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Isomerization of Lactose-Derived Oligosaccharides: A Case Study Using Sodium Aluminate

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Galactooligosaccharides (GOS) obtained during the enzymatic hydrolysis of lactose contain large amounts of glucose, galactose, and unreacted lactose, which do not have prebiotic properties and increase the calorific value of the product. In this work, the isomerization of the GOS mixture by the action of sodium aluminate has been studied. During the reaction, lactose, glucose, and galactose were isomerized to lactulose, fructose, and tagatose, respectively, and in addition allolactose, 6-galactobiose, and 6′-galactosyl-lactose were also converted to the corresponding keto-sugars. The effect of time, temperature, and aluminate/initial lactose ratio has been studied. After 9 h at 40 °C and molar ratio aluminate/lactose 3:1, the isomerization yield was >60%, and the amount of final carbohydrates was close to 90% of the initial product. This process considerably decreases the amount of lactose, glucose, and galactose.

KEYWORDS: Isomerization; GOS; aluminate

INTRODUCTION

Prebiotic carbohydrates are food ingredients that resist hydrolysis in the upper part of the gastrointestinal tract and reach the colon, where they are able to exert their health benefits. The possible benefits of use of prebiotic carbohydrates are supported by the scientific data available on the nutritional effects including the selective stimulation of growth of health-promoting species belonging to the genera *Bifidobacterium* and *Lactobacillus* in colonic microbiota and improvement of bowel habit and calcium bioavailability (*1, 2*). Most research has been conducted with lactose-derived oligosaccharides (GOS), fructo-oligosaccharides, and mixtures of the two types of prebiotics (*3*−*5*), and it has been observed that the chemical structure of the oligosaccharide (glycosidic linkages, degree of polymerization, and monosaccharide composition) may affect their physiological effects such as the rate at which oligosaccharides are fermented, the proportion of the different short-chain fatty acids produced in the cecum, and mineral absorption (*6*).

Prebiotic GOS are usually produced from lactose catalyzed by β-galactosidases from different sources (*7*). Transgalactosylation involves intermolecular as well as intramolecular reactions that may give rise to disaccharide isomers of lactose as well as di-, tri-, tetra-, or higher oligosaccharides (*8*). The number of monosaccharide units and the type of linkage between the monosaccharides are regulated by the source of the enzyme and the operating conditions used in the reaction (*9*). Thus, the preparation of prebiotic carbohydrates under different experimental conditions may provide new ingredients with improved functionalities.

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In basic media, lactose is converted into lactulose by isomerization of the glucose moiety to fructose with yields of 70−80% using amphoteric electrolytes such as aluminum hydroxide (*10*). In contrast to lactose, lactulose is not hydrolyzed by human intestinal enzymes and enters the large intestine, where it exerts a prebiotic effect. Since the 1950s, lactulose has several pharmaceutical applications and is also used as a prebiotic ingredient (*11, 12*).

Because aldose-carbohydrates can be isomerized under basic conditions, it is possible to modify the properties of fermentable prebiotic oligosaccharides by developing economically feasible processes for their conversion into the corresponding ketoses. GOS are reducing carbohydrates susceptible to being isomerized at their reducing glucose end, giving rise to a new type of prebiotic carbohydrates having different fermentation characteristics. Here we report the results obtained on the synthesis and characterization of the isomeric carbohydrates of GOS, catalyzed by the action of sodium aluminate. The influence of factors (temperature, time, and catalyst concentration) affecting the formation of isomeric carbohydrates has been investigated.

MATERIALS AND METHODS

Reagents. Lactose was acquired from Scharlau (Barcelona, Spain). D-Galactose, D-glucose, D-tagatose, D-fructose, and lactulose were from Fluka (Steinheim, Germany). 4-Galactobiose and 6-galactobiose were purchased from Sigma (St. Louis, MO). Sodium aluminate was purchased from Carlo Erba (Divisione Chimica Industriale, Milano, Italy). The commercial enzyme Lactozym 3000 L HP G, a soluble preparation of β-galactosidase from *Kluyveromyces lactis*, was a generous gift from Novozymes (Bagsvaerd, Denmark). High-purity water was produced in-house using a Milli-Q Synthesis A10 system (Millipore, Bellerica, MA) and was used throughout.

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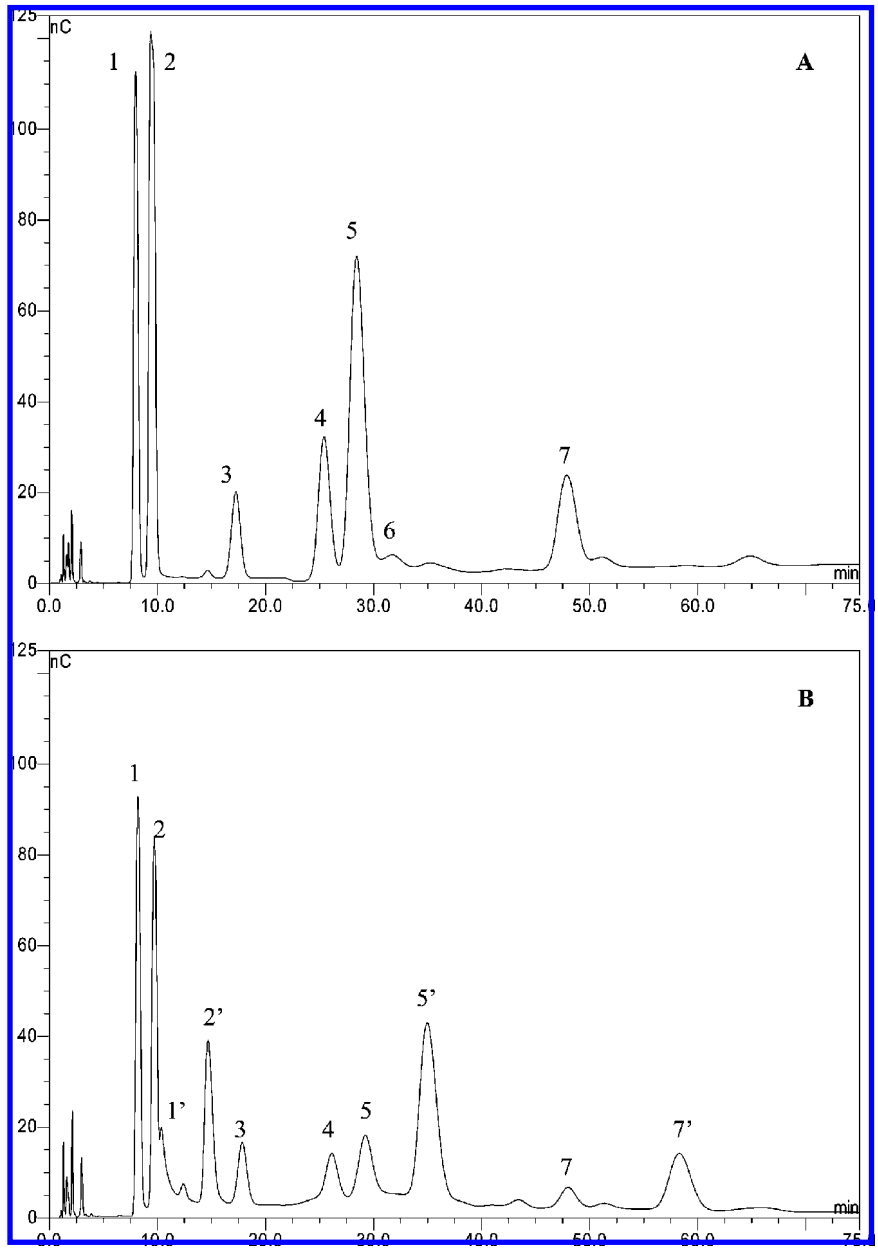

Figure 1. HPAEC-PAD profiles of carbohydrate mixture obtained by enzymatic hydrolysis of lactose by β-galactosidase (Lactozym 3000 L HP G) before (**A**) and after 9 h of isomerization at 40 °C using sodium aluminate/lactose 3:1 (**B**). Peaks: 1, galactose; 2, glucose; 3, 6-galactobiose (β-D-Gal-(1→6)-D-Gal); 4, allolactose (β-D-Gal-(1→6)-D-Glc); 5, lactose; 6, 4-galactobiose (β-D-Gal-(1→4)-D-Gal); 7, 6′-galactosyl-lactose (β-D-Gal-(1→6)-Lac); 1′, tagatose; 2′, fructose; 5′, lactulose; 7′, 6′-galactosyl-lactulose (β-D-Gal-(1→6)-D-Gal-(1→4)-Fru).

Synthesis of GOS from Lactozym 3000 L HP G. The synthesis of GOS was carried out in the optimal conditions previously reported by Martinez-Villaluenga et al. (*13*). Thus, the enzyme (3 units/mL) was added to the lactose solution (250 g/L) in pH 7.5 phosphate buffer (50 mM) containing $MgCl_2$ (1 mM) and incubated for 2 h at 40 °C. Afterward, the mixture was immediately immersed in boiling water for 5 min to inactivate the enzyme.

Isomerization Reactions. The assays of isomerization of the carbohydrate mixture were carried out following the method of Zokaee et al. (*14*). Thus, 1 g of the GOS produced from lactose through enzymatic transgalactosylation was dissolved in a few milliliters of deionized water and mixed with sodium aluminate in different sodium aluminate/carbohydrate molar ratios of 2:1, 3:1, and 4:1 (calculated on the basis of the initial lactose content in the carbohydrate sample, prior to the GOS synthesis). The mixture was made up to 10 mL and immersed into a water bath adjusted to the required temperature (35, 40, 45, and 50 °C) and heated for a specific time period. Isomerization reactions were carried out in duplicate, and samples of 500 μL were taken at 0, 1, 3, 5, 7, 9, 11, and 24 h. The reaction was stopped by placing the tube in an ice bath and then adding a few drops of HCl to neutralize the pH. Afterward, samples were diluted with deionized water to a final volume of 3 mL and then centrifuged at 9030*g* for 5 min. The supernatant was collected, diluted 50 times, and then analyzed by liquid chromatography.

Allolactulose was obtained in the same manner from allolactose and used as a standard. Previously, allolactose was isolated from the GOS mixture, fractionated by high-performance liquid chromatography using a refraction index detector (HPLC-RI) as described below, and NMR characterized.

Isolation of Oligosaccharides. The products obtained by isomerization were purified by HPLC-RI using a refractive index detector RID-10 (Shimazdu) and a semipreparative column Kromasil 100 NH_2 5 μm 25 × 1.0 cm (Teknokroma). Fifty microliters of reaction mixtures was eluted in acetonitrile/water 75:25 as the mobile phase at a flow rate of 3 mL/min, and fractions corresponding to the new compounds were collected, pooled, and evaporated in a rotatory evaporator R-210/215 (Büchi, Switzerland) for their final characterization.

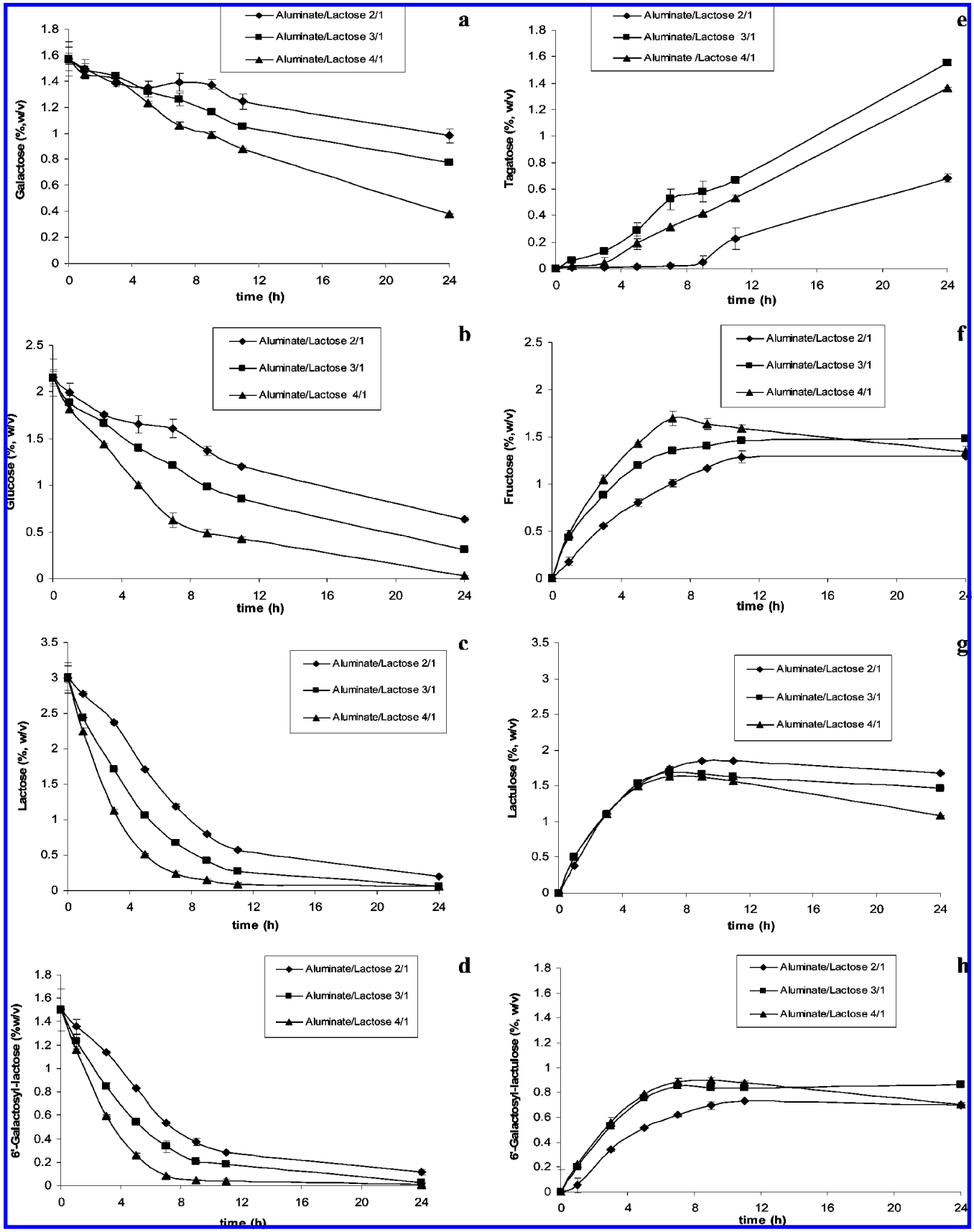

Figure 2. Effect of aluminate content on the isomerization of enzymatic hydrolysates of lactose at 40 °C during 24 h of reaction.

Chromatographic Analysis. Quantitative determination of GOS mixtures was achieved using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Analysis was carried out in a Dionex ICS 2500 system (Dionex Corp., Sunnyvale, CA) incorporating a GP50 gradient pump and an ED50 electrochemical detector with a gold working electrode and a Ag/AgCl reference electrode. Acquisition and processing of data were achieved with Chromeleon 6.7 software (Dionex Corp.) Separations were performed at room temperature. For eluent preparation, Milli-Q water, NaOH 50% (w/v) (Fluka), and sodium acetate (Panreac, Barcelona, Spain) were used.

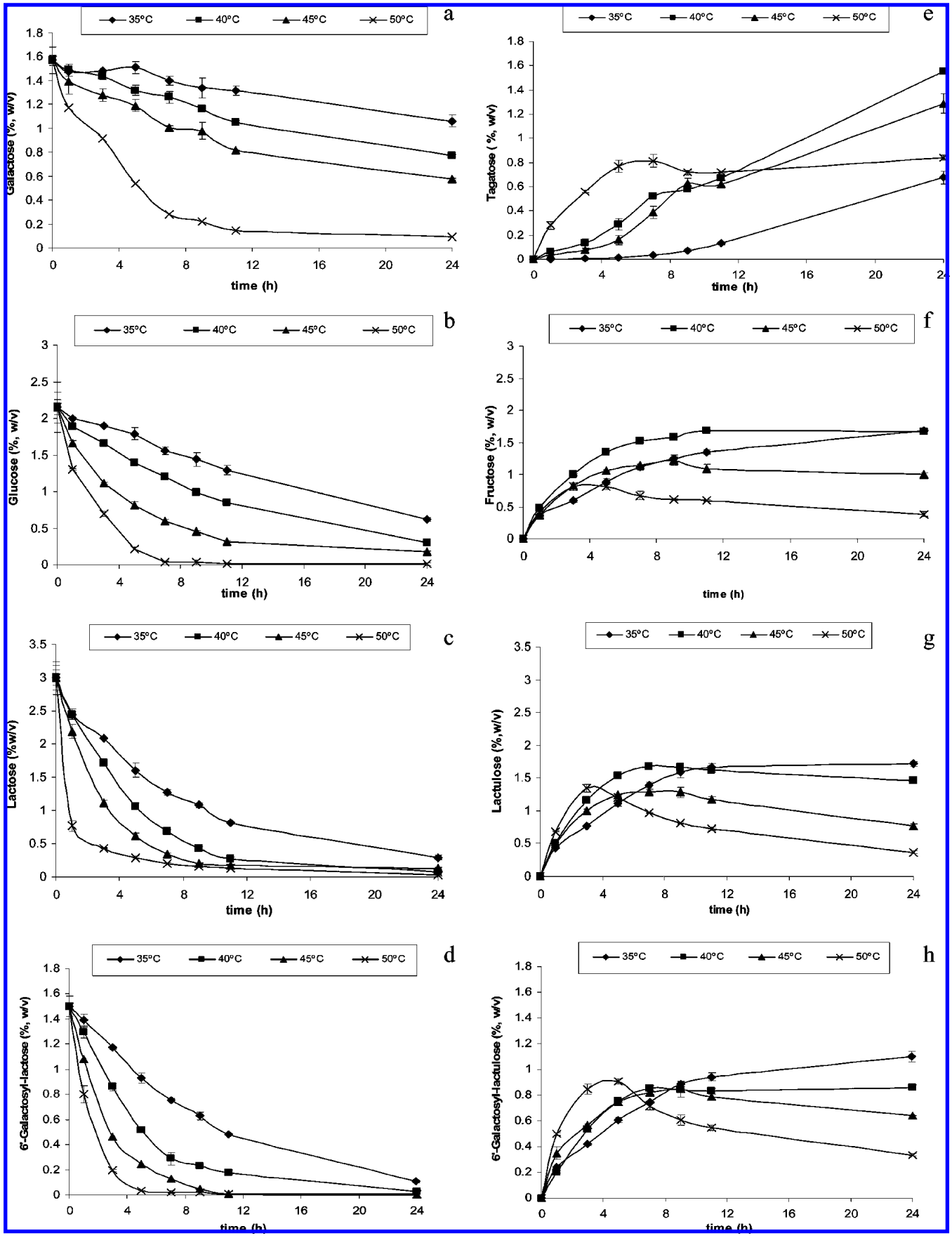

Figure 3. Effect of temperature on the isomerization of enzymatic hydrolysates of lactose using a lactose/aluminate ratio of 3:1.

Analysis of GOS was carried out using a CarboPac PA1 column (4 × 250 mm) connected to a CarboPac PA1 guard column (4 × 50 mm). The elution, at a flow rate of 1.5 mL/min, was in gradient using a combination of three eluents: A (100 mM NaOH), B (water), and C (100 mM NaOH and 1 M NaOAc). The gradient used was 95% B and 5% A from 0 to 20 min and 90% B and 10% A from 23 to 75 min.

After each run, the column was washed for 10 min with 100% C and re-equilibrated for 30 min with the starting conditions of the employed gradient. Detection time and voltage parameters were set according to waveform A (*15*).

Quantification of each sugar was performed by external calibration using solutions of corresponding standards. Samples and standard solutions were filtered through a nylon filter of 0.2 μm (Millipore), and 25 μL of sample was injected using an autosampler. The regression coefficient of the curve for each standard was always >0.99.

Structural Characterization. The molecular weight and the structure of the isomerized GOS were determined by mass spectrometry (MS) and ^{13}C and ^{1}H nuclear magnetic resonance (NMR) spectroscopy, respectively. The mass spectrum was recorded using a quadrupole HP 1100 mass detector in the electrospray positive mode (API-ES) scanning from *m/z* 100 to 1500. The mass spectrometer operated at 4000 V needle potential, 330 °C gas temperature, drying gas flow of 10 L/min, and 40 psi nebulizer pressure. NMR spectra were recorded at 293 K using D_2O as the solvent, on a Varian SYSTEM 500 NMR spectrometer equipped with a 5 mm HCN cold probe.

RESULTS AND DISCUSSION

Products Formed during Basic Isomerization of GOS. **Figure 1** shows the HPAEC-PAD profiles of the products formed during lactose hydrolysis by Lactozym 3000 L HP G, before (**A**) and after treatment with sodium aluminate (**B**). During enzymatic hydrolysis of lactose (peak 5) (**Figure 1A**) in galactose (peak 1) and glucose (peak 2), GOS are also formed (peaks 3, 4, 6, and 7) as a result of the transgalactosidase activity of the commercial enzymatic preparation. With authentic standards and the spike technique, it was possible to identify peak 3 as the disaccharide 6-galactobiose (β-D-Gal-(1→6)-D-Gal), peak 4 as allolactose (β-D-Gal-(1→6)-D-Glc), peak 6 as 4-galactobiose (β-D-Gal-(1→4)-D-Gal), and peak 7 as 6′-galactosyl-lactose (β-D-Gal-(1→6)-Lac).The enzymatic hydrolysis gave a mixture composed of 35% monosaccharides, 11% allolactose, 5% 6-galactobiose, 31% lactose, and 16% 6′-galactosyl-lactose. When the mixture was submitted to the action of sodium aluminate (**Figure 1B**), lactose, glucose, and galactose were isomerized to lactulose (peak 5′), fructose (peak 2′), and tagatose (peak 1′), respectively, and a new major compound (peak 7′) appeared in the trisaccharide region of the chromatogram. MS of the purified compound gave an ion at *m/z* 527 resulting from an intact [trisaccharide + Na]$^+$ ion. ^{1}H NMR and ^{13}C NMR spectroscopy revealed chemical shifts coincident with those previously reported for the trisaccharide 6′-galactosyl-lactulose obtained by transgalactosylation during enzymatic hydrolysis of lactulose (*13*).

With respect to the behavior of 6-galactobiose and allolactose, during basic isomerization of enzymatic hydrolyzate, the area of peak 3 corresponding to galactobiose remains almost unaltered, whereas peak 4 corresponding to allolactose decreased considerably. New peaks in the disaccharide region due to the action of sodium aluminate were not observed. With the aim of detecting the presence of the isomerization products of allolactose and 6-galactobiose, and to identify them, pure allolactose was isomerized under basic conditions, resulting in a new compound with the same retention time as lactulose. The chemical shifts (parts per million) obtained in the ^{13}C NMR spectra for this compound in D_2O were coincident with those reported by Leitner et al. (*16*) for allolactulose (see **Table 1**).

On the other hand, isomerization of 6-galactobiose did not reveal the appearance of any new peaks in the chromatogram. However, further hydrolysis of the isomerization product gave galactose and tagatose, which may indicate that 6-galactobiose was isomerized to β-D-Gal-(1→6)-tagatose, which could coelute with 6-galactobiose.

Table 1. ^{13}C NMR Chemical Shifts (Parts per Million) of Allolactose (β-D-Gal-(1→6)-D-Glc) and Allolactulose (β-D-Gal-(1→6)-D-Fru) in D_2O

	allolactose (β-D-Gal-(1→6)-D-Glc)	allolactulose (β-D-Gal-(1→6)-D-Fru
	glucose	**fructose**
C-1(α,β)	94.25, 98.07	62.7−62.6
C-2	73.58	101.8
C-3	76.16	71.8
C-4	70.7	74.8
C-5	74.8	79.3
C-6	71.59	70.1
	galactose β(1−6)	**galactose β(1−6)**
C-1′	103.75	103.44
C-2′	70.8	70.99
C-3′	74.97	72.77
C-4′	68.6	68.77
C-5′	77.29	75.28
C-6′	61.08	61.15

Optimization of the Isomerization Reaction. During the production of GOS from lactose through the transferase activity of Lactozym 3000 L HP G, significant amounts of free glucose are formed together with some free galactose, and a considerable amount of lactose remains unaltered. These carbohydrates do not have prebiotic properties, because they are absorbed in the small intestine (thereby increasing the caloric value of the product) and they do not exert the necessary fermentation selectivity considered essential for prebiotic carbohydrates (*17*). Isomerization of the remaining lactose and galactose would give rise to the formation of lactulose and tagatose, respectively. This would increase the prebiotic properties of the initial GOS mixture because tagatose and lactulose are considered to be prebiotic carbohydrates and can be used in a wide variety of functional foods and dietary supplements as well as in medicine (*11, 12, 18*).

Because the composition of the isomerized oligosaccharide mixture obtained may affect their prebiotic properties, the determination of factors affecting the final composition of the reaction mixture may be necessary for selecting appropriate experimental conditions. In this study we investigated the influence of various parameters such as temperature, time, and catalyst concentration on the reaction process.

Effect of Carbohydrate/Aluminate Ratio. Preliminary assays were carried out following the method of Zokaee et al. (*14*), who established that at a molar ratio of aluminate to lactose of 2:1 it was possible to reach conversion efficiencies of lactose to lactulose of around 70%. In this work we assayed three aluminate/carbohydrate molar ratios, 2:1, 3:1, and 4:1 (based on initial lactose content). To avoid excessive degradation of carbohydrates, assays were performed at 40 °C. **Figure 2** shows the changes of the carbohydrate mixture during the reaction time.

The rate of disappearance of aldoses (galactose, glucose, lactose, and 6′-galactosyl-lactose) increased with catalyst concentration (**Figure 2a−d**); however, the formation rate of isomers was different depending on carbohydrate. Formation of tagatose (**Figure 2e**) constantly increased during the 24 h studied, at the three studied aluminate/lactose ratios. Using substrate catalyst ratios of 4:1 and 3:1, tagatose reached values close to 1.3 and 1.5%/ respectively, whereas only 0.6 and 0.8% of galactose was consumed. This indicates that part of the tagatose may be originated from other carbohydrates containing galactose units. The maximum amount was reached using a molar ratio of 3:1 after 24 h of reaction. Fructose formation (**Figure 2f**) was higher using an aluminate/lactose molar ratio

of 4:1 and 7 h of reaction; after that, a degradation can be observed. Also, a maximum formation of lactulose (**Figure 2g**) was achieved within the first 9 h of reaction, and slight differences, at the three aluminate/carbohydrate molar ratios assayed, were observed; after reaching a maximum, the degradation rate increased with increasing aluminate content. The formation rates of 6′-galactosyl-lactulose (**Figure 2h**) were similar at 3:1 and 4:1 aluminate/carbohydrate molar ratios, but after reaching the maximum formation, at 9 h of reaction, a progressive degradation of the trisaccharide was observed at a molar ratio of 4:1. According to this, for the subsequent assays, the selected molar aluminate/lactose ratio was 3:1.

Effect of Temperature. The rate of isomerization is influenced not only by the temperature but also by the amount and type of catalyst used (*14*). To study the influence of temperature on GOS isomerization, different assays were carried out at 35, 40, 45, and 50 °C. The obtained results (**Figure 3**) showed that, as expected, the rate of disappearance of aldoses (galactose, glucose, lactose, and 6′-galactosyl-lactose) (**Figure 3a−d**) increased with temperature; however, the formation of the corresponding ketoses did not follow a similar pattern (**Figure 3e−h**).

Maximum formation of tagatose (**Figure 3e**) and fructose (**Figure 3f**) was observed at 40 °C. Tagatose constantly increased during the 24 h studied, whereas the fructose formation reached a maximum at 9 h of reaction. Higher temperatures caused noticeable degradation giving rise to a decrease on monosaccharide content. The rate of lactulose formation (**Figure 3g**) increased from 35 to 40 °C, reaching for both temperatures similar levels in the reaction mixture but at different reaction times; at 35 °C the maximum formation took place at 9 h, whereas at 40 °C this was at 7 h. Higher temperatures cause a decrease in the formation rate of lactulose; this can probably be due to a higher carbohydrate degradation. With regard to 6′-galactosyl-lactulose (**Figure 3h**), maximum formation was attained at 35 °C after 24 h of reaction and progressively decreased with temperature.

In this study a mixture containing a high concentration of prebiotic carbohydrates has been successfully produced through isomerization in basic media of GOS obtained by enzymatic hydrolysis of lactose. Galactose, glucose, and unreacted lactose present in the GOS mixture were converted into tagatose, fructose, and lactulose, respectively. Allolactose, 6-galactobiose, and 6′-galactosyl-lactose, the main GOS formed during enzymatic lactose hydrolysis, were also isomerized into the corresponding ketoses. The optimal conditions of reaction to obtain the highest conversion of lactose and 6′-galactosyl-lactose were 40 °C for 9 h and an aluminate/lactose ratio of 3:1. Under these conditions the isomerization yield was >60%, and the amount of final carbohydrates was close to 90% of the initial product. Therefore, this process considerably decreased the amounts of lactose, glucose, and galactose, which are not recommended for people with diabetes or lactose intolerance.

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