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Purification and Characterization of a Thermostable ß-Glycosidase from *Thermus caldophilus* GK24

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Abstract A β-glycosidase enzyme with β-D-fucosidase, β-D-galactosidase, and β-D-glucosidase activities has been purified from *Thermus caldophilus* GK24. The enzyme was monomeric with a molecular mass of 49 kDa, as evidenced by SDS-PAGE. The K_m values for *p*-nitrophenyl β-D-fucopyranoside (*p*-NPFuc), *p*-nitrophenyl β-D-galactopyranoside (*p*-NPGal), and *p*-nitrophenyl β-D-glucopyranoside (*p*-NPGlu) were 0.23 mM, 6.25 mM, and 0.28 mM, respectively. The enzyme showed optimal pH ranging between 5.5–6.5 and maximum temperature in the range of 85–90°C for all the above mentioned activities. The half-life of the enzyme in sodium phosphate buffer (pH 6.0) at 80°C was approximately 7 h. The *p*-NPGal hydrolyzing activity of *Tca* β-glycosidase was strongly activated by L-histidine, while the *p*-NPFuc and *p*-NPGlu hydrolyzing activities of *Tca* β-glycosidase were not affected at all by the amino acid. These results suggest differences in the conformation or in the reactive residues at the active site of *Tca* β-glycosidase.

Key words: β-Glycosidase, *Thermus caldophilus* GK24, *Tca* β-glycosidase, purification of *Tca* β-glycosidase, characterization of *Tca* β-glycosidase

The thermostable enzymes from thermophiles are capable of catalyzing biochemical reactions at high temperature and they are generally more stable than enzymes of mesophiles, thus having longer half-lives of enzyme preparations. The thermostable enzymes represent not only great stability, but also enhanced activity in the presence of common protein denaturants such as heat, detergents, and organic solvents [14]. Because of the properties mentioned above, thermostable enzymes are now extensively utilized in the industrial processing.

Among possible applications of such thermophilic enzymes, β-glycosidases have potential applications for hydrolysis of lactose and cellobiose, as well as for oligosaccharide synthesis by reversing their hydrolytic action in the food industry [15]. β-Glycosidase is identified as a group of heterogeneous enzymes with broad specificity and distributed in numerous microorganisms, plants, and animal tissues [8, 10]. One of the enzymes under extensive study is the thermostable β-glycosidases from the thermoacidophilic archaeon *Sulfolobus solfataricus* [1, 19]. The enzyme is a tetramer with a subunit molecular mass at 60 kDa and its crystal structure has been determined [1].

In our laboratory, a thermostable β-galactosidase was identified from the extreme thermophilic bacterium *Thermus caldophilus* GK24 by hydrolysis of *p*-NPGal and activity staining with 6-bromo-2-naphthyl-β-galactopyranoside (BNGal) in a nondenaturing polyacrylamide gel [21]. More recently, the enzyme was purified and thoroughly characterized to be a thermostable β-glycosidase with β-D-fucosidase, β-D-galactosidase, and β-D-glucosidase activities. This paper describes the purification and properties of β-glycosidase from *Thermus caldophilus* GK24.

Materials and Methods

Bacterial Strains, Culture Conditions, and Enzyme Activity Assay

T. caldophilus GK24 cells [20] were cultured in 0.3% polypeptone, 0.3% yeast extract, 0.2% cellobiose, and a basal salt at 75°C, and pH of 7.6 [22].

β-Glycosidase activities were assayed with 5 mM *p*-nitrophenyl (*p*NP) glycoside as substrates at 75°C for 5 min in 20 mM sodium phosphate buffer, pH 6.0 (standard assay conditions). After the reaction mixture (0.6 ml) was incubated, 0.4 ml of 1 M Na_2CO_3 was added, and the *p*-nitrophenol (*p*-NP) liberated was measured spectrophotometrically. Absorbance at 410 nm was converted to molar concentrations using E_M=1.77×10^4 M^{-1} cm^{-1} [18]. One unit of β-glycosidase activity is defined as 1 μmol *p*-NP released per minute.

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Hydrolysis of lactose and cellobiose was followed by measuring glucose release with a commercial glucose oxidase kit (Sigma Chemical Co., St. Louis, U.S.A.).

Purification of *Tca* β-Glycosidase

T. caldophilius GK24 cells (24 g) were harvested by centrifugation, and suspended in 50 mM sodium phosphate buffer (pH 6.0) containing 1 mM phenylmethylsulfonyl fluoride (PMSF). The cells were disrupted using an ultrasonic homogenizer, followed by centrifugation. The supernatant was brought to 60% (w/v) saturation by slowly adding ammonium sulfate. After 10 h, crude proteins were precipitated by centrifugation. The pellet was dissolved in a small volume of 10 mM sodium phosphate buffer (pH 6.0), and dialyzed against the same buffer.

The resulting dialysate was applied to a DEAE-cellulose DE32 (Whatman Ltd., Maidstone, Kent, England) column equilibrated in 50 mM sodium phosphate buffer (pH 6.0). Elution of protein was performed with a linear gradient of NaCl (0-0.3 M) in the same buffer mentioned above. The fractions having *p*-NPGal hydrolyzing activity were pooled, and dialyzed against 10 mM sodium phosphate buffer (pH 6.0).

The resulting dialysate was applied to a small column of 4% agarose beads derivatized with p-aminobenzyl β-D-thiogalactopyranoside groups (PABTG, Sigma Chemical Co., St. Louis, U.S.A.) which had been equilibrated in 50 mM of sodium phosphate buffer (pH 6.0), and the column was washed extensively with five bed volumes of the same buffer containing 0.2 M of NaCl. The enzyme fraction was eluted with a linear pH gradient from pH 7.6 to 9.2 in 50 mM boric acid-borax buffer. Fractions with *p*-NPGal hydrolyzing activity were pooled and dialyzed against distilled water.

The resulting dialysate was applied to a Rotofor apparatus (Bio-Rad Laboratories, Hercules, U.S.A.), and fractionated by isoelectric focusing in a Rotofor cell using 6% ampholyte (pH range, pH 3-10) (Amersham Phamarcia Biotech, Uppsala, Sweden) for 5 h at 12 W. The proteins were isolated and picked up by a vacuum-assisted harvesting system in fractions. Each fractions were analyzed for protein content, pH, and *p*-NPGal hydrolyzing activity.

Protein Determination

Protein concentration was determined by the procedure described by Lowry *et al.* [13], using bovine serum albumin as the standard protein.

Determination of Molecular Weight and Electrophoresis Analysis

The molecular weight of the native enzyme was determined by a gel filtration through a Bio-pre Se-100/17 size exclusion column (Bio-Rad Laboratories, Hercules, U.S.A.) applying the Bio-Rad size exclusion stands (catalog number 151-1901).

SDS-PAGE was performed by the method of Laemmli [11], using a 5% (w/v) acrylamide stacking gel and a 10% (w/v) acrylamide separating gel. The molecular weight of the purified enzyme in the denatured state was estimated using a low-molecular marker kit (Amersham Phamarcia Biotech, Uppsala, Sweden). Nondenaturing electrophoresis was performed in 10% of polyacrylamide gels using the standard markers (Bio-Rad Laboratories, Hercules, U.S.A.). In addition, gels were stained for proteins with Coomassie Blue R-250 and for activity with BNGal (Sigma Chemical Co., St. Louis, U.S.A.) in 50 mM sodium phosphate buffer (pH 6.0) at 75°C [6].

Determination of pI and NH_2-Terminal Amino Acid Sequence

Analytical isoelectric focusing was performed in the pH range of 3.5-9.5 on a polyacrylamide gel at 10°C using ampholyte (Amersham Phamarcia Biotech, Uppsala, Sweden) as described by Bollag and Edelstein [3]. The p*I* marker used was the broad p*I* calibration kit (pH 3-10) (Amersham Phamarcia Biotech, Uppsala, Sweden).

NH_2-terminus of the purified enzyme was sequenced with an Applied Biosystems model 470A gas-phase sequencer, and the phenylthiohydantoin derivatives of amino acids were identified according to the recommended procedures described by the manufacturer.

RESULTS AND DISCUSSION

Purification and Analytical Gel Electrophoresis of *Tca* β-Glycosidase

We earlier found a thermostable β-galactosidase from *T. caldophilus* GK24 by hydrolysis of *p*-NPGal [22]. Therefore, in the present study, the enzyme with *p*-NPGal hydrolyzing activity was purified from *T. caldophilus* GK24 cells grown in the presence of 0.2% of cellobiose in the cultured medium. The purification of the enzyme is summarized in Table 1. The specific activity of the purified enzyme was more than 236 times that of the crude extracts, and the recovery was about 12%.

Table 1. Purification summary of *Tca* β-glycosidase. The purification started using 24 g wet weight cells. One unit of enzyme corresponds to 1 μmol of product synthesized in 1 min at 75°C.

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)
Sonicated extract	3,434.0	2,403	0.7	100.0
Ammonium sulfate	2,121.0	2,145	1.0	89.3
DEAE-cellulose	6,28.0	1,748	2.8	72.7
Affinity column	24.0	659	27.4	27.4
Rotofor cell	1.8	298	165.0	12.4

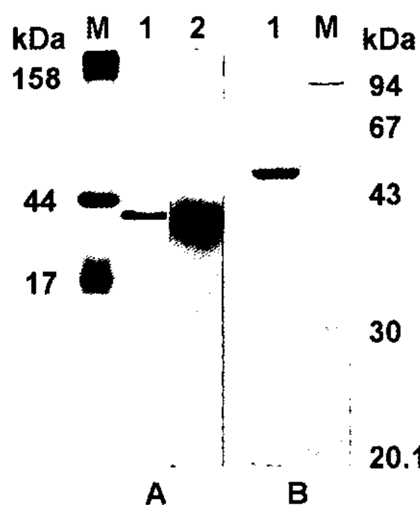

Fig. 1. Polyacrylamide gel electrophoresis of *Tca* β-glycosidase.
(A) A sample of the purified *Tca* β-glycosidase was subjected to electrophoresis under nondenaturing conditions as described in Materials and Methods. Lane M and 1 were stained with Coomassie Brilliant Blue, and lane 2 was stained with BNGal for *Tca* β-glycosidase activity. Lane M, protein markers; lanes 1 and 2, purified enzyme. (B) A sample of the purified *Tca* β-glycosidase was subjected to electrophoresis under denaturing conditions (SDS-PAGE). After an initial electrophoretic run, the gel was stained with Coomassie Brilliant Blue. Lane 1, purified enzyme; lane M, protein markers.

The purified enzyme appeared to be homogeneous as evidenced by gel electrophoresis under nondenaturing conditions (Fig. 1A). A single protein band was evident when the gel was stained with Coomassie Blue. In fact, this band was coincident with the enzyme activity, when the gel was stained with an activity staining mixture using BNGal. An apparent molecular mass of 39,000 Da was determined. For determining the subunit composition, protein samples were boiled in SDS sample buffer. A single protein band corresponding to molecular weight of 49,000 was obtained by SDS-PAGE under denaturing conditions (Fig. 1B). The molecular mass of native enzyme was estimated by employing gel filtration on a Bio-pre Se-100/17 size exclusion column, and approximately 32,000 Da was obtained (data not shown). These results suggest that the enzyme with β-galactosidase activity in its native conformation is a monomer. This property is different from *S. solfataricus* β-glycosidase, which is a tetramer [19], differing also from *Thermus* strain 4-1A β-galactosidase [5] and *Pyrococcus furiosus* glucosidase [9]. The enzyme showed conformation similar to that of *Thermus* sp. Z-1 β-glucosidase [21] and *Thermus* sp. A4 β-galactosidase [17].

Isoelectric focusing of the purified enzyme showed a band corresponding to p*I* 4.8 when the plates were stained for protein and activity. The NH_2-terminal amino acid sequence of the purified enzyme was N-A-E-K-F-L-W-G-V-A-T-S-A-Y-Q.

Table 2. Relative activity of *Tca* β-glycosidase on various substrates. The activity of hydrolysis as determined in 20 mM of sodium phosphate buffer (pH 6.0). Reaction mixtures contained 5 mM of substrate which were incubated at 75°C. Hydrolysis rate was measured by the procedure described in Materials and Methods.

Substrate	Relative hydrolysis rate (%)
p-Nitrophenyl β-D-galactopyranoside	100.0
p-Nitrophenyl β-D-fucopyranoside	80.5
p-Nitrophenyl β-D-glucopyranoside	40.0
p-Nitrophenyl α-D-glucopyranoside	15.0
p-Nitrophenyl β-D-xylopyranoside	5.1
p-Nitrophenyl α-D-fucopyranoside	0.4
p-Nitrophenyl α-D-galactopyranoside	0.7
p-Nitrophenyl α-L-arabinopyranoside	1.2
Lactose	14.8
Cellobiose	43.2

Hydrolytic Activity of Purified Enzyme toward Various Substrates

Table 2 shows the relative activities of the *p*-NPGal hydrolyzing activity in the final concentration of 5 mM substrates. The activity towards *p*-NPFuc was approximately 80.5% when compared with the activity towards *p*-NPGal. The activity towards *p*-NPGlu was about 40% when compared with the activity towards *p*-NPGal. The enzyme was also active on cellobiose and lactose. Accordingly, this enzyme is properly classified as a β-glycosidase with β-fucosidase, β-galactosidase, and β-glucosidase activities rather than as a β-galactosidase. The K_m values were determined by measuring activity at 75°C in 50 mM sodium phosphate buffer (pH 6.0), and calculated by Lineweaver-Burk plots [12]: They were 2.3 mM, 6.25 mM, and 0.28 mM for *p*-NPFuc, *p*-NPGal, and *p*-NPGlu, respectively. The glucoside and fucoside were high-affinity substrates for this enzyme.

Effect of pH on the Activity and Stability of *Tca* β-Glycosidase

Optimum pH for hydrolysis of *p*-NPFuc, *p*-NPGal, and *p*-NPGlu was observed at pH ranges of 6.0-6.5, 5.0-5.5, and 7.0-7.5, respectively. These pH ranges represent an usual phenomenon, since the optimal pH of most β-glycosidases from bacterial sources are in the acidic range (pH 5.0-7.0). The pH stability of the enzyme was determined by incubating the enzyme solution at different pHs for 24 h at 25°C, and the residual activity was then measured under the routine reaction conditions using *p*-NPFuc, *p*-NPGal, and *p*-NPGlu as substrates. The enzyme was found to be fairly stable and had more than 98% activity in the pH range of 5.0-7.0, and about 95% activity remained at the pH ranges of 4.5-5.0 and 7.0-8.0.

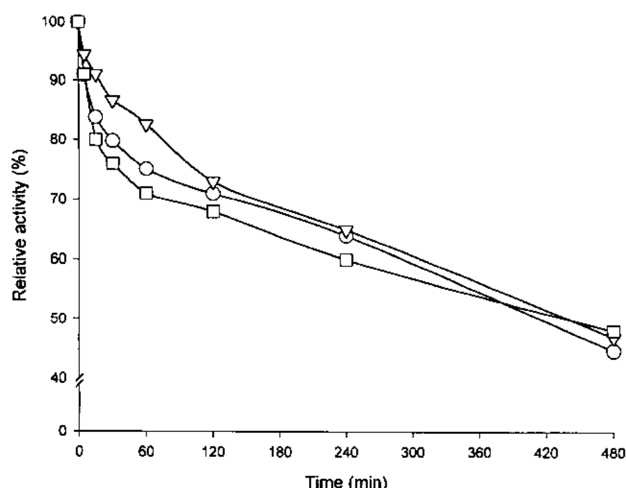

Fig. 2. The thermostability of *Tca* β-glycosidase.
The enzyme was dissolved at the concentration of 2.0 μg protein/ml in 20 mM of sodium phosphate buffer (pH 6.0). After an incubation period at 80°C for the indicated times, the remaining *p*-NPFuc (○), *p*-NPGal (▽), and *p*-NPGlu (□) activities were assayed as described under Materials and Methods.

Effect of Temperature on the Activity and Stability of *Tca* β-Glycosidase

The maximal activities toward *p*-NFuc, *p*-NPGal, and *p*-NPGlu were observed at a temperature range of 85–90°C. The activity increased continuously from 30 to 90°C and dropped at 95°C. Figure 2 shows the thermostability of the enzyme in 50 mM sodium phosphate buffer (pH 6.0). The enzyme seemed to be fairly stable at 80°C, however, at temperatures above 95°C, the stability of the enzyme decreased drastically (results not shown). The half-life was about 7 h at 80°C.

Effects of Various Compounds on the Activity of *Tca* β-Glycosidase

The effects of various compounds on the hydrolysis of *p*-NPFuc, *p*-NPGal, and *p*-NPGlu were tested (Table 3). Most metal ions (with the exception of mercuric and copper compounds) had little or no inhibitory effect on all the activities. All the activities were slightly increased by 1 mM EDTA and 1 mM DTT. The strongest inhibition on *Tca* β-glycosidase was observed with 1 mM $HgCl_2$. Mercuric compounds often inhibit enzymes by reacting with sulfhydryl groups, and various reagents are available for the modification of sulfhydryl side chains in proteins [2]. All the activities of *Tca* β-glycosidase were strongly inhibited by 1 mM iodoacetic acid, *p*-chloromercuribenzoate and *N*-bromosuccinimide, as expected (Table 3). With iodoacetate, *p*-NPFuc and *p*-NPGlu hydrolyzing activities were inhibited more than *p*-NPGal hydrolyzing activity, however, with *p*-chloromercuribenzoate and *N*-bromosuccinimide, *p*-NPGal hydrolyzing activity was inhibited more than the *p*-NPFuc and *p*-NPGlu hydrolyzing activities.

Unexpectedly, we observed that the *p*-NPGal hydrolyzing activity of *Tca* β-glycosidase was strongly activated by 1 mM L-histidine, while the *p*-NPFuc and *p*-NPGlu hydrolyzing activities of *Tca* β-glycosidase were not activated by the amino acid at all (Table 3). Histidine and imidazole are potent competitive inhibitors for β-glycosidase [7], however, the mechanism of an apparent activation of *p*-NPGal hydrolyzing activity by L-histidine is unclear. Nevertheless, these results suggest a difference in the conformation or in the reactive residues at the active site of *Tca* β-glycosidase. β-Fucosidase, β-glucosidase, and β-galactosidase activities have been reported to be catalyzed in the same active site of the enzyme by some investigators [16], while others

Table 3. Effect of various substances on *Tca* β-glycosidase. Substances were tested for their effects on the hydrolytic activity for *p*-NPFuc, *p*-NPGal, and *p*-NPGlu in 20 mM of sodium phosphate buffer (pH 6.0) at 75°C.

Substance	Final concentration (mM)	% Activity remaining		
		p-NPFuc	*p*-NPGal	*p*-NPGlu
Control	-	100	100	100
$MgCl_2$	1	97	103	100
$CaCl_2$	1	96	105	93
$ZnCl_2$	1	99	102	96
$FeCl_2$	1	90	83	95
$CuCl_2$	1	78	58	81
$HgCl_2$	1	0	0	0
EDTA	1	108	103	114
DTT	1	126	108	126
Iodoacetate	1	36	70	40
N-Bromosuccinimide	1	28	9	66
p-Chloromercuribenzoate	1	28	6	21
L-Histidine	1	107	140	106
	5	104	175	106

reported that only β-fucosidase and β-glucosidase activities are catalyzed in the same active site, with β-galactosidase activity in a different active site [4, 23].

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