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A Thermostable Non-xylanolytic α-Glucuronidase of *Thermotoga maritima* MSB8

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A putative α-glucosidase belonging to glycosyl hydrolase family 4 of *Thermotoga maritima* (TM0752) was expressed in *Escherichia coli* and it was found that the recombinant protein (Agu4B) was a *p*-nitrophenyl α-D-glucuronopyranoside hydrolyzing α-glucuronidase, not α-glucosidase. It did not hydrolyze 4-*O*-methyl-D-glucuronoxylan or its fragment oligosaccharides. Agu4B was thermostable with an optimum temperature of 80°C. It strictly required Mn^{2+} and thiol compounds for its activity. The presence of NAD^+ slightly activated the enzyme. The amino acid sequence of Agu4B showed higher identity with Agu4A (another α-glucuronidase of *T. maritima*, 61%) than with AglA (α-glucosidase of *T. maritima*, 48%).

Key words: *p*-nitrophenyl α-D-glucuronopyranoside hydrolyzing α-glucuronidase; non-xylanolytic α-glucuronidase; thermostable α-glucuronidase; *Thermotoga maritima*

An interesting feature of some enzymes from hyperthermophilic organisms is their low sequence similarity to the enzymes with similar activity from other organisms.[1–5] *Thermotoga maritima* is a hyperthermophilic bacterium with an optimum growth temperature of 80°C.[6] Bibel *et al*. found the first α-glucosidase (AglA) belonging to family 4 glycosyl hydrolases in a phenotype screening of a *T. maritima* gene library.[3] Later, the whole genomic sequence of *T. maritima*[7] revealed the existence of three homologue genes of AglA (TM1834), and these genes (TM0434, TM0752, and TM1068) were annotated as putative α-glucosidases. Recently, one of the genes (TM0434) was expressed in *Escherichia coli* and it was found that the expressed enzyme hydrolyzed *p*-nitrophenyl-α-D-glucuronopyranoside (*p*NP-GUA) but did not hydrolyze *p*-nitrophenyl-α-D-glucopyranoside (*p*NP-Glc).[5] Thus, the enzyme was identified to be an α-glucuronidase, not an α-glucosidase.[5] The enzyme was unique in being the only known microbial enzyme that hydrolyzed *p*NP-GUA and in being the first α-glucuronidase to be classified under family 4 of the glycosyl hydrolases.[5] It showed no sequence similarity to any of the known α-glucuronidases.

The α-glucuronidase encoded by TM0434 (Agu4A, formerly named Agu[5] and renamed from this report) was characterized by its distinct thermal instability and unusual cofactor dependency, where it needed a very high concentration of reducing agent for activity.[5] Even though most of the enzymes of *T. maritima* are extremely thermostable,[8,9] one exception was reported. An iron hydrogenase from *T. maritima* was unstable even at ambient temperatures and the stability was recovered by adding glycerol (10% v/v).[10] However, the stability of Agu4A was not recovered under these conditions. The instability was not caused by the additional N-terminal His-tag sequence, evidenced by the fact that the the transfer of the His-tag sequence to the C-terminal did not improve the stability. In the light of the instability of Agu4A, it is of interest to know whether there are any other genes which code for the same enzyme and if so, whether the thermal instability and low substrate affinity are common features of these enzymes. Besides, obtaining a thermostable *p*NP-GUA hydrolyzing α-glucuronidase with better affinity for the substrate may be valuable in order to study the role of this unique enzyme in the bacterial metabolism.

In this paper, cloning and expression of another putative α-glucosidase gene of *T. maritima* (TM0752, GenBank accession number AE001745), which is the first thermostable *p*NP-GUA hydrolyzing α-glucuronidase has been reported.

Materials and Methods

Bacterial strains, plasmids, and medium. The genomic DNA of *T. maritima* MSB8 was kindly provided by Prof. K. O. Stetter, Lehrstuhl für Mikrobiologie, Universität Regensburg, Germany. pDrive cloning vector was obtained from Qiagen (Hilden, Germany). Expression vector pET-28b(+) was from Novagen (Madison, WI, USA). *E. coli* EZ competent cells (Qiagen) and *E. coli* BL21-Codon-

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Plus-RIL competent cells (Stratagene, La Jolla, CA, USA) were used as hosts for cloning and expression, respectively. Luria-Bertani (LB) medium supplemented with kanamycin (50 μg/ml) was used for the cultivation of *E. coli* transformants.

PCR, sequencing, and expression vector construction. Restriction endonucleases were purchased from New England Biolabs, USA. The open reading frame (ORF) encoding the putative α-glucosidase (TM0752) was retrieved from GenBank (accession number AE001745). The gene was amplified by the polymerase chain reaction using the oligonucleotides 5′-CATATGAAGATCTCCATCATCGGA-3′ and 5′-AAGCTTTCAGAGTTTCTCTTTGTAGTATC-3′ (restriction sites are underlined). The restriction sites *Nde*I and *Hin*dIII were chosen so as to insert an N-terminal 6X His tag into the construct. The PCR product was cloned in the TA cloning vector (pDrive cloning kit, Qiagen, Germany) and *E. coli* EZ cells were transformed. Both strands of the isolated plasmid, TM-*agu4B*-pDrive, were sequenced (310 Genetic analyzer, Applied Biosystems, USA) using a BigDye terminator cycle sequencing kit (Perkin-Elmer, Applied Biosystems, Norwalk, CT, USA). The PCR product excised from TM-*agu4B*-pDrive with *Nde*I and *Hin*dIII was ligated to pET-28b(+) vector (High T4 DNA Ligase, Toyobo, Osaka, Japan) and *E. coli* BL21-CodonPlus-RIL competent cells were transformed. The expression construct (TM-*agu4B*-pET) was sequenced to verify the correct insertion of the gene into the cloning site.

Expression and purification. The recombinant *E. coli* BL21-CodonPlus-RIL cells were cultivated in LB broth supplemented with kanamycin (50 μg/ml) under shaking conditions at 37°C. In the exponential growth phase at $OD_{600nm} = 0.6$, expression was induced by adding isopropyl thiogalactoside (IPTG) to a final concentration of 0.4 mM and incubation was continued for 4 h at 25°C. Cells harvested by centrifugation were washed with 50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES; pH 7.5), resuspended in the same buffer and disrupted by sonication. The supernatant was the crude enzyme extract.

The enzyme was purified by Ni-NTA agarose metal chelate chromatography done at room temperature. The crude enzyme extract (2 ml, 8 mg protein/ml) was put on 3 ml of Ni-NTA agarose resin (Qiagen, Germany) equilibrated with 50 mM HEPES buffer (pH 7.5) containing 20 mM imidazole and 300 mM NaCl. The enzyme was eluted with a linear gradient of 20–250 mM imidazole in 50 mM HEPES buffer (pH 7.5) containing 300 mM NaCl. Protein concentrations were routinely measured by the dye binding method.[11]

Electrophoresis. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) was done in 10% acrylamide gels[12] and the proteins were made visible by staining with Coomassie brilliant blue R-250. A BenchMark protein ladder (Invitrogen, USA) was used as the protein molecular mass standard.

Synthesis of substrates. *p*NP-GUA was synthesized by catalytic oxidation of *p*NP-Glc.[13] *p*-Nitrophenyl α-D-glucopyranoside-6-phosphate (*p*NP-G6P) was synthesized as described before.[5]

Enzyme assay. The α-glucuronidase activity was measured by the *p*-nitrophenol (*p*NP) released from *p*NP-GUA at 40°C in 20 min. The assay mixture consisted of 3 mM *p*NP-GUA, 0.2 mM $MnCl_2$, 30 mM DTT, and 0.5 mM NAD^+ in 50 mM HEPES buffer (pH 7.5). Appropriately diluted enzyme in buffer was added to the reaction mixture for a final volume of 300 μl. The reaction was stopped by adding 20 μl of 0.5 M ethylenediaminetetraacetic acid (EDTA). The absorbance at 405 nm was measured after adding 280 μl of 1 M Na_2CO_3. One unit of the enzyme activity corresponded to 1 μmol of *p*NP released/min under the conditions described for the assay.

Characterization of Agu4B. To identify the temperature optimum, an assay of Agu4B was done at different temperatures under standard assay conditions. The pH optimum of Agu4B was identified under standard assay conditions using the standard assay mixture in 50 mM of the following buffers: sodium acetate (pH 5.4–6.6), 2-(*N*-morpholino)ethanesulfonic acid (MES; pH 5.6–6.9), 3-(*N*-morpholino)propanesulfonic acid (MOPS; pH 6.1–7.8), HEPES (pH 5.7–8.1), Tris-HCl (pH 7.8–8.3), 2-(*N*-cyclohexylamino)ethanesulfonic acid (CHES; pH 7.8–9.0) and 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS; pH 8.4–9.4). The pH stability was measured by incubating the enzyme in 10 mM of the above said buffers as well as sodium citrate (pH 2.7–4.3), sodium acetate (pH 4.0–5.4), CAPS (pH 9.1–10.0), and piperidine (pH 10.8–11.8) for 30 min at room temperature. Enzyme activity was measured under the standard assay conditions.

Replacing Mn^{2+} with 1–10 mM of Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+}, or Fe^{2+} in the reaction mixture, we tested the effects of these divalent metal ions on the enzyme activity. Enzyme activity was assayed under standard assay conditions.

Activity of Agu4B on other substrates. Quantitative analysis of the enzyme activity was done with 3 mM *p*NP-Glc, *p*NP-G6P or *p*-nitrophenyl α-D-galactopyranoside in standard reaction mixture. For qualitative analysis of the enzyme activity on maltose (3 mM), 4-*O*-methyl-D-glucuronoxylan (2%;

Table 1. Amino Acid Sequence Similarity in the Four Family 4 Glycosyl Hydrolases of *T. maritima*

Locus	Name	Activity	Thermo stability	% Identity			
				TM0434	TM0752	TM1068	TM1834
TM0434	Agu4A	α-glucuronidase	no		61	99	52
TM0752	Agu4B	α-glucuronidase	yes	61		62	48
TM1068	Agu4C	nd*	nd*	99	62		52
TM1834	AglA	α-glucosidase	yes	52	48	52	

* not done.

Sigma), reduced aldouronic acids (2′-*O*-α-D-glucuronopyranosyl-xylobitol:2″-*O*-α-D-glucuronopyranosyl-xylotritol:2‴-*O*-α-D-glucuronopyranosyl-xylotetraitol = 40:40:20) and aldotriuronic acid (2′-*O*-α-D-glucuronopyranosyl-xylobiose, 0.4%, Megazyme, Ireland), the reaction mixture was incubated at 40°C or 50°C for 72 h. The products of maltose and 4-*O*-methyl-D-glucuronoxylan were analyzed by TLC done on silica gel plates, 60 F_{254} (Merck, Darmstadt, Germany), developed twice with acetonitrile/H_2O 80:20 (vol/vol). For the TLC analysis of the products of reduced aldouronic acids and aldotriuronic acid, the solvent system, acetonitrile/1 M acetic acid 80:20 (vol/vol) was used.

Sequence alignment. Protein sequences were aligned using the GENETYX-WIN program (Genetyx, Tokyo, Japan).

Results and Discussion

Sequence similarity

T. maritima was reported to have a family 4 α-glucosidase gene (TM1834, *aglA*) and three putative α-glucosidase genes (TM0434, TM0752, and TM1068),[7] out of which one (TM0434, *agu4A*) has been shown to be a *p*-nitrophenyl α-D-glucuronopyranoside (*p*NP-GUA) hydrolyzing α-glucuronidase.[5] Another putative α-glucosidase gene (TM0752, *agu4B*) of *T. maritima* cloned and expressed in this study encodes a 471-amino acid protein with a deduced molecular mass of 55 kDa. Table 1 shows the amino acid sequence similarity of Agu4B to α-glucuronidase (Agu4A), α-glucosidase (AglA), and the putative α-glucosidase of *T. maritima*. Agu4B, classified under family 4 of glycosyl hydrolases based on amino acid sequence similarity,[14] showed low homology to the other family 4 enzymes. It showed no sequence similarity to α-glucuronidase (AguA) of *T. maritima*, a family 67 enzyme.[14]

Substrate specificity

Agu4B, a putative α-glucosidase, had no activity on maltose as seen by TLC analysis and on *p*NP-Glc. The enzyme was active on *p*NP-GUA, thus showing that it was an α-glucuronidase rather than an α-glucosidase. A trace of enzyme activity was detected on *p*-nitrophenyl α-D-galactopyranoside (*p*NP-Gal) with a high concentration of the enzyme and long incubation time. The enzyme also showed no activity towards the substrates of other family 4 enzymes (Table 2). While α-glucuronidases (EC 3.2.1.139) are enzymes that hydrolyze the α-1,2-glycosidic linkage between xylose and D-glucuronic acid or its methyl ether,[15,16] most of the α-glucuronidases studied are known to be capable of releasing 4-*O*-methyl-D-glucuronic acid only from fragment oligosaccharides of 4-*O*-methyl-D-glucuronoxylan. A few fungal α-glucuronidases had this activity towards polymeric 4-*O*-methyl-D-glucuronoxylan.[17–19] However, Agu4B was non-xylanolytic, with no detectable activity towards 4-*O*-methyl-D-glucuronoxylan and its fragment oligosaccharides. Activity on *p*NP-GUA has been reported in the enzyme preparation of a limpet (*Patella vulgata*),[20] snail α-glucuronidases,[21] and in snail acetone powder.[22] Reports on *p*NP-GUA hydrolyzing α-glucuronidases are very few since the attachment of uronic acid to at least one xylanopyranosyl residue appears to be essential for the activity of microbial α-glucuronidases.[23,24] To date, the *p*NP-GUA hydrolyzing α-glucuronidase (Agu4A) of *T. maritima* is the only reported microbial enzyme with this activity.[5]

Table 2. Substrate Specificity of Agu4B

+, Substrate hydrolyzed; −, no hydrolysis. Activity on *p*NP α-D-galactopyranoside was estimated after incubating the reaction mixture at 40°C for 24 h.

Substrates	Activity	Specific activity (s^{-1})
*p*NP α-D-Glucuronopyranoside	+	0.28
*p*NP α-D-Galactopyranoside	+	0.0003
4-*O*-Methyl-D-glucuronoxylan	−	
Reduced aldouronic acids	−	
Aldotriuronic acid	−	
*p*NP α-D-Glucopyranoside	−	
Maltose	−	
*p*NP α-D-Glucopyranoside-6-phosphate	−	

Cofactor requirement and biochemical properties

The activity of the enzyme increased significantly in the presence of $MnCl_2$ and DTT, and the addition

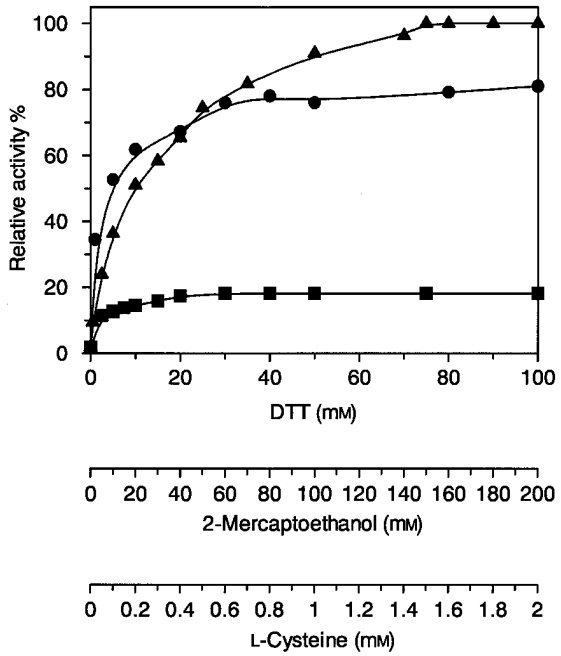

Fig. 1. Effect of DTT (circles), 2-Mercaptoethanol (triangles), and L-Cysteine (squares) on Agu4B Activity.

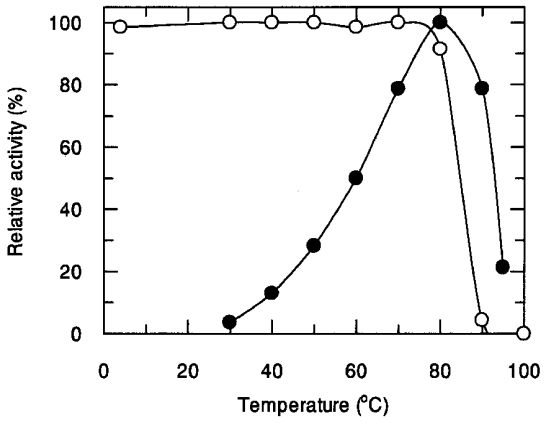

Fig. 2. Temperature Stability and Activity of Agu4B. (open circles) stability; (closed circles) activity. For measuring the stability, the enzyme in 50 mM HEPES (pH 7.5) was incubated at temperatures from 4 to 100°C for 30 min and the residual activity was measured at 40°C under standard assay conditions. For measuring the activity, Assay of Agu4B was done at different temperatures under the standard assay conditions.

of NAD^+ resulted in further enzyme activation. Concentrations of 0.2 mM $MnCl_2$, 30 mM DTT, and 0.5 mM NAD^+ were found to be required for maximum enzyme activity. The absence of NAD^+ in the reaction mixture resulted in only 60% of the enzyme activity. Some of the family 4 enzymes are known to be dependent on Mn^{2+} and NAD^+ for activity[2] and their NAD^+ dependence has been attributed to the NAD^+ binding motif at their N-terminus.[25] While Agu4B showed a weak NAD^+ requirement, Agu4A, which has a similar primary structure at the N-terminus, did not show any need for NAD^+.[5] Replacement of $MnCl_2$ with other divalent metal ions did not result in any appreciable increase in the basal level enzyme activity. Replacement of DTT with 2-mercaptoethanol resulted in the maximum activation of the enzyme at a concentration of 150 mM. The activation of the enzyme by L-cysteine at an optimal concentration of 0.6 mM was only 15% of the activation achieved with 2-mercaptoethanol (Fig. 1).

The optimum temperature of Agu4B was 80°C. It was stable up to 70°C when the enzyme was assayed after a 30-min incubation at temperatures from 4 to 100°C (Fig. 2). It is interesting to note that the property of thermostability of Agu4B was a significant variation from the previously described α-glucuronidase (Agu4A), which displayed distinct thermal instability at all the temperatures in the absence of the substrate and was stable only up to 40°C in the presence of the substrate for a tested period of 30 min.[5] Agu4B showed an optimum pH of 7.8 and was stable from pH 4 to a tested pH of 12 when incubated at room temperature for 30 min. The kinetic parameters of Agu4B for *p*NP-GUA were found to be $K_m = 0.1$ mM and $k_{cat} = 0.28\ s^{-1}$, whereas those of Agu4A[5] are $K_m = 1.0$ mM and $k_{cat} = 0.0018\ s^{-1}$. Moreover, specific activity comparison of Agu4A (unpublished data) and Agu4B, at their respective optimum temperatures, show a 400-times higher value in Agu4B.

Reducing agent requirement

Kinetic analysis of Agu4B was done in the presence of suboptimal levels of the DTT to observe the variation in the kinetic parameters when compared to the kinetics of the enzyme at the optimal DTT concentration. Kinetic analysis in the presence of 1 mM DTT showed $K_m = 0.12$ mM, which was not significantly different from the one observed at the optimal DTT concentration. The k_{cat} under this condition was $0.12\ s^{-1}$. To establish whether the release of *p*NP from *p*NP-GUA was a hydrolysis or a transfer reaction to the thiol group, *p*NP-GUA in the normal reaction mixture containing reducing agents (DTT, 2-mercaptoethanol or L-cysteine) was incubated with the enzyme for 36 h. The product analyzed by TLC did not show any presence of the corresponding thio-glucuronide, thus confirming the hydrolytic action of the enzyme. The replacement of the thiol compounds with the corresponding hydroxyl compounds (erythritol and ethylenglycol) resulted no activity. Similar results have been reported for *T. maritima* α-glucosidase, AglA.[2] The substitution of thiol compounds to other reducing agents such as ascorbic acid or citric acid again caused no activity, suggesting that Agu4B required thiol groups.

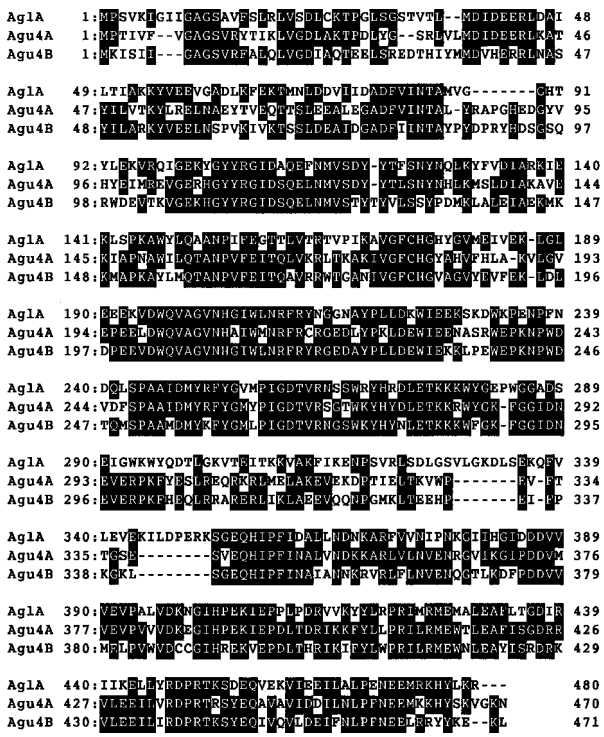

Fig. 3. Alignment of the Sequences of α-Glucosidase (AglA) and α-Glucuronidases (Agu4A and Agu4B) of *T. maritima*.

94 residues are in common between Agu4A and Agu4B while AglA has 56 and 38 residues in common with Agu4A and Agu4B, respectively. The residues common for all the three sequences were 197 and were not taken into consideration.

Sequence alignment

Agu4B shows 61% identity with Agu4A and 48% identity with AglA. The multiple sequence alignment shows that the residues common in between Agu4A and Agu4B are approximately twice compared to the residues common in between Agu4B and AglA or Agu4A and AglA (Fig 3). These residues common in between Agu4A and Agu4B, which are not present in AglA, could have resulted in the absence of α-glucosidase activity in Agu4A and Agu4B, an activity present in AglA, while it is not known whether AglA has the *p*NP-GUA hydrolyzing activity as well as *p*NP-Glc hydrolyzing activity.

On the basis of amino acid sequence similarity and cofactor requirements, especially the high concentrations of reducing agents required for activation, AglA,[2] Agu4A,[5] and Agu4B appear to form a distinct group of proteins. The protein coded by the still to be characterized putative α-glucosidase gene of *T. maritima* (TM1068, GenBank accession number AE001767)[7] that shares 99% identity with Agu4A, may also be a member of this distinct group. It may be interesting to investigate the thermal instability and low substrate affinity of Agu4A in the group of thermostable proteins.

The *p*NP-GUA hydrolyzing α-glucuronidases of *T. maritima*, Agu4A, and Agu4B, are non-xylanolytic. Being the only known microbial α-glucuronidases with the ability to hydrolyze *p*NP-GUA, they constitute a unique group of enzymes with their unusual substrate specificity and in not having amino acid sequence similarity to any of the known α-glucuronidases. Agu4B does not have a signal sequence and is most likely to be intracellular. It may be of interest to know what role an enzyme with this activity plays inside the bacterial cell. Identification of the natural substrate and studies on gene regulation appear to be essential to understand the role of this enzyme in bacterial metabolism. Elucidation of the structure of the protein may throw more light on the unusual thiol dependency. Agu4B being the first thermostable *p*NP-GUA hydrolyzing α-glucuronidase, is apparently a better candidate than the previously characterized Agu4A[5] for such investigations because of its thermostability, higher affinity for the substrate, and better catalytic efficiency.

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