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Research Article

Isolation and characterization of β-galactosidase from the thermophile B1.2

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Abstract

The enzyme β-galactosidase has two main biotechnological applications in milk and dairy products, e.g. the removal of lactose from milk for lactose-intolerant people and the production of galacto-oligosaccharides (GOS) for use in probiotic food. The intracellular β-galactosidase from thermophile B1.2 was isolated from Ta Pai hot spring, Maehongson, Thailand. The enzyme was purified by ion-exchange and affinity chromatography with a fold purification of 2.2 and 3.9, respectively. The activity of purified β-galactosidase was shown to have an optimal operating condition at pH 6.5 and a temperature of 60^0C. The thermostability of the enzyme was in the range of 40-60^0C with the pH stability in the range of 6.0 - 10.0. The K_m and V_{max} values for oNPG were determined as 28.85 mM and 8.38 $\times 10^{-3}$ $mmolL^{-1}min^{-1}$, respectively. An intracellular β-galactosidase from B1.2 was also inhibited by various mono- and divalent cations, including Zn^+ and Mg^{2+}; reagents, including EDTA. It was moderately inhibited by its reaction products; glucose and galactose. The molecular mass of the purified enzyme as determined by native PAGE was approximately 215 kDa, by SDS – PAGE was 75 kDa and by gel filtration was 215 kDa.

Keywords: β-galactosidase, purification, dairy, probiotic, thermophilic bacteria, Thailand.

Introduction

β-galactosidase is a member of glycosyl hydrolyses enzymes (EC 3.2.1–3.2.3) which are known to cleave the glycosidic bond between two or more carbohydrates or between a carbohydrate and another moiety. This group of enzymes was traditionally classified based on functional similarity. β-galactosidase hydrolyses the β-1,4-d-galactosidic linkage of lactose, as well as those of related chromogens, o-nitro-phenyl-β-D-galactopyranoside (oNPG), p-nitrophenyl-β-D-galactopyranoside (PNPG) and 6-bromo-2-naphthyl-galacto-pyranoside (BNG). This enzyme is widely distributed in nature, being found in various types of microorganisms, plant and animal tissues [1]. The enzyme β-galactosidase has two main biotechnological uses in the dairy industry, e.g. the removal of lactose from milk for lactose-intolerant people and the production of galacto-oligosaccharides for use in probiotic food. The application of β-galactosidase to the hydrolysis of lactose in dairy products, such as milk and cheese whey has received much attention. Commercially available galactosidase tablets are being used in industries for producing low lactose milk [2]. Also, whey utilisation for the production of many valuable products has been extensively studied and in this regard, the thermostable β-galactosidases have attracted increasing interest for use in such industrial processes [3]. Many studies have been carried out on the thermophilic β-galactosidases in numerous microorganisms, including Bacteria and Archaea [4, 5, 6], with particular attention focused on those from Antarctica [7]. Thermophilic bacilli grown at extreme environments such as hot springs and geothermal soils are described as aerobic, endospore-forming organisms with optimal growth temperature in the range 45-75^{o}C . By using 16S rDNA sequence analysis, currently they are classified into two genera, Bacillus, the majority being assigned by the former [8]. Although often considered as contaminants of heat-treated food products, the importance of these thermophilic bacilli has increased due to their potential as a source of thermostable enzymes, including β-galactosidase, protease, amylase, lipase and DNA restriction enzymes [8]. In this research, the purification a thermostable β–galactosidase from a microorganism isolated from Ta Pai hot spring, Maehongson, Thailand and also the characterization of some properties of the enzyme are studied.

Materials and Methods

Microorganism and culture conditions

Strains for screening of thermophilic microorganisms were obtained from Ta Pai hot spring, Maehongson, Thailand. The selected strain with which most of the work was performed was isolated from calf (isolate B1.2). This strain was stored in sterile vials at −80°C in lactose broth medium (5 g/L Lactose, 5 g/L Peptone, 3 g/L Beef extract) containing glycerol (15%, v/v) and activated by transfers in lactose broth medium for 24 h. It was grown on lactose broth medium, and incubated at 50°C for 24 h. 1-day culture of the strain B1.2 from lactose broth was aseptically transferred into 50-mL sterile inoculum lactose-mineral salt medium (10 g/L Lactose, 10 g/L Peptone, 10 g/L Yeast extract, 5 g/L $(NH_4)_2SO_4$, 3 g/L K_2HPO_4, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4.7H_2O$) in 250-mL Erlenmeyer flask. The inoculum was incubated at 50°C at 150 rpm for 15 h. The inoculum was then aseptically transferred into 500-mL lactose-mineral salt medium 1% (w/v) lactose in a 1000-mL Erlenmeyer flask at 50°C at 150 rpm and initial pH of 7 for 15 h. To screen for β-galactosidase activity, cells were harvested from a liquid culture by centrifugation (4,000 rpm for 15 min at 4°C) using an Eppendorf centrifuge and resuspended in 50 mM sodium phosphate buffer (pH 6.5).

16s rDNA sequencing

The results of 16s rDNA sequence (partial length) was performed at BIOTEC Culture Collection (BCC), Pathumthani, Thailand.

Enzyme purification

The cells were disrupted by French Pressure Cell Press (SLM-AMINCO® Spectronic Instrument; USA) at 1,500 psi and 4°C. The debris was removed by centrifugation (4,000 rpm for 15 min at 4°C). The crude extract was then applied to a DEAE sepharose (Pharmacia) column (XK, 10 x 26 mm) that had been previously equilibrated with 50 mM phosphate buffer pH 6.5. The protein was eluted by using a linear gradient of 1 M NaCl in buffer at a flow rate of 2 mL min^{-1}. Fractions with significant β-galactosidase activity were pooled, desalted and concentrated using ultrafiltration (Amicon membrane, 10,000 MWCO; Millipore, USE). The concentrated enzyme solution was loaded onto the affinity (Agarose p-aminobenzyl-1-thio-β-D-galactopyranoside, Sigma) column (XK 3.5 x 26 cm) that was pre-equilibrated with 50 mM sodium phosphate buffer. The enzyme was eluted at a rate of 0.5 mL/min by using a linear 1 M NaCl gradient in 50 mM phosphate buffer pH 6.5. The active fractions were pooled, desalted, and concentrated.

Enzyme assays

β-galactosidase activity was determined using o-nitrophenyl β-D-galactopyranoside (oNPG) as a substrate. Unless otherwise specified, β-galactosidase activity was assayed at 40°C by incubating 20 µL of suitably diluted enzyme with 480 µL of 22 mM o-nitrophenyl β-D-galactopyranoside (oNPG) in 50 mM phosphate buffer pH 6.5 as the substrate for 15 min [9]. The reaction was stopped by adding 750 µL of 0.4 M Na_2CO_3 and the o-nitrophenyl (oNP) released was determined by reading the increase in absorbance at 420 nm. One unit of β-galactosidase activity (U) was defined as the amount of enzyme releasing 1 µmol of oNP from oNPG per minute under the given conditions. Protein was determined by the dye binding method [10], with bovine serum albumin (BSA) as a standard protein.

Determination of molecular mass

Native polyacrylamide gel electrophoresis (PAGE) and denatured sodium dodecyl sulphate−polyacrylamide gel electrophoresis (SDS−PAGE) were prepared as described in Nakkharat and Haltrich [9]. For SDS−PAGE, Coomassie brilliant blue staining was used for the visualization of the protein bands. Active staining for the visualization of the bands with β-galactosidase activity was carried out by applying filter paper soaked with the staining solution [50 mM sodium phosphate buffer (pH 6.5) and 3.5 mg/mL 4-methylumbelliferyl β-D-galactoside] onto the gel and incubating at 37°C for 30 min. After the application of 1 M carbonate−bicarbonate buffer (pH 10.0) onto the gel using a filter paper, the protein bands displaying enzyme activity were visualized under UV light, thus detecting the release of 4-methylumbelliferone.

For molecular mass determination by gel filtration, a Biogel P-100 was equilibrated with 50 mM phosphate buffer pH 6.5. The column was calibrated to the standard proteins; Lysozyme (M_r 14600), Trypsin (M_r 23800), Protenase K (M_r 28900), Lipase (M_r 45000) and Amyloglucosidase (M_r 97000) each at 10 mg mL^{-1}. The flow rate for elution was 0.1 ml min^{-1}

Steady-state kinetic measurements

All steady-state kinetic measurements were obtained at 40°C using o-nitrophenyl β-D-galactopyranoside (oNPG) as a substrate in 50 mM sodium phosphate buffer (pH 6.5) with concentrations ranging from 0 to 30 mM for oNPG. The kinetic parameters were calculated by nonlinear regression, and the observed data were fit to the Henri−Michaelis−Menten equation.

pH and temperature dependence of activity and stability

The pH dependence of the enzymatic release of o-nitrophenol (oNP) from oNPG was measured between pH 3 and 10 using Citrate buffers (50 mM; pH 3.0, 4.0 and 5.0), Sodium phosphate buffers (50 mM; pH 6.0, 6.5 and 7.0), Tris buffer (50 mM; pH 8.0) and Glycine-NaOH buffer (50 mM pH 9.0 and 10.0). The activity was determined at different pH values under standard assay conditions. To determine the pH stability of B1.2 β-gal, the enzyme samples were incubated at various pH levels and 40°C for up to 12 h, and the remaining enzyme activity was measured at time intervals using oNPG as the substrate under standard assay conditions. The temperature dependence of enzyme activity was measured by assaying the enzyme samples over the temperature range of 30−80°C for 15 min. The temperature stability of enzymes was studied by incubating the enzyme samples in 50 mM sodium phosphate buffer pH 6.5, at various temperatures (40-80°C). At certain time intervals, samples were withdrawn and the residual activity was measured with oNPG as the substrate under standard assay conditions.

Effects of various reagents, cations and carbohydrates on β-galactosidase activity

The enzyme samples were assayed with 22 mM oNPG solution [in 50 mM sodium phosphate buffer pH 6.5] in the presence of 1 and 10 mM mercaptoethanol, EDTA, DTT and urea, individually, at 40°C for 15 min. The enzyme activity measured without added reagents was used as a control. To evaluate the effects of various cations on β-galactosidase activity, the respective cations were added to the standard enzyme assay and the activity was determined. The final cation concentrations in the assay were 1, 10 and 100 mM. Metal salts tested included NaCl, KCl, $MgCl_2.6H_2O$, $FeSO_4.7H_2O$, $MnCl_2.4H_2O$, $CaCl_2.2H_2O$, $CoCl_2.6H_2O$, $ZnSO_4.7H_2O$ and $CuCl_2.2H_2O$. Similarly, to study the effect of various carbohydrates (final concentrations in the assay of 1, 10 and 100 mM), galactose, glucose and lactose were included.

Results and Discussion

16s rDNA sequencing

Thermophilic bacteria are the chief components of the microflora that are utilized in food and milk industries [11]. They constitute a heterogeneous physiological group of various genera, for example, *Streptococcus thermophilus*, *Fusarium moniliforme* [12, 13]. The intracellular β-galactosidase from thermophile B1.2, were isolated from Ta Pai hot spring, Maehongson, Thailand. Following the screening, one isolate of strain B1.2 was selected for further studies on β-galactosidase. To investigate the species, different identification methods were used: gram staining, endospore staining, catalase test and the direct amplified polymorphic DNA-polymerase chain reaction analysis and the partial sequencing of the 16S rDNA gene. B1.2 was found to be a gram positive bacteria with its endospores located at the terminal end of the vegetative cell. B1.2 is considered to be a new species in the genus *Anoxybacillus* that is close to the strains of *Anoxybacillus kestanbolinensis, Anoxybacillus flavithermu and Anoxybacillus pushchinoensis*, giving results of 97.3%, 98.1% and 98.0%. A phylogenetic tree is represented for B1.2 in Figure 1.

Purification of β-galactosidase

β-Galactosidase was isolated from the cell extracts of B1.2 using a purification protocol DEDE chromatography and affinity chromatography on agarose p-aminobenzyl-1-thio-β-D-galactopyranoside. The result of representative purification procedure for β-galactosidase is summarized in Table 1. The enzyme was purified approximately 3.9-fold and a recovery of 86% from the crude cell extracts and specific activities of 1.1 units/mg of protein using standard assay conditions with oNPG as the substrate. The subunit molecular weight, as determined by SDS–PAGE electrophoresis, was 75 kDa. The molecular mass of the native purified enzyme as

determined by native PAGE was approximately 215 kDa (Figure 2) and by gel filtration was 215 kDa.

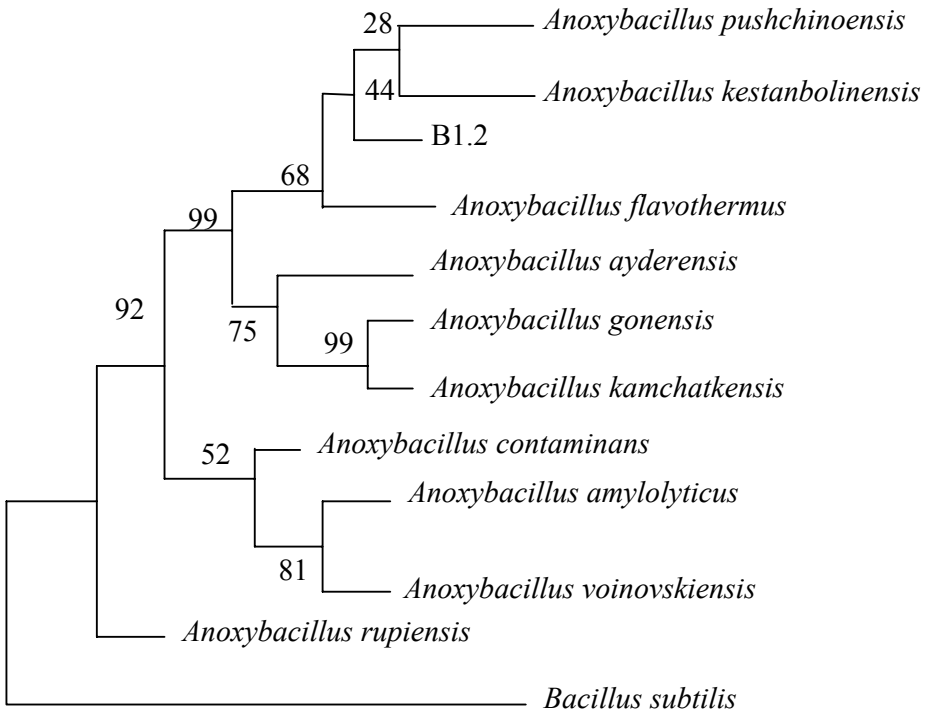

Figure 1. 16s rDNA Phylogenetic Tree.

Table 1. Purification of intracellular β-galactosidase from strain B1.2.

Purification steps	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Purification (flod)	Recovery (%)
Crude extract	25.8	89.6	0.29	1	100
DEAE + Ultrafiltration	24.3	37.1	0.65	2.2	94
Affinity + Ultrafiltration	22.3	20.3	1.1	3.9	86

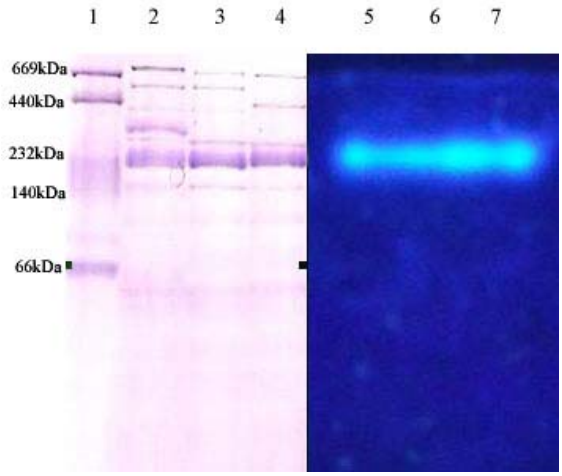

Figure 2. Native PAGE of purified β-galactosidases from B1.2.

Lanes 1−4 Coomassie blue staining of β-galactosidases B1.2 and recombinant molecular mass markers (Amersham) (lane 1). Lanes 5, 6 and 7 contained active staining with 4-methylumbelliferyl β-D-galactoside of β-galactosidases B1.2.

Kinetic parameters

The steady-state kinetic constants and the inhibition constants determined for the hydrolysis of o-nitrophenyl β-D-galactopyranoside (oNPG) were summarized in the values of K_m = 28.85 mM and V_{max} = 8.38×10^{-3} $mmolL^{-1}min^{-1}$.

Effects of pH and temperature on enzyme activity and stability

The pH optimum of B1.2 β-galactosidase was pH 6.5 for oNPG hydrolysis (Figure 3). Similar results have been reported for several β-galactosidase sources such as *Thermus* sp. T2 [6] and *Thermus* sp. A4 [3]. The enzyme was stable at pH 6.0-10, retaining more than 50% of its activity when incubated at pH 6.5 and 40°C for 12 h. Figure 3 shows the optimum temperature of β-galactosidase B.12 of 60 °C when using oNPG as the substrate under standard assay conditions (pH 6.5 for 15 min). Similarly, β-galactosidase from *Streptococcus thermophilus* had the optimal growth temperature of 55^0C [12], and *Fusarium moniliforme* of 50-60°C [13]. For the effect of temperature on the stability of enzyme, β-galactosidase was stable at 40-60°C when incubating at 40-80°C, pH 6.5 for 12 h.

Effects of various reagents, cations and carbohydrates on β-galactosidase activity

Various reagents, cations and carbohydrates were tested with respect to a possible inhibitory or stimulating effect on β-galactosidase activity. Similar effects for the various reagents were found for enzymes from B1.2 (Table 2).

The addition of various reagents (DTT, EDTA, Urea and 2-Mercaptoethanol) was studied at concentrations of 1 and 10 mM. It was found that β-galactosidase from B1.2 was not significantly affected by DTT or Urea, while 2-Mercaptoethanol restored 80-90% of the activity. EDTA was found to strongly inactivate enzymes even at the higher concentration tested (10 mM).

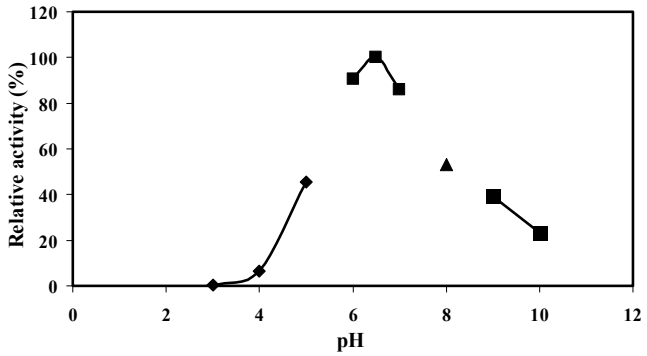

Figure 3. pH optimum of β-galactosidase from B1.2 incubated at 40°C in Citrate buffers (pH 3.0, 4.0 and 5.0), Sodium phosphate buffer (pH 6.0, 6.5 and 7.0), Tris buffer (pH 8.0) and Glycine-NaOH buffers (pH 9.0 and 10.0).

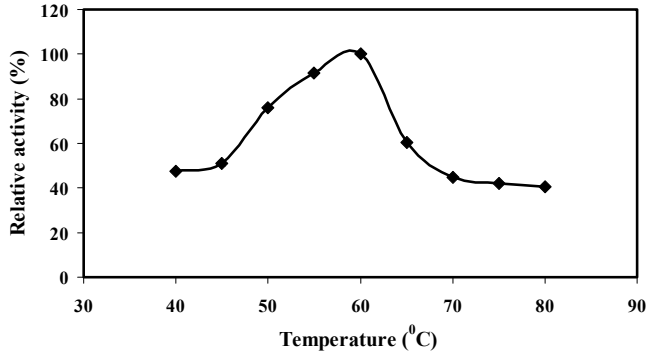

Figure 4. Temperature optimum of β-galactosidases from B1.2.

Table 2. Effect of various reagents on the activity of β-galactosidases from B1.2.

Reagents	Relative β-galactosidase activity (%)	
	1 mM	10 mM
None	100	100
DTT	96	99
EDTA	95	16
Urea	99	96
2-Mercaptoethanol	89	83

The effects of various cations on the activity of β-galactosidase from B1.2, each in final concentrations of 1, 10 and 100 mM were to the standard assay. The addition of monovalent cations Na^+ and K^+ had no affect on enzyme activity. The highest activities of B1.2 β-galactosidases were observed in the presence of 1 mM Fe^{2+} and 10 mM Mg^{2+}, similarly, of 1-10 mM Mn^{2+} and Co^{2+}. The presence of 1 and 10 mM Cu^{2+} decreased B1.2 activity by ~40%. The activity of B1.2 β-galactosidase was also completely inhibited by the addition of 10 mM Fe^{2+}. The inhibitory effects of 10 mM Zn^{2+} and Cu^{2+} was similar in that the enzyme activity was reduced by ~30%. All divalent cations completely inhibited β-galactosidase activity at a final

concentration of 100 mM. A similar result was reported for other thermophilic strains of *Talalomyces thermophilus* CBS 236.58 [9].

The effects of various carbohydrates on hydrolysis of oNPG by β-galactosidases from B1.2, each in final concentrations of 1, 10 and 100 mM to the standard assay conditions (β-galactosidase activity), are shown in Table 3. The addition of all carbohydrates decreased B1.2 activity when up to 100 mM.

Table 3. Effect of Various Carbohydrates on the Activity of β-galactosidase from B1.2.

Carbohydrates	Relative β-galactosidase activity		
	1 mM	10 mM	100 mM
None	100	100	100
Galactose	76	58	20
Glucose	81	77	59
Lactose	86	68	27

Conclusions

In conclusion, this work presented β-galactosidase from isolates of B1.2 which revealed interesting properties for application in the processes of lactose conversion. The applications of the enzyme for the production of galacto-oligosaccharides is also of interest as useful and attractive products for the food and milk industries, helping to avoid undesirable microorganisms.

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