ORIGINAL RESEARCH PAPER

Characterization of an acid-labile, thermostable β-glycosidase from *Thermoplasma acidophilum*

Hye-Jung Kim · Ah-Reum Park · Jung-Kul Lee · Deok-Kun Oh

Abstract A recombinant putative β-galactosidase from *Thermoplasma acidophilum* was purified as a single 57 kDa band of 82 U mg^{-1}. The molecular mass of the native enzyme was 114 kDa as a dimer. Maximum activity was observed at pH 6.0 and 90°C. The enzyme was unstable below pH 6.0: at pH 6 its half-life at 75°C was 28 days but at pH 4.5 was only 13 h. Catalytic efficiencies decreased as *p*-nitrophenyl(*p*NP)-β-D-fucopyranoside (1067) > *p*NP-β-D-glucopyranoside (381) > *p*NP-β-D-galactopyranoside (18) > *p*NP-β-D-mannopyranoside (11 s^{-1} mM^{-1}), indicating that the enzyme was a β-glycosidase.

Keywords Characterization · β-Glycosidase · pH-sensitive enzyme · *Thermoplasma acidophilum* · Thermostable enzyme

Introduction

β-Glucosidase (EC 3.2.1.21) is a key enzyme for carbohydrate metabolism in bacteria, fungi, and yeast. The enzyme catalyzes the hydrolysis of β-glucosidic bond of cellulose to degrade cello-oligosaccharides to glucose. β-Glucosidase plays in many biological processes such as the release of aromatics from flavorless glucosidic precursors, detoxification of cyanogenic glycosides, and synthesis of useful β-glucosides (Bhat and Bhat 1997; Bhatia et al. 2002; Chuankhayan et al. 2007).

Although many β-glucosidases have been isolated from fungi, relatively few have been extracted from archaebacteria. Due to their inherent thermostability, hyperthermophilic β-glucosidases from archaebacteria are more useful for glucose production from cello-oligosaccharides than the enzymes from mesophilic and thermophilic bacteria. The gene sequences of hyperthermophilic β-glycosidases from archaebacteria including *Pyrococcus horikoshii* (Matsui et al. 2000), *P. furiosus* (Kengen et al. 1993), *Sulfolobus solfataricus* (D'Auria et al. 1996), *Thermotoga maritima* (Gabelsberger et al. 1993), and *Thermotoga neapolitana* (Zverlov et al. 1997) have been cloned and their expressed enzymes have been characterized. Family 1 glycoside hydrolases from archaeabacteria exhibit broad substrate specificity (Bhatia et al. 2002). To find a new thermostable β-glucosidase, gene sequences within this family were searched for a β-glycosidase gene, and then we obtained a non-characterized *bglA* gene from *Thermoplasma acidophilum*. However, upon evaluation of substrate specificity, its expressed enzyme (TaBglA) was identified as a β-glycosidase.

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In this study, TaBglA was purified and its biochemical properties, including optimum pH and temperature, pH stability, thermostability, and substrate specificity, were investigated.

Materials and methods

Microorganisms, culture conditions, and media

Thermoplasma acidophilum DSM 1728, used as gene source, was cultivated in medium containing 10 g glucose l^{-1}, 1 g yeast extract l^{-1}, 2 g $(NH_4)_2SO_4$ l^{-1}, 3 g KH_2PO_4 l^{-1}, 0.5 g $MgSO_4 \cdot 7H_2O$ l^{-1}, and 0.25 g $CaCl_2 \cdot 2H_2O$ l^{-1}. The pH of the medium was adjusted to two with 5M H_2SO_4. Cultures were grown at 60°C under aerobic conditions for 2 days. Recombinant *Escherichia coli* for protein expression were cultivated in a LB medium at 37°C with 50 μg ampicillin ml^{-1} for 24 h.

Gene cloning and enzyme expression

The genomic DNA was isolated from harvested cells of *T. acidophilum* using genomic DNA buffer set (Qiagene, Hilden, Germany). The *bglA* gene sequence was obtained from the DNA sequence of *T. acidophilum* previously proposed as *β*-galactosidase (GenBank accession number NP 394779). The PCR product was subcloned into the pHCE plasmid vector (Takara, Tokyo, Japan) using restriction enzyme *Eco*RI and *Hin*dI and then transformed into *E. coli* ER2566 (New England Biolabs, Hertfordshire, UK).

Enzyme assay

The assay of TaBglA was performed in 100 mM citrate/phosphate buffer (pH 6.0) containing 1 mM *p*-nitrophenyl-*β*-D-glucopyranoside (*p*NPGlu) and 0.15 U enzyme ml^{-1} at 90°C for 5 min. The activity was measured by reading the increase in absorbance at 415 nm as a result of *p*-nitrophenol (*p*NP) release. One unit (U) of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol *p*NP per min at 90°C and pH 6.0.

Results and discussion

Enzyme expression and purification

The enzyme investigated in this study was expressed in *Escherichia coli* by a *bglA* gene of 1,452 bp (GenBank accession number NP 394779) from *T. acidophilum*. The amino acid sequence of the resulting enzyme exhibited 49, 48, 47, and 46% identity with those of *Picrophilus torridus* *β*-galactosidase, *Caldivirga maquilingensis* family 1 glycoside hydrolase, *S. solfataricus* *β*-galactosidase, and *P. furiosus* *β*-glycosidase, respectively. These results indicate that the enzyme had no significant similarities with any other glycoside hydrolases isolated from archaeabacteria. The enzyme was purified with a specific activity of 82 U mg^{-1} (Table 1) and as a single band of 57 kDa in SDS-PAGE (Fig. 1a). Using MALDI-TOF-MS (AXIMA-CFR, Shimadzu, Kyoto, Japan) equipped with a 337-nm N_2 laser and calibrated with aldolase (39,212 Da) and albumin (66,429 Da), the subunit molecular mass of the protein was

Table 1 Purification of *T. acidophilum* *β*-glycosidase

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Yield (%)	Purification (fold)
Crude extract	243	299	1.2	100	1
Heat treatment	7.2	264	37	88	31
Hi-Trap HP	2.2	181	82	61	68

The cells were harvested from culture and then resuspended in 50 mM Tris/HCl buffer (pH 7.0). The resuspended cells were disrupted using a sonicator. The crude enzyme from disrupted cell was heated at 85°C for 10 min and then centrifuged at 4°C to remove denatured *E. coli* proteins. The enzyme solution was applied onto a Hi-Trap HP column and eluted with linear gradient to 500 mM NaCl. The active fraction was dialyzed at 4°C for 16 h against 100 mM citrate/phosphate buffer (pH 6.0) and the resulting solution was used as a purified enzyme

estimated as $57{,}071 \pm 65$ Da. The native enzyme existed as a dimer with a molecular mass of 114 kDa as determined by gel filtration (Fig. 1b).
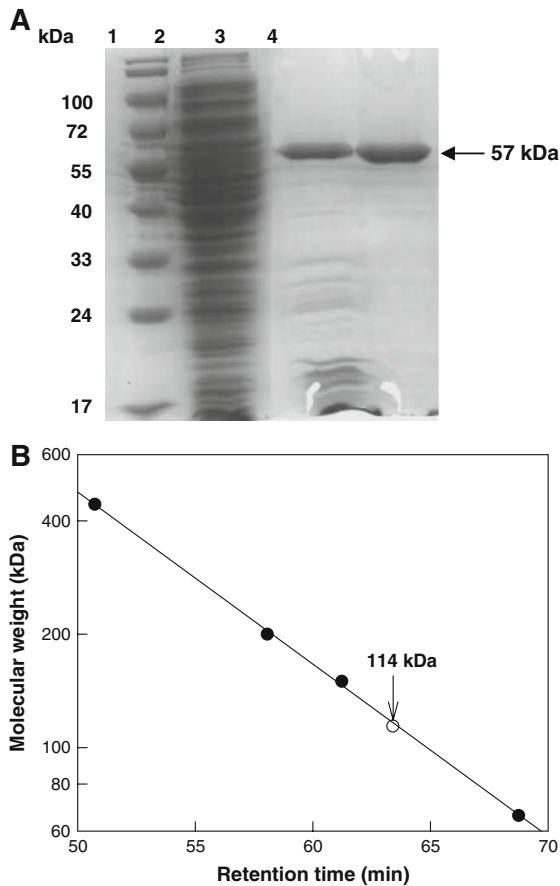

Fig. 1 **a** SDS-PAGE analysis of purified enzyme from each purification step. *Lane 1*, prestained marker proteins (170, 130, 100, 72, 55, 40, 33, 24, and 17 kDa); *lane 2*, crude extract; *lane 3*, supernatant after heat treatment at 85°C for 10 min; *lane 4*, Hi-trap column product (purified enzyme). The subunit molecular mass of β-glycosidase was examined by SDS-PAGE under denaturing conditions, using the proteins of a pre-stained ladder (MBI Fermentas, Hanover, MD) as reference proteins. All protein bands were stained with Coomassie blue for visualisation. **b** Determination of molecular mass of β-glycosidase from *T. acidophilum* by gel-filtration chromatography. The molecular mass of native enzyme was determined using a Sephacryl S-300 preparative-grade column HR 16/60 (Amersham Biosciences, Uppsala, Sweden). The enzyme solution was applied to the column and eluted with 100 mM MacIlvaine buffer (pH 6.0) containing 150 mM NaCl at 1 ml min^{-1}. The column was calibrated with apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa) and albumin (66 kDa) as reference proteins (Amersham Biosciences) and the molecular mass of native enzyme was calculated by comparing with the migration length of reference proteins

Effects of pH and temperature on enzyme stability

The maximum activity of TaBglA at 75°C was initially at pH 4.5 but after 1 h at 75°C was at pH 6.0 (Fig 2a), indicating a relative instability below pH 6.0. Temperature effects were investigated at pH 4.5 and 6.0, with maximum activities observed at 80 and 90°C, respectively (Fig 2b). The decrease of optimum temperature occurred under destabilizing conditions at pH 4.5. Thus, enzyme activity was maximized at pH 6.0 and 90°C.

The amino acid sequence of *S. solfataricus* β-glycosidase exhibited 47% identity with enzyme herein, and the active site residues of both enzymes were highly conserved. The thermostability of β-glycosidase from *S. solfataricus* is determined by a network of ion pairs, located on the tetrameric surface, which results in destabilization of the enzyme under the basic conditions (D'Auria et al. 1997, 1998; Cobucci-Ponzano et al. 2002). Similarly, the destabilization of the enzyme isolated in this study below pH 6.0 may be due to ion pair interactions on the surface of the dimer.

The half-lives of the enzyme for incubation at pH 6.0 were 675, 58, 3.5, and 0.4 h at 75, 80, 85, and 90°C, respectively. These parameters changed significantly at pH 4.5, and were 167, 66, 13, and 0.5 h at 70, 75, 80, and 85°C, respectively (Fig. 3). The half-life of the enzyme at 75°C was decreased from 28 days to 13 h by shifting the pH from 6.0 to 4.5, indicating a significant decrease in thermostability. The half-lives of thermostable β-glycosidases from *P. furiosus*, *S. solfataricus* (Petzelbauer et al. 1999), *Thermus* sp. (Takase and Horikoshi 1988), *Thermus thermophilus* (Dion et al. 1999), *Thermoascus aurantiacus* (Leite et al. 2007), and *Chaetomium thermophilum* var. *coprophilum* (Venturi et al. 2002) have been reported as 60 days at 70°C, 10 days at 70°C, 5 days at 75°C, 30 min at 80°C, and 75 min at 70°C, respectively. Therefore, with the exception of β-glycosidase from *P. furiosus*, the enzyme described here exhibited the highest thermostability. Furthermore, the enzyme was not activated by the presence of monovalent or divalent cations and was not inhibited by EDTA (data not shown).

Substrate specificity

Table 2 shows the specific activities and kinetic parameters of the enzyme for aryl-β-glycosides. The
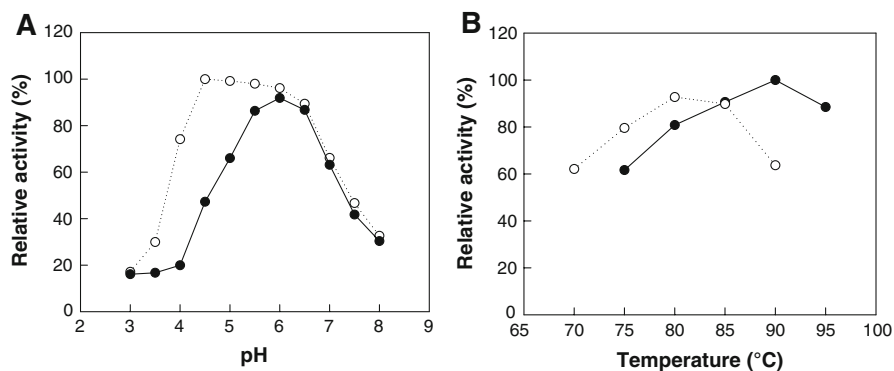
Fig. 2 a pH inactivation of β-glycosidase from *T. acidophilum*. The enzymes were incubated at 4 (*open circle*) and 75°C (*filled circle*) for 1 h using 100 mM citrate/phosphate buffer for pH values between 3.0 and 8.0. After incubation, enzyme activity was measured with 1 mM *p*NPGlu at 80°C for 5 min. The relative activity of 100% was 0.14 U enzyme ml^{-1}.

b Effect of temperature on enzyme activity at pH 4.5 or pH 6.0. The enzyme reactions were performed with 1 mM *p*NPGlu for 5 min at temperature ranging of 70 to 85°C at pH 4.5 (*open circle*) and of 75 to 95°C at pH 6.0 (*filled circle*). The relative activity of 100% was 0.15 U enzyme ml^{-1}
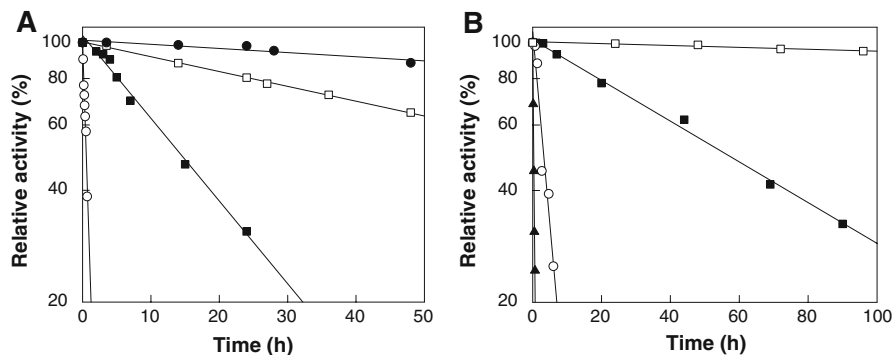

Fig. 3 Thermal inactivation of β-glycosidase from *T. acidophilum*. The enzymes were incubated at (**a**) pH 4.5 and (**b**) pH 6.0 for varying temperatures and periods of time. Temperatures were 70 (*filled circle*), 75 (*open square*), 80 (*filled square*), 85 (*open circle*), and 90°C (*filled triangle*). A sample was withdrawn at each time interval and the relative activity was

determined. The experimental data for thermal deactivation of enzyme were fitted to a first order curve and the half-lives of the enzyme calculated using SigmaPlot 9.0 software (Systat Software, San Jose, CA). After incubation, enzyme activity was measured with 1 mM *p*NPGlu at 90°C for 5 min. The relative activity of 100% was 0.15 U enzyme ml^{-1}

hydrolyzing activity of the enzyme was much higher with substrates containing β-1-4 linkages, such as *p*NPF and *p*NPGlu, than with substrates containing β-1-2 linkages, such as *o*NPF and *o*NPGlu. Catalytic efficiencies decreased as *p*NPF (1067) > *p*NPGlu (381) > *p*NPM (18) > *p*NPGal (11 s^{-1} mM^{-1}). The values of k_{cat}/K_m for *p*NPF and *p*NPGlu were 97- and 35-fold higher than that obtained with *p*NPGal, respectively, indicating that the enzyme investigated herein was not a β-galactosidase as previously proposed, but rather a β-glycosidase.

In conclusion, the non-characterized *bglA* gene from *T. acidophilum* was cloned and expressed. The values of k_{cat}/K_m of the enzyme with *p*NPF and *p*NPGlu were 97- and 35-fold higher than that obtained with *p*NPGal, respectively, indicating that the enzyme was a β-glycosidase. Because the enzyme was unstable below pH 5.5, its half-life at 75°C decreased from 28 days to 13 h upon shifting the pH from 6.0 to 4.5. Thus, TaBglA, when stabilized at pH 6.0, can be used effectively for degradation of cello-oligosaccharides to glucose.

Table 2 Specific activities and kinetic parameters of *T. acidophilum* β-glycosidase

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})	Specific activity (U mg^{-1})
*p*NPGlu	0.4	141	381	83
*p*NPF	0.2	182	1,068	113
*p*NPGal	1.5	27	18	8.5
*p*NPM	1.3	14	11	4.9
*o*NPF	ND	ND	ND	1.7
*o*NPGlu	ND	ND	ND	1.8
*o*NPGal	ND	ND	ND	0.7

ND Not determined

Specific activity is performed that reaction mixture was incubated at 90°C for 5–30 min and the activity was determined by release of *p*-nitrophenol. Various concentration of *p*NPGlu (from 0.05 mM to 1 mM), *p*NPF (from 0.05 mM to 1 mM), *p*NPGal (from 0.1 mM to 2 mM) and *p*NPM (from 0.1 mM to 2 mM) were used to determine kinetic parameters of the enzyme. The reaction was carried out in 100 mM citrate/phosphate buffer (pH 6.0) at 90°C for 5 min. No activity was detected for *p*-nitrophenyl-β-D-xylopyranoside, *p*-nitrophenyl-β-L-arabinopyranoside, *p*-nitrophenyl-α-D-glucopyranoside, *p*-nitrophenyl-α-D-galactopyranoside, and *p*-nitrophenyl-α-L-arabinopyranoside. *p*NPGlu: *p*-nitrophenyl-β-D-glucopyranoside, *p*NPF: *p*-nitrophenyl-β-D-fucopyranoside, *p*NPGal: *p*-nitrophenyl-β-D-galactopyranoside, *p*NPM: *p*-nitrophenyl-β-D-mannopyranoside, *o*NPF: *o*-nitrophenyl-β-D-fucopyranoside, *o*NPGlu: *o*-nitrophenyl-β-D-glucopyranoside, *o*NPGal: *o*-nitrophenyl-β-D-galactopyranoside

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