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

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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],

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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.

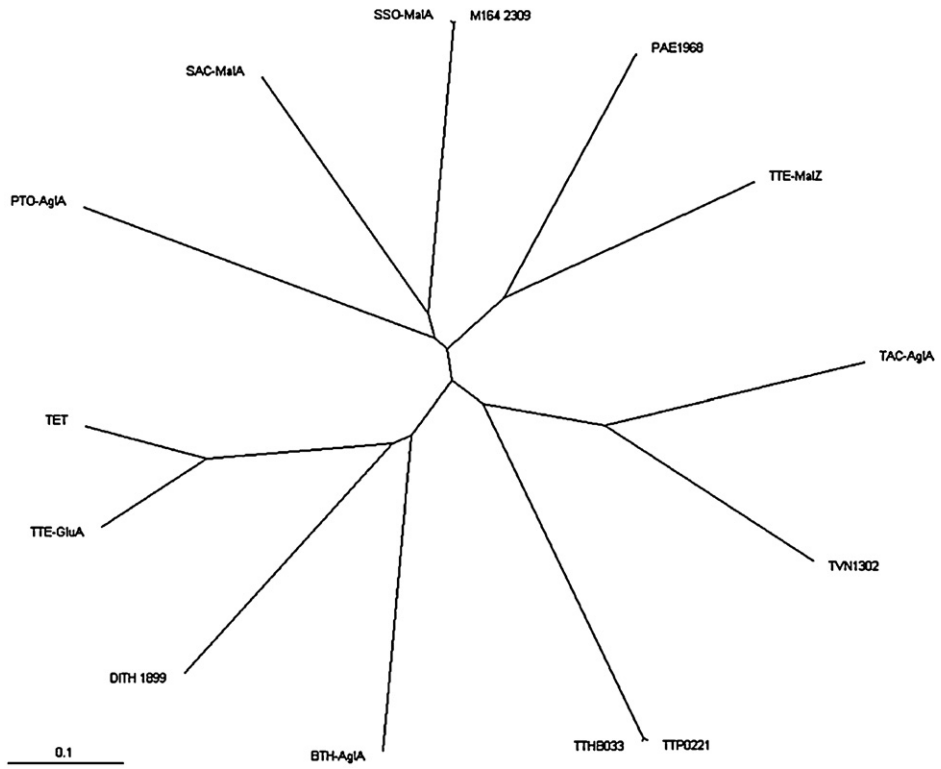

Fig. 1. Phylogenetic tree of selected members of glycoside hydrolase family 31. Phylip format tree outputs from the CLUSTAL X analysis were visualized with TreeViewPPC based on the distance matrix using the neighbor-joining method. The bar at the lower left corner indicates the substitution rate (substitution/site). The following abbreviations are used (accession numbers are in parentheses): TAC-AglA, *T. acidophilum* AglA (CAC11443); TVN1302, *Thermoplasma volcanium* GSS1 TVN1302 (BAB60467); SSO-MalA, *S. solfataricus* P2 MalA (AAK43151); SAC-MalA, *S. acidophilus* MalA (AAY80507); M164_2309, *Sulfolobus islandicus* M.16.4 α-glucosidase (ACR42908); PTO-AglA, *Picrophilus torridus* AglA (AAT42677); PAE1968, *Pyrobaculum aerophilus* str. IM2, PAE1968 (AAL63848); TTE-MalZ, *Thermoproteus tenax* MalZ (CAF18491); TTE-GluA, *Thermoanaerobacter tengcongensis* MB4 GluA (AAM23323); TET, *Thermoanaerobacter ethanolicus* α-glucosidase (ABR26230); DITH_1899, *Dictyoglomus thermophilum* α-glucosidase; BTH-AglA, *Bacillus thermoamyloliquefaciens* AglA (BAA76396); TTHB033, *Thermus thermophilus* HB8 α-glucosidase; and TTP0221 (BAD71829), *Thermus thermophilus* HB27 α-glucosidase (AAS82549).

2.5. Transglucosylation reaction and purification of arbutin derivatives

The transglycosylation activity of AglA was investigated using various glycosides. The reaction (100 μL) was carried out at 80 °C for 3 h with 25 mM maltose as donor and 25 mM arbutin, salicin, or polydatin as an acceptor, 1.5 μg enzyme in 25 mM sodium acetate buffer (pH 5.5). The reaction products acquired by the transglucosylation reaction were collected, freeze-dried, and then concentrated. These products were analyzed by TLC and arbutin transfer products were purified by a recycling preparative HPLC (JAI Korea, Seoul, Korea) as described previously [21].

2.6. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

The mass spectrum was acquired using a Voyager DE-STR matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer (Applied Biosystems, Foster City, CA) as described previously [22].

2.7. Nuclear magnetic resonance analysis

Approximately 5 mg of each purified compound was dissolved in 0.5 mL of deuterium oxide (D_2O) and all NMR experiments were performed using a Varian VNMRS 500 spectrometer (Varian, Palo Alto, CA) at 23 °C. Chemical shifts of proton signals were referenced to a peak due to H_2O at 4.75 ppm and those of carbon signals to the TSP internal standard. Complete spectral assignments of each compound were given on the basis of a combination of 1D and 2D NMR spectra (COSY, TOCSY, HSQC, and HMBC).

3. Results

3.1. Phylogenetic analysis of T. acidophilum AglA

The gene (*aglA*; Ta0298) corresponding to a putative α-glucosidase from *T. acidophilum* was amplified by PCR and the entire ORF was cloned and sequenced. The ORF encoded a protein comprising 749 amino acids with a predicted molecular mass of 85.9 kDa. No detectable signal peptide sequences were found in AglA as revealed by searches with the SignalP 3.0 program [23]. AglA was classified as a member of GH31 based on the conserved active site pattern [GF]-[LIVMF]-W-[HL]-D-M-[NSA]-E-P, which is characteristic of this family [Carbohydrate Active Enzymes database (http://www.cazy.org)]. In archaea, only two enzymes, *S. solfataricus* MalA and *P. torridus* AglA, belonging to this family have been characterized [17,18] *T. acidophilum* AglA showed 35% and 36% identity with *S. solfataricus* MalA and *P. torridus* AglA, respectively. Fourteen thermostable GH31 α-glucosidase orthologs were compared by phylogenetic analysis. The results revealed that *T. acidophilum* AglA is more similar to orthologs from members of the bacterial genus *Thermus* than to sequences from members of the phylogenetically closely related archaeal genera *Picrophilus*, *Sulfolobus*, *Pyrobaculum*, and *Thermoproteus* (Fig. 1).

Table 1
Purification step of recombinant AglA.

	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Fold
Cell-free extract	196	961,000	4900	100	1.0
Heat treatment	15.7	683,000	43,500	71	8.9
Ni-NTA affinity	2.25	101,000	45,000	11	9.2

3.2. Purification and enzyme properties of AglA

The recombinant AglA was expressed in *E. coli* BL21(DE3) cells. The enzyme was purified nine-fold by heat treatment of the crude cell-free extract for 30 min at 70 °C, followed by Ni-NTA affinity chromatography (Table 1). The apparent size of the purified recombinant AglA enzyme estimated by SDS-PAGE was approximately 82 kDa, which is in good agreement with the size calculated from the primary structure (Fig. 2A). The molecular mass of AglA was determined to be about 409 kDa by gel filtration chromatography, revealed that the AglA is a pentamer in its active state (Fig. 2B). The pI value of the recombinant AglA was estimated to 5.29 by Expacy Protparam tool program (http://ca.expasy.org/tools/protparam.html).

The pH range at which AglA was active was determined using *p*NPG as the substrate. AglA was most active at pHs ranging from 5.0 to 6.0, and the optimum pH was found to be 5.5 (Fig. 3A). From the fact that the observed optimum pH range is close to the neutral region, it is evident that AglA is an intracellular enzyme. High enzyme activities (>60%) were still maintained until pH 4.0, whereas the activity decreased sharply at pHs greater than 7.5. The optimal temperature for AglA activity was 80 °C, and relatively low activities were observed below 50 °C and above 95 °C (Fig. 3B). The enzyme exhibited remarkable thermostability, retaining its full activity after 16.8 h incubation at 80 °C (Fig. 3C). The half-life of AglA was determined to be 1.4 h at 85 °C and 12 min at 90 °C. This result implies that AglA was successfully expressed in *E. coli*, and its thermostability preserved. The effects of metal ions and different reagents on enzyme activity were examined (Table 2). Mn^{2+}, as chloride salts at a concentration of 1 mM, slightly enhanced the enzyme activity; however, Cu^{2+} and Zn^{2+} reduced the activity to 34.9 and 28% of control levels, respectively, while Hg^{2+} and *N*-bromosuccinimide strongly inhibited the enzyme. Additionally, the activity of enzyme was completely inactivated by SDS.

Table 2
Effects of metal ions and reagents on the activity of recombinant AglA.

Reagents	Concentration (mM)	Relative activity (%)
None		100
EDTA	1	93 ± 3.3
$MgCl_2$	1	104 ± 0.5
$CaCl_2$	1	103 ± 2.4
$ZnCl_2$	1	28 ± 2.6
$NiCl_2$	1	84 ± 3
$CoCl_2$	1	105 ± 1.9
$MnCl_2$	1	159 ± 0.4
$CuCl_2$	1	35 ± 2.9
$HgCl_2$	1	4.2 ± 0.4
Dithiothreitol	1	107 ± 0.7
2-Mercaptoethanol	1	108 ± 2.7
SDS	1	ND[a]
N-Bromosuccinimide	1	7.8 ± 1.9
p-Chloromercuribenzoic acid	1	95 ± 0.7
Dimethylaminopropyl ethylcarbodiimide	1	107 ± 0.3

The enzyme was preincubated with the above reagents at 80 °C for 5 min. Aliquots were tested for AglA activity by incubating the samples with 0.5 mM *p*NPG in 25 mM sodium acetate buffer (pH 5.5) at 80 °C for 5 min. The metal ions and other reagents were added at a final concentration of 1 mM except for SDS (1%). The relative values shown are the percentages of the activity without additives.
[a] ND, not detected.

The substrate specificity of the enzyme was examined with various types of α-glucans, including di- and oligomaltosaccharides. Broad specificity was observed for the linkages of the glycosidic

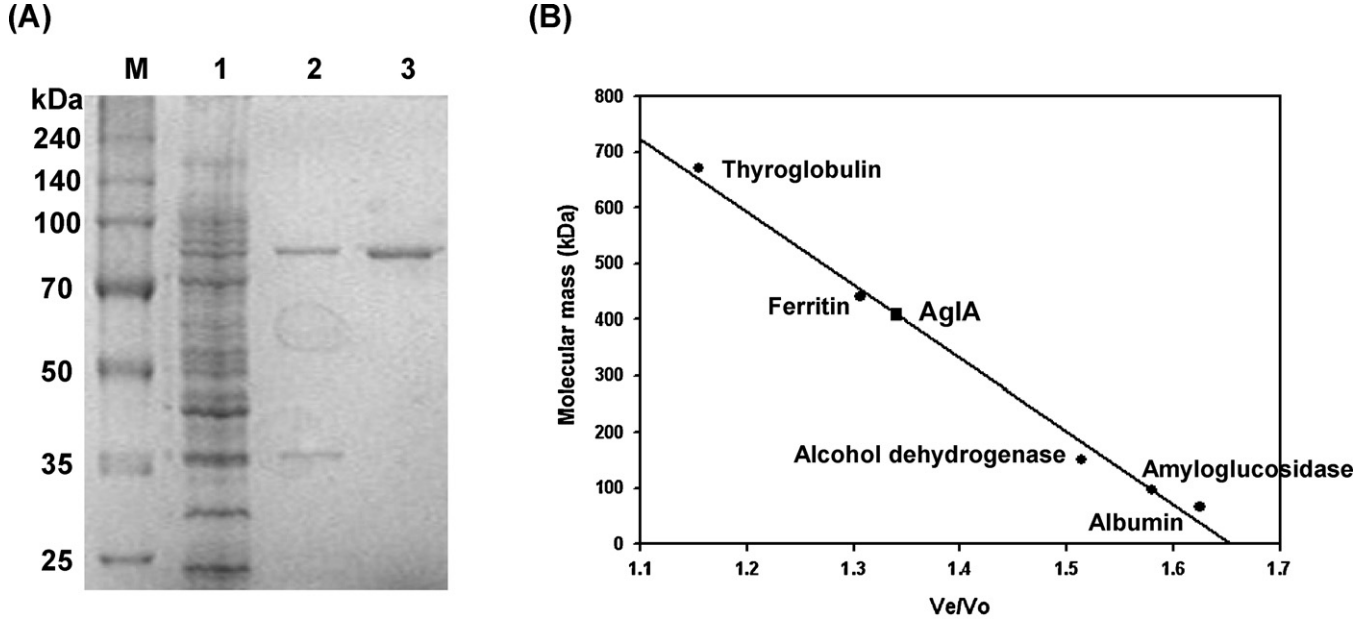

Fig. 2. SDS-PAGE (A) and molecular mass determination (B) of AglA. Lane M, protein size standards; lane 1, cell-free extracts of recombinant AglA; lane 2, proteins after heat treatment; lane 3, proteins after Ni-NTA affinity chromatography. Molecular mass of the native enzyme was estimated by gel filtration chromatography using Sephacryl S-300 HR 16/60 column. The column was calibrated with molecular mass standards: thyroglobulin (670 kDa), ferritin (440 kDa), alcohol dehydrogenase (150 kDa), amyloglucosidase (97 kDa), and albumin (66 kDa).

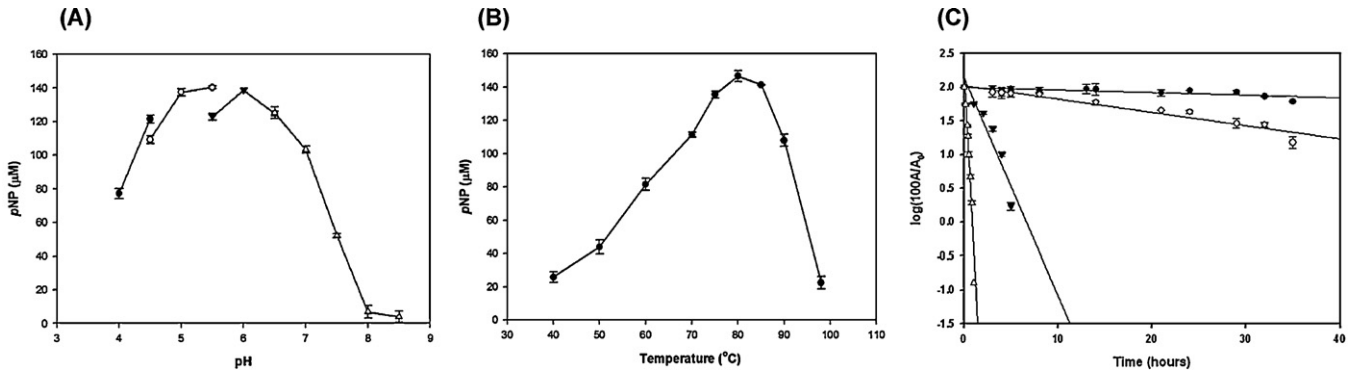

Fig. 3. Effects of pH and temperature on the activity and stability of AglA. (A) The activity was determined at 80 °C for 5 min with *p*NPG in various 25 mM buffer systems: for pH 4.0–4.5, sodium citrate (●); for pH 4.5–5.5, sodium acetate (○); for pH 5.5–6.5, MES (▼); for pH 6.5–8.5, HEPES (△). (B) The effect of temperature on enzyme activity was determined at temperatures ranging from 40 °C to 98 °C at the optimum pH of 5.5. (C) To determine the thermostability of AglA, the enzyme was heated at 75 °C (●), 80 °C (○), 85 °C (▼), and 90 °C (△) in 25 mM sodium acetate buffer (pH 5.5). After various time intervals, samples were withdrawn and the residual activity was measured at 80 °C for 5 min with *p*NPG. Error bars represent the standard deviation from three separate experiments.

Table 3
Substrate specificity of recombinant AglA.

Substrate	Glycosidic linkage	Glucose released (μmol/min μg protein)[a]
Trehalose	α-D-Glc-(1 → 1)-D-Glc	ND[c]
Kojibiose	α-D-Glc-(1 → 2)-D-Glc	445[b]
Nigerose	α-D-Glc-(1 → 3)-D-Glc	233
Maltose	α-D-Glc-(1 → 4)-D-Glc	582
Isomaltose	α-D-Glc-(1 → 6)-D-Glc	153
Cellobiose	β-D-Glc-(1 → 4)-D-Glc	ND
Panose	α-D-Glc-(1 → 6)-α-D-Glc-(1 → 4)-D-Glc	136
Isomaltotriose	α-D-Glc-(1 → 6)-α-D-Glc-(1 → 6)-D-Glc	4[b]
Maltotriose	α-D-Glc-(1 → 4)-α-D-Glc-(1 → 4)-D-Glc	378
Maltotetraose	α-D-Glc-(1 → 4)-α-D-Glc-(1 → 4)-α-D-Glc-(1 → 4)-D-Glc	317
Maltopentaose	α-D-Glc-(1 → 4)-α-D-Glc-(1 → 4)-α-D-Glc-(1 → 4)-α-D-Glc-(1 → 4)-D-Glc	287

[a] Activity was determined with 0.5 μg of pure enzyme and 10 mM of each substrate at 80 °C for 5 min, except where indicated otherwise.
[b] Activity was determined with 0.5 μg of pure enzyme and 1 mM substrate at 80 °C for 5 min.
[c] ND, not detected.

bond. AglA showed the highest activity with maltose (α-1,4 bond), though it also hydrolyzed kojibiose (α-1,2), nigerose (α-1,3), and isomaltose (α-1,6) with lower efficiencies (Table 3). In contrast, cellobiose, which has β-linked glycosidic bond, was not hydrolyzed by AglA. From the kinetic results of AglA with maltooligosaccharides, the rate of maltooligosaccharide hydrolysis decreased with an increasing number of glucose units in the substrate (Table 4). The decreased hydrolytic activities of AglA toward longer maltooligosaccharides were due to the lower k_{cat} values than that of maltose. The observed enzymatic activity with maltotetraose was approximately half of that with maltose. The low catalytic efficiency of AglA against isomaltose, panose, and *p*NPG, which have α-1,6 glycosidic linkages, was due to the high K_m and low k_{cat} values.

TLC analyses of the reaction products of AglA with oligosaccharides showed that glucose was initially released when maltooligosaccharides with different chain lengths were used as substrates and that the hydrolysis of panose yielded glucose and maltose, implying that the enzyme hydrolyzes the maltooligosaccharides in an exo-acting manner from the non-reducing end (Fig. 4). These hydrolysis patterns indicate that AglA is a typical GH31-type α-glucosidase. The overall characteristics of AglA were summarized in Table 5.

Table 4
Kinetic parameters of recombinant AglA.

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
Maltose	0.65	143	220
Maltotriose	0.47	90.2	194
Maltotetraose	0.67	85.5	128
Isomaltose	4.25	22.9	5.4
Kojibiose	1.24	127	103
Panose	6.60	35.9	5.4
*p*NPG	1.77	59.7	33.7

3.3. Inhibition profile of AglA

The effectiveness of α-glucosidase inhibitors on AglA was tested using maltose as a substrate in the presence of aesculin and acarbose, the known inhibitor. The inhibition constants (K_i) of aesculin and acarbose against AglA were determined using the glucose oxidase assay with maltose as a substrate. Lineweaver-Burk plot for AglA in the presence of aesculin and acarbose, respectively, indicates the type of inhibition produced by acarbose and aesculin. The inhibition constants K_i and K_i' represent the respective dissociation constants of the enzyme-inhibitor complex (EI) and the enzyme-substrate-inhibitor complex (ESI). Aesculin inhibition was demonstrated to be a mixed type of noncompetitive inhibition with a K_i value of 4.30 μM and a K_i' of 12.5 μM, whereas acarbose showed a competitive inhibition pattern with a K_i of 2.99 μM (Table 6).

3.4. Transglucosylation activity of AglA

The transglucosylation activity of AglA was investigated using arbutin, salicin, and polydatin as an acceptor and maltose as a donor, respectively. The reaction was allowed to proceed for 3 h before analysis by TLC. Four distinct transglucosylation products, two with maltose-transferred products (MG1 and MG2) and the other two with acceptor-transferred products (A1 and A2) were

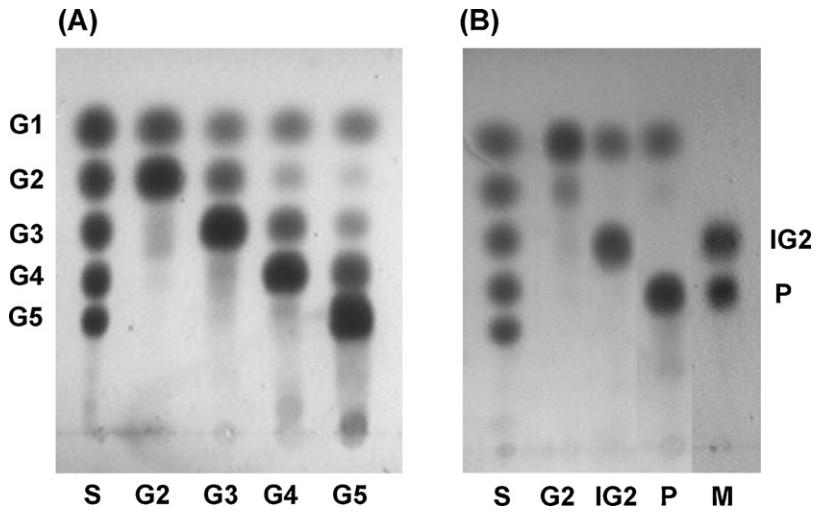

Fig. 4. TLC analysis of the products of AglA hydrolysis. The reaction was carried out at 80 °C for 5 min with maltooligosaccharides (A) and for 30 min with maltose, isomaltose, and panose (B). Lane S, standard markers from G1 (glucose) to G5 (maltopentaose); lane M, IG2 (isomaltose) and P (panose).

Table 5
Characteristics of recombinant AglA.

Substrate	Optimum pH	Optimum temperature (°C)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\ s^{-1}$)	Half-life ($t_{1/2}$)
*p*NPG	5.5	80	1.77	59.7	33.7	1.4 h (85 °C) 12 min (90 °C)

observed (Fig. 5A). In case of arbutin, an arbutin transfer product (AG2) was formed early (within 5 min) and rapidly decreased with an increase in reaction time, whereas AG1 was produced later and retained at 2 h (Fig. 5B). The maximum yields of AG1 and AG2 estimated by densitometry were approximately 30% and 26%, respectively. AG1 and AG2 were purified using recycling preparative HPLC. The isolated AG1 and AG2 appeared as single spots on TLC (Fig. 5C, lanes 17 and 18). Two compounds showed the same molecular ion peaks at *m/z* 457 $[M+Na]^+$ by MALDI-TOF/MS analysis, indicating that the products consisted of a glucose unit and arbutin. This finding was also supported by two characteristic anomeric protons, crowded protons in the range of 3.2–4.0 ppm, and two downfield-shifted doublets in the ^1H NMR spectrum. Furthermore, each anomeric proton in AG1 and AG2 was recognized as α and β positions from the coupling constant values ($J = 7.8$ and 3.7 Hz, respectively). However, the chemical shift of the β-positioned anomeric proton in the two compounds was different. This suggested a difference in the linkage of the glucose unit to the arbutin moiety. At first, all proton and carbon assignments in the two glucose units were made by the COSY, TOCSY, and HSQC experiments to determine the connectivity between the glucose unit and arbutin. In AG1, the β anomeric proton at 4.88 ppm (C-1″) in the glucose unit showed a correlation with the carbon at 65.7 ppm (C-6′) in arbutin in the HMBC spectrum, indicating a α-Glc [1 → 6]-β-Glc linkage. On the other hand, AG2 showed an HMBC correlation of the β anomeric proton at 5.30 ppm (C-1″) in the glucose unit with the carbon at 81.7 ppm (C-3′) in arbutin. This presents the α-Glc [1 → 3]-β-Glc linkage. Accordingly, AG1 and AG2 were established as α-D-glucosyl-(1,6)-arbutin, and α-D-glucosyl-(1,3)-arbutin, respectively (Fig. 5D).

4. Discussion

The purpose of this study was to discover an α-glucosidase that has industrial potential. Extremely thermophilic α-glucosidases, which have strong transglycosylation activity, are ideal for application to the synthesis of functional oligosaccharides, isomaltooligosaccharides and conjugate sugars [24–26]. For the production of isomaltooligosaccharides, α-amylase and β-amylase together with α-glucosidase are required. Thermostable enzymes are advantageous because the liquefaction of starch by α-amylase is considerably faster and gives high yields at higher temperatures. Therefore, thermostable α-glucosidases, isolated from a variety of thermophiles or hyperthermophiles, are potential candidates for improvements in isomaltooligosaccharide production and are a superior replacement for *Aspergillus niger* α-glucosidase, which is used commercially. Thermostable α-glucosidases have previously been characterized from bacteria and archaea [11–15,27,28]; however, the transglucosylation properties of these enzymes are not well defined, particularly in the archaea.

Table 6
Acarbose inhibition pattern of α-glucosidase related enzymes from various sources.

Enzyme	Type of inhibition	K_i[a] (μM)	K'_i[b] (μM)
AglA (archaeal α-glucosidase)	Competitive	2.99 ± 0.02	
Yeast α-glucosidase[c]	Competitive	77.9 ± 11.4	
Rat intestine α-glucosidase[c]	Competitive	0.06 ± 0.006	
Bacillus CGTase[c]	Mixed noncompetitive	2.53 ± 0.30	3.09 ± 0.25
Porcine pancreatic α-amylase[c]	Mixed noncompetitive	0.64 ± 0.20	0.64 ± 0.06

[a] K_i is the inhibition constant, defined as [E][I]/[EI].
[b] K'_i is the inhibition constant, defined as [EI][S]/[ESI].
[c] Data from Ref. [35].

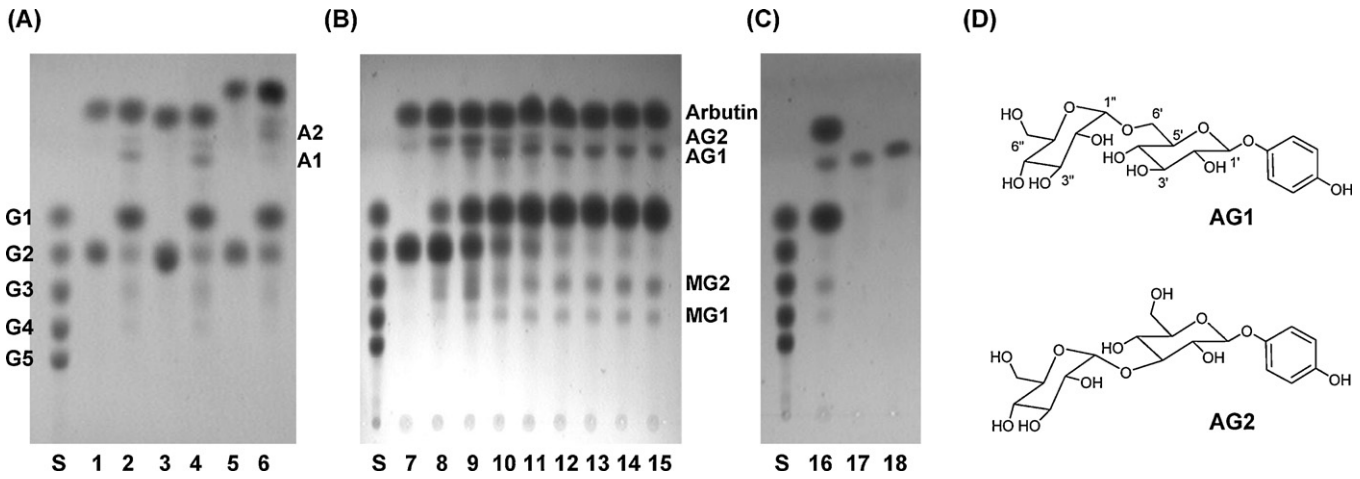

Fig. 5. Identification of the transglucosylation products of AglA with various acceptors. (A) TLC analysis of the arbutin, salicin, and polydatin transglucosylation products. (B) TLC analysis of the arbutin transglucosylation products as a function of time. Lane S, standard markers from G1 (glucose) to G5 (maltopentaose); lane 1, maltose and arbutin; lane 2, reaction products of maltose and arbutin; lane 3, maltose and salicin, lane 4, reaction products of maltose and salicin, lane 5, maltose and polydatin, lane 6, reaction products of maltose and polydatin, lanes 7–15, reaction products at different reaction times (0, 5, 30, 60, 90, 120, 150, 180, and 240 min, respectively). A1, acceptor product 1; A2, acceptor product 2; AG1, arbutin transglucosylation product 1; AG2, arbutin transglucosylation product 2, MG1, maltose transglucosylation product 1, MG2, maltose transglucosylation product 2. (C) The transglucosylation products AG1 and AG2 purified by preparative recycling HPLC. Lane 16, reaction products of the arbutin transglucosylation; lane 17, purified AG1; lane 18, purified AG2. (D) The molecular structures of arbutin transglucosylation products AG1 and AG2.

We cloned and expressed the *T. acidophilum* α-glucosidase in *E. coli*. The size of the *T. acidophilum* α-glucosidase and the multimeric quaternary structure resembles those of other GH31 α-glucosidases [18,29]. The closer relationship of the AglA with bacterial *Thermus* enzymes than phylogenetically related archaeal enzymes based on phylogenetic analysis implies that horizontal gene transfer has occurred between bacteria and archaea. This is consistent with the phylogenetic allocation of the *P. torridus* AglA sequence and supports the hypothesis that there could have been a gene transfer event [18]. The temperature and pH profiles of AglA and its remarkable resistance to thermal inactivation at temperatures up to 85 °C (half-life at this temperature, >1 h) are in agreement with the high optimal growth conditions of *T. acidophilum*.

AglA acts as a typical GH31 α-glucosidase, removing a single D-glucosyl group from the non-reducing end of maltooligosaccharides. The rate of enzyme hydrolysis is affected by substrate molecular size and structure, and also by the next bond in sequence. It preferentially hydrolyzes maltose. The hydrolysis rate increases with a decrease in the chain length of the substrate. AglA has a broad specificity as it is able to attack any glycosidic linkage of disaccharides, but the rate of hydrolysis of α-1,4 linkages is higher than that of other linkages. It is quite distinguishable from crenarchaeal glucoamylases in that the rates of hydrolysis of non-reducing α-1,4 bonds decrease with the increase in the molecular mass of the substrate up to maltopentaose [30]. AglA has higher substrate specificity for smaller molecules such as maltose than for the maltooligosaccharides. The hydrolysis of α-1,6-linked substrates, isomaltose and panose, but not isomaltotriose, was observed and indicates that the enzyme is only able to hydrolyze small α-1,6-linked substrates. The apparent activity against *p*NPG indicates a lack of discrimination for glycosyl moieties in disaccharide substrates.

It has been reported that homology analysis of the complete amino acid sequences divides the α-glucosidases into two groups, namely, family I and family II [31,32]. Family I enzymes show higher activity toward heterogeneous substrates such as sucrose and *p*NPG, and none or less toward homogeneous substrates such as maltooligosaccharides, implying that α-glucosidase I recognizes the "glucosyl structure" in the substrate. In contrast, family II enzymes hydrolyze homogeneous substrates more rapidly than heterogeneous substrates, indicating that this class of α-glucosidases recognizes the "maltosyl structure." It has been reported that family I and II enzymes belong to GH13 and GH31, respectively [33,34]. AglA is much closer to family II enzyme in terms of substrate specificity; however, the amino acid sequence is somewhat distinct from that of both families. The inhibition patterns of α-glucosidase-related enzymes from various sources against acarbose were compared (Table 6). Acarbose showed potent inhibitory action toward α-glucosidases obtained from baker's yeast and rat intestine, CGTase from *Bacillus macerans*, and porcine pancreatic α-amylase, with competitive-type and mixed-type inhibitions, respectively. For rat intestine α-glucosidases, the inhibitory potency of acarbose was found to be highest compared to other enzymes. The inhibitory ability of acarbose toward archaeal α-glucosidase AglA was between that of yeast and rat intestine α-glucosidases. For the yeast enzyme, a large K_i value was obtained for acarbose inhibition compared to that for the intestine enzyme; the ratio being greater than 1000. Since the yeast enzyme belongs to α-glucosidase family I and the intestinal enzyme belongs to α-glucosidase family II, there is a possibility that the two analogues are distinctively recognized by the enzymes of the different families.

The transglycosylation property of AglA was examined using arbutin as an acceptor. AglA successfully synthesized two distinct arbutin derivatives. Identification of the transglucosylation products AG1 and AG2 revealed that AglA catalyzed the regioselective formation of glycosidic bonds through an α-1,6 and α-1,3-linkages. The formation of α-1,6 or α-1,3-linkage is industrially useful for the production of isomaltooligosaccharides and human milk oligosaccharide, such as 3′-sialyllactose, respectively. To the best of our knowledge, the *T. acidophilum* α-glucosidase described here is the first characterized thermostable archaeal member of the GH31 family, which confirms the transglucosylation activity. Therefore, AglA which is able to form an α-1,3-glucosidic linkage as well as α-1,6-glucosidic linkage should be useful for investigating oligosaccharides and their biological functions and for constructing novel glycoconjugates that will be used in therapeutic applications.

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