

## Characterization of *Thermotoga thermarum* DSM 5069 $\alpha$ -Glucuronidase and Synergistic Degradation of Xylan

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$\alpha$ -Glucuronidases are capable of breaking down the  $\alpha$ -1,2-glycosidic bonds of 4-O-methyl-D-glucuronic acid residues. As an accessory enzyme,  $\alpha$ -glucuronidase plays a vital role in xylan degradation. The recombinant  $\alpha$ -glucuronidase from *Thermotoga thermarum* DSM 5069 was heterologously expressed in the *Escherichia coli* system, purified, and characterized. The purified enzyme exhibited optimal activity toward aldouronic acids at pH 6.5 and 80 °C. It was fairly thermostable and maintained 98% residual activity after incubation at 65 °C for 2.0 h. The kinetic parameters  $K_m$ ,  $V_{max}$ , and  $k_{cat}$  were  $3.02 \pm 0.16$  mM,  $88 \pm 2$   $\mu$ mol  $\text{min}^{-1}$   $\text{mg}^{-1}$ , and  $117$   $\text{s}^{-1}$ , respectively. TtAguA had an apparent activation energy of 59.0 kJ/mol. By structure simulation and mutation analyses, Glu<sup>288</sup> was identified as the catalytic proton donor, and Asp<sup>367</sup> and Glu<sup>395</sup> were likely nucleophile bases. The xylan degradation by endoxylanase Xyn10A was enhanced by approximately 10% in the presence of TtAguA.

**Keywords:**  $\alpha$ -Glucuronidase; Degradation; *Thermotoga thermarum*; Xylan

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### INTRODUCTION

Xylan is not only the major heteropolysaccharide present in plant cell walls, but also the second most abundant structural polysaccharide after cellulose in the biosphere. It accounts for up to 35% of the dry weight in wood and agricultural residues. In recent years, much more attention has been paid to xylan degradation and xylose utilization to alleviate global warming, improve environmental quality, and facilitate bioenergy production (Zaldivar *et al.* 2001; Dodd and Cann 2009). Xylan, by definition, is a  $\beta$ -1,4-linked xylose polysaccharide, and great differences with respect to the side groups contribute to the heterogeneity of xylan. The main chain of xylan is often decorated by  $\alpha$ -1,2-linked  $\alpha$ -D-glucuronic acid (GlcA) or 4-O-methyl-glucuronic acid (MeGlcA) and  $\alpha$ -1,3-linked arabinofuranose, ferulate, and acetate (Kolenova *et al.* 2010). Because of its heterogeneity in the composition and structure, effective degradation of xylan polymers to the natural carbon cycle requires a series of glycoside hydrolases and esterases, such as xylanases,  $\beta$ -xylosidases,  $\alpha$ -glucuronidases,  $\alpha$ -L-arabinofuranosidases, feruloyl esterases, and acetylxylan esterases.

Among the xylan-degrading accessory enzymes,  $\alpha$ -glucuronidase hydrolyzes the  $\alpha$ -1,2-glycosidic bond of the GlcA and MeGlcA side chains attached to the xylan backbone. Currently, it is classified into the glycoside hydrolase (GH) families of 67 and 115 by the CAZy (<http://www.cazy.org/>) database according to protein sequence similarity (Cantarel *et al.* 2009). The major difference between GH67 and GH115  $\alpha$ -

glucuronidases is that GH115  $\alpha$ -glucuronidases can remove GlcA or MeGlcA from both the non-reducing end and internal xylose units of xylan; GH67  $\alpha$ -glucuronidases are capable of cleaving the  $\alpha$ -1,2-glycosidic bonds of GlcA and MeGlcA only located at the non-reducing end of glucuronoxylooligosaccharides.

Compared with mesophiles, thermophile-derived enzymes are impressively thermostable and tolerant to unfavorable production conditions such as high salts or solvent concentrations and high operating temperatures. Many efforts have been made to identify thermostable enzymes with attractive properties meeting industrial demands. To date, only three thermophilic  $\alpha$ -glucuronidases, from *Talaromyces emersonii* (Heneghan *et al.* 2007), *Geobacillus stearothermophilus* (formerly *Bacillus stearothermophilus*) (Golan *et al.* 2004), and *Thermotoga maritima* (Suresh *et al.* 2002), have been purified and biochemically characterized. The non-xylanolytic  $\alpha$ -glucuronidase from *T. maritima* is an interesting exception, as it shows hydrolytic activity towards *p*-nitrophenyl  $\alpha$ -D-glucuronopyranoside instead of towards 4-O-methyl-D-glucuronoxylan or its fragment oligosaccharides. Moreover, most studies on thermophilic  $\alpha$ -glucuronidases have been performed in the thermophilic species *B. stearothermophilus* because of its available crystal structure and biochemical information. *T. thermosaccharolyticum* DSM 5069 is a Gram-negative, thermophilic, and obligate anaerobic bacterium. It grows at temperatures between 55 and 84 °C, and the pH range for its growth is 5.5 to 9.0. Even though its genomic information is now accessible in the Genbank database, the putative  $\alpha$ -glucuronidase from *T. thermosaccharolyticum* DSM 5069 has not yet been biochemically characterized.

In the present work, an examination of a putative  $\alpha$ -glucuronidase from *T. thermosaccharolyticum* DSM 5069 (TtAguA) was conducted, and its cloning, expression, biochemical characterization, and site-directed mutation are described. The synergistic hydrolysis of 4-O-methyl-D-glucurono-D-xylan was performed with the endoxylanase (Xyn10A) from the same species. This study will enable exploitation of the biocatalyst for development of biofuel production.

## EXPERIMENTAL

### Chemicals and Enzymes

Reduced aldouronic acids mixture (di: tri: tetra: penta = 10: 60: 20: 10), debranched arabinan, 1,4- $\beta$ -D-mannan, and galactan were purchased from Megazyme (Wicklow, Ireland). 4-O-Methyl-D-glucurono-D-xylan, *p*-nitrophenyl- $\alpha$ -L-arabinofuranoside (*p*NPAF), *p*-nitrophenyl- $\beta$ -D-xylopyranoside (*p*NPX), sodium arsenate dibasic heptahydrate, xylose, xylobiose, xylotriose, xylotetraose, xylopentaose, and xylohexaose were obtained from Sigma Chemical Co., Ltd. (St. Louis, MO). D-glucuronic acid was purchased from Solarbio (Beijing, China). Phusion<sup>®</sup> high-fidelity DNA polymerase and DNA restriction enzymes were purchased from New England BioLabs (Ipswich, MA). All other chemicals were of the highest purity commercially available.

### Bacterial Strains and Growth Conditions

The genomic DNA of *T. thermarum* DSM 5069 was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ). *E. coli* TOP10 strain was used as a general host for plasmid propagation and cloning, and *E. coli* BL21 (DE3) was

employed as a recipient strain in transformation experiments. *E. coli* strains were cultured in Luria-Bertani (LB) medium supplemented with kanamycin ( $100 \mu\text{g mL}^{-1}$ ) when required for plasmid maintenance.

### Construction of Plasmids

DNA manipulation was performed following the standard procedure. A pair of specific primers (*aguAfd*, *aguArv*) for amplifying *aguA* were designed based on the reference genome sequence (Accession No.: CP002351). The *aguA* gene was amplified by polymerase chain reaction (PCR) using the genomic DNA as a template. The PCR mixture was subjected to a 1% (w/v) agarose gel to check the integrity and yield of PCR product. The PCR fragment was digested with *SacI* and *XhoI*, then inserted into the vector pET-28a in frame with the C-terminal hexahistidine ( $\text{His}_6$ ) tag sequence. The ligation mixture was transformed into *E. coli* TOP10 chemically competent cells by heat shock. The ligation transformants were screened by antibiotic kanamycin ( $100 \mu\text{g mL}^{-1}$ ). The authenticity of positive clones was verified by DNA sequencing (Springen Biotechnology, Nanjing, China). Non-essential nucleotide sequences located upstream of the *SacI* site on plasmid pET-28a were completely removed by inverse PCR with the specific oligonucleotide primers (*delfd*, *delrv*). The single mutation of substituting glutamic acid with serine at position 288 was also conducted by inverse PCR using 5' mutant-specific primers (*E288Sfd*, *E288Srv*). All related primers are listed in Table 1.

**Table 1.** Nucleotide Sequences of Designed Primers

Primer	Sequence (5'-3')	Restriction site
<i>aguAfd</i>	CGAGCTCGATGATGCCAAACGGTTATGA	<i>SacI</i>
<i>aguArv</i>	CCGCTCGAGTGGATAAATTTTGGACCTT	<i>XhoI</i>
<i>delfd</i>	ATGATGCCAAACGGTTATGAAATG	
<i>delrv</i>	TGGTATATCTCCTTCTTAAAGTTAAAC	
<i>E288Sfd</i>	TCTTTCAACCCCGGACCG	
<i>E288Srv</i>	GGAATCAGCTTTTATGAGAAATC	

### Expression and Purification of TtAguA

Heterologous expression of *aguA* in *E. coli* was carried out under the regulation of the T7 polymerase system. *E. coli* BL21 (DE3) cells harboring vector pET-28a-*aguA* were cultured in 50 mL of LB medium with  $100 \mu\text{g mL}^{-1}$  kanamycin at  $37^\circ\text{C}$  (200 rpm) until the culture reached the stationary phase ( $\text{OD}_{600} = 0.6$  to  $0.8$ ). Protein expression was induced by treatment with  $0.5 \text{ mM}$  of isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG); the culture was shifted to  $25^\circ\text{C}$  and continuously incubated for an additional 3 h. The cultured cells were harvested by centrifugation at  $4^\circ\text{C}$  ( $5,000 \text{ g}$ , 5 min). The cell pellets were washed twice with ice-cold water to remove residual medium and were suspended in 5 mL of binding buffer ( $5 \text{ mM}$  imidazole,  $0.5 \text{ M}$  NaCl, and  $20 \text{ mM}$  Tris-HCl buffer, pH 7.9). The cells were disrupted by sonication on ice. The crude extract was heat-treated ( $60^\circ\text{C}$ , 30 min) and clarified by centrifugation ( $10,000 \text{ g}$ , 20 min) to remove cell debris and precipitates. The supernatant liquid was filtered through a  $0.22\text{-}\mu\text{m}$  filter and applied to a nickel affinity column (Novagen, USA) equilibrated with  $50 \text{ mM}$  nickel sulfate. The recombinant enzyme was eluted in stepwise manner using elution buffer solutions containing imidazole ( $100 \text{ mM}$ ,  $200 \text{ mM}$ ,  $400 \text{ mM}$ , and  $1000 \text{ mM}$ ). The molecular weight

and homogeneity of the target protein were estimated by sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) using a prestained protein molecular weight ladder (Sangon, Shanghai, China) as a marker. Protein concentration was measured by Bradford kit supplied with bovine serum albumin as a standard (Bio-Rad, USA). All protein purification procedures were performed at 4 °C.

### Biochemical Characterization

$\alpha$ -Glucuronidase activity was assayed in triplicate by measuring the amount of GlcA liberated from aldouronic acids according to the colorimetric method (Milner and Avigad 1967). One unit was defined as the amount of enzyme releasing 1  $\mu$ mol of GlcA per min. Standard calibration curve was plotted against the concentration of GlcA under the tested assay conditions. Activity assay was performed in an aqueous mixture (200  $\mu$ L) containing 100 mM imidazole buffer, 0.475 mg L<sup>-1</sup> aldouronic acids, and the appropriate amount of diluted recombinant  $\alpha$ -glucuronidase. The reaction mixture was incubated at 80 °C for 5 min, chilled in ice water, followed by the addition of 0.8 mL of copper reagent (1.97 M Na<sub>2</sub>SO<sub>4</sub>, 0.68 M NaCl, 0.20 M sodium acetate, 20.8 mM CuSO<sub>4</sub>, pH 6.0) to stop the reaction. The reaction mixture was subsequently incubated in a boiling water bath for 20 min, chilled on ice. Finally, 0.5 mL of arsenomolybdate reagent (19.2 mM sodium arsenate dibasic heptahydrate, 40.5 mM ammonium molybdate tetrahydrate, and 788.0 mM sulfuric acid) was added to develop the color. The absorbance shift at 750 nm after 5 min was monitored, and the mixture was prepared without enzyme as a control.

The optimal pH of TtAugA was determined at 80 °C in three different buffers (100 mM) at various pH values: sodium acetate buffer (pH 4.5 to 6.0), imidazole-HCl buffer (pH 6.0 to 7.5), and Tris-HCl buffer (pH 7.5 to 8.5). The optimal temperature was determined in imidazole-HCl (100 mM, pH 6.5) at temperatures ranging from 65 to 90 °C. The desired pH values were adjusted according to temperature.

To estimate its thermostability, TtAugA was pre-incubated at various temperatures (65 to 80 °C) without the addition of substrate. Samples were collected every 30 min and quickly placed on ice for 10 min before conducting the activity assay. The total time of the enzyme's thermostability assay was 2.0 h. The thermostability of the enzyme was evaluated by measuring the residual activity; the relative activity of unincubated TtAugA was set as 100%.

The effects of various additives on enzymatic activity were examined at the optimal temperature and pH in the presence of 1.0 mM of the test compounds: salts (BaCl<sub>2</sub>, CaCl<sub>2</sub>, CoCl<sub>2</sub>, CuSO<sub>4</sub>, Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub>, MgCl<sub>2</sub>, MnCl<sub>2</sub>, NiSO<sub>4</sub>, and ZnSO<sub>4</sub>), the chelator ethylenediaminetetraacetic acid (EDTA), and surfactants (SDS and Triton X-100). Enzyme activity in the absence of added chemical reagents was defined as 100%.

### Activation Energy and Kinetic Parameters Determination

Activation energy was measured in imidazole-HCl buffer (100 mM, pH 6.5) at temperatures from 40 to 80 °C. The Arrhenius curve was plotted as the relative activity versus temperature (K), and logarithmic transformation was conducted to calculate activation energy.

The apparent Michaelis-Menten constants were measured with various final concentrations of aldouronic acids (0 to 10.0 mM) at 80 °C. All apparent kinetic parameters of TtAugA were calculated by fitting the plots to the Michaelis-Menten equation.

## Synergistic Degradation

Synergistic degradation of xylan was studied in the presence of TtAugA and endoxylanase (Xyn10A) from *T. thermarum* DSM 5069. 4-O-Methyl-D-glucurono-D-xylan ( $4.0 \text{ g L}^{-1}$ ) was prepared as substrate in the imidazole-HCl buffer (100 mM, pH 6.5) at  $80 \text{ }^\circ\text{C}$ . TtAugA and Xyn10A were prepared separately, and recombinant Xyn10A was heterologously expressed and purified as previously described (Shi *et al.* 2013). The specific activities of the purified TtAugA and Xyn10A were determined, 57.5 U/mg and 148 U/mg, respectively. Synergistic degradation was initiated by adding TtAugA (0.132 mg) and Xyn10A (0.0185 mg) into 2.0 mL of substrate solution. As a control, the reaction mixture in the absence of TtAugA was used to estimate the degradation efficiency of synergistic action. Degradation mixtures were taken out at given time points and centrifuged ( $13,000 \text{ g}$ ; 5 min). The degradation supernatants were collected and analyzed by high-performance anion exchange chromatograph (HPAEC; Dionex ICS-3000) equipped with a pulsed amperometric detector (PAD) and an analytical column of CarboPacTMPA200 (Dionex Corp., Sunnyvale, CA, USA). Separation was achieved using a linear gradient of sodium acetate from 0 to 120 mM at a flow rate of 0.3 mL per min.

## Bioinformatics Analysis

The translated protein from the cloned sequence was subjected to SignalP 4.0 (<http://www.cbs.dtu.dk/services/SignalP/>) for signal peptide recognition (Petersen *et al.* 2011). All related protein sequences were retrieved from UniProtKB/Swiss-Prot (<http://www.uniprot.org/>) for the sequence alignment and phylogenetic analysis. Multiple sequences were aligned using ClustalX2 (Larkin *et al.* 2007). The alignment was rendered with ENDscript server (<http://escript.ibcp.fr/ESPrpt/cgi-bin/ESPrpt.cgi>) (Robert and Gouet 2014). The phylogenetic relationship was constructed by MEGA 6.0 (Tamura *et al.* 2013). Protein structure prediction was performed by the web server Phyre 2.0 (<http://www.sbg.bio.ic.ac.uk/phyre2/html>) (Bennett-Lovsey *et al.* 2008; Kelley and Sternberg 2009). The predicted protein structure was visualized and edited using PyMOL Molecular Graphics System.

## Sequence Accession Number

The whole-genome DNA sequence of *T. thermarum* DSM 5069 in GenBank is CP002351. The amino acid sequence of the *T. thermarum* DSM 5069  $\alpha$ -glucuronidase in UniProtKB is F7YUS1.

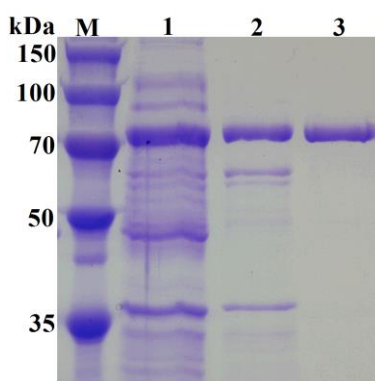
## RESULTS AND DISCUSSION

### Cloning, Expression and Purification of TtAguA

The translated protein of *aguA* is annotated in NCBI's GenBank as a member of the glycoside hydrolase family 67 (GH67) with a GH67 N-terminus, middle domain, and C-terminus. The protein BLAST result demonstrated that TtAguA only exhibited 68% identity with the  $\alpha$ -glucuronidase from *Thermotoga sp.* RQ2 (accession no.: WP\_012310806) and 66% identity with the  $\alpha$ -glucuronidase from *Thermotoga neapolitana* (Accession No.: WP\_015919134). TtAguA is likely located in the cytoplasm, as SignalP 4.0 failed to recognize a signal peptide. Given the ease of protein purification, the His<sub>6</sub> tag originated from the commercial vector pET-28a was adopted.

Hence, the full-length fragment (2043 bp) of *aguA* gene was PCR-amplified from the genomic DNA of *T. thermarum* DSM 5069 and was ligated into pET-28a at sites of *SacI* and *XhoI*, in frame with the C-terminal His<sub>6</sub> tag sequence, and the nucleotide sequence between *NcoI* and *SacI* sites was subsequently removed, generating the new construct pET-28a-*aguA*.

The C-terminal His<sub>6</sub>-tagged TtAguA was heterologously expressed in *E. coli* BL21 (DE3) upon IPTG induction. The enzyme of interest was eluted off using elution buffer supplemented with 400 mM imidazole. The flow through was dialyzed against potassium phosphate buffer (10 mM, pH 7.4). The cell-free preparation was applied to SDS-PAGE to check the homogeneity. A single band was observed on SDS-PAGE, and the molecular weight of the purified protein was ~70 kDa, close to the calculated mass weight of the His<sub>6</sub>-tagged enzyme of 80 kDa (Fig. 1). An 11-fold improvement in activity (57.5 U/mg) was achieved compared with the crude extract (5.3 U/mg). Approximately 28% of the activity measured in the crude extract was recovered after purification by heat treatment and nickel affinity chromatography (Table 2).



**Fig. 1.** SDS-PAGE analysis of recombinant TtAguA expressed in *E. coli* BL21 (DE3). Lane M: protein marker. Lane 1: the crude extract from cell lysate. Lane 2: supernatant after treatment at 60 °C for 30 min. Lane 3: cell-free solution prepared using a Ni-NTA cartridge with 400 mM imidazole

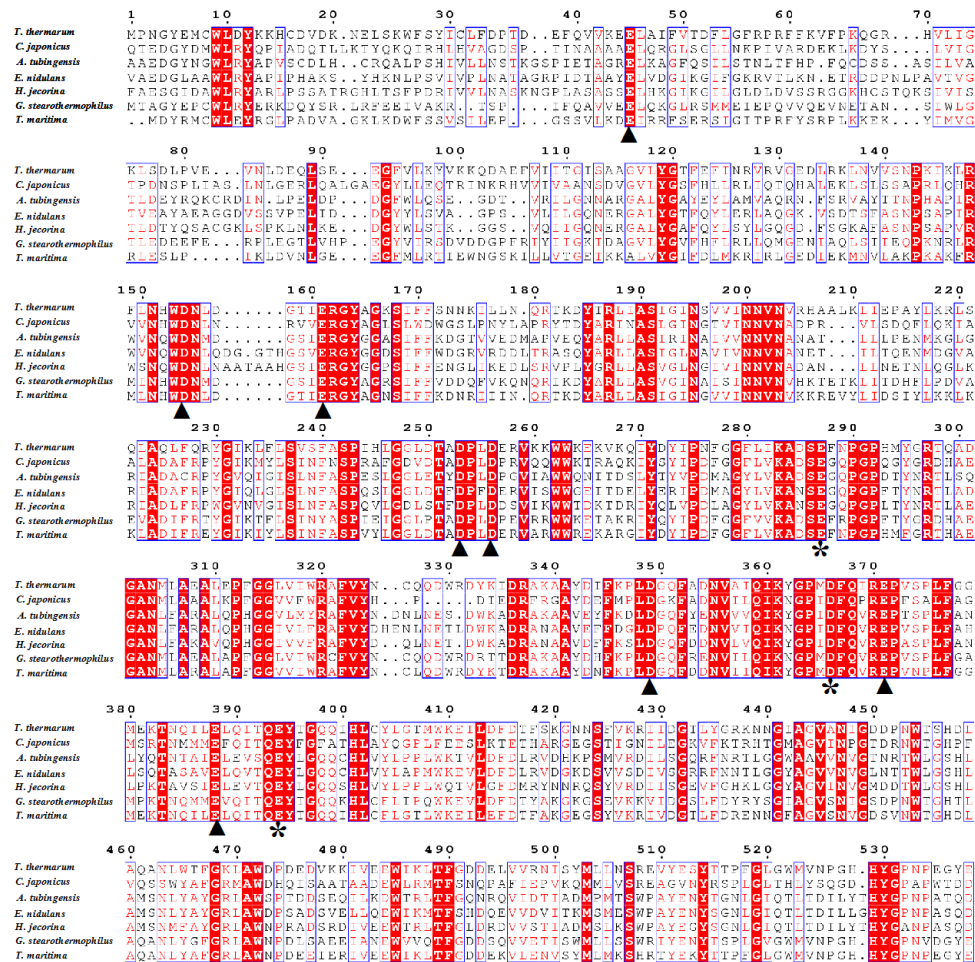
**Table 2.** Purification of  $\alpha$ -Glucuronidase from *T. thermarum* DSM 5069

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Cell extract	2.15	11.3	5.3	1	100
Heat-treatment	0.21	9.8	46.7	9	86
Ni-affinity chromatograph	0.06	3.2	57.5	11	28

### Multiple Sequence Alignment and Phylogenetic Analysis

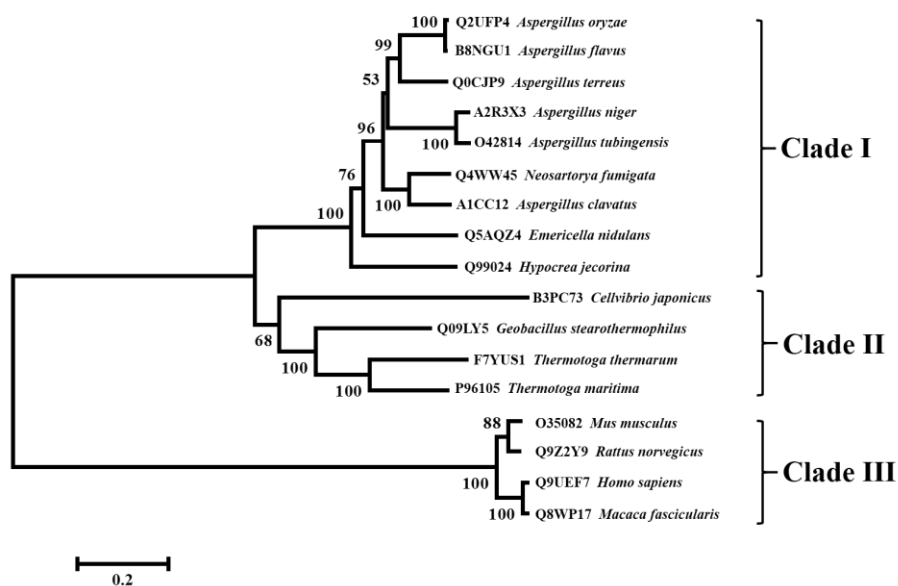
Because the general acid-base catalysis mechanism is extensively accepted for the hydrolytic process of most glycoside hydrolases, multiple sequence alignment was performed to predict the key amino acids participating in the general acid-base catalysis process. As shown in Fig. 2, 11 carboxylic amino acid residues (Glu or Asp) were highly conserved in the aligned regions of GH67  $\alpha$ -glucuronidase sequences from seven species. While a role of these carboxylic amino acids has not yet been proven, some of them appear to be the catalytic residues for TtAguA's hydrolytic ability.

To explore the evolutionary relationship of GH67  $\alpha$ -glucuronidases, a phylogenetic tree was generated using the Poisson substitution model with the neighbor-joining (NJ) method, and the confidence of the tree was evaluated by bootstrap with 1000 replicates. Seventeen GH67  $\alpha$ -glucuronidases were grouped into three distinct clades (Fig. 3). Clade I represented the  $\alpha$ -glucuronidases from fungi including *Aspergillus*, *Emericella*, *Hypocrea*, and *Neosartorya*. Clade II represented the  $\alpha$ -glucuronidases from bacteria including *Cellvibrio*, *Geobacillus*, and *Thermotoga*. Clade III comprised the  $\alpha$ -glucuronidases from animals. TtAguA was grouped into clade II, exhibiting a closer relationship with the  $\alpha$ -glucuronidases from thermophiles *T. maritima* (UniProtKB entry: P96105), and *G. stearothermophilus* (UniProtKB entry: Q09LY5). Based on the phylogenetic analysis, these three  $\alpha$ -glucuronidases are proposed to be distinguished from other  $\alpha$ -glucuronidases because of their thermostability and heat tolerance at higher temperatures.



**Fig. 2.** Sequence alignment of TtAguA and GH67 family members. The amino acid sequences of the aligned  $\alpha$ -glucuronidases are from: *T. thermarum* DSM 5069, *Cellvibrio japonicus* (UniProtKB entry: B3PC73), *Aspergillus tubingenis* (UniProtKB entry: O42814), *Emericella nidulans* (UniProtKB entry: Q5AQZ4), *Hypocrea jecorina* (UniProtKB entry: Q99024), *Geobacillus stearothermophilus* (UniProtKB entry: Q09LY5), and *Thermotoga maritima* (UniProtKB entry: P96105). Catalytic residues are labeled with asterisks. Non-catalytic Glu residues are labeled with triangles

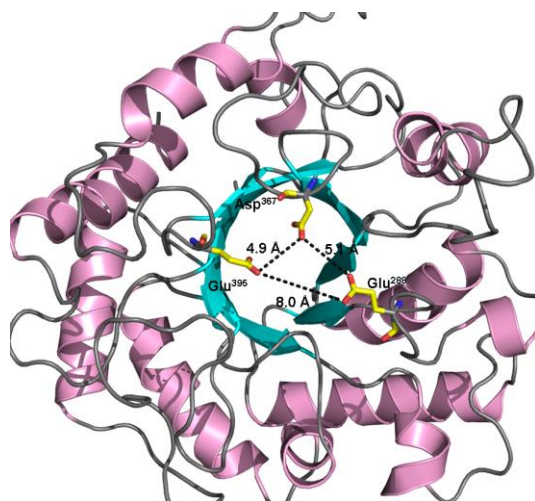




**Fig. 3.** Neighbor-joining tree generated from 17  $\alpha$ -glucuronidases. The values adjacent to the nodes indicate the percentage of bootstrap for 1,000 replicates

### Structure Simulation and Site-Directed Mutagenesis

To identify its active site from the 11 conserved acidic residues, templates of d1|8na1 and c1mqra were selected for the structure prediction of TtAguA using the server Phyre 2.0. The overall arrangement of the middle domain is a  $(\beta/\alpha)_8$  barrel fold, which is one of the most classical folds distributed in hydrolase families. Although eleven conserved carboxylic residues were generated from the multiple sequence alignment, only residues Glu<sup>288</sup>, Asp<sup>367</sup>, and Glu<sup>395</sup> cluster around the substrate channel of the  $(\beta/\alpha)_8$  barrel fold as seen in Fig. 4, the calculated distances between each other are 4.9, 5.1, and 8.0 Å, respectively.



**Fig. 4.** Ribbon representation of TtAguA.  $\alpha$ -Helices,  $\beta$ -strand sheets and random coils are colored pink, cyan, and grey, respectively. Acidic residues (Glu<sup>288</sup>, Asp<sup>367</sup>, and Glu<sup>394</sup>) are shown in sticks and labeled.



A single mutation E288S was introduced to identify the potential proton donor. Compared to the wild-type, approximate 98% of enzymatic activity was lost in the mutant E288S. Based on these results, Glu<sup>288</sup> is likely to act as the proton donor of TtAguA. In addition, Asp<sup>367</sup> and Glu<sup>395</sup> are proposed to be proton acceptors. Glu<sup>288</sup>, Asp<sup>367</sup>, and Glu<sup>395</sup> interact with each other, forming a catalytic triad.

### Substrate Specificity Assay

The substrate specificity of the enzyme was assessed using substrates including aldouronic acids, debranched arabinan, 1,4- $\beta$ -D-mannan, galactan, *p*-nitrophenyl- $\beta$ -D-xylopyranoside (*p*NPX), and *p*-nitrophenyl- $\alpha$ -L-arabinofuranoside (*p*NPAF). The significant hydrolytic activity towards aldouronic acids was achieved under the tested conditions. However, TtAguA showed no detectable activities towards debranched arabinan, 1,4- $\beta$ -D-mannan, galactan, *p*NPX, or *p*NPAF (data not shown). The result of the substrate specificity assay indicates that TtAguA preferentially catalyzes aldouronic acids hydrolysis in this work.

### Biochemical Characterization

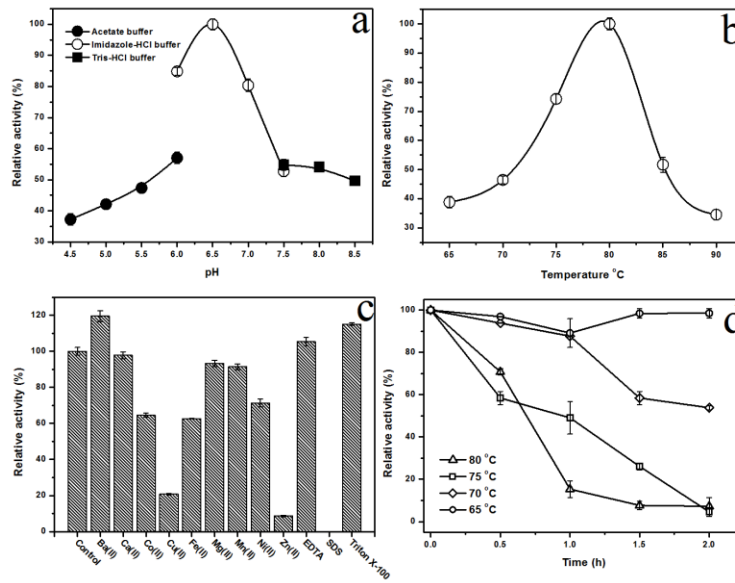
As shown in Fig. 5a, the highest enzymatic activity was achieved at pH 6.5 in imidazole-HCl buffer (100 mM). The enzyme displayed the maximum activity at 80 °C, but it decreased to 54% of its maximum activity at 85 °C (Fig. 5b). The pH optimum of TtAguA was found to be more similar to the  $\alpha$ -glucuronidases from *A. niger* (Kiryu *et al.* 2005), *G. stearothermophilus* (Choi *et al.* 2000), *Phanerochaete chrysosporium* (Castanares *et al.* 1995), and *Bacteroides* J-37 (Kim *et al.* 1997), which have an acidic pH optima of 6.5. Conversely, the  $\alpha$ -glucuronidase from *T. maritima* has an optimal pH of 7.8 (Suresh *et al.* 2002). TtAguA also has a higher optimal temperature of 80 °C than the  $\alpha$ -glucuronidase from *G. stearothermophilus* which is 60 °C (Shallom *et al.* 2004). Likewise, most  $\alpha$ -glucuronidases rapidly lose activity at 80 °C, a temperature at which the TtAguA enzyme is relatively active (Illanes and Wilson 2003).

The effects of various additives on the enzyme activity are shown in Fig. 5c. Activity was slightly enhanced with 1.0 mM of Ba<sup>2+</sup>, EDTA, and Triton X-100, respectively. In contrast, Cu<sup>2+</sup>, Zn<sup>2+</sup>, and SDS inhibited enzymatic activity by over 80%. In particular, enzymatic activity of TtAguA was completely inhibited in the presence of 1.0 mM SDS. Other chemical reagents showed no noticeable influence on TtAguA. Even though the activity of the  $\alpha$ -glucuronidase from *A. niger* is slightly enhanced by 2.0 mM Cu<sup>2+</sup> (Kiryu *et al.* 2005), Cu<sup>2+</sup> inhibits most activity of TtAguA. Hence, copper reagent is used to stop the hydrolysis reaction in this study. These results indicate that TtAguA is not a metal-activated or a metalloenzyme.

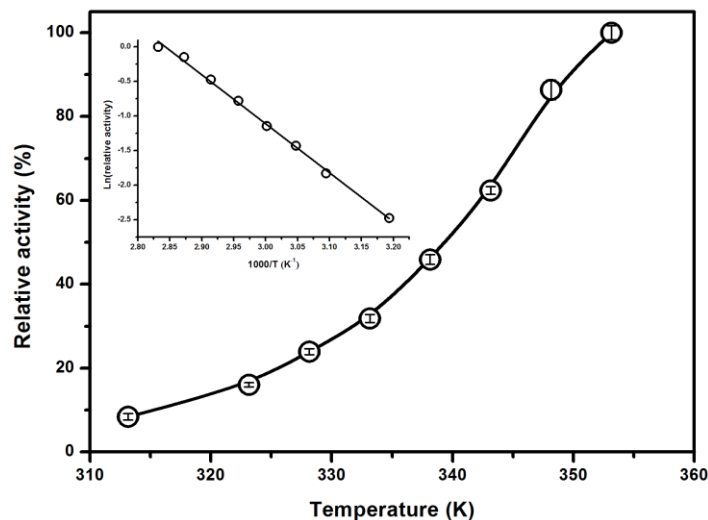
Because enzyme thermostability is one of the limiting factors in industrial applications and processes, the thermostability of TtAguA was estimated at temperatures from 65 to 80 °C within 2.0 h. As shown in Fig. 5d, over 85% of its initial activity was lost after incubating at 75 or 80 °C for 2.0 h. In addition, approximately 55% of its initial activity was retained at 70 °C for 2.0 h. In particular, almost 98% of its initial activity was maintained after a 2-h incubation at 65 °C. The results indicate that the recombinant TtAguA is fairly thermal stable at 65 °C.

The apparent activation energy for glycoside hydrolysis by TtAguA was 59 kJ/mol, according to the logarithmic transformation of the Arrhenius plot from 40 to 80 °C (Fig. 6).

Apparent kinetic parameters on TtAguA were determined using aldouronic acids as a substrate at the optimal temperature and pH. The results showed that TtAguA had a  $K_m$  of  $3.02 \pm 0.16$  mM, a  $V_{max}$  of  $87.6 \pm 2.4$   $\mu\text{mol min}^{-1} \text{mg}^{-1}$  and a  $k_{cat}$  of  $117 \text{ s}^{-1}$ .



**Fig. 5.** Biochemical properties of TtAguA. The effects of pH, temperature and chemicals on the activity and thermal stability of the recombinant TtAguA. (a) The effects of pH on activity. (b) The effects of temperature on activity. (c) The effects of chemical agents on activity. (d) The thermostability of the TtAguA. All values are displayed as the average  $\pm$  SD of three independent replicates



**Fig. 6.** Activation energy of TtAguA. Results are shown as the average  $\pm$  SD of three independent replicates

### Synergistic Degradation

In terms of natural hemicellulose degradation process,  $\alpha$ -glucuronidases cleave the  $\alpha$ -1,2-glycosidic bond between MeGlc and xylooligomers *via* an inverting mechanism. The synergistic hydrolysis of 4-O-methyl-D-glucurono-D-xylan was investigated by adding TtAguA and Xyn10A in an activity ratio of 7.6 U : 2.7 U. In the

presence of TtAguA and Xyn10A, xylobiose and reducing sugars were formed at a 10% faster rate, with the net increase of 400  $\mu\text{g}$  reducing sugar in the synergistic degradation. Moreover, 50% of 4-O-methyl-D-glucurono-D-xylan was synergistically hydrolyzed and converted into xylooligomers including xylobiose, xylotriase, and xyloetraose compared to only 45% in the presence of Xyn10A alone. The content of xylotriase was lower than that of xylobiose or xyloetraose in both the synergistic and non-synergistic degradation systems in the first 0.5 h. Xylotriase gradually became the dominant product with the consumption of xyloetraose after 5 h of degradation (Table 3).

Although GH67  $\alpha$ -glucuronidases do not directly attack the polymeric substrate, synergy was observed in the degradation of 4-O-methyl-D-glucurono-D-xylan with TtAguA and Xyn10A. A likely explanation for this synergy is that the GH10 endoxylanase Xyn10A acts on 4-O-methyl-D-glucurono-D-xylan, generating the acidic fragments, in which the MeGlcA is linked to the non-reducing end xylopyranosyl residue. Subsequently, the GH67  $\alpha$ -glucuronidase TtAguA hydrolyzes  $\alpha$ -1,2-glycosidic bond of the MeGlcA side chains attached to the acidic fragments. Once MeGlcA moiety is liberated, the partial debranching fragments may continue to serve as xylanase substrate, so the slight increase in amount of reducing sugars in the presence of  $\alpha$ -glucuronidase is obvious. Moreover, the transglycosylation mediated by Xyn10A is believed to account for the decrease in amount of xyloetraose which may be converted to xylobiose and xylotriase during the degradation process of 4-O-methyl-D-glucurono-D-xylan.

**Table 3.** Comparison of Synergistic and Non-synergistic Degradation of 4-O-Methyl-D-glucurono-D-xylan

Time (h)	Xylobiose ( $\mu\text{g}$ )		Xylotriase ( $\mu\text{g}$ )		Xyloetraose ( $\mu\text{g}$ )		Reducing sugar ( $\mu\text{g}$ )	
	A	B	A	B	A	B	A	B
0.5	229	212	93	79	123	101	445	392
1	278	263	156	146	20	18	454	427
2	341	341	244	181	10	18	595	540
3	384	347	307	202	0	9	691	558
4	405	368	465	428	6	1	876	797
5	433	400	519	501	0	0	952	901

A, B groups represent synergistic and non-synergistic degradation, respectively

## CONCLUSIONS

1. In this study, the recombinant  $\alpha$ -glucuronidase (TtAguA) from *T. thermarum* DSM 5069 was successfully cloned and expressed in *E. coli*; and its biochemical properties were characterized in detail.
2. Aldouronic acids were shown to be the optimal substrate for TtAguA. TtAguA was fairly thermostable and maintained approximately 98% of its initial activity after incubation at 65 °C for 2 h. Among the conserved acidic residues, Glu<sup>288</sup> was identified as the catalytic proton donor; Asp<sup>367</sup> and Glu<sup>395</sup> were likely nucleophile bases.

3. Xylan degradation by the endoxylanase Xyn10A was enhanced by approximately 10% in the presence of TtAguA.

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