A novel thermostable β -galactosidase from *Bacillus coagulans* with excellent hydrolysis ability for lactose in whey

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ABSTRACT

β -Galactosidase is one of the most important enzymes used in dairy industry. Here, a novel thermostable β -galactosidase was cloned and overexpressed from *Bacillus coagulans* NL01 in *Escherichia coli*. The phylogenetic trees were constructed using neighbor-joining methods. Phylogeny and amino acid analysis indicated that this enzyme belonged to family 42 of glycoside hydrolases. The optimal pH and temperature were, respectively, 6.0 and 55 to 60°C. The purified enzyme had a 3.5-h half-life at 60°C. Enzyme activity was enhanced by Mn^{2+} . Compared with other β -galactosidases from glycoside hydrolase family 42, *B. coagulans* β -galactosidase exhibited excellent hydrolysis activity. The Michaelis constant (K_m) and maximum rate of enzymatic reaction (V_{max}) values for *p*-nitrophenyl- β -D-galactopyranoside and *o*-nitrophenyl- β -D-galactopyranoside were 1.06 mM, 19,383.60 U/mg, and 2.73 mM, 5,978.00 U/mg, respectively. More importantly, the enzyme showed lactose hydrolysis ability superior to that of the commercial enzyme. The specific enzyme activity for lactose was 27.18 U/mg. A total of 104.02 g/L lactose in whey was completely hydrolyzed in 3 h with addition of 2.38 mg of pure enzyme per gram of lactose. In view of the high price of commercial β -galactosidase, *B. coagulans* β -galactosidase could be a promising prototype for development of commercial enzymes aimed at lactose treatment in the dairy industry.

Key words: β -galactosidase, *Bacillus coagulans* NL01, lactose hydrolysis, whey

INTRODUCTION

β -Galactosidase (EC 3.2.1.23), also known as lactase, catalyzes the hydrolysis of β -(1–3), β -(1–4) or β -(1–6)

galactosyl bonds in oligo- and disaccharides, as well as the reverse reaction, transglycosylation (Nath et al., 2013). The natural substrate for most β -galactosidases is lactose; hence they are widely used in food processing, especially in the dairy industry, for the production of lactose-free dairy products such as milk drinks, cream, yogurt, and cheese (Nath et al., 2014). Removal of lactose from milk diminishes the lactose intolerance problem that prevails in more than 70% of the world's adult population. Moreover, due to transglycosylation activity, β -galactosidase can transfer galactosyl moiety to various compounds. It can therefore be used for the production of galactosylated products, such as galactosyl sucralose (Lu et al., 2012), galactosyl fucose (Oh et al., 2019), alkyl galactopyranoside (Yang et al., 2017), and galactosyl furostanoside (Zhang et al., 2016). Presently, thermostable β -galactosidases have attracted considerable attention because of their significant advantages in processing, such as higher substrate solubility and reaction rate, as well as lower probability of microbial contamination.

β -Galactosidases are widely distributed in animals, higher plants, and microorganisms (Das et al., 2015). Based on sequence homology and evolutionary relationships, they can be classified into 4 distinct glycoside hydrolase families (**GH**): GH1, GH2, GH35, and GH42 (Yin et al., 2017). Among these, β -galactosidases of GH42 often derive from organisms that survive in extreme circumstances, such as psychrophilic, thermophilic, and halophilic conditions. Several GH42 β -galactosidases have been crystallized and studied, such as β -galactosidases from *Thermus thermophilus*, *Rahnella* spp., and *Geobacillus stearothermophilus* (Hidaka et al., 2002; Solomon et al., 2013; Fan et al., 2015). Generally, these enzymes consist of 600 to 700 AA residues and have 3 domains: domain A possesses a $(\beta/\alpha)_8$ barrel (TIM barrel), which contains catalytic residues and binding residues; domain B is involved in subunit polymerization; and the function of domain C remains unclear (Godoy et al., 2016).

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Whey is an inexpensive and abundant by-product of cheese production, the main components of which are lactose, proteins, and minerals salts. Unfortunately, it has traditionally been treated as waste and causes an important pollution issue because of its high chemical and biochemical oxygen demand, determined by lactose and whey proteins (Nath et al., 2015a,b). Given that the hydrolyzed products of lactose, glucose and galactose, are important starting materials for production of bulk and value-added chemicals (Zheng et al., 2019; Zhou et al., 2019), recycling the lactose in whey can achieve both whey valorization and environment protection. However, the numbers of microorganisms that can directly use lactose are much fewer than those able to metabolize glucose and galactose. Thus, prior hydrolysis of lactose by β -galactosidase is required for microbial bioconversion of whey (Xavier et al., 2018). Considering the high price of commercial β -galactosidases (approximately US\$0.2 per unit, Sigma-Aldrich, St. Louis, MO), the cost of β -galactosidase will be a big part of the bioconversion process.

Bacillus coagulans is considered a GRAS (generally recognized as safe) strain. In this study, a novel β -galactosidase from *B. coagulans* NL01 (**BcBga**) was reported. Phylogenetic analysis and biochemical characterization were performed. The purified BcBga was used to hydrolyze high concentrations of lactose in whey and compared with the commercially purchased, unpurified BcBga.

MATERIALS AND METHODS

Chemicals

o-Nitrophenyl- β -D-galactopyranoside (**oNPG**), *p*-nitrophenyl- β -D-galactopyranoside (**pNPG**), *p*-nitrophenyl- β -D-xylopyranoside (**pNP-Xyl**), *p*-nitrophenyl- β -D-glucopyranoside (**pNP-Glu**), and *p*-nitrophenyl- α -L-arabinofuranoside (**pNP-Ara**), lactose, and lactulose were purchased from Sigma-Aldrich. Cheese whey powder, including 70.00% (wt/wt) lactose, was purchased from Lactalis Company (Laval, France). Other chemicals were of analytical grade and commercially available.

Cloning, Expression, and Purification

The genes of *BcBga* and *BcBga*^M (encoding a BcBga mutation lacking domain C) were amplified using the genome of *B. coagulans* NL01 as template. The primers of *BcBga*.f (5'-GAATTCGATGTTAAAAA-CAAGAAAAATTTTATTATG-3'; *Eco*RI site underlined) and *BcBga*.r (5'-GTCGACCTATTTTTCAT-

TACCTGCAAAATTTTCA-3'; *Sa*I site underlined) were used to amplify the full length of the *BcBga* gene. The primers of *BcBga*.f and *BcBga*^M.r (5'-GTCGACCTACGCTTCCTTTAAATTTCTTTTAAA-3'; *Sa*I site underlined) were used to amplify the mutant *BcBga*^M. The products were digested by corresponding restriction endonuclease and cloned into a pET-Duet-1 expression vector that had been digested with the same enzymes to obtain recombinant plasmids, pETDuet-*BcBga* and pETDuet-*BcBga*^M. The recombinant plasmids were then transformed into *E. coli* BL21 (DE3) cells to obtain recombinant strains harboring pETDuet-*BcBga* or pETDuet-*BcBga*^M. For protein expression, transformants harboring the recombinant plasmids were inoculated into 5 mL of Luria-Bertani (LB) medium containing 100 μ g/mL of ampicillin and incubated for 10 to 12 h at 37°C, 200 rpm. Then 5 mL of cell suspension was inoculated into 500 mL of LB medium containing 100 μ g/mL of ampicillin. When cells were grown to an optical density at 600 nm of 0.6 to 0.8, 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) was added for protein induction for a further 6 h. For protein purification, cells were harvested, washed twice with phosphate buffer (pH 7.0), and disrupted by sonication. We purified BcBga using Ni-NTA affinity chromatography from supernatant. Protein concentration was determined via the Bradford method, using BSA as the standard, and purity of BcBga was determined using SDS-PAGE.

Sequence Alignment and Phylogenetic Analysis

Database searching was performed using Blast at NCBI (www.ncbi.nlm.nih.gov) and CAZy (www.cazy.org). The multiple sequence alignment tools Clustal X 2.0 and ESPript 3.0 were used for multiple protein sequence alignment. Phylogenetic relationships were built using the neighbor-joining method with MEGA 7.0, and the results were evaluated with 1,000 bootstrap replicates.

Biochemical Assay of BcBga

Typically, enzyme activity was measured using *o*NPG as substrate. We added 0.1 mL of diluted enzyme to 0.9 mL of 50 mM phosphate buffer (pH 6.0) containing 2 mM *o*NPG and incubated at 55°C for 10 min. The reaction was terminated by adding 2 mL of 0.5 M Na₂CO₃, and the amount of *o*NP released was measured at 420 nm. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μ mol of *o*NP per minute.

To determine BcBga activity for lactose, 0.1 mL of diluted enzyme was added to 0.9 mL of 50 mM phos-

phate buffer (pH 6.0) containing 20 mM lactose and incubated at 55°C for 10 min. The reaction was terminated by heating at 100°C for 10 min. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μ mol of glucose per minute.

Optimal pH was determined by measuring enzyme activity at various pH values ranging from 4.0 to 8.0 at 37°C. The temperature dependence of BcBga was assessed by measuring enzyme activity in the range of 40 to 70°C at pH 6.0. Relative enzyme activity was defined as the proportion of activity compared with that obtained at optimal pH or temperature.

To study pH stability, enzymes were incubated at various pH values and 0°C for 1 h, and the remaining enzyme activity was measured according to standard method. For the thermal stability studies, the enzyme was incubated at various temperatures for 1 h, and the residual enzyme activity was then measured according to standard method. The activity of the enzyme without preincubation was defined as 100%.

To investigate the effect of metal ions on BcBga activity, purified BcBga was dialyzed against 50 mM Tris-HCl buffer (pH 7.0) containing 10 mM EDTA at 25°C for 3 h. Then the buffer was changed to 50 mM Tris-HCl (pH 7.0) for another dialysis of 36 h. Metallic ions (Na^+ , K^+ , Mg^{2+} , Mn^{2+} , Ni^{2+} , Ca^{2+} , Zn^{2+} , Fe^{3+} , and Cu^{2+}) were added into reaction mixtures containing EDTA-treated BcBga at a final concentration of 2 mM, respectively. The activity obtained in the absence of metal ions was defined as 100%.

The substrate specificity of BcBga was tested by using *o*NPG, *p*NPG, *p*NP-Xyl, *p*NP-Glu, and *p*NP-Ara. The kinetic parameters [Michaelis constant (K_m) and maximum rate of enzymatic reaction (V_{max})] were determined according to nonlinear regression using various concentrations of *o*NPG and *p*NPG. For inhibition studies, BcBga activity for lactose was measured as mentioned above, with various concentrations of galactose (0 to 100 mM) or glucose (0 to 500 mM) added in reaction system. The inhibition constant (K_i) value of galactose or glucose was defined as the amount needed to inhibit 50% of the BcBga activity.

Hydrolysis of Lactose in Whey

Hydrolysis of lactose in whey was performed in 2 mL of 15% (wt/vol) whey solution containing 0.5 mg of purified enzyme. The flasks were incubated at 55°C and 150 rpm. The concentrations of lactose, glucose, and galactose were determined via HPLC 1260 (Agilent Technologies, Santa Clara, CA) with a differential refraction detector using an Aminex HPX-87H column (Bio-Rad, Hercules, CA). The mobile phase was 5 mM H_2SO_4 at a flow rate of 0.6 mL/min. The temperature

of the column was maintained at 55°C, and the detector temperature was maintained at 35°C. All experiments were performed in triplicate, and error bars indicate standard deviation.

RESULTS

Sequence and Phylogenetic Analysis of BcBga

Based on the draft genome sequences of *B. coagulans* NL01 (Zheng et al., 2015), a gene, designated as *BcBga*, encoding β -galactosidase, was detected. Analysis revealed that the complete β -galactosidase sequence contained an open reading frame of 1,998 bp, which encoded 665 AA with a calculated molecular mass of 76.04 kDa. Multialignment of BcBga with other GH42 β -galactosidase indicated that the enzyme can be divided into 3 domains. Domain A comprises 389 residues (M1-L382), domain B is an α/β fold domain (E383-A594), and domain C contains AA residues from D595 to K665. Generally, 2 glutamic acid residues are the catalytic nucleophile and proton donors in β -galactosidase. Sequence alignment revealed that E149 and E303 in domain A are the pivotal catalytic residues of BcBga (Figure 1). In domain A, R110, W311, E351, and H354 are the lactose-binding residues (Figure 1). Up to now, the function of domain C of GH42 β -galactosidase has been unclear. In this study, the domain C truncation mutant of BcBga was constructed and expressed in *E. coli* BL21 (DE3). The results showed that the mutant BcBga^M was expressed as the inclusion bodies in cells (Supplemental Figure S1; <https://doi.org/10.3168/jds.2019-16654>), which indicates that domain C is an indispensable part of the enzyme.

The AA sequence analysis indicated that BcBga shared 46% sequence identity with β -galactosidase from *Bacillus circulans* ssp. *alkalophilus* (PDB: 3TTS_A), 36% of sequence identities with β -galactosidase from *Bifidobacterium animalis* ssp. *lactis* BI-04 (PDB: ACS45863.1), and 32% of sequence identities with β -galactosidase from *Thermus thermophilus* A4 (PDB: O69315.1). To gain deeper insight into the relationship among the β -galactosidases, the phylogenetic tree generated from 20 candidate sequences was constructed using the neighbor-joining method. The result revealed the presence of 4 clades, each consisting of a monophyletic group. Clade I was the GH42 galactosidases, mainly derived from extremophilic microorganisms. Clade II was the GH2 galactosidases from the genera *Escherichia*, *Arthrobacter*, *Bifidobacterium*, and *Lactobacillus*. Clade III was the GH35 galactosidases, derived from fungi, and clade IV was the GH1 galactosidases. Clades I and II had a relatively close relationship and were distant from the other 2 clades (Figure 2). We

Figure 1. Multialignment of domain A of *Bacillus coagulans* NL01 β -galactosidase (BcBga) with other glycoside hydrolase family 42 (GH42) β -galactosidases. The catalytic residues and binding residues are indicated as red stars and blue circles, respectively, at the bottom of each alignment. BcaBga = *Bacillus circulans* ssp. *alkalophilus* β -galactosidase; BalBga = *Bifidobacterium animalis* ssp. *lactis* BI-04 β -galactosidase; BbBga = *Bifidobacterium bifidum* S17 β -galactosidase; TtA4Bga = *Thermus thermophilus* A4 β -galactosidase.

found that BcBga belonged to clade I (GH42), closest to the β -galactosidase from *G. stearothermophilus*.

Biochemical Properties of BcBga

The biochemical properties of BcBga were investigated using the purified enzyme. At pH 6.0, BcBga displayed its highest activity, and more than 70% of its maximum activity occurred at pH ranging from 5.0 to 7.0. Activity fell sharply at pH 4.0 and 8.0 (Figure 3a). BcBga was very stable at pH 6.5 to 8.0 and retained more than 60% of its initial activity after incubation at pH 5.5 for 1 h (Figure 3b). Optimal BcBga activity occurred at 55 to 60°C, and BcBga exhibited more

than 60% of the maximum activity at 65°C (Figure 3c). After incubation at 50 to 55°C, even for as long as 5 h, BcBga retained more than 65% of its initial activity, and the half-life of the enzyme was approximately 3.5 h at 60°C (Figure 3d).

After treatment with EDTA, no significant activity changes were observed in the enzyme assay. The EDTA-treated enzyme was then incubated with different metallic ions (Na^+ , K^+ , Mg^{2+} , Mn^{2+} , Ni^{2+} , Ca^{2+} , Zn^{2+} , Fe^{3+} , and Cu^{2+}); their effects are shown in Figure 4. The activity of BcBga was greatly stimulated by Mn^{2+} and slightly enhanced by Fe^{3+} , resulting in increases of 96.28 and 21.25%, respectively. Dramatic loss of enzyme activity (82.93 and 86.65%, respectively) was observed

in the presence of Cu²⁺ and Zn²⁺. No pronounced effect was observed in the presence of Ca²⁺, Mg²⁺, Na⁺, or K⁺.

Substrate Specificity and Kinetic Parameters

The substrate specificity of BcBga was tested. The highest activity was toward *p*NPG, followed by *o*NPG, which exhibited 11.4% of the activity shown with *p*NPG. Almost no activity was detected over *p*NP-Glu, *p*NP-Xyl, or *p*NP-Ara, suggesting that BcBga has strict substrate specificity for galactosyl compounds. The dependence of the enzymatic reaction on the substrate concentration followed Michaelis-Menten kinetics, with *K_m* and *V_{max}* values of 1.06 mM and 19,383.60 U/mg for *p*NPG, and 2.73 mM and 5,978.00 U/mg for *o*NPG, respectively.

Hydrolysis of Lactose in Whey

We studied the lactose hydrolysis ability of BcBga because this is its most important property for ap-

plication in the dairy industry. First, enzyme activity toward lactose was determined. The specific activity of purified BcBga was 27.18 U/mg (Supplemental Table S1; <https://doi.org/10.3168/jds.2019-16654>). Effects of galactose and glucose on BcBga activity were also investigated with lactose as substrate. Figure 5 shows that galactose gave a much stronger inhibition than did glucose. When galactose concentration was increased, the enzyme activity of BcBga was gradually inhibited, with a *K_i* of approximately 20 mM galactose. In contrast, the *K_i* of glucose was up to 400 mM.

We further investigated the hydrolysis of lactose in whey to assess whether BcBga is appropriate for whey pretreatment. The initial concentration of cheese whey powder was 145 g/L (corresponding to 104.02 g/L of lactose). Even at a low enzyme concentration (2.38 mg/g of lactose), BcBga was adequate to hydrolyze all the lactose in whey. As shown in Figure 6, 62.45% lactose was hydrolyzed in the first 60 min. Over time, the rate of lactose hydrolysis slowed. All lactose was completely hydrolyzed in 3 h. The complete hydrolysis of lactose in whey indicated the suitability of BcBga for whey treatment.

Figure 2. The neighbor-joining tree based on AA sequences of 20 different β-galactosidases. Numbers at nodes indicate the percentage of neighbor-joining bootstrap analyses with 1,000 replicates. The scale bar indicates the level of AA sequence divergence. GH = glycoside hydrolase family.

DISCUSSION

On the basis of sequence homology, β -galactosidases are mainly grouped into GH1, GH2, GH35, and GH42 glycoside hydrolyse families. These families have distinctly different sequences and exhibit different substrate specificities. In general, GH1 and GH2 β -galactosidases demonstrate prominent lactose hydrolysis activity and have been the most widely used in food processing, water assessment, and molecular experimentation (Rodrigues and Cunha, 2017). β -Galactosidases belonging to GH35 and GH42 usually show either a very low level or a complete absence of activity toward lactose (Solomon et al., 2013). For example, Li reported GH 42 β -galactosidases from *Thermotoga maritima* could not cleave lactose in vitro (Li et al., 2009). In contrast, we reported a GH 42 β -galactosidase, from GARS strain *B. coagulans*, NL01, with outstanding lactose hydrolysis ability.

GH 42 β -galactosidases often derive from microorganisms under environmental extremes and exhibit different optimal temperatures. β -Galactosidase from *Arthrobacter* showed the highest hydrolysis activity

at 10°C (Pawlak-Szukalska et al., 2014), whereas the enzyme from *Thermotoga naphthophila* had an optimal temperature at 90°C (Kong et al., 2014). In our study, BcBga exhibited the highest activity at a relatively mild temperature, 55 to 60°C, which was similar to GH42 β -galactosidases from *B. circulans* sp. *alkalophilus*, and *Marinomonas* sp. BSi20414, whose optimal temperatures are 55°C and 60°C, respectively (Maksimainen et al., 2012; Ding et al., 2017). These enzymes also share a similar optimal pH with BcBga, near 6.0. For BcBga, the pH curve showed a peak with 80% of maximal β -galactosidase activity in the pH range of 5.5 to 6.5, suitable for lactose hydrolysis of whey (pH 5.9 to 6.6).

Metal cations had various effects on BcBga activity. No significant enzyme activity change was observed with the addition of Mg^{2+} , Ca^{+} , Na^{+} , or K^{+} . Studies have shown that Mg^{2+} is beneficial to most β -galactosidase activity, such as enzymes from *Kluyveromyces lactis*, *Lactobacillus plantarum*, *Thermotoga maritima*, *Pediococcus pentosaceus*, and *Pediococcus acidilactici* (Iqbal et al., 2010; Katrolia et al., 2011; Lee et al., 2017; Chanalia et al., 2018; Wojciechowska et al., 2018). However, the β -galactosidase from *Thermotoga*

Figure 3. Effects of pH and temperature on the activity and stability of *Bacillus coagulans* NL01 β -galactosidase (BcBga). (a) Effect of pH on BcBga activity. (b) pH stability of BcBga. (c) Effect of temperature on BcBga activity. (d) Thermostability of BcBga over time. Symbol key: Black squares (■) = 45°C; red circles (●) = 50°C; blue upward-pointing triangles (▲) = 55°C; green downward-pointing triangles (▼) = 60°C; pink diamonds (◆) = 65°C. The highest activity was defined as 100%. Error bars indicate standard deviation.

Figure 4. Effects of metal ions on *Bacillus coagulans* NL01 β -galactosidase (BcBga) activity. None = purified BcBga without EDTA treatment; EDTA = purified BcBga treated by EDTA. The EDTA-treated enzyme was used to determine the effects of metal ions. Error bars indicate standard deviation.

naphthophila RUK-10 was inhibited by Mg^{2+} (Kong et al., 2014). Figure 4 shows that our findings contradict these reports. It should be noticed that BcBga was not inhibited by Ca^{2+} , which is beneficial for its use in the dairy industry.

In the crystal structure, the GH42 β -galactosidase polypeptide chain forms 3 domains. Domain A is the catalytic domain, containing an $(\alpha/\beta)_8$ barrel. Domain B has a complicated (α/β) structure, often with a

central 7-stranded mixed β -sheet and 5 α -helices. The function of domain B is structural, as it forms a major interface with other monomers. Domain C is the smallest, and its function has thus far been unclear (Godoy et al., 2016). In this study, we constructed a domain C truncation mutant of GH42 β -galactosidase (BcBga^M) for the first time and found that the mutant generated insoluble protein products, suggesting that domain C might be indispensable to the correct folding of BcBga.

Figure 5. Effects of (a) galactose and (b) glucose on *Bacillus coagulans* NL01 β -galactosidase (BcBga) activity with lactose as substrate. Error bars indicate standard deviation.

Figure 6. Time course of hydrolysis of lactose in whey by the purified *Bacillus coagulans* NL01 β -galactosidase (BcBga). Symbol key: (black ■), lactose; (red ●), galactose; (blue ▲), glucose. Error bars indicate standard deviation.

The ability of β -galactosidase to hydrolyze lactose is crucial to its application in the dairy industry. Previous studies have revealed that most GH42 β -galactosidases prefer to hydrolyze chromogenic substrates and show weak lactose hydrolysis activity. Few GH42 β -galactosidases have thus far been applied to hydrolyze the lactose in milk or whey. Unexpectedly, the β -galactosidase of *B. coagulans* NL01 exhibited admirable lactose hydrolysis ability. Generally, GH2 β -galactosidase is the best-known enzyme possessing good lactose hydrolysis activity. Zhou et al. (2019) employed the commercial β -galactosidase produced by *K. lactis* (GH2, Sigma-Aldrich, G3665) for hydrolysis of lactose in whey. About 140 g/L of lactose in whey was hydrolyzed by β -galactosidase, with 3.8 mg/g of lactose. However, the hydrolysis rate of this commercial β -galactosidase is much slower than BcBga, as it took 72 h to achieve a hydrolysis yield of 93.9% (Zhou et al., 2019).

CONCLUSIONS

In this study, a novel β -galactosidase from *B. coagulans* NL01 was cloned and analyzed. Based on sequence homology, BcBga was assigned to GH42, but it exhibited excellent hydrolysis activity, with activities toward oNPG, pNPG, and lactose reaching 5,978.00 U/mg, 19,383.60 U/mg, and 27.18 U/mg, respectively. Compared with commercial β -galactosidase from *K. lactis*, BcBga is a superior candidate for wide application in whey treatment, which can offset the high production cost of whey valorization.

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