35].

The hydrolyzing activity of PCGI was detected at different temperatures from 20 to 80 °C. The rate of the enzyme-catalyzed reaction increases to an optimum value with increasing temperature until inactivation of the enzyme occurs. PCGI shows maximal activity at 50 °C (Fig. 3c), which was in good agreement with reported α-galactosidases from other sources [23,34]. Moreover, the enzyme maintained about 70% of its original activity at 40 °C after incubated 1 h, but most of the activity was lost at 50 °C (Fig. 3d), indicating that PCGI was a mesophilic α-galactosidases. Many mesophilic α-galactosidases from fungi have been purified

Table 1
Purification of PCGI.

Purification step	Protein (mg)	Activity (U/mL)	Total activity(U)	Specific activity (U/mg)	Recovery (%)	Purification fold
Extracts	3865.67	3.06	557.48	0.14	100.0	1.00
CM3	132.24	0.19	109.87	0.83	19.71	5.76
D2	13.65	0.88	40.06	2.93	7.19	20.35
SP2	1.13	2.51	27.17	24.07	4.87	166.92
SU1	0.26	13.03	10.42	39.59	1.87	274.53

Table 2
Effects of metal ions on the activity of PCGI.

Metal ion concentration	Relative α-galactosidase activity[a] (%)(mean ± SD, $N = 3$)			
	10 mM	5 mM	2.5 mM	1.25 mM
Fe^{2+}	89.8 ± 0.02	82.6 ± 0.02	72.9 ± 0.01	73.3 ± 0.02
K^{+}	85.2 ± 0.03	86.5 ± 0.03	74.2 ± 0.00	77.3 ± 0.02
Ca^{2+}	95.6 ± 0.01	90.2 ± 0.01	90.4 ± 0.01	90.9 ± 0.01
Cd^{2+}	8.3 ± 0.01	6.9 ± 0.01	6.6 ± 0.01	4.3 ± 0.01
Cu^{2+}	20.3 ± 0.01	9.3 ± 0.01	8.8 ± 0.01	6.0 ± 0.01
Hg^{2+}	0.0 ± 0.01	0.0 ± 0.03	0.0 ± 0.02	0.0 ± 0.02
Mg^{2+}	85.8 ± 0.02	87.8 ± 0.02	98.0 ± 0.02	96.6 ± 0.00
Mn^{2+}	80.9 ± 0.01	82.7 ± 0.02	89.0 ± 0.02	98.4 ± 0.01
Pb^{2+}	45.9 ± 0.01	50.0 ± 0.01	51.9 ± 0.04	54.0 ± 0.05
Zn^{2+}	26.1 ± 0.01	46.7 ± 0.01	51.6 ± 0.01	64.3 ± 0.00
Al^{3+}	9.6 ± 0.01	15.7 ± 0.01	16.1 ± 0.01	23.6 ± 0.01
Fe^{3+}	0.0 ± 0.01	0.0 ± 0.01	0.0 ± 0.02	0.0 ± 0.01
Ag^{+}	0.0 ± 0.00	0.0 ± 0.01	0.0 ± 0.01	0.0 ± 0.01

[a] Relative activities were calculated in relation to pNPGal activity, determined in the reaction without effectors, which was considered as 100%.

and characterized. And for this reason, we take 40 °C as reaction temperature of PCGI to various substrate. To sum up, the enzyme showed significant stability for a wide range of pH but narrow temperature levels, which would limit its industrial applications to some extent.

3.4. Effects of metal ions and chemical reagents on PCGI

The sensitivity of purified PCGI to various concentrations of metal ions was tested (Table 2). When the concentration was increased from 1.25 mM to 10 mM, K^{+} (74.2–86.5%), Fe^{2+} (72.9–89.8%), Mn^{2+} (80.9–98.4%), Mg^{2+} (85.8–98.0%) and Ca^{2+} (95.6–90.2%) ions had only a slight effect on enzyme activity. Pb^{2+} ions suppressed the activity of PCGI (45.9–54.0%) moderately. As the concentration increased, the inhibition of Zn^{2+} (26.1–64.3%) and Al^{3+} (9.6–23.6%) ions on PCGI increased. Mercuric chloride is a strong thiol-specific inhibitor [36] and also reacts with amino and imidazolium groups of histidine and with peptide linkages [37]. Ag^{+} ions can react with carboxyl and/or histidine residues. As shown in Table 2, the activity of PCGI (0.0%) was strongly inhibited by Hg^{2+} ions, likewise Fe^{3+} and Ag^{+} ions. Reduction in the α-galactosidase activity by Hg^{2+} and Ag^{+} ions was similar to those reported for α-galactosidases from *T. multijuga* Benth. seeds, *Streptococcus mutans*, *Lactobacillus fermentum*, which harbor sensitive sulfhydryl groups [23,38,39]. The marked reduction of PCGI activity was also seen with Cu^{2+} (6.0–20.3%) and Cd^{2+} (4.3–8.3%) ions, but the change was not regular. Reduction in the α-galactosidase activity by Ag^{+}, Cu^{2+} and Hg^{2+} ions was reported for α-galactosidases purified from *Bacillus megaterium* VHM1 [40] and *Torulaspora delbrueckii* IFO 1255 [41].

The sensitivity of purified PCGI to various concentrations of chemical reagents was tested (Table 3). SDS is an extremely denaturing reagent through disrupt tertiary and quaternary structures of proteins [42]. Due to loss of tertiary, SDS leads to loss of 83–90% activity of PCGI with the concentration of SDS increased from 2–200 mM. No loss of activity was observed in the presence of the chelating agent EDTA (100.9%), suggesting that PCGI is not dependent on metal ions for its activity. NaCl (107.5%), sodium acetate (97.4%) and $(NH4)_2SO_4$ (119.6%) at 200 mM concentration had no effect on PCGI, which may be the result of a salt stabilization effect [43].

The enzyme activity was also not affected by incubation with 200 mM glucose (120.6%), sucrose (121.2%), lactose (118.6%), xylose (107.3%) and fructose (97.4%), but was highly inhibited by melibiose, indicating melibiose was an inhibitor to PCGI.

As determined by the Dixon plot, the mode of inhibition by galactose and melibiose was found to be competitive and the Ki value was 0.92 mM and 7.13 mM respectively, which was similar to those of most α-galactosidases that are competitively inhibited by galactose, for example *T. multijuga* [23].

3.5. Resistance to proteases

After treatment at 37 °C for 1 h with acid protease, neutral protease, α-chymotrypsin, and trypsin at a concentration of 10 mg/mL, PCGI exhibited 139% ± 0.02, 163% ± 0.00, 157% ± 0.03 and 142% ± 0.01 of its initial activity, respectively, and was markedly activated in the presence of these proteases. However, proteinase K almost completely inhibited PCGI leading to a residual activity of 9.16%. α-Galactosidases which have the ability of resistance to protease were purified from new *sphingomonas* strain [44], *Rhizopus* sp. F78 ACCC 30795 [19], *Gibberella* sp. F75 [45], and *Rhizomucor miehei* [46]. Food and feed supplements/additives, or digestants often combine α-galactosidase with protease to wipe off oligosaccharides, mainly raffinose and stachyose, improve the nutritional value of fodder and make it more digestible. Due to better resistance to proteases, PCGI could be considered as a new candidate additive in the food and feed industries.

3.6. Effects of chemical modification reagents on PCGI

Under acidic conditions, N-bromosuccinimide (NBS) can selectively modify the tryptophan residues of protein [47]. As shown in Fig. 4, the activity of PCGI was drastically inhibited by NBS. Incubation of the enzyme with 0.5 mM NBS for 60 min resulted

Table 3
Effect of chemical reagents on the activity of PCGI.

Effector concentration	Relative α-galactosidase activity[a] (%) (mean ± SD, $N = 3$)		
	200 mM	20 mM	2 mM
Fructose	97.4 ± 0.00	116.0 ± 0.00	121.6 ± 0.02
Glucose	120.6 ± 0.02	125.7 ± 0.02	123.1 ± 0.01
Lactose	118.6 ± 0.03	134.9 ± 0.01	162.2 ± 0.02
Meliobiose	49.7 ± 0.01	92.9 ± 0.02	94.4 ± 0.00
Sucrose	121.2 ± 0.00	109.0 ± 0.02	112.7 ± 0.03
Xylose	107.3 ± 0.01	114.8 ± 0.05	119.5 ± 0.02
Sodium acetate	97.4 ± 0.01	99.1 ± 0.02	104.6 ± 0.01
$(NH4)_2SO_4$	119.6 ± 0.03	128.0 ± 0.04	130.1 ± 0.01
EDTA	100.9 ± 0.02	106.9 ± 0.01	116.0 ± 0.03
SDS	9.8 ± 0.00	12.4 ± 0.00	16.5 ± 0.00
NaCl	107.5 ± 0.00	145.8 ± 0.00	130.2 ± 0.00

[a] Relative activities were calculated in relation to pNPGal activity, determined in the reaction without effectors, which was considered as 100%.

Table 4
Hydrolysis of substrates by PCGI.

Substrate	Concentration (mM)	Relative activity[a] (%)(mean ± SD, N = 3)
4-Nitrophenyl α-D-galactopyranoside (pNPG)	10	100 ± 0.07
2-Nitrophenyl β-D-galactopyranoside (oNPG)	10	0.1 ± 0.00
4-Nitrophenyl β-D-glucuronide	10	0.1 ± 0.00
Melibiose	100	10.3 ± 0.02
Maltose	100	1.1 ± 0.07
Lactose	100	0.6 ± 0.05
Raffinose	100	8.3 ± 0.01
Stachyose	100	4.5 ± 0.01
Sucrose	100	0.0 ± 0.00
Locust bean gum	1%	2.7 ± 0.00
Guar gum	1%	2.7 ± 0.00

Note: [a]Relative activities were calculated in relation to pNPGal activity, which was considered as 100%.

in total abolition of α-galactosidase activity, indicating that tryptophan was the necessary chemical groups of PCGI. Inhibition of α-galactosidase activity by NBS was also observed in enzymes of *Coriolus versicolor* [48] and *A. terreus* [49]. Diethylpyrocarbonate (DEPC) has been shown to react with a single histidyl residue in certain proteins [50]. The activity of α-galactosidase was increased two-fold by DEPC, indicating that histidine residues were probably loaded in the active center of PCGI. 2,3-butanedione (DIC) was used for the modification of arginine residues [51,52]. Carbodiimide (EDC) and trinitrophenol (TNBS) react with the carboxyl groups [53] and lysine residues [47], respectively. EDC, TNBS and DIC had slight effect on the activity of PCGI, demonstrating that carboxyl groups, lysine and arginine may be not necessary or located in the active site of PCGI.

3.7. Substrate specificity and kinetic parameters of PCGI

Purified PCGI was used to hydrolyze various natural and synthetic substrates. As shown in Table 4, pNPGal showed the highest specificity for pNPG (100%), but did not show any activity for other nitrophenyl derivatives (oNPGal, 4-nitrophenyl β-D-glucuronide), suggesting this enzyme showed high specificity for anomeric carbon and also seemed to be regiospecific for the galactoside configuration.

Natural substrates, such as raffinose and stachyose, were also good substrates for this enzyme. Reduction of 10.3% melibiose, 8.3% raffinose, 4.5% stachyose, 2.7% locust bean gum, and 2.7% guar gum was observed after treatment with PCGI, suggesting that the hydrolytic ability of PCGI to natural substrates was melibiose > raffinose > stachyose > locust bean gum, guar gum. PCGI exhibited the ability to act on locust bean gum and guar gum, indicating its potential industrial application for an improvement of the gelling properties of polysaccharide. Locust bean gum is more functional and more expensive than guar gum. The removal of a quantitative proportion of galactose moieties from guar gum by α-galactosidase could improve gelling properties of guar gum rendering them comparable to those of locust bean gum [17]. Furthermore, it is of commercial interest to modify the structure of guar gum to convert it into a material with the desired functional properties of locust bean gum.

The Km values of PCGI determined for pNPGal, stachyose, raffinose, and melibiose was 0.21, 16.71, 18.91, and 6.33 mM respectively (Table 5). The lowest Km value was determined for pNPGal, which indicated that PCGI displayed the highest affinity for pNPGal. The catalytic efficiency expressed by the Vmax/Km ratio showed that the substrate pNPGal was used most efficiently by the enzyme, followed by melibiose, raffinose, stachyose in sequence, which correspond to their rate of hydrolysis detected by TLC mentioned below.

Table 5
Kinetic parameter for PCGI.

Substrate	Km (mM)	Vmax[a]	Vmax/Km(min^{-1})
pNPG	0.21	29.76	140.31
Stachyose	16.71	161.29	9.25
Raffinose	18.91	434.78	22.99
Melibiose	6.33	166.67	26.32

Note: [a]Vmax is expressed in μM pNP/min for pNP Gal, μM reducing sugar/min for stachyose, rafinose and melibiose.

3.8. Hydrolysis of RFOs by PCGI

The degradation of RFOs was performed and the products were analyzed by TLC (Fig. 5). During the initial hydrolysis, the degradation of stachyose resulted in formation of a mixture of raffinose, sucrose and galactose, while the degradation of raffinose resulted in formation of a mixture of sucrose and galactose.

Raffinose was completely hydrolyzed in 9 h, which was in accordance with the result of time-course of hydrolysis of raffinose (Fig. 6). However, stachyose was completely hydrolyzed in 18 h, in accordance with the results of time-course of hydrolysis of stachyose. The difference in hydrolysis might be due to accumulation of raffinose, which is formed after stachyose hydrolysis. Particularly worth mentioning is the observation that no invertase activity was detected, indicating that hydrolysis of raffinose and stachyose was catalyzed exclusively by PCGI. However, reducing sugar content was found to decrease after complete hydrolysis of raffinose and stachyose. It is possible that the α-galactosidase had become denatured and appeared in the precipitate which then absorbed part of the galactose (reducing sugar) thus leading to a reduction of the reducing sugar content observed.

At the same time, the rapid hydrolysis of raffinose could also be explained by the fact that PCGI displayed greater substrate specificity and catalytic efficiency for this oligosaccharide as compared to stachyose.

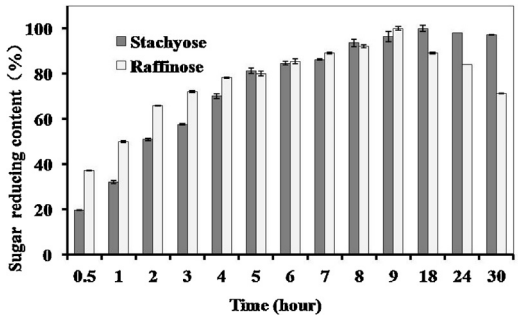

Fig. 6. The content of reducing sugar after treatment with PCGI.

The nutritional value of soy foods could be upgraded by procedures using microbial or plant α-galactosidases to hydrolyze the α-galactosides prior to consumption. As is well known, microbial α-galactosidases can be used to degrade RFOs in soy milk [54]. However, the fungal enzymes are relatively easy to produce, have better safety, and are the most suitable for technological applications, mainly due to their extracellular localization, acidic pH optima, and broad stability profiles. *P. citrinopileatus* has an easily accessible supply, good biocompatibility, innocuity and causes no pollution to the environment. Previous studies on fungal α-galactosidases for removal of RFOs from soymilk suffered from disadvantages such as prolonged incubation time due to low thermostability or incomplete hydrolysis by the enzyme [24]. As indicated above, PCGI exhibited good thermal and pH stabilities and remarkable protease-resistance, and efficiency in completely hydrolyzing the RFOs. These results suggest that PCGI has great potential in the food and feed industries, and they are particularly interesting for biotechnological applications for removal of non-digestible and flatulence-causing RFOs from legumes. In addition, PCGI could be also used for converting guar gum to a material having the desired functional properties of locust bean gum.

Author contributions

Y.H. performed the experiments, and analysed the data. G.T., X.G., W.Z. and L.Z. provided the materials and reagents. H.W. conceived and designed the study. T.B.N. wrote the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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