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Journal of Molecular Catalysis B: Enzymatic 15 (2001) 45–53

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

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Characterization of a thermostable β-glucosidase (BglB) from *Thermotoga maritima* showing transglycosylation activity

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Received 25 November 2000; received in revised form 22 December 2000; accepted 22 December 2000

Abstract

A β-glucosidase gene (*bglB*) of an extremely thermophilic eubacterium, *Thermotoga maritima* was expressed in *Esherichia coli* to yield the active enzyme. The cloned enzyme was purified to homogeneity by heat treatment and ion exchange chromatographies. The purified enzyme gave a single band on SDS-PAGE with a molecular weight of 81 kDa. The estimated K_m and k_{cat} values for *p*-nitrophenyl β-D-glucopyranoside were 0.0039 mM and 6.34 s^{-1}, respectively. The purified enzyme was optimally active at pH 5.0 (85°C), however, it also displayed higher activity at acidic pH (optimum pH 3.5) at a lower temperature (70°C). An investigation into the effect of straight chain alcohols and organic compounds on the activity of enzyme revealed that alcohols had a stimulatory effect, possibly due to the occurrence of transglycosylation. Because of its thermostability and transglycosylation properties, this enzyme displays potential as a catalyst for biotechnological applications.

Keywords: *Thermotoga maritima*; β-Glucosidase; Thermostable; Transglycosylation

1. Introduction

Thermotoga maritima is a fermentative marine hyperthermophilic eubacterium that can be grown in temperatures of up to 90°C with an optimal temperature of around 80°C [1]. Three species of *Thermotoga* have been characterized to date, viz. *T. thermarum*, *T. neapolitana* and *T. maritima* [1,2]. Compared to other hyperthermophilic organisms, *Thermotoga* species exhibit special characteristics in that they can be plated with 100% efficiency at temperatures of 75–80°C and can form colonies in 2 days on defined minimal media [3]. In the last few years, a variety of proteins have been purified from *Thermotoga* species, such as histone like protein [4], chemotaxis proteins [5], D-glyceraldehyde-3-phosphate dehydrogenase [6], pyruvate feredoxin oxidoreductase enzymes catalyzing the oxidation of pyruvate to acetyl-coA [7], 4-α-gluconotransferase [8], lactate dehydrogenase [9], glucose isomerase [10], cellobiohydrolases [11], xylanases and β-glucosidases [12–17]. All of these proteins are exceptionally thermophilic and resistant to high temperatures and denaturing agents [18]. Generally, in the presence of common denaturants, these enzymes show increased stability when compared with their counterparts from mesophilic organisms [19]. On the basis of their remarkable stability against thermal denaturation, enzymes extracted from such organisms represent an attractive source of catalyst for the biotechnology industries [20,21].

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1381-1177/01/$ – see front matter
PII: S1381-1177(01)00003-0

β-Glucosidases (β-glucoside hydrolases) are enzymes that hydrolyse the β-glucosidic linkages of low molecular weight glycosides and are considered to be an important component of the cellulase system of enzymes [22]. For most bioconversion processes, endo (1-4)-β-D-glucan glucanhydrolases and exo-1-4-β-D-glucan cellobiohydrolases catalyze the random hydrolysis of cellulose to produce cellobiose, which is finally, hydrolysed by β-glucosidase to give glucose. Therefore, the complete degradation of cellulose requires the synergistic action of all three enzymes. Due to its broad substrate specificity, β-glucosidases can be classified into three groups: the first group shows a high degree of specificity towards aryl-β-glucosidases while the second group, the cellobiases, hydrolyse only oligosaccharides. The members of the third group are more useful because they show significant activity toward both substrate types [13]. On the basis of amino acid similarities, β-glucosidases have been divided into two families: family 1 (β-glucosidases and phospho-β-glucosidases from bacteria to mammals) and family 3 (β-glucosidases from yeast, molds and rumen bacteria) [23]. The species *T. maritima* has four different exo-acting, β-specific glycosyl hydrolases, including a β-xylosidase [13], a β-galactosidase [12] and two β-glucosidases (BglA and BglB) [12]. The β-glucosidase gene, *bglA*, of *T. maritima* has already been sequenced, and the deduced amino acid sequence resembles that of the family 1 glycosyl hydrolases. On the other hand, the other β-glucosidase gene, *bglB,* of the same strain is similar to that of the family 3 glycosyl hydrolases.

The present report describes the purification and characterization of family 3 β-glucosidase, BglB of *T. maritima*. It has previously been demonstrated that some β-glucosidase enzymes also display transglycosylation activity [24–26]. Therefore, in view of the commercial importance of β-glucosidase enzymes, an effort was made to study the effect of organic solvents on the activity of the β-glucosidase.

2. Materials and methods

2.1. Bacterial strains and plasmids

The genomic DNA of *T. maritima* MSB8 was obtained from Prof. Dr. Stetter (Lehrstuhl fuer Mikrobiologie, Universitaet Regensburg, Universitaetsstrasse 31, D-93053 Regensburg, Germany). Topo-XL TOP 10 [F^- *mcr* AΔ(*mrr-hsd*RMS-*mcr*-BC)Φ80*lac*ZΔM15 Δ*lac*X74 *rec*AI *deo*R *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (*str*^{R}) *end*A1 *nup*G] was used as the host for the β-glucosidase gene from *T. maritima* (TOPO®XL PCR Cloning kit, Invitrogen, USA). The *Escherichia coli* strain BL21(DE3) harboring the pET28a(+) plasmid was used for the expression and purification of the enzyme. Recombinant DNA techniques as described by Sambrook et al. [27], were employed to perform DNA manipulations. All other chemicals were commercially obtained and were of the highest purity.

2.2. PCR and cloning

The coding regions of the β-glucosidase from *T. maritima* (abbreviation, TM) was engineered by PCR to incorporate a *Nde* I site upstream, and a *Sac* I site downstream of the termination codon by employing the following conditions: 98°C for 5 min, 60°C for 60 sec and 72°C for 2.5 min, repeated for 25 cycles.

The PCR products were purified from agarose gels using a QIAquick gel extraction kit (QIAGEN, Germany). The amplified DNA fragments were cloned using a PCR-TOPO cloning kit (Invitrogen, USA) and the resulting recombinant PCR-TOPO plasmid containing β-glucosidase gene was extracted using a QIAminiprep kit (QIAGEN, Germany) and was then sequenced. A Big Dye Terminator Cycle Sequencing Kit (Perkin-Elmer, Applied Biosystems, CA, USA) was used as per the supplier's instructions to prepare sequencing samples and these were assayed with a DNA sequencer (Model 373A, Applied Biosystems, CA, USA). The sequence data were analyzed using the GENETIX program (Software Development Co., Tokyo, Japan). The resulting plasmid was designated TM-glu-TopoXL.

2.3. Expression and preparation of the target protein

The amplified gene, without any mutations in its amino acid sequence, was hydrolysed using the *Nde* I and *Sac* I restriction enzymes and ligated with the pET28a(+) vector which had been previously digested with the same restriction enzymes. Ligation-High T4 DNA ligase (TOYOBO, Osaka, Japan) was used for

gene-vector ligations during the subclonings. Plasmid DNA was prepared from *E. coli* by using a Qiagen gel extraction kit. The host *E. coli* BL21(DE3) competent cells were transformed with the recombinant pET28a(+) vector to obtain the active enzyme. Out of more than ten thousand clones, five were identified as positive clones. Among these five positive clones, only one of them was sequenced to confirm that the DNA sequence is identical to the database sequences for *T. maritima* [28].

2.4. Enzyme purification

Using a rotary shaker, the recombinant *E. coli* cells were cultivated at 37°C in Luria–Bertani broth (LB) medium (1000 ml) containing kanamycin (30 μg ml^{-1}). Upon reaching an optimal density of 0.6 at 600 nm, protein production was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cultivation was then continued overnight and the *E. coli* cells were then harvested by centrifugation (11,000 × *g*, 10 min at 4°C) and suspended in 50 mM MOPS buffer, pH 6.5. The disruption of the cells was accomplished by sonication (Branson sonifier 250D) and remaining any intact cells and debris were removed by centrifugation (15,000 × *g*, 10 min at 4°C). The crude cell extract was heated at 80°C for 10 min and the precipitate was removed by centrifugation (20,000 × *g* for 20 min, 4°C). Purification was achieved by binding the heat-treated crude cell lysate with Ni-NTA agarose slurry. The resin was then packed into a column and the subsequent washing and elution steps were performed using the batch procedure described in the QIA expressionistTM kit (QIAGEN, Germany) with a step gradient of 250 mM imidazole in 50 mM Na-phosphate buffer, pH 8.0. The active fractions were combined and dialyzed overnight at 4°C against 5 mM MOPS buffer, pH 6.5. The dialyzed enzyme solution was further purified by ion exchange chromatography on a Q-Sepharose 16/10 column (Pharmacia), previously equilibrated with 25 mM MOPS buffer, pH 6.5. The protein was eluted by linear salt gradient of 0–500 mM of NaCl. The removal of salt from pooled active fractions was achieved by dialysis against 5 mM MOPS buffer, pH 6.5. For the final purification step, the dialyzed sample was loaded onto an anion exchange Mono Q HR 5/5 (Pharmacia) column pre-equilibrated with 20 mM MOPS buffer, pH 6.5. The adsorbed proteins were eluted from the column with two successive gradients of 0–200 and 200–400 mM NaCl (in 20 mM MOPS, pH 6.5), at a flow rate of 0.5 ml min^{-1}, monitoring the eluant at 280 nm. The fractions with β-glucosidase activity eluted as a single protein peak and the purity of the enzyme were assessed by SDS-PAGE.

2.5. Enzyme assay

The enzyme activity of the β-glucosidase was determined by measuring *p*-nitrophenyl release from the *p*-nitrophenyl β-D-glucopyranoside at 30°C. The assay mixture, consisting of 5 mM *p*-nitrophenyl β-D-glucopyranoside in 50 mM MOPS buffer (pH 6.5) was incubated with the enzyme for 10 min in a total volume of 0.5 ml. The reaction was stopped by the addition of 0.5 ml of 0.2 M glycine-NaOH (pH 10.5), and the amount of *p*-nitrophenyl released was determined by measuring the absorbance at 405 nm. One unit of β-glucosidase is defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenyl per minute under the conditions described above.

2.6. SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted on 1 mm thick 12% acrylamide gel slabs. The samples were dissolved in a Tris–HCl loading buffer containing 1% (w/v) SDS, 20% (v/v) glycerol and 2% (v/v) 2-mercaptoethanol, and heated in boiling water for 3 min [29]. Proteins were stained with 1% (w/v) Coomassie Brilliant blue R 250 in methanol/acetic acid/water (50:10:40, v/v) and destained in methanol/acetic acid/water (30:10:60, v/v). An amount of 10 kDa protein ladder (LIFE TECHNOLOGIES, GIBCO BRL, Rockville, USA) is used as molecular weight marker.

2.7. Effect of alcohols on the enzyme activity

The purified enzyme fractions were used to investigate the effect of the presence of alcohols. Relative rates of hydrolysis at various alcohol concentrations were determined using two different buffer systems, viz. 25 mM MOPS (pH 6.5) and 25 mM Na-formate

(pH 3.5) at 30 and 70°C. To improve the solubility of the alcohols, 15% DMSO was also added.

2.8. Kinetic parameters

Michaelis–Menten constants were determined from a Lineweaver–Burk plot. The data were obtained by measuring the initial rate of hydrolysis by incubating the enzyme with appropriate concentrations of the substrate in 25 mM MOPS at 30°C. The reaction was monitored at 405 nm on a Beckman spectrophotometer (model DU 640) equipped with a temperature controlled cell holder. Initial hydrolysis rates were determined at six different concentrations ranging from approximately 0.5 to 2.0 times of the K_m value. Values for K_m and k_{cat} and their standard errors were obtained by using the nonlinear regression analysis program "Grafit" [30].

2.9. Effect of pH and temperature

The temperature optimum of the enzyme was determined using the standard assay in the temperature range from 0 to 100°C at two different pH levels, pH 3.0 and 5.0. Similarly, the thermal stability of the enzyme was also determined by incubating the purified protein for 30 min at temperatures ranging from 0 to 100°C. After cooling the sample on ice for 10 min, the remaining activity was determined using the standard procedure.

To determine the effect of pH, 50 mM concentrations of various buffers such as sodium phosphate (pH 1.1–3.1), sodium citrate (pH 3.16–4.12), sodium formate (pH 2.8–4.85), sodium acetate (pH 3.75–5.71), 2-[*N*-morpholino]ethane sulfonic acid (MES) (pH 5.14–7.17), 3-[*N*-morpholino]propanesulfonic acid (MOPS) (pH 6.2–8.18) and *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES) (pH 6.48–8.56) were used at two different temperatures (30 and 85°C). Tests of the pH stability of the enzyme at 30 and 85°C were performed by preincubating the enzyme with the above buffers as well as 2-[*N*-cyclohexylamino]ethanesulfonic acid (CHES) (pH 3.16–4.12), 3-[cyclohexylamino]-1-propanasulfonic acid (CAPS) (pH 9.34–11.41) and piperidine (10.5–13.0) for 30 min and the remaining activity was determined using the standard procedure. All buffers were prepared by adjusting pH at room temperature and subjected to the assay at 30 and 85°C, all chemicals used were obtained by nacalai tesque.

3. Results and discussion

3.1. Sequence similarity between TM-β-glucosidase (BglB) and other β-glucosidases

Thermotoga maritima possesses two different β-glucosidase genes (*bglA* and *bglB*). Several other organisms, including some thermophiles, also contain the genes for two or more exo-acting, β-specific glycosyl hydrolases. For example, the thermophilic bacterium *Clostridium thermocellum* has two β-glucosidase genes, *bglA* and *bglB* [31]. The deduced amino acid sequence for β-glucosidase A is similar to that of the family 1 glycosyl hydrolases [31], while that of the β-glucosidase B resembles the family 3 glycosyl hydrolases [32]. *Bacillus polymyxa* also contains the genes for β-glucosidases, *bglA* and *bglB* [33] belonging to two different families. Although the deduced amino acid sequences of BglA and BglB are 44.7% identical, the enzymes display distinctly different biochemical characteristic [33]. The deduced amino acid sequence of the encoded protein (BglB) of *T. martima* is similar to that of the family 3 glycosyl hydrolases, while the amino acid sequence for BglA (accession no. X74163) of *T. maritima* is similar to that of the family 1 glycosyl hydrolases [34]. The deduced amino acid sequence for the *bglB* gene of *T. maritima* shows the highest degree of similarity (82% homology) with the β-glucosidase gene *bglB* (accession no. Z77856) of *T. neopolitana.*

The homology of the BglB from *T. maritima* with other glycosyl hydrolases such as *Prevotella bryantii* (U35425), *C. Thermocellum* (X15644), *Ruminococcus albus* (U92828 and X15415), *Clostridium stercorarium* (Z94045), *Agrobacterium tumefaciens* (M59852), *Butyrivibrio fibrisolvens* (M31120), *Schizosaccharomyces pombe* (AL355920) and *Streptomyces coelicolor* (AL031013) ranges between 50 and 60%.

3.2. Purification of the β-glucosidase

The *T. maritima* β-glucosidase was purified to homogeneity from the cellular extracts of this strain. The first step in the purification of the β-glucosidase

Table 1
Summary of β-glucosidase purification

Purification step	Protein (mg)	Total activity (unit)	Specific activity (unit mg^{-1})	Purification factor (-fold)	Recovery (%)
Crude extract	321	63.3	0.197	1.0	100
Heat precipitation	205	45.4	0.22	1.13	72.7
Ni-NTA	13.0	29.1	2.21	11.2	46.0
Q-Sepharose	1.86	16.8	9.05	46.0	26.5
Mono-Q	0.82	8.98	11.0	56.0	14.2

from the crude cell lysate was heat treatment at 80°C for 10 min. To trap the His-tagged protein, Ni-NTA agarose slurry (QIAGEN) was added to the crude extract and the resin was packed into a column. The enzyme was then eluted with buffer containing 250 mM imidazole. The remaining impurities were removed by two subsequent purification steps employing ion exchange chromatography (a Q-Sepharose column followed by a Mono-Q column) as described in Section 2. The activity of the enzyme was monitored at each step of the purification process and these activities are summarized in Table 1.

To assess the purity and molecular mass of the enzyme, the active fraction was analyzed by SDS-PAGE as depicted in Fig. 1. The final preparation shows a single protein band of 81 kDa.

3.3. Effect of pH and temperature on enzyme activity

The influence of pH on the β-glucosidase activity was determined using a series of various buffers at either 30 or 85°C. At 30°C in the Na-citrate buffer, a broad pH maximum was observed between pH 3 and 4, peaking at pH 3.6 as shown in Fig. 2. The β-glucosidase activity increased approximately two-fold in presence of Na-formate buffer compared with Na-citrate buffer at 30°C. In contrast, at 85°C, no significant increase in activity was observed in Na-formate buffer relative to Na-citrate buffer. On the other hand, the pH profile of the enzyme displayed an optimum pH value between 5 and 5.2 in 50 mM of Na-succinate buffer (Fig. 3).

The stability test of the purified enzyme at different pH levels indicated that it was stable in the pH ranges from 5 to 9 at 85°C and the enzyme retained 68% of its original activity at pH 10. The enzyme demonstrates a relatively broad pH stability range, i.e. pH 3.0 upwards at 30°C, as shown in Fig. 4. At higher temperature such as 85°C, the enzyme is less stable at pH 3.6 then pH 5.1 as shown in Fig. 4. Thus, the optimum pH for the enzyme reaction has shifted from 3.6 measured at 30°C to 5.1 at 85°C.

Correlation of the enzyme activity with increasing reaction temperature resulted in peaks at two different pH values as shown in Fig. 5. The temperature optimum at pH 3.0 was 70°C, while at pH 5.0 it was approximately 85°C. It is remarkable that this β-glucosidase displays two activity maxima under

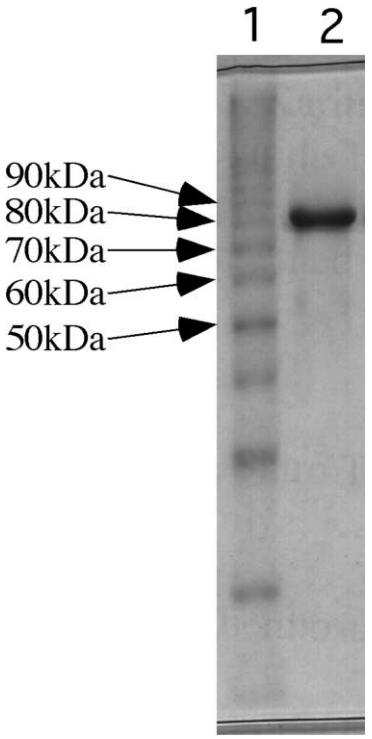

Fig. 1. SDS-PAGE of a purified β-glucosidase fraction. Lane 1: 10 kDa protein marker; lane 2: purified β-glucosidase.

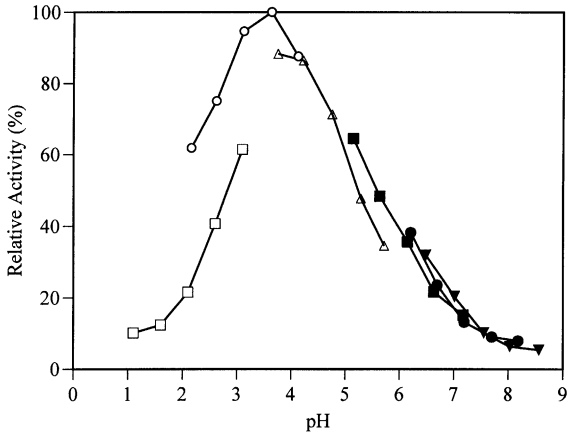

Fig. 2. Effect of pH on β-glucosidase activity. The influence of pH on β-glucosidase activity was determined at 30°C using 50 mM of the following buffers: phosphate (□); citrate (○); acetate (△); MES (■); MOPS (●); HEPES (▲). The highest activity observed at pH 3.6 is defined as the 100% activity to calculate the relative activity.

such different environmental conditions. The encoded enzyme exhibits marked thermal stability which is evident from the activity remaining after preincubation in 50 mM Na-succinate (pH 5.0) for 30 min at various temperatures (0–100°C). A plot of incubation temperature versus relative enzyme activity demonstrates that the β-glucosidase is stable at pH 5.0 at temperatures of up to 75°C (Fig. 6).

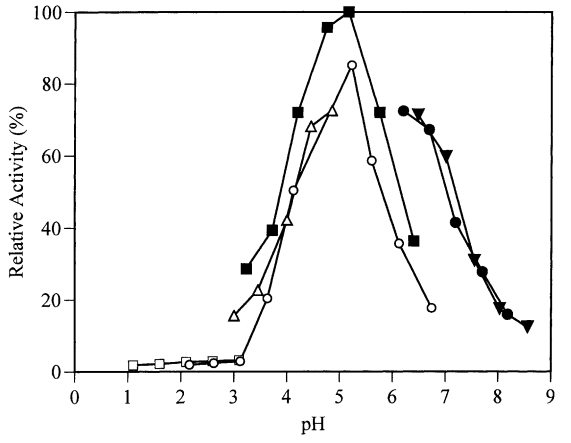

Fig. 3. Effect of pH on β-glucosidase activity. The influence of pH on β-glucosidase activity was determined at 85°C using 50 mM of the following buffers: phosphate (□); citrate (○); formate (△); succinate (■); MOPS (●); HEPES (▲). The highest activity observed at pH 5.1 is defined as the 100% activity to calculate the relative activity.

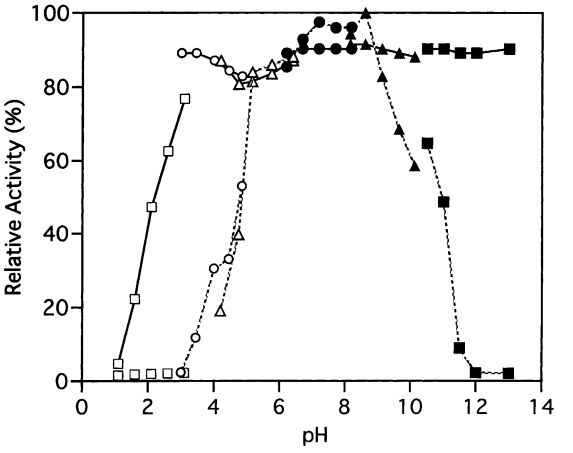

Fig. 4. Effect of pH on β-glucosidase stability. To study the pH stability of the enzyme, the activity remaining after incubation for 30 min at 30°C (—) and 85°C (···) was determined over different pH ranges. The following buffers were used: 50 mM phosphate (□); formate (○); succinate (△); MOPS (●); HEPES (▲); piperidine (■).

3.4. Substrate specificity

The substrate specificity of the purified β-glucosidase was examined with a variety of β-linked

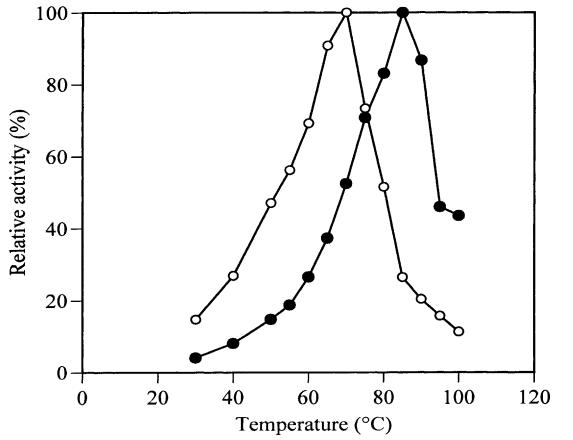

Fig. 5. Effect of temperature on β-glucosidase activity. The temperature profile of the β-glucosidase was determined at various temperatures using the standard assay as described in the Material and Methods Section employing the following buffers: (○) formate (pH 3.0) and (●) succinate (pH 5.0).

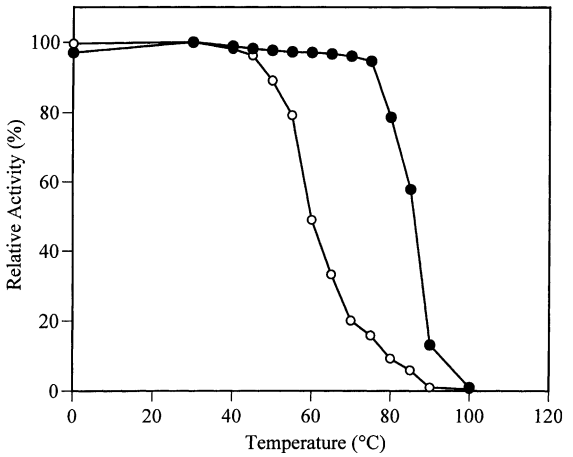

Fig. 6. Effect of temperature on the thermal stability of the β-glucosidase. For estimation of the heat stability of the β-glucosidase, residual activity was determined using the standard assay after a 30 min preincubation at various temperatures: (○) formate (pH 3.0) and (●) succinate (pH 5.0).

saccharide derivatives. The kinetic parameters K_m and k_{cat} were determined using the non-linear regression analysis program, "Grafit" [30]. The data in Table 2 demonstrate the broad substrate specificity of the β-glucosidase. The K_m value of the β-glucosidase for *p*-nitrophenyl β-D-glucopyranoside is 0.0039 ± 0.0002 mM and it is worth noting that this K_m value is one of the lowest ever reported for a β-glucosidase acting on this substrate [13,14,26,35–37]. In addition, the estimated k_{cat} values for *p*-nitrophenyl β-D-glucopyranoside (6.34 ± 0.094 s^{-1}) is also lower than that of the β-glucosidase from *A. tumefacians* (95.4 s^{-1}) [36].

It is interesting to note that the enzyme hydrolyse *p*-nitrophenyl β-D-galactopyranoside with a very low lavel of activity compared to that observed for the *p*-nitrophenyl β-D-glucopyranoside. This is also reflected in a higher K_m value for the β-glucosidase against *p*-nitrophenyl β-D-galactopyranoside (124 ± 24.4 mM) relative to *p*-nitrophenyl β-D-glucopyranoside as the substrate. The K_m values for *p*-nitrophenyl β-D-xylopyranoside, *p*-nitrophenyl α-L-arabinofuranoside and *p*-nitrophenyl β-D-fucopyranoside were 2.64 ± 0.1, 18.9 ± 3.3 and 42.66 ± 6.6 mM, respectively. Despite showing a high level of activity with *p*-nitrophenyl α-L-arabinofuranoside, the β-glucosidase was not able to act on *p*-nitrophenyl α-L-arabinopyranoside or *N*-acetyl-*p*-nitrophenyl β-D-glucosaminide. The substrate specificity of this β-glucosidase is different from those previously reported for other thermophilic β-glucosidases [3,14], however, the enzyme is similar to other broad substrate specificity glucosidases in that it hydrolyses *p*-nitrophenyl α-L-arabinofuranoside and *p*-nitrophenyl β-D-fucopyranoside.

3.5. Transglycosylations

In addition to displaying thermostability and activity at elevated temperatures, enzymes isolated from hyperthermophilic microorganisms are often also resistant to, and active in, the presence of organic solvents and detergents [19]. It is common for glucosidases to be activated by alcohols [36], and this is largely attributed to the occurrence of transglycosylation, although in some cases it has been attributed to allosteric interaction [38]. Herein we studied the effect of various compounds on the activity of the *T. maritima* β-glucosidase. From Fig. 7, it can be seen that the enzyme was activated in the presence of alcohols, with the highest rate of consumption of *p*-nitrophenyl β-D-glucopyranoside occurring in the presence of 0.8 M 1-propanol.

Table 2
Kinetic parameters for the *T. maritima* β-glucosidase[a]

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\,s^{-1}$)
*p*NP-β-D-glucopyranoside	0.0039 ± 0.0002	6.34 ± 0.094	1.63×10^3
*p*NP-β-D-xylopyranoside	2.64 ± 0.12	18.4 ± 0.4	6.97
*p*NP-α-L-arabinofuranoside	18.9 ± 3.37	9.02 ± 1.1	0.47
*p*NP-β-D-fucopyranoside	42.66 ± 6.68	27.7 ± 3.4	0.65
*p*NP-β-D-galactopyranoside	124 ± 24.4	18.2 ± 2.7	0.15

[a] No activity to *p*-nitrophenyl α-L-arabinopyranoside and *N*-acetyl-*p*-nitrophenyl β-D-glucosaminide were observed.

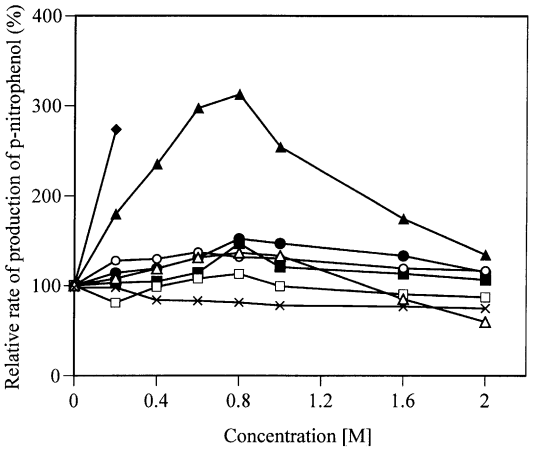

Fig. 7. Relative rates of *p*-nitrophenol production from *p*-nitrophenyl β-D-glucopyranoside by *T. maritima* β-glucosidase in the presence of various compounds; (■) methanol; (●) ethanol; (▼) propanol; (◆) 1-butanol; (□) iso-propanol; (○) ethylene glycol; (△) glycerol; (×) DMSO.

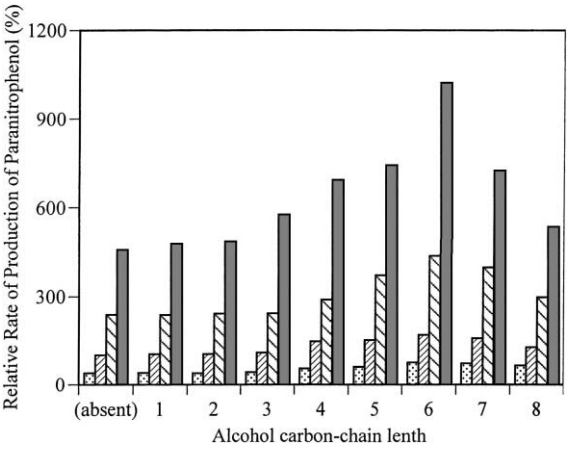

Fig. 8. Relative rates of *p*-nitrophenol production from *p*-nitrophenyl β-D-glucopyranoside with *T. maritima* β-glucosidase in the presence of 15% DMSO and 50 mM concentrations of the series of straight-chain alcohols from methanol to octanol under the following conditions: (⊡) pH 6.5 at 30°C, (▨) pH 3.5 at 30°C, (▧) pH 6.5 at 70°C, (■) pH 3.5 at 70°C.

In order to investigate the effect of straight chain alcohols on the activity of the β-glucosidase, a further study was undertaken. Since, the higher alcohols are not miscible in water, 15% DMSO was used to increase their solubility, as reported by Watt et al. [36]. As shown in Fig. 7, a concentration of 15% DMSO had little effect on the rate of hydrolysis of the *p*-nitrophenyl β-D-glucopyranoside. Therefore, a series of straight chain aliphatic alcohols (50 mM) were studied in the presence of 15% DMSO at two different temperatures, viz. 30 and 70°C. Fig. 8 demonstrates that as the chain length of the alcohol was increased, the rate of release of *p*-nitrophenol also increased. At 70°C, an approximately 225% increase in relative activity was observed in the presence of hexanol when compared to the activity in the absence of alcohol at pH 3.5. Maximal activity was induced by the presence of hexanol; higher carbon chain alcohols reducing the rate of activation. Similar trends were also observed at higher pH (6.5) with a lower temperature (30°C). As shown in Fig. 8, at a pH level of 6.5, an approximately 184% enhancement in activity was observed in the presence of hexanol at both 30 and 70°C while at the lower pH level of 3.5, around 170% activation at 30°C was observed. Maximal enzyme activation was achieved in the presence of hexanol with an optimum temperature of 70°C at pH 3.5. These results indicate that in each case, enzyme activation is a function of the length of the alkyl chain.

4. Conclusions

The β-glucosidase from the hyperthermophilic eubacterium *T. maritima* has been expressed to high levels in *E. coli*, and purified to homogeneity by heat precipitation and ion exchange chromatographies. Similar to other enzymes from the *Thermotoga* species, the β-glucosidase is extremely thermophilic, thermostable and resistant to common protein denaturants. In addition to the observed thermostability, the activity of the β-glucosidase is also stimulated in the presence of alcohols and organic compounds. These properties make this β-glucosidase a good candidate as an enzyme for use in industrial applications.

Acknowledgements

The authors wish to convey their sincere thanks to Prof. Karl O. Stetter and Prof. Robert Huber for the supply of genomic DNA of *T. maritima.* We also thank Dr. M. Kitaoka and Dr. E. Rajashekhara for useful discussions. This work was supported in part by a grant

from the program for promotion of Basic Research Activities for Innovative Biosciences.

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