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Purification and Characterization of a Novel β-Galactosidase with Transglycosylation Activity from *Bacillus megaterium* 2-37-4-1

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Abstract A novel β-galactosidase of 120 kDa (BgaBM) from *Bacillus megaterium* 2-37-4-1 was purified, and its gene (*bgaBM*) was analyzed and expressed. It displayed wide acceptor specificity for transglycosylation with a series of acceptors, including pentose, hexose, hydroxyl, and alkyl alcohol using *o*-nitrophenyl-β-D-galactoside (ONPG) as a donor. BgaBM preferentially hydrolyzed ONPG in all tested substrates and showed maximum activity at pH 7.5–8.0 and 55 °C. It was stable at pH 6.0–9.0 below 40 °C. The K_m and V_{max} values for ONPG and lactose were 9.5 mM, 16.6 mM/min and 12.6 mM, 54.4 mM/min, respectively. The nucleotide sequence of the *bgaBM* gene consists of an ORF of 3,105 bp corresponding to 118 kDa protein, which indicates that BgaBM is a modular enzyme in the glycosyl hydrolase family 2, including conserved sugar-binding domain, acid–base catalyst, and immunoglobulin-like beta-sandwich domain. The possible acid/base and nucleophile sites of BgaBM were estimated to be E481 and E547, respectively. Furthermore, expression of the *bgaBM* gene in *Escherichia coli* and purification of the recombinant enzyme were performed. The recombinant enzyme showed similar biochemical characteristics to natural enzyme.

Keywords β-Galactosidase · Purification · *Bacillus megaterium* · Transglycosylation · Gene

Introduction

β-Galactosidases (EC 3.2.1.23) are present in various sources, including plants, animals, and microorganisms. These enzymes have attracted particular interest in industrial applications owing to their capability of catalyzing transglycosylation and hydrolysis reaction [1].

Usually, β-galactosidases hydrolyze glycosidic linkages, but when the reaction conditions are manipulated in vitro, molecules other than water can be accepted as nucleophiles, and new glycosides are obtained [2]. This method is called transglycosyla-

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tion. Using this technique, galacto-oligosaccharides (GOS) and some galactosyl compounds have been synthesized by β-galactosidases. Splechtna and Nguyen produced GOS, which are important pre-biotics that selectively stimulate the growth of *bifidobacteria* in the lower part of the human intestine and inhibit potentially harmful bacteria in the gut, such as *Chostridia* and *Bacteriodes* species [3]. Katsumi and Mariko synthesized NeuAcα-(2→3) Galβ-(1→3)-GalNAc, which is regarded as an important tumor marker as a glycopeptides unit, and is called the sialyl *T*-antigen [4]. Takeomi and Masaki synthesized 6′-sulfo*N*-acetyllactosamine and its positional isomer 6′-sulfo*N*-acetylisoactosamine, which have been known to play various roles in biological events such as selectin binding, laminin binding, neural cell migration, bacteria binding, and activation of macrophages [5]. Higashiyama and Watanabe synthesized *cyclo*-{→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→}, which is a low-calorie sweetener [6].

Recently, interest in β-galactosidases have gained more momentum owing to their biosynthetic abilities of producing alkyl galactosides and modifying antibiotics. Stevenson reported the large-scale production of alkyl galactosides, which are prepared from naturally occurring renewable sources (sugars and fatty alcohols), and are easily biodegradable and more stable under alkaline conditions than the corresponding sugar fatty acid esters. They are a group of non-ionic surfactants with a variety of applications in food products, detergents and cleaning agents, personal health products, fine chemicals, and pharmaceuticals [7]. Christian reported galactosylation of antibiotics such as chloramphenicol, which is a phosphodiesterase inhibitor and is applicable against vaginal mycoses [8].

So far, β-galactosidases from numerous microorganisms have been used to produce GOS, but few of them have been applied in synthesizing bio-active galactosides. Alkyl galactosides and galactosyl antibiotics were mainly synthesized by two β-galactosidases from *Bacillus circulans* and *Aspergillus oryzae*, respectively. It is necessary to find new enzymes for synthesizing valuable galactosides.

In this paper, transglycosylation activity for β-galactosidase was first reported from *Bacillus megaterium* 2-37-4-1. BgaBM was purified, and its gene was analyzed and expressed. The enzyme was endowed with good thermo-stability and wide acceptor specificity for transglycosylation reactions capable of the synthesis of important galactosyl compounds such as alkyl glycoside. These excellent characteristics suggested that the enzyme may be a novel tool for enzymatic synthesis with applications in the food, healthcare, and pharmaceutical industries in the future.

Materials and Methods

Bacterial Strain and Media

B. megaterium 2-37-4-1 was isolated from soil and was cultivated at 37 °C in medium consisting of 10 g lactose, 10 g peptone, 5 g yeast extracts, and 5 g NaCl in 1 l of water at an initial pH of 7.2.

Enzyme and Protein Assay

β-Galactosidase activity was measured by adding 50 μl enzyme solution to 450 μl of 2 mM *o*-nitrophenyl-β-D-galactoside (ONPG). The reaction was performed at 50 °C for 10 min and then stopped by adding 1 ml 500 mM Na_2CO_3. The amount of *o*-nitrophenol released was measured by spectrophotometer at 400 nm. One unit of enzymatic activity (U) was

defined as the amount of enzyme required to liberate 1 μM of *o*-nitrophenol per minute under the assay conditions. Assays for the other nitrophenyl glycosides (Sigma) were performed under the same conditions. Protein was determined by means of Coomassie Brilliant Blue G-250 with bovine serum albumin as a standard.

Enzyme Purification

All the procedures described below were performed in 10 mM phosphate buffer, pH 7.5 at 4 °C. Cells were harvested from a 2-l culture and disintegrated by sonication. The resulting crude enzyme solution was concentrated by ammonium sulfate precipitation (20–60% saturation), followed by desalting, and sequentially applied to a 1×20-cm DEAE Sepharose Fast Flow column (Amersham, USA), a 2.5×30-cm Gigapite K-100 S column (Seikagaku, Japan) and a 1×10-cm para-aminophenyl-1-thio-β-D-galactopyranoside (APTG) affinity chromatography column (Sigma).

Protein Electrophoresis

Sodium dodecyl sulfate (SDS)-ploy acrylamide gel electrophoresis (PAGE) and Native gradient PAGE were performed to determine the molecular weight of the pure enzyme. The enzyme samples were applied to 7% (*w*/*v*) SDS-PAGE and 5–10% (*w*/*v*) native PAGE. The denatured protein was stained by means of quick silver stain, and the native protein was detected by means of 4-methylumbelliferyl-β-D-galactopyranoside staining at 37 °C for 10 min; the fluorescent strand was observed using UV scanning at 365 nm and photographed.

Biochemical Properties

The optimal temperature for enzymatic reaction was determined by incubating the enzyme with ONPG for 10 min at temperatures ranging from 0 °C to 70 °C. The thermo-stability was determined by assaying residual enzymatic activity after incubating the enzyme for 60 min at 4 °C, 20 °C, 30 °C, 40 °C, 50 °C, and 60 °C, sampling periodically and assaying enzymatic activity. The optimal pH was determined by assaying the enzymatic activity at pH values ranging from 3.0 to 10.0 (50 mM citric acid-sodium citrate for pH 3.0 to 6.0, 50 mM K_2HPO_4–KH_2PO_4 for pH 6.0 to 8.0, and 50 mM KCl–H_3BO_3 for pH 8.0 to 10.0). The pH stability was determined by incubating the enzyme in the presence of the above different pH buffers at 4 °C for 24 h and assaying the residual activity under standard reaction conditions. K_m and V_{max} values of the enzyme were determined according to the Lineweaker–Burke method (1934) using different concentrations of ONPG and lactose as substrates. The hydrolytic substrate specificity in response to various nitrophenyl glycosides was detected in the same way as the enzyme assay condition. The characterization of recombinant enzyme was investigated using the above methods.

Acceptor Specificity for Transglycosylation

Transglycosylation reactions were performed at 55 °C for 4 h by incubating 5 μl pure enzyme (20 U/ml), 20 μl of each acceptor (100 mM), and 5 μl ONPG (50 mM) in 50 mM phosphate buffer (pH 7.5) as the tested group. Under the same conditions, two corresponding reactions, including enzyme, each acceptor and enzyme, ONPG was performed as control groups. All the reactions were stopped by boiling for 10 min.

Carbohydrates were separated by thin layer chromatography using butanol–enthanol-distilled water [5:3:2 (*v*/*v*/*v*)] as a mobile phase. Detection was completed by spraying with 0.05% (*w*/*v*) 3′5-dihydroxytoluene in 20% (*v*/*v*) sulfuric acid and heating to show color at 120 °C, and the yields were quantified by Image J, a software for dot analysis and quantification (http://www.ebi.ac.uk/ clustalw/).

Gene Cloning and Expression

The genomic DNA of *B. megaterium* 2-37-4-1 was used as a template for PCR amplification. A 3.1-kb DNA fragment was amplified by PCR using sense (5′-GATC *GGATCC*ATGTTAAAAACCGGCAAGAAA-3′) and antisense (5′-AG*CTCGAG*TAGAGG TTTTAGCGTAAAGTCG-3’) primers with *Bam*HI and *Xho*I recognition sites. The amplification fragment was inserted into the pMD18-T vector and sequenced. The amplified ORF fragment of the *bgaBM* gene was digested by *Bam*HI and *Xho*I and cloned into corresponding sites of pET15b resulting in pET15/*bgaBM*, which was over-expressed in *E. coli* BL21 (DE3) pLysS by isopropyl-β-D-thiogalactopyranoside induction at 37 °C for 4 h. Cells were harvested and disintegrated by sonication. The resulting crude enzyme solution was applied to Ni^{2+} chelation chromatography for one-step purification.

Results

Enzyme Purification and its Molecular Weight

A novel β-galactosidase, designated BgaBM, was purified about 10.2-fold by a five-step procedure with a 7.1% recovered activity yield (Table 1). The molecular weight of the enzyme was determined by SDS-PAGE and native gradient PAGE. They both were about 120 kDa (Fig. 1). The results indicated that the enzyme was a monomeric protein.

Characterization of the Enzyme

The enzyme showed the highest hydrolysis activity at pH 7.0–8.0. The optimal reaction temperature was 55 °C. It was stable in the range of pH 6.0–9.0 for 24 h at 4 °C. Without substrate protection, the enzyme could remain above 95% activity at 4 °C and 20 °C, about 80% activity at 30 °C, about 70% activity at 40 °C, about 50% activity at 50 °C for 60 min, but below 10% activity at 60 °C for 15 min. K_m and V_{max} values of the enzyme were 9.5 mM and 16.6 mM/min for ONPG and 12.6 mM and 54.4 mM/min for lactose,

Table 1 Purification procedures of β-galactosidase (BgaBM).

Step	Total protein (mg)	Enzymatic activity (U)	Specific activity (U/mg)	Activity yield (%)
Crude extract	398.8	2,354	5.9	100
Ammonium sulfate	322.5	2,315	7.18	98.3
DEAE Fast Flow	48.2	1,559	32.3	66.2
Gigapite K-100S	17.4	847.1	48.7	35.9
APTG	2.8	165.9	59.9	7.1

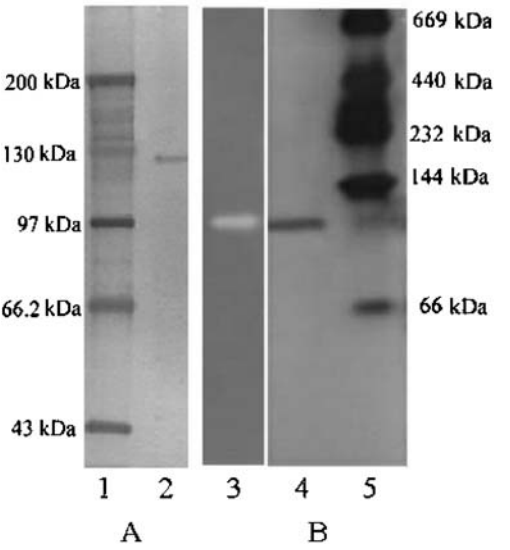


Fig. 1 Native gradient PAGE (**A**) and SDS-PAGE (**B**) of BgaBM. Lanes *1* and *5*, marker proteins; *2*, denatured enzyme with silver staining; *3* and *4*, native enzyme with active staining and silver staining

respectively. The hydrolytic activity of BgaBM was assayed using a series of chromogenic substrates (Table 2). The enzyme showed highest activity for ONPG and displayed 89% ONPG activity using *p*-nitro-phenyl-β-D-galactopyranoside as the substrate. No activity was detected for other tested substrates. The recombinant enzyme was similar.

Acceptor Specificity

Acceptor specificity of the enzyme is shown in Table 3. Novel transglycosylation products appeared in the tested group, but no products appeared in the control groups. It is suggested that the enzyme is endowed with broad acceptor specificity for transglycosylation.

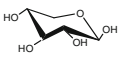
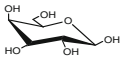
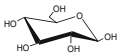
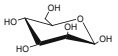
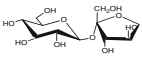
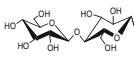
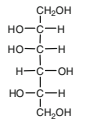
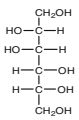
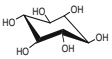
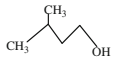
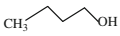
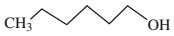
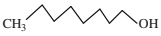
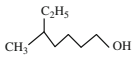
Gene Sequence Analysis and Expression

The size of 3,105 bp DNA fragments were amplified and sequenced. It encodes a protein of 1,034 amino acids with a predicted molecular weight of 118 kDa, similar to the calculated molecular weight by SDS-PAGE (120 kDa). The nucleotide sequence was submitted to GenBank with accession no. EF200842. The result from NCBI Blastn showed that the *bgaBM* gene had several conserved regions for glycosyl hydrolase family 2 (GH2) such as

Table 2 Relative hydrolytic activities of various substrates by β-galactosidase (BgaBM).

Substrate	Relative activity (%)
o-Nitrophenyl-β-D-galactoside	100
p-Nitrophenyl-β-D-galactoside	89.0
p-Nitrophenyl-α-D-galactoside	0
o-Nitrophenyl-α-D-galactoside	0
o-Nitrophenyl-α-D-glucoside	0
o-Nitrophenyl-β-D-glucoside	0
p-Nitrophenyl-*N*-acetyl-β-D-galactosaminide	0
p-Nitrophenyl-*N*-acetyl-α-D-galactosaminide	0

Table 3 Results of transglycosylation in the presence of ONPG.

Acceptor	Structure	Novel product yields(%)	Acceptor	Structure	Novel product yields(%)
Xylose		0-1.0	Galactose		1.0-5.0
Glucose		5.0-10.0	Mannose		0-1.0
Fructose		5.0-10.0	Sorbose		10.0-15.0
Sucrose		15-20	Cellobiose		15-20
Sorbitol		5.0-10.0	Mannitol		5.0-10.0
Inositol		1.0-5.0	Amyl-alcohol		10.0-15.0
Butanol		15-20	Hexanol		15-20
Octanol		15-20	2-Ethyl-hexanol		5.0-10.0

sugar-binding domain, immunoglobulin-like beta-sandwich domain, acid–base catalyst, and several other GH2-conserved regions (http://www.ncbi.nlm.nih.gov/BLAST/). For determining catalysis sites, all aligned β-galactosidases possess residues corresponding to known active site of β-galactosidase from *E. coli* (amino acid residues: H357, H391, E 416, H418, E461, Y503, E537, H540) [9, 10]. The possible acid/base and nucleophile sites of

Fig. 2 Multiple alignment of possible acid/base, nucleophile, and other active sites. The active sites of *E. coli* in the *black region* have already been determined. E481 and E547 are possible acid/base and nucleophile sites of BgaBM, respectively. *F*, *H*, *C*, *Y*, and *N* residues are possibly important sites to possess wide acceptor specificity for transglycosylation. All aligned sequences are available in GenBank and are from *Bacillus halodurans* C-125, *Clostridium beijerincki*, *B. megaterium* 2-37-4-1, *Clostridium perfringens*, and *E. coli*, respectively

BgaBM were estimated to be E481 and E547, respectively. In addition, special attention was devoted to F, H, C, Y, and N residues near the catalytic sites different from aligned β-galactosidases because these amino acids might play an important role in positioning the substrate molecule and catalytic acceptor molecule through stacking interactions and/or H-bonds, which could result in wide acceptor specificity [11]. These results are shown as Fig. 2. The *bgaBM* gene was successfully expressed in *E. coli* BL21 (DE3) pLysS, and the recombinant enzyme was purified and showed similar activity to the natural enzyme.

Discussion

This paper reports on the purification, biochemical properties, gene sequence analysis, and expression of a novel β-galactosidase (BgaBM) with transglycosylation activity from *B. megaterium* 2-37-4-1. The enzyme exhibited favorable synthesized characteristic by transglycosylation. Thus, it will be a novel tool for enzymatic synthesis.

BgaBM is a monomeric protein different from other bacterial β-galactosidases, which are commonly multimeric. They are dimeric in *Planococcus* sp. and *Lactobacillus reuteri*, trimeric in *Bifidobacterium adolescentis* DSM 20083, and tetrameric in *Pseudoalteromonas* sp. 22b, *Pseudoalteromonas haloplanktis*, and *E. coli* (LacZ β-galactosidases family) [12–17].

BgaBM showed a relatively high optimal temperature (55 °C), which only occurs in *Bacillus coagulans* (55 °C) and some lactic acid bacteria such as *Lactobacillu thermophilus* (55 °C) and *Lactobacillu acidophilus* R22 (55 °C) [17–19]. It displayed favorable thermo-stability within a broad range of pHs (6.0–9.0), and its activity decreased below 10% for 15 min at 60 °C without substrate protection; similar features were reported for the enzymes of *Bifidobacterium bifidum*, *B. circulans*, and *Lactobacillus crispatus* [20–23].

It is worth noting that the enzyme could utilize a wide range of acceptors for transglycosylation, including hexose, pentose, β- or α- disaccharides, hexahydroxy alcohol, cyclitol glycosides, and alkyl alcohol in the presence of ONPG. It displayed different transglycosylation efficiencies under the same reaction conditions (Table 2). It preferred fructose, glucose, sorbitose, sucrose, cellobiose, sorbitol, mannitol, and alkyl alcohol as acceptor than other saccharides. Differences in the structure of tested acceptors may affect its interaction with the enzyme and influence the binding with anticipant glycosyl moiety (Table 3). In similar reports, transglycosylation to galactose, mannose, and xylose by β-galactosidase from *E. coli* and *B. circulans* were mentioned [24, 25]. But transglycosylation to so wide range of acceptors under the same conditions have rarely been reported. It is suggested that BgaBM is a potential tool for synthesis of novel galactosyl compounds, including food ingredients, pharmaceuticals, and other bio-active substances owing to its broad acceptor specificity. Due to inevitable drawbacks of glycosidase reactions, in which the products are always substrates for enzymes and undergo hydrolysis, the yields of carbohydrate by glycosidases from all sources are modest (BgaBM also follows this rule). Modern molecular evolution is useful to overcome the above problem. By directed mutation at nucleophile or acid/base sites, many glycosidases have been modified such as glycosynthases or thioglycoligases [26, 27].

In conclusion, a novel β-galactosidase from *B. megaterium* 2-37-4-1 showed a favorable thermo-stability and displayed a broad range of acceptor specificity for transglycosylation. It will be a promising tool for synthesizing galactosyl compounds. The *bgaBM* gene was successfully cloned and analyzed. Subsequent molecular evolution to enhance trans-glycosylation activity specificity is being performed.

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