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Article

Properties of β-galactosidase purified from *Bifidobacterium longum* subsp. *longum* JCM 7052 grown on gum arabic

Naoko Saishin[1], Masami Ueta[2], Akira Wada[2], and Isamu Yamamoto[3]*

[1]Department of Food Nutrition, Kobe Women's Junior College, Chuo-ku, Kobe 605-0046 Japan, [2]Yoshida Biological Laboratory, Yamashina-ku, Kyoto 607-8081, Japan, and [3]Laboratory of Molecular Microbiology, Graduate School of Life Science, Kobe Women's University, Suma-ku, Kobe 654-8585, Japan

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β-Galactosidase was purified and characterized from *Bifidobacterium longum* subsp. *longum* JCM 7052 grown on gum arabic. The molecular masses of the enzyme were estimated to be 77 and 110 kDa by polyacrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulfate, respectively. MALDI-TOF-MS showed that the purified β-galactosidase was a homologue of the *lacA1* gene product found in the genome of *B. longum* subsp. *longum* DJO10A. This β-galactosidase could hydrolyze 4-nitrophenyl (NP)-β-D-galactopyranoside but neither lactose nor gum arabic. The K_m value for 4-NP-β-D-galactopyranoside was 0.42 ± 0.015 mM. Transglycosylation was observed from 4-NP-β-D-galactopyranoside to galactose and melibiose, but not from lactose. Maximal activities of β-galactosidase were obtained at pH 7.0 and 50~55°C. The enzyme was stable at 35°C for 5 h, but was gradually inactivated at higher than 40°C. Galactose was an effective competitive inhibitor: its inhibition constant K_i was 5.8 ±0.38 mM.

Keywords: β-galactosidase, purification, *Bifidobacterium longum* subsp. *longum*

Introduction

Bifidobacteria are strictly anaerobic, Gram-positive, and saccharolytic bacteria which predominantly live in the intestinal tract of humans and animals. Some strains of this genus are known to have many health-promoting or probiotic activities [1, 2]. The growth of bifidobacteria has been shown to be selectively stimulated by various dietary carbohydrates including gum arabic [3-6]. The genome sequence of *Bifidobacterium longum* subsp. *longum* is now available [7, 8], and it has revealed many genes for carbohydrate utilization. More than 30 genes coding for glycoside hydrolases are found there and some of them are predicted to be involved in degradation of complex carbohydrates.

Gum arabic is a soluble dietary fiber obtained from the stems and branches of *Acacia senegal* and *Acacia seyal*. It is composed of three main fractions, arabinogalactan protein complex (AGP, 10%), arabinogalactan (AG, 90%), and glycoprotein (1%) [9]. AG and carbohydrate block of AGP consist of a branched β-1,3-linked galactose backbone with branches linked through the 1,6-positions, and with arabinose, rhamnose, and glucuronic acid in ramified side chains [10, 11]. It is known that some strains of *Bifidobacterium adolescentis* and *B. longum*

Corresponding author: Isamu Yamamoto.
Phone: +81-78-737-2312, Fax: +81-78-732-5161
E-mail: iyamamoto@suma.kobe-wu.ac.jp

subsp. *longum* can ferment gum arabic [3]. For the fermentation some glycoside hydrolytic enzymes would be required to degrade gum arabic: β-galactosidase, α-arabinopyranosidase, α-arabinofuranosidase, arabinogalactan-endo-β-galactosidase and so on.

β-Galactosidase (EC 3.2.1.23) catalyzes hydrolysis of β-D-galactoside linkage, and some β-galactosidases may have an activity of transferring one or more D-galactosyl units onto lactose [12]. Various β-galactosidases have been characterized in *Bifidobacterium* species, *B. adolescentis* [13, 14], *Bifidobacterium bifidum* [15-17], *Bifidobacterium longum* subsp. *infantis* [16, 18, 19], and *B. longum* subsp. *longum* [20-22]. Glycoside hydrolases (GHs) including β-galactosidase constitute 113 protein families [23]. Lactose-degrading β-galactosidases are classified as GH2 family and others classified as GH42 family which show activity toward β-galactosyl linkages in oligosaccharides [14, 24].

We have previously shown that *B. longum* subsp. *longum* JCM 7052 is able to grow on gum arabic as carbon source and contains a high activity of β-galactosidase as well as α-galactosidase [25]. These enzymes are considered probably to contribute to degradation of the polysaccharide. In this paper, some properties of β-galactosidase purified from *B. longum* subsp. *longum* JCM 7052 grown on gum arabic are described.

Materials and Methods

Chemicals Nitrophenyl (NP)-glycosides, and 3,5-dinitrosalitylic acid were purchased from Sigma-Aldrich (St. Louis, Mo, USA). Gum arabic (*Acacia senegal*) was obtained from Wako Pure Chemical Industries (Osaka, Japan). Other chemicals of reagent grade were products of Wako Pure Chemical Industries.

Bacterial strain and growth conditions *Bifidobacterium longum* subsp. *longum* JCM 7052 was used in this study. Bacterial cells were grown in a medium of slightly modified Bifidobacterium medium, which contained (g/l) Polypepton (Nihon Seiyaku, Japan), 10; yeast extract (Oriental Yeast Co. Japan), 5; meat extract (Wako Pure Chemical Industries, Japan), 5; K_2HPO_4, 3; sodium ascorbate, 10; cysteine hydrochloride, 0.5; and Tween 80, 1 ml/l. Gum arabic (10 g/l) was added as carbon source instead of glucose. pH was adjusted to 6.8. Cells were anaerobically grown at 37°C for 16 h. Anaerobic conditions were obtained by filling the neck of screw-capped bottles with the medium.

Assay of enzyme activities Standard assay of β-galactosidase activity was performed with 4-nitrophenyl (4-NP)- β-D-galactopyranoside as substrate. The reaction mixture (1 ml) contained an enzyme preparation, 80 mM K-phosphate (pH 7.0) and 1 mM 4-NP-β-D-galactopyranoside. The reactions were carried out at 30°C for 10 min and were terminated by the addition of 0.5 ml of 0.2 M Na_2CO_3. The amount of 4-nitrophenol released was determined at 400 nm with an extinction coefficient of 18.3 mM^{-1} cm^{-1}. One unit of enzyme activity is defined as the amount of enzyme that releases 1 μmol of 4-nitrophenol per min from 4-NP-β-D- galactopyranoside.

For assay of activities which hydrolyze oligosaccharides, reducing sugar produced from oligosaccharide was determined in the reaction mixtures containing 80 mM K-phosphate (pH 7.0), enzyme preparation, and 100 mM lactose or 0.1% gum arabic. After incubation at 30°C for 60 min, 3,5-dinitrosalitylic acid was added to stop the reaction. The absorbance was measured at 540 nm. Galactose was used as the standard for calibration. One unit of enzyme activity is defined as the amount of enzyme that produces 1 μmol of reducing sugar per min from the oligosaccharide.

Transglycosylation activity was assayed by detection of product in HPTLC (Silica gel 60; Merck Ltd., Germany). The Reaction mixtures (1 ml) contained 80 mM K-phosphate buffer (pH 7.0), 15 mM 4-NP-β-D-galactopyranoside, 80 mM saccharide, and 0.2 units of purified β-galactosidase, and were incubated at 30°C for 3, 6, and 12 h. Saccharides were separated in *n*-butanol:acetic acid:water (5:4:1), and detected with spray of 5% sulfuric acid in 20% ethanol.

Thermal stability was assayed by incubating a partially purified enzyme at 35, 40, 45, 50 and 55°C and drawing aliquots at intervals. The enzyme activity was then determined according

to the standard assay procedure.

The protein concentrations were determined by using Coomassie brilliant blue (Bio-Rad Laboratories, USA). Bovine serum albumin was used as the standard. All reactions were carried out in triplicate.

Purification of β-galactosidase Wet cells (10 g) were suspended in four volumes of 50 mM Tris-HCl buffer (pH 7.2). The cells were sonicated (TOMY Ultrasonic Disruptor UD-200, Japan) in an ice bath at 20 kHz, 140 W for 5 min and centrifuged at 20,000 x *g* for 30 min at 4°C. The supernatant was used as crude extract (Step 1. Crude extract, 40 ml). The precipitate obtained between 30 and 70% saturation of ammonium sulfate was dissolved in 50 mM Tris-HCl, pH 7.2 (Step 2. Ammonium sulfate fraction, 4.4 ml). Proteins dissolved were loaded on a column (2.5 x 90 cm) of Sepharose 4B (GE Healthcare, Sweden) equilibrated in 50 mM Tris-HCl (pH 7.2) containing 0.1 M NaCl and eluted at a flow rate of 0.4 ml/min. Fractions containing β-galactosidase activity were pooled (Step 3. Sepharose 4B, 77 ml). The pooled fractions were loaded on a column (1.5 x 7 cm) of Q-Sepharose (GE Healthcare, Sweden) equilibrated in 50 mM Tris-HCl (pH 7.2) containing 0.1 M NaCl. Proteins were eluted at a flow rate of 0.4 ml/min with a linear concentration gradient of NaCl (0.1~0.7 M). Fractions containing β-galactosidase activity were pooled (Step 4. Q-Sepharose, 28 ml). The solution pooled was mixed with ammonium sulfate at a concentration of 1.84 M, then was loaded on a column (1.5 x 7 cm) of Butyl-S Sepharose 6 Fast Flow (Amersham Biosciences, Sweden) equilibrated with 50 mM Tris-HCl (pH 7.2) containing 1.84 M ammonium sulfate. Proteins were eluted using a decreasing stepwise gradient of ammonium sulfate (1.84~0 M) at a flow rate of 1 ml/min. Fractions active with β-galactosidase were pooled (Step 5. Butyl-S Sepharose, 6.0 ml). The enzyme pooled was desalted and concentrated by centrifugation with a filtration tube Vivaspin 4 (Sartorius, Germany). Concentrated proteins were loaded on 6% polyacrylamide gel electrophoresis (PAGE) without sodium dodecyl sulfate (SDS). An area stained with β-galactosidase activity was cut out of the polyacrylamide gel and was soaked in 50 mM Tris-HCl (pH 7.2). The enzyme was filtrated using a gel extraction spin column (Bio-Rad, USA) and was concentrated by centrifugation with a filtration tube Vivaspin 4 (Step 6. Native PAGE, 4.0 ml).

Molecular mass determination SDS-PAGE was carried out with 12.5% (w/v) polyacrylamide gel according to Laemmli [26]. Molecular weight marker proteins for SDS-PAGE (GE Healthcare, UK) were used for calibration. Native PAGE was done using a 5~15% (w/v) linear gradient slab gel (Wako Pure Chemical Industries). Molecular weight marker proteins for native PAGE (HMW calibration kit, GE Healthcare, UK) were used for calibration. Activity staining of β-galactosidase on the gel was done using 4-NP- β-D-galactopyranoside as substrate. Protein bands in gels were stained with Coomassie blue R-250 (Bio-Rad Laboratories, USA).

Protein identification by mass spectrometry The Coomassie-stained protein bands were excised from the SDS-PAGE gel, and subjected to tryptic digestion as previously described [27]. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) and tandem mass spectrometry (MS/MS) were performed on an Ultraflex MS system (Bruker Daltonik GmbH, Bremen, Germany). Peptide mass fingerprinting (PMF) searches were performed by using the Mascot Search engine against the NCBI protein database.

Results

Purification of β-galactosidase and its molecular mass

A summary of a purification of β-galactosidase from *B. longum* subsp. *longum* JCM 7052 grown on gum arabic is given in Table 1. When the enzyme was separated by means of chromatography, β-galactosidase activity was eluted by 0.55 M NaCl and by 1.1 M $(NH_4)_2SO_4$ in ion exchange chromatography with Q-Sepharose and in hydrophobic chromatography with Butyl-S Sepharose, respectively. At final

Table 1. Purification of β-galactosidase from *B. longum* subsp. *longum* JCM 7052 grown on 1% gum arabic

Purification step	Protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Yield (%)
1. Crude extract	283	128	0.45	100
2. Ammonium sulfate fraction	104	72.5	0.70	57
3. Sepharose 4B	73.4	65.2	0.89	51
4. Q Sepharose	26.6	38.1	1.43	30
5. Butyl-S Sepharose	1.76	13.1	7.41	10
6. Native PAGE	0.52	6.3	12.1	5

step of purification, the enzyme obtained was obviously homogenous in SDS-PAGE (Fig. 1A).

The molecular mass of the enzyme was determined by SDS-PAGE and native gradient PAGE. SDS-PAGE showed a single band of protein and its molecular mass to be 77 kDa (Fig. 1A). Native gradient PAGE also showed a single protein with a molecular mass of about 110 kDa (Fig. 1B). These results suggested that the enzyme was a dimeric protein.

Identification of β-galactosidase by mass spectrometry

The purified enzyme was subjected to PMF analysis in order to identify its putative gene. The PMF result (Accession number, gi|189439789; sequence coverage, 43.7%; score, 210) and two MS/MS results (1795.875 (637-651), ADETGENHFVFLFNR; score, 150) and (1882.879 (60-74), LEPEEGVYDFDWLDR; score, 92) were obtained. These results indicate that the purified β-galactosidase is evidently identical with the product of the *lacA1* gene (the locus tag BLD 0926) in the genomic DNA of *B. longum* subsp. *longum* DJO10A [8]. The same amino acid sequence is also found as BL 1168 in the genome of *B. longum* subsp. *longum* NCC 2705 [7]. This gene product consists of 691 amino acids and has the molecular mass of 77,414 Da. This value is consistent with the molecular mass of 77 kDa determined by SDS-PAGE. The BLD 0926 β-galactosidase belongs to GH42 family. β-Galactosidase encoded in the *lacZ* gene is another enzyme which has been found in *B. longum* subsp. *longum* [7, 8].

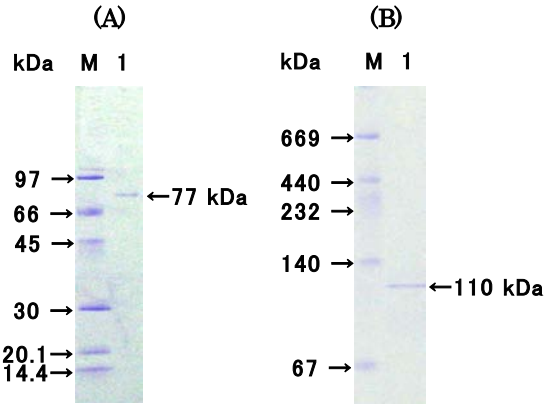

Fig 1. Molecular mass determination of β-galactosidase by SDS-PAGE **(A)** and native gradient PAGE **(B)**. Lanes: M, marker proteins; 1, purified enzyme. Proteins were stained by Coomassie blue R-250.

Substrate specificity and transglycosylation activity of β-galactosidase

The β-galactosidase purified from *B. longum* subsp. *longum* JCM 7052 displayed a narrow substrate range. The enzyme hydrolyzed 4-NP-β-D-galactopyranoside (100% of activity), 2-NP-β-D-galactopyranoside (31%), and 4-NP-β-D-fucopyranoside (8%), but not 4-NP-β-D-glucopyranoside and 4-NP-α-linked compounds with L-arabinofuranoside, L-arabinopyranoside, D-galactopyranoside, D-glucopyranoside, D-mannopyranoside, L-rhamnopyranoside, and L-fucopyranoside. The K_m values for 4-NP-β-D-galactopyranoside and 2-NP-β-D-galactopyranoside were determined by double reciprocal plots to be 0.42 ±0.015 and 0.78 ±0.039 mM, respectively.

Maximal velocities (V_{max}) for 4-NP- and 2-NP-β-D-galactopyranosides were also shown to be 5.21 ±0.38 and 2.16 ±0.062 μmol/min/mg of protein, respectively.

Lactose and gum arabic were not hydrolyzed by β-galactosidase during 1 h incubation at 30°C and pH 7.0.

Transgalactosylation assay with the purified β-galactosidase was performed as described in Materials and Methods. When 4-NP-β-D-galactopyranoside was used as a donor of galactosyl group, an apparently single product was formed with galactose and melibiose as acceptors (data not shown). Transgalactosylation from lactose to these acceptor sugars was not observed.

Effects of pH, temperature, and chemicals on the enzyme activity

In these experiments on characterization of the catalytic properties, the enzyme preparation obtained at Butyl-S Sepharose step of purification was used. To observe pH-dependency of enzyme activity, β-galactosidase was incubated in 80 mM K-phosphate buffer with 1 mM 4-NP-β-D-galactopyranoside at 30°C for 10 min. The highest activity was obtained at pH 7.0 (Fig. 2). In the assay in 80 mM Tris-HCl buffer enzyme activities obtained were slightly low.

The optimal temperature for β-galactosidase activity was 50~55°C at pH 7.0 in a 5 min reaction (Fig. 3A). The enzyme was stable during 5 h incubation at 35°C, but very instable at higher than 40°C (Fig. 3B). NaCl of 0.15 M concentration was slightly effective to stabilize the enzyme.

Of metal chloride salts tested (Table 2) 1 mM Cu^{2+} inhibited the β-galactosidase activity by 50%, while Ba^{2+}, Ca^{2+}, Co^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+} showed almost no effects on the enzyme activity. No effects of 10 mM NaCl and 10 mM KCl were observed on the enzyme activity.

A metal chelator EDTA did not affect the activity of β-galactosidase.

The enzyme activity was determined in the presence of 20 mM galactose, glucose, or lactose (Table 2). Galactose showed about 60% inhibition of the activity, and glucose and lactose 17% and

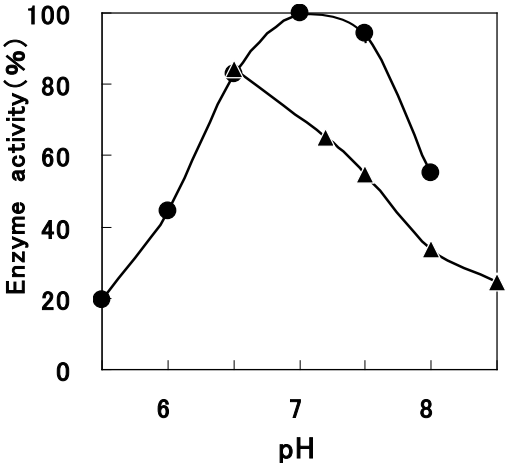

Fig 2. Effects of pH on the activity of the purified β-galactosidase. Buffers used were, ●, 80 mM K-phosphate (pH 5.5–8.0); and ▲, 80 mM Tris-HCl (pH 6.5–8.5).

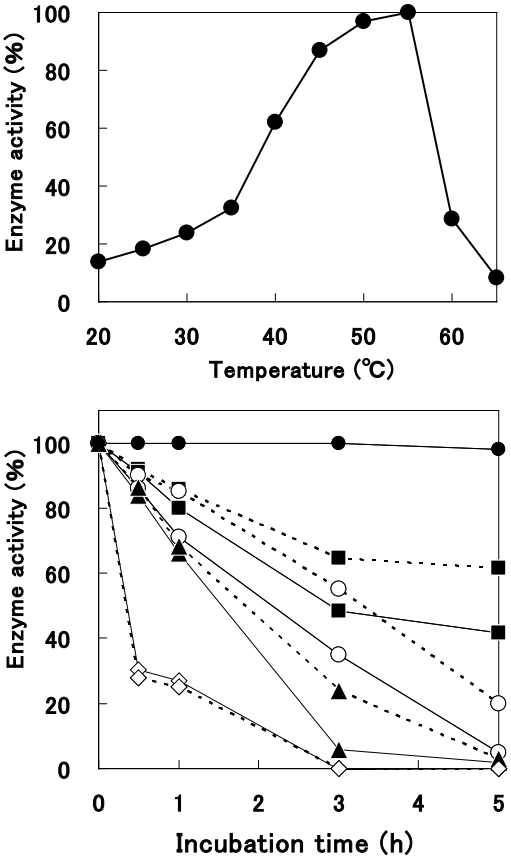

Fig 3. Effects of temperature on the activity **(A)** and stability **(B)** of the purified β-galactosidase. Symbols in **(B)**, ●, 35℃; ■, 40℃; ○, 45℃; ▲, 50 ℃; and ◇, 55 ℃. Solid lines indicate activities in the absence of NaCl, and dotted lines in the presence of 0.15 M NaCl.

Table 2. Effects of various reagents on the β-galactosidase activity

Reagents		Relative activity (%)
None		100
$BaCl_2$	1 mM	100
$CaCl_2$	1 mM	99
$CoCl_2$	1 mM	90
$CuCl_2$	1 mM	50
$FeCl_3$	1 mM	100
$MgCl_2$	1 mM	98
$MnCl_2$	1 mM	100
$NiCl_2$	1 mM	100
$ZnCl_2$	1 mM	100
NaCl	10 mM	98
KCl	10 mM	96
EDTA	1 mM	100
Galactose	20 mM	43
Glucose	20 mM	83
Lactose	20 mM	81
Tris*	50 mM	73
	100 mM	54

*Tris (hydroxymethyl) aminomethane

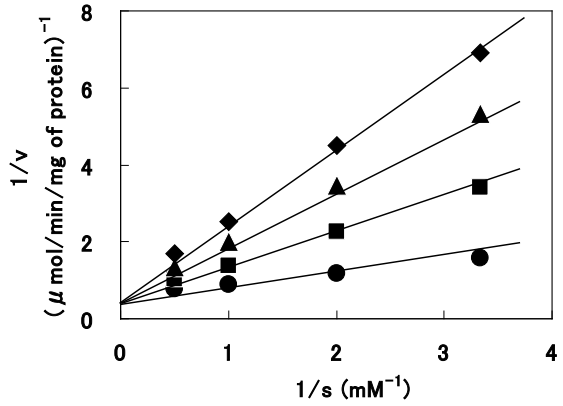

Fig 4. Inhibitory effects of D-galactose on the activity of the purified β-galactosidase. Double reciprocal plots of β-galactosidase activity against 4-NP- β-D-galactopyranoside in the presence of D-galactose. For the enzyme activity assay, K-phosphate buffer (pH 7.0) was used. The concentrations of galactose were 0 (●), 10 (■), 20 (▲), and 30 mM (◆).

19%, respectively. The inhibitory effect of galactose was competitive and its inhibition constant K_i was 5.8 ±0.38 mM (Fig. 4).

Effect of Tris (hydroxymethyl) aminomethane was also investigated because Tris has been known to inhibit competitively some glycoside hydrolases [25, 28, 29]. β-Galactosidase activity was inhibited 27% and 46% in the presence of 50 and 100 mM Tris, respectively. A possible inhibition type was shown to be competitive by double reciprocal plots and an inhibition constant K_i of Tris was about 98.0 ± 1.16 mM in Dixon plots. These results suggested weak interaction between β-galactosidase and Tris.

Discussion

β-Galactosidase was purified from *B. longum* subsp. *longum* JCM 7052 grown on gum arabic. Its primary protein structure was partly determined by mass spectrometry and showed that the purified β-galactosidase was a homologue of BLD 0926 and BL 1168, which have been found in the genomes of *B. longum* subsp. *longum* strains DJO10A and NCC 2705, respectively [7, 8]. This enzyme is encoded in the *lacA1* gene, but not in the *lacZ* gene. The purified β-galactosidase showed activities hydrolyzing 4-NP-β-D-fucopyranoside with 8% of reactivity to 4-NP-β-D-galactopyranoside, but did not hydrolyze lactose and gum arabic. Maximal activities were obtained at pH 7.0 and at 55°C. However, the enzyme was completely inactivated during 3 h incubation at 55°C. Neither activation nor inhibition of β-galactosidase activity occurred in the presence of monovalent and divalent cations except for Cu^{2+}, which inhibited by 50% at 1 mM concentration. β-Galactosidase was competitively inhibited by D-galactose with K_i of 5.8 mM. Transglycosylation activity was also observed from 4-NP-β-D-galactopyranoside, but not from lactose.

So far, β-galactosidases have been purified and characterized from two strains of *B. longum* subsp. *longum*. However, the molecular mass and catalytic properties of each enzyme are quite different from those of the β-galactosidase purified in this study. β-Galactosidase with an

activity of hydrolyzing 4-NP-β-D-fucoside has been purified from *B. longum* subsp. *longum* 401 [20]. Its molecular mass is 330 kDa. The enzyme activity is not activated by any cations but inhibited by 1 mM $CuCl_2$, $ZnCl_2$, and $MgCl_2$ about 90%, 55%, and 43%, respectively. This enzyme is thermo-instable: the enzyme loses 75% of its initial activity during incubation at 45°C for 10 min. The sodium-stimulated β-galactosidase has been isolated from *B. longum subsp. longum* CCRC 15708 [22]. This enzyme has the molecular mass of 357 kDa as a native form, and its activity is inhibited by lactose in a competitive manner but not by galactose. There are no information about subunit structures in both studies.

The *lacZ* gene encoding β-galactosidase has been characterized in *B. longum* subsp. *longum* MB219 [21]. Transformation with a *lacZ*-carrying plasmid can make some strains that do not grow on lactose use this sugar as a carbon source. The genome sequence of *B. longum* subsp. *longum* [7, 8] clearly shows that there are three genes, *lacZ*, *lacA1*, and *lacA2*, for β-galactosidases whose deduced molecular masses are 114, 77.4, and 78.9 kDa, respectively. It is recently revealed that the *lacZ* gene is inducible in *B. longum* subsp. *longum* NCC2705 by lactose, maltose, and fructo-oligosaccharide [29]. On the other hand, the *lacA2* gene and genes coding for an ABC sugar permease transporter within the same gene cluster are induced when *B. longum* subsp. *longum* LMG 13197 is grown in the presence of galacto-oligosaccharide [31]. β-Galactosidase activity is increased about 4-fold when *B. longum* subsp. *longum* JCM 7052 is grown on gum arabic [25]. In this study we identified the gum arabic-inducible β-galactosidase was the product of the *lacA1* gene, which was classified as GH42 family. This enzyme could not hydrolyze lactose and gum arabic, though the whole cell of *B. longum* subsp. *longum* JCM 7052 was capable of hydrolyzing gum arabic [25]. Galacto-oligosaccharides produced by hydrolyzing gum arabic would be imported into the cell. If one of physiological roles of the purified β-galactosidase would be to hydrolyze the galacto-oligosaccharides inside the cell, the β-galactosidase may have specificity for β-1,3- or β-1,6-linked galactosides because gum arabic consists of a β-1,3-linked galactose backbone with branches of β-1,6-linked galactose chains.

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