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Characterization of a Heterodimeric GH2 β-Galactosidase from *Lactobacillus sakei* Lb790 and Formation of Prebiotic Galacto-oligosaccharides

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ABSTRACT: The *lacLM* genes from *Lactobacillus sakei* Lb790, encoding a heterodimeric β-galactosidase that belongs to glycoside hydrolase family GH2, were cloned and heterologously expressed in *Escherichia coli*. Subsequently, the recombinant β-galactosidase LacLM was purified to apparent homogeneity and characterized. The enzyme is a β-galactosidase with narrow substrate specificity because *o*-nitrophenyl-β-D-galactopyranoside (*o*NPG) was efficiently hydrolyzed, whereas various structurally related *o*NP analogues were not. The K_m and k_{cat} values for *o*NPG and lactose were 0.6 mM and 180 s^{-1} and 20 mM and 43 s^{-1}, respectively. The enzyme is inhibited competitively by its two end-products D-galactose and D-glucose (K_i values of 180 and 475 mM, respectively). As judged by the ratio of the inhibition constant to the Michaelis constant, K_i/K_m, this inhibition is only very moderate and much less pronounced than for other microbial β-galactosidases. β-Galactosidase from *L. sakei* possesses high transgalactosylation activity and was used for the synthesis of galacto-oligosaccharides (GalOS), employing lactose at a concentration of 215 g/L. The maximum GalOS yield was 41% (w/w) of total sugars at 77% lactose conversion and contained mainly non-lactose disaccharides, trisaccharides, and tetrasaccharides with approximately 38, 57, and 5% of total GalOS formed, respectively. The enzyme showed a strong preference for the formation of β-(1→6)-linked transgalactosylation products, whereas β-(1→3)-linked compounds were formed to a lesser extent and β-(1→4)-linked reaction products could not be detected.

KEYWORDS: β-galactosidase, lactase, *Lactobacillus sakei*, transgalactosylation, galacto-oligosaccharides

INTRODUCTION

Lactic acid bacteria (LAB) have been used extensively for the preservation and processing of foods such as milk, meat, and vegetables. *Lactobacillus sakei*, an important member of LAB, belongs to the main bacterial flora of fresh meat and fish and is best known for its use in the fermentation of various meat products, improving properties that allow better preservation and storage of fresh meat. *L. sakei* has furthermore been isolated from several other raw fermented food products of plant and animal origin, including sauerkraut, sourdough, smoked fish, or silage.[1−4] To speed and improve the ripening process of fermented meat products, glucose and sometimes lactose are added as exogenous substrates. The utilization of the milk sugar lactose is a primary function of lactobacilli and other LAB. The mechanism by which lactose is transported into the cell determines largely the subsequent pathway for the hydrolysis of this disaccharide. In several *Lactobacillus* spp. lactose is transported via phosphotransferase systems, which results in phosphorylation of lactose concurrent with its uptake. The resulting lactose-6′-phosphate is then hydrolyzed by phospho-β-galactosidase. Alternatively, lactose is taken up by secondary transport systems, and lactose is further metabolized by β-galactosidase within the cell.[5,6] The organization of these genes involved in lactose metabolism, which often form operons or operon-like structures with modular organization, has been studied in considerable detail.[5,7,8] The β-galactosidase (EC 3.2.1.23) of *L. sakei* is encoded by two genes, *lacL* and *lacM*, which are partly overlapping and out of frame, which suggests a coordinate regulation at the level of translation to produce an equal amount of both subunits that together form the active heterodimeric β-galactosidase.[8] Despite this previous study on the genes encoding β-galactosidase in *L. sakei*, this lactose-hydrolyzing enzyme from a technologically important organism has not been studied in detail, and interestingly only very few lactobacillal β-galactosidases have been characterized in depth with regard to their biochemical properties.[9−13] This is quite surprising considering the importance of lactobacilli in food technology and especially in dairy applications. The complete genome sequences of a number of fermentative and commensal LAB species including *L. sakei* [1] have been published by now, which opens new possibilities for comprehensive studies of a variety of biological processes.

β-Galactosidases catalyze the hydrolysis of the β-1,4-D-glycosidic linkage of lactose and structurally related substrates.

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β-Galactosidases have two main biotechnological uses in the food industry—the removal of lactose from milk and dairy products[14,15] as well as the production of galacto-oligosaccharides (GalOS) exploiting the transglycosylation potential of these enzymes.[16−18] GalOS belong to the prebiotics, which are defined as a "selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health".[19] GalOS have been classified as one of the few proven prebiotics fulfilling the following three criteria: (i) resistance to gastric acidity, hydrolysis by mammalian enzymes, and gastrointestinal absorption; (ii) fermentation by intestinal microflora; and (iii) selective stimulation of the growth and/or activity of intestinal bacteria associated with health and well-being.[19]

GalOS are complex mixtures of different oligosaccharides and are attracting increasing attention as prebiotic ingredients in various food applications including infant milk formula, as is evident from a number of reviews on GalOS that have been published recently.[16−18] The spectrum of the oligosaccharides making up these mixtures strongly depends on the source of the β-galactosidase used for the biocatalytic reaction and on the conversion conditions used in their production. Because these differences in GalOS spectrum and yield are a result of structural and/or mechanistic differences between β-galactosidases from different sources, a detailed knowledge of novel, yet-unexplored β-galactosidases from various strains can be of significant interest.[16,18] Rabiu et al.[20] and Tzortzis et al.[21] produced various GalOS mixtures using lactose as substrate and β-galactosidases from different probiotic bifidobacteria. Subsequently, they showed that these different mixtures typically resulted in better growth of that strain that had served as the source of the enzyme for GalOS production. This concept can serve as the basis for a new generation of functionally enhanced, targeted oligosaccharides and has increased the interest in β-galactosidases from beneficial probiotic organisms.[22]

The objective of this work was to characterize in detail the β-galactosidase from *L. sakei* Lb790 and to evaluate the transgalactosylation activity of this enzyme. Recent data are indicating that *L. sakei* might be of interest as a beneficial LAB, making it attractive as a probiotic strain,[23] and hence prebiotic oligosaccharides produced by its enzyme might be of functional interest.

MATERIALS AND METHODS

Chemicals and Enzymes. All chemicals were of the highest quality available and were purchased from Sigma (St. Louis, MO) unless otherwise stated. Phenylmethanesulfonyl fluoride (PMSF) and glucose oxidase (GOD) from *Aspergillus niger* (lyophilized, 205 U/mg of enzyme preparation) were obtained from Fluka (Buchs, Switzerland), whereas isopropyl-β-D-thiogalactoside (IPTG) and 1,4-dithiothreitol (DTT) were from Roth (Karlsruhe, Germany). The test kit for the determination of D-galactose was purchased from Megazyme (Wicklow, Ireland). All chromatographic materials were from Amersham Biosciences (Uppsala, Sweden), and restriction enzymes and corresponding buffers were from Fermentas (Vilnius, Lithuania).

Bacterial Strains and Culture Conditions. *L. sakei* Lb790, the original source of the β-galactosidase genes, was isolated from meat as described in Schillinger and Lücke[24] and was received from the culture collection of the Norwegian University of Life Sciences, Ås, Norway. *Escherichia coli* BL21 Star (DE3) (Invitrogen, Carlsbad, CA) was used as expression host for the expression vector carrying the two overlapping genes encoding the large and small subunits (*lacLM*) of β-galactosidase from *L. sakei* Lb790. *E. coli* cells were grown on Luria−Bertani (LB) agar plates (10 g/L peptone, 5 g/L yeast extract, 10 g/L NaCl, and 15 g/L agar) containing the appropriate antibiotics (100 μg/mL ampicillin or 50 μg/mL kanamycin) required for maintaining the plasmids or in Terrific-Broth (TB) medium (12 g/L peptone, 24 g/L yeast extract, 0.4% (v/v) glycerol, and 13.6 g/L KH_2PO_4) supplemented with 100 μg/mL ampicillin. *E. coli* DH5α electrocompetent cells were from Invitrogen.

DNA Amplification and Subcloning of the β-Galactosidase Genes. Plasmid pEH9S, which contains the complete genes *lacLM* encoding heterodimeric β-galactosidase from *L. sakei* Lb790, the construction of which was described previously,[25] was used as template for the amplification of these *lacLM* genes. The oligonucleotides used for PCR amplification, *LacLM*Sak_Forward (5′-ACCATGGAACCTAATATTCAATGGTTAG-3′) and *LacLM*Sak_Reverse (5′-TTTTCTCGAGTGAAAACGAAATTTCAA-3′), were designed on the basis of the published sequence of the β-galactosidase gene from *L. sakei* subsp. *sakei* (GenBank accession no. X82287) and were obtained from VBC Biotech (Vienna, Austria). These primers created a restriction site (underlined above), *Nco*I and *Xho*I, at the 5′ and 3′ ends of the gene fragment, respectively. The amplification was performed in a total volume of 25 μL containing 0.2 mM of each deoxynucleotide triphosphate, 0.5 μM of each primer, 12.5 μL of 5× Phusion HF buffer (final concentration of $MgCl_2$ was 1.5 mM), 1 U of Phusion DNA Polymerase, and 1−40 ng of plasmid DNA. The initial denaturation step at 98 °C for 30 s was followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 60 °C for 20 s, and extension at 72 °C for 1 min. The final cycle was followed by an additional 5 min elongation step at 72 °C. The amplified PCR product was visualized by gel electrophoresis in an 0.8% agarose gel (containing 0.2 μg/mL ethidium bromide) in 1× TAE (Tris−acetate−EDTA) electrophoresis buffer (4.8 g/L Tris base, 1.2 g/L acetic acid, 1 mM EDTA, pH 8.0) under UV light. The amplified product was purified from the agarose gel using the Wizard SV gel and PCR Clean-up System (Promega, Madison, WI). The Zero Blunt TOPO PCR Cloning Kit containing the vector pCR-Blunt II-TOPO (Invitrogen) was used for subcloning of PCR-amplified blunt end-products. The resulting plasmid pBT*lacLM*Sak contained the complete genes (*lacL* and *lacM*) of β-galactosidase from *L. sakei* Lb790 as was confirmed by sequencing performed by a commercial provider.

Construction of the *lacLM* Expression Vector. A DNA fragment containing the β-galactosidase genes was obtained from the pBT*lacLM*Sak plasmid after digestion with *Nco*I and *Xho*I restriction enzymes. The resulting fragments were then ligated into the *Nco*I−*Xho*I fragment of expression vector pET21d (Novagen, Darmstadt, Germany) so that a C-terminal His_6-tag was added. The resulting plasmid was transformed into *E. coli* DH5α electrocompetent cells, and the correct sequence was verified by DNA sequencing. The expression vector pSI*lacLM*Sak thus obtained was then transformed into *E. coli* BL21 Star (DE3) for the heterologous overexpression of the β-galactosidase LacLM.

Expression of β-Galactosidase. *E. coli* BL21 Star (DE3) carrying pSI*lacLM*Sak was grown at 37 °C in 30 mL of TB medium containing 100 μg/mL ampicillin until an optical density at 600 nm (OD_{600}) of 0.3 was reached. IPTG or lactose, added to the culture medium in final concentrations of 0.1, 1, or 10 mM for IPTG and 5 or 10 g/L for lactose, was then used as inducer, and the cultures were incubated further at 18 °C for 24 h until OD_{600} of 8.5 ± 1. Cells were then harvested by centrifugation (3300*g*, 10 min, 4 °C), washed, resuspended in sodium phosphate buffer (50 mM, pH 6.5), and disrupted by using a French press (Aminco, Silver Spring, MD). Cell debris was removed by centrifugation (16000*g*, 15 min, 4 °C) to obtain the crude cell extract.

Protein Purification. The crude cell extract was loaded onto a 10 mL Ni^{2+}-Sepharose fast flow column that was pre-equilibrated with

buffer A (20 mM sodium phosphate, 0.5 M NaCl, and 20 mM imidazole, pH 6.5). Proteins were eluted at a rate of 1.5 mL/min by using a linear 100 mL gradient of 20−500 mM imidazole. The active fractions were pooled, desalted, and concentrated (Amicon Ultra Centrifugation filter tubes, 10 kDa cutoff; Millipore, Billerica, MA). The purified β-galactosidase was stored in 50 mM sodium phosphate buffer (pH 6.5) at 4 °C for further analysis. Protein concentrations were determined according to the method of Bradford using bovine serum albumin as standard.

Gel Electrophoresis and Activity Staining. Native polyacrylamide gel electrophoresis (PAGE), denaturing sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE), and activity staining using 4-methylumbelliferyl β-D-galactoside (MUG) as the substrate, as well as isoelectric focusing in the range of pH 3−10, were carried out as previously described.[26]

Enzyme Assays. β-Galactosidase (β-gal) activity was determined using *o*-nitrophenyl-β-D-galactopyranoside (*o*NPG) and lactose as the substrate, as described previously.[26,27] In brief, the standard assay of β-gal activity was performed at 30 °C using *o*NPG as the substrate. The reaction was started by adding 20 μL of enzyme sample to 480 μL of 22 mM *o*NPG in buffer (50 mM sodium phosphate buffer, pH 6.5) and stopped after exactly 10 min by adding 750 μL of 0.4 M Na_2CO_3. The release of *o*-nitrophenol (*o*NP) was assessed by measuring the absorbance at 420 nm. One unit of β-galactosidase (U_{oNPG}) is defined as the amount of enzyme releasing 1 μmol of *o*NP per minute under the reaction conditions given above. When β-gal activity was determined with its natural substrate lactose, 20 μL of enzyme sample was added to 480 μL of substrate solution (600 mM lactose in 50 mM sodium phosphate buffer, pH 6.5) and incubated at 30 °C for 10 min. The reaction was stopped by boiling the sample for 5 min. Glucose released was measured with an enzymatic assay based on glucose oxidase (GOD) and peroxidase (POD).[28] One unit of lactase activity (U_{Lac}) refers to the amount of enzyme necessary for the formation of 1 μmol of D-glucose per minute under the described conditions. All measurements and experiments were performed at least in duplicate, and the experimental error was always below 5%.

Characterization of Recombinant β-Galactosidase. Steady-state kinetic data were obtained at 30 °C using either *o*NPG or lactose as the substrate in 50 mM sodium phosphate buffer (pH 6.5) with concentrations ranging from 0 to 25 mM for *o*NPG and from 0 to 600 mM for lactose. Furthermore, the inhibition of *o*NPG hydrolysis by D-glucose as well as the inhibition of lactose hydrolysis by D-galactose was investigated, and the respective inhibition constants were determined. The kinetic parameters and the inhibition constants were calculated using nonlinear regression, fitting the observed data to the Henri−Michaelis−Menten equation using SigmaPlot (SPSS, Chicago, IL).

The pH dependence of the enzymatic release of *o*NP from *o*NPG as well as that of D-glucose from lactose was assessed in the range of pH 4−9 using Britton−Robinson buffer (containing 20 mM each of phosphoric, acetic, and boric acid adjusted to the required pH with NaOH). The temperature dependence of β-gal activity (both *o*NPG and lactase activity) was determined by measuring activity in the range of 20−70 °C for 10 min. To determine the pH stability of *L. sakei* β-galactosidase, enzyme samples were incubated at 30 °C and at pH values ranging from 4 to 9 for up to 24 h, and the remaining β-gal activity was measured for different time points using the standard *o*NPG assay. The temperature stability of the enzyme was studied by incubating enzyme samples in sodium phosphate buffer (50 mM, pH 6.5) at various temperatures (4, 22, 30, 37, and 42 °C). At certain time intervals, samples were withdrawn and the residual β-gal activity was measured with *o*NPG as the substrate under standard assay conditions.

Substrate Specificity. To determine the specificity of recombinant β-galactosidase from *L. sakei* Lb790, several structurally related chromogenic substrates, that is, *p*-nitrophenyl-α-D-galactopyranoside, *o*-nitrophenyl-α-D-glucopyranoside, *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-β-D-mannopyranoside, and *p*-nitrophenyl-β-D-xylopyranoside, were tested as potential substrates. Enzyme activities were measured using 22 mM of each of the substrates under otherwise standard β-gal assay conditions.

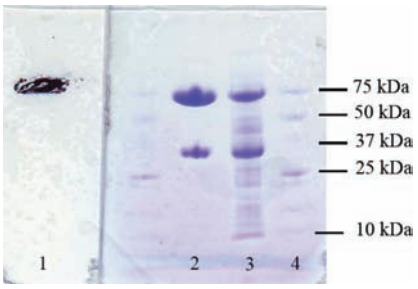

Figure 1. SDS-PAGE analysis of recombinant β-galactosidase from *L. sakei* Lb790. Lane 1 shows active staining of the purified β-galactosidase with MUG (marked on the gel); lanes 2, 3, and 4 show Coomassie blue staining of purified β-galactosidase, crude enzyme extract, and molecular mass marker (Amersham), respectively.

Formation of Galacto-oligosaccharides. The synthesis of galacto-oligosaccharides (GalOS) was carried out in discontinuous mode at 37 °C using purified recombinant β-galactosidase from *L. sakei* Lb790 (1.2 lactase U per mL of reaction mixture). Reaction conditions were 215 g/L initial lactose concentration in sodium phosphate buffer (50 mM, pH 6.5) containing 1 mM $MgCl_2$. Continuous agitation was applied at 300 rpm. Samples were withdrawn periodically, and the composition of the GalOS mixture was analyzed by thin layer chromatography (TLC), capillary electrophoresis (CE), and high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), following methods described previously.[29] Individual GOS compounds were identified and quantified by using authentic standards and the standard addition technique.[29,30]

RESULTS

Overexpression in *E. coli* and Purification of β-Galactosidase from *L. sakei*. A T7 RNA polymerase-based expression system was used for the overexpression of the *lacLM* genes of *L. sakei* in *E. coli*. The coding region of these two overlapping genes, *lacL* and *lacM*,[8] was cloned into the *Nco*I- and *Xho*I-cloning sites of pET21d, resulting in the expression plasmid pSI*lacLM*Sak. Gene expression in *E. coli* BL21 Star (DE3) was induced after OD_{600} reached 0.3, using different concentrations of IPTG (0−10 mM) and lactose (5 and 10 g/L), and the cultivation was continued at 18 °C for 20 h. The highest levels of β-gal activity were obtained when 0.1 mM IPTG was used for induction. Approximately 2800 U of β-gal activity/L of fermentation medium was produced under these conditions with a specific activity of 17 U/mg of protein. The His-tagged enzyme was subsequently purified directly from the crude cell extract by a single-step purification procedure using a Ni^{2+}-Sepharose fast flow column, which gave an overall yield of ∼70% and an apparently homogeneous β-galactosidase preparation with a specific activity of 83 U/mg of protein.

Gel Electrophoresis Analysis. β-Galactosidase from *L. sakei* is a heterodimer of ∼110 kDa, consisting of a large subunit of ∼72 kDa and a small subunit of ∼35 kDa as analyzed by SDS-PAGE (Figure 1). This compares well to the calculated molecular masses of the two subunits (72 457 and 35 254 Da, respectively) deduced from their sequences. Activity staining of purified

Table 1. Kinetic Parameters for Recombinant, His-Tagged β-Galactosidase from *L. sakei* Lb790 Overexpressed in *E. coli* for the Hydrolysis of *o*NPG and Lactose

	substrates	
kinetic parameter	lactose	*o*NPG
K_m (mM)	20 ± 2	0.60 ± 0.19
v_{max} (μmol min^{-1} mg^{-1})	24 ± 1	98 ± 8
k_{cat} (s^{-1})	43 ± 1	180 ± 14
k_{cat}/K_m (M^{-1} s^{-1})	2100	290000
$K_{i,Gal}$ (mM)	180 ± 17	
$K_{i,Gal}/K_{m,Lac}$	9.0	
$K_{i,Glc}$ (mM)		475 ± 200
$K_{i,Glc}/K_{m,oNPG}$		790

β-galactosidase (after preincubation with SDS buffer at 60 °C for 5 min to separate the subunits) directly on the SDS-PAGE gel using 4-methylumbelliferyl β-D-galactoside as the substrate showed that only the larger subunit exhibited activity with this substrate (Figure 1), whereas the smaller subunit did not. In addition, nondissociated β-galactosidase of M_r ~110 000 showed activity on the SDS-PAGE gel under these conditions (not shown). The treatment of the enzyme preparation with denaturing SDS buffer at 99 °C for 5 min apparently resulted in complete denaturation of both subunits, as is evident from the total loss of activity on the gel. The isoelectric point (p*I*) of recombinant β-galactosidase from *L. sakei* was determined by isoelectric focusing, and it was found to be in the range of 5.2 (data not shown), which is in agreement with the calculated theoretical p*I* of 5.05.

Enzyme Kinetics. The steady-state kinetic constants determined for the hydrolysis of the substrates lactose and *o*NPG as well as the inhibition constants for both end-products, D-galactose and D-glucose, for β-galactosidase from *L. sakei* are summarized in Table 1; the k_{cat} values were calculated on the basis of the theoretical v_{max} values obtained from the experimental data by nonlinear regression and using a molecular mass of 108.5 kDa for the enzyme (LacLM plus His-tag). The catalytic efficiencies (k_{cat}/K_m) for these two substrates, lactose and *o*NPG, indicate that *o*NPG is the preferred substrate, because of the more favorable K_m and k_{cat} values. As is evident from the Michaelis–Menten plots (Figure 2), β-galactosidase from *L. sakei* is inhibited by elevated concentrations of the substrate *o*NPG, whereas no comparable substrate inhibition could be observed for lactose when present in concentrations of up to 600 mM.

The hydrolysis end-product D-galactose was found to competitively inhibit the hydrolysis of lactose. This inhibition, however, is not pronounced as is obvious from the ratio of the Michaelis constant for lactose and the inhibition constant for D-galactose ($K_{i,Gal}/K_{m,Lac}$ = 9.0). *o*NPG was used as the substrate for investigation of the inhibition by the second end-product, D-glucose. The inhibitory effect of D-glucose is found to be even less pronounced ($K_{i,Glc}/K_{m,oNPG}$ = 790), and D-glucose is also a competitive inhibitor of β-gal activity.

The effects of D-galactose and D-glucose on β-gal activity during the hydrolysis of *o*NPG were investigated in more detail (Figure 3). Again, it is obvious that D-galactose is the more efficient inhibitor. When added at a concentration of 200 mM to the reaction mixture, β-galactosidase activity was reduced by approximately 50%, whereas D-glucose in similar concentrations

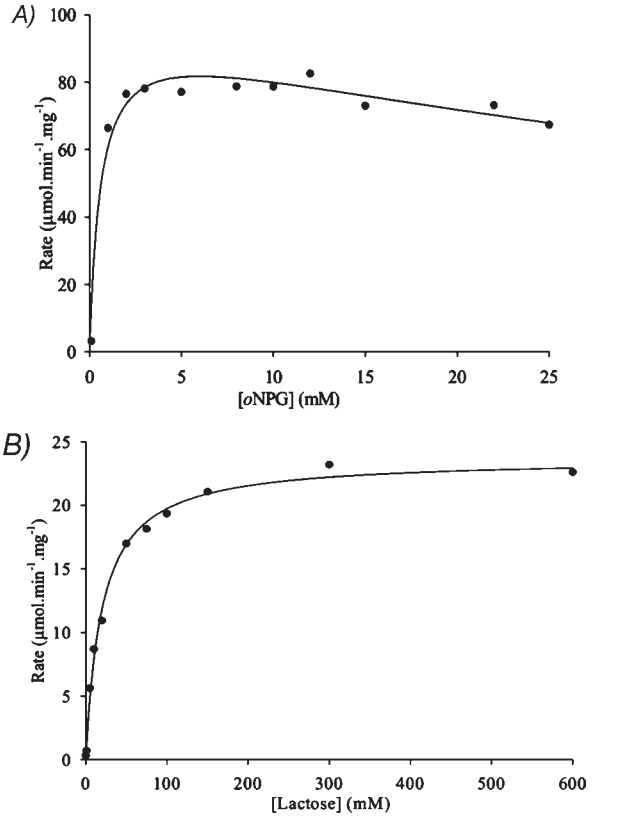

Figure 2. Kinetic analysis of β-galactosidase activity from *L. sakei* with (A) chromogenic *o*NPG as the substrate and (B) lactose as the substrate.

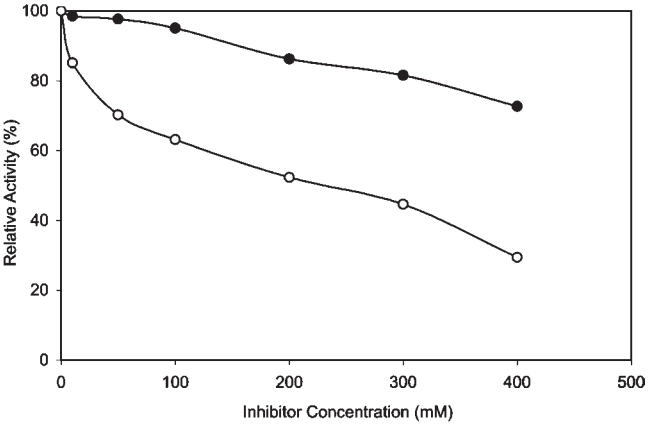

Figure 3. Inhibition of β-galactosidase activity from *L. sakei* Lb790 by its reaction products D-galactose (○) and D-glucose (●). *o*NPG (2 mM) was used as substrate for assaying β-gal activity.

reduced the activity by only 14% compared to reactions without the addition of the monosaccharides.

Effect of Temperature and pH on Enzyme Activity and Stability. The optimum temperature of the activity of β-galactosidase from *L. sakei* is 55 °C for both *o*NPG and lactose hydrolysis (Figure 4A) for the 10 min assay. The Arrhenius plot of the temperature dependence of lactase activity was found to be linear in the range of 25–55 °C (data not shown), and the activation energy E_a for β-galactosidase was calculated as 41 kJ/mol. The pH optimum of β-gal activity is pH 6.5 when using

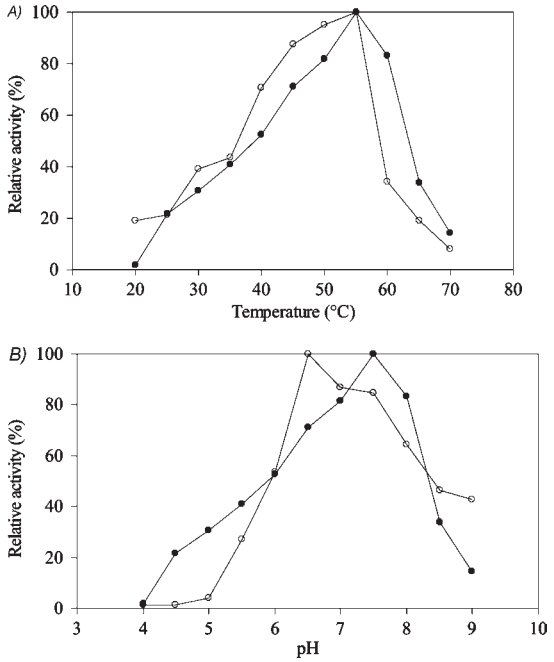

Figure 4. Temperature optimum (A) and pH optimum (B) of recombinant β-galactosidase from *L. sakei*: (○) *o*NPG as the substrate; (●) lactose as the substrate.

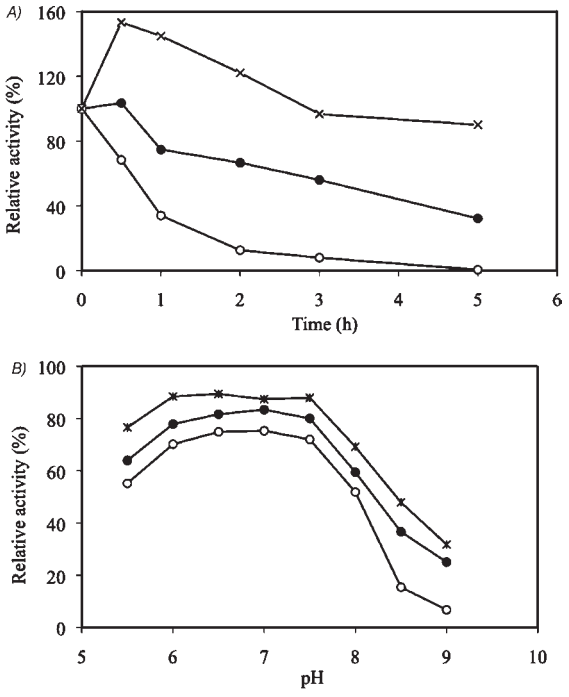

Figure 5. (A) Stability of recombinant β-galactosidase from *L. sakei* at 42 °C without the presence of $MgCl_2$ (○) and with the presence of 1 mM (●) and 10 mM (×) $MgCl_2$. (B) pH stability of recombinant β-galactosidase from *L. sakei* Lb790 at 30 °C in Britton−Robinson buffer (pH 5−9): residual activity after 2 h (×), 5 h (●), and 10 h (○).

*o*NPG as substrate, whereas an optimum pH of 7.5 was found for lactose (Figure 4B). Overall, the pH curve is rather narrow for this β-galactosidase, showing 80% of its maximal activity in a pH range of only 1−1.5 units.

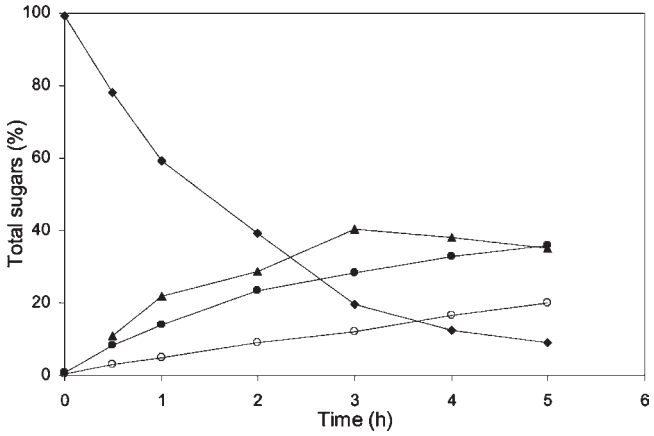

Figure 6. Composition of the sugar mixture during lactose conversion by recombinant β-galactosidase from *L. sakei*. The reaction was carried out at 37 °C using 215 g/L initial lactose concentration in 50 mM sodium phosphate buffer (pH 6.5), 1 mM $MgCl_2$, and 1.2 U_{Lac}/mL of homogeneous enzyme: (◆) lactose; (●) glucose; (○) galactose; (▲) GalOS. Monosaccharides were measured enzymatically, and lactose and GalOS were quantified by HPAEC-PAD and CE.

The temperature stability of recombinant β-galactosidase from *L. sakei* was analyzed at different temperatures under otherwise standard assay conditions (pH 6.5). The enzyme has a half-life time of activity ($t_{1/2}$) of approximately 20 days at 4 °C, and after a 1 h incubation at 37 and 42 °C without the addition of any stabilizing reagents, the enzyme retained 56 and 34% of its activity, respectively. Furthermore, the effect of various concentrations of Mg^{2+} on the thermal stability of the enzyme was investigated. In the presence of 10 mM Mg^{2+}, the enzyme retained 90% of its activity after a 5 h incubation at 42 °C. A lower concentration of Mg^{2+} of only 1 mM proved to be less effective with respect to stabilization, and >60% of the activity was lost after 5 h at 42 °C, whereas the enzyme completely inactivated within 5 h at that temperature without the addition of Mg^{2+} (Figure 5A). These experiments not only showed a significant stabilization but also an activation of β-galactosidase activity in the presence of Mg^{2+} (Figure 5A). β-Galactosidase from *L. sakei* is most stable in the pH range of 6.0−7.5, retaining more than 80 and 60% of its activity after incubations of 2 and 10 h, respectively, in this pH range and at 30 °C (Figure 5B). The enzyme is not stable at pH values below 5 and loses >85% of its activity when kept at this pH and 30 °C within 1 h (data not shown).

Substrate Specificity. To study the substrate specificity of β-galactosidase from *L. sakei* Lb790, various structurally related chromogenic substrates were used under standard assay conditions. No activity (<1% relative to the standard substrate *o*NPG) was detected when using *p*-nitrophenyl-α-D-galactopyranoside, *o*-nitrophenyl-α-D-glucopyranoside, *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-β-D-mannopyranoside, and *p*-nitrophenyl-β-D-xylopyranoside as substrates, indicating that the enzyme is a β-galactosidase with narrow substrate specificity.

Synthesis of GalOS. Lactose conversion and product formation of a typical discontinuous conversion reaction, using 215 g/L initial lactose in 50 mM sodium phosphate buffer + 1 mM $MgCl_2$, pH 6.5, and 1.2 U_{Lac}/mL of β-gal activity at 37 °C, are shown in Figure 6. As the reaction proceeded, D-glucose and D-galactose, the primary hydrolysis products, were formed but

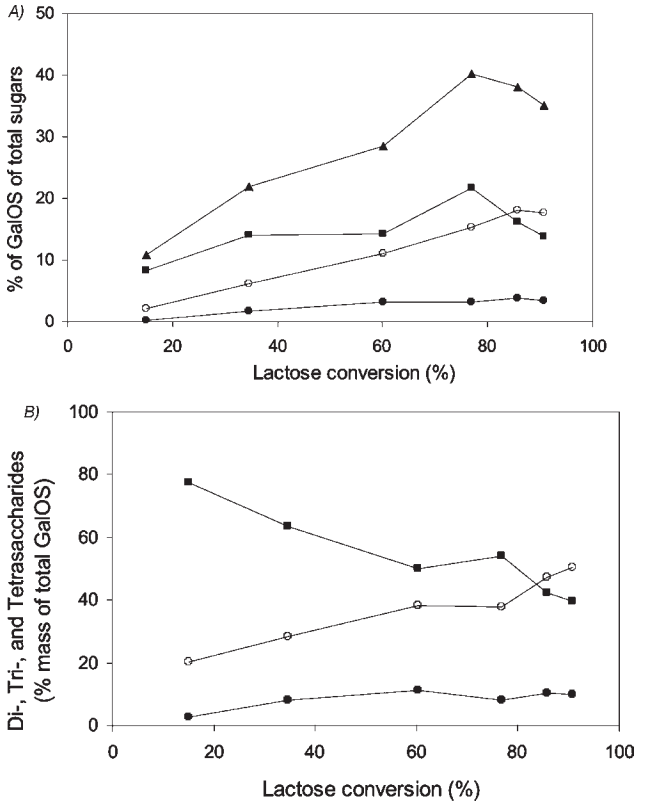

Figure 7. Formation and degradation of GalOS during lactose conversion by β-galactosidase form *L. sakei* Lb790 with 215 g/L initial lactose concentration in 50 mM sodium phosphate buffer (pH 6.5) at 37 °C and 1 mM $MgCl_2$: (○) disaccharides; (■) trisaccharides; (●) tetrasaccharides; (▲) total GalOS.

also GalOS as a result of the transgalactosylation reaction catalyzed by the enzyme. Between 3 and 4 h of reaction, the concentration of total GalOS reached at maximum of 89 g/L, corresponding to approximately 41% of the total sugars. Thereafter, the concentration of GalOS decreased because these oligosaccharides are not stable end-products of a thermodynamically controlled reaction; they are only transiently formed and are subject to hydrolysis, which became more pronounced when the substrate lactose was depleted or their concentration reached a higher, critical concentration for hydrolysis (Figure 6). In addition to hydrolysis of these newly formed oligosaccharides, GalOS can also serve as acceptor for galactosyl transfer as can the primary hydrolysis products D-glucose and D-galactose, and therefore the amount and composition of GalOS change dramatically during the course of the reaction, as is illustrated in more detail in Figure 7. Up to ~80% lactose conversion, the amount of GalOS, expressed by their relative concentration (percentage of GalOS of total sugars), was constantly rising. The maximal concentration of GalOS was found at 77% lactose conversion (Figure 7A), and the GalOS mixture at this reaction point was composed of mainly non-lactose disaccharides, trisaccharides, and tetrasaccharides with approximately 38, 57, and 5% of total GalOS formed, respectively. At the beginning of the reaction, trisaccharides were predominantly formed, constituting up to ~80% of total GalOS during this initial reaction phase (Figure 7B). This is not unexpected because lactose is the most abundant carbohydrate species in the reaction mixture acting as the main galactosyl acceptor during the first phase of the reaction.

Table 2. Individual GalOS Components Produced by β-Galactosidase from *Lactobacillus sakei* Lb790[a]

		degree of lactose conversion					
		15%	35%	60%	77%	85%	90%
	GalOS component (g/L)						
1	D-Gal*p*-(1→3)-D-Glc	nd	0.44	0.44	0.94	1.82	2.20
2	D-Gal*p*-(1→6)-D-Glc (allolactose)	3.31	8.00	15.1	19.3	21.9	20.0
3	D-Gal*p*-(1→3)-D-Gal	nd	0.41	1.15	1.70	3.01	2.21
4	D-Gal*p*-(1→6)-D-Gal	1.40	5.02	7.36	12.4	13.5	15.1
5	D-Gal*p*-(1→6)-D-Lac	17.5	27.1	26.6	33.2	26.4	21.7
6	D-Gal*p*-(1→3)-D-Lac	0.69	0.90	2.59	5.08	2.41	2.10
	GalOS component (mM)						
1	D-Gal*p*-(1→3)-D-Glc	nd	1.29	1.29	2.75	5.32	6.43
2	D-Gal*p*-(1→6)-D-Glc (allolactose)	9.68	23.4	43.9	56.4	64.0	58.5
3	D-Gal*p*-(1→3)-D-Gal	nd	1.19	3.36	4.97	8.80	6.46
4	D-Gal*p*-(1→6)-D-Gal	4.09	14.7	21.5	36.3	39.5	44.3
5	D-Gal*p*-(1→6)-D-Lac	34.6	53.8	52.8	65.9	52.4	43.1
6	D-Gal*p*-(1→3)-D-Lac	1.37	1.79	5.14	10.1	4.78	4.17
	GalOS (% mass of total GOS)						
1	D-Gal*p*-(1→3)-D-Glc	nd	0.93	0.71	1.05	2.19	2.87
2	D-Gal*p*-(1→6)-D-Glc (allolactose)	14.1	16.9	24.4	21.6	26.3	26.1
3	D-Gal*p*-(1→3)-D-Gal	nd	0.86	1.86	1.90	3.61	2.87
4	D-Gal*p*-(1→6)-D-Gal	5.97	10.7	12.0	13.9	16.2	19.7
5	D-Gal*p*-(1→6)-D-Lac	74.4	57.6	43.2	37.2	31.7	28.3
6	D-Gal*p*-(1→3)-D-Lac	2.95	1.90	4.20	5.69	2.89	2.74

[a] The reaction was carried out at 37 °C using 215 g/L initial lactose concentration in 50 mM sodium phosphate buffer (pH 6.5), 1 mM $MgCl_2$, and 1.2 U_{Lac}/mL of homogenous enzyme.

As the conversion of lactose proceeded further, the concentrations of the main hydrolysis products, D-galactose and D-glucose, increased, and these in turn became prominent acceptors for the galactosyl moiety. Hence, disaccharides other than lactose were also formed to a significant extent at higher lactose conversion. Furthermore, disaccharides can result from β-gal-catalyzed hydrolysis of trisaccharides, which are present in higher concentrations at latter phases of the conversion. Non-lactose disaccharides were dominant by weight at above 85−90% lactose conversion.

A detailed analysis of the main GalOS components, which were identified and quantitated by HPAEC-PAD using authentic standards and the standard addition technique, over the time course of the reaction is given in Table 2. The *L. sakei* β-galactosidase shows a strong preference for the formation of β-(1→6) linkages in its transgalactosylation mode, whereas β-(1→3)-containing compounds are formed to a lesser extent, and β-(1→4)-containing GalOS compounds could not be identified among the major reaction products. The main components of this GalOS mixture were identified as D-Gal*p*-(1→6)-D-Glc (allolactose), D-Gal*p*-(1→3)-D-Glc, D-Gal*p*-(1→6)-D-Gal, D-Gal*p*-(1→3)-D-Gal, D-Gal*p*-(1→6)-Lac, and D-Gal*p*-(1→3)-Lac.

■ DISCUSSION

L. sakei is an important member of the LAB because it is widely used as a starter for the manufacture of fermented sausages and other meat products. Meat has a low carbohydrate content, with glucose and ribose, originating from glycogen and ATP, respectively, being the main sugars,[1,2,31] and exogenous glucose

or lactose is usually added to accelerate the ripening process of these fermented meat products. β-Galactosidases represent one possible way of introducing lactose into the metabolism of lactobacilli through intracellular cleavage of this disaccharide to galactose and glucose. *Lactobacillus* spp. can carry β-galactosidases belonging to two different glycoside hydrolase (GH) families, 2 and 42.[13,16] Recent genome projects revealed that certain species such as *L. acidophilus* or *L. plantarum* can carry at least two genes encoding β-galactosidases belonging to GH2 (*lacLM*) and GH42 (*lacA*), whereas other strains such as *L. sakei* with its modestly sized genome possess only the genes encoding the β-galactosidase of the LacLM type.

β-Galactosidase from *L. sakei* was produced heterologously because the yields obtained with the wild-type strain were very low (data not shown). Recently, we compared the expression of four different lactobacillal β-galactosidases, all of the LacLM type, in *L. plantarum* and *L. sakei*[25] as well as in *Lactococcus lactis*.[32] Whereas the expression levels of β-gal from *L. plantarum* and *L. reuteri* were generally high with these different hosts, expression of *L. acidophilus* β-gal was high only in *Lactococcus lactis*, reaching expression levels of ∼85 mg of recombinant protein/L without further optimization. Interestingly, *L. sakei* β-gal expressed relatively poorly in these three hosts, even when homologously overexpressed in *L. sakei* with typical expression levels found in the range of 10−15 mg/L. Whereas overexpression in *E. coli* yielded sufficient enzyme for the subsequent characterization and transglycosylation experiments, the yields of 33 mg/L are also only rather modest for *E. coli* as expression host. When using the identical expression system, *E. coli* BL21 Star (DE3) and an expression plasmid based on pET21d carrying *lacLM*, we obtained expression levels of ∼690 mg/L for the overexpression of *L. reuteri* β-gal.[27] We previously proposed that rarely used and unfavorable codons in the start of the *lacLM* genes from *L. sakei* could be responsible for the inefficient expression in the two *Lactobacillus* species and *Lactococcus lactis*,[25,32] and this assumption also seems to hold for *E. coli*.

We recently characterized several β-galactosidases of the LacLM type isolated from different lactobacilli.[26,33−35] The enzyme from *L. sakei* shows properties that are in general quite similar to those found for the other lactobacillal β-galactosidases, for example, with respect to pH and temperature optima or kinetic properties. One noteworthy aspect of *L. sakei* β-gal is its K_m value of 20 mM (corresponding to approximately 6.8 g/L lactose), which is relatively low when compared to those of other microbial β-galactosidases (see, e.g., the BRENDA Enzyme Database; http://www.brenda-enzymes.org). This low Michaelis constant ensures efficient utilization of lactose by *L. sakei* even when this disaccharide is present in low concentrations as, for example, in sausage production. An additional attractive property that is of interest for a potential application of *L. sakei* β-gal is its very slight inhibition by the end-products D-galactose and D-glucose. This end-product inhibition of β-gal activity can be a severe limitation in technical processes, especially when lactose-free or lactose-reduced products are the goal, because complete lactose hydrolysis is very difficult to achieve when this inhibition is pronounced. We determined an inhibition constant $K_{i,Gal}$ of 180 mM for *L. sakei* β-gal and D-galactose, which is considerably higher (and hence indicates less inhibition) than reported for most other microbial β-galactosidases, with $K_{i,Gal}$ values typically found in the range of 0.93 mM for β-gal from *Bacillus licheniformis*[36] to 366 mM for the enzyme from *Sulfolobus solfataricus*.[37] Arguably a better way to compare this inhibition, however, is by considering the ratio of the inhibition constant $K_{i,Gal}$ to the Michaelis constant $K_{m,Lac}$, which can be interpreted as a specificity constant that determines preferential binding of the substrate lactose versus that of the competitive inhibitor D-galactose.[17,38] Therefore, a high value for this ratio is important for an efficient hydrolysis, with only moderate product inhibition. This ratio, $K_{i,Gal}/K_{m,Lac}$, was calculated to be 9.0 for β-gal from *L. sakei*, which is higher than the respective ratio for most other β-galactosidases (e.g., 0.0055 for β-gal from *B. licheniformis*,[36] 0.4 for *Caldicellulosiruptor saccharolyticus*,[38] 4.0 for β-gal from *S. solfataricus* [37]). This ratio is also higher than that previously reported by us for β-gal from *L. reuteri* ($K_{m,Lac}$ = 31 mM; $K_{i,Gal}$ = 89 mM; $K_{i,Gal}/K_{m,Lac}$ = 3.0),[26] indicating that β-gal from *L. sakei* could be advantageous for complete lactose hydrolysis because of this very slight product inhibition. This weak inhibition is also obvious in the comparison of the inhibition constant $K_{i,Gal}$ and the Michaelis constants for the artificial substrates *o*NPG or *p*NPG $K_{m,NPG}$, which can be retrieved more frequently from the scientific literature. The ratio $K_{i,Gal}/K_{m,NPG}$ was calculated to be 300 for β-gal from *L. sakei*, whereas the corresponding ratio for the commercially important enzymes from *Kluyveromyces lactis* and *Aspergillus niger* are 12.5[39] and 0.36,[40] indicating again the much less pronounced inhibition by D-galactose of the *L. sakei* enzyme.

Another aspect that makes this particular enzyme attractive is its application in the synthesis of oligosaccharides. Using β-gal from *L. sakei* and lactose at an initial concentration of 215 g/L, we obtained GalOS in a total yield of 41%. This efficiency in producing GalOS in high yields seems to be superior to that found for other β-galactosidases from various LAB, which have reported yields ranging from 28% for β-gal from *L. plantarum* to ∼38% for *L. acidophilus*, *L. reuteri*, and *Streptococcus thermophilus* when using comparable initial concentrations of lactose.[11,13,34,35] The lactose concentration of 215 g/L that was used here is relatively low compared to other studies.[16−18] However, this concentration was selected deliberately because it corresponds to the solubility limit of lactose under ambient conditions and reflects the realistic technological situation. Lactose concentration is one of the most important parameters affecting oligosaccharide yields,[16−18] and hence supersaturated lactose solutions as used by others in concentrations of up to 600 g/L could further improve GalOS yields when using β-gal from *L. sakei*.

β-Galactosidase from *L. sakei* formed GalOS structurally similar to those obtained with other β-galactosidases from LAB,[13,16,29,33,35] yet proportions of individual components varied to some extent. The predominant oligosaccharide products were identified as β-D-Gal*p*-(1→6)-D-Glc (allolactose), β-D-Gal*p*-(1→6)-D-Gal, β-D-Gal*p*-(1→3)-D-Glc, β-D-Gal*p*-(1→3)-D-Gal, β-D-Gal*p*-(1→6)-Lac, and β-D-Gal*p*-(1→3)-Lac, indicating that this β-galactosidase has a propensity to synthesize β-(1→6)- and β-(1→3)-linked GalOS. At maximal GalOS production (77% lactose conversion), β-D-Gal*p*-(1→6)-D-Lac was the most abundant oligosaccharide species formed, comprising 37% of total GalOS, followed by β-D-Gal*p*-(1→6)-D-Glc as the second most important GalOS compound produced (23% of total GOS). Especially, this yield of the trisaccharide is significantly higher than that obtained with β-gal from, for example, *L. reuteri* or *L. plantarum*.[33] Hence, different enzymes can be used to tailor GalOS mixtures to contain certain desired oligosaccharide structures. Current commercial GOS products contain structures with predominant β-(1→4)-linkages,[18,29,41] whereas the

lactobacillal enzymes show a strong propensity to form β-(1→6)-linked transgalactosylation products. In a study on the structure−function relationship of various disaccharides with respect to their prebiotic effect, it was shown that among a group of galactose-containing disaccharides, those containing a (1→6)-linkage supported growth of bifidobacteria best in mixed culture populations.[42] Because of this strong bifidogenic effect of these compounds, a GalOS mixture produced with β-galactosidase from *L. sakei* that is rich in these β-(1→6)-linked oligosaccharides should therefore be of considerable interest as a prebiotic oligosaccharide mixture.

■ ABBREVIATIONS USED

β-gal, β-galactosidase; CE, capillary electrophoresis; DTT, 1,4-dithiothreitol; GalOS, galacto-oligosaccharides; GOD, glucose oxidase; HPAEC-PAD, high-performance anion exchange chromatography with pulsed amperometric detection; IPTG, isopropyl-β-D-thiogalactoside; Lac, lactose; MUG, 4-methylumbelliferyl β-D-galactoside; *o*NP, *o*-nitrophenol; *o*NPG, *o*-nitrophenyl-β-D-galactopyranoside; *p*NPG, *p*-nitrophenyl-β-D-galactopyranoside; PMSF, phenylmethanesulfonyl fluoride.

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