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Research Note

Broad substrate specificity of a hyperthermophilic α-glucosidase from *Pyrobaculum arsenaticum*

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Abstract *Pyrobaculum arsenaticum* is a hyperthermophilic archaeon that thrives at 95°C. This strain encodes a putative GH31 intracellular α-glucosidase (Pars_2044, PyAG) in its genome. The recombinant PyAG (rPyAG) was optimally expressed in *Escherichia coli* at 37°C for 4 h after IPTG induction. The purified rPyAG is a homotetrameric α-glucosidase that exhibited highly thermostable properties. Maximum *p*-nitrophenyl-α-D-glucopyranoside (*p*NPG) hydrolysis activity was observed at 90°C and pH 5.0. The enzyme mainly recognized the non-reducing end of the substrate, releasing the glucose unit. rPyAG also had broad substrate specificity, cleaving maltose (α-1,4-linkage), kojibiose (α-1,2-linkage), and nigerose (α-1,3-linkage) with similar efficiency. Based on these results, rPyAG can be used to modify health-relevant sugar conjugates linked by α-1,2- or α-1,3-bonds.

Keywords: α-glucosidase, glycoside hydrolase family 31, hyperthermophile, *Pyrobaculum arsenaticum*

Introduction

α-Glucosidase (EC 3.2.1.20) is an exo-type hydrolase that recognizes α-glucosidic linkage substrates and can hydrolyze the glucose unit from the non-reducing end of the substrate. This enzyme is widely distributed in most organisms including plants, animals, bacteria, and archaea and is classified into three main groups (type I, II, and III) based on substrate specificity (1). Type I α-glucosidases mainly attack aryl-glucosides such as *p*-nitrophenyl-α-D-glucopyranoside (*p*NPG), Type II α-glucosidases prefer the hydrolysis of disaccharides including maltose, and Type III α-glucosidases are more active on polymer substrates such as starch. According to sequence homology-based classification of the glycoside hydrolase (GH) family (CAZy, www.cazy.org), α-glucosidases are mainly observed in GH13 and GH31 (1). Family GH13 is a large polyspecific family that contains various types of enzymes including α-amylase, cyclodextrin glucanotransferase, sucrose isomerase, and α-glucosidase (2). This family is divergent, containing α-glucosidase, α-xylosidase, and α-glucan lyase enzymes (2). A small number of GH31 α-glucosidases have been found in bacteria and archaea (1). Physiologically, α-glucosidases play several roles such as utilization of carbon molecules like maltooligosaccharides and modification of glycoconjugates (1,3). Industrial interest in α-glucosidase is focused on glucose production from starch and modification of therapeutic biomolecules such as 2′-fucosyllactose and 3′-sialyllactose (1,4).

Hyperthermophilic archaea are microorganisms that thrive in extreme environments such as high temperature (>80°C). To adapt to such harsh conditions, many aspects of their metabolic systems evolved differently from those of mesophilic bacteria (5). One of the outstanding properties of archaeal proteins is their hyperthermostability. For instance, a kojibiose phosphorylase from *Pyrococcus* sp. ST04 maintains most of its activity at 95°C, and its half-life was measured as 1.9 h at 100°C (6). The optimum temperature of maltogenic amylase from *Staphylothermus marinus* is 100°C, and its melting temperature was determined to be 109°C (7). Moreover, some archaeal proteins show unique enzymatic activity that has not been observed in mesophilic bacteria. Maltose-forming amylase from *Pyrococcus* sp. ST04 and *Thermococcus cleftensis* CL1 recognizes maltose units linked by both α-1,4- and α-1,6-glycosidic bonds (8,9). The activity of amylopullulanase from *Staphylothermus marinus* was not reduced by the typical amylopullulanase inhibitor cyclodextrin (10). These unique properties of archaeal enzymes provide potential interesting applications in the sugar industry.

In this study, an α-glucosidase-encoding gene was isolated from

the hyperthermophilic crenarchaeon *Pyrobaculum arsenaticum,* which can grow at temperatures higher than 95°C. When the gene was heterologously expressed in *E. coli*, the purified recombinant protein showed not only thermostability, but also high enzyme activity toward various α-1,4-, α-1,2-, and α-1,3-linked disaccharides.

Materials and Methods

Bacterial strains and reagents The *Pyrobaculum arsenaticum* used in this study was kindly obtained from Dr. James Holden (Department of Microbiology, University of Massachusetts, Amherst, MA, USA). It was grown anaerobically at 95°C in 50 mL media in serum bottles sealed with a butyl rubber stopper and flushed with Ar gas (11). *E. coli* DH10B [F⁻ *araD139 Δ*(*ara leu*)*7697 ΔlacX74 galU galK rpsL deoR* Φ80*lacZΔM15 endA1 nupG recA1 mcrA Δ*(*mrr hsdRMS mcrBC*)] and *E. coli* BL21-CodonPlus(DE3)-RP [F⁻ *ompT hsdS*(r_B^- m_B^-) *dcm*$^+$ Tet^r *gal λ(DE3) endA* Hte *(argU proL* Cam^r*)*] strains were employed as hosts for gene manipulation and expression, respectively. *E. coli* strains were grown in lysogeny broth (LB) medium (BD, Franklin Lakes, NJ, USA) supplemented with ampicillin (100 μg/mL). The pGEM T-easy vector (Promega, Madison, WI, USA) and pET-21a vector (Novagen, Darmstadt, Germany) were used for PCR-cloning and expression of the α-glucosidase enzyme, respectively. The various maltooligosaccharides used in this study were purchased from Wako Pure Chemical Industries (Osaka, Japan). The *p*-nitrophenyl-α-glucopyranoside (*p*NPG) was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Cloning and expression of *P. arsenaticum* α-glucosidase The gene encoding α-glucosidase (Pars_2044) was amplified using the following primers: PyAG_F: 5'-CATATGTCCCTAGAGCTTGGGAGGAAG-3' and PyAG_R: 5'-CTCGAGGGTCGTCTCTAGATAGGTGCCGAG-3' (underline indicates *Nde*I and *Xho*I restriction enzyme sites, respectively). *Pfu*Ultra polymerase (Agilent, Santa Clara, CA, USA) was used to amplify low-error PCR products, and the PCR was carried out under the following conditions: an initial denaturation step for 40 s at 94°C; 20 cycles of 40 s at 94°C, 40 s at 57°C, and 3 min at 72°C for amplification; followed by a final elongation step for 5 min at 72°C. The amplified PCR product was ligated with the pGEM-T-easy vector to confirm the sequence. For expression in *E. coli*, the insert was excised from the pGEM-T-easy vector using *Nde*I and *Xho*I and ligated into the corresponding restriction sites in the pET21a-vector to construct pET-PyAG.

Expression and purification of recombinant PyAG *E. coli* CodonPlus-RP cells harboring pET-PyAG were grown in 500 mL LB medium (BD) supplemented with ampicillin (100 μg/mL) and chloramphenicol (34 μg/mL) to an OD_{600} of 0.5 and were induced by adding 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) at 18, 25, and 37°C. Cultures were collected using centrifugation at 4,000×*g* for 20 min, and pellets were suspended in 20 mL lysis buffer [50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole (pH 7.5)]. The pelleted cells were disrupted by sonication (4×5 min, output control 4, 40% duty cycle; VC-600; Sonics & Materials, Inc., Newtown, CT, USA) and centrifuged at 12,000×*g* for 20 min. To remove the heat-labile proteins of the *E. coli* host strain, heat treatment was carried out at 70°C for 20 min. After centrifugation, the crude extracts containing recombinant PyAG (rPyAG) with a C-terminal six-histidine tag were applied to nickel–nitrilotriacetic acid (Ni–NTA) resin (Qiagen). The resins were washed with 20 mL washing buffer [50 mM NaH_2PO_4, 300 mM NaCl, and 20 mM imidazole (pH 7.5)] and eluted with elution buffer [50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole (pH 7.5)].

Determination of enzyme activity The hydrolysis activity of rPyAG was determined using *p*NPG as a substrate. The reaction mixture contained 1 mM *p*NPG in 20 mM sodium citrate buffer (pH 5.0) with 0.9 μg PyAG, which was then incubated at 90°C for 10 min. The reaction was stopped by addition of 0.1 N NaOH. The absorbance of *p*-nitrophenol (*p*NP) was measured spectrophotometrically at 400 nm. One unit of activity (U) was defined as the amount of enzyme that released 1 μmol *p*NP per min. To determine the effect of temperature on enzyme activity, the same reaction mixtures were incubated at various temperatures ranging from 60 to 95°C. The effect of pH was examined at various pH levels between 4.0 and 9.0. The buffers used in this study were 20 mM sodium acetate (pH 4.0-5.0), sodium citrate buffer (pH 5.0-6.0), and sodium phosphate buffer (pH 6.0-8.0). Substrate specificity of rPyAG was determined at a 1 mM concentration of various disaccharides with different linkages. The reaction was implemented at 90°C for 10 min. Glucose released from the reaction was measured by high-performance anion exchange chromatography (HPAEC).

Gel permeation chromatography (GPC) A purified protein sample (200 μL of a 1.0 mg/mL solution) was applied to a Superdex 200 HR column (10 mm×300 mm, GE Healthcare Bio-Sciences Ltd., Buckinghamshire, England) to estimate the apparent molecular weight of rPyAG under non-denaturing conditions. The column was equilibrated with 20 mM sodium phosphate buffer (pH 7.5), and the protein was eluted with the same buffer at a flow rate of 0.4 mL/min. The molecular mass was estimated through protein standards including carbonic anhydrase (29 kDa), bovine serum albumin (BSA, 66 kDa), β-amylase (200 kDa), and thyroglobulin (669 kDa).

High performance anion exchange chromatography (HPAEC) HPAEC analysis was used to confirm and calculate the amount of glucose released due to enzymatic activity. The reaction mixture was dissolved in a 150 mM NaOH solution and analyzed using a CarboPac^{TM} PA-1 column (Dionex Co., Sunnyvale, CA, USA) and an electrochemical detector (ED40; Dionex Co.). Solution A (150 mM NaOH) and Solution B (150 mM NaOH and 600 mM sodium acetate)

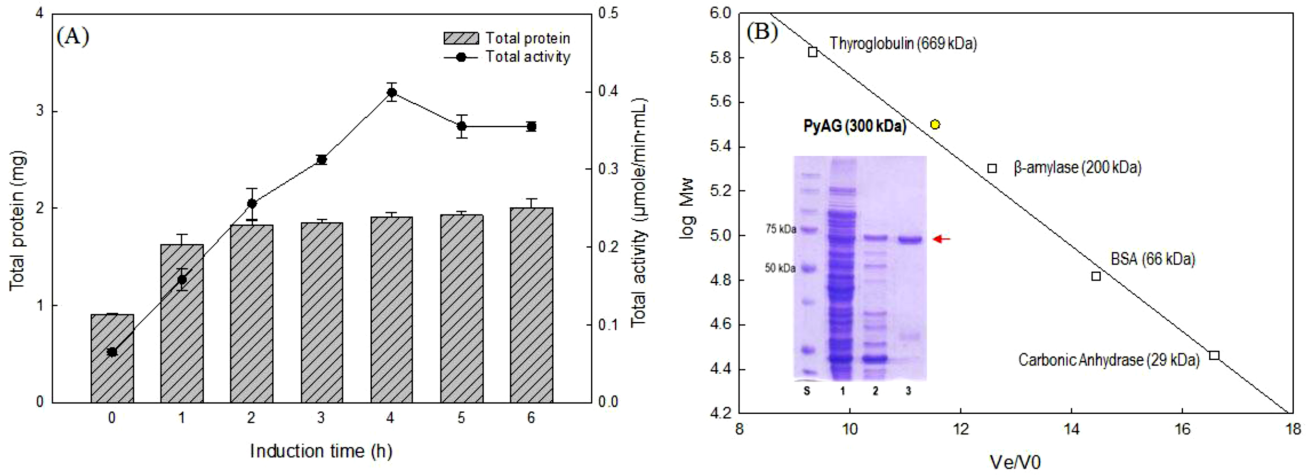

Fig. 1. Optimum induction conditions for recombinant PyAG (A) and determination of molecular mass of rPyAG using SDS-PAGE and GPC analysis (B). Lane S, protein size standard; Lane 1, crude extract; Lane 2, proteins after heat-treatment at 70°C for 20 min; Lane 3, protein after purification using Ni-NTA affinity chromatography.

were used for separation of products at a flow rate of 1.0 mL/min. The samples were eluted with a linear gradient from 100% solution A to 35% solution B over 35 min at 1.0 mL/min.

Results and Discussion

Heterologous expression of a hyperthermostable α-glucosidase from *P. arsenaticum* The gene encoding *P. arsenaticum* α-glucosidase (PyAG) contains 685 amino acid residues, which showed low amino acid identity to known archaeal α-glucosidases from *Sulfolobus solfataricus* (36%), *Sulfolobus tokodaii* (36%), and *Ruminococcus obeum* (25%) (12-14). PyAG seems to be a cytoplasmic glucosidase since it lacks a predicted signal peptide sequence at the N-terminal end of the protein. To avoid codon usage issues, we used *E. coli* CodonPlus-RP as the host for heterologous expression. For soluble protein production, induction at low temperature was usually implemented (15); however, we found that IPTG induction at 37°C showed much higher hyperthermostable rPyAG yield in *E. coli* than under low temperature conditions (<18°C) (data not shown). The highest total *p*NPG hydrolysis activity was observed with a 4 h induction at 37°C (Fig. 1A), indicating that high temperature is required for the soluble production of rPyAG. Similar properties have been reported for the heterologous expression of thermophilic enzymes (16). Enzyme purification was established through a two-step process of heat treatment and Ni-NTA affinity chromatography. The cell pellet was disrupted and heat-treated at 70°C for 20 min. Most of the heat-labile proteins originating from *E. coli* were denatured and precipitated (Fig. 1B). After purification with Ni-NTA chromatography, we observed one prominent protein band in SDS-PAGE (Fig. 1B), the size of which did not match with the calculated molecular mass (76 kDa) despite high enzyme activity. This characteristic is commonly observed in other reports of hyperthermophilic enzymes (17). The molecular mass of rPyAG under non-denaturing conditions was calculated to be 300 kDa by gel filtration chromatography, which indicated that PyAG exists naturally as a homotetramer (Fig. 1B). This homotetrameric property of rPyAG is distinct from the GH31 α-glucosidase (MalA) from *Sulfolobus solfataricus*, which forms a hexameric assembly in crystals and α-glucosidase from *Ruminococcus obeum* and *Thermoplasma acidophilum,* which are dimeric and pentameric, respectively (12,14,18).

Enzymatic properties of hyperthermostable α-glucosidase from *P. arsenaticum* Many hyperthermophilic enzymes expressed in *E. coli* exist in an insoluble form and therefore lack enzymatic activity (19), limiting their industrial application. Until now, only six archaeal GH31 α-glucosidases have been investigated, whereas 26 bacterial α-glucosidases have been investigated. Here, we characterized an active hyperthermophilic α-glucosidase. The optimal hydrolysis activity of rPyAG was measured using *p*NPG as the sole substrate over a range of temperatures (60 to 95°C) and pH conditions (pH 4.0 to 8.0). The highest enzyme activity of rPyAG was observed in sodium citrate buffer at pH 5.0 and 90°C (Fig. 2). The enzyme activity was greater than 90% between pH 4.0 and 6.0. More than 70% of the rPyAG activity remained at 80°C, indicating that rPyAG expressed in *E. coli* was active and exhibited high thermostability, which is advantageous for industrial applications.

The broad substrate specificity of the hyperthermostable α-glucosidase from *P. arsenaticum* The multiple alignment analysis of amino acid sequences of PyAG and various glucosidases indicated that PyAG has similar conserved regions (^{322}GIWLDMNEP^{330} and ^{453}GADVGGF^{459}) of Type II α-glucosidase mainly hydrolyzing the short length sugars such as maltose (13). The enzyme activity of rPyAG was investigated on various substrates including maltooligosaccharides and was monitored using thin layer chromatography (TLC). The

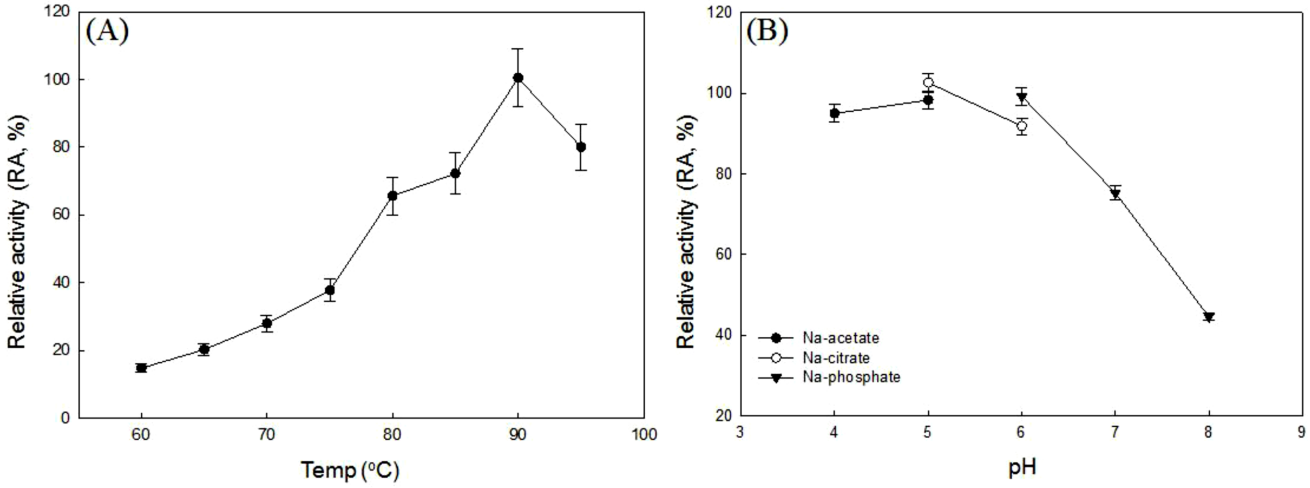

Fig. 2. Effects of temperature (A) and pH (B) on rPyAG activity

production of glucose was commonly observed in the reaction with maltooligosaccharides ranging from maltose to maltoheptaose. The observed activity was in good agreement with properties of α-glucosidase from *Pyrobaculum aerophilum,* which has high amino acid similarity with PyAG (77%) (17). rPyAG also can hydrolyze the *p*NPG6 producing *p*NPG5 and *p*NPG4 (data not shown), which suggested that this enzyme is an exo-type α-glucosidase that releases glucose units from the non-reducing end of the substrate. The determination of substrate preference for rPyAG showed that this enzyme had higher hydrolysis activity with short maltooligosaccharides such as maltose (5.84±0.10 μmol/min·mg) and maltotriose (2.58±0.19 μmol/min·mg). The specific activities with other long maltooligosaccharides (G4, G5, G6, and G7) were much lower than that of maltose (data not shown). Maltose is composed of two glucose units that are linked by α-1,4-glycosidic bonds. To investigate the effect of glycosidic linkage in enzyme activity, a variety of disaccharides formed by different linkages were reacted with rPyAG. Among the substrates, rPyAG efficiently hydrolyzed not only α-1,4-glycosidlic linkages, but also α-1,2- and α-1,3-glycosidic linkages. Jeon *et al.* (17) have reported that α-glucosidase from *P. aerophilum* can hydrolyze maltose and isomaltose; however, isomaltose hydrolysis activity (α-1,6-linkage) of rPyAG was determined to be 1.53±0.10 μmol/min·mg, which is much lower than those for kojibiose and nigerose, respectively (Table 1). Interestingly, rPyAG displayed similar activity with the maltose, kojibiose, and nigerose disaccharides. Moreover, rPyAG can hydrolyze α-D-glucosyl-1,3-D-fructose, known as turanose (Table 1). Most GH31 α-glucosidases prefer maltose and maltooligo- saccharides bonded by α-1,4-glycosidic linkages (1), which is different from rPyAG. For example, *Thermoplasma acidophilus* α-glucosidase is one of the thermophilic GH31 α-glucosidases that favors maltose and kojibiose over nigerose by about 2-fold (18). The *Lactobacillus johnsonii* α-glucosidase is an α-1,3-glucosidase displaying high activity for nigerose; however, its maltose hydrolysis activity is much lower than that of nigerose (20). Generally, the different +1 subsite environment affects disaccharide substrate specificity (20,21). Multiple sequence alignment and structure analysis have suggested that subdomain B (B'1 to B'2) has the most distinctly different sequence between α-1,3-glucosidases and α-1,4-specific enzymes (12,20), and these regions are closed in an active site pocket. Interestingly, PyAG also shows a different length in this region compared to other GH31 α-glucosidases (data not shown), suggesting that the variation in this region explains the broad substrate specificity of PyAG.

In conclusion, the *P. arsenaticum* α-glucosidase is a hyperthermostable

Table 1. Substrate specificities of rPyAG

Substrate (1 mM)	Linkage	Specific activity (μmol/min·mg)	Relative activity (%)
Maltose	O-α-D-glucosyl-(1→4)-D-glucose	5.84±0.10	100
Maltotriose	O-α-D-glucosyl-(1→4)-α-D-glucosyl-(1→4)-D-glucose	2.58±0.19	44
Isomaltose	O-α-D-glucosyl-(1→6)-D-glucose	1.53±0.10	26
Trehalose	O-α-D-glucosyl-(1→1)-D-glucose	<0.1	0
Kojibiose	O-α-D-glucosyl-(1→2)-D-glucose	4.86±0.13	83
Nigerose	O-α-D-glucosyl-(1→3)-D-glucose	5.24±0.14	89
Sucrose	O-α-D-glucosyl-(1→2)-β-D-fructose	<0.1	0
Isomaltulose	O-α-D-glucosyl-(1→6)-D-fructose	<0.1	0
Turanose	O-α-D-glucosyl-(1→3)-D-fructose	1.36±0.07	23
*p*NPG	α-glycosidic linkage	4.67±0.04	80

enzyme that exhibits high catalytic activity toward α-1,4-, α-1,2-, and α-1,3-glycosidic linkages. The α-1,3- and α-1,2-glycosidic bonds are valuable structural components in therapeutic biomolecules such as milk oligosaccharides and glycoconjugates. Therefore, the broad substrate specificity of PyAG provides opportunity for the modification of biomolecules, allowing production of a variety of health-relevant sugar conjugates.

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Disclosure The authors declare no conflict of interest.

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