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A protease-resistant α-galactosidase characterized by relatively acid pH tolerance from the Shitake Mushroom *Lentinula edodes*

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ABSTRACT

A monomeric α-galactosidase with a molecular weight of 64 kDa was purified from fresh fruiting bodies of *Lentinula edodes*. The purification protocol involved ion-exchange chromatography on DEAE-cellulose, CM-cellulose and Q-Sepharose and a final gel-filtration on Superdex 75. The purified α-galactosidase (LEGI) was identified by LC-MS/MS. It demonstrated the optimum pH of 5.0 and temperature optimum of 60 °C towards pNPGal. It was inhibited by Cd^{2+}, Fe^{3+}, Pb^{2+}, Zn^{2+}, Al^{3+}, Hg^{2+}, Cr^{2+}, Ba^{2+}. The LEGI activity was strongly abolished by the chemical modification *N*-bromosuccinimide (NBS) at 1 mM, while significantly enhanced by the thiol-reducing agents dithiothreitol (DTT). Moreover, LEGI showed strong resistance to protease pepsin, papain, acid protease and neutral protease. LEGI demonstrated hydrolysis towards melibiose (13.27%), raffinose (4.75%), stachyose (2.58%), locust bean gum (0.82%) and guar gum (1.29%). The Km values of LEGI for pNPGal, stachyose, raffinose, and melibiose were found to be 1.08, 17.24, 13.80 and 8.05 mM, respectively. Results suggest that LEGI demonstrates potential for elimination of indigestible oligosaccharides.

1. Introduction

Recently, enzyme especially glycoside hydrolases plays an important role in various fields. As a glycoside hydrolase, α-Galactosidases (α-Gal) (EC3.2.1.22) cleave the nonreducing α-linked galactosyl residues in galacto-oligosaccharides as well as polymeric galactomannans [1]. It has been found great potential for bioindustrial applications, such as feed [2], food [3], medicine [4], and paper pulp processing [5] etc. α-Gal has been wildly reported among plant, mammals and microorganisms. Comparing amino acid similarity, the majority of fungi α-galactosidase are categorized into GH families 27 and 36 [6].

Lentinula edodes is widely cultivated and consumed in the Orient for its nutritional values and pharmacological effects. Variety of bioactive compounds isolated from *L. edodes* (e.g. laccase, lectin, lentinan and phytase) have shown different functions, especially the effect of antioxidant, antitumor, antiviral and antimicrobial [7–13]. As one of the famous edible fungi, *L. edodes* is a good source of functional food or drugs due to its biological activities and no toxicity nor serious side effects. However, α-Gal from edible fungi have been relatively less explored compared to other microbial sources. Therefore, the objective of this study was to purify a novel α-Gal from the fresh shitake mushroom *L. edodes* and to compare the enzymatic properties and the hydrolyzation ability with other mushroom α-Gal.

2. Materials and methods

2.1. Materials and chemicals

Fresh shitake mushroom was acquired from the edible fungus center of Shanxi Agricultural University. Carboxymethyl (CM)-cellulose, Diethylaminoethyl (DEAE)-cellulose, Quaternary amine (Q)-Sepharose and Superdex 75HR10/30 were purchased from GE Healthcare. All chemicals were of analytic reagent grade from China unless otherwise noted.

2.2. Enzyme activity assay

Assay of α-Gal activity was carried out with slightly modifications [14]. In short, 40 μL enzyme solution and 40 μL 10 mM pH 4.6 *p*-Nitrophenyl-α-D-galactopyranosides (pNPGal) were incubated at 40 °C for 10 min. The reaction was terminated by adding 320 μL 0.5 mM Na_2CO_3. Then the released *p*-nitrophenol (pNP) was measured spectrophotometrically at 405 nm. One α-Gal unit was defined as the amount that released one micromole pNP per min.

All assays were repeated three times.

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Table 1
Summary of purification procedure of LEGI.

Purification step	Protein (mg)	Activity (U/mL)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Purification fold
Extracts	4851.37	3.78	756.28	0.16	100.0	1.00
D3	403.20	0.54	288.15	0.71	38.10	4.44
C1	59.52	1.57	157.08	2.64	20.77	16.53
Q1	3.72	2.05	40.10	10.77	5.30	67.29
SU1	0.08	16.82	13.46	168.25	1.78	1051.56

2.3. Isolation of LEGI

Fresh shitake mushroom was homogenized in distilled water (4 mL/g), kept at 4 °C overnight without agitation and then centrifuged (15 min, 10,000 g). The 1 M pH 5.2 acetate buffer was added to the clear supernatant to 10 mM. The supernatant was loaded on a 2.5 cm × 30 cm column of DEAE-cellulose. The active fraction D3 enriched in α-Gal activity was eluted with the 250 mM NaCl. After dialyzed against 10 mM pH 4.6 acetate buffer, fraction D3 was subjected to a 2.5 cm × 20 cm column of CM-cellulose. The unbounded fraction CM1 containing α-Gal activity was collected and dialyzed. The dialyzed CM1 was injected into a 1 cm × 10 cm Q-Sepharose column with 10 mM pH 5.2 acetate buffer. A linearity gradient elution was carried on with 0–300 mM NaCl. The unadsorbed solution Q1 with α-Gal activity was finally put on a Superdex 75 gel filtration column by an AKTA Purifier (GE Healthcare). The buffer used for gel filtration was 10 mM NaAc-HAc + 150 mM NaCl. The first eluted peak (SU1) with the highest specific activity was collected.

2.4. Estimation of molecular weight

Molecular weight estimation of LEGI was performed by the reducing SDS-PAGE and gel filtration mentioned above. In gel filtration, the molecular weight of the active α-Gal was calculated by the standard curve of elution volume-lg*Mr*. The reducing SDS-PAGE was carried out by following the method of Laemmli [15]. The gels were visualized by Coomassie brilliant blue staining. The molecular weight of inactive monomer was calculated by anther standard curve of relative mobility-lg*Mr*.

2.5. Analysis of amino acid sequence

The purified LEGI band was excised, digested then dissolved in 0.1% formic acid for LC-MS/MS analysis. The inner amino acid sequences of LEGI were compared with α-Gal from other sources by Mascot. Sequence homologues were obtained by NCBI database.

2.6. Effect of pH and temperature on LEGI

To evaluate the optimal pH of LEGI, a series of pNPGal solution in 100 mM, pH 2.0 to 8.0 Na_2HPO_4-citric acid buffers were tested to replace the standard pNPGal solution at pH 4.6. The pH stability of LEGI was assayed by incubating LEGI in the above-mentioned buffers for 60 min at 4 °C. The residual activity of α-Gal was tested with standard method. The temperature optima of LEGI was analyzed at temperatures varying

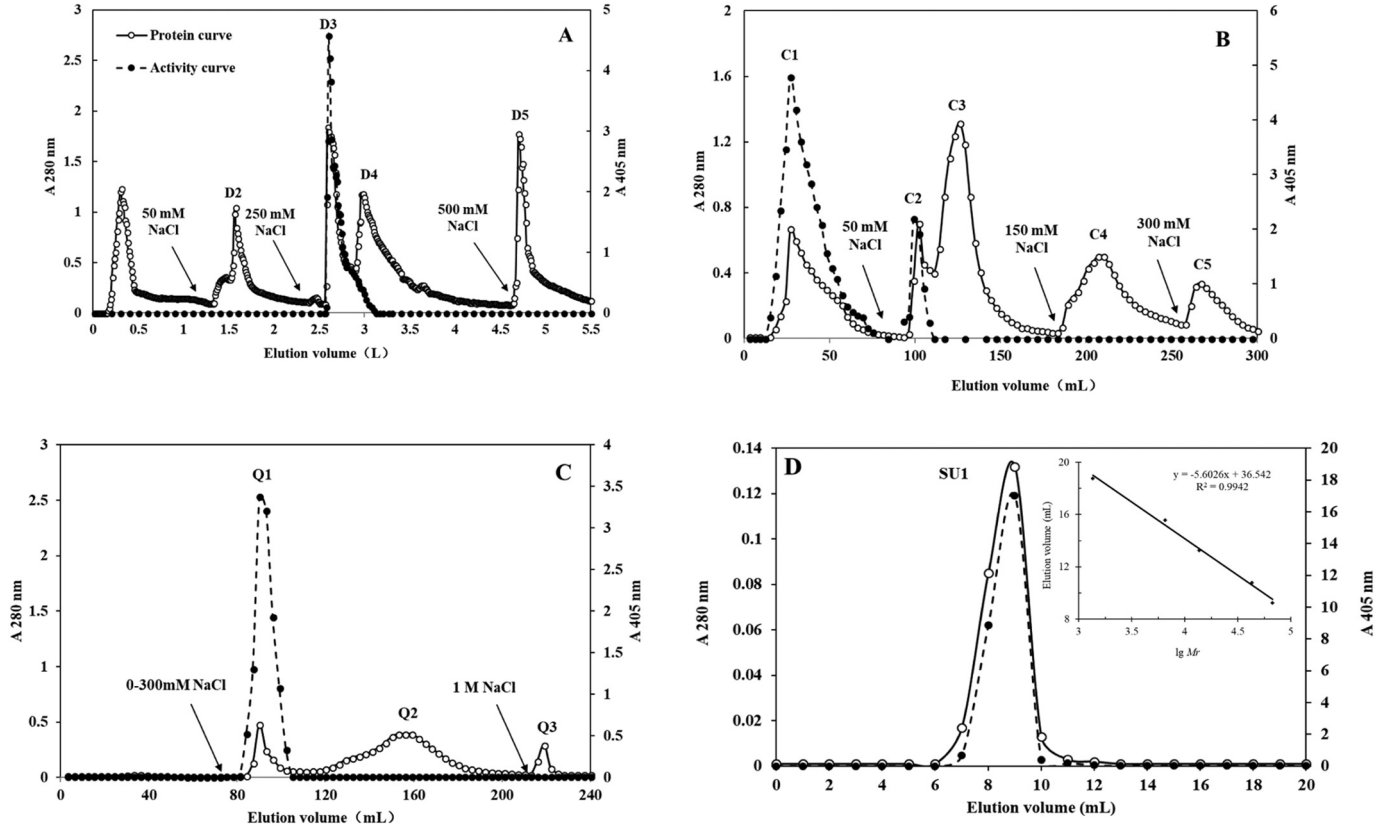

Fig. 1. Elution curves of LEGI. (A) Anionic exchange chromatography on DEAE-cellulose column (pH 5.2), (B) Cation exchange chromatography of fraction D3 on CM-cellulose column (pH 4.6), (C) Anion exchange chromatography of fraction C1 on Q-Sepharose (pH 5.2), (D) Gel-filtration chromatography of fraction Q1 by FPLC on a Superdex 75 column and the standard curve of elution volume-lg*Mr*.

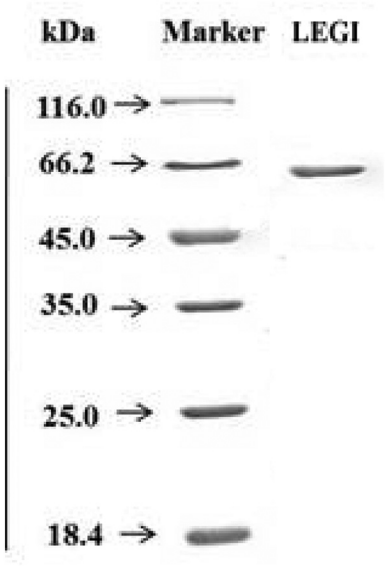

Fig. 2. SDS-PAGE of LEGI. Lanes: Marker, molecular mass standards; LEGI, fraction SU1 from FPLC.

from 20 to 90 °C in buffers. The thermal inactivation was studied by incubating LEGI at diverse temperatures (20 °C, 30 °C, 40 °C, 50 °C, 60 °C, 70 °C, 80 °C and 90 °C) for 60 min. The residual activity of α-Gal was tested with standard method.

2.7. Effects of metal ions, chemical reagents and chemical modification reagents on LEGI activity

Various metal ions (1.25, 2.5, 5 and 10 mM), chemical reagents (2, 20 and 200 mM), and chemical modification reagents (0, 0.2, 0.4, 0.6, 0.8 and 1.0 mM) were incubated with appropriately diluted LEGI and 10 mM pMPGal at 37 °C for 1 h, respectively. The control was measured without above mentioned reagents.

2.8. Substrate specificity

To determine the hydrolyzation ability of LEGI, various synthetic substrates (*o*NPGal, pNPGal, 4-nitrophenyl β-D-glucuronide and 4-nitrophenyl α-D-glucopyranoside) and natural substrates (raffinose, stachyose, guar gum and locust bean gum) were added to the assay solution. The α-Gal activity towards synthetic substrates was estimated by the abovementioned assay. While the amount of reducing sugar was measured to get the α-Gal activity against the natural substrates. Based on the substrates of melibiose, maltose, lactose and galactose, a glucose oxidase kit (Solarbio Life Science Co., Beijing) was used to estimate the hydrolyzation ability of LEGI.

2.9. Protease treatments

A series of protease with different optimal pH values including α-chymotrypsin, acid protease, neutral protease, papain, pepsin, proteinase K, trypsin and subtilisin were used to assay the resistance of LEGI to protease mentioned above, respectively. 2 mg/mL protease was mixed with the purified LEGI (0.05 U/mL) for 1 h at 37 °C. The control was measured without above mentioned protease.

2.10. Enzyme kinetic

The kinetic parameters (*K*m and *k*cat) of LEGI towards pNPGal, raffinose and stachyose were determined from the plot of the reciprocal of initial velocities against corresponding reciprocal of substrate concentrations using Lineweaver-Burk graphs. The inhibition constants (Ki) of LEGI with respect to galactose and melibiose were measured using the same method.

3. Results and discussion

3.1. LEGI purification and molecular weight determination

The LEGI purification protocol was shown in Table 1, resulting in the specific activity of 168.25 U mg^{-1} against pNPGal with a purification fold of 1051. The crude extract was applied on DEAE-cellulose eluting

Table 2
Comparison of the inner peptide sequence of LEGI with other mushroom α-galactosidase.

Peptide fragment	Peptide sequence	Microorganism containing similar sequence		
		Microorganism	Identity	Accession number
Gal1	LPLTVTVPR	*Jiangella* sp. DSM 45060	100%	SDS81292.1
Gal2	GFSAFLNGAQVASGASVDDR	*Nocardioides lianchengensis*	100%	SDD63066.1
Gal3	MIAPNTMGAVSPR	*Lechevalieria fradiae*	100%	SDF34224.1
Gal4	AVGKAGIADVFNLDK	*Actinopolymorpha singaporensis*	87%	SDS43309.1
Gal5	KASAATFDILDNK	*Streptomyces avermitilis*	100%	WP_010982913.1
		Streptomyces sp. OV198	100%	SOE77868.1
		Streptomyces sp. Ag82_O1-15	100%	PBD01055.1
		Streptomyces sp. Tue6028	100%	WP_095932580.1
Gal6	KVVLGTGETAAGR	*Amycolatopsis tolypomycina*	100%	WP_091304565.1
		Amycolatopsis vancoresmycina	100%	WP_081655742.1
		Amycolatopsis sp. CA-128772	100%	WP_103353016.1
Gal7	GIADLFVSSGLR	*Kutzneria* sp. 744	100%	WP_043724796.1
Gal8	LTATIGIDDK	*Lentzea waywayandensis*	100%	SFR22691.1
		Lechevalieria deserti	100%	PWK80739.1
Gal9	VTGASATVPVEVDVAGARHVHLK	*Streptomyces* sp. Ncost-T10-10d	100%	SCF58490.1
Gal10	TVALFNESGSPRR	*Streptomyces sviceus* ATCC 29083	100%	EDY60641.2
		Streptomyces sviceus	100%	WP_037903073.1
		Streptomyces sp. Ag109_O5-10	100%	SED90961.1
Gal11	TWTVRPSSPGGDRVEVAGR	*Amycolatopsis decaplanina*	100%	WP_007028446.1
Gal12	LSLNSGNLTLDVSRQGR	*Lentzea flaviverrucosa*	100%	WP_090070319.1
		Lentzea albidocapillata	100%	WP_030477203.1
Gal13	ISAQFTLDEGER	*Saccharomonospora cyanea*	100%	WP_005456311.1
Gal14	SVADTLVSR	*Kribbella* sp. ALI-6-A	100%	WP_077015633.1
Gal15	VEAASPTR	*Streptomyces* sp. CB02460	100%	WP_073969731.1
		Streptomyces sp. CB01249	100%	WP_073865608.1
Gal16	TVAPVRVTR	*Streptomyces rubidus*	100%	SEO80168.1

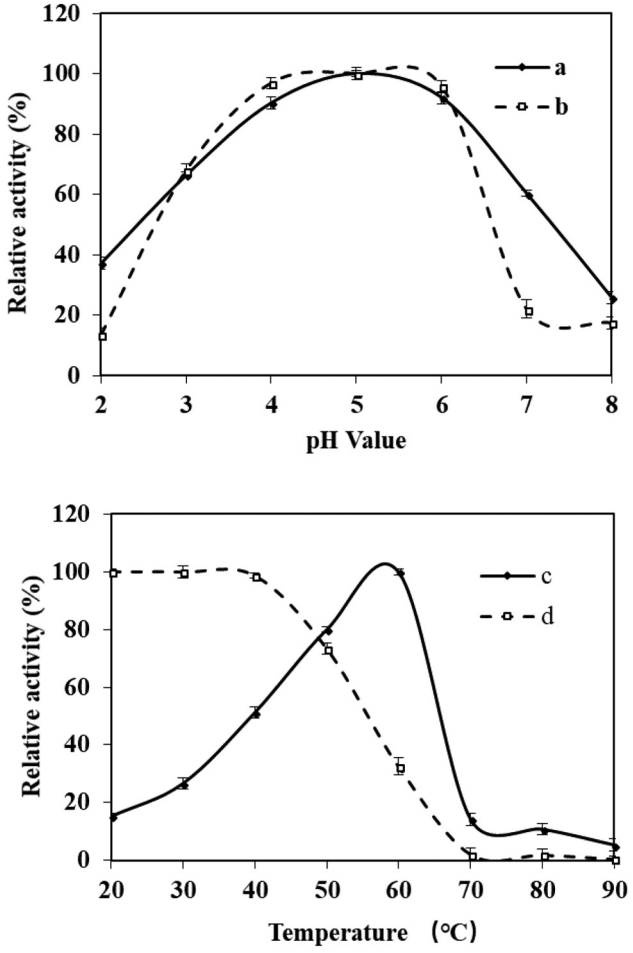

Fig. 3. Effects of pH and temperature on the activity and stability of LEGI. (a) optimal pH; (b) pH stability; (c) optimal temperature; (d) thermal stability.

fraction D3 containing α-Gal activity (Fig. 1A). Subsequently, fraction D3 was subjected to CM-cellulose. The specific activity of fraction C1 was higher than C2 (Fig. 1B). The unbounded fraction C1 was applied to Q-Sepharose. And a linearity gradient elution was carried on with 0–300 mM NaCl. As illustrated in Fig. 1C, the activity resided in fraction Q1. The unadsorbed solution Q1 was finally put on a Superdex 75 gel filtration column and enriched in a single peak designed as SU1 (Fig. 1D). It was shown in Fig. 2 as a single band on SDS-PAGE and deduced to be 64 kDa. Based on its elution volume of FPLC and SDS-PAGE, it is indicated LEGI was a monomer enzyme. This was in good agree with most of mushroom α-Gal, such as α-Gal from *Pleurotus djamor* (60 kDa) *Pleurotus citrinopileatus* (60 kDa) [16], and *Pseudobalsamia microspore* (62 kDa) [17]. Although most of microbial α-Gal were monomeric proteins, a thermotolerant α-Gal from *Ganoderma lucidum* [18] is a 249 kDa multimeric enzyme [18]. Besides, α-Gal from *P. citrinopileatus* is a heterodimer with two subunits (33 kDa, 27 kDa) [16] (Table 3).

3.2. Amino acid sequence of inner peptide

After tryptic digestion, the peptides of LEGI were elucidated by LC-MS/MS. Among homologues sequences were gained by blast search (Table 2), peptide **LPLTVTVPR**, **GFSAFLNGAQVASGASVDDR**, **MIAPNTMGAVSPR**, **VTGASATVPVEVDVAGARHVHLK**, **TVAPVRVTR** demonstrated 100% homology to NPCBM-associated NEW3 domain of α-galactosidase from *Jiangella* sp. DSM 45060 (SDS81292.1), *Nocardioides lianchengensis* (SDD63066.1), *Lechevalieria fradiae* (SDF34224.1), *Streptomyces* sp. Ncost-T10-10d (SCF58490.1) and *Streptomyces rubidus* (SEO80168.1), respectively. Peptide **AVGKAGVTDVFNLDK** shared 87% identical to NPCBM-associated, NEW3 domain of alpha-galactosidase from *Actinopolymorpha singaporensis* (SDS43309.1). Although the function of this domain is still unknown. It is associated with the NPCBM family (pfam08305), a novel putative carbohydrate binding module found at the N-terminus of glycosyl hydrolases and typically located between the GH27 and NPCBM domains. Therefore, it may be concluded that LEGI belongs to GH family 27.

3.3. Effects of pH and temperature

The maximal activity of LEGI was obtained at pH 5.0 (Fig. 3), which was identical to that of *P. djamor* [14], *P. microspora* [17], *Pleurotus florida* [19], *Termitomyces eurrhizus* [20], *Penicillium* sp. F63 CGMCC [21] and *Aspergillus terreus*$_{GR}$ [22] (Table 3). Generally, microbial α-galactosidase possessed the optimal pH values from pH 4.5 to pH 5.5, which has been found to be able to survive and effectively catalyze in the gastric. LEGI showed high-stability between pH 4.0–6.0 for 1 h, retaining more than 95% activity (Fig.3). The optimal temperature of LEGI was determined to be 60 °C and underwent a sharp decline when the temperature was elevated to 70 °C (Fig. 3). The optimal temperature was the same with *Agaricus bisporus* [23], *Coriolus versicolor*

Table 3
Comparison of biochemical characteristics and activities of α-galactosidase from *Lentinus edodes* and other mushrooms.

	Molecular mass (kDa) & subunit	Optimum temperature (°C) & pH	Inhibitor	Protective agent	Substrate
L. edodes	64, monomer	60, 5.0	EDTA, NBS, meliobiose, galactos, Ag^{+}, Hg^{2+}, Fe^{3+}, Cd^{2+} etc.	DTT, Cu^{2+}	pNPG, oNPG, melibiose, raffinose
A. bisporus [23]	45, monomer	60, 4.0	NBS, PCMB, DTT meliobiose galactose Ag^{+}, Hg^{2+}, Fe^{3+}, Cu^{2+}	EDTA	pNPG, stachyose, locust bean gum, guar gum
C. versicolor [24]	40, monomer	60, 3.0	NBS, Pb^{2+}, Hg^{2+}, Cu^{2+} Cd^{2+}	–	pNPG, oNPG, melibiose, raffinose, stachyose
G. lucidum [18]	249 (56), teramer	70, 6.0	galactose, xylose, Ag^{+}, Hg^{2+}	Cu^{2+}	pNPG, oNPG, melibiose, raffinose, stachyose
P. citrinopileatus [16]	60, (33, 27), heterodimer	50, 4.4	NBS, melibiose Cd^{2+}, Cu^{2+} Hg^{2+}, Al^{3+}, Fe^{3+}, Ag^{+}	DEPC	pNPG, melibiose, raffinose
P. djamor [14]	60, monomer	53.5, 5.0	NBS, PCMB, meliobiose, galactose, K^{+}, Cd^{2+}, Cu^{2+}, Hg^{2+}, Al^{3+}, Fe^{3+}, Ag^{+}	DEPC, DIC, TNBS	pNPG, melibiose, stachyose
P. florida [19]	99, monomer	55, 4.6–5.0	NBS, galactose, glucose, maltose, lactose, Ag^{+}, Hg^{2+}	–	pNPG, raffinose
P. microspore [17]	62, monomer	55, 5.0	DTT, Hg^{2+}, Cd^{2+}, Cu^{2+}, Fe^{3+}	EDC, DIC, DEPC	pNPG, melibiose, raffinose, stachyose
T. eurrhizus [20]	72, monomer	60, 5.0	Hg^{2+}, Cd^{2+}, Fe^{3+}, Cu^{2+}, Mn^{2+}, Al^{3+}	K^{+}, Ca^{2+}	pNPG, oNPG, melibiose, raffinose, stachyose
T. matsutake [25]	47, monomer	55, 4.5	NBS, Fe^{3+}, Cu^{2+}, Pb^{2+}, Mn^{2+}, Mg^{2+}	–	pNPG, melibiose, raffinose, stachyose, locust bean gum, guar gum

Table 4
Effect of different metal ions on the activity of LEGI.

Metal ion	Relative α-galactosidase activity (%)			
	10 mM	5 mM	2.5 mM	1.25 mM
Ag^{+}	16.05 ± 0.00	19.86 ± 0.01	23.34 ± 0.01	26.04 ± 0.01
Al^{3+}	62.14 ± 0.01	84.39 ± 0.01	90.01 ± 0.01	94.54 ± 0.02
Ba^{2+}	49.54 ± 0.00	71.01 ± 0.01	85.67 ± 0.02	98.11 ± 0.01
Ca^{2+}	87.56 ± 0.01	88.16 ± 0.01	89.97 ± 0.01	95.66 ± 0.00
Cd^{2+}	0.00 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Cr^{2+}	33.46 ± 0.01	36.16 ± 0.01	43.14 ± 0.04	57.02 ± 0.05
Cu^{2+}	133.05 ± 0.00	129.72 ± 0.00	123.47 ± 0.01	115.39 ± 0.02
Fe^{2+}	57.18 ± 0.00	70.31 ± 0.00	95.66 ± 0.00	101.38 ± 0.00
Fe^{3+}	0.00 ± 0.01	0.00 ± 0.01	0.00 ± 0.02	0.00 ± 0.01
Hg^{2+}	32.89 ± 0.01	33.71 ± 0.00	44.33 ± 0.00	60.51 ± 0.00
K^{+}	89.04 ± 0.00	85.28 ± 0.01	84.07 ± 0.01	82.41 ± 0.00
Mg^{2+}	81.40 ± 0.02	89.80 ± 0.02	90.83 ± 0.02	95.92 ± 0.00
Mn^{2+}	100.67 ± 0.01	100.21 ± 0.01	99.87 ± 0.00	98.79 ± 0.02
Na^{+}	96.51 ± 0.01	97.77 ± 0.03	99.62 ± 0.02	100.31 ± 0.02
Pb^{2+}	36.26 ± 0.00	37.23 ± 0.01	42.63 ± 0.02	43.32 ± 0.01
Zn^{2+}	35.31 ± 0.01	38.61 ± 0.01	41.6 ± 0.01	54.30 ± 0.00

α-Galactosidase activity in the absence of metal ions was regarded as 100%. Results represent mean ± standard deviation (n = 3).

[24] and *T. eurrhizus* [20]. It was higher than that of α-galactosidase from *P. djamor* [14], *P. microspore* [17], *Tricholoma matsutake* [25] and *P. florida* [19], but lower than that of α-galactosidase from *G. lucidum* [18] (Table 3). The LEGI was stable at 40 °C for 1 h without loss of activity. When the temperature is up to 70 °C, most of the α-Gal was inactivated.

3.4. Effect of metal ions and chemical reagents on LEGI

With the increase of the metallic ions' concentration, the activity of LEGI were gradually inhibited by Ag^{+}, Al^{3+}, Ba^{2+}, Cd^{2+}, Cr^{2+}, Fe^{2+}, Fe^{3+}, Hg^{2+}, Pb^{2+}, and Zn^{2+}(Table 4). In general, most of the α-galactosidase can be inactivated by metal ions especially Hg^{2+} and Ag^{+} suggesting that thiol groups and carboxyl group are required for the adequate function of LEGI. Among those metallic ions, Cd^{2+} and Fe^{3+} were the strongest inhibitor towards LEGI, because the enzyme activity was completely lost by at 1.25 mM. LEGI was depressed by Cd^{2+} and Fe^{3+}, which was similar to that purified from *A. bisporus* (Fe^{3+}) [23], *P. djamor* (Fe^{3+}) [14], *T. matsutake* (Fe^{3+}) [25] and *P. microspore* (Cd^{2+}) [17]. The influence of Ca^{2+}, K^{+}, Mg^{2+}, Mn^{2+} and Na^{+} on LEGI was negligible. LEGI was found to be significantly stimulated by Cu^{2+}. Various studies have been reported the inhibition of α-galactosidase activity by Cu^{2+} except α-galactosidase purified from *G. lucidum* [18] which was slightly stimulated [26].

The effect of chemical reagents on LEGI was displayed in Table 5. The activity of LEGI was significantly inhibited by EDTA. As one of the most popular chelating agents, EDTA inhibits enzyme activity by chelating metal in enzyme active site and affecting the advanced structure. Moreover, its catalytic activity was enhanced by Cu^{2+}, it can be concluded that LEGI was a metalloenzyme. About 92% of its activity losses were also detected with SDS because of the enzyme's denaturation. There was no effect on LEGI by NaCl and $(NH4)_2SO_4$.

The LEGI was affected negligibly by glucose, lactose, maltose, sodium acetate, sucrose and xylose. Whereas it was significantly inhibited by melibiose and the reaction end products, galactose, which suggested they were competitive inhibitors of LEGI. This was similar to α-galactosidase from *A. bisporus* [23] and *T. multijuga*. Ki values were 1.33 and 33.95 mM for galactose and melibiose, respectively.

Table 5
Effect of chemical reagent on activity of LEGI.

Chemical reagent	Relative α-galactosidase activity (%)		
	200 mM	20 mM	2 mM
EDTA	78.03 ± 0.02	82.18 ± 0.01	99.38 ± 0.03
SDS	7.89 ± 0.00	15.15 ± 0.00	23.22 ± 0.00
NaCl	96.84 ± 0.00	99.50 ± 0.00	100.27 ± 0.00
(NH4)2SO4	99.96 ± 0.03	99.37 ± 0.04	99.12 ± 0.01
Sucrose	118.38 ± 0.00	108.87 ± 0.02	100.87 ± 0.03
Lactose	92.65 ± 0.03	100.54 ± 0.01	103.17 ± 0.02
Meliobiose	45.20 ± 0.01	76.24 ± 0.02	87.41 ± 0.00
Glucose	106.50 ± 0.02	108.69 ± 0.02	112.25 ± 0.01
Galactose	30.31 ± 0.02	57.62 ± 0.01	78.82 ± 0.03
Sodium acetate	91.32 ± 0.01	94.16 ± 0.02	99.01 ± 0.01
Xylose	84.90 ± 0.01	97.13 ± 0.05	99.45 ± 0.02
Maltose	96.55 ± 0.00	102.57 ± 0.00	104.38 ± 0.00

α-Galactosidase activity in the absence of chemical reagents was regarded as 100%. Results represent mean ± standard deviation (n = 3).

3.5. Effects of chemical modification reagents on LEGI

The association of amino acid in the active site and catalytic mechanism of LEGI was investigated by the modification of amino acid by chemical reagents (Fig. 4) The LEGI activity was strongly abolished by *N*-bromosuccinimide (NBS) at 1 mM in the acidic condition, which indicated that tryptophan may participate in substrate binding. The catalytic activity of LEGI was dramatically increased by dithiothreitol (DTT) which indicating that the catalytic activity is thiol group dependent. This was similar to the observation on the alkaline α-galactosidase from *Oryza sativa* L. cv. Tainong 67 [27]. The single histidyl residue, arginine residue and carboxyl groups can be modified by 2,3-butanedione (DIC), diethylpyrocarbonate (DEPC) and carbodiimide (EDC), respectively. Incubation LEGI with the above reagents merely resulted in slight changes in enzyme activity, suggesting that active center of LEGI contained no histidyl residue, arginine residue nor carboxyl groups.

3.6. Resistance to proteases

As is shown in Table 6, LEGI showed strong resistance to protease pepsin (102.80% residual activity), papain (95.65% residual activity), acid protease (88.25% residual activity) and neutral protease (75.07% residual activity) at 5 mg/mL. It suggested that LEGI demonstrated the excellence resistant to proteases with optimum pH range from neutral to acid. Since the acidic environment in animal gut, LEGI exhibited its application potentials in the industries of food and animal feed. The enzyme activity decreased drastically by trypsin and protease K at concentration of 0.5 to 5 mg/mL. LEGI were hydrolyzed efficiently by α-chymotrypsin and subtilisin at a lower concertation. This indicated that LEGI was sensitive to trypsin, protease K, α-chymotrypsin and subtilisin.

RmGal36 displayed better antiprotease activity of trypsin, proteinase K and subtilisin [28]. The α-galactosidase from *Gibberella* sp. F57 was observed strong resistance to α-chymotrypsin, trypsin, collagenase

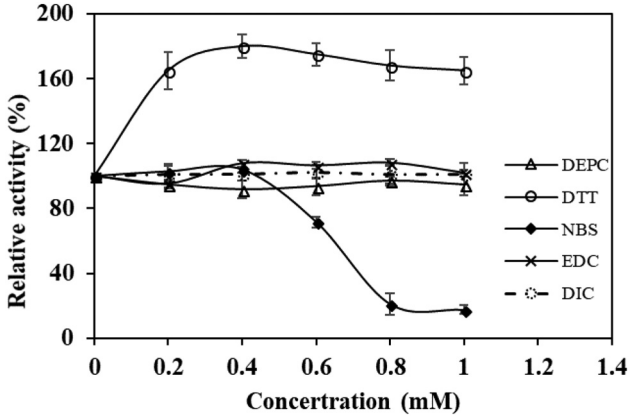

Fig. 4. Effect of chemical reagents on the activity of LEGI.

Table 6
The effect of protease on LEGI.

Protease	Relative α-galactosidase activity (%)		
	5 mg/mL	2.5 mg/mL	0.5 mg/mL
Pepsin (pH 3.0)	115.71 ± 0.02	108.95 ± 0.01	102.80 ± 0.03
Papain (pH 6.0)	95.65 ± 0.00	98.57 ± 0.00	101.98 ± 0.00
Subtilisin (pH 7.5)	24.92 ± 0.00	31.72 ± 0.00	46.06 ± 0.00
Acid protease (pH 4.0)	88.25 ± 0.03	95.72 ± 0.04	97.52 ± 0.01
Trypsin (pH 7.0)	39.32 ± 0.00	67.27 ± 0.02	100.98 ± 0.03
α-Chymotrypsin (pH 7.0)	32.48 ± 0.03	35.16 ± 0.01	37.54 ± 0.02
Neutral protease (pH 7.0)	75.07 ± 0.01	83.76 ± 0.02	91.75 ± 0.00
Protease K (pH 7.5)	57.05 ± 0.02	62.97 ± 0.02	80.15 ± 0.01

α-Galactosidase activity in the absence of protease was regarded as 100%. Results represent mean ± standard deviation (n = 3).

and subtilisin [29]. It seems that different sources of α-galactosidase had difference proteases resistance.

3.7. Substrate specificity

As shown in Table 7, LEGI hydrolyzed pNPG (100%) more efficiently than other synthetic nitrophenyl derivatives such as oNPG (9.2%), 4-nitrophenyl α-D-glucopyranoside (8.1%) and 4-nitrophenyl β-D-glucuronide (4.97%). It indicated that anomeric carbon of the substrate is better access to the active site of LEGI. Compare to synthetic substrates, raffinose family oligosaccharides such as melibiose (13.27%), raffinose (4.75%), stachyose (2.58%) and branched polysaccharides such as locust bean gum (0.82%) and guar gum (1.29%) were partially hydrolyzed. This suggested that the relative activity of LEGI towards composite substrates was higher than that to natural substrates, consistent with α-galactosidase from *P. djamor* [14], *Aspergillus terreus* [30]. In general, most purified α-galactosidase did not act on the polysaccharides [17,20,24,28]. While LEGI demonstrated meager hydrolytic activity to guar gum and locust bean gum.

3.8. Kinetic parameters of LEGI

The kinetic constants were determined through Michaelis-Menten equation towards pNPGal, melibiose, stachyose and raffinose (Table 8). The Michaelis-Menten constants (*K*m) of LEGI on pNPGal were lowest, which indicated that the optimum substrate of LEGI was pNPGal rather than natural oligosaccharides. The *K*m value of LEGI was similar to α-galactosidase from *T. matsutake* [25] (0.99 mM) and *P. florida* [19] (1.1 mM), lower than that of *Ruminococcus gnavus* E1 [31] (*K*m = 1.8 mM) and higher than that of most mushroom, such as *C. versicolor* [24] (0.01 mM), *G. lucidum* [18] (0.4 mM) and *P. djamor* [14] (0.76 mM) etc.

Table 7
Substrate specificity of LEGI.

Substrate	Concentration	Relative activity (%)
4-Nitrophenyl α-D-galactopyranoside (pNPG)	10 mM	100.00 ± 0.07
2-Nitrophenyl β-D-galactopyranoside (oNPG)	10 mM	9.20 ± 0.00
4-Nitrophenyl β-D-glucuronide	10 mM	4.97 ± 0.00
4-Nitrophenyl α-D-glucuronide	10 mM	8.10 ± 0.00
Melibiose	100 mM	13.27 ± 0.02
Maltose	100 mM	0.02 ± 0.07
Lactose	100 mM	3.49 ± 0.05
Raffinose	100 mM	4.75 ± 0.01
Stachyose	100 mM	2.58 ± 0.01
Sucrose	100 mM	0.14 ± 0.00
Locust bean gum	1%	0.82 ± 0.00
Guar gum	1%	1.29 ± 0.00

α-Galactosidase activity towards pNPG was regarded as 100%. Results represent mean ± standard deviation (n = 3).

Table 8
Kinetic parameter for LEGI.

Substrate	*K*m (mM)	*k*cat (s^{-1})	*k*cat/*K*m ($mM^{-1}\cdot s^{-1}$)
pNPGal	1.08	14.57	13.49
Stachyose	17.24	21.29	1.23
Raffinose	13.80	43.93	3.18
Melibiose	8.05	33.81	4.20

Among the natural substrates, LEGI exhibited the highest value of the *k*cat/*K*m ratio for pNPGal followed by melibiose, raffinose and stachyose. The higher *k*cat/*K*m ratio indicated higher hydrolytic efficiency of pNPGal than RFOs.

4. Conclusion

Lentinula edodes is rich with α-galactosidase, which was firstly purified by various chromatography. LEGI is a 64 kDa monomeric metalloenzyme. Considering the enzymatic properties of LEGI such as degradation of RFOs, strong resistance to protease and good acid-tolerant, LEGI possesses potential applications on degradation of antinutritional factors in food and feed industries. However, the yield of LEGI purification is low, which will limit its applications in industries. Therefore, efforts must be made to increase the enzyme recovery. For example, using the aqueous two-phase extraction to make the purification process simple and efficient. Based on the reported genome sequence of shiitake mushroom, we plan to clone and express its α-galactosidase gene in heterologous hosts for further study.

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