Journal of Molecular Catalysis B: Enzymatic 72 (2011) 305–312

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

# Characterization of the catalytic and kinetic properties of a thermostable *Thermoplasma acidophilum* α-glucosidase and its transglucosylation reaction with arbutin

Seong-Hwa Seo[a], Kyoung-Hwa Choi[a], Sungmin Hwang[a], Jieun Kim[a], Cheon-Seok Park[b], Jung-Rae Rho[c], Jaeho Cha[a,*]

[a] Department of Microbiology, College of Natural Sciences, Pusan National University, Busan 609-735, Republic of Korea
[b] Graduate School of Biotechnology, and Institute of Life Science and Resources, KyungHee University, Yongin 446-701, Republic of Korea
[c] Department of Oceanography, Kunsan National University, Jeonbuk 573-701, Republic of Korea

ARTICLE INFO

*Article history:*
Received 16 February 2011
Received in revised form 11 July 2011
Accepted 11 July 2011
Available online 20 July 2011

*Keywords:*
GH31 α-glucosidase
Hyperthermophiles
Thermostability
*Thermoplasma acidophilum*
Transglucosylation

ABSTRACT

The gene (Ta0298) encoding a putative α-glucosidase from hyperthermophilic archaeon *Thermoplasma acidophilum* (AglA) was cloned and expressed in *Escherichia coli*. Gel filtration chromatography of the purified enzyme indicated that the native form was a pentamer with strong maltose (α-1,4 linkage)-hydrolyzing activity. AglA was optimally active at pH 5–6 and 80 °C and had a half-life of 16.8 h and 1.4 h at 80 °C and 85 °C, respectively. The enzyme also hydrolyzes kojibiose (α-1,2), nigerose (α-1,3), and isomaltose (α-1,6) to a lesser extent. Analysis of the reaction with maltooligosaccharides and panose as substrates show that AglA specifically liberates glucose from the non-reducing end indicating that it is typical of a glycoside hydrolase family 31 (GH31) α-glucosidase. Kinetic analyses revealed that the hydrolytic activity of AglA was greatly affected by the chain length of the substrate and the regiospecificity of the glucosidic linkages. The enzyme showed highest specificity for maltose and decreasing values of catalytic efficiency ($k_{cat}/K_m$) toward higher maltooligosaccahrides, although these still serve as substrates. The inhibition profile of AglA toward aesculin was revealed to be a mixed type of noncompetitive inhibition with a $K_i$ value of 4.30 μM and $K_i'$ of 12.5 μM, whereas that toward acarbose showed a competitive inhibition pattern with a $K_i$ of 2.99 μM. Structural analyses of two arbutin transglucosylation products using NMR indicated that the glucose unit of maltose was transferred to the C-3 and C-6 position in the glucose moiety of arbutin.



## 1. Introduction

α-Glucosidases (EC 3.2.1.20) are a widespread group of enzymes that catalyze the hydrolysis of the α-glucosidic bond from the non-reducing end of a chain as well as the α-glucosidic bond of free disaccharides [1,2]. With a high concentration of substrate, the enzyme also displays a transferring reaction (transglucosylation) to yield maltooligosaccharides [3,4]. Commercially available oligosaccharides, comprising an isomaltosyl or nigerosyl structure, are produced by α-glucosidase-catalyzed transferring reactions [5,6]. The application of these enzymes in the biosynthesis of bioactive compounds using the transglucosylation activity is rapidly attracting considerable interest due to the advantages of the specificity, efficiency, and safety of enzymatic reaction [5,7].

α-Glucosidases found in various organisms show diverse substrate specificities. Glycoside hydrolase family 13 (GH13) enzymes show considerably more activity toward heterogeneous substrates than to homogeneous substrates (i.e. maltooligosaccharides), whereas GH31 enzymes hydrolyze homogeneous substrates more rapidly than heterogeneous substrates. In prokaryotes, GH31 enzymes play an important role in nutrient uptake and utilization [8]. Although a number of α-glucosidases belonging to the GH31 family have been isolated from bacteria, relatively few are known from the archaea. Due to their inherent thermostability, hyperthermophilic α-glucosidases isolated from archaea have great potential for the biosynthesis of bioactive glycosides. These hyperthermophilic α-glucosidases can catalyze reactions at high temperatures with higher substrate concentrations, lower viscosity, fewer risks of microbial contamination, and often higher reaction rates [9,10]. The previously characterized archaeal representatives are the enzymes from *Sulfolobus solfataricus* [11,12],

* Corresponding author at: Department of Microbiology, College of Natural Sciences, Pusan National University, San 30, Jangjeon-dong, Geumjeong-gu, Busan 609-735, Republic of Korea. Tel.: +82 51 510 2196; fax: +82 51 514 1778.
*E-mail address:* jhcha@pusan.ac.kr (J. Cha).

1381-1177/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2011.07.006

*Pyrococcus furiosus* [13], *Thermococcus* sp. [14], and *Picrophilus torridus* [15]. Among these, only *S. solfataricus* and *P. torridus* α-glucosidases have been produced recombinantly [16–18]. There has been a constant effort to discover a functional, thermostable α-glucosidase with industrial potential. The archaeal genome was scanned for genes encoding GH31 α-glucosidase and a putative α-glucosidase gene (*aglA*) from *Thermoplasma acidophilum* DSM 1728 was chosen. This *aglA* gene was cloned and expressed to study the catalytic properties of the gene product. Herein, we show that the putative α-glucosidase of *T. acidophilum* is indeed a thermo- and acidophilic α-glucosidase that has specific α-1,3 and α-1,6-transferring activities as well as hydrolytic activity.

## 2. Materials and methods

### 2.1. Cloning and expression of the gene encoding AglA

Genomic DNA of *T. acidophilum* DSM 1728 was obtained from Prof. Sang-Hyeon Lee (Silla University, Busan, Korea). Cloning of the *T. acidophilum* ORF Ta0298 was accomplished by amplifying the genomic region surrounding the *aglA* gene with primers TA-NdeI-FP (5′-CATATGCTTGACGATATCCGAGGTTTTATGAAT-3′) and TA-XhoI-RP (5′-CTCGAGCTTCAACCTTATTATGCCATCG-3′) using the genomic DNA as the template. The 2270-bp product was cloned into a pGEM-T vector using a T-vector cloning kit (Promega). The vector obtained (pGEM-*aglA*) was digested with *Nde*I and *Xho*I and ligated with pET29b (Novagen) that had been digested with the same restriction enzymes. After sequence analysis, one clone containing no PCR errors was selected and designated pET-TAAGL, and this was used for the production of a six histidine-tagged target enzyme.

### 2.2. Purification of the recombinant AglA

The recombinant *Escherichia coli* BL21(DE3) carrying pET-TAAGL was cultured in Luria-Bertani broth [1% (w/v) Bacto-tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl] supplemented with kanamycin (30 μg/mL) overnight at 37 °C, and 1 mL of cells was transferred to 1 L fresh medium and cultured at 30 °C until an $OD_{600nm}$ of between 0.6 and 0.8 was reached. At this point, protein expression was induced by the addition of 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). After 6 h of induction, the cells were harvested by centrifugation (10,000 × *g*, 20 min, 4 °C) and resuspended in 20 mM sodium phosphate buffer (pH 7.0). Cells were disrupted by a two-fold passage through a French pressure cell (American Instruments, Silver Spring, MD). The crude cell extract was centrifuged at 12,000 × *g* (20 min, 4 °C) to remove cell debris. The supernatant was then incubated at 70 °C for 30 min to denature thermolabile host proteins and centrifuged again at 12,000 × *g* (20 min, 4 °C) to remove denatured proteins from the extract. The resulting supernatant was dialyzed against 20 mM sodium phosphate buffer (pH 7.0) and 0.5 M NaCl at 4 °C overnight and subjected to nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography (Qiagen, Hilden, Germany) using the same equilibration buffer. The bound proteins were eluted out using 100 mM imidazole in the same buffer at a flow rate of 1.0 mL/min. The protein solution was concentrated using a Centricon-10 filter from Amicon (Millipore, Bedford, MA) and dialyzed using Spectra/Pormolecular porous membrane tubing (Spectrum Laboratories, Rancho Dominguez, USA) against 20 mM sodium phosphate buffer (pH 7.0). The molecular mass of the native enzyme was estimated by gel filtration chromatography using a Sephacryl S-300 HR 16/60 column equilibrated with 20 mM sodium phosphate (pH 7.0), 150 mM NaCl. The purity of the recombinant AglA was checked by SDS-PAGE using a 12% (w/v) gel. The protein concentration was determined according to the Bradford method, using bovine serum albumin as a standard [19]. An extinction coefficient at 280 nm of 136,140 $M^{-1}$ $cm^{-1}$ was determined for the purified enzyme. Determination of the subsequent protein concentration was based on the absorbance at 280 nm.

### 2.3. Enzyme assay

The enzyme activity was determined using *p*-nitrophenyl-α-D-glucopyranoside (*p*NPG; Sigmal–Aldrich). The reaction mixture, containing 0.5 mM *p*NPG, 25 mM sodium acetate buffer (pH 5.5), and enzyme in a total volume of 200 μL, was incubated at 80 °C for 5 min. The enzymatic reaction was terminated by the addition 100 μL of 1 M $Na_2CO_3$, and the *p*-nitrophenol liberated was measured at 415 nm. The influence of pH on the activity of AglA was measured at 80 °C in sodium citrate (pH 4.0–4.5), sodium acetate (pH 4.5–6.0), MES (pH 5.5–7.0), and HEPES (pH 6.5–8.5) buffers. The pH of each buffer system was adjusted at 80 °C using the standard enzyme assay conditions described above. The influence of temperature on the activity of AglA was determined at a constant pH of 5.5 using 25 mM sodium acetate buffer (pH 5.5), and temperatures ranging from 40 to 100 °C were studied. The thermostability of the enzyme was analyzed by incubating the enzyme (1 μg) at 75, 80, 85, or 90 °C. Aliquots were taken at hourly interval and placed immediately in an ice-water bath to halt the enzymatic activity. The residual activities of the aliquots were measured with 0.5 mM *p*NPG under standard conditions. One unit of the enzyme activity was defined as the amount of enzyme that releases 1 μmol of *p*-nitrophenol per minute.

The effects of various metal ions and chemical reagents on AglA activity were examined after preincubation of the purified enzyme with metal ions or other reagents at 1 mM concentration at 80 °C for 5 min. The activity assayed in the absence of metal ions or reagents was taken to be 100%. The activity with natural substrates were analyzed by thin-layer chromatography (TLC) on Whatman K6F silica gel plates (Whatman, Maidstone, UK) using 1-butanol/ethanol/$H_2O$ (5:5:3, v/v/v) as the mobile phase as described previously [20].

The enzyme activity with natural substrates was measured quantitatively by determining the amounts of glucose released after enzyme treatment. The reaction mixtures (100 μL) containing 10 mM substrate were incubated with 0.5 μg of purified enzyme for 5 min. The reaction was stopped by the addition of 200 μL of 12 N $H_2SO_4$, and 200 μL of glucose oxidase assay reagent (Sigma) was added to the stopped reaction. Aliquots of 200 μL that were transferred to a 96-well plate were developed for 30 min, and absorbance was measured at 490 nm to determine the amount of glucose produced by enzyme activity in the reaction. One unit of the enzyme activity was defined as the amount of the enzyme that hydrolyzes the substrate to release 1 μmol of the respective product per minute. All reactions were performed in triplicate.

### 2.4. Kinetic parameters

The kinetic parameters of recombinant AglA were determined using the glucose oxidase assay to follow the production of glucose upon the addition of enzyme (0.15 μg) at increasing concentrations (0.1–4.0 mM) of substrates, such as maltose, maltotriose, maltotetraose, isomaltose, kojibiose, panose, and *p*NPG, with a reaction time of 6 min. Reactions were linear within this time frame. The program Sigmaplot was used to fit the data to the Michaelis–Menten equation and estimate the kinetic parameters, $K_m$ and $V_{max}$, of the enzyme. All experiments were performed in triplicate. $K_i$ values for aesculin and acarbose were determined by measuring the rate of maltose hydrolysis by AglA at varying inhibitor concentrations (1–20 μM). Inhibition types for the inhibitors were determined by Lineweaver-Burk plot.