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Article

Structural Comparison of Different Galacto-oligosaccharide Mixtures Formed by β-Galactosidases from Lactic Acid Bacteria and Bifidobacteria

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ABSTRACT: The LacLM-type β-galactosidase from *Lactobacillus helveticus* DSM 20075 expressed in both *Escherichia coli* (*EcoliBL21Lhβ*-gal) and *Lactobacillus plantarum* (*Lp609Lhβ*-gal) was tested for their potential to form galacto-oligosaccharides (GOS) from lactose. The Lh-GOS mixture formed by β-galactosidase from *L. helveticus*, together with three GOS mixtures produced using β-galactosidases of both the LacLM and the LacZ type from other lactic acid bacteria, namely, *L. reuteri* (Lr-GOS), *L. bulgaricus* (Lb-GOS), and *Streptococcus thermophilus* (St-GOS), as well as two GOS mixtures (Br-GOS1 and Br-GOS2) produced using β-galactosidases (β-gal I and β-gal II) from *Bifidobacterium breve*, was analyzed and structurally compared with commercial GOS mixtures analyzed in previous work (Vivinal GOS, GOS I, GOS III, and GOS V) using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), high-performance size-exclusion chromatography with a refractive index (RI) detector (HPSEC-RI), and one-dimensional ^{1}H NMR spectroscopy. β-Galactosidases from lactic acid bacteria and *B. breve* displayed a preference to form β-(1→6)- and β-(1→3)-linked GOS. The GOS mixtures produced by these enzymes consisted of mainly DP2 and DP3 oligosaccharides, accounting for ∼90% of all GOS components. GOS mixtures obtained with β-galactosidases from lactic acid bacteria and *B. breve* were quite similar to the commercial GOS III mixture in terms of product spectrum and showed a broader product spectrum than the commercial GOS V mixture. These GOS mixtures also contained a number of GOS components that were absent in the commercial Vivinal GOS (V-GOS).

KEYWORDS: *β-galactosidase, galacto-oligosaccharides, prebiotic, lactic acid bacteria, bifidobacteria*

INTRODUCTION

β-Galactosidases (lactases; EC 3.2.1.23), which catalyze the hydrolysis of the β-1,4-D-glycosidic linkage of milk sugar lactose and its structurally related compounds to yield the monosaccharides glucose and galactose, are of importance to several biotechnological processes in the food industry.[1] The well-known applications of β-galactosidases in lactose hydrolysis include development of low-lactose and lactose-free dairy products for lactose-intolerant consumers, improvement of technological and sensory properties of foods containing lactose, and utilization of cheese whey to diminish economic and environmental problems.[2−5] In addition to their hydrolytic activity, β-galactosidases possess transgalactosylation activity to form galacto-oligosaccharides (GOS) from lactose. The mechanism of these enzymes involves two steps of which the cleavage of lactose and the formation of a covalently linked galactosyl−enzyme intermediate while releasing glucose occurs in the first step, followed by the transfer of galactosyl moieties from the donor sugar lactose to an acceptor, which can be water, lactose, or any of the sugar products in the reaction mixture.[6,7] In the trans-galactosylation mode, galactosyl moieties of lactose are transferred to other saccharides instead of water to yield GOS with different degrees of polymerization as reaction products.[8,9]

GOS are known as a mixture of carbohydrates consisting of non-lactose disaccharides, various trisaccharides, and higher oligosaccharides with the degree of polymerization up to eight (DP8).[10] Sugar units in GOS typically join together with β-(1→4) and β-(1→6) linkages, but β-(1→2) and β-(1→3) linkages are also reported.[11−15] Currently, GOS are considered as dominant functional food ingredients fulfilling the criteria of "prebiotics"[16] as reported in the literature on their modulatory effects on gut microbiota including stimulation of beneficial bacteria such as bifidobacteria and lactobacilli as well as inhibition of "undesirable" bacteria, maintenance of gut health, beneficially affecting the bowel functions, and colitis prevention.[17,18] In addition to these confirmed beneficial effects, postulated physiological benefits of GOS include suppression of intestinal disturbances and colorectal cancer, lowering serum cholesterol levels, reducing risk of cardiovascular diseases, increasing absorption and retention of divalent

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minerals, and promoting immune responses.[10,16,19,20] Although GOS are not a significant element in human breast milk, they were shown to have a similar bifidogenic effect[21] and therefore are used as key fortificants in infant formulas.[19]

A number of β-galactosidases from microbial sources have been isolated and purified for the production of GOS.[3] Among the β-galactosidase-producing microorganisms, some species of lactic acid bacteria, predominantly lactobacilli, and bifidobacteria have attracted great attention and have been studied intensively because of their probiotic potential and their generally recognized as safe (GRAS) status. It is anticipated that GOS synthesized by lactobacillal and bifidobacterial β-galactosidases would have structural characteristics, which will be preferentially utilized by this group of beneficial intestinal microorganism that facilitates the growth and metabolic activity of gut microbiota.[8,22]

In the present study, we describe the formation of GOS from lactose using the recombinant LacLM-type β-galactosidase from the industrially important lactic acid bacterium *Lactobacillus helveticus* DSM 20075. Individual GOS components of the resulting GOS mixtures were identified and structurally compared with different GOS mixtures formed by β-galactosidases from several lactic acid bacteria and bifidobacteria, including *Lactobacillus reuteri*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus salivarius* subsp. *thermophilus*, and *Bifidobacterium breve*, in comparison with some commercial GOS products studied previously.[23] Significantly, the information on the GOS profiles obtained from this study will provide deeper insights into lactic acid bacterial and bifidobacterial β-galactosidases with respect to the spectrum of their GOS products.

MATERIALS AND METHODS

Chemicals. All chemicals and analytical standards of D-glucose, D-galactose, and lactose used in this study were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise and were of the highest purity available. Vivinal GOS (V-GOS; 59% GOS, 21% lactose, 19% glucose, and 1% galactose) and the GOS mixtures, GOS I, GOS III, and GOS V are the commercial GOS standards from previous work.[23] Isopropyl-β-D-thiogalactopyranoside (IPTG) was obtained from Roth (Karlsruhe, Germany). Peptide pheromone IP-673 was synthesized by Caslo (Lyngby, Denmark). The Glucose (HK) Assay Kit for the determination of D-glucose was obtained from Megazyme (Wicklow, Ireland).

β-Galactosidase Assays. The determination of β-galactosidase activity was performed in 50 mM sodium phosphate buffer (NaPB; pH 6.5) at 30 °C for 10 min using 22 mM *o*-nitrophenyl-β-D-galactopyranoside (*o*NPG) or 600 mM lactose as previously described.[24] When the artificial chromogenic substrate *o*NPG was used, the change in absorbance due to the release of *o*-nitrophenol (*o*NP) was measured at 420 nm using a spectrophotometer (Beckman DU 800, Palo Alto, CA). One unit of *o*NPG activity was defined as the amount of β-galactosidase liberating 1 μmol of *o*NP per minute under the specified conditions. When the natural substrate lactose was used, the release of glucose in the reaction mixture was determined using the Glucose (HK) Assay Kit according to the manufacturer's instructions. One unit of lactose activity referred to the amount of β-galactosidase liberating 1 μmol of glucose per minute under the specified condition.

Preparation of Recombinant β-Galactosidases from *L. helveticus*. *Escherichia coli* BL21 Star (DE3) carrying pET21-lacLMLh and *Lactobacillus plantarum* TLG02 carrying p609lacLMLh, which contain the β-galactosidase encoding genes *lacLM* of *L. helveticus* DSM 20075, were cultivated and used for production of recombinant *L. helveticus* β-galactosidases as described previously.[25] Heterologous expression of β-galactosidase in *E. coli* BL21 Star (DE3) was performed in 300 mL of Luria-Bertani (LB) broth containing 100 μg/mL ampicillin. The recombinant *E. coli* strain was grown at 37 °C with shaking to an OD_{600} of 0.6. IPTG was then added into the culture to a final concentration of 1 mM, and the culture was further incubated at 25 °C for 18 h. The expression of β-galactosidase in *L. plantarum* TLG02 carrying p609lacLMLh was performed in 1 L of MRS broth. The recombinant *L. plantarum* strain was grown at 30 °C to an OD_{600} of 0.3. Inducing peptide pheromone IP-673[26] was then added to a final concentration of 25 ng/mL, and the culture was further incubated at 30 °C for 4 h. The cultures were harvested by centrifugation (4000*g*, 4 °C for 15 min), washed twice with 50 mM NaPB (pH 6.5), and resuspended in the same buffer containing 1 mM PMSF. Cell disruption was performed using a French press (Aminco, Silver Spring, MD). After centrifugation (10,000*g* for 15 min at 4 °C), the crude recombinant enzymes from *E. coli* BL 21 (DE3) (*EcoliBL21Lhβ*-gal) and *L. plantarum* TLG02 (*Lp609Lhβ*-gal) were collected and stored at −20 °C. *EcoliBL21Lhβ*-gal was then purified to apparent homogeneity. The purification was carried out by immobilized metal affinity chromatography (IMAC) using a prepacked 1 mL HisTrap HP column with Ni-Sepharose resin (GE Healthcare, Uppsala, Sweden) as described previously.[25] Active fractions were collected, desalted, and concentrated by ultrafiltration using an Amicon ultra centrifugal filter unit with a 30 kDa cutoff membrane (Millipore, MA, USA). The purified *EcoliBL21Lhβ*-gal was kept in 50 mM NaPB (pH 6.5) at 4 °C.

GOS Formation by Recombinant β-Galactosidases from *L. helveticus*. Batch conversions of lactose for the formation of GOS were carried out using recombinant β-galactosidases from *L. helveticus*, crude enzyme *Lp609Lhβ*-gal, and purified enzyme *EcoliBL21Lhβ*-gal. The reactions were performed based on the enzyme properties obtained from our previous study.[25] Reaction conditions were 205 g/L initial lactose concentration in 50 mM NaPB (pH 6.5) containing 1 mM $MgCl_2$, β-galactosidase (1.5 U_{Lac}/mL of reaction mixture), and constant agitation (300 rpm). Different reaction temperatures varying from 37−50 °C were applied for crude enzyme *Lp609Lhβ*-gal, while those varying from 30−50 °C were applied for purified enzyme *EcoliBL21Lhβ*-gal. Samples were withdrawn periodically to monitor their residual enzyme activities with *o*NPG as the substrate. Sugar compositions and size distribution of the GOS mixtures were analyzed by HPAEC-PAD, HPSEC-RI, and one-dimensional ^{1}H NMR spectroscopy.

Other GOS Preparations. The purified GOS mixtures Lr-GOS, free from monosaccharides and unconverted lactose, produced using recombinant LacLM-type β-galactosidase from *L. reuteri* L103 were prepared as previously described by Maischberger et al.[14] Preparation of the GOS mixtures, Lb-GOS and St-GOS, using LacZ-type β-galactosidases from *L. delbrueckii* subsp. *bulgaricus* DSM 20081[15] and *S. salivarius* subsp. *thermophilus* DSM 20259,[2] respectively, and Br-GOS1 and Br-GOS2 using purified LacZ-type β-galactosidases β-gal I and β-gal II from *B. breve* DSM 20213,[12] respectively, was carried out under the conditions that yielded the highest GOS for each enzyme as reported previously. The GOS mixtures were analyzed by HPAEC-PAD, HPSEC-RI, and one-dimensional ^{1}H NMR spectroscopy.

High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD). Carbohydrate contents in the reaction mixtures were analyzed by HPAEC-PAD at the Food Biotechnology Lab (BOKU; Vienna). HPAEC-PAD analyses were performed on a Dionex DX-500 system (Dionex Corp., Sunnyvale, CA) as previously described in detail.[27] The GOS yield (%) was defined as the percentage of GOS produced in the samples compared to initial lactose.

Detailed structural analyses of the GOS mixtures were also performed using HPAEC-PAD at the laboratory of Groningen Biomolecular Sciences and Biotechnology Institute (University of Groningen). GOS syrup samples were diluted by pipetting 20.2 ± 0.40 μL of GOS syrup into 1.990 ± 0.007 mL of Milli-Q water containing 1.01 ± 0.02 mM fucose as an internal standard. Reference GOS samples, used to identify peaks, were diluted ∼400 times first by diluting a volume of thick GOS syrup 1:1 with Milli-Q and then diluting it 200 times by adding ∼10 μL in 1.990 ±0.007 mL of Milli-

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Q water containing a 1.01 ± 0.02 mM fucose internal standard. GOS samples (10 μL, ∼1.0 mg/mL) were profiled on a Dionex ICS-3000 workstation (Dionex, Amsterdam, The Netherlands) equipped with a CarboPac PA-1 column (250 × 2 mm, Dionex) and an ICS-3000 ED pulsed amperometric detector (PAD). Oligosaccharides were eluted using a complex gradient of A: 100 mM NaOH, B: 600 mM NaOAc in 100 mM NaOH, C: Milli-Q water, and D: 50 mM NaOAc. The fractionations were performed at 0.25 mL/min with 10% A, 85% C, and 5% D in 25 min to 40% A, 10% C, and 50% D followed by a 35 min gradient to 75% A and 25% B directly followed by 5 min washing with 100% B and reconditioning for 7 min with 10% A, 85% B, and 5% D.[23,28,29]

High-Performance Size-Exclusion Chromatography with a Refractive Index Detector (HPSEC-RI). Size distribution analysis of GOS samples (10 μL injections of ∼10 mg/mL GOS) was performed on a Rezex RSO-01 oligosaccharide Ag^+ (4%) column (200 × 10 mm; Phenomenex, Utrecht, The Netherlands) using a Waters 2690XE Alliance HPLC system (Waters, Etten-Leur, The Netherlands) equipped with a Waters 2410 RI detector. Elutions were carried out with Milli-Q water at a flow rate of 0.3 mL/min. The peaks were identified and quantified in relation to a calibration curve (0.05−20 mg/mL) of Glc, Gal, Lac, and isolated GOS fractions up to DP5.

NMR Spectroscopy. Samples of ∼10 mg of GOS were exchanged two times with 300 μL of 99.9 atom % D_2O with intermediate lyophilization and finally dissolved in 650 μL of D_2O containing 25 ppm acetone as an internal standard (δ^1H 2.225). One-dimensional ^{1}H NMR spectra were recorded at a probe temperature of 25 °C on a Varian Inova 500 spectrometer (NMR Department, University of Groningen, The Netherlands). Spectra were recorded with a 4500 Hz spectral width at 16,000 complex data points using a WET1D pulse to suppress the HOD signal. All spectra were processed using MestReNova 12 (Mestrelabs Research SL, Santiago de Compostela, Spain), applying manual phase corrections and Whittaker Smoother baseline corrections.

Statistical Analysis. All experiments and measurements were conducted at least in duplicate, and the standard deviation (SD) never exceeded 5%. The data are expressed as the mean ± SD with significant digits when appropriate.

RESULTS AND DISCUSSION

Lactose Conversion and GOS Formation by Recombinant *β*-Galactosidases from *L. helveticus*. The thermophilic lactic acid bacterium *L. helveticus* is extensively used as starter cultures for various milk fermentation processes. The strain is capable of utilizing lactose by exhibiting intracellular *β*-galactosidase activity.[30,31] It was shown previously that two recombinant *β*-galactosidases from *L. helveticus* DSM 20075, *EcoliBL21Lhβ*-gal and *Lp609Lhβ*-gal, were able to convert lactose using a batch mode of conversion and were found to be promising candidates for the production of prebiotic GOS.[25] It was also shown in our previous study that both enzymes are stable in the presence of high concentrations of lactose, retaining more than 75 and 60% of the initial activity, respectively, after 24 h of incubation at 50 °C.[25] Therefore, there is great potential for application of these recombinant *β*-galactosidases in lactose conversion processes at temperatures up to 50 °C.

In this study, we looked in more detail at the lactose conversions catalyzed by these two recombinant *β*-galactosidases from *L. helveticus* DSM 20075 at various process temperatures and performed a detailed analysis of the GOS mixtures formed. As shown in Figure 1A,B, lactose conversions catalyzed by the crude enzyme *Lp609Lhβ*-gal and the purified enzyme *EcoliBL21Lhβ*-gal approached completion (>98%) within 12 h at the examined process temperatures varying from 30 to 50 °C. The rate of lactose conversion considerably

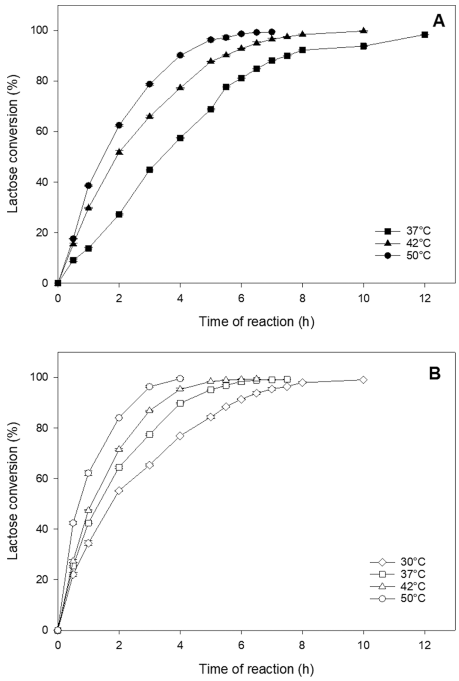

Figure 1. Time course of lactose conversion catalyzed by (A) crude enzyme *Lp609Lhβ*-gal and (B) purified enzyme *EcoliBL21Lhβ*-gal. The reactions were carried out with an initial lactose concentration of 205 g/L in 50 mM sodium phosphate buffer (pH 6.5) containing 1 mM $MgCl_2$ and 1.5 U_{Lac}/mL of enzyme.

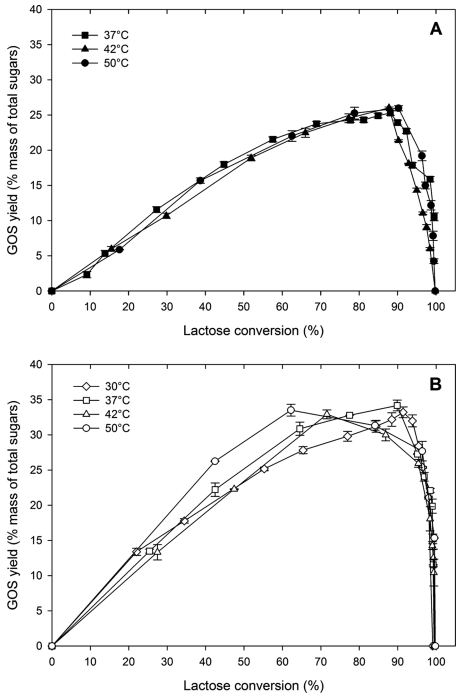

Figure 2. GOS formation and degradation during lactose conversion catalyzed by (A) crude enzyme *Lp609Lhβ*-gal and (B) purified enzyme *EcoliBL21Lhβ*-gal. The reactions were carried out with an initial lactose concentration of 205 g/L in 50 mM sodium phosphate buffer (pH 6.5) containing 1 mM $MgCl_2$ and 1.5 U_{Lac}/mL of enzyme.

increased with increasing reaction temperature. The conversions at 50 °C occurred most rapidly and achieved ∼99% lactose conversion after 6 and 4 h with the crude enzyme *Lp609Lhβ*-gal and the purified enzyme *EcoliBL21Lhβ*-gal,

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Table 1. Individual GOS Components in the Different GOS Mixtures Analyzed[a,b]

peak[c]	GOS component	DP	V-GOS	GOS I	GOS III	GOS V	Lh-GOS	Lr-GOS	Lb-GOS	St-GOS	Br-GOS1	Br-GOS2
1	D-Gal*p* (D-galactose)		●	●	●	●	●	○	●	●	●	●
2	D-Glc*p* (D-glucose)		●	●	●	●	●	○	●	●	●	●
3	*β*-D-Gal*p*-(1→6)-D-Gal*p*	2	●	●	●	●	●	●	●	●	●	●
4	*β*-D-Gal*p*-(1→6)-D-Glc*p* (allolactose)	2	●	●	●	●	●	●	●	●	●	●
5	*β*-D-Gal*p*-(1→4)-D-Glc*p* (lactose)	2	●	●	●	●	●	○	●	●	●	●
6a	*β*-D-Gal*p*-(1→4)-[*β*-D-Gal*p*-(1→6)-]D-Glc*p*	3	●	●	●	●	●	●	●	●	●	●
6b	*β*-D-Gal*p*-(1→6)-*β*-D-Gal*p*-(1→4)-D-Glc*p*	3	●	●	●	●	●	●	●	●	●	●
7	*β*-D-Gal*p*-(1→4)-D-Gal*p*	2	●	●	●	●	○	○	○	○	○	○
8a	*β*-D-Gal*p*-(1→2)-D-Glc*p*	2	●	●	●	●	○	○	○	●	●	●
8b	*β*-D-Gal*p*-(1→3)-D-Glc*p*	2	●	●	●	●	●	●	●	●	●	●
9	*β*-D-Gal*p*-(1→2)-[*β*-D-Gal*p*-(1→4)-]D-Glc*p*	3	●	●	●	○	○	○	○	○	○	○
10a	*β*-D-Gal*p*-(1→2)-[*β*-D-Gal*p*-(1→6)-]D-Glc*p*	3	●	●	●	○	○	○	○	○	○	○
10b	*β*-D-Gal*p*-(1→3)-[*β*-D-Gal*p*-(1→6)-]D-Glc*p*	3	●	●	●	○	●	○	●	●	○	●
11	*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→4)-D-Glc*p*	3	●	●	●	●	○	○	○	○	○	○
12	*β*-D-Gal*p*-(1→3)-*β*-D-Gal*p*-(1→4)-D-Glc*p*	3	●	○	●	●	●	●	●	●	●	●
13a	*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→2)-D-Glc*p*	3	●	●	○	○	○	○	○	○	○	○
13b	*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→3)-D-Glc*p*	3	●	●	○	○	○	○	○	○	○	○
14a	*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→6)-[*β*-D-Gal*p*-(1→4)-]D-Glc*p*	4	●	○	○	○	○	○	○	○	○	○
14b	*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→4)-[*β*-D-Gal*p*-(1→6)-]D-Glc*p*	4	●	○	○	○	○	○	○	○	○	○
15a	*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→2)-[*β*-D-Gal*p*-(1→4)-]D-Glc*p*	4	●	●	○	○	○	○	○	○	○	○
15b	*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→4)-[*β*-D-Gal*p*-(1→2)-]D-Glc*p*	4	●	●	○	○	○	○	○	○	○	○
16a	*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→2)-[*β*-D-Gal*p*-(1→6)-]D-Glc*p*	4	●	○	○	○	○	○	○	○	○	○
16b	*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→6)-[*β*-D-Gal*p*-(1→2)-]D-Glc*p*	4	●	○	○	○	○	○	○	○	○	○
16c	*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→6)-D-Glc*p*	4	●	○	○	○	○	○	○	○	○	○
17	*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→4)-D-Glc*p*	4	●	●	○	○	○	○	○	○	○	○
18a	*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→2)-D-Glc*p*	4	●	○	○	○	○	○	○	○	○	○
18b	*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→3)-D-Glc*p*	4	●	○	○	○	○	○	○	○	○	○
19a	*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→6)-[*β*-D-Gal*p*-(1→4)-]D-Glc*p*	5	●	○	○	○	○	○	○	○	○	○
19b	*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→4)-[*β*-D-Gal*p*-(1→6)-]D-Glc*p*	5	●	○	○	○	○	○	○	○	○	○
19c	*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→4)-[*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→6)-]D-Glc*p*	5	●	○	○	○	○	○	○	○	○	○
20a	*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→4)-[*β*-D-Gal*p*-(1→2)-]D-Glc*p*	5	●	○	○	○	○	○	○	○	○	○
20b	*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→4)-[*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→2)-]D-Glc*p*	5	●	○	○	○	○	○	○	○	○	○
20c	*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→2)-[*β*-D-Gal*p*-(1→4)-]D-Glc*p*	5	●	○	○	○	○	○	○	○	○	○
21a	*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→6)-[*β*-D-Gal*p*-(1→2)-]D-Glc*p*	5	●	○	○	○	○	○	○	○	○	○
21b	*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→2)-[*β*-D-Gal*p*-(1→6)-]D-Glc*p*	5	●	○	○	○	○	○	○	○	○	○
21c	*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→6)-[*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→2)-]D-Glc*p*	5	●	○	○	○	○	○	○	○	○	○
22	*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→4)-D-Glc*p*	5	●	○	○	○	○	○	○	○	○	○
23a	*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→2)-D-Glc*p*	5	●	○	○	○	○	○	○	○	○	○
23b	*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→3)-D-Glc*p*	5	●	○	○	○	○	○	○	○	○	○
24	*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→4)-D-Glc*p*	6	●	○	○	○	○	○	○	○	○	○
25	*β*-D-Gal*p*-(1→6)-*β*-D-Gal*p*-(1→3)-D-Glc*p*	3	○	●	●	○	●	●	●	●	○	○
26	*β*-D-Gal*p*-(1→6)-*β*-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→4)-D-Glc*p*	4	○	●	○	○	○	○	○	○	○	○

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Table 1. continued

peak[c]	GOS component	DP	V-GOS	GOS I	GOS III	GOS V	Lh-GOS	Lr-GOS	Lb-GOS	St-GOS	Br-GOS1	Br-GOS2
27	β-D-Galp-(1→6)-β-D-Galp-(1→4)-β-D-Galp-(1→3)-D-Glcp	4	○	●	○	○	○	○	○	○	○	○
28	β-D-Galp-(1→3)-β-D-Galp-(1→6)-D-Glcp	3	○	○	●	○	●	●	●	●	●	●
29	β-D-Galp-(1→3)-β-D-Galp-(1→3)-D-Glcp	3	○	○	●	○	○	○	●	○	○	●
30	β-D-Galp-(1→3)-β-D-Galp-(1→2)-D-Glcp	3	○	○	●	○	○	○	○	○	○	○
31	β-D-Galp-(1→3)-β-D-Galp-(1→3)-β-D-Galp-(1→4)-D-Glcp	4	○	○	●	○	●	●	●	●	●	●
32	β-D-Galp-(1→3)-β-D-Galp-(1→3)-β-D-Galp-(1→3)-D-Glcp	4	○	○	○	○	○	○	○	○	○	○
33	β-D-Galp-(1→3)-β-D-Galp-(1→3)-β-D-Galp-(1→2)-D-Glcp	4	○	○	○	○	○	○				
34	β-D-Galp-(1→6)-β-D-Galp-(1→6)-β-D-Galp-(1→4)-D-Glcp	4	○	○	●	○	●	●	●	●	○	○
35	β-D-Galp-(1→3)-β-D-Galp-(1→6)-β-D-Galp-(1→4)-D-Glcp	4	○	○	●	○	●	●	●	●	○	●
36	β-D-Galp-(1→3)-[β-D-Galp-(1→6)-]β-D-Galp-(1→4)-D-Glcp	4	○	○	●	○	●	●	●	●	○	●
37	β-D-Galp-(1→6)-β-D-Galp-(1→3)-β-D-Galp-(1→4)-D-Glcp	4	○	○	●	○	●	●	●	●	○	●
38	β-D-Galp-(1→3)-D-Galp	2	●	●	●	●	●	●	●	●	●	●
x	UNK	UNK	○	○	○	○	○	●	○	●	○	○

[a]Graphical presentations of individual GOS structures are shown in;[24] [ ] represents branched elongation. [b]● structure present; ○ structure absent; and UNK is abbreviated for unknown structure. [c]Peak numbers correspond to the HPAEC-PAD chromatograms presented in Figure 3.

respectively. The conversion of lactose was thus faster when using the purified enzyme *EcoliBL21Lhβ*-gal than when using the crude enzyme *Lp609Lhβ*-gal under similar process conditions. The suppressed catalytic efficiency of the crude enzyme may be caused by the interference of the impurities, such as other proteins and substances in the cell-free extract.[32]

The highest total GOS yield was ∼26% (∼53 g/L) at approximately 90% lactose conversion at 37, 42, and 50 °C (Figure 2A) when the crude enzyme *Lp609Lhβ*-gal was used in batch conversions with an initial lactose concentration of 205 g/L. The time needed to obtain 90% lactose conversion decreased from a reaction time of 7 h at 37 °C to 5 h at 42 °C and 4 h at 50 °C (Figure 1A). Trans-galactosylation may thus become more pronounced than hydrolysis at higher reaction temperatures[33] although the increased temperature hardly affected the maximum GOS yield.[15,33] Interestingly, the maximum GOS yield increased to ∼33% (∼68 g/L) when using the purified enzyme *EcoliBL21Lhβ*-gal under reaction conditions similar to those used for the crude enzyme *Lp609Lhβ*-gal. This highest GOS yield was reached when approximately 90% of the initial lactose was converted after 6 and 4 h of reaction at 30 and 37 °C, respectively (Figures 1B and 2B). The maximum GOS yield obtained with the purified enzyme *EcoliBL21Lhβ*-gal was achieved in a much shorter time at elevated reaction temperatures after 2 h at 42 °C (∼70% lactose conversion) and only after 1 h at 50 °C (∼60% lactose conversion) (Figures 1B and 2B). The highest GOS yield of approximately 33% obtained with the purified enzyme *EcoliBL21Lhβ*-gal (Figure 2B) is comparable to the reported GOS yields for other lactobacillal β-galactosidases, for instance 31% for *L. pentosus* β-galactosidase,[34] 38% for *L. reuteri* β-galactosidase,[27] and 41% for *L. sakei* β-galactosidase.[13]

Synthesis of GOS by β-galactosidases is kinetically controlled as a consequence of the competition between hydrolysis and trans-galactosylation,[9] and the GOS formed via trans-galactosylation are also potential substrates for hydrolysis. The total amount of GOS decreased dramatically after reaching their maximum yields as a result of the degradation of GOS by crude *Lp609Lhβ*-gal and purified *EcoliBL21Lhβ*-gal (Figure 2A,B, respectively), and consequently, hydrolysis prevailed over trans-galactosylation when the substrate lactose was depleted. This phenomenon was well observed and reported in the literature.[15,35]

Identification of Individual GOS Components Formed Using Recombinant β-Galactosidases from *L. helveticus*. The main products of the GOS mixture (Lh-GOS) formed via trans-galactosylation of lactose using recombinant β-galactosidases from *L. helveticus* were identified to be β-D-Galp-(1→6)-D-Galp (peak 3), β-D-Galp-(1→6)-D-Glcp (allolactose) (peak 4), β-D-Galp-(1→6)-D-Lac (peak 6), β-D-Galp-(1→3)-D-Lac (peak 12), β-D-Galp-(1→3)-D-Glcp (peak 8b), and β-D-Galp-(1→3)-D-Galp (peak 38) (Table 1, Figure 3). The enzyme thus showed a strong preference for the formation of β-D-(1→3) and β-D-(1→6) linkages in accordance with other lactobacillal β-galactosidases reported previously.[13−15,34] The composition of the GOS mixture formed in batch conversion at 30 °C with an initial lactose concentration of 205 g/L using the purified enzyme *EcoliBL21Lhβ*-gal was determined to be 19.3% disaccharides (DP2), 11.5% trisaccharides (DP3), and 1.2% tetrasaccharides (DP4) (Table 2).

Structural Comparison of Different GOS Mixtures. GOS mixtures produced using β-galactosidases from different lactic acid bacteria (Lh-GOS, Lr-GOS, Lb-GOS, and St-GOS) and *B. breve* (Br-GOS1 and Br-GOS2) were analyzed and structurally compared with previously studied commercial GOS of known compositions (V-GOS, GOS I, GOS III, and GOS V).[23] All the GOS samples, except Lr-GOS, contain the monosaccharides (galactose, peak 1 and glucose, peak 2) and unconverted lactose (peak 5) (Figure 3). The Lr-GOS mixture was purified after the conversion to remove the monosaccharides and unconverted lactose.[14] A major component found in all GOS samples is the disaccharide allolactose (peak 4, Figure 3). Trans-galactosylation is known to involve intermolecular and intramolecular reactions of which the latter reaction is the result of direct galactosyl transfer to D-glucose to

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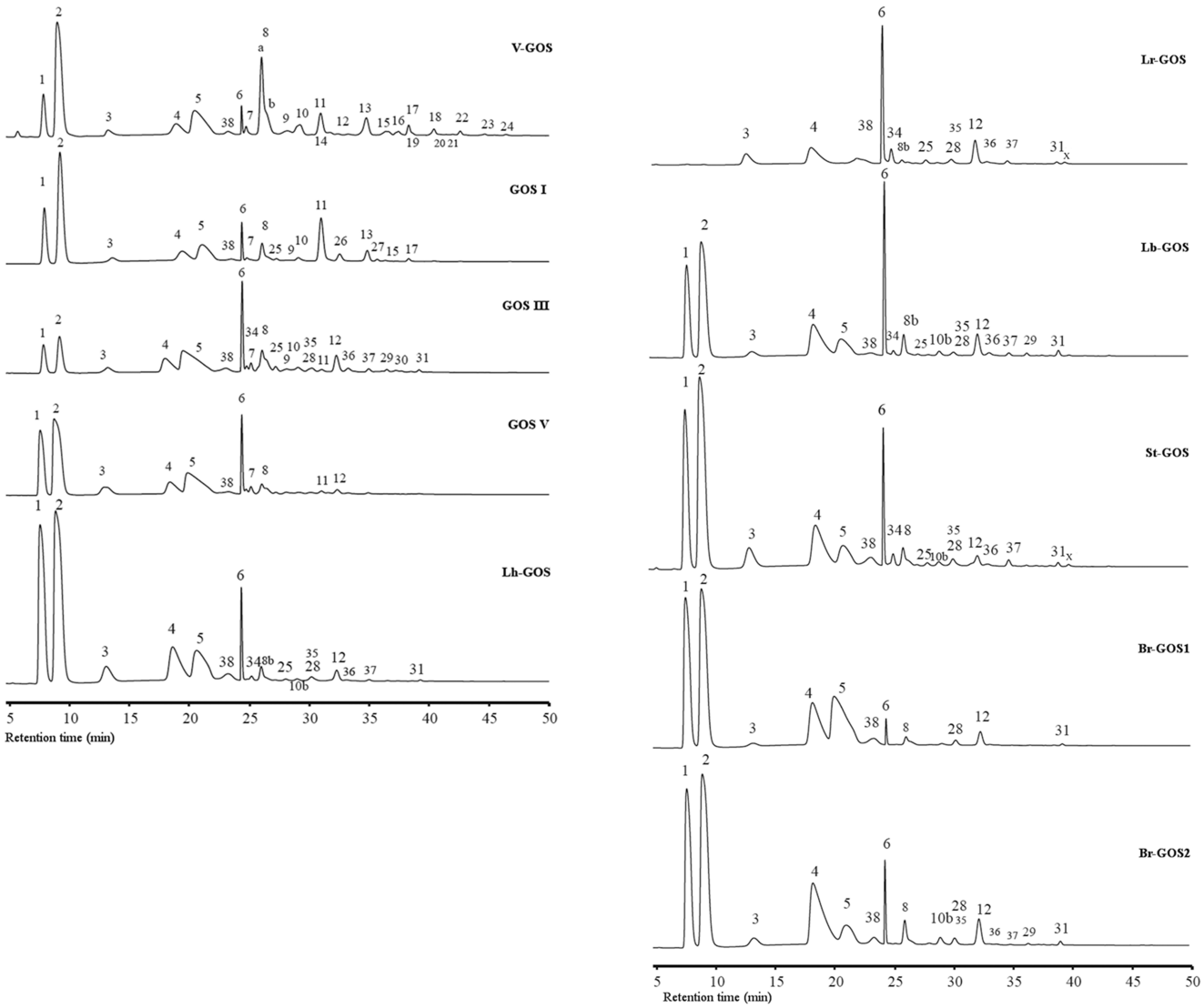

Figure 3. HPAEC-PAD profiles of different GOS mixtures. Assigned peaks are numbered and correspond with the GOS components presented in Table 1.

Table 2. Composition (as % Mass of Total Sugars) of the GOS Mixtures Prepared Using β-Galactosidases from Lactic Acid Bacteria and *B. breve* as Analyzed by HPAEC-PAD and HPSEC-RI

GOS mixture	DP4	DP3	DP2 (non-lactose)	glucose	galactose	lactose
Lh-GOS[a]	1.20 ± 0.13	11.53 ± 0.51	19.28 ± 0.04	33.32 ± 0.28	26.07 ± 0.30	8.59 ± 0.10
Lr-GOS 60%[b]	5.11 ± 0.15	71.17 ± 0.45	23.36 ± 0.09	0.37 ± 0.09	0.00 ± 0.00	0.00 ± 0.00
Lr-GOS 80%[c]	9.12 ± 0.14	71.23 ± 0.64	19.07 ± 0.27	0.53 ± 0.44	0.05 ± 0.07	0.00 ± 0.00
Lb-GOS[d]	3.57 ± 0.05	35.11 ± 0.03	13.12 ± 0.37	23.50 ± 0.04	12.29 ± 0.08	12.41 ± 0.40
St-GOS[e]	7.12 ± 0.76	19.88 ± 0.37	18.22 ± 0.11	29.58 ± 0.13	16.40 ± 0.07	8.80 ± 0.08
Br-GOS2[f]	1.84 ± 0.15	17.05 ± 0.07	15.79 ± 0.79	27.07 ± 0.04	21.07 ± 0.04	17.18 ± 0.87

[a]Lh-GOS is the GOS mixture formed with purified enzyme *EcoliBL21Lhβ*-gal (recombinant β-galactosidase from *L. helveticus*). The reactions were carried out at 30 °C under the conditions as described in Materials and Methods. [b]Lr-GOS is the GOS mixture formed with β-galactosidase from *L. reuteri*, collected at 60% lactose conversion and purified to remove lactose and monosaccharides.[14] [c]Lr-GOS is the GOS mixture formed with β-galactosidase from *L. reuteri*, collected at 80% lactose conversion and purified to remove lactose and monosaccharides.[14] [d]Lb-GOS is the GOS mixture formed with β-galactosidase from *L. bulgaricus*.[15] [e]St-GOS is the GOS mixture formed with β-galactosidase from *S. thermophilus*.[2] [f]Br-GOS2 is the GOS mixture formed with β-galactosidase (β-gal II) from *B. breve*.[12]

yield regioisomers of lactose, such as allolactose. In intramolecular trans-galactosylation, the glycosidic bond of lactose is cleaved and immediately formed again at a different position of the glucose.[27,36,37] All commercial GOS mixtures contained β-(1→6), β-(1→4), β-(1→3), and β-(1→2) transfer products.[23] The β-(1→4)-linked oligosaccharides are predominant in V-GOS produced using β-galactosidase from *B. circulans*, which was previously reported to produce predominantly β-

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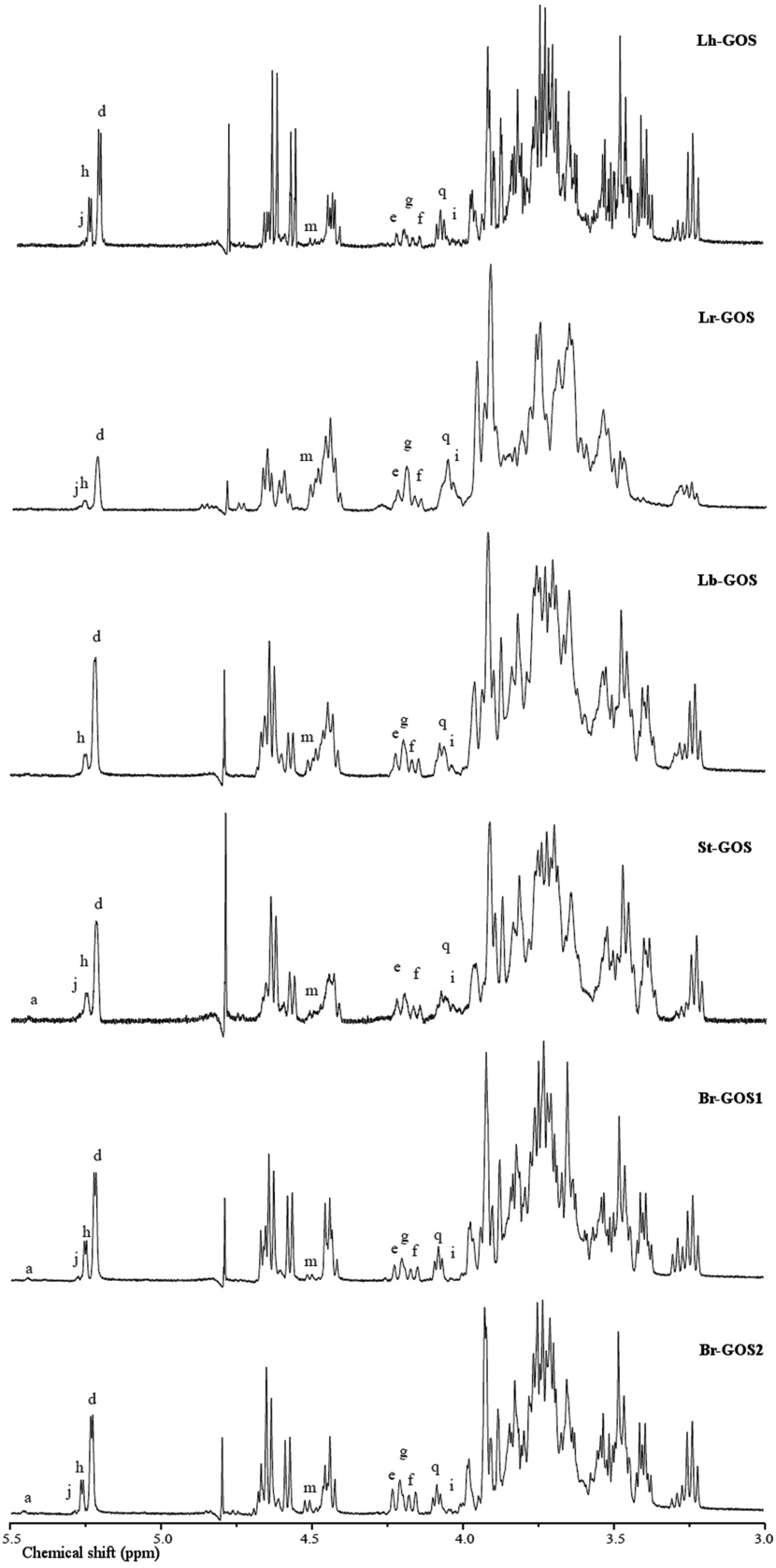

Figure 4. One-dimensional ^{1}H NMR spectra of GOS mixtures prepared using β-galactosidases from lactic acid bacteria and *B. breve*. Peaks of structural-reporter-group signals have been previously explained.[23,28,29]

(1→4) transfer products.[23,38] GOS mixtures were also analyzed by HPSEC-RI and DP distribution data of the previously studied commercial V-GOS, GOS I, GOS III, and GOS V[23] and were included for comparison. V-GOS provided

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Table 3. ^{1}H NMR Structural-Reporter-Group Signals Employed for the Evaluation of One-Dimensional ^{1}H NMR Spectra of GOS Mixtures[a]

NMR signal	chemical shift (ppm)	explanation	HPAEC-PAD signal
a	5.45	H-1 of a 2-substituted α-D-Glc*p* unit; [β-D-Gal*p*-(1→2)-α-D-Glc*p*]	8a, 9, 10a, 15b, 16b, 20a, 21a, 30, 33
d	5.23−5.22	H-1 of a 4-, 6-, and/or 1-substituted α-D-Glc*p* unit; [β-D-Gal*p*-(1→4/6)-α-D-Glc*p*; α-D-Glc*p*-(1↔1)-β-D-Gal*p*] H-1 of free α-D-Glc*p*	4, 5, 6ab, 11, 12, 14ab, 16c, 17, 19a, 22, 24, 26, 28, 31, 34, 35, 36, 37, 2
e	4.22−4.21	H-6a of a 6-substituted β-D-Glc*p* unit; [β-D-Gal*p*-(1→6)-β-D-Glc*p*]	4, 10ab, 16abc, 21abc, 28
f	4.17−4.16	H-6a of a 6-substituted α-D-Glc*p* unit; [β-D-Gal*p*-(1→6)-α-D-Glc*p*]	4, 10ab, 16abc, 21abc, 28
g	4.21−4.17	H-4 of a 3- and/or 4-substituted (reducing) β-D-Gal*p* unit; {β-D-Gal*p*-(1→3/4)-β-D-Gal*p*[−(1→]}	7, 11, 12, 13ab, 14ab, 15ab, 16abc, 17, 18ab, 19abc, 20abc, 21abc, 22, 23ab, 24, 26, 27, 28, 29, 30, 31, 32, 33
h	5.26	H-1 of free α-D-Gal*p*	1
i	4.08−4.05	H-6a of a 6-substituted (reducing) β-D-Gal*p* unit; {β-D-Gal*p*-(1→6)-β-D-Gal*p*[−(1→]}	3, 6b, 25, 26, 27
j	5.27	H-1 of a 4- and/or 6-substituted reducing α-D-Gal*p* unit; [β-D-Gal*p*-(1→4/6)-α-D-Gal*p*]	3, 7
m	4.51−4.52	H-1 of a 3-substituted β-D-Gal*p*-(1→4)- unit in a β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)- D-Glc*p* sequence	12, 31
q	4.10	H-5 of a 3-substituted (reducing) α-D-Gal*p* unit; [β-D-Gal*p*-(1→3)-α-D-Gal*p*]	38

[a]NMR signals reflect the structure element of GOS components.[23,28,29]

the broadest spectrum of oligosaccharide components with a DP ranging from DP2 to DP6, while other commercial GOS samples (GOS I, GOS III, and GOS V) and the GOS mixtures produced using lactic acid bacterial and bifidobacterial β-galactosidases contain GOS components with the DP up to DP4. The compositions of the latter GOS mixtures in terms of DP2, DP3, and DP4 are summarized in Table 2.

Interestingly, GOS mixtures obtained with β-galactosidases from lactic acid bacteria and *B. breve* are quite similar to the commercial GOS III in terms of the product spectrum. They contain several GOS components that are not present in V-GOS, such as β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→3)-D-Glc*p* (peak 25), β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→6)-D-Glc*p* (peak 28), β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Glc*p* (peak 31), and β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Glc*p* (peak 37), and clearly provided a more varied GOS composition than GOS V (Figure 3). Lactic acid bacterial and bifidobacterial β-galactosidases clearly showed a preference for β-D-Gal*p*-(1→6) transfer to form 6′-galactobiose (peak 3), allolactose (peak 4), 6′-galactosyllactose (peak 6), and 6′-6′-digalactosyllactose (peak 34 in the cases of Lh-, Lr-, Lb-, and St-GOS mixtures) as well as for the formation of β-D-Gal*p*-(1→3) structural elements including β-D-Gal*p*-(1→3)-D-Glc*p* (peak 8b), 3′-galactosyllactose (peak 12), 3′-galactosylallolactose (peak 28), 3′-3′-digalactosyllactose (peak 31), and 3′-galactobiose (peak 38) (Figure 3). Notably, two trisaccharides, 6′-galactosyllactose and 3′-galactosyllactose, which are the two main components of GOS mixtures produced using lactic acid bacterial and bifidobacterial β-galactosidases, are present in the V-GOS and GOS I samples only in minor and trace amounts, respectively. Furthermore, Lr-GOS and St-GOS mixtures also showed the presence of an unknown component (peak x, after peak 31) in the HPAEC-PAD chromatograms (Figure 3), which was not present in any commercial GOS sample.

Generally, β-(1→6) and β-(1→3) linkages were formed predominantly in all GOS mixtures prepared using β-galactosidases from lactic acid bacteria and *B. breve*, not only with linear elongations but also with branched elongations (Table 1). There also was no evidence of β-(1→4) elongation for oligomers formed when using these enzymes. The results obtained from HPAEC-PAD analyses were supported by one-dimensional ^{1}H NMR spectroscopy data. A reference library of ^{1}H NMR spectroscopy data for GOS was established previously.[23,28,29] In the one-dimensional ^{1}H NMR spectra (Figure 4, Table 3), the presence of the major components allolactose (HPAEC-PAD peak 4, Figure 3) and 6′-galactosyllactose (HPAEC-PAD peak 6, Figure 3) was confirmed by the presence of peak **d** (∼δ 5.22 ppm). The occurrence of Gal*p*-(1→6)-Gal*p* elements was reflected by the presence of the structural-reporter-group signal at ∼4.06 ppm (peak **i**). The presence of 6-substituted Glc was shown by the presence of peak **e** (δ 4.22) and peak **f** (δ 4.16).[23,28,29] The presence of 3-substituted reducing Gal (HPAEC-PAD peak 38, Figure 3) was confirmed by the NMR peak **q** (δ 4.10), corresponding to the H-5 signal of a 3-substituted reducing Gal residue in α-configuration.[28] The presence of 2-substituted reducing Glc was shown by the one-dimensional ^{1}H NMR peak at δ 5.45 (peak **a**) and occurred only in minor levels in St-GOS, Br-GOS1, and Br-GOS2.

The structural building blocks of different GOS mixtures strongly depend on the β-galactosidases used during their synthesis. β-Galactosidases from lactic acid bacteria and *B. breve* show the preference to form β-(1→6)- and β-(1→3)-linked GOS. It was reported that the administration of a GOS mixture containing β-(1→3) as well as β-(1→4) and β-(1→6) linkages proved to have a better bifidogenic effect than a mixture containing GOS with β-(1→4) and β-(1→6) linkages.[39] Recently, we have reported the specific growth stimulation of certain desired intestinal bacteria by one of our GOS mixtures, which contained mainly oligosaccharides of β-(1→6) and β-(1→3) glycosidic linkages, and our GOS mixture stimulated the growth of several probiotic strains more than the two commercial preparations, which contain mainly β-(1→4)-linked GOS.[40] GOS mixtures produced with these enzymes consist mainly of DP2 and DP3 oligosaccharides, accounting for ∼90% of all GOS components. The major disaccharides

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present in these GOS mixtures are allolactose, 6′-galactobiose, 3′-galactobiose, and 3′-galactosyl glucose. Sanz et al.[41] investigated the prebiotic potential of a number of disaccharides and found that β-D-Galp-(1→6)-D-Gal (6′-galactobiose), one of the major disaccharides in our GOS mixtures, is a highly prebiotic molecule. In our recent study, we have reported that some bifidobacteria preferentially utilized DP2 GOS and some lactobacilli often utilized only the DP2 and DP3 molecules.[42] Trisaccharides are the main constituents in Lr-GOS and Lb-GOS of which 6′-galactosyllactose and 3′-galactosyllactose are the major components. The tetrasaccharide 3′-3′-digalactosyllactose is present in all GOS mixtures produced using lactic acid bacterial and bifidobacterial β-galactosidases, whereas 6′-6′-digalactosyllactose is only found in the GOS mixtures formed with lactic acid bacterial β-galactosidases (Lh-GOS, Lr-GOS, Lb-GOS, and St-GOS). The two tetrasaccharides, which are the β-D-Galp-(1→3) elongation of 6′-galactosyllactose and the β-D-Galp-(1→6) elongation of 3′-galactosyllactose, are found in the GOS mixtures formed with lactic acid bacterial β-galactosidases and also in Br-GOS2. These GOS mixtures contain a number of GOS components that are not present in the commercial Vivinal GOS mixture. The information on individual GOS obtained in this study as analyzed by HPAEC-PAD and confirmed with ^{1}H NMR spectroscopy has given more insights into the basic structural elements of the GOS mixtures produced using lactic acid bacterial and bifidobacterial β-galactosidases, and it will be of interest for studies on the correlation between individual GOS structures and their prebiotic potential.

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Notes

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