Thermostable Manganese (II) Dependent α-Glycosidase from *Pseudothermotoga thermarum*

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Alpha-glycosidase degrades polysaccharides and oligosaccharides and participates in the synthesis of oligosaccharides through a process called transglycosylation. In this study, an α-glycosidase gene *pthgly* from *Pseudothermotoga thermarum* was cloned using pET-20b as a vector and was expressed in *E. coli* BL21(DE3). After heat treatment and affinity chromatography, the resulting recombinant enzyme was purified. The purity of the enzyme reached a single band at a molecular weight of approximately 55 kDa. The properties of the recombinant enzyme were determined. The optimal temperature of α-glycosidase (Pthgly) was 90 °C and the optimal pH was 7.5. In addition, Pthgly exhibited good thermal stability at 70 °C and 75 °C. The relative molecular mass of the recombinant enzyme was 116 kDa, as determined by a protein purification system with a gel filtration column. Furthermore, α-glycosidase possessed Michaelis-Menten kinetics with a K_m and V_{max} of 0.29 ± 0.01 mmol l^{-1} and 22.12 ± 1.31 μmol min^{-1} mg^{-1}, respectively.

Keywords: α-Glycosidase; Glycoside hydrolases; Polysaccharides; Thermostability; Transglycosylation

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INTRODUCTION

Enzymes have been used in industrial applications for a very long time. However, the existence of these enzymes was not known or understood during early usage. It was not until the 20th century that the role and importance of enzymes for applications in diverse industrial markets, such as animal feed, detergents, food and beverages, leather, biofuels, and textile were acknowledged (Cherry and Fidantsef 2003; Sarmiento *et al*. 2015). Based on amino acid sequence similarity, there are many glycoside hydrolases classified within the glycoside hydrolase (GH) family. At this time, 162 GH families are recognized (http://www.cazy.org/). Among all the GHs, those acting on α-glucosidic linkage hydrolases belong to GH 4, 13, 15, 31, 63, and 122 (Okuyama 2011).

Alpha-glucosidase (EC 3.2.1.20) is a member of the glycosidases group, which was called maltase in the past. Glycosidases are exo-acting glycoside hydrolases of varying specificity that catalyze the specific hydrolysis of α-1,4-linked polysaccharides or oligosaccharides (amylopectin, oligomaltose, trehalose, *etc*.), liberating them from the non-reducing end (Chiba 1997). These molecules are widely distributed in almost all organisms. However, the fungal α-glucosidases from *A. niger* (Tsujisaka and Fukumoto 1963), and *A. oryzae* (Sugawara *et al*. 1961) have low or no activity toward glucans; whereas, an enzyme obtained from the mycelia of *Mucor javanicus* (Yamasaki *et al.* 2014) has higher activity.

Meanwhile, many α-glucosidases also act in transglycosylation reactions, and they can catalyze transglycosylation under certain conditions (Okuyama *et al.* 2002; Miyazaki *et al.* 2016). Also, the enzymes can be efficient tools in the synthesize of oligosaccharides and glycoconjugates through the conversion of maltose, maltotriose, or other oligosaccharides (Song *et al*. 2013). These converted oligosaccharides and glycoconjugates may have physical functions in humans and animals. The α-glucosidase from *Aspergillus niger* induces a reaction by converting maltose into isomaltose and panose (Duan *et al*. 1995). Similarly, α-glucosidase from *Xantophyllomyces dendrorhous* yields trisaccharides and tetrasaccharides as a final product (Fernández-Arrojo *et al*. 2007). Glucosidases play a vital physiological function in regards to glycogen biodegradation in humans and the carbohydrate metabolism of microorganisms, plants, and animals. The molecular mass of α-glucosidases varies among different organisms. Most glucosidases exhibit high thermal stability and as such, at a relatively high temperature, they are very beneficial in industrial applications.

The high activity and thermostability of specific hydrolases have practical value and are worth exploration for further applications. Extreme thermostable hydrolases can simplify the production process. Additionally, hydrolases can reduce bacterial contamination and production costs. As the economy develops, more hydrolases will be utilized in industrial applications. *Pseudothermotoga thermarum* (named *Thermotoga thermarum* before) is a hyperthermophile that was isolated from hot springs containing sulfur (Windberger *et al*. 1989); it grows well at a high temperature (around 80 °C). *P. thermarum* produces glycoside hydrolases, such as β-glucosidase, β-xylanase, and β-xylosidase (Shi *et al*. 2013; Pei *et al*. 2015). *P. thermarum* is of great value due to its enzymes' high thermostability. Extremely thermostable α-glycosidases, which are produced by thermophilic bacteria, have outstanding advantages as enzyme.

This study reports the expression and enzymatic properties of the extremely thermostable α-glycosidase derived from *P. thermarum*, including its kinetic parameters, optimum temperature and pH, and substrate specificity.

EXPERIMENTAL

Materials

Microbial strains, plasmid, growth medium, substrates, and enzymes

The plasmid pET-20b, purchased from Novagen (Madison, WI, USA) served as a vector and was used to express α-glycosidase. *Escherichia coli* Top10 and *E. coli* BL21 (DE3) purchased from Novagen were routinely grown in Luria-Bertani medium (LB) with a rotary shaking rate of 200 rpm at 37 °C.

Primers for PCR were synthesized by Sangon Biotech (Shanghai, China). DNA polymerase, ligase, and endonucleases were purchased from Takara (Dalian, China). Substrates, *p*-nitrophenyl-α-D-glucopyranoside (*p*NPG1), *p*-nitrophenyl-α-D-manno-pyranoside (*p*NPM), *p*-nitrophenyl-α-D-galactopyranoside (*p*NPG2), *p*- nitrophenyl -α-D-nitrophenyl glucuronide (*p*NPG3), *p*-nitrophenyl-β-D-galactopyranoside (*p*NPG4), and *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG5) were purchased from Sigma Aldrich (St. Louis, MO, USA).

Methods

DNA manipulation

Genomic DNA of recombinant *Thermotoga maritima* was used as a template, and the following primers (Table 1) were used for α-glucosidase gene *pthgly* cloning. PCR conditions were as follows: pre-denaturation at 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 90 s; then extensions at 72 °C for 10 min. Plasmids and PCR products and recombinant plasmids were purified through a Biomiga Gel/PCR Kit and a Biomiga Plaminiprep Kit, respectively (Shanghai, China). DNA transformation was performed by electroporation using GenePulser (Bio-Rad, California, USA).

Table 1. Nucleotide Sequences of Used Primers

Primers	Nucleotide Sequences
Primer 1	5'-GGAATTCCATATGGCTTCGGTGAAAATTGCCATTA-3'
Primer 2	5'-CCGCTCGAGTAATTTGATTCGTTTTTCATAGTGT-3'

Construction of recombinant vector pET20b-pthgly

PCR products were collected and purified using a Biomiga PCR Kit (Shanghai, China). Purified PCR products and plasmid pET-20b were digested with restriction endonucleases NdeI and XhoI. T4 DNA ligase was used to obtain fused pET20b-*pthgly*, followed by a transformation into *E. coli* Top10 cells. The positive transformants were screened using colony PCR, followed by a double digestion verification. After that, the positive colonies were sent to Sango Biotech so that the sequencing company could ensure that the correct recombinant vectors were obtained.

Expression and purification of recombinant Pthgly

The correct pET20b-*pthgly* plasmid was transformed into competent *E. coli* BL21(DE3) and was incubated into a LB plate at 37 °C overnight. From there, 5 mL of the LB medium with a single colony was incubated in a shaker at 200 rpm and 37 °C overnight. Then, 2 mL of the above inocula was mixed with 200 mL fresh LB medium and was incubated at 200 rpm at 37 °C. Within each of the mediums and plates, 100 μg mL^{-1} ampicillin was added when necessary. When the OD_{600} reached around 0.6 to 0.8, 0.1 mM of isopropyl β-D-thiogalactoside (IPTG) (Sangon Biotech, Shanghai, China) was added to induce the target protein expression. After another 4 h of incubation, cells were harvested by centrifugation at 5000 rpm for 10 min. Cell pellets were washed with a Tris-HCl buffer (20 mM, pH 7.9), followed by the re-suspension of cells with 5 mL of 20 mM Tris-HCl (including 0.5 M NaCl and 5 mM imidazole). Cell debris were disrupted by sonication (with 40% duty cycle 6 s, 15% output, 12 min), followed by centrifugation at 5000 rpm and a temperature of s4 °C for 10 min. The supernatant was then loaded onto a nickel affinity column (Novagen, USA) for affinity chromatography though a series of elution buffers (20 mM Tris-HCl, 0.5 mM NaCl, 50 mM to 1000 mM imidazole, pH 7.9). Purified proteins were desalted and concentrated by an Amicon Ultra-4 10K centrifugal filter with an appropriate buffer. Fractions were then stored in 30% glycerol at -20 °C for use in the next step.

Purity and yield of recombinant proteins were measured through a 12% SDS-PAGE gel electrophoresis by using standard markers (MBI Fermentas, USA). Bovine serum albumin (BSA) was used as a standard to determine protein concentration. A gel imaging system was utilized to analyze the electrophoretic results.

Native molecular weight of Pthgly

The native molecular weight of α-glycosidase was measured with a Sephacryl S200 HR gel filtration column (GE Healthcare, Boston, USA) using 100 mM NaCl in a 50 mM Tris-HCl buffer (pH7.5) via AKTA Pure system (GE Healthcare, Boston, USA). A series of proteins were used, containing cytochorome c (12.4 kDa), carbonic anhydrase (29 kDa), bovine albumin (66 kDa), alcohol dehydrogenase (150 kDa), and α-amylase amylase (200 kDa) (Sigma Aldrich, St. Louis, MO, USA).

Enzyme assay

α-Glycosidase activity was assayed in a mixture (200 μL) containing 1 mM *p*NPG1, 1 mM NAD^+, 1 mM Mn^{2+}, and 10 μL of diluted enzyme solution in 50 mM of an imidazole potassium phthalate buffer (pH 7.5) at 80 °C for 10 min. Then, 0.6 mL of 1 M Na_2CO_3 was added to the reaction system to stop the reaction, followed by boiling for 10 min. The pNP absorbance was measured at 405 nm.

One unit of α-glycosidase activity was defined as an enzyme release of 1 μmol *p*NP per min. α-Glycosidase activity values with other *p*NP glycosides were also determined using the same method.

Properties of Pthgly

The optimal pH values for recombinant Pthgly were assayed by incubation at 85 °C for 10 min in an imidazole potassium phthalate buffer adjusted to a series of pH (4.5 to 9). To determine the optimal temperature, the enzymes were reacted with the substrate in the same buffer (pH 7.5) for 10 min at various temperatures (50 °C to 100 °C, at 5 °C intervals). For thermostability test, enzymes were incubated for 0 to 120 min (30 min intervals) at 70 °C to 90 °C (5 °C intervals). The maximum activity was defined as 100 % (A, C) or initial activity of 100 % (B, D).

Mean values were calculated from triplicate experiments. The effects of chemical reagents on the activity of Pthgly were also determined. For the enzyme assay, the final concentration of Zn^{2+}, Mg^{2+}, Ba^{2+}, Cu^{2+}, Ni^{2+}, Ca^{2+}, Co^{2+}, or Al^{3+} was 1 mM; while, the final concentration of Tris, Tween 60, or SDS were 0.05%, 0.05% and 0.1%. In the reaction system, Pthgly and chemical reagents were added into a 50 mM imidazole potassium phthalate buffer and incubated at 75 °C for 1 h. From there, 1 mM of *p*NPG1 was added to start the reaction. Under the same conditions, the enzyme activity with 1 mM of Mn^{2+} and without any other chemical reagents was defined as 100%.

RESULTS AND DISCUSSION

Verification of recombinant proteins and amino acids analysis

The molecular weight of the protein was calculated to be 116 kDa. Because the theoretical molecular weight of the protein was 55.32 kDa (Fig. 1), it was determined that this recombinant protein was a dimeric protein. The isoelectric points (pI) of almost all α-glucosidases lie in acid range, ranging from 3.0 to 5.0, while the pH of α-glycosidase, consisting of 481 amino acids, was deduced to be 6.5 via the uniprot website (https://www.uniprot.org).

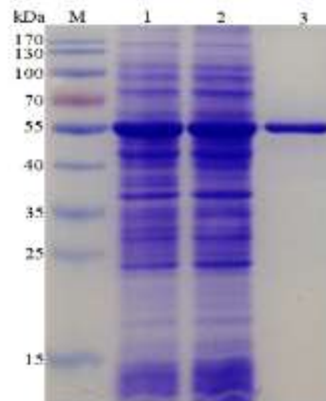

Fig. 1. SDS-PAGE of α-glycosidase. Lane M: marker; lane 1: the total protein of recombinant E. coli BL21 (DE3); lane 2: the fractions by heat treatment (60 °C, 30 min); lane 3: the purified proteins via nickel affinity column chromatography

Properties of Recombinant Pthgly

Recombinant Pthgly was optimally active at a pH of 7.5 and a temperature of 90 °C (Fig. 2A, C). Enzyme activity decreased dramatically below pH 6.5 and above 90 °C. Moreover, activity was stable from a pH of 6.5 to 8.5 and a temperature of 70 °C and 75 °C (Fig. 2B, D).

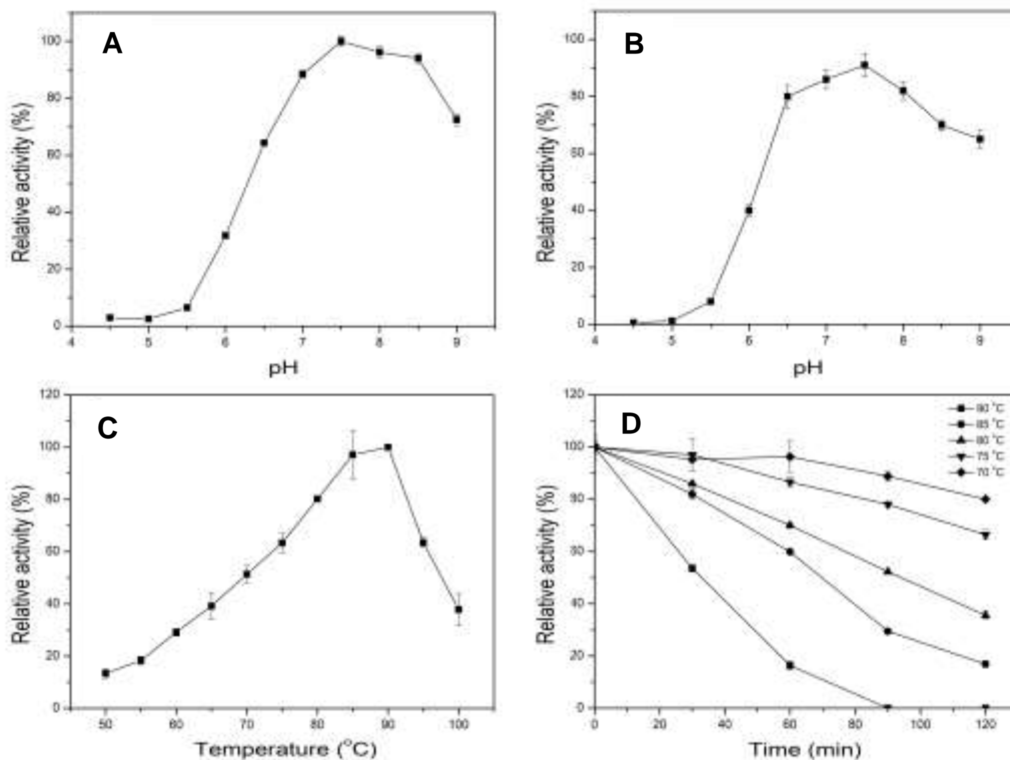

Fig. 2. Temperature and pH on Tthgly activity. A) pH optimum (pH 4.5 to 9, 85 °C for 10 min); B) pH stability (pH 4.5 to 9, 70 °C for 1 h); C) Temperature optimum (pH 7.5, 50 °C to 100 °C for 10 min); D) Thermal stability (pH 7.5, 70 °C to 90 °C for 0, 30, 60, 90 and 120 min).

As shown in Fig. 2D, the residual activity retained more than 60% of its initial activity at 70 °C or 75 °C and only 40% at 80 °C through a 2 h incubation. However, it was

quite unstable at 90 °C. In industrial processes, α-glucosidases obtained from the genus *Aspergillus* have been attracting more attention. These α-glucosidases display high activity at temperatures ranging from 45 °C to 60 °C (Michelin *et al*. 2008; Kumar and Satyanarayana 2009). The *A. niger* possesses an optimum pH within the range of 3.5 to 6.5. However, the pH optimum for α-glucosidases can exceed 7.0 (Aquino *et al*. 2001). In this study, Pthgly exhibited obvious advantages of thermal stability at a relatively high temperature.

Pthgly was only active when NAD^+ and Mn^{2+} were present. It is well known that cofactors and cations are not necessary for the hydrolysis reaction in most glycoside hydrolases. The GH4 family was found to be a requirement of the NAD^+ and divalent metal cations (Lodge *et al.* 2003), while the GH31, GH 63, and GH 97 α-glycosidases were not (Okuyama 2011; Miyazaki *et al*. 2016). Pthgly, one of GH4 α-glycosidases, confirmed that NAD^+ and Mn^{2+} were needed for the reaction. In addition, Pthgly activity was significantly inhibited by SDS and partially inhibited by Ni^{2+} and Zn^{2+}, while other molecules showed almost no influence on activity (Fig. 3).

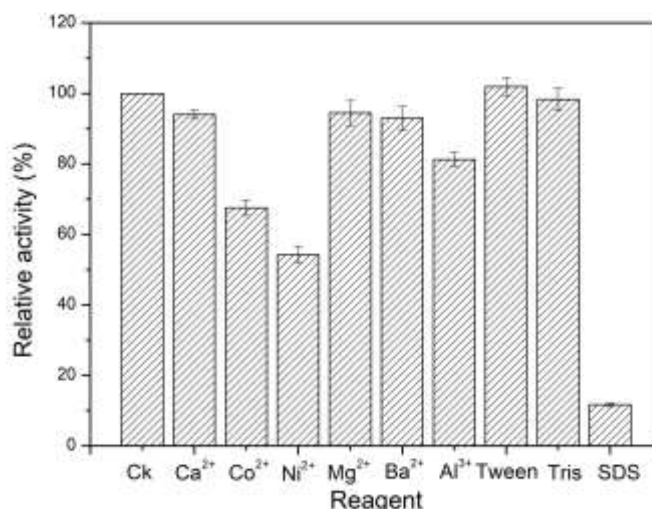

Fig. 3. Effects of cations and detergents on Pthgly activity

Substrate specificity and kinetics

Pthgly was only active towards pNPG1 (Table 2). This result indicated that the Pthgly can be classed as an α-glycosidase, consistent with its hypothesized α-glucosidase/α-galacosidase; however, no α-galacosidase activity was detected.

Table 2. Substrate Specificity

Substrates	Specific activity (μmol min^{-1} mg^{-1})
p-nitrophenyl-α-D-glucopyranoside (*p*NPG1)	18.91 ± 0.96
p-nitrophenyl-α-D-galactopyranoside (*p*NPG2)	ND
p-nitrophenyl-α-D-nitrophenyl-glucuronide (*p*NPG3)	ND
p-nitrophenyl-β-D-glucopyranoside *(pNPG4)*	ND
p-nitrophenyl-β-D-glucopyranoside (*p*NPG5)	ND
p-nitrophenyl-α-D-mannopyranoside (*p*NPM)	ND

ND: Not detected

α-Glucosidase from *Schizosaccharomyces pombe* has been reported as active to several *p*NP substrates (Okuyama *et al.* 2002). Kinetic parameters were calculated from the Michaelis-Menten equation at the optimal pH and temperature. The results showed an apparent V_{max} of 22.12 ± 1.31 µmol min^{-1} mg^{-1}, K_m of 0.29 ± 0.01 mmol l^{-1}, and a k_{cat} of 42.77 ± 2.01 s^{-1} (Table 3).

Table 3. Characteristics of Recombinant Pthgly

Property	Recombinant Pthgly
Molecular weight	116 kDa
Specific enzyme activity	18.9 ± 0.96 µmol min^{-1} mg^{-1}
Optimal pH	7.5
Optimal Temperature	90 °C
pH stability (6.5-8.0, 1h)	Residual activity: above 80%
Thermostability (70 °C - 75 °C, 2 h)	Residual activity: above 60%
k_m	0.29 ± 0.01 mmol l^{-1}
V_{max}	22.12 ±1.31 µmol min^{-1} mg^{-1}
k_{cat}	42.77±2.01 s^{-1}

Industrial application of α-glucosidases

α-Glucosidases are very useful in the degradation of glucans, the transglycosylation needed for the synthesis of oligosaccharides, and the utilization of biomass. As commonly known, single substrate specificity usually has great benefits for industrial use. Pthgly only had activity towards a single substrate, and according to its high activity and its thermostability at a relatively high temperature, it represents a potential candidate for industrial applications in the future.

CONCLUSIONS

1. The recombinant pET-20b harboring the *ptthgly* gene was introduced into *E. coli* for expression, which was followed by cell disruption using a sonicator. From there, the recombinant enzyme was further purified using a nickel column. The molecular weight of the protein was deduced to be 116 kDa by the ATKA system and was thus confirmed as a dimer.
2. The optimum temperature of α-glucosidase was 90 °C and the optimum pH was 7.5. The α-glucosidase showed good thermal stability and the recombinant enzyme maintained above an 60% residual activity after a 2 h incubation at 70 °C and 75 °C.
3. Pthgly was only active when NAD^+ and Mn^{2+} were present, and it was significantly inhibited by SDS and partially inhibited by Ni^{2+} and Zn^{2+}. The kinetic parameters of α-glucosidase were a K_m of 0.29±0.01 mmol l^{-1}, V_{max} of 22.12 ±1.31 µmol min^{-1} mg^{-1}, and a k_{cat} of 42.77±2.01 s^{-1}. These characteristics implied that this recombinant α-glycosidase may possess desirable characteristics for future industrial applications.

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