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Purification and Characterization of Two Novel β-Galactosidases from *Lactobacillus reuteri*

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The intracellular β-galactosidase (β-gal) enzymes from two strains of *Lactobacillus reuteri*, L103 and L461, were purified by ammonium sulfate fractionation, hydrophobic interaction, and affinity chromatography. Both enzymes are heterodimers with a molecular mass of 105 kDa, consisting of a 35 kDa subunit and a 72 kDa subunit. Active staining of *L. reuteri* L103 and L461 β-gal with 4-methylumbelliferyl β-D-galactoside showed that the intact enzymes as well as the larger subunits possess β-galactosidase activity. The isoelectric points of *L. reuteri* L461 and L103 β-gal were found to be in the range of 3.8−4.0 and 4.6−4.8, respectively. Both enzymes are most active in the pH range of 6−8; however, they are not stable at pH 8. The *L. reuteri* β-galactosidases are activated by various mono- and divalent cations, including Na^+, K^+, and Mn^{2+}, and are moderately inhibited by their reaction products D-glucose and D-galactose. Because of their origin from beneficial and potentially probiotic lactobacilli, these enzymes could be of interest for the synthesis of prebiotic galacto-oligosaccharides.

KEYWORDS: Lactobacilli; β-galactosidase; lactase; galacto-oligosaccharides; transgalactosylation

INTRODUCTION

Lactobacilli are considered to be one of the most beneficial to human health among the species of bacteria present in the human intestine. Several beneficial functions such as vitamin production, production of digestive enzymes, and stimulation of the immune system have been suggested for the members of this genus as well as for other probiotic strains (*1*−*3*). Many species of *Lactobacillus* (*Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Lactobacillus casei*, *Lactobacillus plantarum*, and *Lactobacillus reuteri*) have been evaluated for clinical effects in humans, including modulation of intestinal flora, lowering fecal enzyme activities, prevention and treatment of antibiotic-associated diarrhoea, and effects on superficial bladder cancer and cervical cancer (*1*, *4*). Because of these proven and assumed positive effects, it is desirable to increase their number in the colon of human hosts.

The concept of prebiotics has been employed in achieving an increased number of beneficial microorganisms in the intestines. A prebiotic is defined as "a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health" (*5*). Certain oligosaccharides, specific types of dietary carbohydrates, are nondigestible for humans and monogastric animals, promote selectively the growth of desirable bacteria in the colon at the expense of less desirable bacteria, and thus are classified as prebiotics (*5*, *6*). The physiological importance and health benefits of nondigestible oligosaccharides have been reported (*1*, *7*−*10*). Because of these benefits, prebiotic oligosaccharides are of great interest for both human and animal nutrition.

To be utilized by the target group of microorganisms, these oligosaccharides have to be cleaved by suitable glycosidases. The enzyme of particular interest in this work is β-galactosidase (β-gal, EC 3.2.1.23). β-Galactosidases catalyze the hydrolysis and transgalactosylation of β-D-galactopyranosides (such as lactose) (*11*−*13*) and are widespread in nature. An attractive biocatalytic application is found in the transgalactosylation

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potential of these enzymes which is based on the catalytic mechanism of β-galactosidases (*12*, *14*). The products of transgalactosylation, galacto-oligosaccharides, are nondigestible carbohydrates which meet the criteria of "prebiotics" and therefore have attracted an increasing amount of attention. β-Galactosidases have been isolated and characterized from many different sources (*12*), yet the information about these important enzymes from probiotic strains, such as the lactobacilli or bifidobacteria, is still sparse.

L. reuteri is a dominant strain of the hetero-fermentative lactobacilli in the gastrointestinal tract (GIT) of humans and animals (*15*−*17*); in fact, it is the only *Lactobacillus* species thought to inhabit the GIT of all vertebrates and mammals (*18*). In this paper, we describe the purification and characterization of two novel β-galactosidases extracted from *L. reuteri* strains L103, originating from calf, and L461, isolated from suckling pigs.

MATERIALS AND METHODS

Chemicals. All chemicals were purchased from Sigma (St. Louis, MO) and were of the highest quality available unless otherwise stated. MRS broth powder (for *Lactobacillus* broth according to De Man, Rogosa, and Sharpe) was obtained from Merck (Darmstadt, Germany), and 1,4-dithiothreitol (DTT) was from Roth (Karlsruhe, Germany). Transgalacto-oligosaccharide powder (TOS) was from Yakult Honsha (Tokyo, Japan), and galacto-oligosaccharides Elix'or (different brand name Vivinal) were from Borculo Domo Ingredients (Zwolle, The Netherlands). Glucose oxidase (GOD) from *Aspergillus niger* (lyophilized, 205 units/mg enzyme preparation) was from Fluka (Buchs, Switzerland). Horseradish peroxidase (POD) (lyophilized, 210 units/mg) and the test kit for the determination of the amount of lactose were from Boehringer (Mannheim, Germany).

Strains and Culture Conditions for Screening. Fourteen strains of *Lactobacillus* spp. were obtained from Lactosan (Starterkulturen GmbH & Co. KG). The selected strains, with which most of the work was performed, were isolated from calf (isolate L103) and suckling pig (isolate L461). The strains were stored in sterile vials at −70 °C in MRS broth medium containing glycerol (15%, v/v) and activated by three successive transfers every 24 h in MRS broth medium. They were grown on MRS broth medium (10 g/L peptone, 2 g/L dipotassium hydrogen phosphate, 8 g/L meat extract, 2 g/L diammonium hydrogen citrate, 4 g/L yeast extract, 5 g/L sodium acetate, 0.2 g/L magnesium sulfate, 1 g/L Tween 80, and 0.04 g/L manganese sulfate), in which glucose, lactose, or galacto-oligosaccharides served as the carbon source (1 or 2%, w/v), and incubated anaerobically at 37 °C for 24 h. The optical densities (OD_{600}) of the cultures were measured with a microplate reader (GENious, Tecan, Maennedorf, Switzerland) with the readings being taken every 20 min for 24 h. To screen for β-galactosidase activity, cells were harvested from a liquid culture by centrifugation (10 000 rpm for 10 min at 4 °C) using an Eppendorf centrifuge and resuspended in 50 mM sodium phosphate buffer (pH 6.8) containing 20% (w/v) glycerol and 1 mM dithiothreitol (buffer P) (*19*). The cells were disrupted by ultrasonication, and debris was removed by centrifugation (10 000 rpm for 15 min at 4 °C) to obtain the cell-free extract.

Fermentation of the Selected *Lactobacillus* Strains. The strains were grown anaerobically in MRS broth medium containing 1% (w/v) lactose in a 50 L laboratory fermentor (strain *L. reuteri* L461) and in a 220 L industrial fermentor (strain *L. reuteri* L103) at 37 °C and with an initial pH of 6.5. Precultures were grown overnight in MRS broth medium [1% (w/v) lactose] and inoculated into the fermentors to a final concentration of 0.5% inoculum. The pH was allowed to drop to 5.5 and was then regulated at this value.

Enzyme Purification. Approximately 50 g of biomass (wet weight) was suspended in 100 mL of buffer P. The cells were disrupted by using a French press (Aminco), and debris was removed by centrifugation (25000*g* for 15 min at 4 °C) to obtain the crude extract.

Ammonium Sulfate Precipitation. Ammonium sulfate was slowly added to crude extract to 60% saturation. The precipitate was obtained by centrifugation as described above. The supernatant was discarded; the pellet was dissolved in 50 mM sodium phosphate buffer containing 1 M ammonium sulfate (buffer A).

Hydrophobic Interaction Chromatography (HIC). The dissolved pellet (200−400 mg of protein) was applied to a 400 mL phenyl-Sepharose fast flow column (50 mm × 200 mm; Amersham, Uppsala, Sweden) that was pre-equilibrated with buffer A. β-Galactosidase was eluted at a rate of 15 mL/min with a 4000 mL linear gradient from 0 to 100% buffer B (50 mM sodium phosphate). The active fractions were pooled and concentrated by ultrafiltration with a membrane with a 10 kDa cutoff (Amicon, Beverly, MA).

Affinity Chromatography. The concentrated enzyme solution (≈40 mg of protein) was loaded onto the affinity column (10 mL of *p*-aminobenzyl 1-thio-β-D-galactopyranoside immobilized on cross-linked 4% beaded agarose; Sigma) that was pre-equilibrated with 50 mM sodium phosphate buffer (buffer B). The enzyme was eluted at a rate of 0.5 mL/min by using a linear 0 to 1 M NaCl gradient in 10 column volumes. The active fractions were pooled, desalted, and concentrated.

The pH values of the buffers used during purification of the β-galactosidases from L103 and L461 (buffer A and buffer B) were 6.0 and 6.5, respectively. The purified enzymes were stored in 50 mM sodium phosphate buffer (pH 6.5) (L461) and 50 mM sodium phosphate buffer (pH 6.0) containing 1 mM DTT (L103) at 4 °C.

Protein Determination. The protein concentration was determined by the method of Bradford (*20*) using bovine serum albumin as a standard.

Enzyme Assays. β-Galactosidase activity was determined using *o*-nitrophenyl β-D-galactopyranoside (*o*NPG) and lactose as the substrates.

Assay with Chromogenic Glycoside. When chromogenic *o*NPG was used as the substrate, the determination of β-galactosidase activity was carried out at 30 °C with 22 mM *o*NPG in 50 mM sodium phosphate buffer (pH 6.5). The reaction was initiated by adding 20 μL of enzyme solution to 480 μL of the substrate solution, and then the mixture was incubated for 10 min using an Eppendorf (Hamburg, Germany) thermomixer compact. Agitation was carried out at 600 rpm. After the incubation time, the reaction was stopped by adding 750 μL of 0.4 M Na_2CO_3. The release of *o*-nitrophenol (*o*NP) was assessed by determining the absorbance at 420 nm. One unit of *o*NPG activity was defined as the amount of enzyme releasing 1 μmol of *o*NP per minute under the described conditions.

Assay with Lactose. When lactose was used as the substrate, 20 μL of an enzyme solution was added to 480 μL of a 600 mM lactose solution in 50 mM sodium phosphate buffer (pH 6.5). The reaction mixture was incubated at 30 °C using an Eppendorf thermomixer compact (600 rpm). After 10 min, the reaction was stopped by heating the reaction mixture at 99 °C for 5 min. After the sample had been cooled to room temperature, the release of D-glucose was assessed colorimetrically using the GOD/POD assay (*21*) by adding 60 μL of this reaction mixture to 600 μL of a solution containing GOD (94 μg/mL), POD (6.1 μg/mL), 4-aminoantipyrine (157 μg/mL), and phenol (1.95%, v/v) in 0.1 potassium phosphate buffer (pH 7.0). This assay mixture (660 μL) was incubated in the dark at room temperature for 40 min, and the absorbance at 546 nm was measured. One unit of lactase activity was defined as the amount of enzyme releasing 1 μmol of D-glucose per minute under the given conditions.

All measurements and experiments were performed at least in duplicate, and the experimental error never exceeded 5%.

Determination of Molecular Masses. *Electrophoretic Analysis and Active Staining*. Native polyacrylamide gel electrophoresis (PAGE) and denaturing sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) were performed on a PhastSystem unit (Amersham) using precast polyacrylamide gels (PhastGel 8-25). For SDS−PAGE, the enzyme was preincubated with SDS buffer [47 mM Tris-HCl (pH 6.8) containing 34 mg/mL SDS, 0.1 mg/mL bromophenol blue, 5% (v/v) mercaptoethanol, and 15% (v/v) glycerol] at 60 °C for 5 min. Coomassie blue staining was used for the visualization of the protein bands. Active staining for the visualization of the bands with β-galactosidase activity was carried out by applying filter paper soaked with the staining solution [50 mM sodium phosphate buffer (pH 6.5) and

Table 1. β-Galactosidase Activity of *Lactobacillus* Strains Grown on Galacto-Oligosaccharides

		growth on GOS[a]		β-galactosidase activity[b]	
strain	species	Elix'or (1%, w/v)	TOS (1%, w/v)	activity (units/mL)	specific activity (units/mg of protein)
L41	*L. reuteri*	+	+	4.44	10.10
L103	*L. reuteri*	+++	++	3.29	21.91
L104	*L. reuteri*	+++	+++	3.91	8.70
L264	not yet identified	+	+++	5.08	10.17
L281	*L. reuteri*	–	–	1.42	3.38
L305	*L. reuteri*	++	++	0.33[c]	6.55
L457	*L. reuteri*	++	++	2.66	5.91
L461	*L. reuteri*	+++	++	2.65	11.51
L655	not yet identified	+++	+++	1.39	4.64
L657	*Lactobacillus amylovorus*	++	+	1.24	2.14
L662	not yet identified	+++	++	5.01	4.96
L665	*L. amylovorus*	++	++	1.85	2.20
L674	*L. amylovorus*	–	–	0.43	1.08
L722	*Lactobacillus brevis*	+++	++	0.96	5.34

[a] The ability of these strains to grow on GOS at 37 °C after 24 h was evaluated as no growth (–), slow growth (+), moderately good growth (++), and good growth (+++). [b] Intracellular β-galactosidase activity shown here was measured from cells harvested from liquid medium with Elix'or (1%, w/v) as a carbon source after 24 h. Enzyme activity was measured at 30 °C with *o*NPG as the substrate under standard assay conditions and is expressed as units per milliliter of crude enzyme extract. [c] Intracellular β-galactosidase activity shown was measured from cells harvested from liquid medium with Elix'or (2%, w/v) as a carbon source after 24 h.

3.5 mg/mL 4-methylumbelliferyl β-D-galactoside] onto the gel and incubation at 37 °C for 30 min. After application of 1 M carbonate−bicarbonate buffer (pH 10.0) onto the gel using a filter paper, the protein bands displaying enzyme activity were visualized under UV light, thus detecting the release of 4-methylumbelliferone (*22*).

MALDI Mass Spectrometry. Selected protein bands from SDS−PAGE and native PAGE were digested with trypsin as described elsewhere (*23*, *24*). The resulting peptides were analyzed by MALDI Q-TOF MS on a Q-TOF (quadrupole-time-of-flight) Ultima Global (Waters Micromass) in positive ion mode laser desorption ionization (LDI+ mode). Spectra were analyzed using MassLynx version 4.0 (Waters Micromass).

Determination of the Isoelectric Point. Isoelectric focusing in the range of pH 3−10 was performed using the Multiphor System (Amersham) and precast, dry polyacrylamide gels (CleanGel IEF, Amersham) rehydrated with carrier ampholytes. The rehydration solution contained 100 μL of Pharmalyte at pH 2.5−5 (Amersham), 290 μL of Ampholine at pH 3−10 (Fluka, Buchs, Switzerland), and 10% (w/v) sorbitol in 5.2 mL of water as recommended by the supplier. Enzyme samples were desalted by using ultrafree-MC centrifugal filter units (Amicon). Both the low and the broad pI marker protein kit [pH 3.6−6.6 (Sigma) and pH 3−10 (Amersham), respectively] were used to determine pI values. Protein bands were Coomassie stained following the instructions of the manufacturer.

Steady-State Kinetic Measurements. All steady-state kinetic measurements were obtained at 30 °C using *o*-nitrophenyl β-D-galactopyranoside (*o*NPG) and lactose as the substrates in 50 mM sodium phosphate buffer (pH 6.5) with concentrations ranging from 0.5 to 22 mM for *o*NPG and from 1 to 600 mM for lactose. The inhibition of *o*NPG hydrolysis by D-galactose and D-glucose as well as that of lactose hydrolysis by D-galactose was investigated. The kinetic parameters and inhibition constants were calculated by nonlinear regression, and the observed data were fit to the Henri−Michaelis−Menten equation (SigmaPlot, SPSS Inc., Chicago, IL).

pH and Temperature Dependence of Activity and Stability. The pH dependence of the enzymatic release of *o*-nitrophenol (*o*NP) from *o*NPG and D-glucose from lactose was measured between pH 4 and 9 using sodium citrate (50 mM, pH 4.0−5.5), sodium phosphate (50 mM, pH 6.0−7.5), and borate (50 mM, pH 8.0−9.0) buffers. The activity was determined at different pH values under standard assay conditions. To determine the pH stability of L103 and L461 β-gal, the enzyme samples were incubated at various pHs and 37 °C for up to 20 h, and the remaining enzyme activity was measured at time intervals using *o*NPG as the substrate under standard assay conditions. The temperature dependence of enzyme activity (both *o*NPG activity and lactase activity) was measured by assaying the enzyme samples over the range of 20−60 °C for 10 min. The temperature stability of both enzymes was studied by incubating the enzyme samples in 50 mM sodium phosphate buffer at pH 6.0 for L103 β-gal and pH 6.5 for L461 β-gal, at various temperatures (4, 20, 30, 37, and 45 °C). At certain time intervals, samples were withdrawn and the residual activity was measured with *o*NPG as the substrate under standard assay conditions.

Effects of Various Reagents and Cations. The enzyme samples were assayed with 22 mM *o*NPG solution [in 50 mM sodium phosphate buffer (pH 6.5)] in the presence of 1 and 10 mM mercaptoethanol, EDTA, DTT, and urea, individually, at 30 °C for 10 min. Enzyme activity measured without added reagents was used as a control. To study the effect of various cations on the release of *o*NP from *o*NPG, the enzyme samples were assayed with 22 mM *o*NPG [in 10 mM Bis-Tris (pH 6.5)] in the presence of various cations with final concentrations of 1.0, 10, and 100 mM (chloride or sulfate form), at 30 °C for 10 min. The measured activities were compared with the activity of the enzyme solution without added cations under the same conditions.

RESULTS

Screening of *Lactobacillus* Strains. Fourteen *Lactobacillus* strains were screened for the ability to grow on galacto-oligosaccharides and for β-galactosidase activity. The ability of these strains to grow on galacto-oligosaccharides (GOS) was judged on the basis of the optical densities of the cultures (OD_{600}) measured during the course of their growth. Two strains of *L. reuteri*, isolates L103 and L461, were selected for further studies since these two strains grow very well on both galacto-oligosaccharides that were tested (TOS and Elix'or) and showed significantly higher specific β-galactosidase activities than the other strains (**Table 1**).

Fermentation of Selected *L. reuteri* Strains L103 and L461. Batch fermentations of the two selected *L. reuteri* strains, L461 and L103, were carried out in a 50 L laboratory fermentor and a 220 L industrial fermentor, respectively, using lactose-based MRS medium, to produce sufficient biomass for the subsequent enzyme purification and characterization.

Figure 1 shows the time course of the cultivation of *L. reuteri* L103. As is obvious from this figure, the formation of β-gal activity is associated with growth of the organism and only starts after a long lag phase, even though the same lactose-based medium was used for the preculture. The biomass was harvested after 22 h when lactose was almost depleted and the volumetric β-gal activity reached its maximum. Intracellular β-gal activity rapidly decreased when lactose was completely exhausted from the medium (data not shown). In the case of *L. reuteri* L461, the biomass was harvested after fermentation for 14 h (data not shown).

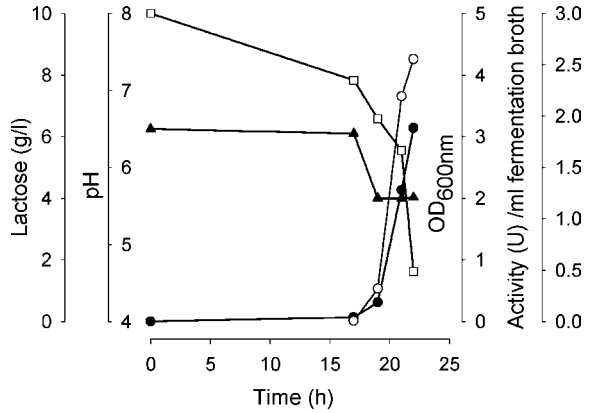

Figure 1. Time course of an industrial fermentation of *L. reuteri* strain L103 in a 220 L stirred tank reactor using lactose-based MRS medium: pH (▲), lactose concentration (grams per liter) (□), OD_{600} (●), and β-galactosidase activity (units) per liter of fermentation broth (○).

The biomass yield and β-galactosidase activity obtained in the fermentor cultivation were 6 g (wet biomass) and 350 units of β-galactosidase activity per liter of fermentation broth for strain *L. reuteri* L461 and 9 g (wet biomass) and 2500 units of β-galactosidase activity per liter for strain *L. reuteri* L103.

Purification of β-Galactosidases from *L. reuteri* L103 and L461. β-Galactosidase was isolated both from the cell extracts of *L. reuteri* L103 and L461 using a purification protocol based on ammonium sulfate precipitation, hydrophobic interaction chromatography, and affinity chromatography on *p*-aminobenzyl thiogalactoside agarose. The results of representative purification procedures for both β-galactosidases are summarized in **Table 2**. The two enzymes were purified approximately 16- and 50-fold from the crude cell extracts with an overall yield of 7 and 12% for L103 β-gal and L461 β-gal, respectively, and specific activities of 158 and 180 units/mg of protein, respectively, using standard assay conditions with *o*NPG as the substrate. The three-step purification procedure yielded homogeneous β-galactosidase from both sources as judged by SDS−PAGE (**Figure 2**).

Properties of β-Galactosidases from *L. reuteri* L103 and L461. *(i) Molecular Mass.* As judged by SDS−PAGE (**Figure 2A**) and native PAGE (**Figure 2B**), both L103 and L461 β-galactosidases are heterodimers of approximately 105 kDa consisting of a large subunit of 72 kDa and a smaller one of 35 kDa.

Several bands were found in native PAGE with the largest band corresponding to approximately 105 kDa. To investigate the nature of the smaller bands, peptide mapping and MALDI Q-TOF MS analysis were performed (data not shown). This analysis revealed that the two bands on SDS−PAGE are different and that each band on native PAGE contains components of both subunits. Hence, the first band of approximately 105 kDa represents the intact dimeric enzyme, and the other bands with lower molecular masses result from degradation of

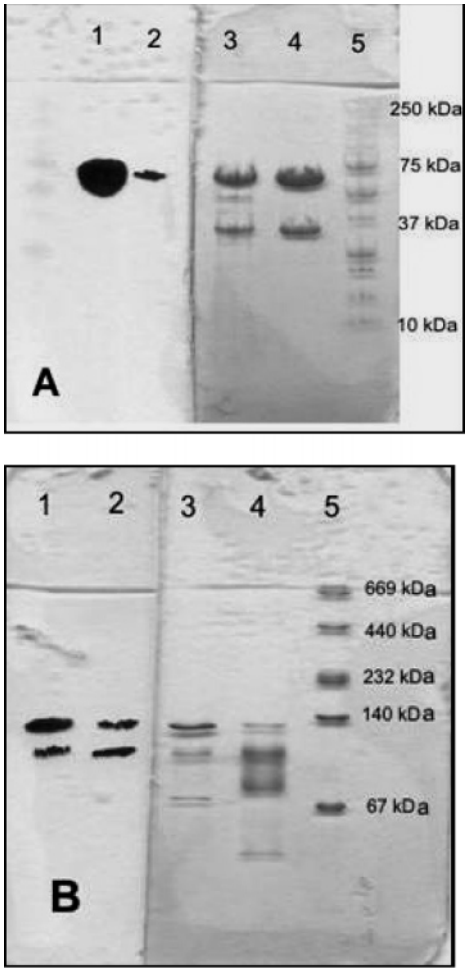

Figure 2. SDS–PAGE (**A**) and native PAGE (**B**) of purified β-galactosidases from *L. reuteri* L103 and L461. (**A**) Lanes 1 and 2 contained active staining with 4-methylumbelliferyl β-D-galactoside of β-galactosidases L461 (lane 1) and L103 (lane 2) and lanes 3–5 Coomassie blue staining of β-galactosidases L461 (lane 3) and L103 (lane 4) and recombinant molecular mass markers (Bio-Rad) (lane 5). (**B**) Lanes 1 and 2 contained active staining with 4-methylumbelliferyl β-D-galactoside of β-galactosidases L461 (lane 1) and L103 (lane 2), lane 3 β-galactosidase L461, lane 4 β-galactosidase L103, and lane 5 molecular mass markers (Amersham).

Table 2. Purification of β-Galactosidases from *L. reuteri* Strains L103 (**A**) and L461 (**B**)

purification step	total activity (units)	total protein (mg)	specific activity (units/mg)	purification factor	recovery (%)
(**A**) L103					
enzyme extract	4700	490	9.6	1.0	100
$(NH_4)_2SO_4$ precipitation	3260	210	15.3	1.6	69
HIC (phenyl-Sepharose)	2100	43	49	5.1	45
affinity chromatography (*p*-aminobenzyl thiogalactoside agarose)	325	2.1	158	16.5	7
(**B**) L461					
enzyme extract	4500	1370	3.3	1.0	100
$(NH_4)_2SO_4$ precipitation	2530	370	6.8	2.1	56
HIC (phenyl-Sepharose)	1130	40	28	8.5	25
affinity chromatography (*p*-aminobenzyl thiogalactoside agarose)	525	2.9	180	54	12

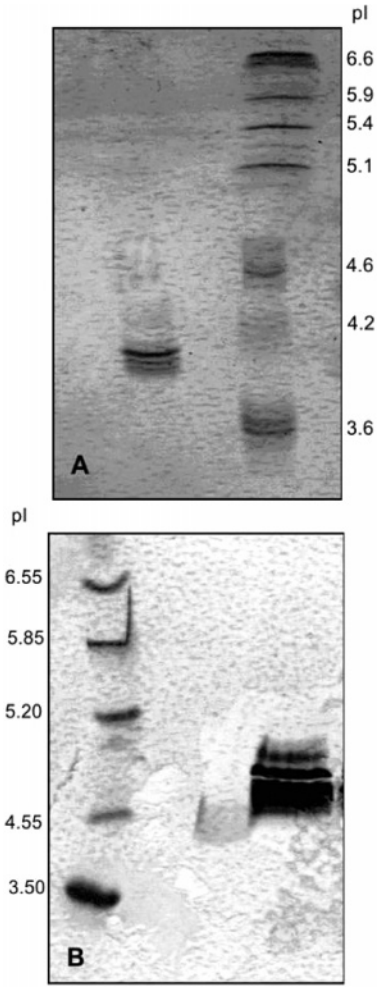

Figure 3. Isoelectric focusing of purified β-galactosidases from *L. reuteri* (**A**) β-gal L461 with the marker protein kit at pH 3.6–6.6 (Sigma) and (**B**) β-gal L103 with the marker protein kit at pH 3–10 (Amersham).

this intact protein. This confirmed that both *L. reuteri* L103 and L461 β-galactosidases are heterodimeric enzymes consisting of a 35 and 72 kDa subunit. Active staining of the purified β-galactosidases of *L. reuteri* L103 and L461 directly on the SDS−PAGE gel after preincubation of the enzymes with denaturing SDS buffer at 60 °C for 5 min (see Materials and Methods) and using 4-methylumbelliferyl β-D-galactoside as the substrate showed that one band corresponding to the larger subunit exhibited activity with this substrate. In contrast, the smaller subunit did not show any activity (**Figure 2A**). The treatment with denaturing SDS buffer at 60 °C resulted in the dissociation of the two subunits, while the native structure of the subunits was apparently maintained. A similar treatment at 98 °C resulted in complete denaturation and loss of activity on the gel. In accordance, active staining on native PAGE yielded two bands with β-galactosidase activity (**Figure 2B**), one band of approximately 105 kDa, corresponding to the intact heterodimer, and a second band representing the degradation product, which contains components of both subunits.

(ii) Isoelectric Point. The isoelectric point of β-galactosidase L461 was determined by isoelectric focusing, and it was found to be in the range of 3.8−4.0 (**Figure 3A**). In contrast, the isoelectric point of β-galactosidase L103 was in the range of 4.6−4.8 (**Figure 3B**). Interestingly, several tightly spaced bands were observed on the gel for both homogeneous β-galactosidase preparations. This could indicate several isoforms of β-galactosidase; however, the nature of the difference between these isoenzymes is not known at present.

Kinetic Parameters. The steady-state kinetic constants and the inhibition constants determined for the hydrolysis of lactose and *o*-nitrophenyl β-D-galactopyranoside (*o*NPG) are summarized in **Table 3**. The β-galactosidase from *L. reuteri* L461 has a higher affinity for *o*NPG than for lactose as indicated by the K_m values. The k_{cat} values were calculated on the basis of theoretical V_{max} values obtained by nonlinear regression using SigmaPlot (SPSS Inc.). The catalytic efficiencies (k_{cat}/K_m) for the two substrates, lactose and *o*NPG, again indicate that *o*NPG is the preferred substrate of β-gal L461, mainly because of the more favorable K_m value.

The end product D-galactose was found to competitively inhibit the hydrolysis of lactose by both enzymes. This inhibition, however, is only moderate as is obvious from the ratio of the Michaelis constant for lactose and the inhibition constant for D-galactose that were calculated for both enzymes (β-gal L461, $K_{i,Gal}/K_{m,Lac} = 2.9$; β-gal L103, $K_{i,Gal}/K_{m,Lac} = 14.5$). D-Galactose was also found to be a competitive inhibitor against *o*NPG with an inhibition constant of 115 mM. On the basis of the ratio of K_i to K_m, this inhibition is even less pronounced ($K_{i,Gal}/K_{m,oNPG} = 117$). *o*NPG was also used as the substrate for studying inhibition by the second end product, D-glucose. Again, glucose is a competitive inhibitor of β-gal, but this inhibiting effect is not pronounced ($K_{i,Glu}/K_{m,oNPG} = 697$). These moderate inhibitory effects of both end products glucose and galactose during *o*NPG hydrolysis catalyzed by β-galactosidase from *L. reuteri* L461 are also shown in **Figure 4**. When using a substrate concentration of 2 mM *o*NPG, the relative β-gal activity was reduced to 45% at a concentration of 200 mM galactose; on the other hand, glucose had an almost negligible inhibitory effect with 95% of the initial activity remaining at a concentration of 170 mM glucose.

Table 3. Kinetic Parameters for β-Galactosidases from *L. reuteri* L103 and L461 for the Hydrolysis of Lactose and *o*-Nitrophenyl β-D-Galactopyranoside (*o*NPG)

substrate	method for determination of enzyme activity	kinetic parameter	β-gal L103	β-gal L461
lactose	release of D-Glc	$v_{max,Glc}$ (μmol min^{-1} mg^{-1})	34	33
		$K_{m,Lac}$ (mM)	13 ± 2	31 ± 5
		k_{cat} (s^{-1})	60	58
		k_{cat}/K_m (mM^{-1} s^{-1})	4.6 ± 0.7	1.9 ± 0.2
		$K_{i,Gal}$ (mM)	188 ± 34	89 ± 27
oNPG	release of *o*NP	$v_{max,oNP}$ (μmol min^{-1} mg^{-1})		190
		$K_{m,oNPG}$ (mM)	not determined	0.98 ± 0.14
		k_{cat} (s^{-1})		338
		k_{cat}/K_m (mM^{-1} s^{-1})		345 ± 48
		$K_{i,Glc}$ (mM)		683 ± 249
		$K_{i,Gal}$ (mM)		115 ± 21

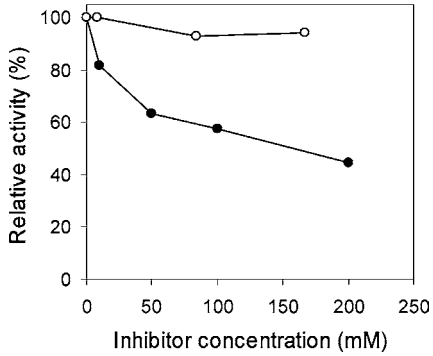

Figure 4. Inhibition by glucose (○) and galactose (●) of *o*NPG hydrolysis catalyzed by β-galactosidase *L. reuteri* L461 at an *o*NPG concentration of 2 mM.

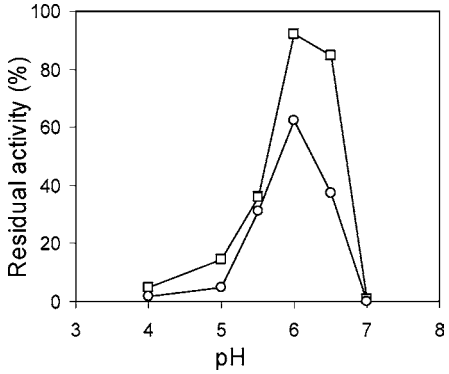

Figure 6. pH stability of β-galactosidase from *L. reuteri* L103 incubated at 37 °C in sodium citrate buffer (pH 4–5.5), sodium phosphate buffer (pH 6.0–7.5), and borate buffer (pH 8.0–9.0). The residual activity after 3 (□) and 20 h (○).

Effects of pH and Temperature on Enzyme Activity and Stability. The pH optimum of L103 β-galactosidase is pH 8.0 for both lactose and *o*NPG hydrolysis (**Figure 5A,B**); however, the enzyme is unstable at this pH. L103 β-galactosidase is most stable at pH 6.0, retaining more than 90 and 60% of its activity when incubated at pH 6.0 and 37 °C for 3 and 20 h, respectively (**Figure 6**). The pH optimum of L461 β-galactosidase is pH 8.0 for *o*NPG and 6.5 for lactose hydrolysis. A similar profile of pH stability was observed for L461 β-gal, and the enzyme is most stable at pH 6.0−6.5.

The optimum temperature of both β-galactosidases L103 and L461 was 50 °C when using *o*NPG as the substrate under standard assay conditions (pH 6.5 for 10 min). For lactose hydrolysis, the optimum temperatures of L103 and L461 β-gal were found to be 45 and 50 °C, respectively. The Arrhenius plots of the temperature dependence of lactase activity were found to be linear in the range of 20−45 °C for the L103 enzyme and 20−50 °C in the case of L461 β-gal (**Figure 7**). The activation energies are 30 and 15 kJ/mol for L103 and L461 β-gal, respectively. Both enzymes exhibit a significant loss of activities at temperatures above 50 °C.

The effect of temperature on the stability of both enzymes was investigated in more detail. Both β-galactosidases, L103 and L461, are very stable at 4 °C. L461 completely retained its activity after 3 months, and 60% of its activity was observed after 6 months at 4 °C; L103 has a half-life time of activity ($t_{1/2}$) of approximately 15 days at the same temperature. However, in the presence of 1 mM 1,4-dithiothreitol (DTT), more than 80% of the activity of L103 β-gal was observed after 6 months at 4 °C.

Figure 8 shows the effect of temperature on the stability of both enzymes, L103 β-gal and L461 β-gal, after incubation for 48 h at different temperatures. No remaining activity was found after an incubation at 45 °C; in fact, both enzymes were

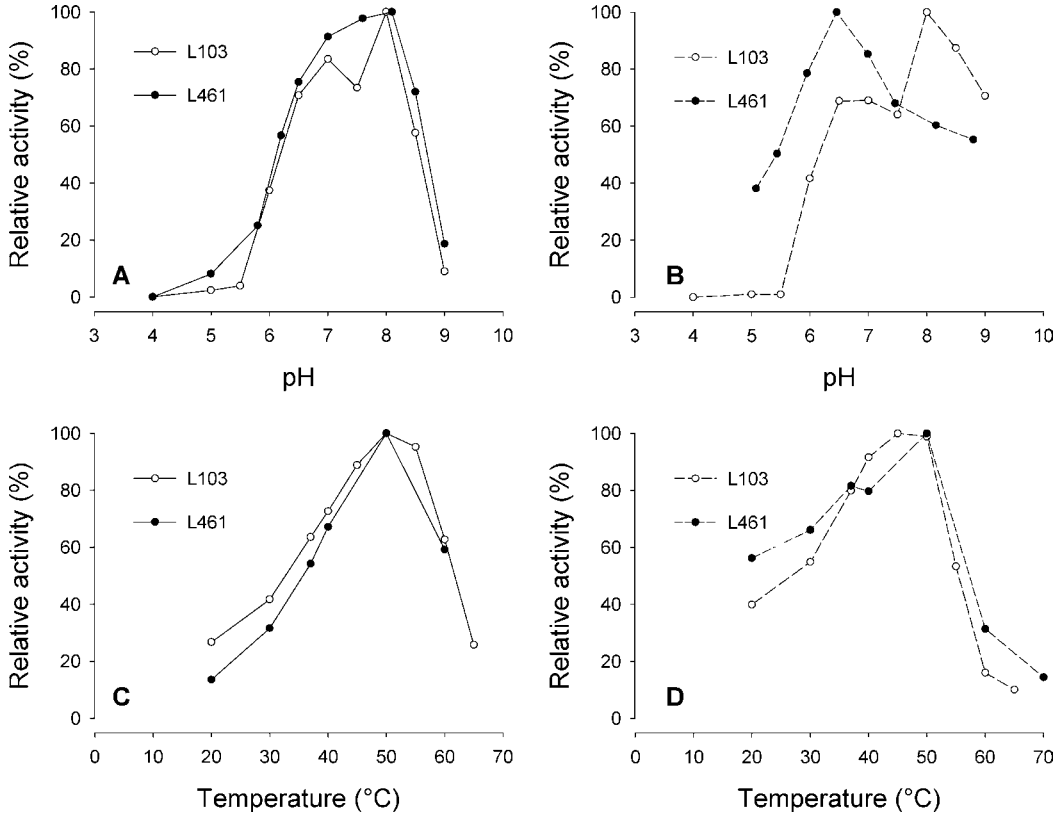

Figure 5. pH optimum (**A** and **B**) and temperature optimum (**C** and **D**) of β-galactosidases from *L. reuteri* L103 and L461 (**A** and **C**, *o*NPG as the substrate; **B** and **D**, lactose as the substrate).

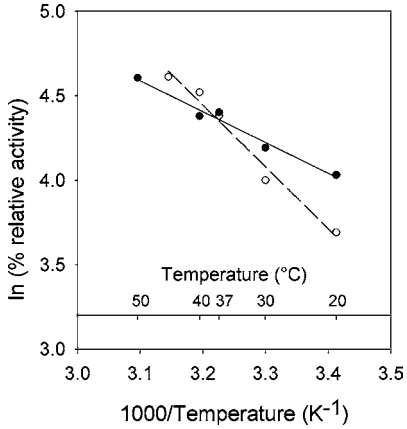

Figure 7. Effect of temperature on the lactase activity of β-galactosidases from *L. reuteri* L103 (○) and L461 (●).

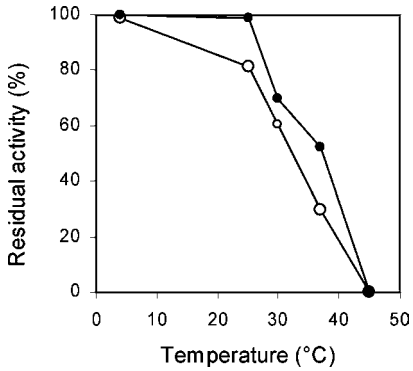

Figure 8. Temperature stability of β-galactosidase from *L. reuteri* L103 (○) and L461 (●). Residual activity after an incubation for 48 h at different temperatures in sodium phosphate buffer at pH 6.0 (L103) or 6.5 (L461).

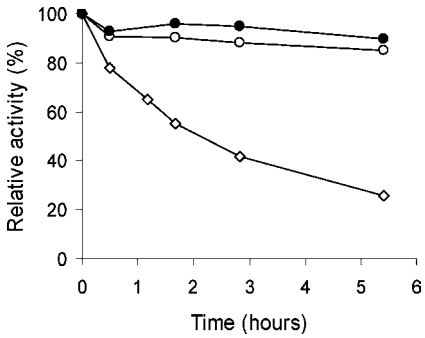

Figure 9. Stability of β-galactosidase from *L. reuteri* L461 at 42 °C without the presence of $MgCl_2$ (◇) and with the presence of 1 (○) and 10 mM (●) $MgCl_2$. Experiments were performed in sodium phosphate buffer (pH 6.5).

inactivated when held at this temperature for only 3 h. L103 and L461 β-galactosidases retained 60 and 70% of their activities, respectively, when they were kept at 30 °C for 48 h.

The thermal stability of both β-galactosidases L103 and L461 was significantly improved in the presence of $MgCl_2$. **Figure 9** shows the effect of 1 and 10 mM $MgCl_2$ on the thermostability of β-gal L461 at 42 °C. Both concentrations of $MgCl_2$ (1 and 10 mM) increased the thermal stability to approximately the same extent. In the presence of 10 mM $MgCl_2$, the enzyme retained 90% of its activity after incubation for 6 h at 42 °C, and its half-life time ($t_{1/2}$) of activity at this temperature was increased to approximately 24 h, compared to less than 3 h without Mg^{2+}. Comparable results were obtained for the enzyme from strain L103.

Table 4. Effect of Various Reagents on the Activity of β-Galactosidases from *L. reuteri* L461 and L103

	relative activity (%)					
	L103			L461		
	0.1 mM	1 mM	10 mM	0.1 mM	1 mM	10 mM
none	100	100	100	100	100	100
2-mercaptoethanol	–	109	98	–	106	78
1,4-dithiothreitol (DTT)	–	96	59	–	86	42
EDTA	10	8	–	15	8	5
urea	–	94	90	–	95	89

Table 5. Effect of Various Cations on the Activity of β-Galactosidases from *L. reuteri* L461 and L103

(**A**) Effect of Na^+ and K^+

	relative activity (%)[a]					
	Na^+			K^+		
	1 mM	10 mM	100 mM	1 mM	10 mM	100 mM
L103	269	554	529	392	630	609
L461	288	345	329	256	295	241

(**B**) Synergistic Effect of Different Cations

	relative activity (%)[a]					
	10 mM Na^+, 1 mM K^+	10 mM Na^+, 10 mM K^+	10 mM Na^+, 1 mM Mn^{2+}	10 mM Na^+, 10 mM Mn^{2+}	10 mM Na^+, 1 mM Mg^{2+}	10 mM Na^+, 10 mM Mg^{2+}
L103	550	593	745	268	633	618
L461	305	261	570	911	357	333

[a] The relative activity of no added cations was 100%.

Effects of Various Reagents and Cations on Enzyme Activity. Various reagents and cations were tested with respect to a possible inhibitory or stimulating effect on β-galactosidase activity. Similar effects for the various reagents were found for both enzymes L103 and L461 (**Table 4**).

Both enzymes were slightly activated by 1 mM 2-mercaptoethanol, while this compound was found to be slightly inhibiting when employed at the higher concentration (10 mM). This inhibitory effect on L103 β-galactosidase was not pronounced, while a significant inhibition of more than 20% was observed for L461 β-galactosidase activity. Similarly, a certain inhibitory effect was detected for 1,4-dithiothreitol (DTT) that was more pronounced for L461 than for L103 β-galactosidase activity. EDTA was found to strongly inactivate both enzymes even at the lowest concentration that was tested (0.1 mM).

Both enzymes were significantly activated by Na^+ and K^+ (**Table 5A**). Either 10 mM K^+ or 10 mM Na^+ is required for maximum activities of L103 and L461 β-galactosidase, but concentrations of these ions in the range of 1−100 mM exerted comparable stimulating effects.

The effects of K^+, Mn^{2+}, and Mg^{2+} in the presence of 10 mM Na^+ were tested to determine whether a possible synergism exists with respect to the activation of the lactobacillus β-galactosidases by cations (**Table 5B**). The highest activities of L103 and L461 β-gal were observed in the presence of 1 and 10 mM Mn^{2+}, respectively, together with 10 mM Na^+. This stimulating and synergistic effect of 10 mM Mn^{2+} is especially noteworthy with L461 β-gal, for which the activity was increased 9-fold compared to the blank containing no metal ions and 3-fold compared to the assay containing only 10 mM Na^+. Interestingly, the presence of 10 mM Mn^{2+} together with 10 mM Na^+ decreased L103 β-gal activity by ∼50% compared to that of the sample containing only Na^+. Other divalent cations

tested in the presence of 10 mM Na^+ (Fe^{2+}, Ca^{2+}, Cu^{2+}, and Zn^{2+}) inhibited the activities of both enzymes to varying extents. Complete inhibition of the activities of both enzymes was observed when 10 mM Cu^{2+} was added in the presence of 10 mM Na^+. The activity of L461 β-gal was also completely inhibited by the addition of 10 mM Zn^{2+}. The inhibitory effects of 1 mM Fe^{2+} and Ca^{2+} were similar in that the enzyme activity was reduced by ~30%.

DISCUSSION

It is now well established that the colonic microbiota exert an important influence on health. Consequently, oligosaccharides or prebiotics are increasingly being recognized as useful dietary tools for the modulation of the colonic microflora to improve health (*6*). Even though there are several prebiotic oligosaccharides on the market today, there is great interest in the development of novel prebiotics with tailor-made functions. Conceivably, these may possess improved and desired attributes that are not present in the prebiotic substances currently available. To date, the possibility of rationally targeting prebiotics at specific groups of bacteria such as certain known and approved probiotics has not been studied in detail; however, this could be a promising future prospect. One potential approach to this end is the use of enzymes, such as a β-galactosidase, obtained from a probiotic strain for the synthesis of oligosaccharides (*9*). β-Galactosidases from different microorganisms differ in their rate for hydrolysis of specific glycosidic linkages. This cleavage of the different glycosidic linkages as found in galacto-oligosaccharide mixtures to varying extents is an important prerequisite for an oligosaccharide mixture to be efficiently utilized by targeted probiotics and hence to exert their specific prebiotic effect. It is conceivable that these probiotic β-galactosidases, which rapidly hydrolyze certain galacto-oligosaccharide structures, can preferentially form these glycosidic linkages when acting in transgalactosylation mode. Therefore, β-galactosidases from probiotic microorganisms might produce galacto-oligosaccharide structures that have special prebiotic effects, specifically targeting selected probiotic strains (*9*, *25*).

Following a screening, two isolates of *L. reuteri*, strains L103 and L461, were selected from 14 strains of *Lactobacillus* spp. for further studies on their β-galactosidases. The ability to grow well on galacto-oligosaccharides (on both TOS and Elix'or) and the high β-galactosidase activity were the criteria for the selection of these strains.

The two β-galactosidases from *L. reuteri* are both heterodimers of approximately 105 kDa. Dimeric structures are a common feature found in many β-galactosidases described in the literature; however, most of them are homodimers such as β-gal from *Lactobacillus delbrückii* subsp. *bulgaricus* (two 110 kDa subunits) (*26*) or *Sterigmatomyces elviae* CBS8119 (two 86 kDa subunits) (*27*). Heterodimeric structures are less frequently encountered and were found for some other lactobacillus β-gal, e.g., from *Lactobacillus sakei* (*28*), *L. acidophilus* (*29*), and *Lactobacillus helveticus* (*30*). In accordance with our results on *L. reuteri* β-gal, these enzymes also consist of two subunits of approximately 35 and 72 kDa. However, *Lactobacillus* β-galactosidases are not restricted to functional dimers. The enzyme from *L. helveticus* (*31*) is a homotetramer of four subunits of 65 kDa each. Both β-galactosidases from *L. reuteri* are acidic proteins with pI values of 4.6−4.8 for L103 and 3.8−4.0 for L461 β-gal. Several tightly spaced bands were observed on the IEF for both homogeneous β-galactosidase preparations. This could indicate different isoforms of β-gal; however, it is currently not known whether these are true isoforms encoded by separate genes or result from different modifications of one polypeptide.

Kinetic constants were determined for the two substrates lactose and *o*NPG. In accordance with β-galactosidases from a number of different sources (*32*), the Michaelis constants are significantly lower for the chromogenic model substrate *o*NPG than for the natural substrate lactose. The kinetic parameters of L103 β-galactosidase with respect to *o*NPG were not determined due to the similarity of both enzymes, L461 and L103, but it can be expected that the $K_{m,oNPG}$ is also significantly lower than the K_m value determined for lactose. These K_m values determined for lactose, 13 and 31 mM for L103 and L461 β-galactosidase, respectively, compare favorably to the values reported for fungal and yeast enzymes that are commonly employed in technological applications (36−180 mM for *Aspergillus oryzae*, 54−99 mM for *Aspergillus niger*, 15−52 mM for *Kluyveromyces fragilis*, and 35 mM for *Kluyveromyces lactis*) (*33*, *34*). These relatively low K_m values of the *L. reuteri* β-galactosidases can be an advantage, e.g., when the complete hydrolysis of lactose is desired.

An additional advantage for lactose hydrolysis is the moderate inhibition by the two end products, galactose and glucose, which is evident from the ratio of K_i to K_m calculated for both of these competitive inhibitors. This ratio can be interpreted as a specificity constant which determines preferential binding of the substrate lactose versus that of the monosaccharide end products. Therefore, a high value for this ratio is desirable for efficient hydrolysis of lactose. The *L. reuteri* β-galactosidases display $K_{i,Gal}/K_{m,Lac}$ ratios of 14.5 and 2.9 for L103 and L461 β-gal, respectively, indicating moderate product inhibition by galactose that is still acceptable even when aiming at a high degree of lactose hydrolysis. Since the lactase assay using lactose as a substrate is based on the determination of the amount of released glucose, $K_{i,Glc}$ was not determined, yet from the inhibition constants measured for *o*NPG hydrolysis, it can be concluded that the value of $K_{i,Glu}/K_{m,Lac}$ will be significantly higher, indicating very moderate to negligible inhibition.

Both β-galactosidases, L103 and L461, were strongly activated by monovalent ions Na^+ and K^+ as well as by 1 mM Mg^{2+} and Mn^{2+}. This requirement for mono- and divalent metal ions for optimal activity and stability is well-known for a number of different β-galactosidases, most prominently the *Escherichia coli lacZ* β-galactosidase (*12*), yet the exact role that is played by these metal ions in catalysis is still unclear. However, a recent structural study showed binding sites for Na^+ and Mg^{2+} in the vicinity of the active site of *lacZ* which are thought to be consistent with stabilization and activity (*35*). Ca^{2+} is a known inhibitor of β-galactosidases (*36*, *37*), and this was also found for both enzymes L103 and L461. However, the application of these enzymes in fluid milk should not be affected by this inhibitory effect since Ca^{2+} is bound to casein. Applications of these enzymes in some other lactose conversion processes might require the modification of lactose-rich substrate to reduce the level of free Ca^{2+} in solution.

Even though both enzymes were purified from two different isolates of *L. reuteri*, they showed distinct differences with respect to some of their properties. Some of these differences are the varying Michaelis constants for lactose (13 mM for L103 β-gal and 31 mM for L461 β-gal) or inhibition constants for galactose (188 mM for L103 β-gal and 89 mM for L461 β-gal) which could indicate slight differences in the active site or the substrate binding in the active site. Other distinct differences that are obvious are the varying isoelectric points (4.6−4.8 for

β-gal L103 and 3.8−4.0 for β-gal L461) or varying effects of sulfhydryl protecting agents such as DTT mainly on stability. This could indicate differences in the primary structures between the two enzymes; this can be corroborated by cloning of both β-galactosidases which is ongoing in our laboratory.

In conclusion, this work presented two β-galactosidases from different isolates of *L. reuteri* which revealed interesting properties for applications in the processes of lactose conversion. Both enzymes also exhibited high transgalactosylation activity, and the application of these two enzymes for the production of galacto-oligosaccharides also interests us. A detailed knowledge about the properties of these two enzymes can serve as the basis for applications in the food industry.

ABBREVIATIONS USED

β-gal, β-galactosidase; *o*NPG, *o*-nitrophenyl β-D-galactopyranoside; *o*NP, *o*-nitrophenol; GOS, galacto-oligosaccharides.

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