Evidence that the putative α-glucosidase of *Thermotoga maritima* MSB8 is a *p*NP α-D-glucuronopyranoside hydrolyzing α-glucuronidase

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Abstract The gene (*agu*) encoding *p*-nitrophenyl α-D-glucuronopyranoside (*p*NP-GUA) hydrolyzing α-glucuronidase of the hyperthermophilic bacterium *Thermotoga maritima* was cloned and expressed in *Escherichia coli*. The recombinant enzyme was purified and characterized. The gene previously designated as putative α-glucosidase was found to code for a protein that had no α-glucosidase activity. It showed a rare activity profile with its ability to hydrolyze *p*NP-GUA, an activity not known in the α-glucuronidases from microbial sources. This is the first report on the occurrence of an α-glucuronidase which belongs to the family 4 of glycosyl hydrolases.

Key words: Hyperthermophile; *p*-Nitrophenyl α-D-glucuronopyranoside hydrolyzing α-glucuronidase; Family 4 glycosyl hydrolase; *Thermotoga maritima*

1. Introduction

α-Glucosidases (EC 3.2.1.20) are enzymes that hydrolyze the α-glucosidic linkages from the non-reducing end of oligosaccharides and polysaccharides with the release of α-glucose. α-Glucosidases are present in a number of organisms with varying substrate specificities [1,2]. According to amino acid sequence similarity, α-glucosidases are classified under the families 13 and 31 of glycosyl hydrolases [3].

Thermotoga maritima, one of the most hyperthermophilic bacteria with an optimum growth temperature of 80°C [4] is known to utilize a number of carbohydrates fermentatively [5]. Bibel et al. [6] reported the cloning and sequencing of an α-glucosidase gene (*aglA*) of *T. maritima*, which was identified by phenotypic screening of the *T. maritima* gene library. The amino acid sequence of AglA was not related to other typical α-glucosidases classified under families 13 and 31. It shared a moderate similarity with the enzymes of family 4 [6,7], which comprises of 6-phospho-β-glucosidase (EC 3.2.1.86), 6-phospho-α-glucosidase (EC 3.2.1.122) and α-galactosidase (EC 3.2.1.22). Characterization of AglA revealed a cofactor dependency similar to that of some of the enzymes of family 4 [7]. Later, the whole genome sequence of *T. maritima* revealed the existence of three putative α-glucosidase genes TM0434, TM0752 and TM1068 [8] with GenBank accession numbers AE001722, AE001745 and AE001767, respectively. *aglA* (TM1834) showed 52% similarity to TM0434 and TM1068 and 48% similarity to TM0752.

Even though α-glucosidases have been extensively studied in a number of bacteria, very little is known about the enzyme in hyperthermophilic organisms [7]. In addition, the lack of sequence similarity of α-glucosidases of hyperthermophilic organisms *T. maritima* [6,7] and *Thermococcus hydrothermalis* [9] to any other α-glucosidases makes the study of α-glucosidases from hyperthermophiles more interesting. In order to study the putative α-glucosidase genes of *T. maritima*, one of the genes was cloned and characterized. In this paper, cloning of a putative α-glucosidase gene of *T. maritima* (TM0434) is reported and evidence is presented to show that the gene actually codes for a *p*-nitrophenyl α-D-glucuronopyranoside (*p*NP-GUA) hydrolyzing α-glucuronidase and not an α-glucosidase.

2. Materials and methods

2.1. Bacterial strains, plasmids and media

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). *Escherichia coli* EZ competent cells (Qiagen) and *E. coli* BL21-CodonPlus-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.

2.2. Cloning and sequencing of agu

Restriction endonucleases were purchased from New England Biolabs, USA. The open reading frame (ORF) encoding the putative α-glucosidase (TM0434) was retrieved from GenBank (accession number AE001722). The ORF was amplified by polymerase chain reaction (PCR) using a 5′ forward primer containing a restriction site for *Nco*I (5′-CCATGGCTACCATAGTATTTGTAGG-3′, the restriction site is underlined) and a 3′-reverse primer with a *Hin*dIII site (5′-CAAGCTTATTCTTTCCTACTTTTGAGTAA-3′). The restriction sites were chosen so as to insert a C-terminal 6× His tag into the construct. PCR amplification was performed for 25 cycles using the following conditions: 98°C, 1 min; 55°C, 30 s; 68°C, 1.5 min. The PCR product was ligated to the TA cloning vector (pDrive cloning kit, Qiagen) and the plasmid was transformed into *E. coli* EZ cells. The plasmid TM-*agu*-pDrive was isolated from a positive clone and DNA sequencing was done in both the strands (310 Genetic analyzer, Applied Biosystems, USA) using BigDye terminator cycle sequencing kit (Perkin-Elmer, Applied Biosystems, Norwalk, CT, USA).

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Abbreviations: *p*NP, *p*-nitrophenol; *p*NP-GUA, *p*-nitrophenyl α-D-glucuronopyranoside; *p*NP-Glc, *p*-nitrophenyl α-D-glucopyranoside; *p*NP-G6P, *p*-nitrophenyl α-glucopyranoside-6-phosphate; DTT, dithiothreitol

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2.3. Construction of expression vector

The PCR product excised from TM-*agu*-pDrive with *Nco*I and *Hin*dIII was ligated to pET-28b(+) vector using High T4 DNA Ligase (Toyobo, Osaka, Japan) and transformed into *E. coli* BL21-CodonPlus-RIL competent cells. The expression construct (TM-*agu*-pET) from a positive colony was sequenced to verify the correct insertion of the ORF into the cloning site.

2.4. Expression and purification

For expression of *agu*, recombinant *E. coli* BL21-CodonPlus-RIL cells harboring TM-*agu*-pET 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 30°C. Cells were harvested by centrifugation, washed with 50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES; pH 7.5) containing 1 mM dithiothreitol (DTT), resuspended in the same buffer and disrupted by sonication. The supernatant obtained by centrifugation at $10\,000 \times g$ for 15 min was the crude enzyme extract.

The enzyme was purified by Ni-NTA agarose metal chelate chromatography performed at room temperature. All buffers used for purification contained 1 mM DTT. 2 ml of the crude enzyme extract (6 mg protein/ml) was applied to 4 ml Ni-NTA agarose resin (Qiagen) equilibrated with 50 mM HEPES buffer (pH 7.5) containing 20 mM imidazole and 300 mM NaCl. The enzyme was eluted in a linear gradient of 20–250 mM imidazole in 50 mM HEPES buffer (pH 7.5) containing 300 mM NaCl. The active fractions were dialyzed overnight at 4°C against 50 mM HEPES buffer (pH 7.5). Protein concentrations were routinely determined by dye-binding method [10].

2.5. Electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed in 10% acrylamide gels [11] and the proteins were visualized by staining with Coomassie brilliant blue R-250. Benchmark protein ladder (Invitrogen, USA) was used as the protein molecular mass standard.

2.6. Synthesis of substrates

*p*NP-GUA was synthesized by a catalytic oxidation of *p*-nitrophenyl α-D-glucopyranoside (*p*NP-Glc) [12]. *p*-Nitrophenyl α-glucopyranoside-6-phosphate (*p*NP-G6P) was prepared by the reaction of hexokinase (Sigma, St. Louis, MO, USA) with *p*NP-Glc and ATP.

2.7. Enzyme assay

The α-glucuronidase activity was determined by measuring 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, 1 mM $MnCl_2$ and 50 mM DTT 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.

2.8. Effect of temperature and pH

The temperature optimum was determined under standard assay conditions by incubating the reaction mixture at temperatures ranging from 30 to 90°C. For estimating the thermal stability in the presence of the substrate, time course was done at various temperatures and the velocity was calculated. For determining the optimum pH for enzyme activity, standard assay mixture in 50 mM of the following buffers were used: sodium acetate (pH 5.2–5.6), 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 determined by preincubating the enzyme in 10 mM of the above mentioned buffers as well as sodium citrate (pH 2.7–4.3), sodium acetate (pH 4.0–4.7), CAPS (pH 9.1–10.0) and piperidine (pH 10.8–11.8) for 30 min at room temperature. Enzyme activity was then measured under the standard assay conditions.

For determining the enzyme activity on other substrates, standard assay procedure was used. To check the activity of the enzyme on *p*NP-Glc and *p*-nitrophenyl α-D-galactopyranoside, 3 mM of each was used. Reaction was carried out in the absence of DTT as well. For qualitative analysis of the enzyme activity on maltose (3 mM) and 4-*O*-methyl-D-glucuronoxylan (2%; Sigma) the reaction mixture was incubated at 25°C for 72 h. The products were analyzed by TLC performed on silica gel plates, 60 F_{254} (Merck, Darmstadt, Germany), developed twice with acetonitrile/H_2O 80:20 (vol/vol).

3. Results and discussion

3.1. Sequence similarity

T. maritima was reported to have three putative α-glucosidase genes [8]. The amino acid sequence similarity of the putative α-glucosidase (Agu) cloned and expressed in this study to the α-glucosidases of *T. maritima* and *Thermotoga neapolitana* is shown in Table 1. The enzyme showed 52% similarity to the α-glucosidase of *T. maritima* the sequence of which is not related to other typical α-glucosidases [6,7]. It showed 26% similarity to α-galactosidase of *Bacillus halodurans* and 24% similarity to the 6-phospho-β-glucosidase of *Vibrio cholerae*. Agu had no similarity to the α-glucuronidase (AguA) of *T. maritima*, an enzyme with a novel primary structure [13].

3.2. Expression and purification

On the basis of the nucleotide sequence of the putative α-glucosidase (TM0434), primers were synthesized and the open reading frame was cloned and expressed in *E. coli* BL21-CodonPlus-RIL under the control of the inducible phage T7 promoter of the vector, pET-28b(+). SDS–PAGE analysis of the cell lysate indicated the production of a protein of molecular mass 56 kDa as the major protein. The apparent molecular mass of the overexpressed protein as seen by SDS–PAGE was consistent with that of the molecular mass 56129 Da, calculated based on the deduced amino acid sequence. The 56 kDa protein was not found in the transformants which did not carry the insert. The overproduced enzyme was purified from the cell lysate. Approximately 6 mg of pure protein was obtained from 1 l culture. Due to apparent instability of the enzyme only 10% activity was recovered after purification.

3.3. Substrate specificity and cofactor requirement

Even though the sequence similarity suggested this enzyme to be a putative α-glucosidase, no activity was observed on *p*NP-Glc. Thin layer chromatographic (TLC) analysis showed

Table 1
Amino acid sequence similarity of Agu to the α-glucosidases of *T. maritima* and *T. neapolitana*

Organism	Locus	GenBank accession no.	% Identity
T. maritima	TM0752[a]	AE001745	61
	TM1068[a]	AE001767	99
	TM1834	AJ001089	52
T. neapolitana	TNE9832	AJ009832	50

[a]Putative proteins.

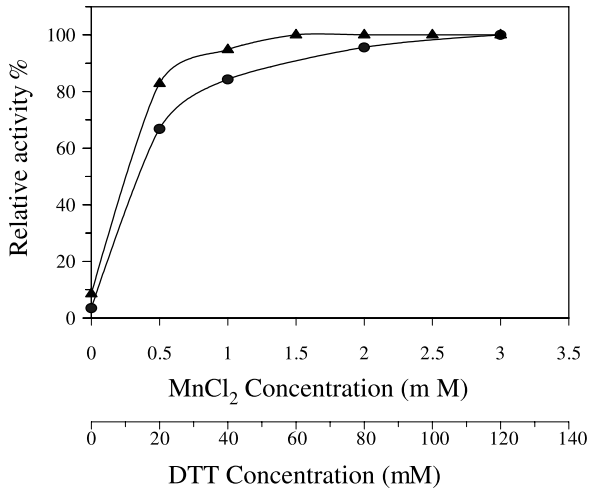

Fig. 1. Effects of Mn^{2+} (circles) and DTT (triangles) on purified Agu in the presence of 50 mM DTT and 1 mM $MnCl_2$, respectively.

that Agu was not active against maltose either. But the enzyme displayed the ability to hydrolyze *p*NP-GUA, an activity not present in the host cells harboring the vector without insert. This activity pattern suggested that Agu was an α-glucuronidase. TLC analysis showed that the enzyme lacked activity on 4-*O*-methyl-D-glucuronoxylan. In view of these observations, further investigation was carried out with *p*NP-GUA as substrate. The enzyme however showed low activity on this substrate. Since some of the family 4 enzymes are known to require Mn^{2+} and NAD^+ as cofactors for maximum activity [7], the activity of Agu was examined in the presence of these cofactors. While $MnCl_2$ resulted in slight increase in the activity, significant increase in activity was observed when the enzymatic reaction was carried out under reducing conditions in the presence of DTT. NAD^+ did not have any effect on the activity of the enzyme on any of the substrates. Concentrations of 1 mM $MnCl_2$ and 50 mM DTT in the reaction mixture were needed for maximum activity of the enzyme (Fig. 1). If either of the two cofactors was not provided the activity reduced to almost basal level. The reason for the requirement of high concentration of reducing agent for maximum activity of the enzyme is not known. However, a similar concentration of DTT requiring α-glucosidase of *T. maritima* is known to literature [7]. Substitution of DTT by 2-mercaptoethanol resulted in maximum activity of the enzyme at a concentration of 50 mM. Replacement of $MnCl_2$ with other divalent metal ions, Li^{2+}, Ca^{2+} and Mg^{2+} did not result in any appreciable increase in the basal enzyme activity. The other metal ions checked, Zn^{2+}, Cu^{2+}, Co^{2+} and Fe^{2+}, were not compatible with the reaction mixture. The enzyme did not show any ability to hydrolyze *p*NP-Glc and maltose even in the presence of the metal ions mentioned above and also under reducing conditions.

The ability of Agu to hydrolyze *p*NP-GUA is rather unusual as none of the microbial α-glucuronidases tested exhibited any significant activity towards this substrate [14–16]. Though α-glucuronidases (EC 3.2.1.139) are responsible for the hydrolysis of α-1,2-glycosidic linkage between xylose and D-glucuronic acid or its methyl ether [17,18], most of the α-glucuronidases studied are known to liberate 4-*O*-methylglucuronic acid from substituted xylo-oligosaccharides or from polymeric xylan [19–21]. The attachment of uronic acid to at least one xylanopyranosyl residue appears to be essential for the activity of microbial α-glucuronidases [14,22]. However, Fontana et al. [23] have reported *p*NP-GUA hydrolyzing activity of the snail α-glucuronidases. Earlier this activity was reported in the enzyme preparation from limpet (*Patella vulgata*) [24]. Kawabata et al. [15] later characterized an α-glucuronidase from snail acetone powder with *p*NP-GUA hydrolyzing activity. To our knowledge, prior to the current study, no microbial α-glucuronidase that had *p*NP-GUA hydrolyzing activity was known.

3.4. Properties of Agu

The enzyme showed a temperature optimum of 60°C. But the enzyme when assayed after a 30 min preincubation at temperatures ranging from 15 to 100°C showed partial inactivation at all the temperatures (Fig. 2). Incubation at 50°C for 30 min resulted in a loss of approximately 70% of the enzyme activity, thus displaying a distinct thermal instability. It is surprising to note that the α-glucuronidase under investigation is thermolabile as all the enzymes of *T. maritima* studied to date are shown to be extremely thermostable [5,25]. However, the enzyme displayed an interesting feature where the activity loss with every increment in the preincubation temperature was very high, up to 50°C. Above 50°C the activity loss was gradual and the enzyme retained 5–10% residual activity after incubation at 80°C for 30 min. This suggests a role for the enzyme in the hyperthermophilic bacterium *T. maritima* that has an optimum growth temperature of 80°C. The enzyme inactivation pattern did not vary in the absence or under different concentrations of DTT tested (up to 50 mM). In the presence of the substrate (3 mM) the enzyme was stable up to 40°C for a tested period of 30 min. During storage at 4°C, loss of approximately 30% activity was observed in the first 72 h. Subsequently no significant activity loss was seen for a tested period of 10 days. Agu has two cysteine residues with a possibility of an S–S bond. Since the structure of the protein is unknown, the reason for lack of thermostability is not known. As a detailed analysis of thermostability of *T. maritima* proteins has been made [5] it may be interesting to study the relative instability of Agu. Agu displayed maximum activity at pH 7.8. When incubated at room temperature for 30 min the enzyme was stable from pH 5 to pH 11 retaining 50%

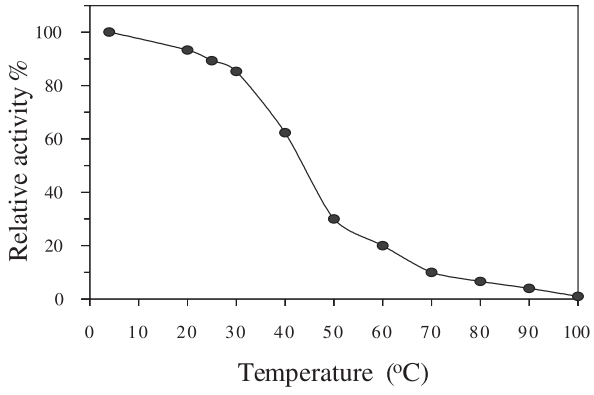

Fig. 2. Thermal inactivation of Agu in the absence of substrate. Purified enzyme (0.36 mg ml^{-1}) in 50 mM HEPES buffer, pH 7.5 was incubated at various temperatures for 30 min. Residual activity was measured under standard assay conditions. Activity of the enzyme measured prior to incubation was considered as 100% activity.

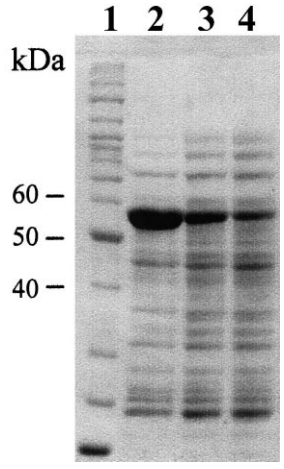

Fig. 3. Differential expression of Agu by inducing with varying IPTG concentrations. Lane 2, 0.4 mM IPTG; lane 3, 0.05 mM IPTG; lane 4, 0.005 mM IPTG; lane 1, molecular mass standards (10 μg protein loaded into each lane).

activity at pH 11.5. The kinetic parameters for *p*NP-GUA were determined to be $K_m = 1.0$ mM and $k_{cat} = 0.0018$ s^{-1}. The α-glucuronidases studied so far are reported in general to have low specific activities and substrate affinities [13]. The *p*NP-GUA hydrolyzing enzyme of *Helix pomatia* displayed a K_m of 0.13 mM [15].

3.5. Differential protein expression

The possibility of the enzyme losing α-glucosidase activity due to the protein overexpression was taken into consideration since misfolding during the cellular expression in the host cell due to high-level protein expression is known [26]. In order to study this possibility, differential levels of enzyme expression were obtained by inducing the expression with varying concentrations of IPTG (Fig. 3). Even in the lowest expression level the enzyme lacked α-glucosidase activity on the substrates *p*NP-Glc and maltose whereas the enzyme expressed at all levels showed *p*NP-GUA hydrolyzing activity.

3.6. Agu belongs to family 4 of glycosyl hydrolases

According to the classification of glycosyl hydrolases based on amino acid sequence similarities, Agu belongs to the family 4. This family comprises of 6-phospho-β-glucosidase, 6-phospho-α-glucosidase, α-galactosidase and α-glucosidase. To date, no α-glucuronidases are known to belong to family 4 as they are generally assigned to family 67 of glycosyl hydrolases [27,28]. Therefore, this is the first report of an α-glucuronidase which belongs to family 4 of glycosyl hydrolases. Agu showed no activity of 6-phospho-α-glucosidase and α-D-galactosidase, both family 4 enzymes, when *p*NP-G6P and *p*-nitrophenyl α-D-galactopyranoside were used as substrates.

The α-glucuronidase characterized in this study appears unique in having an activity that is not known in the family of enzymes to which it belongs, rare substrate specificity, relative thermal instability and the requirement of high concentration of reducing agent for activity. Studies on the gene regulation and structure–function relationship will help in the better understanding of the nature of this enzyme and its role in the bacterium.

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