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Characteristics of a *Bifidobacterium longum* LL04 β-Galactosidase (recombinant) Produced in *Escherichia coli*

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Abstract Recombinant β-galactosidase from *Bifidobacterium longum* LL04 was expressed in *Escherichia coli* and partially purified by ammonium sulphate precipitation and anion-exchange chromatography (Mono-Q). The optimum temperature and pH of the partially purified enzyme were 50°C and pH 7.0-8.0, respectively, when o-nitrophenyl-β-D-galactopyranoside was used as a substrate. The enzyme was stable over the pH range of 5.0-9.0, and was active at 40°C for more than 60 min at pH 7.0. The enzyme was significantly activated by Na^+ and K^+. Maximal activity was observed at the concentration of 10 mM for both Na^+ and K^+. The enzyme activity was strongly inhibited by most bivalent metal ions. The Km and Vmax on ONPG at 37 and 50°C were 0.72, 167.9, and 0.507 mM, 310.9 U/mL, respectively.

Keywords: *Bifidobacterium longum*, recombinant β-galactosidase, characteristics

Introduction

Lactose, a disaccharide consisting of galactose and glucose with a β-1,4 linkage, is the main carbohydrate in the milk of all mammals, and plays an important role in milk and milk products. Lactose can be hydrolyzed to glucose and galactose by an enzyme (β-galactosidase, commonly called lactase) or by acids. Commercial sources of β-galactosidase (β-gal) are the fungi (*Aspergillus* spp.) and yeasts (*Kluyveromyces* spp.) which produce the enzymes with acid and neutral pH optima, respectively. Two main commercial applications of β-gal (free or immobilized form) are in producing low-lactose milk for treating lactose intolerance and generating a lactose-hydrolyzed whey directed at reducing whey disposal and transforming whey into value-added products. The production technology for lactose-hydrolyzed whey (glucose-galactose syrup) has been well developed and the use of whey sweeteners has expanded to include various applications in the food industry to enable economic competition with other syrups e.g., glucose-fructose. The relationship between healthy breast-fed infants and a high level of bifidobacteria indicates the health advantage to maintaining a high bifidobacteria count in the gastrointestinal tract. Much research has also been carried out investigating the probiotic effects of bifidobacteria. These multiple effects can be categorized into decreasing the lactose intolerance because of the digestion of lactose by microbial β-gal; inhibiting the growth of pathogens and putrefactive bacteria in the intestine by competing for nutrients and for binding sites or by the production of acids and bacteriocins (1-3); anticarcinogenic effects by direct or indirect removal of procarcinogens, or the activation of the host's immune system (4-7); anticholesterolemic effects (8, 9); and synthesis of B-complex vitamins (10, 11). Due to their overall beneficial effects, bifidobacteria have been used in the dairy and health foods industries. Molecular cloning of the β-gal genes from bifidobacteria strains has been reported by several investigators [*Bacillus longum* (12, 13), *Bacillus infantis* (14), *Bacillus breve* (15), *Bacillus bifidum* (GenBank accession number AJ224434)]. We have cloned a β-gal gene from *B. longum* LL04 in *Escherichia coli*, determined the nucleotide sequence (data not shown) and in this report, describe the purification process and further detailed characterization of the recombinant form.

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Materials and Methods

Chemicals and reagents All chemicals used in this study were of analytical reagent grade and were purchased from Sigma (St. Louis, MO, USA) other suppliers as specified. Bacto-tryptone, yeast extract and agar were purchased from Difco Laboratories (Detroit, MI, USA).

Polymerase chain reaction (PCR) The PCR studies were performed with genomic DNA or plasmid DNA from β-gal-positive clones used as templates for amplifying the target genes. When appropriate, restriction sites were added at the 5 end of the primers to facilitate future cloning steps. Template DNA and primers were added to 50 μL of a PCR mixture containing 200 μM of each deoxynucleoside triphosphate, PCR buffer, and 2.5 U of HotStarTaq DNA polymerase (QIAGEN, Hilden, Germany). The PCR was conducted in a Perkin Elmer GeneAmp system (Boston, MA, USA) with an initial activation step at 95°C for 15 min, followed by 35 cycles each consisting of a denaturation step at 94°C for 1 min, an annealing step at 55°C for 30 sec, and an extension step at 72°C for 1 min. A final extension step of 10 min at 72°C was

performed to ensure complete amplification of all DNA fragments. PCR products were analyzed by electrophoresis in 0.8% agarose gels containing ethidium bromide (1 μg/mL) and purified with the QIAquick PCR purification kit (QIAGEN).

Overexpression vector construction The gene encoding β-gal from *B. longum* LL04, a strain isolated from the feces of infants, was cloned into pET36b(+) (Novagen, Madison, WI, USA) by PCR using pBRgal (pBR322 with 6.5-kb *EcoR*I-*EcoR*I insert from *B. longum* LL04 chromosomal DNA) as template. To create the overexpression plasmid, pBgal36b, the β-gal gene was amplified with the primers β-gal-F and β-gal-R (5'-AGC ATA TGA CAG ACG TCA CAC ATG TCG-3' and 5'-ATA AGC TTC A GA TCA GCT CGA GGT C-3', respectively). An *Nde*I site was designed in primer β-gal-F and a *Hind*III site was created in primer β-gal-R to include the start codon sequence and the stop codon (TGA) sequence, respectively. Cloning into the *Nde*I-*Hind*III sites of pET36b(+) resulted in the translational fusion of the β-gal gene (3-kb of PCR product) to the T7 promoter and *E. coli* ribosome binding site of the plasmid. Plasmid pBGal36b was created in *E. coli* DH5α and transformed into *E. coli* ER2566 (*F– l– fhuA2 [lon] ompT lacZ::T7 gene1 gal sulA11D(mcrC-mrr)114::IS10 R (mcr-73:: mini Tn10–Tet^S)2 R(zgb-210::Tn10–Tet^S) endA1 [dcm],* New England BioLabs Inc., Ontario, Canada) for the overexpression studies.

Overproduction of β-Gal and the preparation of cell extracts For partial purification of β-gal, the cell extract of ER2566 (pBgal36b) was prepared by the following method. ER2566 (pBGal36b) was grown in 2YT medium (1.5% tryptone, 0.75% yeast extract, 0.5% NaCl, 0.5% glycerol) until an optical density at 600 nm of 0.7 was reached. For overexpression, the cells were induced with 1.0 mM isopropyl-β-d-thiogalactopyranoside (IPTG; Roche Diagnostics, Basel, Switzerland) and incubated at 37°C continued for 5 hr. The induced cells were harvested, washed twice, suspended in 50 mM sodium phosphate buffer (pH 7.0), and disintegrated by sonication (Sonic dismembrator, model 300; Fisher Scientifique, Ontario, Canada) for 5 min at 30 sec intervals. The disrupted cells were centrifuged at 15,000×g for 20 min (4°C), and the supernatant was used as the cell-free extract.

Enzyme partial purification A cell free extract of 100 mL was precipitated at 4°C with ammonium sulfate (46-70%), the pellet was resuspended in 5 mL of 20 mM sodium phosphate buffer (pH 7.0) and dialysis conducted over-night against the same buffer. The suspended enzyme solution was then concentrated using polyethylene glycol 8000 (American Chemical Ltd., Quebec, Canada). Portions (500 μL) of the concentrated sample were applied to an anion-exchange column (Mono Q HR 5/5; Pharmacia, Quebec, Canada) using a FPLC system (Pharmacia) equilibrated with 20 mM Bis-Tris propane buffer (pH 6.0). Elution was performed using a linear gradient of 1 M NaCl in 20 mM Bis-Tris propane buffer (pH 6.0) at a flow rate of 0.5 mL/min and 1 mL fractions were collected. Fractions exhibiting β-gal activity collected from several chromatographic runs were pooled, desalted, and further concentrated using the Centriplus system (Millipore, MA, USA). The active fraction was used for subsequent enzyme assays.

Enzyme and protein assays β-Gal activity was measured by incubating enzymes with 10 mM o-nitrophenyl-β-D-galactopyranoside (ONPG; Sigma) in 50 mM sodium phosphate buffer (pH 7.0) at 37°C for 10 min and the reaction was stopped by adding 1.0 M sodium carbonate. The released o-nitrophenol was quantitatively determined by measuring the A_{420} of the reaction solution. One unit of activity was defined as that amount of enzyme liberating 1 μmol of o-nitrophenol per min (14). Specific activity was defined in units per mg of protein. The protein concentration was determined by the method of Bradford (16) using bovine serum albumin as standard.

Gel electrophoresis and activity staining In accordance with the method of Laemmli (17), proteins were analyzed by polyacrylamide gel electrophoresis (PAGE) using 10% (wt/v) polyacrylamide gel and staining by Coomassie blue or activity staining. For activity staining, the crude cell lysates were loaded and electrophoresed in a non-denaturing PAGE system. β-Gal activity was then detected by incubating the gel in a 4-o-methylumbelliferyl-β-d-galactoside (4MeUmG; Sigma) solution (0.2 mg/mL in phosphate buffer, pH 7.0) for 30 min (14). Fluorescent bands were visualized under UV light on a transilluminator.

Effects of pH, temperature and metal ions on β-galactosidase Three buffer systems, citrate buffer (50 mM, pH 3-6), sodium phosphate buffer (50 mM, pH 6-9), and sodium carbonate buffer (50 mM, 9-11) were used for determining the optimal pH for enzyme activity. To estimate pH stability, enzyme samples were preincubated in different buffers at room temperature for 3 hr before adding substrate in 50 mM sodium phosphate buffer (pH 7.0). The optimum temperature was determined over the range of 40-60°C by incubating for 10 min in 50 mM sodium phosphate buffer (pH 7.0). Thermal stability was estimated by incubating the enzyme at the desired temperature and residual activity was measured at 37°C. To study the effect of various cations, the cations (final concentration 1.0, 10, or 100 mM; chloride or sulfate form) were incubated with enzyme and 10 mM ONPG in water at 37°C for 10 min. Enzyme activity measured without added cations as the control.

Enzyme kinetics The Km and Vmax of the partially purified β-gal were determined using the substrate ONPG in 50 mM sodium phosphate buffer (pH 7.0). Namely, β-gal activity was measured by incubating 0.51 mL of mixture [(0.50 mL of 0.156-10.0 mM ONPG in 50 mM sodium phosphate buffer (pH 7.0) and 0.01 mL of enzyme (9.47 μg/mL; 221.9 U/mL)] at 37 or 50°C for 5 or 2 min, respectively; the reaction was stopped by adding 0.5 mL of 1.0 M sodium carbonate. The released o-nitrophenol was quantitatively determined by measuring the A_{420} of the reaction solution. The kinetic constants were computed

from the slope and intercept of the regression line on Lineweaver-Burk plots.

Results and Discussion

Production and partial purification of recombinant β-galactosidase To characterize the recombinant β-galactosidase from *B. longum* LL04, a β-gal gene expression plasmid (pBgal36b) was constructed, and the plasmid was introduced into *E. coli* ER2566. Gene expression in *E. coli* was induced by IPTG. After the IPTG was added, a dramatic increase of enzyme activity was noted at 3 hr for β-gal, and the activity slowly increased thereafter. Cell extracts from IPTG-induced ER2566 (pBgal36b) cells were partially purified. The β-gal was partial purified by ammonium sulfate precipitation and Mono Q anion-exchange chromatography (Fig. 1). The enzyme activity detected by precipitation with ammonium sulfate was 46-70% and the results are summarized in Table 1. The specific activity of partially purified β-gal was approximatly 234 U/mg, which is about 12.3-fold higher than that of the crude extract. The purification sequence allowed for a recovery of 66.5% of the original crude enzyme activity.

Effect of pH and temperature The optimal pH range of β-gal was determined to be pH 7.0-8.0 for ONPG (Fig. 2). The enzyme lost 1.7% of its maximum activity at pH 7.0 and at pH 9.0, it lost only 14% of its maximum activity. Pre-incubation of the enzyme in different buffer (pH 3.0-11.0) had no effect on the pH optimum profile. The enzyme retained more than 90% of its activity in the range of pH 5.0-9.0 after 3 hr incubation, suggesting that this enzyme is quite stable in the acidic and alkaline pH ranges. This optimal pH (7-8) of β-gal was slightly higher than that of the general microbial β-gal containing *Lactobacillus acidophilus* (19), which ranges around pH 3-7.2, but the pH optimum was the same as that reported for β-gal from *B. infantis* HL96(20), *Leuconostoc* spp. (21), *Streptococcus thermophilus* (22), *Arthrobacter* B7 (23), *E. coli* (24), *Bacillus* sp. (25), and *Saccharopolyspor rectivirgula* (26). Optimum temperature for the activity with ONPG was 50°C (Fig. 3). After pre-incubation in 50 mM sodium phosphate buffer (pH 7.0) at 40°C for 60 min, the enzyme retained over 90% of its activity, whereas the

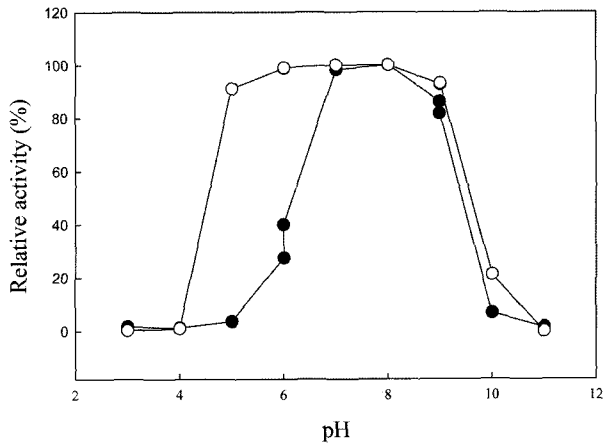

Fig. 2 Optimal pH (black circles) and pH stability (open circles) of recombinant β-galactosidase using ONPG as substrate. The buffers used are citrate buffer (pH 3-6), Na-phosphate buffer (pH 6-9), and sodium carbonate buffer (pH 9-11).

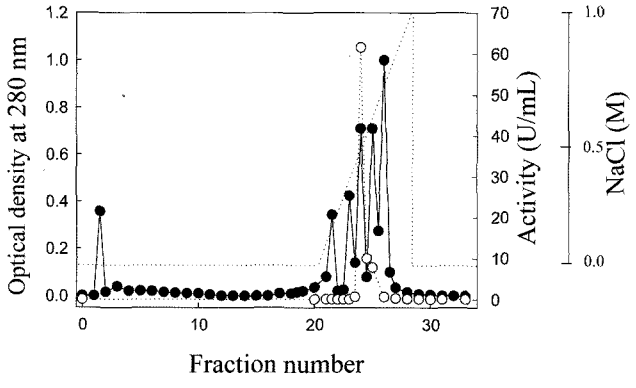

Fig. 1. Mono Q HR 5/5 anion-exchange column chromatography. Absorbance at 280 nm (black circles), activity (open circles), and sodium chloride concentration (dotted line). Applied sample volume, 500 μL; flow rate, 1 mL/min; fraction volume, 1 mL/tube; elution buffer, 20 mM Bis-Tris propane buffer (pH 6.0); active fractions, 24-25.

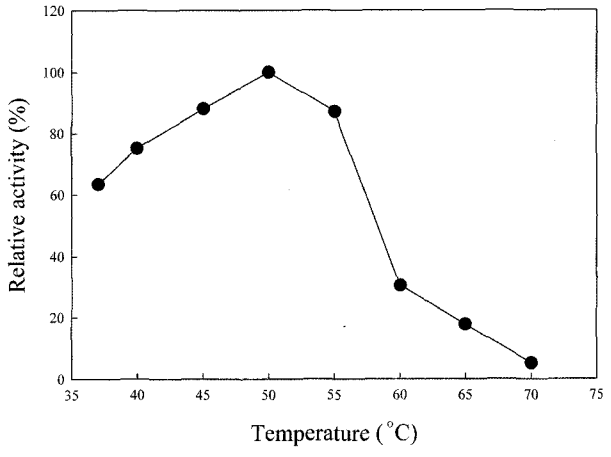

Fig. 3. Optimal temperature of recombinant β-galactosidase using ONPG as substrate.

Table 1. Purification of β-galactosidase from ER2566 (pBgal36b)

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification	Recovery factor (%)
Crude extract	388.7	7,512	19	1.0	100
Ammonium sulfate precipitation	144.6	5,840	40	2.1	77.7
Mono Q ion-exchange	21.3	4,992	234	12.3	66.5

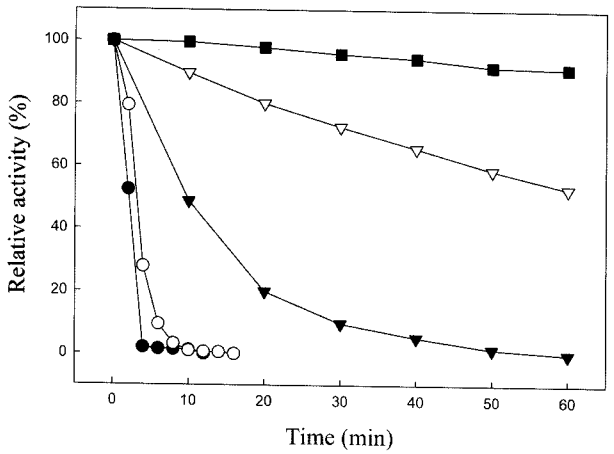

Fig. 4 Stability of recombinant β-galactosidase at different temperatures. black circles 60°C, open circles 55°C, black triangles 50°C, open triangles 45°C, black squares 40°C.

Table 2. Effects of metallic cations on the activity of β-Gal in distilled water and 50 mM sodium phosphate buffer (pH 7.5)

Metal ions[1)]	Relative activity (%)[3)] in dH_2O		
	1 mM	10 mM	100 mM
None	100		
Na^+	186	263	261
K^+	142	175	165
Mg^{2+}	155	109	53
Ca^{2+}	64	41	29
Cu^{2+}[b2)]	0	0	0
Fe^{2+}	0	0	0
Cr^{3+}	0	0	2
Zn^{2+}	0	0	0
Mn^{2+}	62	38	12

[1)]Added as chloride.
[2)]Added as sulphate.
[3)]Activity measured in triplicate samples ($p<0.05$).

activity were significantly decreased after pre-incubation at 50-60°C for 10 min (Fig. 4). Most β-gals hydrolyzed ONPG and lactose at the same optimum temperature. Although β-gals from *B. infantis* (20), lactic acid bacteria such as *Luconostoc citrovorum*, thermophilic organisms such as *Bacillus stearothermophilus*, and *Thermus aquaticus* (27) are found to have the optimum temperature of 60, 60, 65, and 80°C, respectively, this β-gal of *B. longum* showed different characteristic. This β-gal showed relatively low temperature optima (50°C), that is similar to those mesophilic bacteria such as *Pediococcus pentosaceus* (45°C)(28).

β-Gals from microbial sources are of higher commercial value as compared with that of plant and animal origin. β-gals from plants and some animals have little or no activity on lactose. Current commercial β-gals are those from *Kluyveromyces fragilis*, *Candida pseudotropicalis*, *Aspergillus niger*, *Aspergillus oryzae* and *Bacillus* spp. (29). They differ widely in their properties, particularly the optimum pH, temperature, and the activity. Fungal β-gals have the pH optima in the range of 3.0-5.0 and optimum temperature at around 50°C, depending on the strain and the substrate used, and they are stable over a wide pH range of 2.4-8.0 and at a higher temperature than the yeast enzymes. Yeast β-gals are most active at the pH range between 6.0-7.0, and temperature at 30-40°C, and they are stable over pH 6.5-7.5, but not stable over 50°C. Fungal enzymes are suitable for processing acid whey, acid whey permeate or fermented dairy products. The β-gals (immobilized form) from *A. oryzae* is now the most commonly used enzyme for whey treatment. Yeast enzymes are useful in treating fluid milk or sweet whey at near neutral pH, mostly used in batch process. Although the β-gals from *K. fragilis*, *Kluyveromyces lactis*, *A. oryzae*, and *A. niger* have been applied in food industry for decades, there is a continuing interest of characterizing new β-gals from new sources with academic and/or potential application values. The primary characteristics that determine the application ability are the operation pH and temperature range, the stability, the substrate specificity, cofactor and the product inhibition, and the enzyme activity. The β-gal of *S. thermophilus* has the advantage of safety and low sensibility to product inhibition, but it is not stable at temperature above 55°C (22). The β-gal of *B. stearothermophilus* is more stable, but its yield is rather low, about ten times lower than that attained from yeast in batch culture (29). A thermostable enzyme with high optimum temperature is advantageous for minimizing the microbial growth during reaction; however, a high reaction temperature will create off-flavor and color changes. When considering this aspect, a cold active β-gal is favorable. A cold active β-gal has been characterized from *Arthrobacter* species (23), which unfortunately are generally not considered as safe for food processing. Other cold temperature active β-gal (30) have also been reported.

No commercial application using β-gal of bifidobacteria has been taken place so far. However, it is possible to expect the use of β-gal of *B. longum* in treating fluid milk or sweet whey at near neutral because the optimum condition of β-gal of *B. longum* is similar to that of yeast enzyme.

Effect of metal ions The recombinant β-gal was significantly activated by Na^+ and K^+. At 10 mM concentration, the enzyme activity was increased 2.6 (Na^+) and 1.7 (K^+) times higher than the control (Table 2). Although the enzyme activity enhanced by 1 mM of Mg^{2+}, in the presence of 100 mM of all the divalent cations tested, as well as Cr^{3+}, the activity was markedly inhibited. The enzyme was strongly stimulated by monovalent ions and similar to that of *Lactobacillus bulgaricus* (31), which was inhibited by various bivalent ions, including Mg^{2+}, Mn^{2+}, Ca^{2+}. In contract, Mg^{2+} or/and Mn^{2+} were found to enhance the β-gal activity in *Arthrobacter* B7 (23), *E. coli* (24), *B. bifidum* 1901 (32), *K. lactis* (33), and *Bacillus* sp. MTCC 3088 (25). β-Gals of *L. casei* 20094, *B. bifidum* 1901 and *S. thermophilus* were also activated by Na^+ and K^+ (34).

Enzyme kinetics The kinetic constant (Km) and maximum reaction velocity (Vmax) of the partially purified β-gal at 37 and 50°C on synthetic substrate ONPG were calculated

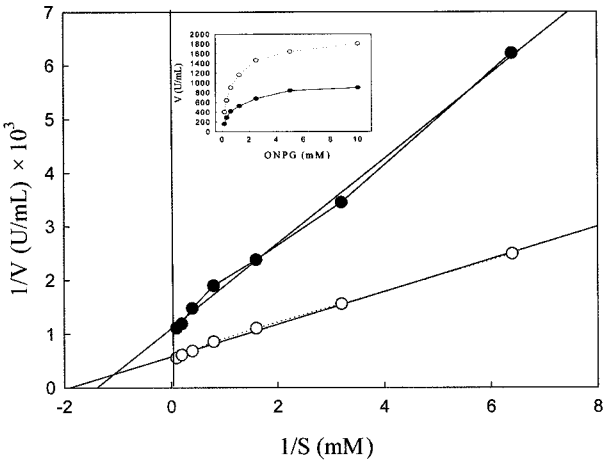

Fig. 5. Lineweaver-Burk plots of recombinant β-galactosidase activity with ONPG at 37 and 50°C (B). Black circles 37°C, open circles 50°C

to be 0.72, 167.9 and 0.51 mM, 310.9 U/mL, respectively (Fig. 5), suggesting that temperature increase had more influence on catalytic activity than substrate binding. The Km value at 37 or 50°C was similar to that of *A. oryzae* (0.77 mM) (35), *Leuconostoc* spp. (0.68 mM) (21), *Arthrobacter* B7 (0.4 mM) (23), and *S. thermophilus* (0.98 mM) (22) and was less than that found for the bifidobacteria such as *B. infantis* (2.6 mM) (20) and *B. bifidum* (2.0 mM) (36), *L. Bulgaricus* (79 mM) (32), *Cryptococcus laurentil* (18.2 mM) (37), and *K. lactis* (17.3 mM) (33). The β-gal from *B. longum* LL04 had maximum reaction velocity (V_{max}) of 310.9 U/mL at 50°C, which was higher than that of as *B. infantis* (262 U/mL) (20) and was less than that of *Bacillus* sp. MTCC3088 (9351 U/mL) (25).

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