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Hydrolysis and Transglycosylation Activity of a Thermostable Recombinant β-Glycosidase from *Sulfolobus acidocaldarius*

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Abstract We expressed a putative β-galactosidase from *Sulfolobus acidocaldarius* in *Escherichia coli* and purified the recombinant enzyme using heat treatment and Hi-Trap ion-exchange chromatography. The resultant protein gave a single 57-kDa band by SDS-PAGE and had a specific activity of 58 U/mg. The native enzyme existed as a dimer with a molecular mass of 114 kDa by gel filtration. The maximum activity of this enzyme was observed at pH 5.5 and 90ºC. The half-lives of the enzyme at 70, 80, and 90ºC were 494, 60, and 0.2 h, respectively. The hydrolytic activity with *p*-nitrophenyl(*p*NP) substrates followed the order *p*-nitrophenyl-β-D-fucopyranoside>*p*NP-β-D-glucopyranoside>*p*NP-β-D-galactopyranoside>*p*NP-β-D-mannopyranoside>*p*NP-β-D-xylopyranoside, but not toward aryl-α-glycosides or *p*NP-β-L-arabinofuranoside. Thus, the enzyme was actually a β-glycosidase. The β-glycosidase exhibited transglycosylation activity with *p*NP-β-D-galactopyranoside, *p*NP-β-D-glucopyranoside, and *p*NP-β-D-fucopyranoside in decreasing order of activity, in the reverse order of its hydrolytic activity. The hydrolytic activity was higher toward cellobiose than toward lactose, but the transglycosylation activity was lower with cellobiose than with lactose.

Keywords β-Glycosidase · *Sulfolobus acidocaldarius* · Hydrolytic activity · Transglycosylation activity · Thermostable enzyme

Introduction

β-Glycosidases (β-glucoside glucohydrolases; EC 3.2.1.21) cleave β-glucosidic bonds in disaccharides or between saccharide and aglycon groups. These enzymes have broad

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specificity toward β-glucosides, β-galactosides, β-fucosides, and β-xylosides [1], and they also catalyze transglycosylation between donor and acceptor glycosides. In the transglycosylation reaction, a donor glycoside such as a disaccharide or aryl-linked glycoside is hydrolyzed concomitant with the formation of an enzyme–glycosyl intermediate. The glycosyl group is then transferred to a nucleophile acceptor glycoside other than water, such as a monosaccharide, disaccharide, or an aryl-, amino-, or alkyl- alcohol, to yield a new elongated product [2]. Thus, the transglycosylation activity of β-glycosidases can be exploited in the synthesis of a variety of biologically important compounds [3].

Enzymes from hyperthermophilic bacteria are particularly attractive for industrial uses because of their thermostability and resistance to organic solvents [4]. High temperature promotes high initial productivity of these enzymes and increases the solubility of their substrates in the aqueous phase. Thus, hyperthermophilic β-glycosidases can provide superior hydrolysis and transglycosylation activity at high temperatures [5–8]. Several β-glycosidases from hyperthermophiles, including *Pyrococcus furiosus* [9], *Pyrococcus kodakaraensis* [10], *Sulfolobus solfataricus* [9], and *Thermus thermophilus* [11] exhibit hydrolysis and transglycosylation activity, but the details of their activity profiles toward various substrates have not yet been reported.

In the present study, a gene encoding a putative β-galactosidase from *S. acidocaldarius* was expressed in *E. coli*, and the expressed protein was purified and characterized. The purification scheme, molecular mass, and biochemical properties of the recombinant enzyme, as well as its hydrolytic and transglycosylation activities toward various substrates, are reported here.

Materials and Methods

Bacterial Strains, Plasmid, and Culture Conditions

The genomic DNA from *S. acidocaldarius* DSMZ 639 (DSMZ, Brauschweig, Germany), *E. coli* ER2566 (New Englands Biolabs, Herfordshire, UK), and pTrc-99a(+) plasmid were used as a source of β-glycosidase gene (*bgaS*), a host, and a vector for expression, respectively. *S. acidocaldarius* was grown anaerobically at 70ºC in a 7-l fermentor (Biotron, Bucheon, Korea) on *Sulfolobus* medium (DSM media formulation No.88) for 5 days. The recombinant *E. coli* for protein expression was cultivated in a 2-L flask containing 500 ml of Luria-Bertani (LB) medium and 50 μg/ml of ampicillin at 37ºC with agitation at 200 rpm until the optical density of bacteria reached 0.8 at 600 nm. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture medium at a final concentration of 0.1 mM, and the enzyme was induced and expressed at 37ºC for 9 h.

Cloning and Expression

The gene encoding β-glycosidase (*bgaS*) was amplified from the genomic DNA of *S. acidocaldarius* by PCR using *Pfu* DNA polymerase (Solgent, Daejeon, Korea). The sequence of the oligonucleotide primers used for gene cloning was based on the DNA sequence of the *S. acidocaldarius bgaS* gene (GenBank accession number, YP 256448). Forward (SA-F, 5′-GAATTCATGTTATCATTCCCAAAGGGTTTC-3′) and reverse primers (SA-R, 5′-CTGCAGTTAATGTCTCAAAGGTTTTATTGGTGG-3′) were designed to introduce the *Eco*RI and *Pst*I restriction sites (underlined) for *bgaS* gene cloning. The amplified DNA fragment obtained by PCR was purified and digested with both *Eco*RI and

*Pst*I endonucleases (New England Biolabs, Hertfordshire, UK). The digested DNA fragment was extracted, and then inserted into the pTrc-99a(+) vector digested with the same restriction enzymes. *E. coli* ER2566 strain was transformed with the ligation mixture and plated on LB agar containing ampicilin. An ampicilin-resistant colony was selected, and plasmid DNA from the transformant was isolated using a plasmid purification kit (Promega, Madison, WI, USA). DNA sequencing was performed at the facility of Solgent (Daejeon, Korea). The expression of *bgaS* gene was determined by both SDS-PAGE and used on the assay of enzyme activity.

Enzyme Assay

The assay of β-glycosidase was performed in 50 mM citrate buffer (pH 5.5) containing 1 mM *p*-nitrophenyl-β-D-glucopyranoside (*p*NPGlu) and 0.23 U/ml enzyme at 90ºC 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 of *p*NP/min at 90ºC and pH 5.5. The assay of the other aryl glycosides was determined under the same conditions. The reactions using were stopped by adding HCl with a final concentration of 0.2 M. When the stopping reactions with 0.2 M HCl at 90ºC were performed without the enzyme, *p*NP was not formed.

Enzyme Purification

The grown cells were harvested and disrupted by sonication in 50 mM Tris–HCl buffer (pH 7.0) containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF) as protease inhibitor. The unbroken cells and cell debris were removed by centrifugation at 15,000×*g* for 20 min at 4ºC, and the supernatant obtained was used as a crude extract. The crude extract was then heated at 80ºC for 5 min to remove denaturated *E. coli* proteins, and the suspension was centrifuged at 15,000 × *g* for 20 min. The enzyme solution was applied onto a Hi-Trap Q HP column (Amersham Biosciences, Uppsala, Sweden) equilibrated with 50 mM Tris–HCl buffer (pH 7.0). The column was washed with 50 ml of the same buffer and eluted with the Tris–HCl (pH 7.0) buffer containing 500 mM NaCl. Each fraction was analyzed in SDS-PAGE and the fractions containing the enzyme were collected. The active fraction was dialyzed at 4ºC for 16 h against 50 mM citrate buffer (pH 5.5) and the resulting solution was used as a purified enzyme. The purification step using the column was carried out by a fast protein liquid chromatography (FPLC) system (Bio-rad, Hercules, CA, USA) in a cold room. *S. acidocaldarius* cell-free extract was purified as a previous reported method [12].

Molecular Mass Determination

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), USA as reference proteins. All protein bands were stained with Coomassie blue for visualization. The molecular mass of native enzyme was determined by gel filtration chromatography using a Sephacryl S-300 HR 16/60 preparative-grade column (Amersham Biosciences). The enzyme solution was applied to the column and eluted with 50 mM citrate buffer (pH 5.5) buffer containing 150 mM NaCl at a flow rate of 1 ml/min. The column was calibrated with β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa), and ovalbumin (43 kDa) as reference proteins (Amersham Biosciences)

and the molecular mass of the native enzyme was calculated by comparing with the migration length of reference proteins.

Effects of pH and Temperature on Enzyme Activity

To examine the effects of pH and temperature on the enzyme, pH values were varied from 4.5 and 7.0 using 50 mM citrate buffer (pH 4.5−6.0) and 50 mM sodium phosphate buffer (pH 6.0−7.0) at 90ºC and temperature were varied from 80 to 100ºC at pH 5.5. The reactions were performed with 1 mM *p*NPGlu and 0.23 U/ml enzyme for 5 min. The influence of temperature on enzyme stability was monitored as a function of incubation time by placing the enzyme solution at five different temperatures (70, 75, 80, 85, and 90ºC) in 50 mM citrate buffer (pH 5.5). To test the thermostability of *S. acidocaldarius* β-glycosidase, 0.23 U/ml enzyme was incubated in 70, 75, 80, 85, and 90ºC for varying periods of time. A sample was withdrawn at time interval and was assayed in 50 mM citrate buffer (pH 5.5) at 85ºC for 5 min. The half-lives of the enzyme were calculated using Sigma Plot 9.0 software (Systat Software, San Jose, CA, USA).

Hydrolytic Activity

The hydrolytic activity of β-glycosidase was determined using various aryl glycosides including *o*-nitrophenyl-β-D-fucopyranoside (*o*NPFuc), *o*-nitrophenyl-β-D-glucopyranoside (*o*NPGlu), *o*-nitrophenyl-β-D-galactopyranoside (*o*NPGal), *p*-nitrophenyl-β-D-fucopyranoside (*p*NPFuc), *p*-nitrophenyl-β-D-glucopyranoside (*p*NPGlu), *p*-nitrophenyl-β-D-galactopyranoside (*p*NPGal), *p*-nitrophenyl-β-D-mannopyranoside, *p*-nitrophenyl-β-D-cellobiose, *p*-nitrophenyl-β-D-xylopyranoside, *p*-nitrophenyl-β-D-lactopyranoside, *p*-nitrophenyl-β-D-maltopyranoside, *p*-nitrophenyl-α-D-glucopyranoside, *p*-nitrophenyl-α-D-galactopyranoside, *p*-nitrophenyl-α-L-arabinofuranoside, and *p*-nitrophenyl-β-L-arabinofuranoside (Sigma, St. Louis, MO, USA). The reactions were performed in citrate buffer (pH 5.5) at 90ºC for 5 min and the activity was measured by release of *p*NP and *o*NP.

Kinetic Analysis

Various concentrations of *o*NPFuc (from 0.05 mM to 1 mM), *p*NPFuc (from 0.05 mM to 1 mM), *o*NPGlu (from 0.1 mM to 2 mM), *p*NPGlu (from 0.25 mM to 2 mM), *o*NPGal (from 0.25 mM to 4 mM), and *p*NPGal (from 0.25 mM to 4 mM) were used to determine kinetic parameters of the enzyme. The reactions were performed in citrate buffer (pH 5.5) at 90ºC for 5 min. The enzyme kinetic parameters, K_m (mM) and k_{cat} (s^{-1}) values were determined by fitting to the Michaelis–Menten equation.

Transglycosylation Activity

The transglycosylation activity of the recombinant β-glycosidase from *S. acidocaldarius* was determined using the donor/acceptor *p*NPFuc/D-fucose, *p*NPGlu/D-glucose, or *p*NPGal/D-galactose. The reactions were performed in 50 mM citrate buffer containing 23 U/ml enzyme, 100 mM each acceptor, and 50 mM each donor at 85ºC for 1 h. Transglycosylation activity was determined by measuring the total amounts of di- and trisaccharide products. The time course of transglycosylation was examined using reactions performed in 50 mM citrate buffer (pH 5.5) with 200 g/L lactose or cellobiose as the substrate and 23 U/ml enzyme 85ºC for 5 h.

Analytical Methods

The concentrations of lactose, glucose, galactose, galacto-oligosaccharides, and cello-oligosaccharides were analyzed with a Aminex HPX-87 k column (Bio-Rad Laboratories, Hercules, CA, USA), operated by an HPLC system (Agilent 1100 series, Santa Clara, CA, USA) equipped with a Agilent G1362A RID detector. The eluant was water at a flow rate of 0.4 ml/min. The authentic standard sugars of 3-*O*-(4-*O*-[3-*O*-α-D-galactopyranosyl-β-D-galactopyranosyl]-α-D-galactopyranosyl)-D-galactopyranose and 4-*O*-(3-*O*-α-D-galactopyranosyl-β-D-galactopyranosyl)-D-galactopyranose were purchased from Sigma and showed the same retention times of trisaccharide and tetrasaccharide of the reaction products, respectively [13–15]. Cellotriose and cellotetraose were purchased from Carbosynth (Berkshire, UK).

The molecular masses of trisaccharide and tetrasaccharide fractions were confirmed by LC-MS with ESI mode (1100 series LC/MSD, Agilent). Furthermore, after hydrolysis with trifluoroacetic acid and neutralization with ammonia water, the ratio of galactose to glucose of each galacto-oligosaccharide fraction was also determined by a Bio-LC with an electrochemical detector (Dionex ED-50) using a Dionex CarboPac MA1 column with 612 mM sodium hydroxide at a flow rate of 0.4 ml/min [14].

Results

Gene Cloning and Enzyme Expression

A gene of 1,476 bp encoding a putative β-galactosidase (*bgaS*) from *S. acidocaldarius*, with the same sequence as that reported in GenBank (accession number YP 256448), was cloned and expressed in *E. coli*. The amino acid sequence of the resultant enzyme exhibited 72, 55, 52, and 51% identities with β-glycosidases from *S. solfataricus*, *Thermoplasma volcanium*, *P. furiosus*, and *Thermosphaera aggregans*, respectively. The results suggest that the enzyme is a β-glycosidase.

Enzyme Purification and Molecular Mass

The enzyme was purified from crude extract obtained from harvested cells as a soluble protein via heat treatment and Hi-Trap ion-exchange chromatography (Table 1). The β-glycosidase from *S. acidocaldarius* was purified with a final purification of 41-fold, with a yield of 74%, and a specific activity of 58 U/mg (micromole per milligram per minute).

Proteins obtained at each purification step were analyzed by SDS-PAGE and the final purified enzyme showed a single band with a molecular mass of approximately 57 kDa (Fig. 1a), consistent with the calculated value of 57,143 Da based on the 491-residue amino

Table 1 Purification of *S. acidocaldarius* β-glycosidase.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	240	330	1.4	100	1
Heat treatment	5.1	262	51	79	36
Hi-Trap HP	4.2	244	58	74	41

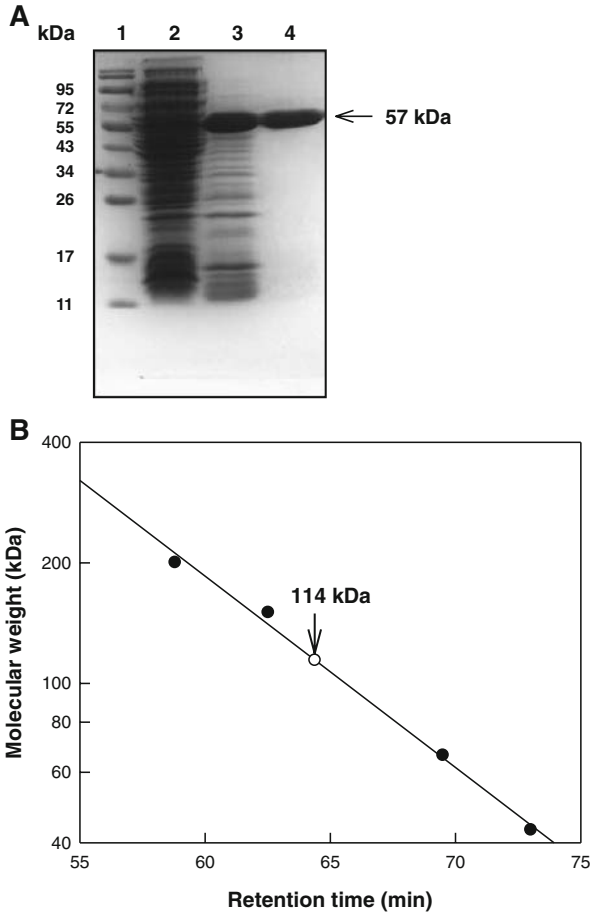

Fig. 1 SDS-PAGE analysis of purified enzyme. *Lane 1* marker proteins (170, 130, 95, 72, 55, 43, 34, 26,17, and 11 kDa); *lane 2* crude extract; *lane 3* supernatant after heat treatment at 80ºC for 5 min; *lane 4* Hi-Trap column product (purified enzyme). **b** Determination of molecular mass of *S. acidocaldarius* β-glycosidase by gel filtration chromatography

acid sequence. The native enzyme existed as a dimer with a molecular mass of 114 kDa as determined by gel filtration chromatography, using a Sephacryl S-300 HR 16/60 column, based on the masses of reference proteins (Fig. 1b).

Effects of pH and Temperature on the Enzyme Activity

The activity of the β-glycosidase from *S. acidocaldarius* was examined over a pH range of 4.5 to 7.0. The maximal enzyme activity was observed at pH5.5 (Fig. 2a). At pH 5.0 and 6.0, the activity was approximately 80% of the maximum. The effect of temperature on enzyme activity was investigated, and the maximum activity was recorded at 90ºC (Fig. 2b). At temperatures of 80 and 100ºC, the activities were 72% and 39% of the maximum, respectively.

The thermostability was examined by measuring the activity over time (Fig. 3). β-Glycosidase displayed first-order kinetics for thermal inactivation, and the half-lives of the enzyme at 70, 75, 80, 85, and 90ºC were 494, 453, 60, 9, and 0.2 h, respectively.

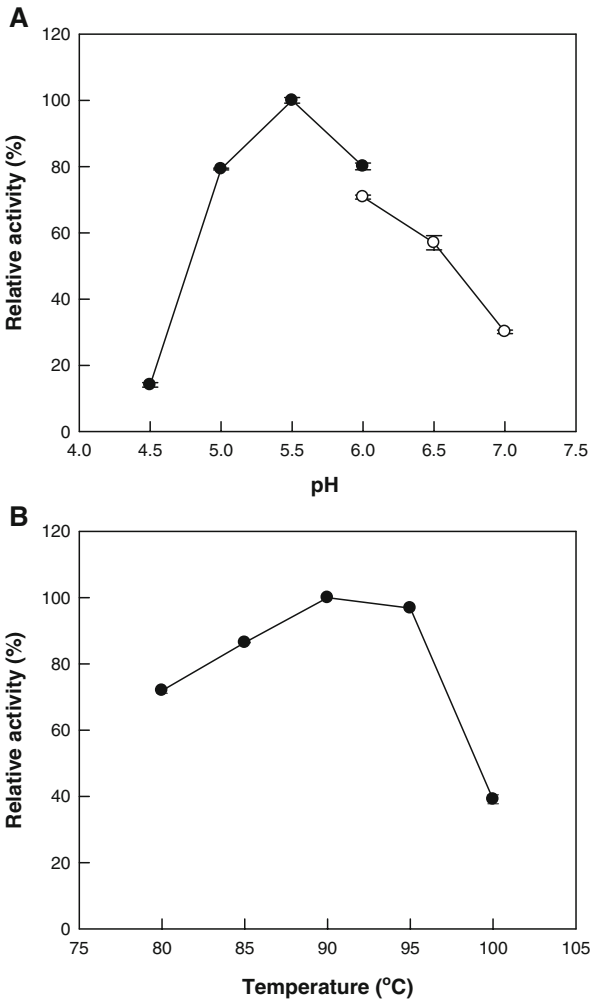

Fig. 2 Effects of temperature and pH of *S. acidocaldarius* β-glycosidase. **a** pH effect. The used buffer systems was 50 mM sodium citrate buffer in the range of pH 4.5–6.0 (*filled circles*) and 50 mM sodium phosphate buffer in the range of pH 6.0–7.0 (*empty circles*). **b** Temperature effect. The temperature range was 80–100°C in 50 mM sodium citrate buffer (pH 5.5). Data shown represent the means of three experiments and *error bars* represent standard deviation

Hydrolytic Activity

The hydrolytic activity of β-glycosidase from *S. acidocaldarius* was investigated with various aryl glycosides (Table 2). The hydrolytic activity for the *o*NP substrates followed the order *o*NPFuc>*o*NPGlu>*o*NPGal. For the *p*NP substrates, the activity followed the order *p*NPFuc>*p*NPGlu>*p*NPGal>*p*NP-β-D-mannopyranoside>*p*NP-β-D-xylopyranoside. However, the enzyme exhibited no activity for *p*NP-β-L-arabinofuranoside, and aryl-α-glycosides, including *p*NP-α-D-glucopyranoside, *p*NP-α-D-galactopyranoside, and *p*NP-α-L-arabinofuranoside. The hydrolytic activities of the enzyme for *o*NPGlu and *o*NPGal were 1.2- and 1.7-fold higher than for *p*NPGlu and *p*NPGal, respectively. The hydrolytic activities of

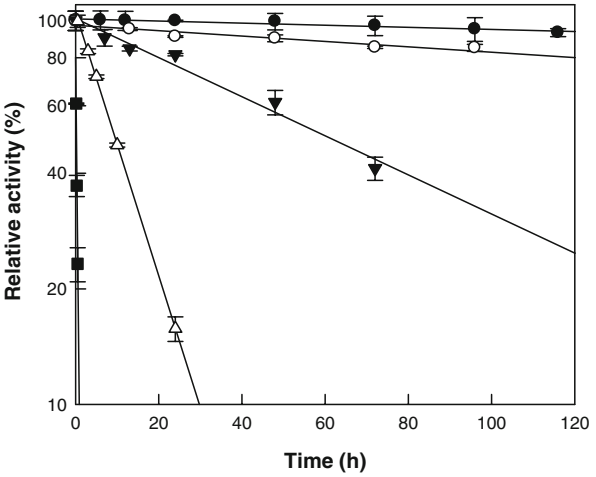

Fig. 3 Thermal inactivation of *S. acidocaldarius* β-glycosidase. The enzymes were incubated at 70 (*filled circles*), 75 (*empty circles*), 80 (*filled down triangles*), 85 (*empty triangles*), and 90ºC (*filled squares*) for varying periods of time. A sample was withdrawn at each time interval and the relative activity was determined

the β-galactosidases from *Bacillus megaterium* and *Arthrobacter* sp. for *o*NPGal were 12 and 19% higher than those for *o*NPGal, respectively [16, 17]. In contrast, the hydrolytic activities of the β-glycosidase from *Thermoplasma acidophilum* and the β-glucosidase from *Caldicellulosiruptor saccharolyticus* for *p*NPGlu (and *p*NPGal) were 46- and 43-fold (and 12- and 22-fold) higher than those for *o*NPGlu (and *o*NPGal), respectively [18, 19].

The Michaelis–Menten constants (K_m), turnover numbers (k_{cat}), and catalytic efficiencies (k_{cat}/K_m) for *o*NPFuc, *o*NPGlu, *o*NPGal, *p*NPFuc, *p*NPGlu, and *p*NPGal are presented in

Table 2 Hydrolytic activity of *S. acidocadarius* β-glycosidase.

Substrate	Relative activity (%)
o-Nitrophenyl-β-D-fucopyranoside	100±0.12
p-Nitrophenyl-β-D-fucopyranoside	73±0.43
o-Nitrophenyl-β-D-glucopyranoside	56±0.68
p-Nitrophenyl-β-D-glucopyranoside	47±0.21
o-Nitrophenyl-β-D-galactopyranoside	22±0.42
p-Nitrophenyl-β-D-galactopyranoside	13±0.19
p-Nitrophenyl-β-D-mannopyranoside	5.3±0.03
p-Nitrophenyl-β-D-xylopyranoside	1.6±0.01
p-Nitrophenyl-α-D-glucopyranoside	N.D
p-Nitrophenyl-α-D-galactopyranoside	N.D
p-Nitrophenyl-α-L-arabinofuranoside	N.D
p-Nitrophenyl-β-L-arabinofuranoside	N.D

Data represent the means of three separate experiments

ND activity is not detected by the analytical method used in this study.

Table 3 Kinetic parameters of *S. acidocaldarius* β-glycosidase for nitrophenyl-β-D-glycopyranosides.

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)
o-Nitrophenyl-β-D-fucopyranoside	116±4.2	26.4±0.54	228±9.1
p-Nitrophenyl-β-D-fucopyranoside	139±5.1	18.6±0.26	134±5.2
o-Nitrophenyl-β-D-glucopyranoside	138±1.8	16.7±0.47	121±3.8
p-Nitrophenyl-β-D-glucopyranoside	318±9.8	14.6±0.31	46±1.7
o-Nitrophenyl-β-D-galactopyranoside	3180±79	31.5±4.16	9.9±0.39
p-Nitrophenyl-β-D-galactopyranoside	1449±11	9.01±0.07	6.2±0.06

Data represent the means of three separate experiments

Table 3. The catalytic efficiencies decreased as *o*NPF (228), *p*NPF (133), *o*NPGlu (122), *p*NPGlu (46), *o*NPGal (10), and *p*NPGal (6 $s^{-1}mM^{-1}$). The k_{cat}/K_m values of the enzyme for *p*NPF and *p*NPGlu were 22- and eightfold higher than for *p*NPGal, respectively, indicating that the enzyme investigated herein was a β-glycosidase.

The native *S. acidocaldarius* β-glycosidase showed K_m values of 112 and 135 μM for *o*NPF and *o*NPGlu, respectively, k_{cat} values of 25.8 and 17.0 s^{-1} for *o*NPF and *o*NPGlu, respectively. The values of kinetic parameters determined for the recombinant β-glycosidase were similar to the native enzyme, with K_m values of 116 and 138 μM for *o*NPF and *o*NPGlu, respectively, k_{cat} values of 26.4 and 16.7 s^{-1} for *o*NPF and *o*NPGlu, respectively. The k_{cat}/K_m values for *o*NPF and *o*NPGlu of the native *S. acidocaldarius* β-glycosidase were 230 and 126 $mM^{-1}s^{-1}$, and those of the recombinant β-glycosidase were 228 and 121 $mM^{-1}s^{-1}$.

Transglycosylation Activity

The transglycosylation activity of the recombinant β-glycosidase from *S. acidocaldarius* was investigated with the donor/acceptor *p*NPF/D-fucose, *p*NPGlu/D-glucose, or *p*NPGal/D-galactose (Table 4). The transglycosylation activity followed the order *p*NPGal/D-galactose> *p*NPGlu/D-glucose>*p*NPF/D-fucose, which was the reverse of the order for the hydrolytic activity. The donor–acceptor with *p*NPGal/galactose exhibited 22-fold higher activity than that with *p*NPGlu/glucose.

We examined the time course of the transglycosylation reaction in experiments using 200 g/L lactose or cellobiose as the substrate in enzyme reactions performed at 85ºC for 5 h at pH 5.5 (Fig. 4). Glucose, galactose, and galacto-oligosaccharides (tri- and tetrasaccharides) were formed from lactose by hydrolysis and transgalactosylation.

Table 4 Transglycosylation activity of *S. acidocaldarius* β-glycosidase using a donor/acceptor.

Donor/acceptor	Relative activity of transglycosylation (%)
p-Nitrophenyl-β-D-galactopyranoside/D-galactose	100±0.56
p-Nitrophenyl-β-D-glucopyranoside/D-glucose	8±0.27
p-Nitrophenyl-β-D-fucopyranoside/D-fucose	ND

ND activity is not detected by the analytical method used in this study.

Data represent the means of three separate experiments

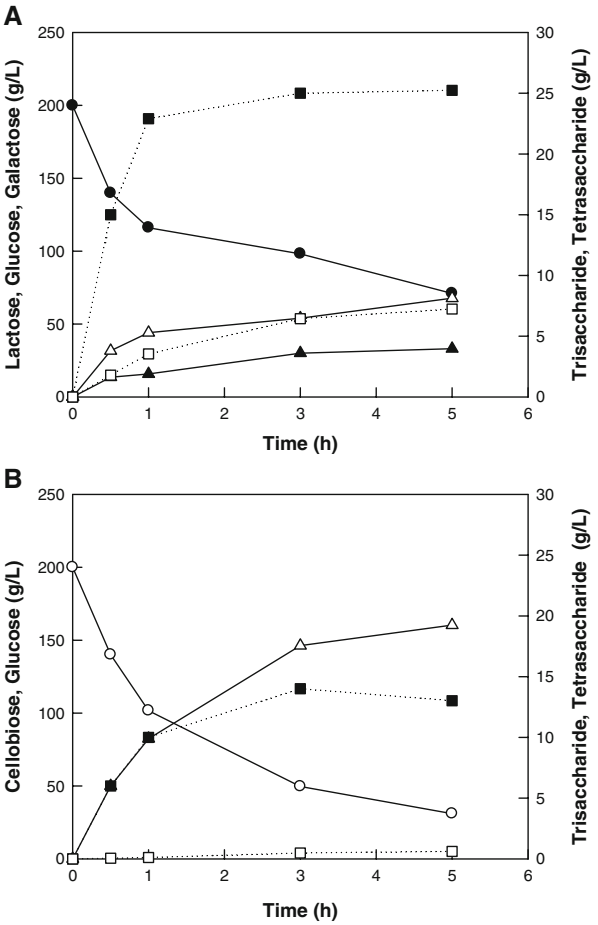

Fig. 4 Time course of transglycosylation activity determined using lactose or cellobiose by β-glycosidase from *S. acidocaldarius*. **a** Lactose, **b** cellobiose. Sugars were lactose (*filled circles*), cellobiose (*empty circles*), galactose (*filled triangles*), glucose (*empty triangles*), trisaccharide (*filled squares*), and tetrasaccharide (*empty squares*)

Trisaccharide and tetrasaccharide of galacto-oligosaccharides were galactosyl-galactosyl-glucose and galactosyl-galactosyl-galactosyl-glucose, respectively, due to the ratio of galactose to glucose based on the previous results [9, 20]. The glucose concentration was higher than the galactose concentration, suggesting that some of the galactose produced was used in the transgalactosylation to form galacto-oligosaccharides. Glucose and cello-oligosaccharides (cellotriose and cellotetraose) were formed from cellobiose by hydrolysis and transglucosylation. After the 5-h reaction, the concentrations of residual lactose and cellobiose were 71 and 31 g/L, respectively, indicating that the hydrolytic activity with cellobiose is higher than that with lactose. At the same time, the concentrations of the lactose-derived tri- and tetrasaccharide were 25 g/L and 7.2 g/L, respectively, and the concentrations of cellobiose-derived cellotriose and cellotetraose were 13 g/L and 0.6 g/L, respectively. Thus, the transglycosylation activity of the enzyme with lactose was higher than that with cellobiose.

Discussion

In this study, we expressed and characterized a thermostable recombinant β-glycosidase from *S. acidocaldarius*. The β-glycosidase activity of the purified recombinant enzyme toward the substrate *p*NPGlu was maximal at 90 °C and pH 5.5 (Fig. 2). Thus, the β-glycosidase from *S. acidocaldarius* can be regarded as an acidic, thermostable β-glycosidase. The activities of other β-glycosidases from hyperthermophiles toward *p*NPGlu, including *T. thermophilus* [7], *S. solfataricus* [4], *P. furiosus* [21], and *P. kodakaraensis* [10] are most active at 88ºC and pH 7.0, 95ºC and pH 6.5, 100ºC and pH 5.0, and 100ºC and pH 6.5, respectively.

The half-lives of the recombinant β-glycosidase from *S. acidocaldarius* at 70, 75, 80, 85, and 90ºC were 494, 453, 60, 9, and 0.2 h, respectively. In comparison, the half-lives of the hyperthermophilic β-galactosidases from *P. furiosus* [21], *P. kodakaraensis* [10], *S. solfataricus* [4], and *T. thermophilus* [7] were previously reported to be 85 h at 100ºC, 18 h at 90ºC, 10 h at 80ºC, and 10 min at 90ºC, respectively. In terms of thermal inactivation, the β-glycosidase from *S. acidocaldarius* showed a higher stability than those from *S. solfataricus* and *T. thermophilus* but a lower stability than those from *P. furiosus* and *P. kodakaraensis*.

S. acidocaldarius β-glycosidase, as a glycoside hydrolase family 1, showed broad substrate specificity because family 1 enzymes from the archaebacteria exhibit broad substrate specificity [22]. The hydrolytic activity of *S. acidocaldarius* β-glycosidase followed the order *p*NPFuc>*p*NPGlu>*p*NPGal>*p*NP-β-D-mannopyranoside>*p*NP-β-D-xylopyranoside (Table 2). In comparison, the hydrolytic activities of the β-glycosidases from other thermophiles followed these orders: *S. solfataricus*, *p*NPGal>*p*NPFuc>*p*NPGlu>*p*NP-β-D-arabinopyranoside>*p*NP-β-D-xylopyranoside [12]; *P. kodakaraensis*, *p*NPGlu>*p*NP-β-D-mannopyranoside>*p*NPGal>*p*NP-β-D-xylopyranoside [10]; *P. furiosus*, *p*NPGlu>*p*NPFuc>*p*NPGal>*p*NP-α-D-arabinopyranoside>*p*NP-β-D-xylopyranoside>*p*NP-β-D-mannopyranoside [23]; and *T. thermophilus*, *p*NPFuc>*p*NPGlu>*p*NPGal [11]. Thus, the substrate specificity of *S. acidocaldarius* β-glycosidase is similar to that of the enzyme from *T. thermophilus* despite the low level of amino acid sequence identity (22%) between the two proteins. The β-glycosidase from *S. acidocaldarius* exhibited higher hydrolytic activity for the substrates *p*NPGlu and cellobiose than for *p*NPGal and lactose (Table 2), but the transglycosylation activity for *p*NPGlu and cellobiose was lower than that for *p*NPGal and lactose (Fig. 4, Table 4).

Although the β-glycosidases from *S. acidocaldarius* and *S. solfataricus* had 72% amino acid sequence identity, the properties of *S. acidocaldarius* β-glycosidase such as form of association, thermostability, and substrate specificity were different from *S. solfataricus* β-glycosidase. The native β-glycosidase from *S. acidocaldarius* existed as a dimer, whereas that from *S. solfataricus* was a tetramer [24]. The substrate specificity of the β-glycosidase from *S. acidocaldarius* β-glycosidase followed the order *p*NPF>*p*NPGlu>*p*NPGal, whereas that from *S. solfataricus*, *p*NPGal>*p*NPF>*p*NPGlu [12]. The half-lives of the β-glycosidase from *S. acidocaldarius* at 75, 80, and 85ºC were 19-, six-, and threefold higher, respectively, than those from *S. solfataricus* [4]. Moreover, the comparison about the hydrolytic and transglycosylation activities of β-glycosidase for substrates has been not reported.

The production of a thermostable β-glycosidase in *E. coli* has great advantages for applications in commercial processes. The culture procedure for the production of a thermostable β-glycosidase in the mesophilic host is straightforward. The enzyme is overproduced with high activity, the cloned enzyme can be improved genetically by

directed evolution, and the thermostability and transgalactosylation activity of the enzyme are particularly favorable. In this study, a gene encoding a putative β-galactosidase from *S. acidocaldarius* was expressed in *E. coli*. As the recombinant enzyme exhibited broad substrate specificity for β-glycosides, it was determined that the enzyme is actually a β-glycosidase. The recombinant *S. acidocaldarius* β-glycosidase showed higher hydrolytic activity toward cellobiose than toward lactose, but its transglycosylation activity with cellobiose was lower than that with lactose. The results of this study indicate that this novel enzyme may be useful as a β-glycosidase for cellobiose hydrolysis and galacto-oligosaccharide production.

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