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Characterisation of a thermostable family 42 β-galactosidase from *Thermotoga maritima*

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ABSTRACT

A β-galactosidase gene (TM_0310) of *Thermotoga maritima* MSB8 was expressed in *Escherichia coli*. The recombinant β-galactosidase (designated BgalB) was purified to homogeneity by heat treatment and Ni-NTA affinity chromatography. BgalB belongs to the glycoside hydrolase family 42. Its molecular mass was estimated to be 78 kDa and 76 kDa by SDS–PAGE and gel filtration, respectively. The enzyme was optimum at pH 5.5, and it was quite stable over the pH range 5.0–11.4 at 70 °C. It was optimally active at 80 °C and was stable up to 75 °C. Besides, BgalB exhibited broad substrate specificity with a preference for *p*-nitrophenyl-β-galactopyranoside (*p*NPGal). K_m values of the purified enzyme for *p*NPGal, *o*-nitrophenyl-β-galactopyranoside (*o*NPGal) and *p*NP-β-fucopyranoside were 2.7 mM, 12.5 mM and 1.4 mM, respectively. These properties make this enzyme an interesting candidate for biotechnological applications. This is the first report of the family 42 β-galactosidases from *T. maritima*.

1. Introduction

β-Galactosidases (β-D-galactoside galactohydrolase or lactase; EC 3.2.1.23) catalyze the hydrolysis of the β-D-galactosyl bond. They have two main biotechnological uses in the diary industry, e.g., removal of lactose from milk for lactose-intolerant people and the production of galacto-oligosaccharides for use in probiotic food stuffs (Gaur, Pant, Jain, & Khare, 2006; Martínez-Villaluenga, Cardelle-Cobas, Corzo, Olano, & Villamiel, 2008; Onishi & Tanaka, 1995; Sako, Matsumoto, & Tanaka, 1999). On the basis of amino acid similarities, β-galactosidases have been divided into four glycoside hydrolase (GH) families: 1, 2, 35 and 42. Most of β-galactosidases belong to family 2, whereas those from thermophilic, psychrophilic and halophilic organisms belong to GH family 42 (Henrissat & Davies, 1997; Holmes & et al., 1997; Kosugi, Murashima, & Doi, 2002; Ohtsu, Motoshima, Goto, Tsukasaki, & Matsuzawa, 1998; Sheridan & Brenchley, 2000; Shipkowski & Brenchley, 2006).

β-Galactosidases have been isolated from various bacteria, fungi and yeasts (Gul-Guven, Guven, Poli, & Nicolaus, 2007; Kang & et al., 2005; Ladero et al., 2002; Møller, Jørgensen, Hansen, Madsen, & Stougaard, 2001; Phan Trân et al., 1998; Sako et al., 1999; Shaikh, Khire, & Khan, 1999). Thermostable β-galactosidases have significant advantages, either in the production of low-lactose dairy products, or in glycoconjugate synthesis, compared to thermolabile enzymes. Thus, it is highly desirable to search for thermostable β-galactosidases as catalysts. Whereas many available β-galactosidases are relatively thermolabile, enzymes derived from thermophilic microorganisms demonstrate a higher degree of thermostability than do β-galactosidases derived from mesophilic ones (Dabrowski, Maciuńska, & Synowiecki, 1998; Fischer, Scheckermann, & Wagner, 1995; Gabelsberger, Liebl, & Schleifer, 1993). Even though β-galactosidases have been extensively studied in a number of bacteria, including thermophilic bacteria, relatively little is known about the β-galactosidases in hyperthermophilic organisms (Grogan, 1991; Gul-Guven et al., 2007; Kang et al., 2005; Kim, Ji, & Oh,2004). The hyperthermophilic bacterium, *Thermotoga maritima*, was originally isolated from geothermal heated marine sediment at Vulcano, Italy. The organism grows between 55 °C and 90 °C with an optimal temperature of 80 °C (Huber & et al., 1986). Inspection of the *Thermotoga maritima* genome located three open reading frames identified as TM_0310, TM_1193

Abbreviations: CAPS, (cyclohexylamino)-1-propanesuhinic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; GH, glycoside hydrolase; IPTG, isopropyl-β-D-thiogalactoside; LB, Luria–Bertani broth; MES, 2-(*N*-morpholino)ethane sulfonic acid; *o*NP, *o*-nitrophenyl; *o*NPGal, *o*NP-β-galactopyranoside; ORF, open reading frame; PCR, polymerase chain reaction; PDA, potato dextrose-agar; *p*NP, *p*-nitrophenyl; *p*NPFuc, *p*NP-β-fucopyranoside; *p*NPGal, *p*NP-β-galactopyranoside; TLC, thin-layer chromatography; BgalB, the recombinant β-galactosidase B (TM_0310) from *Thermotoga maritima* MSB8.

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and TM_1195 that encode three β-galactosidases (Nelson & et al., 1999). The *BgaA* gene for β-galactosidase in *T. maritima* was cloned and characterised. BgaA appears to be a homodimer with native molecular mass of approximately 240 kDa (Gabelsberger et al., 1993). The *lacZ* gene, encoding a β-galactosidase in *T. maritima*, was cloned on an 11 kb fragment by complementation of an *E. coli* deletion strain (Moore, Markiewicz, & Miller, 1994). The *lacZ* gene was found to be truncated on both amino and carboxyl termini, probably as a result of sequencing errors. Recently, a β-galactosidase gene (TM_1193, i.e., *lacZ* gene, accession no. 08186) of *T. maritima* was cloned and expressed in *E. coli*. Purification and characterisation of a 120-kDa β-galactosidase similar to BgaA has been reported (Gabelsberger et al., 1993; Kim et al., 2004). In the present study, a β-galactosidase gene (TM_0310) from genomic DNA of *T. maritima* MSB8 was isolated, cloned and expressed in *E. coli* as a soluble carboxyl terminal His-tagged enzyme. Moreover, purification and characterization of the recombinant β-galactosidase were investigated. The data show that, in addition to β-galactosidase activity, the enzyme displays β-fucosidase activity.

2. Materials and methods

2.1. Materials

The *o*-nitrophenyl (*o*NP)-β-galactopyranoside (*o*NPGal), *p*-nitrophenyl (*p*NP) substrates, *p*NP-β-galactopyranoside (*p*NPGal), *p*NP-α-galactopyranoside, *p*NP-β-glucopyranoside, *p*NP-α-glucopyranoside, *p*NP-β-D-xylopyranoside, *p*NP-α-arabinofuranoside, *p*NP-β-fucopyranoside (*p*NPFuc) and *p*NP-β-mannopyranoside were purchased from Sigma Chemical Company (St. Louis, MO, USA). Ni-NTA agarose resin was obtained from Qiagen (Hilden, Germany). All other chemicals used were analytical grade reagents unless otherwise stated.

2.2. Bacterial strains and plasmids

The genomic DNA from *T. maritima* MSB8 (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, DSM 3109) was used as the source of β-galactosidase gene (TM_0310). *E. coli* DH5α strain was used for DNA manipulations and amplification. *E. coli* BL21 (DE3) was used as host for the recombinant plasmid harbouring pET28a (+) (Novagen, Madison, WI, USA) and expression. Recombinant DNA techniques, including plasmid extraction, restriction endonuclease digestion and DNA ligation, were performed using standard methods (Sambrook & Russell, 2001).

2.3. PCR and cloning the gene into expression vector

The open reading frame (ORF) encoding a β-galactosidase (TM_0310, designated *BgalB*) was retrieved from GenBank (accession no. AAD35398.1). The gene was amplified by polymerase chain reaction (PCR) using a 5′-forward primer containing a restriction site for *Nco I* (5′-CCATGGTAAATCCGAAACTTCCTGT-3′; the restriction site is underlined) and 3′-reverse primer with a *Hind III* site (5′-AAGCTTTTCTTTTAGAAGGATCAGAAC-3′). The restriction sites were chosen so as to insert a C-terminal 6 × His-tag into the construct. PCR conditions were performed as follows: a hot start at 94 °C for 5 min, 30 repeated cycles of 94 °C for 30 s, 52.5 °C for 30 s and 72 °C for 2 min, followed by one cycle of 72 °C for 10 min. The PCR products were purified from agarose gels. The purified DNA fragment was ligated to the pMD18-T and the plasmid was transformed into *E. coli* DH5α cells. The resulting recombinant plasmids (TM-*BgalB*-pMD18-T) were isolated from a positive clone and DNA sequencing was performed using BigDye terminator cycle sequencing kit (Perkin–Elmer, Applied Biosystems, Norwalk, CT, USA).

2.4. Expression of BgalB gene in E. coli

The *BgalB* gene was excised from the recombinant plasmid TM-*BgalB*-pMD18-T using one pair of restriction enzymes, *Nco I* and *Hind III*, and ligated with the pET28a (+) vector, which had been previously digested with the same pair of restriction enzymes. Ligation of DNA-vector was conducted overnight at 16 °C, using the ligation High T_4 DNA ligase kit (TOYOBO Co., Osaka, Japan). The host *E. coli* BL21 (DE3) competent cells were transformed with the ligated *BgalB*-pET28. The positive colonies were screened out from LB (Luria–Bertani broth)-plates containing kanamycin (50 μg/ml) by the direct colony PCR, using the vector-specific primers (T7 promoter and T7 terminator primer). One of the positive clones was sequenced to confirm that the DNA sequence was identical to the database sequence for *T. maritima*.

2.5. SDS–PAGE and protein determination

Electrophoresis of protein samples was performed according to the method described by Laemmli (1970). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) was conducted on 1 mm thick 10% acrylamide gel slabs. The proteins were stained with Coomassie brilliant blue R-250 in SDS–PAGE. The molecular weight standard used was the low molecular weight calibration kit for SDS electrophoresis (Amersham): phophorylase *b* (97.0 kDa), albumin (66.0 kDa), ovalbumin (45.0 kDa) and carbonic anhydrase (30.0 kDa). Protein concentrations were measured by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951) with BSA (bovine serum albumin) as the standard.

2.6. Enzyme assay

Unless mentioned otherwise, β-galactosidase activity was determined as follows: mixtures containing 5 mM *p*NPGal as the substrate dissolved in 225 μl of 50 mM phosphate buffer (pH 7.0) and an appropriate amount of enzyme in a total volume of 25 μl, were incubated for 10 min at 70 °C. The reaction was terminated by adding 0.75 ml of 2.0 M Na_2CO_3, and then the liberated *p*-nitrophenol was measured spectrophotometrically at 410 nm. One unit of enzyme activity was defined as 1 μmol of *p*-nitrophenol released per minute under the described conditions. Specific activity was expressed as U/mg of protein.

2.7. Purification of BgalB in E. coli

To express *T. maritima* β-galactosidase gene (*BgalB*), 5 ml of freshly prepared seed culture of *E. coli* BL21, harbouring recombinant pET28, was inoculated to LB medium (100 ml) containing kanamycin (50 μg/ml) which was cultured at 30 °C with a rotary shaker (200 rpm) until the an optimal density at 600 nm ($OD_{600\ nm}$) reached 0.5–0.6. IPTG was added up to 1 mM, and incubation was then continued 12 h. The cells were harvested by centrifugation at 12,000*g* for 15 min at 4 °C, and suspended in 50 mM phosphate buffer, pH 8.0. The disruption of the cells was accomplished by sonication, to extract enzyme. The cell lysate was then centrifuged at 12,000*g* for 10 min at 4 °C and the supernatant was collected. For denaturing some *E. coli* proteins, the crude cell extract was heated at 70 °C for 10 min. The heat-treated crude cell extract was centrifuged at 12,000*g* for 10 min at 4 °C. The β-galactosidase remained in the clear supernatant. The enzyme was purified by Ni-NTA agarose metal chelate chromatography, performed at room temperature. The crude enzyme extract was applied to a Ni-NTA agarose resin column equilibrated with 50 mM phosphate buffer (pH 8.0)

containing 20 mM imidazole and 300 mM NaCl. The enzyme was eluted in a linear gradient of 20–250 mM imidazole in 50 mM phosphate buffer (pH 8.0) containing 300 mM NaCl. The active fractions were eluted as a single protein peak, dialyzed overnight at 4 °C against 50 mM phosphate buffer (pH 8.0) and used as a purified BgalB. The homogeneity of the purified BgalB was monitored by SDS–PAGE.

2.8. Determination of pH and thermal profiles

The pH-dependence of enzyme activity was determined at 70 °C in 50 mM concentrations of various buffer systems: sodium citrate (pH 3.0–6.0), sodium acetate (pH 4.0–5.5), sodium phosphate (pH 6.0–8.0), MOPS (pH 6.5–8.5), CHES (pH 8.0–11.0) and CAPS (pH 9.0–11.0), were used instead of the usual pH buffer to assay enzyme activity. To determine the pH stability, the enzyme was pre-incubated with the above buffers for 30 min at 70 °C, and then the residual activities of these treated enzymes were measured by the standard assay procedure.

The temperature optimum was determined under standard assay conditions by incubating the reaction mixture at temperatures ranging from 30 °C to 100 °C in 50 mM sodium citrate (pH 5.5). For estimating the thermostability, the enzyme was pre-incubated for 30 min in 50 mM sodium citrate (pH 5.5) at different temperatures. After cooling the treated enzymes on ice for 30 min, the remaining activity was then measured according to the standard assay method.

Purified enzyme samples were pre-incubated with different metal ions, the chelator (EDTA) and some agents (4 mM) at 70 °C for 30 min and were checked for enzyme activity compared to control without metal ions or agents addition.

2.9. Substrate specificity and analysis of kinetic parameters

Tests on the chromogenic substrates were performed at 70 °C for 10 min. Activity was measured by the rate of *p*-nitrophenyl or *o*-nitrophenyl formed during hydrolysis of 5 mM substrates in 50 mM sodium citrate (pH 5.5), detected at 410 nm. One unit of activity towards chromogenic substrates was defined as the amount of enzyme needed to produce 1 μmol *p*-nitrophenyl or *o*-nitrophenyl per minute under the defined conditions. The activity of the β-galactosidase against disaccharides was estimated spectrophotometrically. The reactions were carried out with 5 mM substrate for 10 min at 70 °C in 50 mM sodium citrate (pH 5.5). After the reactions were terminated by boiling for 5 min, β-galactosidase activity was measured by determining glucose release using a glucose oxidase kit (Beijing BHKT Clinical Reagent Co., Ltd.) after converting absorbance at 505 nm to mM concentration of glucose using a standard curve. One unit of enzyme activity is defined as the amount of enzyme needed to produce 1 μmol of glucose per minute under the defined conditions.

The kinetic parameters were determined using different concentrations of substrates. All the experiments were carried out at 70 °C for 5 min in 50 mM citrate buffer (pH 5.5). Initial hydrolysis rates were determined at six different concentrations ranging from approximately 0.5–2.0 times the K_m values. K_m and k_{cat} and their standard errors were calculated by using the nonlinear regression analysis programme "Grafit".

2.10. Analysis of hydrolysis products

The hydrolysis reactions consisted of 5 mM chromogenic substrates and diluted β-galactosidase (0.02 U/ml) in 50 mM citrate buffer (pH 5.5), and were incubated at 80 °C for 4 h. The hydrolysis of lactose was evaluated by incubating 2.5% (w/v) of lactose with 5 U/ml of the purified enzyme in 50 mM citrate sodium (pH 5.5) at 80 °C for 8 h. The aliquots were withdrawn at different time intervals. After deionization, the hydrolysis products were analyzed by thin-layer chromatography (TLC). The reaction mixtures were deionized and spotted onto silica gel plates (Merck Silica Gel 60F 254; E. Merck, Darmstadt, Germany), and then developed twice in a solvent system containing butan-1-ol–acetic acid–water (2:1:1, v/v). Saccharides were detected by heating for a few minutes in a hot dry oven after spraying the plates with a methanol:concentrated sulfuric acid mixture (95:5, v/v).

3. Results and discussion

3.1. Cloning and expression of a β-galactosidase gene from T. maritima

A β-galactosidase gene (designed as *BgalB*) from genomic DNA of *T. maritima* (TM_0310) (Nelson et al. 1999) was cloned and expressed in *E. coli*. The amplified β-galactosidase-coding DNA fragment was inserted into the pET-28a(+) plasmid with both *Nco I* and *Hind III* restriction sites, resulting in an expression vector designated *BgalB*-pET28. The expressed β-galactosidase (i.e., BgalB) was a soluble protein that contained a hexa-histidine tag at the carboxyl terminus, and consisted of 672 amino acid residues. According to the homology search of the deduced amino acid sequence, the enzyme showed the highest degree of similarity (95% identity) to the putative β-galactosidase of *Thermotoga petrophila* RKU-1 (accession no. ABQ46628) and *Thermotoga* sp. RQ2 (accession no. EDQ29256) is next close to the putative β-galactosidase from *Bacillus halodurans* C-125 (49% identity, accession no. BAB07420). The rest of the β-galactosidases showed identities of less than 48%. Comparison of the recombinant β-galactosidase protein sequence to other β-galactosidases in the Genbank database revealed that the enzyme belonged to family 42 of the glycosyl hydrolases.

3.2. Purification of the recombinant β-galactosidase

A summary of the purification of the recombinant β-galactosidase (BgalB) is presented in Table 1. BgalB was purified to homogeneity by heat treatment and Ni-NTA affinity chromatography. The finally purified enzyme yielded 44.4% of the initial total activity, with an approximate 10.4-fold increase in specific activity. The purified enzyme revealed a single protein band with an apparent molecular mass of 78 kDa by SDS–PAGE analysis (Fig. 1); this is consistent with that of the molecular mass 79,080.5 Da, calculated on the basis of the deduced amino acid sequence (Nelson et al., 1999). The molecular mass of the native enzyme was determined to be approximately 76 kDa by gel filtration (data not shown), indicating that it was a monomeric protein.

Multimeric nature is common among microbial β-galactosidases (Fischer et al., 1995; Gul-Guven et al., 2007; Onishi & Tanaka, 1995; Shaikh et al., 1999; Sheridan & Brenchley, 2000). Most of recombinant β-galactosidases reported so far are multimeric en-

Table 1
Summary of the recombinant β-galactosidase (BgalB) purification

Purification step	Protein[b] (mg)	Total activity[a] (U)	Specific activity (U/mg)	Purification factor (-fold)	Recovery (%)
Crude enzyme	945	1964	2.08	1	100
Heat precipation	204	1501	7.36	3.5	76.4
Ni-NTA	40.4	873	21.6	10.4	44.4

[a] Activity was measured in 50 mM phosphate buffer (pH 7.0) at 70 °C using 5 mM *p*NPGal as the substrate.
[b] The protein was measured by the Lowry method (Lowry et al., 1951), using BSA (bovine serum albumin) as the standard.

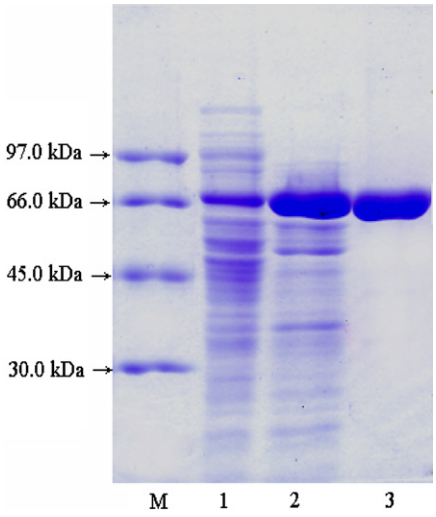

Fig. 1. SDS–PAGE of fractions from the purification of the recombinant β-galactosidase (BgalB) from *T. maritima*. Lane M, low molecular weight calibration kit; lane 1, crude enzyme; lane 2, heat precipation and lane 3, Ni-NTA chromatography (purified β-galactosidase).

zymes with subunit moleculear mass of 70–80 kDa (Gabelsberger et al., 1993; Holmes et al., 1997; Lu, Xiao, Xu, Li, & Li, 2007; Møller et al., 2001; Wanarska, Kur, Pladzyk, & Turkiewicz, 2005). The halophilic β-galactosidase from *Haloferax alicantei* is dimeric with two subunits of 78 kDa (Holmes & et al., 1997). However, BgalB is a monomeric enzyme, akin to a thermostable β-galactosidase from *Thermus* sp. A4 (Ohtsu et al., 1998). The subunit mass of purified enzyme in the present study was also closer to those of β-galactosidases from *Thermomyces lanuginosus* (75–80 kDa; Fischer et al., 1995), *H. alicantei* (78 kDa; Holmes et al., 1997), *Thermus* sp. A4 (75 kDa; Ohtsu et al., 1998), *Planococcus* isolate (75 kDa; Sheridan & Brenchley, 2000), *Alicyclobacillus acidocaldarius* subsp. *Rittmannii* (76 kDa; Gul-Guven et al., 2007). Besides, the molecular mass of this enzyme was relatively low, compared to the 120 kDa β-galactosidases reported from *Achatina achatina* (Leparoux, Padrines, Placier, & Colas, 1997), *Rhizomucor* sp. (Shaikh et al., 1999), *T. maritima* (Gabelsberger et al., 1993; Kim et al., 2004) and *Enterobacter agglomerans* B1 (Lu et al., 2007).

3.3. Effect of pH and temperature on enzyme activity

The effects of pH and temperature on the β-galactosidase activity are shown in Figs. 2 and 3, respectively. pH optimum of BgalB was pH 5.5 at 70 °C, with 60% of maximum activity being retained between pH 5.0 and pH 7.0 (Fig. 2a). BgalB exhibited high pH stability (more than 90%) with the range of 4.5–9.5 at 70 °C (Fig. 2b). As shown in Fig. 3, maximum activity was observed at 80 °C for pH 5.5. It was stable and displayed 90% activity at 75 °C.

The purified β-galactosidase showed maximal activity at pH 5.5, a pH value comparable with that reported for the recombinant β-galactosidase from *Bacillus licheniformis* (Phan Trân et al., 1998), the thermostable β-galactosidase from *Pyrococcus woesei*

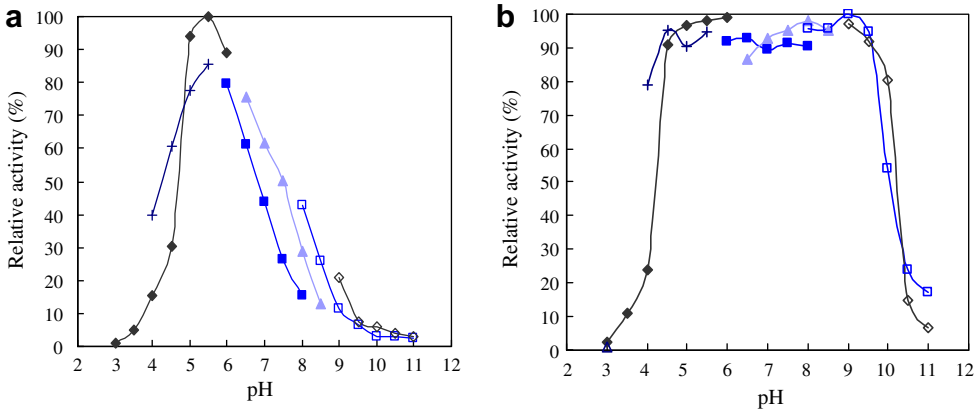

Fig. 2. Effect of pH on activity (a) and pH stability (b) of BgalB. The influence of pH on β-galactosidase activity was determined at 70 °C using 50 mM of different buffers: acetate (+), citrate (♦), phosphate (■), MOPS (▲), CHES (□) and CAPS (◇). The remaining activity was measured after incubation for 30 min at 70 °C over various pH ranges.

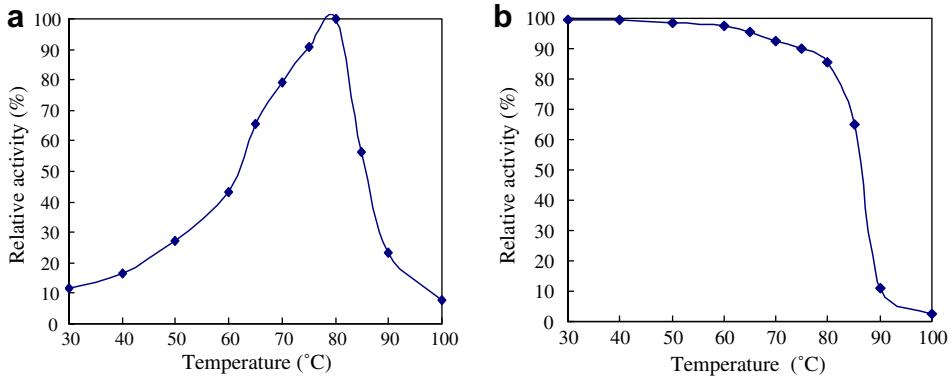

Fig. 3. Effects of temperature on activity (a) and thermostability (b) of BgalB. The temperature profile was measured at different temperatures using the standard assay at optimum pH 5.5, 50 mM citrate buffer. For determination of thermostability, the residual activities of the treated enzymes were measured according to the standard assay after a 30 min preincubation at different temperatures in 50 mM citrate buffer (pH 5.5).

Table 2
Kinetic parameters for the BgalB[a]

Substrate	V_{max} (μmol min^{-1} mg^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
*p*NP-β-Galactopyranoside	351 ± 11.3	2.74 ± 0.22	5.85	2.13
*o*NP-β-Galactopyranoside	79.3 ± 2.6	12.5 ± 1.1	1.32	0.11
*p*NP-β-Fucopyranoside	58.1 ± 2.1	1.44 ± 0.12	0.97	0.67

[a]Enzymatic reactions were carried out for 5 min at 70 °C in 50 mM citrate buffer (pH 5.5).

(Dabrowski et al., 1998) and 120 kDa BgaA from *T. maritima* (Gabelsberger et al., 1993), but slightly lower than that for 120 kDa β-galactosidase (pH 6.5) from *T. maritima* (Kim et al., 2004), and β-galactosidase (pH 6.0) from *A. acidocaldarius* subsp. *Rittmannii* (Gul-Guven et al., 2007). The enzyme exhibited a temperature optimum of 80 °C, and 65% of the maximum activity was detected after treatment at 85 °C for 30 min. These characterizations with regard to temperature indicated that BgalB was a highly thermostable β-galactosidase. It is apparent that the BgalB was extremely thermophilic and thermostable, like other β-galactosidases from the *Thermotoga* sp. (Gabelsberger et al., 1993; Kim et al., 2004). A better thermostability would be a significant advantage for prospective biotechnological applications, either in the food industry or in glycoconjugate synthesis. Therefore, these may be advantageous for its applications in the industry.

The influence of different metal ions and other reagents was investigated (data not shown). β-Mercaptoethanol, DTT, EDTA, Ni^{2+}, Ca^{2+}, Sr^{2+}, Co^{2+}, Zn^{2+}, Fe^{3+} and Mg^{2+} increased the activity by 127% (of control), 121%, 121%, 117%, 115%, 114%, 113%, 113%, 112% and 111%, respectively, whereas SDS (4.2%), Ag^{+} (1.2%), and Hg^{2+} (0.7%) strongly inhibited the enzyme. In addition, Mn^{2+} (81.3%), and Cu^{2+} (88.9%) showed moderate inhibition.

Generally, β-galactosidases can be inhibited or activated by metal ions or other reagents (Lu et al., 2007; Shaikh et al., 1999). It is known that Mg^{2+} is required for enzyme activity in most β-galactosidases (Kim et al., 2004; Lu et al., 2007; Ohtsu et al., 1998; Wanarska et al., 2005), whereas $Hg^{2+}$and Cu^{2+} evidently deactivated the enzyme activity as previously reported (Ladero et al., 2002; Ohtsu et al., 1998; Onishi & Tanaka, 1995; Shaikh et al., 1999; Shipkowski

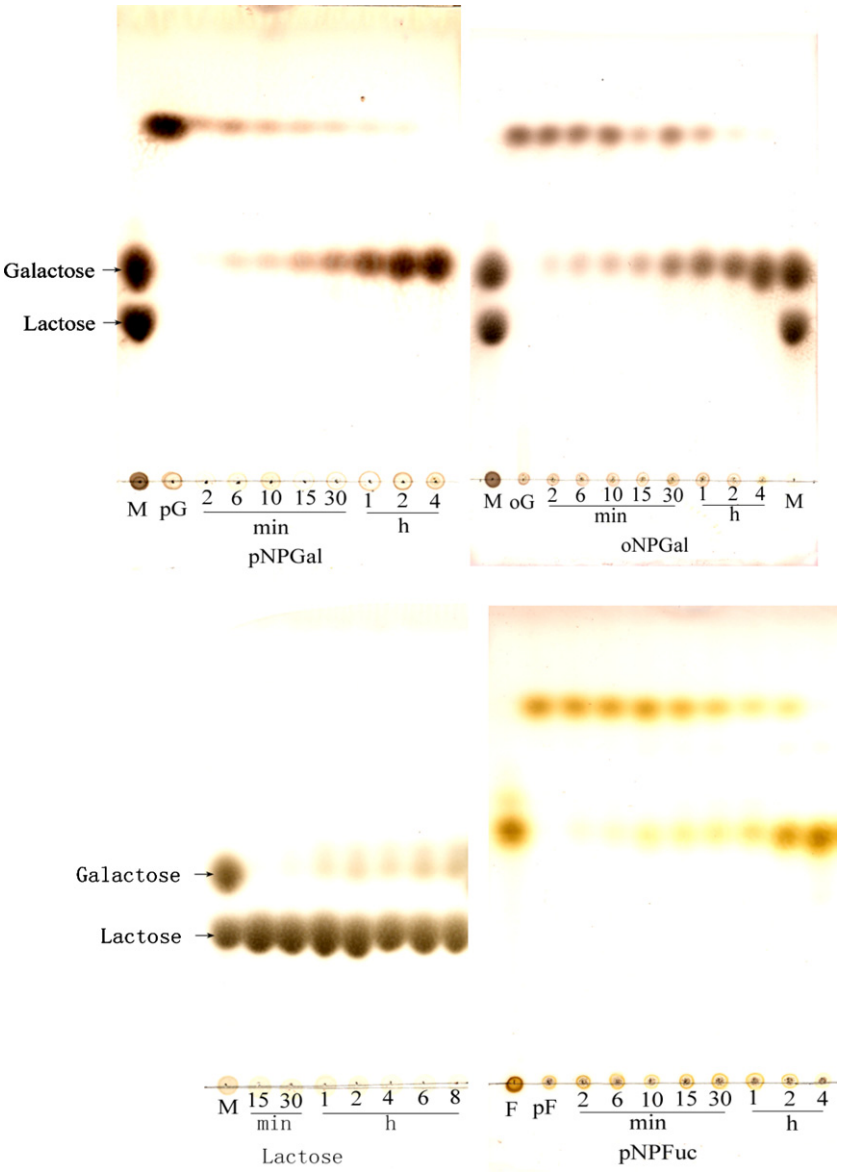

Fig. 4. TLC analysis of hydrolysis products from various substrates by BgalB. Incubation times (h or min) and substrates are indicated. Lanes M, a mixture of galactose and lactose; lane pF, *p*NPFuc; lane pG, *p*NPGal and lane oG, *o*NPGal.

& Brenchley, 2006; Wanarska et al., 2005). Most of the metal ions activated the enzyme activity in this study, which is similar to the 120 kDa β-galactosidases from *T. maritima* and *E. agglomerans* B1 (Kim et al., 2004; Lu et al., 2007). The addition of EDTA activated β-galactosidase activity by 20.6%, suggesting that no metals are needed for enzymatic reactions. The enzyme was activated in the presence of SH reagents, namely, β-mercaptoethanol and DTT. These results are similar to the findings for a thermostable β-galactosidase from *Thermus* sp. A4 (Ohtsu et al., 1998).

3.4. Substrate specificity and kinetic parameters

The substrate specificity of the enzyme was examined using various substrates (data not shown). BgalB was active towards *p*NPGal, *o*NPGal, *p*NPFuc and lactose. Especially, *p*NPFuc was hydrolyzed at a rate of approximately 35.6% relative to *p*NPGal hydrolysis. Thus, the enzyme exhibited broad substrate specificity. Additionally, this enzyme had no activity on other substrates tested, such as *p*NP-α-galactopyranoside, *p*NP-β-glucopyranoside, *p*NP-α-glucopyranoside, *p*NP-β-xylopyranoside, *p*NP-α-arabinofuranoside and *p*NP-β-manopyranoside.

The substrate specificity studies indicated that the enzyme hydrolyzed *p*NPGal, *o*NPGal, *p*NPFuc and lactose. No β-glucosidase, β-xylosidase, β-mannosidase, α-glucosidase, α-galactosidase or α-arabinosidase activities were detected. Generally, most characterised GH family 42 β-galactosidases hydrolyze lactose weakly and prefer to hydrolyze the β-linked galactosidic substrates *p*NPGal or *o*NPGal, with less than 10% relative activity on nongalactosidic chromogens (Kosugi et al., 2002; Møller et al., 2001; Phan Trân et al., 1998; Shipkowski & Brenchley, 2006). It is reported that two GH family 42 β-galactosidases do not cleave lactose *in vitro* (Holmes et al., 1997; Van Laere, Abee, Schols, Beldman, & Voragen, 2000). Associated activities, e.g., β-glucosidase and/or α-arabinosidase activity, are also known to be present in some β-galactosidases (Fischer et al., 1995; Grogan, 1991; Kosugi et al., 2002; Onishi & Tanaka, 1995). It is interesting to note that the enzyme possesses a high level of β-fucosidase activity. Low levels of activity for some β-galactosidases were observed with *p*NP-β-fucopyranoside or *o*NP-β-fucopyranoside (Grogan, 1991; Holmes et al., 1997; Sheridan & Brenchley, 2000; Shipkowski & Brenchley, 2006). However, the β-galactosidase from *Sterigmatomyces elviae* CBS8119 hydrolysed *p*NPFuc 6.3 times faster than *p*NPGal (Onishi & Tanaka, 1995).

As shown in Table 2, K_m values for *p*NPGal, *o*NPGal and *p*NPFuc were 2.74 ± 0.22 mM, 12.5 ± 1.1 mM and 1.44 ± 0.12 mM, respectively, suggesting that BgalB possessed higher affinity for β-fucopyranoside residues than for β-galactopyranoside residues. The K_m values show that the purified BgalB has a weak affinity for *o*NPGal, compared to the 120 kDa enzyme from *T. maritima* (K_m value of 0.33 mM; Kim et al., 2004) and other members of the glycosidase family 42, e.g., K_m value of 0.87 mM for the β-galactosidase in *H. alicantei* (Holmes et al., 1997), K_m value of 5.9 mM for the β-galactosidase in *Thermus* sp. A4 (Ohtsu et al., 1998), and K_m value of 4.9 mM for the β-galactosidase of *Planococcus* isolate (Sheridan & Brenchley, 2000).

3.5. Hydrolytic properties

The hydrolysis of various substrates catalyzed by purified BgalB was followed by analyzing the reaction products by TLC (Fig. 4). Transglycosylation reactions were not detected in TLC hydrolysis patterns using *p*NPGal, *o*NPGal, lactose and *p*NPFuc as substrates. This suggests that the enzyme may be used in industries for producing low-lactose milk. Many β-galactosidases have transglycosylation activity and have been used for the enzymatic synthesis of oligosaccharides and galactosyl-amino acid and -dipeptide derivatives (Kim et al., 2004; Leparoux et al., 1997; Lu et al., 2007; Martínez-Villaluenga et al., 2008; Onishi & Tanaka, 1995; Sako et al., 1999). A thermostable β-galactosidase from *Thermus* sp. A4 has no transfer activity (Ohtsu et al., 1998).

4. Conclusions

The study demonstrated that a β-galactosidase gene (TM_0310) of *T. maritima* MSB8 was expressed in *E. coli*. The described purification procedure of the recombinant β-galactosidase (designated BgalB) is simple and rapid. Sequence analysis indicates that BgalB is a member of the glycoside hydrolase family 42. The optimal temperature for the enzyme was 80 °C and it was stable up to 75 °C. The enzyme displayed a wide range of substrate specificity. The combination of thermostability and broad substrate specificity may make the enzyme useful in a variety of applications.

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