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ORIGINAL ARTICLE

Purification, characterization and gene analysis of a new α-glucosidase from *shiraia* sp. SUPER-H168

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Abstract A new α-glucosidase from *Shiraia* sp. SUPER-H168 under solid-state fermentation was purified by alcohol precipitation and anion-exchange and by gel filtration chromatography. The optimum pH and temperature of the purified α-glucosidase were 4.5 and 60 °C, respectively, using *p*-nitrophenyl-α-glucopyranoside (α-*p*NPG) as a substrate. Ten millimoles of sodium dodecyl sulfate, Fe^{2+}, Cu^{2+}, and Ag^{+} reduced the enzyme activity to 0.7, 7.6, 26.0, and 6.2 %, respectively, of that of the untreated enzyme. The K_m, V_{max}, and k_{cat}/K_m of the α-glucosidase were 0.52 mM, 3.76 U mg^{-1}, and 1.3×10^4 L s^{-1} mol^{-1}, respectively. K_m with maltose was 0.62 mM. Transglycosylation activities were observed with maltose and sucrose as substrates, while there was no transglycosylation with trehalose. DNA and its corresponding full-length cDNA were cloned and analyzed. The α-glucosidase coding region consisted of a 2997-bp open reading frame encoding a 998-amino acid protein with a 22-amino acid signal peptide; one 48-bp intron was located. The α-glucosidase was a monomeric protein with a predicted molecular mass of 108.2 kDa and a predicted isoelectric point of 5.08. A neighbor-joining phylogenetic tree demonstrated that *Shiraia* sp. SUPER-H168 α-glucosidase is an ascomycetes α-glucosidase. This is the first report of α-glucosidase from a filamentous fungus that had good glycoside hydrolysis with maltose and α-*p*NPG, transglycosylation and conversion activity of maltose into trehalose.

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Introduction

Glycoside hydrolases (EC 3.2.1.-) are a widespread group of enzymes that hydrolyze the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety. α-glucosidases (EC 3.2.1.20, α-D-glucoside glucohydrolase) constitute a group of exo-acting glycoside hydrolases of diverse specificities that catalyze the release of α-D-glucose from the non-reducing end of α-linked substrates, and they are widely distributed among microorganisms, plants, and mammals (Chiba 1997). Oligo-1,6-glucosidase (EC 3.2.1.10) and sucrase-isomaltase (EC 3.2.1.48/EC 3.2.1.10) are also categorized as α-glucosidases.

Glycoside hydrolases are currently classified into 134 families (CAZy database at http://www.cazy.org/CAZY, October 2015), while α-glucosidases belong to families 13 and 31 (Henrissat and Davies 1997). Family 13 enzymes are more active on heterogeneous substrates, such as phenyl α-glucoside and sucrose, than on maltose. Family 13 includes enzymes designated as type I. In contrast, family 31 enzymes prefer homogeneous substrates such as maltose, maltotriose, and maltotetraose. Type II and III α-glucosidases are classified as family 31. Finally, type III α-glucosidases hydrolyze polysaccharides such as amylose and starch (Chiba 1997; Frandsen and Svensson 1998; Marin et al. 2006). In general, α-glucosidases have broad specificities, and a given substrate is not strictly connected to a single enzyme type.

Synergistic effects between the α-amylase/pullulanase and α-glucosidase enzymes of *Thermococcus hydrothermalis* have been reported (Legin et al. 1998). Legin et al. showed

that many (43–68 %) of the products generated by α-amylase and pullulanase were converted into glucose by the α-glucosidase of *T. hydrothermalis*. Some α-glucosidases are capable of transglycosylation, an activity that has applications in the industrial-scale production of isomalto-oligosaccharides and in the conjugation of sugars to biologically useful materials (Kato et al. 2002). Another type of α-glucosidase converts malto-oligosaccharides into trehalose via intramolecular transglycosylation, and this ATP-independent enzymatic route for trehalose synthesis was first described for two prokaryotes: a *Pimelobacter* sp. and an *Arthrobacter* sp. (Maruta et al. 1995; Nishimoto et al. 1995). This activity, which is required for the conversion of maltose into trehalose, has also been observed in an α-glucosidase from the filamentous fungus *Chaetomium thermophilum* var. *coprophilum* (Giannesi et al. 2006).

In our previous studies, hypocrellin A production by *Shiraia* sp. SUPER-H168 was studied under solid-state fermentation. Corn was found to be the best substrate after evaluating eight kinds of agro-industrial crops and residues (Cai et al. 2010a). Starch is the major carbohydrate storage product in corn kernels, and it accounts for 70–72 % of the kernel weight on a dry weight basis (Bothast and Schlicher 2005); more than 80 % of the world starch market originates from corn (Lacerda et al. 2008). To increase the yield of hypocrellin A and the utilization of corn starch, *Shiraia* sp. SUPER-H168 α-glucosidase was studied, and it was found that α-glucosidase could be expressed at a high level during solid-state fermentation. Three extracellular polysaccharides from *Shiraia* sp. SUPER-H168 were purified in our previous studies (Cai et al. 2010b) and here we studied transglycosylation activity of α-glucosidase.

This is the first report of a new α-glucosidase from *Shiraia* sp. SUPER-H168. The biochemical properties of the purified enzyme, including its optimum pH and temperature, its thermal stability, and the effects of inhibitors and metal ions on its activity, were investigated. In addition, transglycosylation activities were observed with maltose and sucrose as substrates, while there was no transglycosylation with trehalose. Furthermore, to fully understand the structural and catalytic characteristics of *Shiraia* sp. SUPER-H168 α-glucosidase for industrial applications, the corresponding gene was sequenced and its protein structure was analyzed.

Materials and methods

Materials

Corn was purchased from a local supermarket (self-brand; Wuxi, Jiangsu, China). *p*-Nitrophenyl-α-glucopyranoside (α-*p*NPG) was obtained from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade which were obtained from Sinopharm Chemical Reagent (Shanghai, China).

Microorganism, medium, and culture conditions

Shiraia sp. SUPER-H168 was obtained from a stock culture at the Laboratory of Biochemistry, School of Biotechnology, Jiangnan University, Wuxi, Jiangsu Province, China (Liang et al. 2009). *Shiraia* sp. SUPER-H168 was routinely maintained on potato dextrose agar (PDA) slants at 4 °C by regular sub-cultivation (no longer than 6 months) (Yang et al. 2013).

Growth in seed culture medium (50 mL), consisting of 20 % potato and 2 % glucose (w/v), was conducted in a 250-mL Erlenmeyer flask. Growth on solid-state medium was conducted in a 250-mL Erlenmeyer flask containing 30 g of dry corn which was moistened with a solution consisting of (g/l of distilled water): glucose, 16.5; $NaNO_3$, 4.3; K_2HPO_4, 1; KCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; and $FeSO_4$, 0.01 (Cai et al. 2010a). The seed culture and solid-state media were sterilized by autoclaving at 121 °C for 20 min.

Inoculum preparation

A spore suspension was obtained as follows: *Shiraia* sp. SUPER-H168 was grown on PDA slants in the dark at 30 °C for 7 days. Black, massive spores were harvested from the surface by using sterile distilled water to wash off the spores, which were homogenized aseptically in a Sorvall Omni mixer for 10 min. The spore concentration was measured by counting with a hemocytometer under a microscope (Shi et al. 2009). The spore suspension was immediately used as the inoculum in a subsequent fermentation.

The seed culture was inoculated with 3 mL of the spore suspension (10^6 spores mL^{-1}) and cultured on a rotary shaker at 200 rpm at 30 °C for 48 h. Then, the solid-state medium was inoculated with 3 mL of the seed culture and grown at an initial moisture content of 50 % at 30 °C for 10 days under 97 % relative humidity.

α-glucosidase assay and protein estimation

α-Glucosidase activity was determined by a spectrophotometric method (Kurihara et al. 1995) using α-*p*NPG as the substrate in a 20-mM phosphate buffer (pH 4.5). Five millimoles of α-*p*NPG (0.1 mL) and buffer (2.3 mL) were added to a test tube. They were pre-incubated at 37 °C for 5 min, and an α-glucosidase solution (0.1 mL) was added and incubated at 37 °C for 5 min. The reaction was stopped by adding 0.2 M sodium carbonate (1.5 mL). Enzyme activity was quantified by measuring the absorbance at 405 nm. One unit (U) of α-glucosidase activity was defined as the amount of enzyme liberating 1.0 μmol of *p*-nitrophenol per min under the conditions described above. The protein concentration was

estimated by the Bradford method (Bradford 1976), with bovine serum albumin as the standard.

When maltose and soluble starch were used as substrates, enzyme activity was determined by measuring the production of reducing sugars using 3,5-dinitrosalicylic acid as described by Miller (1959). One U of α-glucosidase activity was defined as the amount of enzyme required to release 1.0 μmol of glucose per minute under the assay conditions.

Enzyme extraction and purification

For enzyme extraction, 10 mL of phosphate buffer (pH 7.0, 20 mM) per g of fermentation medium was added and agitated for 4 h at 30 °C. Subsequently, the material was filtered and centrifuged (10,000*g* for 10 min) at 4 °C, and the supernatant (the enzyme extract) was used as the crude extract. The supernatant was precipitated by alcohol, and the precipitate was collected by centrifugation at 10,000*g* at 4 °C for 10 min and dissolved in a small amount of buffer A (20 mM phosphate buffer, pH 7.0). The sample was loaded onto a HiTrap™ DEAE FF column with buffer A, and eluted with a step gradient of 20, 35, and 100 % buffer B (1 M NaCl in 20 mM phosphate buffer, pH 7.0). The fractions containing α-glucosidase activity were collected, and the protein content and α-glucosidase activity of each fraction were determined. The collected samples were concentrated and desalted by an ultrafiltration concentrator (30-kDa molecular weight cutoff; Amicon; EMD Millipore, Billerica, MA, USA). The concentrated enzyme solution was loaded onto a pre-equilibrated Sephadex G-200 column and eluted with 0.15 M NaCl in 20 mM phosphate buffer (pH 7.0) at a flow rate of 0.5 mL min^{-1}. The fractions containing α-glucosidase activity were collected and concentrated.

Gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970) using a 12 % resolving gel and 5 % stacking gel in a mini-electrophoresis apparatus, and a low-molecular-mass protein marker was used to determine the molecular weight of the purified protein and the homogeneity of the enzyme following each purification step. Proteins were visualized by staining with Coomassie Brilliant Blue G-250.

Enzyme activity and stability

To estimate the optimum reaction temperature, purified α-glucosidase was tested at temperature range of 20–80 °C for 5 min using α-*p*NPG as the substrate. To determine its thermostability, purified α-glucosidase was incubated at a temperatures range of 40–70 °C for various times (0–110 h). The residual activity was determined using α-*p*NPG as the substrate.

The optimum pH of the purified α-glucosidase was tested at its optimal temperature at a pH range of 2.5–9.0 for 5 min using α-*p*NPG as the substrate. The pH was adjusted using 0.05 M citric acid phosphate buffer (pH 2.5–8.0) and 0.05 M phosphate buffer (pH 7.5–9.0). To analyze its pH stability, purified α-glucosidase was incubated at 25 °C at a pH range of 3.5–7.5, and the residual activity was determined using α-*p*NPG as the substrate.

Enzyme activity in the presence of inhibitors and metal ions

The effects of metal ions (K^+, Fe^{2+}, Fe^{3+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+}, and Ag^+) and inhibitors [SDS, NaN_3, NaF, dithiothreitol (DTT), L-cysteine, ethylene glycol tetraacetic acid (EGTA), and ethylenediaminetetraacetic acid (EDTA)] on α-glucosidase activity were investigated using α-*p*NPG as the substrate and by incorporating these metal ions and inhibitors into the assay mixture prior to determining the residual activity. The effects of different concentration NaCl on α-glucosidase activity were investigated. All metal ions and inhibitors were mixed with 0.05 M citric acid phosphate buffer (pH 4.5) to final concentrations of 1 and 10 mM. α-Glucosidase activity was determined after incubation with different metal ions and inhibitors at 25 °C for 10 min. The α-glucosidase activity without metal ions and inhibitors was recorded as 100 %.

Kinetic parameters

The initial reaction rate was determined at pH 4.5 and 60 °C for various α-*p*NPG concentrations (0.5–5.0 mM), starch, and maltose. The V_{max} and K_m were calculated by Lineweaver–Burk (Lineweaver and Burk 1934) plots.

Transglycosylation activity analysis

The transglycosylation activity was determined using 5 μg of purified enzyme and 800 mM maltose, sucrose, or trehalose in the standard reaction buffer at 40 °C for 15 h. The reaction was terminated in a boiling water bath for 10 min. The reaction mixture was precipitated by alcohol, and then the supernatant was collected by centrifugation at 10,000*g* at 4 °C for 10 min. Transglycosylation product analysis was conducted by HPLC using a 4.6 mm × 250 mm HITACHI LaChorm NH_2 Analysis column (Hitachi, Japan) with acetonitrile/water (70/30, v/v) as the mobile phase at 1 mL/min and a refractive index detector. The column temperature was kept constant at 30 °C (Zhou et al. 2015).

In-gel trypsin digestion and mass spectrometry analysis

Following SDS-PAGE, the Coomassie Brilliant Blue-stained protein band corresponding to α-glucosidase was excised and cut into small (1 mm × 1 mm) pieces. Gel pieces were washed and destained with 0.1 M NH_4HCO_3 and 30 % acetonitrile (ACN), reduced by 10 mM DTT at 56 °C for 30 min, and alkylated by 100 mM iodoacetamide in the dark for 20 min. The reduced and alkylated gel pieces were rehydrated in 50 mM NH_4HCO_3, dehydrated with ACN, and hydrated with 10 μL of 25 mM NH_4HCO_3 containing 200 ng of sequencing grade trypsin (Promega, Fitchburg, WI, USA). Once this solution was fully absorbed by the gel pieces, 25 μL of enzyme-free 25 mM NH_4HCO_3 buffer was added, and the samples were digested at 37 °C for 20 h. After digestion, the supernatant was removed and dried in a Thermo Savant SpeedVac concentrator (Thermo Fisher Scientific, Waltham, MA, USA). Dry peptides were dissolved in 3 μL of 0.1 % trifluoroacetic acid, and manually spotted onto a stainless steel plate for mass spectrometric analysis.

A matrix-assisted laser desorption/ionization tandem time-of-flight (MALDI-TOF/TOF) mass spectrometer (TSQ Quantum Ultra EMR; Thermo Fisher Scientific) was used to obtain mass spectrometry (MS) and tandem MS (MS/MS) data. Protein identification was performed using GPS explorer software (Applied Biosystems, Waltham, MA, USA) equipped with the MASCOT (Matrix Science, Boston, MA, USA) search engine using peptide sequencing data obtained from the MS/MS mode (MALDI-TOF/TOF). The search was performed using the default settings for the MALDI-TOF/TOF instrument, as supplied by Matrix Science (a peptide mass tolerance of 300 ppm and a fragment mass tolerance of 0.9 Da).

Isolation of genomic DNA and total RNA

Shiraia sp. SUPER-H168 was grown under the culture conditions (Yang et al. 2014) and separated from the culture supernatant by centrifugation at 10,000*g* for 20 min. Genomic DNA and RNA were isolated using the Trizol reagent (Tiandz, Beijing, China) and stored at −80 °C.

Polymerase chain reaction (PCR) amplification

The 5× All-in-One RT MasterMix (in the AccuRT Genomic DNA Removal Kit) (Applied Biological Materials, Richmond, BC, Canada) was used to eliminate contaminating genomic DNA in the RNA preparations. Six microliters of RNA template and 2 μL of AccuRT Reaction Mix (4×) were mixed and incubated at 42 °C for 2 min, and the reaction was stopped by adding AccuRT Reaction Stopper (5×). The first-strand cDNA synthesis reaction contained 4 μL of 5× All-In-One RT MasterMix and 6 μL of nuclease-free H_2O. The reaction was incubated at 25 °C for 10 min and at 42 °C for another 50 min (for PCR), and it was inactivated by heating at 85 °C for 5 min.

Degenerate primers were designed based on the MS analysis sequences GSPYESASHGVYYR and WASVAEASR. All primers used (sequences are shown in Table 1) were purchased from Sangon Biotech (Shanghai, China), and the fragments generated by PCR were sequenced by Sangon Biotech. PCR regents were purchased from TaKaRa (Dalian, China). PCR was performed in a total volume of 50 μL containing 0.5 μL of *Taq* polymerase (5 U/μL), 5 μL of 10× PCR buffer, 5 μL of $MgCl_2$ (25 mM), 8 μL of dNTP mixture, 200 ng of DNA, 2 μL of each degenerate primer (100 μM) or 1 μL of each specific primer (20 μM), and PCR-grade water up to 50 μL. PCR conditions included an initial denaturation step at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C (depending on the T_m value) for 30 s, and elongation at 72 °C for 3 min (1 min/kb). A final elongation step was performed at 70 °C for 10 min. The PCR fragments were cloned into the pMD18-T vector.

To obtain the full-length cDNA sequence of α-glucosidase, 3′- and 5′- rapid amplification of cDNA ends (3′-RACE and

Table 1 Oligonucleotide primers used in this study.

Primer	Nucleotide sequence
5′-RACE outer primer	5′-CATGGCTACATGCTGACAGCCTA-3′
5-′RACE inner primer	5′-CGCGGATCCACAGCCTACTGATGATCAGTCGATG-3′
G-F	5′-TACGAGTCKGCITCYCAYGGCGT-3′
G-R	5′-ACGCCRTGRGAIGCMGACTCGTA-3′
W-F	5′-TGGGCDTCIGTBGCBGARGC-3′
W-R	5′-GCYTCVGCVACIGAHGCCCA-3′
3′-RACE outer primer	5′-TACCGTCGTTCCACTAGTGATTT-3′
3′-RACE inner primer	5′-CGCGGATCCTCCACTAGTGATTTCACTATAGG-3′
5-F	5′-ATGGCGCGCTCAAGCTTCT-3′
3-R	5′-CTACGTCCAACTCAAACTCCACCC-3′

R = A/G, *Y* = C/T, *M* = A/C, *K* = G/T, *S* = C/G, *W* = A/T, *H* = A/C/T, *B* = C/G/T, *V* = A/C/G, *D* = A/G/T, and *I* = rare base

Table 2 A summary of the purification of α-glucosidase from *Shiraia* sp.SUPER-H168

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	649.85	201.40	0.31	1.00	100.00
Alcohol precipitation	209.91	94.86	0.45	1.45	47.10
Anion exchange	88.31	78.66	0.89	2.87	39.06
Superdex G-200	17.23	68.86	3.99	12.87	34.19

5′-RACE, respectively) was performed to obtain the 3′- and 5′- ends, respectively, of the cDNA according to the 3′-Full RACE Core Set Ver. 2.0 and 5′-Full RACE Kit protocols (TaKaRa). Degenerate primers used for the 3′-RACE were G-F and W-F. G-R and W-R were used for the 5′-RACE. Finally, based on the 5′- and 3′- end sequences of the cDNA, primer 5-F was designed to match the start codon ATG, and primer 3-R was designed to match the sequence immediately downstream of the stop codon TAG. PCR and reverse transcription polymerase chain reaction (RT-PCR) were performed using 5-F and 3-R primers to amplify the full-length cDNA and DNA sequences of the α-glucosidase gene.

Bioinformatic analysis of the gene sequence

The Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and ClustalX were used to analyze the homology between the α-glucosidase encoded by *Shiraia* sp. SUPER-H168 and other known α-glucosidases. The computed parameters, including the molecular weight, theoretical pI, amino acid composition, atomic composition, instability index, aliphatic index, and grand average of hydropathicity (GRAVY), were predicted with the ProtParam tool (http://web.expasy.org/protparam/). *N*-glycosylation sites (Asn-Xaa-Ser/Thr) were predicted using the program NetGlyc 1.0. Signal peptides were predicted using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/). The conserved domains of the protein were predicted and analyzed using the Conserved Domain Database (CDD) (http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi). The theoretical structure of *Shiraia* sp. SUPER-H168 α-glucosidase was obtained by homology modeling with SWISS-MODEL (http://www.swissmodel.expasy.org/) (Benkert et al. 2011; Guex and Peitsch 1997). Based on this alignment, a neighbor-joining phylogenetic tree was constructed using MEGA v.2. The sequences were aligned using ClustalX in MEGA. After manual adjustments, the conserved regions from 20 α-glucosidase protein sequences, for which the assignment of positional homology was possible, were used for the tree construction. All other regions were excluded.

Results

Purification of α-glucosidase

The *Shiraia* sp. SUPER-H168 α-glucosidase was purified from a crude extract using a three-step purification procedure as summarized in Table 2. In the first step, proteins were precipitated by alcohol precipitation and dissolved in phosphate buffer (pH 7.0, 20 mM). In the second step, α-glucosidase was purified by anion-exchange chromatography (HiTrap™ DEAE FF) with a step gradient using 0.2, 0.35, 0.5, and 0.65 M NaCl. Next, fractions with protein

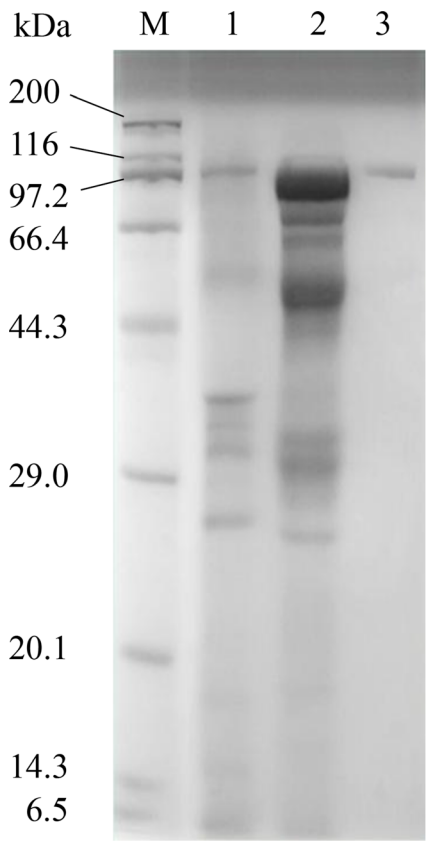


Fig. 1 SDS-PAGE analysis of *Shiraia* sp. SUPER-H168 α-glucosidase: *M* molecular weight ladder; *lane 1* the degree of purification following the alcohol precipitation step; *lane 2* the degree of purification after anion-exchange chromatography; *lane 3* purified α-glucosidase after Superdex G-200 chromatography

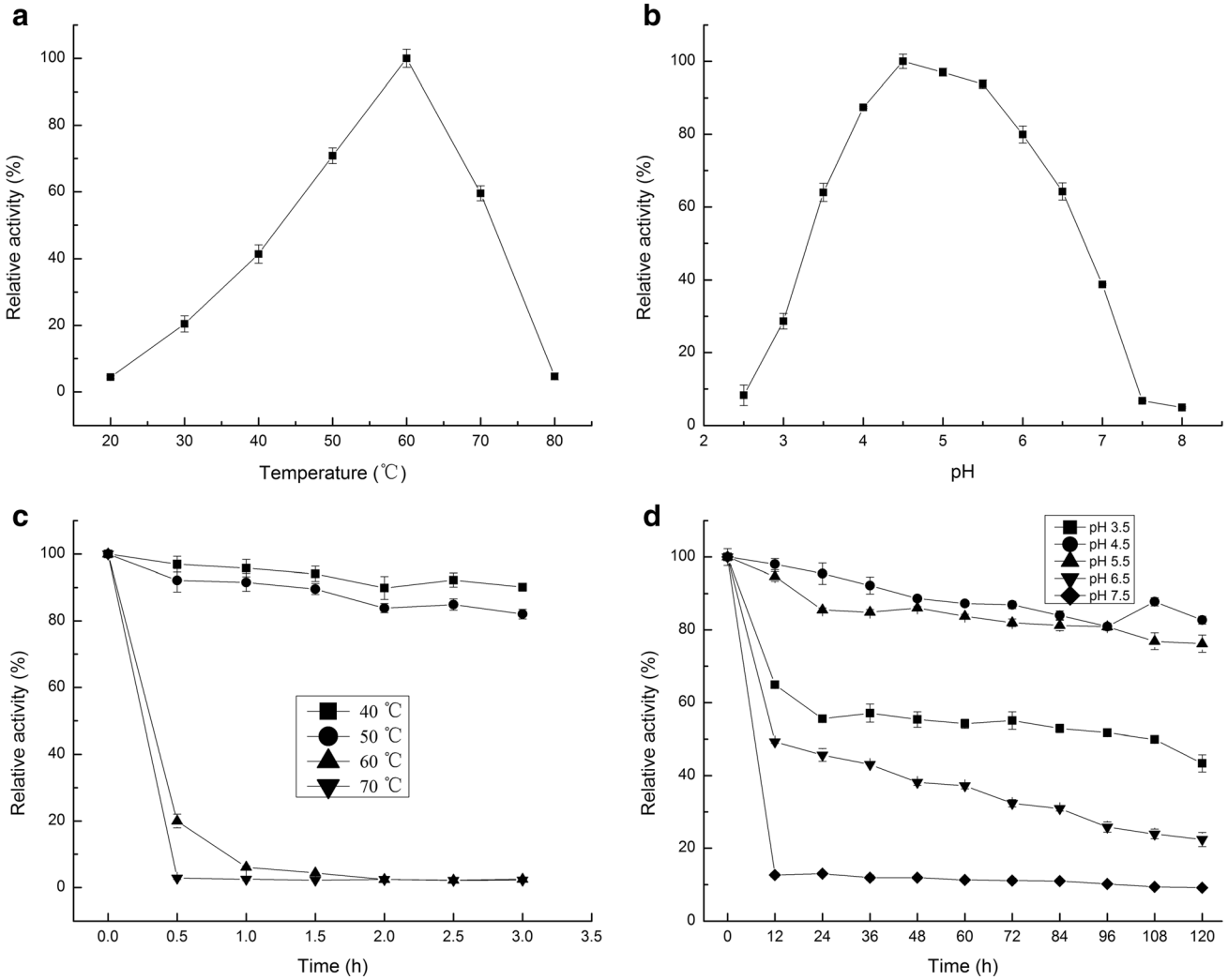


Fig. 2 Effects of temperature and pH on α-glucosidases activity and stability. **a** optimal temperature; **b** optimal pH; **c** stabilities at 40, 50, 60, and 70 °C; **d** stabilities at different pH values

Table 3 Effect of metal ions on the activity of the purified α-glucosidase

Metal ions	Relative activity (%)	
	Ion concentration (1 mM)	Ion concentration (10 mM)
Control	100.0 ± 1.9	100.0 ± 1.9
K^+	95.0 ± 0.7	104.3 ± 2.9
Fe^{2+}	105.6 ± 0.4	7.6 ± 1.8
Fe^{3+}	105.4 ± 0.7	117.0 ± 1.2
Ca^{2+}	97.7 ± 0.6	56.7 ± 2.3
Mg^{2+}	94.9 ± 0.4	142.3 ± 3.2
Mn^{2+}	101.2 ± 1.1	60.3 ± 2.1
Cu^{2+}	93.9 ± 1.0	26.0 ± 0.3
Zn^{2+}	94.6 ± 0.6	56.8 ± 0.5
Ag^+	70.0 ± 3.2	6.2 ± 0.2

Table 4 Effect of inhibitors on the activity of the purified α-glucosidase

Various inhibitors	Relative activity (%)	
	Inhibitor concentrations (1 Mm)	Inhibitor concentrations (10 Mm)
Control	100.0 ± 3.0	100.0 ± 3.0
SDS	0.6 ± 0.1	0.7 ± 0.1
NaN3	99.8 ± 0.5	102.1 ± 3.2
NaF	98.7 ± 1.0	98.4 ± 0.9
DTT	98.4 ± 1.6	98.4 ± 1.3
L-lys	99.0 ± 0.3	97.6 ± 0.8
EGTA	88.4 ± 1.3	78.1 ± 0.8
EDTA	98.1 ± 0.5	101.3 ± 2.0

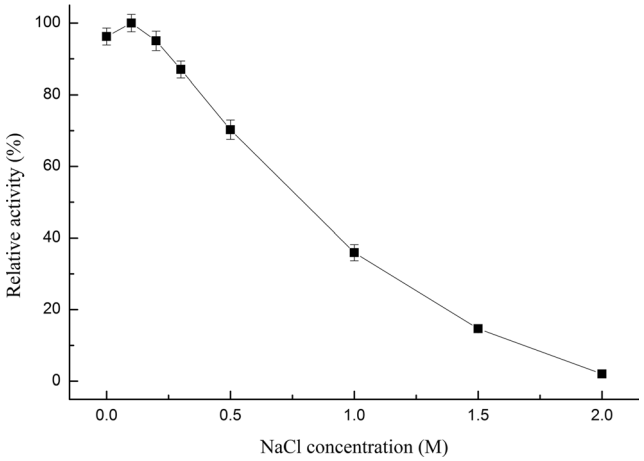


Fig. 3 Effects of different concentrations of NaCl on α-glucosidase activity

peaks were collected, and peak 2 displayed α-glucosidase activity. In the third step, Sephadex G-200 gel chromatography was used to purify the α-glucosidase, and one fraction with a single protein peak was collected, which suggests that α-glucosidase exists as a monomeric protein. SDS-PAGE analysis of the purifications steps is shown in Fig. 1. Finally, α-glucosidase was purified by approximately 12.9-fold from the crude extract, with a yield of 34.2 %.

Effect of temperature and pH on α-glucosidase activity and stability

The optimum temperature of α-glucosidase was determined using α-*p*NPG as a substrate, and the maximum activity was observed at 60 °C (Fig. 2a). The α-glucosidase was stable at 40 °C, and the enzyme activity retained 90.1 % of its initial activity after 3 h. The enzyme activity rapidly declined when the temperature was greater than 60 °C, and the enzyme retained 2.4 % of its initial activity after 2 h (Fig. 2c).

The optimum pH of the α-glucosidase was determined using α-*p*NPG as the substrate, and the maximum activity was observed at pH 4.5 (Fig. 2b). Moreover, at pH 7.5, the enzyme displayed only 6.7 % of its initial activity. The α-glucosidase was stable at pH 4.5–5.5, and it retained 82.7 % of its initial activity after 120 h (Fig. 2d).

Effects of metal ions and inhibitors on enzyme activity

The effects of several metal ions and inhibitors of different concentrations (1 mM and 10 mM) on α-glucosidase activity are summarized in Tables 3 and 4. As shown in Table 3, in the presence of 1 mM Fe^{2+} and Fe^{3+}, the relative enzyme activity increased to 105.6 and 105.4 %, respectively, of that of the untreated enzyme, and it decreased to 70.0 % in the presence of 1 mM Ag^{+}. In the presence of 10 mM K^{+}, Fe^{3+}, and Mg^{2+}, the relative enzyme activity increased to 104.3, 117.0, and 142.3 %, respectively, and it decreased to 7.9, 26.0, and 6.2 % in the presence of 10 mM Fe^{2+}, Cu^{2+}, and Ag^{+}, respectively. As shown in Table 4, α-glucosidase was obviously inhibited by SDS and EGTA, as the relative enzyme activity decreased to 0.6 and 0.7 % of that of the untreated enzyme when the SDS concentrations were 1 and 10 mM, respectively; the relative activity decreased to 88.4 and 78.1 % when the EGTA concentrations were 1 and 10 mM, respectively. This suggested that 1 mM SDS could almost completely inhibit the activity.

Effects of NaCl on enzyme activity

The effects of NaCl of different concentrations on α-glucosidase activity were investigated (Fig. 3). The maximum activity was observed at 0.1 M NaCl. At 2 M NaCl, the activity was only 2 % of that observed at 0.1 M NaCl.

Kinetic properties

The kinetics of α-glucosidase were analyzed using α-*p*NPG, starch, and maltose as substrates, and the results are summarized in Table 5. At 60 °C and pH 4.5, using α-*p*NPG as the substrate, the K_m and V_{max} of α-glucosidase were 0.52 mM and 3.76 U mg^{-1}, respectively. K_m of the α-glucosidase with maltose as substrate was 0.62 mM.

Transglycosylation of α-glucosidase

To determine whether α-glucosidase could perform transglycosylation, it was incubated with 800 mM maltose, sucrose and trehalose for 15 h. HPLC analysis revealed a distinct peak indicating a transglycosylation product and a minor peak indicating trehalose was detected after reaction with maltose (Fig. 4b); similarly, three major peaks corresponding to

Table 5 Kinetic parameters of α-glucosidase from *Shiraia* sp. SUPER-H168

Substrate	K_m (mM)	V_{max} (U mg^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (L s^{-1} mol^{-1})
α-*p*NPG	0.52	3.76	6.78	1.30×10^4
Starch	2.38 (mg mL^{-1})	1.80	3.24	1.36 (L s^{-1} g^{-1})
Maltose	0.22 (mg mL^{-1})	0.61	1.11	4.95 (L s^{-1} g^{-1})
	0.62	0.61	1.11	1.79×10^3

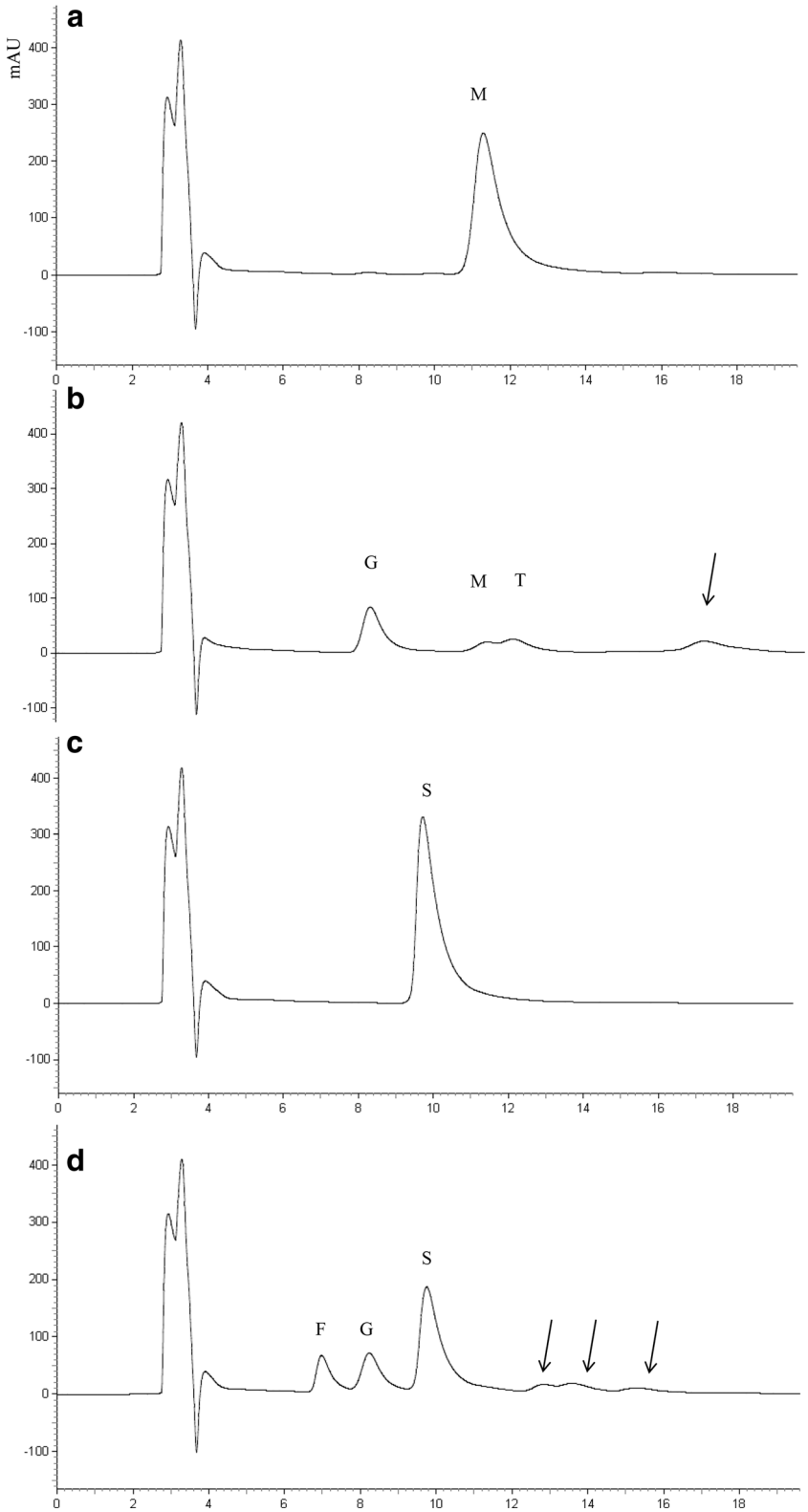


Fig. 4 HPLC chromatograms of the reaction mixture after 15 h of incubation when maltose (**a**, **b**), sucrose (**c**, **d**) or trehalose (**e**, **f**) were used as substrates; (**a**, **c** and **e** used activated enzyme, **b**, **d** and **f** used inactivated enzyme as control). *F* fructose, *G* glucose, *S* sucrose, *M* maltose, *T* trehalose. The *arrows* indicate the new transglycosylation products

fructose, glucose and sucrose, and three minor peaks corresponding to the products of a transglycosylation reaction with sucrose (Fig. 4d); a minor peak corresponding to glucose and no transglycosylation activity was observed with trehalose (Fig. 4f). Whereas typical α-glucosidase favored the α-1.4-glycosidic bonds of α-*p*NPG or maltose, *Shiraia* sp. SUPER-H168 α-glucosidase can hydrolyse the α-1.2 bonds in sucrose and had little hydrolysis activity on α-1.1 bonds in trehalose.

MALDI TOF/TOF mass spectrometry analysis

The band of purified α-glucosidase was excised, digested in-gel with trypsin, and analyzed by MALDI TOF/TOF MS. The peptide masses obtained from the MALDI-TOF/TOF combined with MS/MS were used to search the entire non-redundant database at the National Center for Biotechnology Information (NCBInr) using the MASCOT search engine. As a result, α-glucosidase was matched with the hypothetical protein SNOG_11356 (accession no. gi|169616370) from *Phaeosphaeria nodorum* SN15 by the peptide GSPYESASHGVYYR, and an α-glucosidase (predicted) (accession no. gi|63054510) from *Schizosaccharomyces pombe* 972 h- by the peptide WASVAEASR. The two peptides are shown in Table 6.

Cloning of full-length cDNA and DNA of the *shiraia* sp. SUPER-H168 α-glucosidase, and bioinformatic analysis

To better study and use the *Shiraia* sp. SUPER-H168 α-glucosidase, the α-glucosidase gene and its full-length cDNA were cloned and analyzed. Starting with *Shiraia* sp. SUPER-H168 total RNA as template, degenerate primers were designed based on the peptide sequences obtained from the MS analysis, and 3′-RACE and 5′-RACE were performed to obtain the 3′- and 5′- ends of the cDNA, respectively. A 2997-bp, full-length cDNA was obtained based on the 5′- and 3′-end sequences.

A BLAST search of the NCBI database revealed that the α-glucosidase from *Shiraia* sp. SUPER-H168 is a member of glycoside hydrolase family 31. As shown in Fig. 5, the complete genomic DNA and full-length cDNA sequences of *Shiraia* sp. SUPER-H168 α-glucosidase containing the intact open reading frame (ORF) were cloned and sequenced. The coding region of *Shiraia* sp. SUPER-H168 α-glucosidase consisted of a 2997-bp ORF encoding a 998-amino acid protein with a 22-amino acid signal peptide. A 48-bp intron was detected by comparing the DNA and cDNA sequences.

The parameters of the deduced, mature α-glucosidase were predicted with the ProtParam tool. The predicted molecular weight is 108,213.7 Da, the theoretical pI is 5.08, the total number of negatively charged residues (Asp+Glu) is 107, and the total number of positively charged residues (Arg+Lys) is 69. The instability index was computed to be 30.31. This classifies the protein as stable. The aliphatic index is 72.21, and the GRAVY score is −0.321. *Shiraia* sp. SUPER-H168 α-glucosidase contained nine potential *N*-glycosylation sites (Asn-Xaa-Ser/Thr), which are underlined in Fig. 3.

Conserved domains of the protein were predicted and analyzed using the CDD (http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi). A model structure of *Shiraia* sp. SUPER-H168 α-glucosidase (Fig. 6) was constructed using the crystal structure of α-glucosidase (PDB code: 3w37.1.A) (Tagami et al. 2013a) as a template (http://swissmodel.expasy.org/interactive). The structure consisted of an amino-terminal conserved domain, GH31_N, and a carboxyl-terminal domain, GH31_MGAM_SI_GAA.

The protein contained 14 active sites that contained two catalytic sites, and two aspartic acid residues (Asp 503 and Asp 672) were identified as the catalytic nucleophile and the acid/base, respectively. A loop of the amino-terminal beta-sandwich domain is part of the active site pocket. The active site pocket of α-glucosidase is formed mainly by $(\beta/\alpha)_8$ barrel domain and is extended by the N-loop. Only the C-terminal domain has any interaction with the active site pocket. It appears to contribute to stabilization of the $(\beta/\alpha)_8$ barrel catalytic domain rather than substrate binding (Tagami et al. 2013a).

The deduced α-glucosidase protein had low amino acid sequence similarities to α-glucosidases from other fungi, such as *Bipolaris maydis* C5 (76 %) with 99 % of query cover, *Phaeosphaeria nodorum* SN15 (74 %), *Neofusicoccum parvum* UCRNP2 (65 %), *Histoplasma capsulatum* H143 (63 %), and *Exophiala aquamarina* CBS 119918 (57 %).

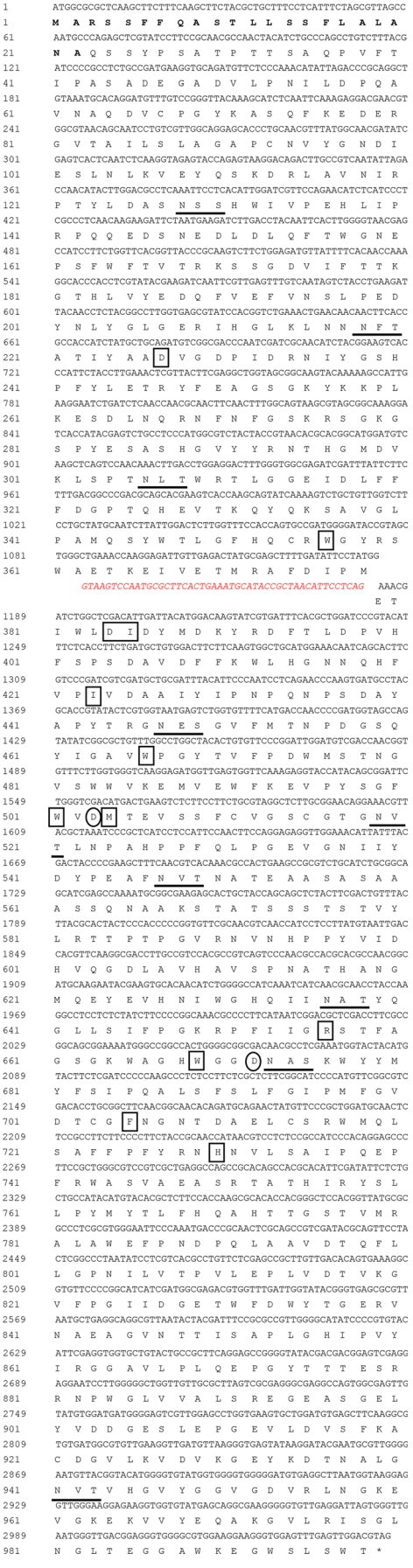
To further investigate the relationships between fungal α-glucosidases, a phylogenetic analysis (Fig. 7) was performed using 20 fungal α-glucosidase amino acid sequences from GenBank. The phylogenetic relationships between *Shiraia* sp. SUPER-H168 α-glucosidase and α-glucosidases from different fungal sources were evaluated by constructing a neighbor-joining phylogenetic tree. The phylogenetic analysis indicated that *Shiraia* sp. SUPER-H168 α-glucosidase was closely related to α-glucosidases from ascomycetes; thus, we can conclude that *Shiraia* sp. SUPER-H168 α-glucosidase is an ascomycetes α-glucosidase.

Discussion

Identifying α-glucosidases with broad substrate specificities, improved thermostabilities, and glucose tolerances is an important area of research, since α-glucosidases surmount feedback inhibition by hydrolyzing starch into glucose.

Table 6 MALDI-TOF/TOF mass spectrometric analysis of the tryptic digests of the purified α-glucosidase

Calculated peptide mass	Expected peptide mass	Observed peptide mass	Delta	Matched peptides
1571.7005	1571.6794	1572.6867	−0.0210	GSPYESASHGVYYR
975.4774	975.4757	976.4829	−0.0017	WASVAEASR



◀ **Fig. 5** Nucleotide sequence and deduced amino acid sequence of the *Shiraia* sp. SUPER-H168 α-glucosidase gene. The putative signal peptide sequence is indicated in *bold*. The intron sequence is in *italics*. Possible *N*-glycosylation sites are *underlined*. Fourteen active sites are indicated by *squares*, and the two catalytic sites are indicated by *circles*

Glucosidases from filamentous fungi are highly preferred for industrial applications because of their stabilities and broad substrate specificities (Ramani et al. 2015). A novel α-glucosidase was purified from *Shiraia* sp. SUPER-H168, and its enzymatic characteristics were investigated. *Shiraia* sp. SUPER-H168 α-glucosidase was a monomeric protein with a molecular mass of approximately 108.2 kDa and an isoelectric point of 5.08.

The effects of metal ions and inhibitors on α-glucosidase were investigated. In the presence of 10 mM K^+, Fe^{3+}, and Mg^{2+}, the relative enzyme activities were 104.3, 117.0, and 142.3 %, respectively, of that of the untreated enzyme; it has been reported that Mg^{2+} can enhance enzyme activity by 4 % (Zhang et al. 2011). *Shiraia* sp. SUPER-H168 α-glucosidase was not significantly inhibited by EGTA, which is similar to *Geobacillus thermodenitrificans* HRO10 α-glucosidase, whose activity was not affected by 10 mM EGTA (Ezeji and Bahl 2006).

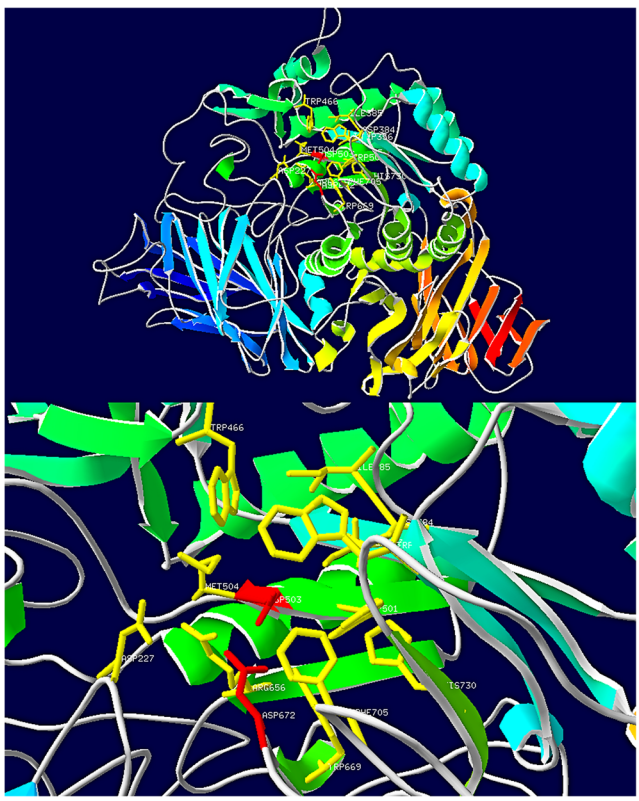


Fig. 6 A model structure of *Shiraia* sp. SUPER-H168 α-glucosidase was constructed using the crystal structure of α-glucosidase (PDB code: 3w37.1.A) as a template. Active sites residues (Asp 227, Trp 356, Asp 384, Ile 385, Ile 423, Trp 466, Trp 501, Met 504, Arg 656, Trp 669, Phe 705, and His 730) are shown in *yellow*, and catalytic sites residues (Asp 503 and Asp 672) are shown in *red*

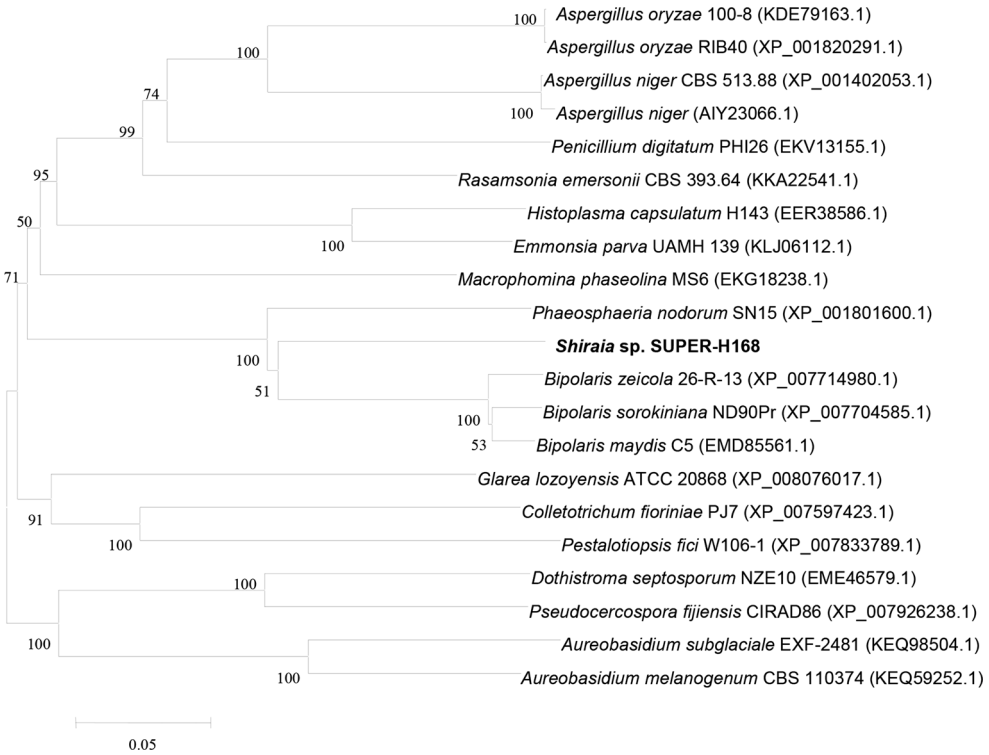


Fig. 7 Phylogenetic tree of 21 fungal α-glucosidase amino acid sequences

Measuring the conversion rate of a specific substrate is a way to evaluate enzyme efficiency, which is defined by a low K_m and high V_{max}. *Shiraia* sp. SUPER-H168 α-glucosidase showed the highest specificity constant (k_{cat} K_m^{-1}) using α-*p*NPG as the substrate, compared with maltose and starch as substrates (Table 5). Thus, this enzyme was classified as type I, and it may belong to the GH13 family based on its substrate specificity; however, α-glucosidases generally have broad specificities, and a given substrate is not strictly connected to a single enzyme type (Frandsen and Svensson 1998).

The properties of α-glucosidases isolated from different sources and using α-*p*NPG as the substrate are shown in Table 7. Most α-glucosidases have their highest activities at pH 4–5, but a few exhibit their highest activities at pH 6–7.5. Optimal temperatures of α-glucosidases vary between 40 and 65 °C (da Silva et al. 2009; Wongchawalit et al. 2006; Yamasaki et al. 2005; Kita et al. 1991; Frandsen et al. 2000; Krohn and Lindsay 1991). *Shiraia* sp. SUPER-H168 α-glucosidase exhibited a K_m of 0.52 mM with α-*p*NPG as the substrate; this is the lowest reported K_m, except for that from an *Aspergillus niger* α-glucosidase, whose other kinetic parameters were not reported, and it also revealed that the affinity of *Shiraia* sp. SUPER-H168 α-glucosidase for α-*p*NPG is greater than that of other α-glucosidases.

RACE is a method based on PCR techniques, and it is used frequently to amplify the 5′- and 3′- ends of cDNAs. Frohman et al. (1988) first cloned the phenol oxidase 1 (*pox1*) gene from the white-rot fungus *Pleurotus ostreatus* using RACE. Using this technique, *Shiraia* sp. SUPER-H168 α-glucosidase was successfully cloned in this study.

Table 7 Properties of α-glucosidases from different sources using α-*p*NPG as a substrate

Source	Mr (kDa)	Optimal pH	Optimal Temp. (°C)	K_m (Mm)	V_{max} (U mg^{-1})	k_{cat} (S^{-1})	k_{cat}/K_m (L s^{-1} mol^{-1})	Reference
Aspergillus niveus	56.0	6.0	65	0.55	8.84	8.25	1.50×10^4	(da Silva et al. 2009)
Apis cerana japonica	82.0	5.0	40	1.00	18.00	24.60	2.46×10^4	(Wongchawalit et al. 2006)
Germinating millet seeds	86.0	5.0	55	3.90	2.65	3.80	9.70×10^2	(Yamasaki et al. 2005)
Aspergillus niger	131.0	4.3	NR	0.34	NR	NR	NR	(Kita et al. 1991)
Barley malt	96.6	4.5	NR	0.60	1.36	2.20	3.60×10^3	(Frandsen et al. 2000)
Bacillus subtilis	110.0	7.5	65	1.46	NR	NR	NR	(Krohn and Lindsay 1991)
Shiraia sp. SUPER-H168	108.2	4.5	60	0.52	3.76	6.78	1.30×10^4	This study

NR not reported

The α-glucosidase gene was identified by a BLAST search of the NCBI database. The gene contains one intron, whose position was identified accurately by comparing the DNA and cDNA sequences of *Shiraia* sp. SUPER-H168, and the intron follows the GT-AG rule. The deduced *Shiraia* sp. SUPER-H168 α-glucosidase protein product displays lower similarities to α-glucosidases from other ascomycetes according to phylogenetic analyses.

Concerning the classification of glycoside hydrolases, the International Union of Biochemistry and Molecular Biology enzyme nomenclature of glycoside hydrolases is based on their substrate specificities and, occasionally, on their molecular mechanisms; such a classification does not reflect (and was not intended to reflect) the structural features of these enzymes. In this study, the K_m of purified α-glucosidase with α-*p*NPG and maltose were 0.52 mM and 0.62 mM, respectively. Classifying glycoside hydrolases into families based on their amino acid sequence similarities was proposed a few years ago. Because there is a direct relationship between amino acid sequences and protein folding similarities, such a classification: (1) reflects the structural features of these enzymes better than their substrate specificities; (2) helps to reveal the evolutionary relationships between these enzymes; (3) provides a convenient tool to derive mechanistic information (Henrissat 1991; Henrissat and Bairoch 1993); and (4) illustrates the difficulty of deriving relationships between family memberships and substrate specificities (Cantarel et al. 2009). Because α-glucosidases generally have broad specificities and a given substrate is not strictly connected to a single enzyme type, e.g., honey α-glucosidase II hydrolyzes phenyl α-glucoside and α-*p*NPG more rapidly than maltooligosaccharides (Takewaki et al. 1993), *Shiraia* sp. SUPER-H168 α-glucosidase was classified based on its amino acid sequence similarities to other α-glucosidases. The presence of a GH31_N domain in the amino-terminal region and a GH31_MGAM_SI_GAA domain in the carboxyl-terminal region of the protein suggest that this enzyme belongs to the GH31 family; thus, we classified this enzyme as belonging to the GH31 family based on its amino acid sequence similarities.

In the Protein Data Bank (PDB), eukaryotic complete structures of α-glucosidases (EC 3.2.1.20) in GH31 were only from Beta vulgaris (PDB: 3 W37, 3 W38, 3WEL, 3WEM, 3WEN and 3WEO), *Chaetomium thermophilum* var. *termophilum* DSM 1495 (5DKX, 5DKY, 5DKZ and 5DL0) and *Homo sapiens* (3TON, 3TOP, 2QLY, 2QMJ, 3CTT, 3L4T, 3L4U, 3L4V, 3L4W, 3L4X, 3L4Y and 3L4Z). The model structure of *Shiraia* sp. SUPER-H168 α-glucosidase was constructed using the crystal structure of α-glucosidase (PDB code: 3w37.1.A) with 37.42 % of amino acid identity. It is of interest that *Beta vulgaris* α-glucosidase showed higher substrate specificity for longer substrate and *Shiraia* sp. SUPER-H168 α-glucosidase for maltose, α-*p*NPG and sucrose, despite the fact that the enzymes in GH31 share similar amino acid sequences.

Transglycosylation and conversion of maltose into trehalose was first described for two prokaryotes: a *Pimelobacter* sp. and an *Arthrobacter* sp. (Maruta et al. 1995; Nishimoto et al. 1995). This activity has also been observed in an α-glucosidase from the filamentous fungus *Chaetomium thermophilum* var. *coprophilum* (Giannesi et al. 2006), but did not have glycoside hydrolysis with α-*p*NPG. Thus, this is the first report of α-glucosidase from a filamentous fungus that had good glycoside hydrolysis with maltose, and α-*p*NPG, transglycosylation and conversion activity to produce trehalose with maltose.

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