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β-Galactosidase with transgalactosylation activity from *Lactobacillus fermentum* K4

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ABSTRACT

The LacLM β-galactosidase of *Lactobacillus fermentum* K4 is encoded by 2 consecutive genes, *lacL* (large subunit) and *lacM* (small subunit), that share 17 overlapping nucleotides. Phylogenetic analysis revealed that this enzyme was closely related to other *Lactobacillus* β-galactosidases and provided significant insight into its common and distinct characteristics. We cloned both the *lacL* and *lacM* genes of *L. fermentum* K4 and heterologously expressed each in *Escherichia coli*, although the recombinant enzyme was only functional when both were expressed on the same plasmid. We evaluated the enzymatic properties of this species-specific LacLM β-galactosidase and discovered that it acts as both a hydrolase, bioconverting lactose into glucose and galactose, and a transgalactosylase, generating prebiotic galacto-oligosaccharides (GOS). The recombinant β-galactosidase showed a broad pH optimum and stability around neutral pH. The optimal temperature and Michaelis constant (K_m) for the substrates *o*-nitrophenyl-β-D-galactopyranoside and lactose were, respectively, 40°C and 45 to 50°C and 1.31 m*M* and 27 m*M*. The enzyme activity was stimulated by some cations such as Na^+, K^+, and Mg^{2+}. In addition, activity was also enhanced by ethanol (15%, wt/vol). The transgalactosylation activity of *L. fermentum* K4 β-galactosidase effectively and rapidly generated GOS, up to 37% of the total sugars from the reaction. Collectively, our results suggested that the β-galactosidase from *L. fermentum* K4 could be exploited for the formation of GOS.

Key words: β-galactosidase, *Lactobacillus fermentum*, lactose, galacto-oligosaccharides

INTRODUCTION

The carbohydrate-active enzymes (CAZymes) are divided among 5 functional classes: glycoside hydrolases (**GH**), glycosyltransferases, polysaccharide lyases, carbohydrate esterases, and carbohydrate-binding modules (Cantarel et al., 2009). The β-galactosidases (**β-gal**, EC 3.2.1.23) belong to 4 different GH families (GH1, GH2, GH35, and GH42; http://www.cazy.org/) and catalyze the hydrolysis and the transgalactosylation of β-D-galactopyranoside substrates such as lactose. β-Galactosidases are widely distributed throughout nature and have been characterized in animals, plants, and microorganisms, including bacteria, fungi, and yeast. The β-gal from *Escherichia coli* has been particularly well described because of the universal application of the lactose operon as a molecular tool. Furthermore, the transgalactosylation activity of β-galactosidases has gained considerable attention for its ability to produce galacto-oligosaccharide (**GOS**) prebiotics (Otieno, 2010; Park and Oh, 2010).

Galacto-oligosaccharides are enymatically produced upon lactose conversion, and they vary in saccharide chain length (between 2 and 8 monomeric units) and the types of linkages between the units. Recently, however, certain invariable characteristics were described. The saccharide chain is composed of a single terminal glucose, galactose monosaccharides, and disaccharides comprising 2 galactose units (Tzortzis and Vulevic, 2009). Industrial processes aimed at producing low-lactose or lactose-free items are concerned with undesirable GOS byproducts, for fear of unknown side effects that may stimulate symptoms of lactose intolerance. However, GOS have demonstrated beneficial effects that are distinct from lactose. The GOS can increase the numbers of *Bifidobacterium* strains and other probiotics (Onishi and Tanaka, 1995; Rabiu et al., 2001; Rastall and Maitin, 2002; Macfarlane et al., 2008) and contribute to metabolic activity of colon microbiota (Knol et al., 2005). As such, GOS have been proposed as an emerging special class of prebiotics and have gained popularity as supplemental components to

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Table 1. Bacterial strains and plasmids included in this study

Strain or plasmid	Characteristic[1]	Source
Strain		
Lactobacillus fermentum K4	Isolated from Chinese traditional dairy products	This work
Escherichia coli Origami B (DE3)	F′ *ompT hsdS*$_B$(r_B′ m_B′) *gal dcm lacY1 ahpC*(DE3) *gor522*:: Tn *10* *trxB* (Kan^r, Tet^r); derived from a *LacZY* mutant of DE3 and carries *trxB/gor* mutations for cytoplasmic disulfide bond formation	Novagen, Germany
Plasmid		
pET-22b(+)	Amp^r, 5.5 kb, C-terminal His-Tag, T7 promoter/lac operator, *pelB* leader	Novagen, Germany
pET Duet-1	Amp^r, 5.4 kb, T7 promoter/lac operator, *ColE1* replicon, two MCS, His-Tag, S-Tag	Novagen, Germany
p22bLM	Amp^r, 8.3 kb, pET 22b(+) derivative with *lacLM* genes inserted before His-Tag	This work
pDuetL	Amp^r, 7.3 kb, pET Duet-1 derivative with *lacL* gene inserted after His-Tag	This work
pDuetLM	Amp^r, 8.2 kb, pET Duet-1 derivative with *lacL* gene inserted after His-Tag and *lacM* gene inserted before S-Tag	This work

[1]Amp^r = ampicillin resistant; Kan^r = kanamycin resistant; Tet^r = tetracycline resistant; MCS = multiple clone site.

infant formula powder, wherein they replicate the oligosaccharide effect of human milk (Torres et al., 2010). It is now believed that combining prebiotic GOS with probiotics in food sources will strongly benefit overall human health.

Lactic acid bacteria (**LAB**) are an established and crucial component of modern dairy processing and the food industry. The most common species applied are from the genera *Lactobacillus*, *Lactococcus*, *Bifidobacterium*, and *Streptococcus*. *Lactobacillus fermentum* is a heterofermentative LAB that acts within a broad range of environmental niches, including dairy, meat, cereal, and vegetable fermentations, and even in the human gastrointestinal tract (Walter, 2008). The probiotic properties of some *L. fermentum* strains have been described, such as that of the ME-3 strain, which is also considered to elicit a prebiotic effect (Calderon Santoyo et al., 2003; Songisepp et al., 2004, 2005; Mikelsaar and Zilmer, 2009).

In recent years, whole-genome sequencing studies of LAB model strains have provided significant insights into the molecular mechanisms by which these bacteria affect biological processes. The principal objective of this study was to investigate the transgalactosylation properties of β-gal from *L. fermentum* K4. To this end, the LacLM β-gal was heterologously expressed and the recombinant protein purified. The amino acid sequences of LacLM and putative active sites were analyzed, and homology with other GH2 β-gal from various LAB strains was investigated. Our results indicated that the β-gal from *L. fermentum* K4 could be used to yield GOS.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Lactobacillus fermentum strain K4 (16S rDNA GenBank accession no. EU621851; Table 1) was grown anaerobically at 37°C in standard Lactobacillus de Man, Rogosa, and Sharpe broth (Difco, Detroit, MI) containing 2% lactose (wt/vol). *Escherichia coli* Origami B (DE3) (Table 1) was grown at 37°C under aeration in Luria-Bertani broth, supplemented with 100 μg/mL ampicillin and 30 μg/mL kanamycin for plasmid maintenance.

Gene Cloning and Vector Construction

Chromosomal DNA was extracted from *L. fermentum* K4 using the TIANamp bacteria genomic DNA extraction kit (Tiangen, Beijing, China). Amplification primers for the *lacL* and *lacM* genes encoding β-gal were designed according to the complete genome sequences of *L. fermentum* IFO 3956 (GenBank accession no. AP008937) and *L. fermentum* CECT 5716 (GenBank accession no. CP002033; Table 2). Amplification of the *lacLM* genes using Lf22b-F and Lf22b-R primers resulted in introduction of (5′) *Nco*I and (3′) *Xho*I restriction enzyme recognition sites, respectively. Likewise, amplification of the large subunit (*lacL*) gene using LfDuetL-F and LfDuetL-R primers introduced (5′) *Bam*HI and (3′) *Pst*I sites, and amplification of the small subunit (*lacM*) gene using LfDuetM-F and LfDuetM-R primers introduced (5′) *Nde*I and (3′) *Bgl*II sites.

Expression vectors pETDuet-1 and pET-22b(+) (Novagen, Darmstadt, Germany) were restructured with digested PCR products of *lacL* and *lacLM* genes, respectively, to generate pDuetL and p22bLM. Subsequently, pDuetL was used to construct the pDuetLM plasmid containing the complete *lacLM* genes. The restructured plasmids (Table 1) were confirmed by restriction enzyme digestion and sequencing.

Expression and Purification

The recombinant plasmids p22bLM, pDuetL, and pDuetLM were transformed into *E. coli* Origami B

Table 2. Sequences of the primers used in this study

Primer	Target fragment	Annealing temperature (°C)	Size (bp)	Restriction enzyme	Sequence[1] (5′ to 3′)
Lf22b-F	*lacLM*	61	2,838	*Nco*I	GCACCATGGAAGCAGAGCTGAAATG
Lf22b-R				*Xho*I	TAGCTCGAGGTTAAGCTCGGGCAC
LfDuetL-F	*lacL*	60	1,887	*Bam*HI	GGTGGATCCTATGGAAGCAGAGCTGA
LfDuetL-R				*Pst*I	GCGCTGCAGTTTGTGTAATCCATAGT
LfDuetM-F	*lacM*	58	975	*Nde*I	GCTCATATGGATTACACAAATAAGCTG
LfDuetM-R				*Bgl*II	TTGAGATCTGTTAAGCTCGGGCAC

[1]Restriction enzyme sites are underlined.

(DE3) for expression. The transformants were grown at 37°C in antibiotic-supplemented Luria Bertani medium with shaking until an optical density of 0.5 at 600 nm was reached. Isopropyl-β-D-thiogalactoside (**IPTG**, 1 m*M*) was then added to the culture medium and incubation continued at 25°C for 12 h. The induced cells were then harvested by centrifugation at 12,000 × *g* for 10 min at 4°C.

The cell pellet was suspended with 50 m*M* sodium phosphate buffer (pH 6.5) and disrupted by sonication, after which the cell debris was pelleted by centrifugation (16,000 × *g* for 30 min at 4°C). The supernatant was then applied to a His-Trap HP column (GE Healthcare, Uppsala, Sweden) that had been pre-equilibrated with buffer A (20 m*M* sodium phosphate, 0.5 *M* NaCl, 20 m*M* imidazole, pH 7.4). Nonspecific adsorbed materials were removed by washing with buffer B (20 m*M* sodium phosphate, 0.5 *M* NaCl, 40 m*M* imidazole, pH 7.4). The recombinant β-gal was eluted with elution buffer (20 m*M* sodium phosphate, 0.5 *M* NaCl, 500 m*M* imidazole, pH 7.4). The active fractions were desalted and collected by ultrafiltration with Amicon Ultra-4 (Millipore, Billerica, MA). The concentration of protein was determined by the Bradford method using BSA as standard (Bradford, 1976). The expression level and purity of recombinant β-gal were evaluated by resolution by 12% SDS-PAGE and compared with a protein molecular weight marker (TaKaRa, Shiga, Japan) after visualization with Coomassie Brilliant Blue staining.

Enzyme Assays

β-Galactosidase activity was determined using *o*-nitrophenyl-β-D-galactopyranoside (***o*NPG**) and lactose as the substrates. The *o*NPG reaction was carried out in 100 μL of 50 m*M* sodium phosphate buffer (pH 6.5) containing 40 μL of 20 m*M* *o*NPG and 10 μL of diluted enzyme solution. After 10 min of incubation at reaction temperature, 100 μL of 1 *M* Na_2CO_3 was added to terminate the reaction. Activity of β-gal was determined by the amount of *o*-nitrophenol (***o*NP**) released, as measured by absorbance at 405 nm on a microplate reader (Bio-Rad Laboratories, Hercules, CA). One unit of *o*NPG activity was defined as the amount of enzyme releasing one micromole of *o*NP per minute under the described conditions.

The lactose substrate reaction was initiated by adding 50 μL of diluted enzyme solution to 150 μL of 50 m*M* sodium phosphate buffer (pH 6.5) with 200 m*M* lactose. After 10 min of incubation at reaction temperature, the reaction was stopped by heating at 100°C for 5 min. Activity of β-gal was determined by measuring the amount of D-glucose released using a commercially available glucose oxidase kit (Biosino, Beijing, China) and reading absorbance at 490 nm. One unit of lactase activity was defined as the amount of enzyme releasing one micromole of D-glucose per minute under the given conditions.

Characterization of the Recombinant β-gal Enzyme

pH and Temperature Dependence of Activity and Stability. Both *o*NPG and lactose assays were variably performed so as to determine the optimum pH and temperature of the respective enzyme activity. The optimum pH was determined for the range of pH from 2.5 to 11.0 by using 50 m*M* McIlvaine buffer (pH 2.5–5.5), 50 m*M* sodium phosphate buffer (pH 5.5–8.0), or 50 m*M* glycine-NaOH buffer (pH 8.5–11.0). The optimum temperature was determined by measuring the respective enzyme activity over a range from 20 to 70°C (Juajun et al., 2011). All other assay conditions remained unchanged.

The release of *o*NP from *o*NPG was measured to determine pH and thermal stability. For determination of pH stability, the enzyme samples were diluted with buffers of various pH values and incubated at 4°C for 3 d. Temperature stability was determined by incubating at various temperatures in a range from 4 to 55°C for more than 120 min. The samples were separated at the desired time intervals, and the residual activity was measured under standard assay conditions.

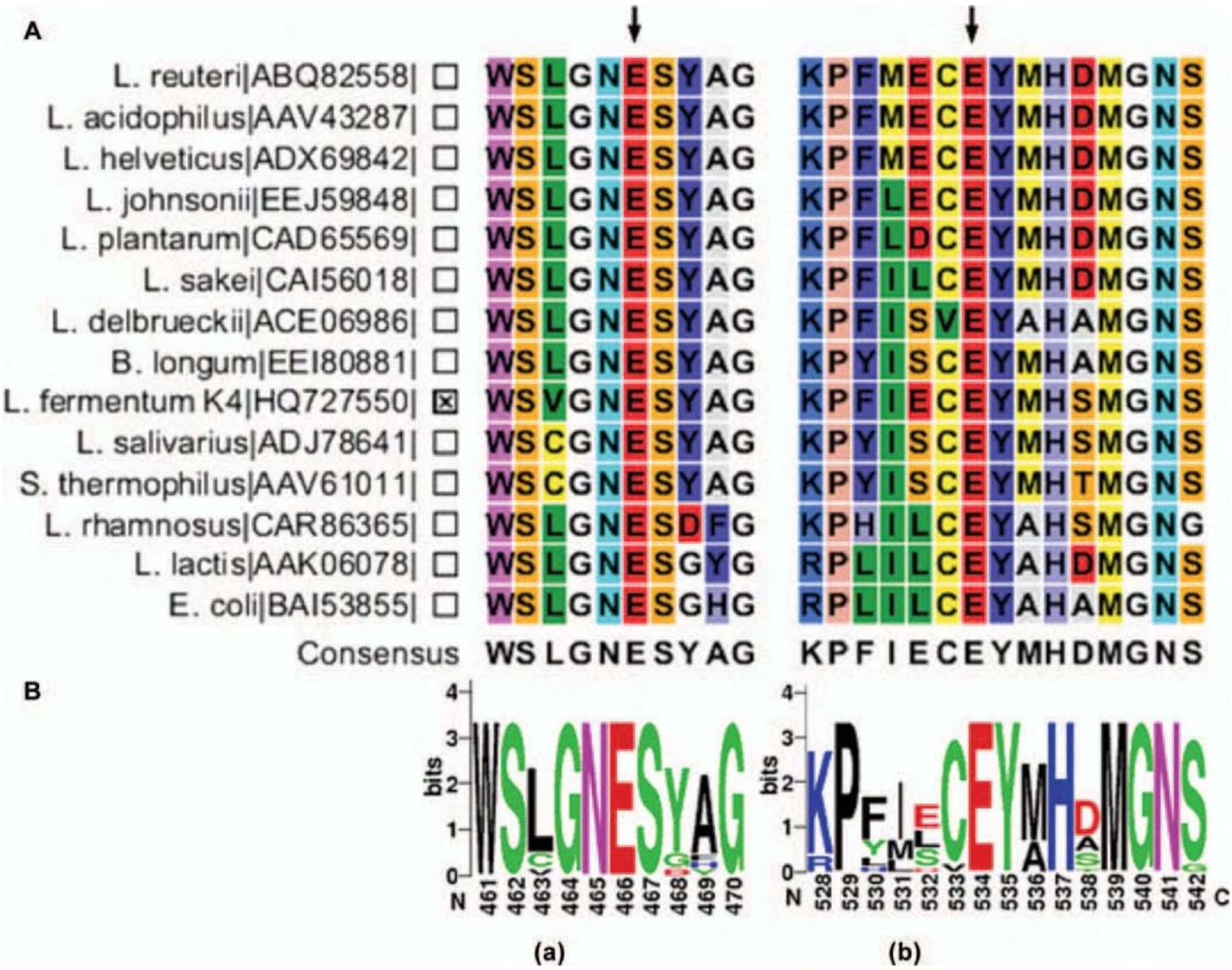

Figure 1. Multiple alignments of the possible active sites (A) and conservative frequency (B) of β-galactosidases of different species (genera: L. = *Lactobacillus* spp., B. = *Bifidobacterium* spp., S. = *Streptococcus* spp., and E. = *Escherichia*). GenBank accession numbers follow the species names. Conserved catalytic amino acids proposed to be the key residues in the active sites are indicated with black arrows. Color version available in the online PDF.

Determination of Kinetic Parameters. Kinetic parameters were evaluated by performing the *o*NPG and lactose assays at 30°C using 50 m*M* sodium phosphate buffer (pH 6.5) with substrate concentrations ranging from 0.5 to 22 m*M* for *o*NPG and from 1 to 600 m*M* for lactose (Nguyen et al., 2006).

Effect of Various Cations and Reagents. To study the effect of various cations and reagents on the activity of β-gal, the enzyme samples were assayed with aqueous solution containing 20 m*M* *o*NPG at the optimum temperature for 10 min in the presence of various cations and reagents added at a final concentration of 5 m*M*, or at 15% (vol/vol) for ethanol and glycerol. The measured activities were compared with the activity of the enzyme solution under the same conditions but without added cations or reagents.

Formation of GOS

Cell extracts were incubated for 48 h at 45°C in 50 m*M* sodium phosphate buffer (pH 6.5) with either lactose solution (20% or 40%, wt/vol) or milk containing 5% (wt/vol) lactose, respectively. Samples were withdrawn at certain time intervals and immediately heated at 100°C for 5 min to inactivate the enzyme. The compositions of GOS mixtures were analyzed using thin-layer chromatography (**TLC**) and an HPLC system. The TLC was carried out on silica-gel 60 plates

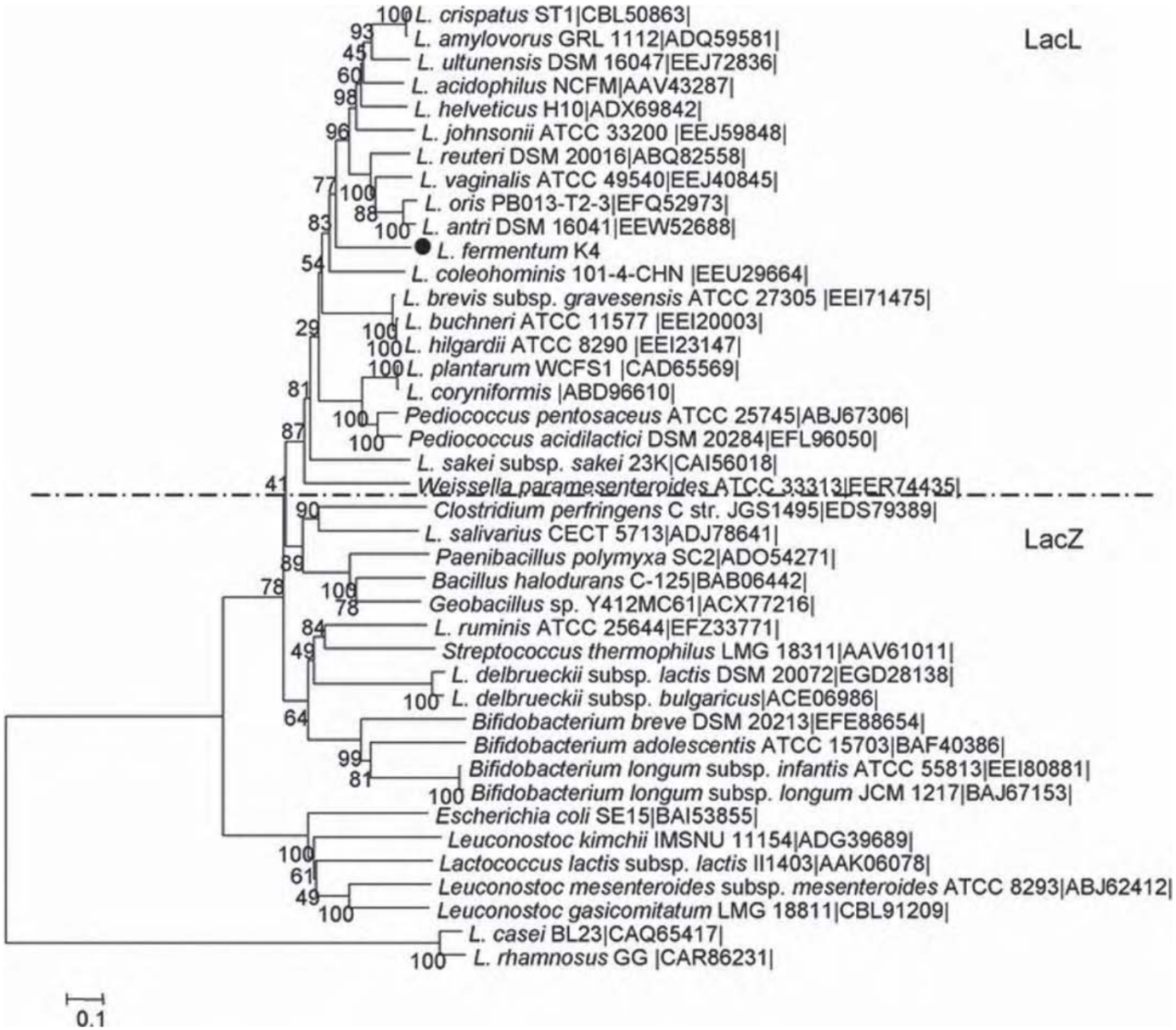

Figure 2. Phylogenetic tree of β-galactosidase from some lactic acid bacteria (where L. = *Lactobacillus*). *Lactobacillus fermentum* K4 is marked with a black circle. GenBank accession numbers follow the species names. The β-galactosidases above the dotted line pertain to the LacLM type, and those below the dotted line pertain to the LacZ type, except the β-galactosidases of glycoside hydrolase group (GH)42 from *Lactobacillus rhamnosus* GG and *Lactobacillus casei* BL23.

(Merck, Darmstadt, Germany) in a solvent composed of *n*-butanol:*n*-propanol:ethanol:water (2:3:3:2, vol/vol/vol/vol), as described previously (Splechtna et al., 2006). For further analysis of GOS, the samples were diluted appropriately, filtered, and injected into the HPLC system on a column of Aminex HPX 87H (Bio-Rad Laboratories) at 50°C using 5 m*M* H_2SO_4 solution as the mobile phase (0.3 mL/min) and refractive index detection. The yield of GOS was calculated by the previously described method (Jørgensen et al., 2001).

Nucleotide Sequence Accession Numbers

The genes of *lacL* and *lacM* were submitted to the GenBank database under accession numbers HQ727550 and HQ727551, respectively.

RESULTS

Sequence Analysis of β-gal from L. fermentum K4

The *L. fermentum* K4 genome sequences of *lacL* and *lacM* share an overlapping region of 17 nucleotides.

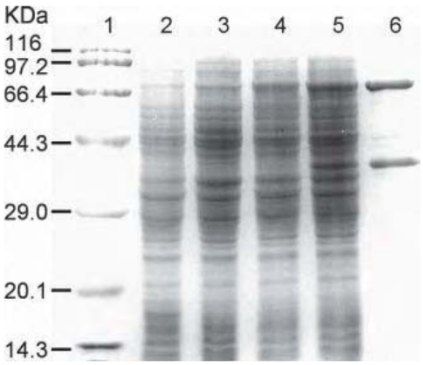

Figure 3. Sodium dodecyl sulfate-PAGE analyses of β-galactosidase (LacLM) from *Lactobacillus fermentum* K4 expression in *Escherichia coli* Origami B (DE3). Lane 1 = protein molecular weight marker; lane 2 = cells of *E. coli* Origami B (DE3); lane 3 = cells of *E. coli* containing p22bLM without induction; lanes 4 and 5 = cells grown with pDuetL (lane 4) and p22bLM (lane 5) for 12 h with 1 m*M* isopropyl-β-D-thiogalactoside induction, respectively; lane 6 = purified protein of β-galactosidase LacLM.

Sequence alignment by the basis local alignment tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed that these 2 genes have 99.81% identity to those published from *L. fermentum* strains IFO 3956 and CECT 5716. Based on the deduced amino acid sequences of the β-gal large subunit LacL and small subunit LacM, the theoretical molecular weights were estimated to be 72.29 and 35.8 kDa, respectively (http://au.expasy.org/tools/pi_tool.html). The *L. fermentum* K4 β-gal resembles the GH2 family members that are classified as LacLM type as opposed to LacZ type (Schwab et al., 2010). The potential active sites in *L. fermentum* K4 LacLM were identified by comparison with those defined for the other major LAB by using the CLC sequence viewer (Figure 1A) and WebLogo (Figure 1B; http://weblogo.berkeley.edu/logo.cgi). The *E. coli* LacZ acid/base and nucleophile regions are located at residues Glu461 and Glu537 (Cupples et al., 1990;, Gebler et al., 1992; Henrissat and Bairoch, 1993; Hung et al., 2001; Matthews, 2005). These regions were located in *L. fermentum* K4 LacLM at Glu466 (Figure 1Ba) and Glu534 (Figure 1Bb) and exhibited remarkably high identity with the corresponding ones from *E. coli* LacZ. However, when the entire AA sequence of *L. fermentum* K4 LacLM was compared with that of *E. coli* LacZ, only 31.66% identity was observed.

Phylogenetic trees were constructed for LacL and LacM of *L. fermentum* K4 using MEGA 5 software (www.megasoftware.net) with the bootstrap method and using all of the putative β-galactosidases discovered thus far in *Lactobacillus* spp. and some of the LAB strains that are prevalent in food manufacturing. Figure 2 shows the LacL phylogenetic tree of *L. fermentum* K4. Both LacL and LacM (data not shown) were most closely related to the β-gal from other *Lactobacillus* spp.

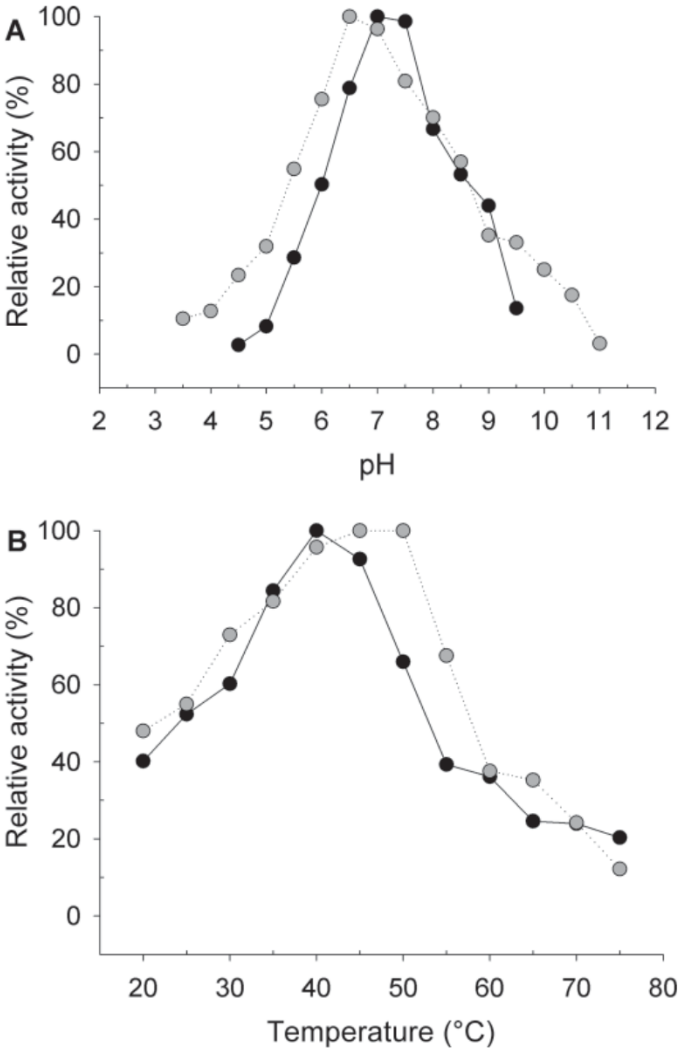

Figure 4. pH (A) and temperature (B) optima of β-galactosidase from *Lactobacillus fermentum* K4 using *o*-nitrophenyl-β-D-galactopyranoside (black circles) and lactose (gray circles) as the substrates, respectively. For all the graphs, the values are the mean of 3 determinations.

Expression of β-gal from L. fermentum in E. coli

The β-gal LacLM from *L. fermentum* K4, which is encoded by the *lacLM* operon, was amplified and cloned into pET-22b(+), resulting in the p22bLM expression vector. To study the detailed characteristics of this strain-specific β-gal, the large subunit gene *lacL* was cloned and expressed as an IPTG-inducible recombinant protein (as described in Materials and Methods). Sodium dodecyl sulfate-PAGE analysis of the β-gal samples at various steps of the expres-

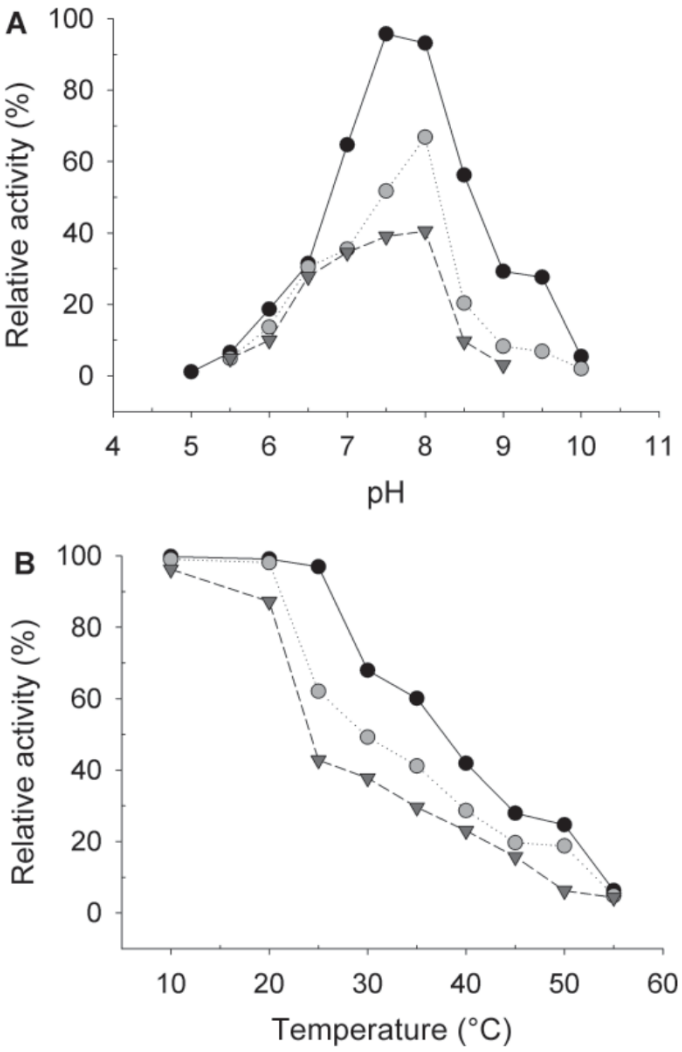

Figure 5. pH (A) and temperature (B) stability of β-galactosidase from *Lactobacillus fermentum* K4. The enzyme was incubated for different periods: A: 24 h (black circles), 48 h (gray circles), 72 h (gray triangles); B: 20 min (black circles), 40 min (gray circles), 100 min (gray triangles). For all graphs, the values are the mean of 3 determinations.

sion and purification process are shown in Figure 3. The purified recombinant LacLM consisted of a large subunit and a small subunit, which were estimated to be approximately ~72 and 35 kDa, respectively (lane 6). The large subunit LacL, which was encoded by the *lacL* gene from vector pDuetL, was expressed (lane 4) with no activity. However, the whole protein LacLM encoded by the recombinant plasmid pDuetLM (and generated by inserting the *lacM* gene before the S-tag of pDuetL) was active.

Properties of β-gal

The optimal pH of β-gal LacLM were determined to be pH 6.5 and 7.0 for lactose and *o*NPG substrates, respectively (Figure 4A). The enzyme exhibited very low activity outside the range of pH from 5.5 to 8.5. Optimum temperature was 40°C for the transgalactosylation activity with *o*NPG (Figure 4B) and 45 to 50°C for lactose hydrolysis. The kinetic parameters K_m (Michaelis constant) and V_{max} (maximum rate) of the purified enzyme were determined to be approximately 1.31 m*M* and 184.4 μmol·min^{-1}·mg^{-1} for *o*NPG, and 27 m*M* and 41 μmol·min^{-1}·mg^{-1} for lactose hydrolysis.

The purified recombinant β-galactosidase protein was determined to be more stable at pH 8.0 after 3 d of incubation at 4°C (Figure 5A), which was distinct from the optimal pH. The protein was also found to retain about 40% of its enzyme activity in neutral pH after incubation for 72 h. Thermal stability was observed in the range of 10 to 20°C (Figure 5B), and 35 to 50% of the maximum activity was retained after incubation in the temperature range for 2 d (data not shown). Incubation at 55°C inactivated the enzyme within 20 min (Figure 5B).

Effect of Various Cations and Reagents

The activity of *L. fermentum* K4 recombinant β-gal was enhanced upon exposure to 15% (wt/vol) ethanol and 5 m*M* Na^+, K^+, and Mg^{2+} (Figure 6). The reagent dithiothreitol had almost no effect on enzymatic activity. The activity was moderately inhibited by glycerol, 2-mercaptoethanol, and urea, and was clearly inhibited by Fe^{2+}, Mn^{2+}, and Zn^{2+}. Glutathione, Cu^{2+}, and Fe^{3+} completely deactivated the enzyme. Thus, the cations K^+ and Mg^{2+} were considered and applied as cofactors to enhance the efficiency of *L. fermentum* β-gal.

Bioconversion of Lactose

Certain microbial β-gal can mediate the transfer of their hydrolyzed galactose products onto lactose to yield GOS (Panesar et al., 2006; Park and Oh, 2010). The β-gal LacLM from *L. fermentum* K4 exhibits such transgalactosylation activities. During lactose conversion, we observed that the transgalactosylation reaction was rapidly initiated, as demonstrated by the formation of GOS in 0.5 h (Figure 7A). The weight of GOS as a percentage of the total sugars in the reaction mixture was determined by HPLC. The value reached a maximum of 37% when the incubation involving 50 m*M* sodium phosphate buffer (pH 6.5) at 45°C with 40% (wt/vol) lactose solution was extended to 9 h. Beyond 9 h, however, hydrolysis prevailed over transgalactosylation, and the total amounts of GOS trended downwards, accompanied by an increase in galactose content (Figure 7B). When a lower lactose content solution was used (as in milk), the amount of bioconverted GOS was less.

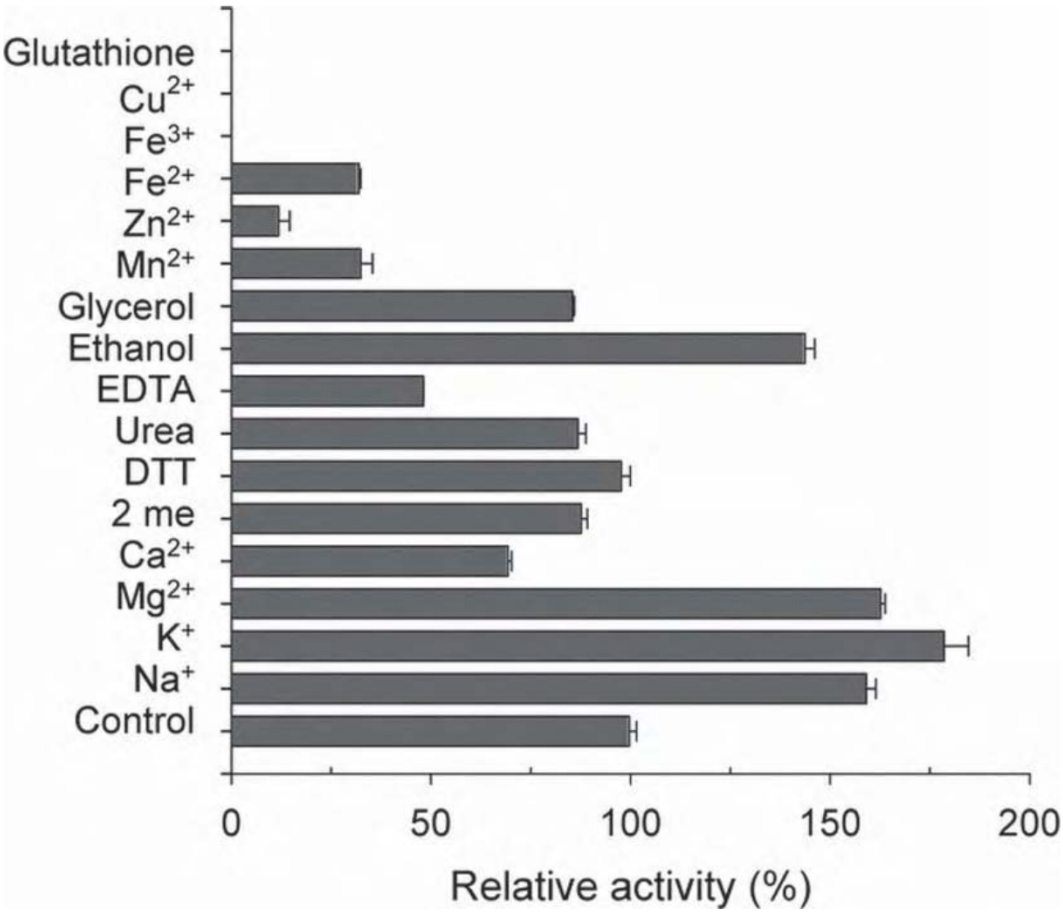

Figure 6. Effect of various cations and reagents on the activity of β-galactosidase from *Lactobacillus fermentum* K4. DTT = dithiothreitol.

Likewise, as the lactose concentration was increased in the reaction solution, more and larger GOS were produced (Figure 7). This result was consistent with that from a previous report (Albayrak and Yang, 2002).

DISCUSSION

The β-gal from *L. fermentum* strain K4 was cloned, expressed, purified, and analyzed to determine its distinctive enzymatic properties and indicate its potential as a manipulable molecular tool for bioconversion of GOS. The recombinant β-gal showed a broad pH optimum and stability around neutral pH (6.5–8.5), preferably utilized lactose between 45 and 50°C, and was quickly inactivated at 55°C. The cations Na^+, K^+, and Mg^{2+} improved enzymatic activity, consistent with findings from previous studies on other LacLM-type β-gal (Nguyen et al., 2006, 2007; Iqbal et al., 2010). The effect of Mn^{2+} was especially noteworthy, because it increases β-gal activity from both *L. fermentum* K4 and *Lactobacillus plantarum* WCFS1 (Iqbal et al., 2010), but inhibits that from *Lactobacillus acidophilus* (Nguyen et al., 2007). Another interesting finding was that ethanol was a stimulator of β-gal LacLM enzyme, a finding yet to be reported with any other of the LAB. This may be a reflection of the relatively broad range of environmental niches in which *L. fermentum* is known to function; it is possible that a symbiotic relationship evolved with other ethanol-producing strains, such as *Saccharomyces cerevisiae* or *Zymomonas mobilis*. In addition, the types and total amounts of GOS that were produced by the *L. fermentum* LacLM were mediated by the concentration of lactose solution, not the temperature or pH.

The β-gal enzyme is known to catalyze the hydrolysis and transglycosylation of its substrates through a double-displacement reaction involving both galactosylation and degalactosylation steps (Brás et al., 2010). The preference for transglycosylation activity can be enhanced by exposure to high concentrations of lactose, as demonstrated by measuring the difference between glucose and galactose products that arise from specific reaction conditions. In our study, we observed that the greatest yield of GOS was achieved when the differ-

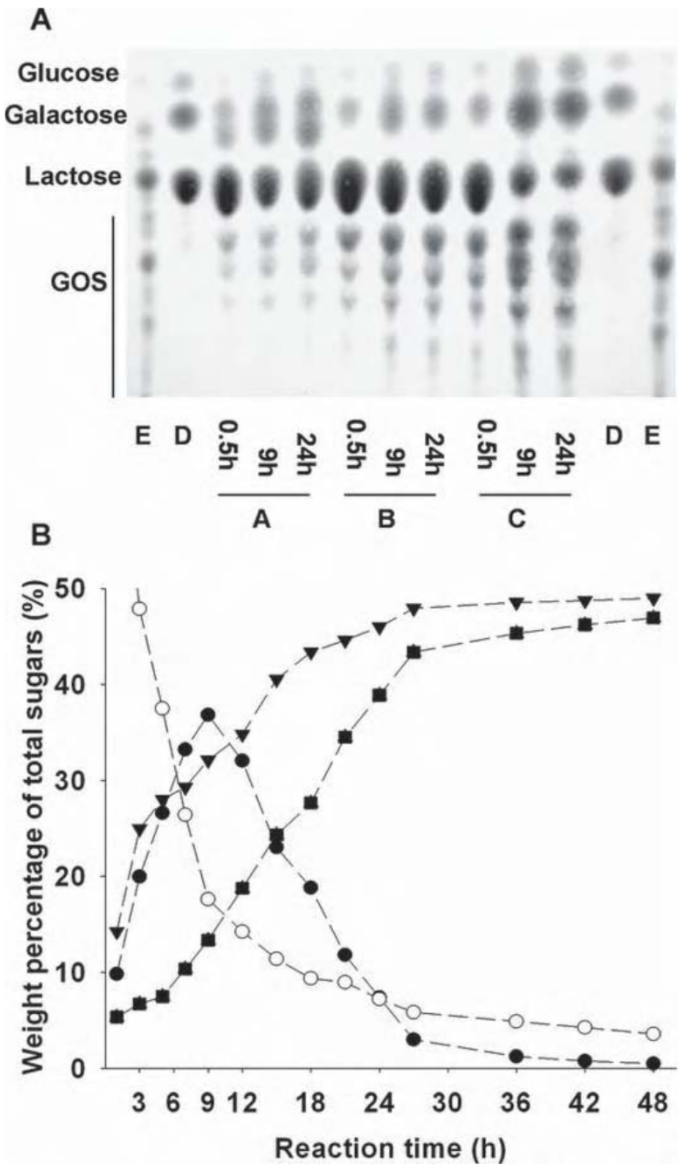

Figure 7. Thin layer chromatography (A) and HPLC (B) analysis of transgalactosylation products. (A) A = milk substrate; B = 20% lactose solution; C = 40% lactose solution; D = standard substance; E = commercial galacto-oligosaccharides (GOS). (B) The enzyme was incubated in 50 m*M* sodium phosphate buffer (pH 6.5) at 45°C with 40% (wt/vol) lactose solution: lactose (○), galactose (■), glucose (▲) and GOS (●). For all graphs, the values are the mean of 3 determinations.

ence value was greatest. Fortunately, the recombinant LacLM was able to bioconvert GOS from milk lactose (which exists at very low concentrations).

As mentioned above, the β-gal from *L. fermentum* K4 is composed of a large subunit (LacL) and a small subunit (LacM) and belongs to the GH2 family of carbohydrate-active enzymes. Most *Lactobacillus* strains contain the LacLM type β-gal, and some LAB that are involved in fermentation (particularly in the food industry) pertain to the LacZ type, such as *Bifidobacterium* spp., *Lactococcus* spp., and *Streptococcus* spp. (Hung et al., 2001; Jørgensen et al., 2001; Hung and Lee, 2002; Lamoureux et al., 2002; Hsu et al., 2007). Phylogenetic analysis revealed that both the large subunit LacL and small subunit LacM of *L. fermentum* K4 β-galactosidase had high homology with most of the β-galactosidase from other *Lactobacillus* spp. It should be noted that the probiotic *Lactobacillus rhamnosus* GG contains the β-gal ebgA (CAR86365) and bgaC (CAR86231), which belong to GH2 and GH42, respectively, which is distinct from the other *Lactobacillus* spp.. Furthermore, β-gal from the genus *Pediococcus* were also represented in the LacLM group. In general, 4 subgeneric groups were generated based on the phylogenies of *Lactobacillus*. *Lactobacillus fermentum* was classified into group B, which contained some *Lactobacillus* spp. strains, such as *Lactobacillus salivarius*, *L. plantarum*, *Lactobacillus reuteri*, *Lactobacillus brevis*, *Lactobacillus antri*, and even *Pediococcus pentosaceus* (Claesson et al., 2008).

Through this study, we were able to confirm that the *L. fermentum* β-gal would be functionally activated in its complete form, including both the small subunit LacM and the large subunit LacL. Because LacL was not active in the absence of LacM, we presumed that LacM might be the unit essentially mediating the activity of LacLM type β-gal. Interestingly, the functional LacM subunit from *L. antri* DSM 16041 β-gal (EEW52689) is only 229 AA long, unlike most of the other *Lactobacillus* β-gal that are approximately 320 AA long. This suggested to us that *L. fermentum* β-galactosidase activity may be manipulable by altering (truncating) the LacM sequence. This strategy, if successful (experiments ongoing in our laboratory), would be a useful method for customizing the enzyme according to specific industrial needs. In addition to the lower lactose concentration, the inhibition of glucose seriously restricted the production of GOS, and we presume that inhibition could be reduced through immobilization of the substrate or structural modification of the enzyme at the product-binding site (Kim et al., 2004; Mateo et al., 2004; Park and Oh, 2010). Thus, the findings from this study will aid future research focused on enhancing the transgalactosylation activity of β-gal and developing more efficient enzymes to generate high yields of GOS.

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