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Synthesis of Oligosaccharides Derived from Lactulose and Pectinex Ultra SP-L

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The β-galactosidase activity of the commercial enzymatic preparation Pectinex Ultra SP-L derived from *Aspergillus aculeatus* has been used to hydrolyze and transgalactosylate the prebiotic carbohydrate lactulose. During this reaction, new oligosaccharides derived from lactulose have been detected by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The presence of the trisaccharide 6′-galactosyl-lactulose, the major compound formed, has been confirmed by NMR. In addition, disaccharides and other oligosaccharides with higher retention times have been also detected. The effect of transgalactosylation conditions such as time, temperature, pH, and initial lactulose and enzyme concentrations, as well as product inhibition on oligosaccharide synthesis, has been studied. The optimal conditions for the formation of tri and higher oligosaccharides were 60 °C, pH 6.5, 450 g/L lactulose, 16 units/mL of enzyme, and 7 h of reaction. Selective formation of disaccharides was achieved under the same conditions with the exception of pH (4.5). The present work provides additional knowledge on the synthesis of new oligosaccharides with potential prebiotic properties.

KEYWORDS: Lactulose hydrolysis; Pectinex Ultra SP-L; oligosaccharides synthesis; HPAEC-PAD

INTRODUCTION

The use of dietary carbohydrates as prebiotic food ingredients has gained interest with the increased awareness about the relationship between the activity of colon bacteria and health. Prebiotic carbohydrates escape digestion in the upper gastrointestinal tract and are fermented by bacteria in the colon, leading to the proliferation of bacteria that are beneficial for health in humans. Because the place where fermentation mainly occurs (proximal or distal colon) is an important factor influencing the extent of the prebiotic effect (*1*), the development of new types of functional carbohydrates with specific fermentation properties seems to be of interest.

Galactooligosaccharides (GOS) are commercially available prebiotics produced from lactose by a transfer of galactose residues onto the galactose moiety of lactose catalyzed by β-galactosidases. The composition of a GOS mixture depends on several factors including the source of the enzyme, the concentration and nature of the substrate, the degree of conversion, and the reaction conditions (*2*). Lactose has been the substrate mainly used for the production of GOS; however, recent studies carried out by our research group have proved that other carbohydrates such as lactulose are also good substrates for the production of oligosaccharides (OS) when treated with β-galactosidase from the commercial enzyme preparation Lactozym 3000L HP G (*3*). Lactulose is a well-known disaccharide with excellent prebiotic activity (*4, 5*); however, a drawback of this carbohydrate is the production of gas during its fermentation, due to the fact that it is mainly consumed by the bacteria of the proximal colon (*6*). Therefore, it is reasonable to assume that lactulose-derived oligosaccharides originated during enzymatic hydrolysis of lactulose might be bioactive carbohydrates slowly fermented and, therefore, with higher colonic persistence than lactulose, so that the obtention of new oligosaccharides derived from lactulose is of great interest.

Pectinex Ultra SP-L is a commercial enzyme preparation derived from *Aspergillus aculeatus* consisting predominantly of pectinolytic enzymes and commonly used in fruit juice processing (*7, 8*); moreover, this enzymatic preparation possesses fructosyltransferase (*9*) and β-galactosidase (*10*) activities. The optimum transgalactosylation conditions to obtain GOS during lactose hydrolysis with Pectinex Ultra SP-L have been previously determined (*11, 12*).

To the best of our knowledge, no data have been reported on the synthesis of OS derived from lactulose using Pectinex Ultra SP-L. Therefore, in this work, we have investigated the possible formation of new oligosaccharides during the hydrolysis of lactulose by the β-galactosidase activity of the commercial enzyme preparation Pectinex Ultra SP-L. The effect of transgalactosylation conditions such as temperature, pH, time, and initial lactulose and enzyme concentrations, as well as product inhibition on oligosaccharide synthesis, has been studied.

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MATERIALS AND METHODS

Chemicals. Lactulose, D-fructose, and 6′-galactobiose were purchased from Sigma-Aldrich (Steinheim, Germany). D-Galactose, raffinose, and *o*-nitrophenyl-β-D-galactopyranoside (oNPG) were obtained from Fluka (Steimheim, Germany). The commercial enzyme preparation of β-galactosidase from *A. aculeatus* (Pectinex Ultra SP-L) was a generous gift from Novozymes (Bagsvaerd, Denmark).

Determination of Enzyme Activity. The β-galactosidase activity of Pectinex Ultra SP-L was measured at 40 °C using oNPG as substrate. The reaction was started by adding 50 μL of enzyme to 800 μL of oNPG 4.99 mM in 0.1 M sodium acetate buffer, pH 5. The rate of formation of free oNP was recorded spectrophotometrically using a 1 cm path length cuvette provided with magnetic stirring. The absorbance was measured at 410 nm (ϵ for oNP = 442 M^{-1}/cm). The hydrolysis products are galactose and oNP, and the enzymatic mechanism is similar to that of lactose hydrolysis.

The enzyme was diluted to an appropriate concentration using the same buffer. oNPG solution was kept at 40 °C in a water bath before the enzyme was added to initiate the reaction.

Pectinex Ultra SP-L expressed a β-galactosidase activity of 82.07 units, where 1 unit is defined as the amount of enzyme releasing 1 μmol of oNP per minute per milliliter under the assayed conditions. Enzyme activity measurements were repeated five times, and the experimental error (RSD relative standard deviation) was lower than 3%.

Determination of Specific Protein Content. The protein content in the enzymatic preparation was determined using the bicinchoninic acid (BCA) assay with bovine serum albumin (BSA) as standard (*13*). The specific activity of the enzyme was calculated from the fraction of the activity unit (units per milliliter) over protein concentration (milligrams per milliliter).

The protein content in the commercial preparation Pectinex Ultra SP-L was 82 mg/mL. Therefore, the enzyme expressed a specific activity of 1 μmol of oNP released/min/mg (1 unit/mg).

Production of Lactulose-Derived Oligosaccharides. Batch assays for oligosaccharide production were carried out with the commercial preparation Pectinex Ultra SP-L. The influence of process conditions such as time (1, 3, 5, 7, and 24 h), temperature (40, 50, and 60 °C), pH (0.1 M acetate buffer at pH 4.5 or 5.5 and 0.1 M phosphate buffer at pH 6.5), lactulose concentration (250, 450, 650, and 850 g/L), and enzyme concentration (8, 16, and 24 units/mL) on oligosaccharide synthesis was studied.

Lactulose solutions were heated before the enzyme was added and maintained at the required temperature throughout all of the experiments. Reactions were performed in individual Eppendorf tubes incubated in an orbital shaker at 300 rpm, using a final volume of solvent of 525 μL. Aliquots (50 μL) of sample were withdrawn from the reaction mixture at different times for 24 h and immediately immersed in boiled water for 5 min to inactivate the enzyme. The samples were stored at −18 °C for subsequent analysis. After appropriate dilution, 25 μL was injected in the chromatograph. Control samples were prepared in the same manner except no enzyme was added, and no changes in lactulose were detected. All assays were performed in duplicate.

Inhibition on Oligosaccharide Synthesis. The potentially inhibiting effects of fructose and galactose on oligosaccharide formation were separately investigated. The procedure above-described was repeated after adding fructose or galactose at concentrations of 50, 100, and 150 g/L to reaction mixtures with 250 g/L of lactulose and 16 units/mL of the enzyme solution. Reactions were carried out at 60 °C and monitored for 6 h. Samples (50 μL) were withdrawn after 2, 4, and 6 h and immediately immersed in boiled water for 5 min to inactivate the enzyme. The samples were stored at −18 °C for subsequent analysis.

Determination of Carbohydrates. The carbohydrate composition of the reaction mixtures was determinated by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) on an ICS2500 Dionex system consisting of a GP50 gradient pump and an ED50 electrochemical detector with a gold working electrode (Ag/AgCl reference electrode). Acquisition and processing of data were achieved with Chromeleon software version 6.7 (Dionex Corp., Sunnyvale, CA). Separations were performed at room temperature, and for eluent preparation Milli-Q water, 50% (w/w) NaOH (Fluka, Steinheim, Germany), and sodium acetate (NaOAc) (Panreac, Barcelona, Spain) were used. All eluents were degassed by flushing helium for 25 min.

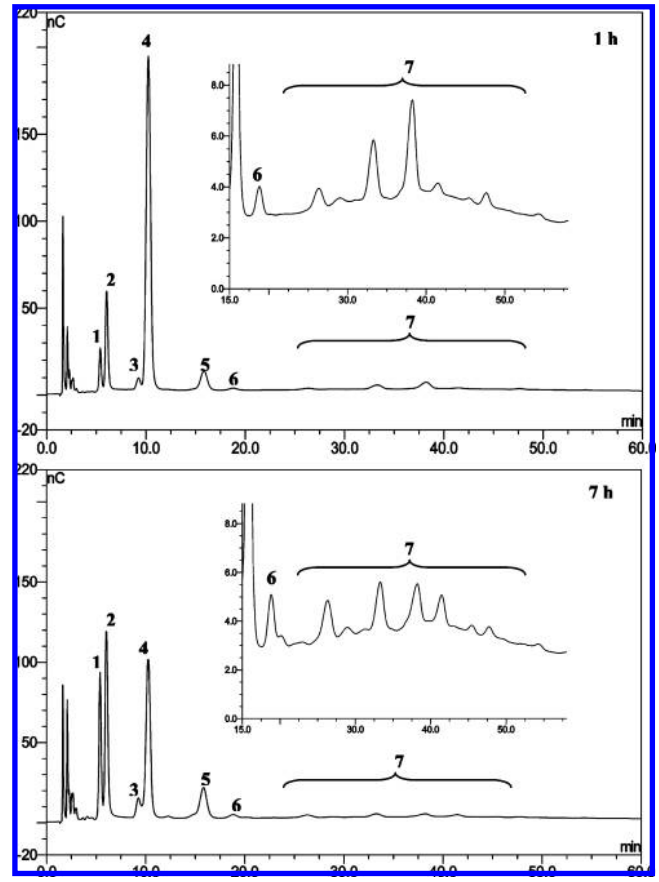

Figure 1. HPAEC-PAD carbohydrate profiles of oligosaccharides formed from hydrolysis of lactulose and β-galactosidase from Pectinex Ultra SP-L at pH 6.5, 60 °C, 650 g/L of lactulose, and 16 units/mL of enzyme after 1 and 7 h of reaction. Identified compounds: (1) galactose; (2) fructose; (3) Gal-β(1,6)-Gal; (4) lactulose; (5) β-D-Gal-(1→6)-β-D-Gal-(1→4)-β-D-Fru (6′-galactosyl-lactulose); (6) β-D-Gal-(1→4)-β-D-Fru-(1→1)-β-D-Gal; (7) oligosaccharides with DP ≥ 3.

Chromatographic separations were performed following the method of Splechtna et al. (*14*). Elution of carbohydrates was at room temperature on a CarboPac PA-1 column (4 × 250 mm) connected to a CarboPac PA-1 guard column (4 × 50 mm). Eluent A (100 mM NaOH), eluent B (100 mM NaOH and 50 mM NaOAc), and eluent C (100 mM NaOH and 1 M NaOAc) were mixed as follows: 100% A from 0 to 20 min and from 0 to 100% B from 20 to 70 min. After each run, the column was washed for 10 min with 100% C and re-equilibrated for 15 min with the starting conditions of the employed gradient. Separations were performed at a flow rate of 1 mL/min. Detection time and voltage parameters were set according to waveform A: $E_1 = 0.1$ V ($t_1 = 400$ ms); $E_2 = -2$ V ($t_2 = 10$ ms); $E_3 = 0.6$ V; $E_4 = -0.1$ ($t_3 = 60$ ms) (*15*).

Quantification of each sugar was performed by external calibration using solutions of the corresponding standards. Each carbohydrate was expressed as the weight percentage of total carbohydrates.

Purification and Characterization of Lactulose-Derived Oligosaccharides. The main trisaccharide formed in the synthesis with lactulose and Pectinex Ultra SP-L was purified by high-performance liquid chromatography with refractive index detector (HPLC-RI) following the methodology described by Martínez-Villaluenga et al. (*3*) using an amino Kromasil 4.6 × 250 mm i.d., 5 μm size column (Alltech Associates, Inc.). A mixture of acetonitrile/water (75:25, v/v) was used as mobile phase at a flow rate of 1 mL/min. Fractions from 10 runs

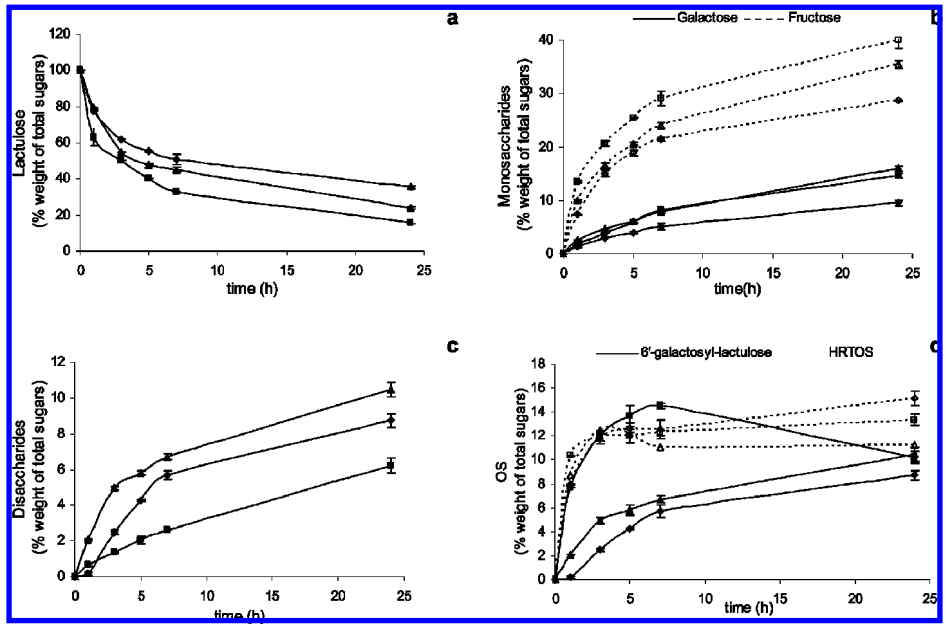

Figure 2. (a–d) Effect of temperature on oligosaccharide production during the enzymatic hydrolysis of lactulose (650 g/L) with Pectinex Ultra SP-L (16 units/mL) in 0.1 M buffer phosphate, pH 6.5: (◆) 40 °C; (▲) 50 °C; (■) 60 °C. HRTOS, high retention time oligosaccharides; OS, oligosaccharides.

corresponding to the oligosaccharide were collected, evaporated in a rotatory evaporator (Büchi), pooled, and freeze-dried for MS and NMR characterization.

The mass spectrum was acquired in a quadrupole HP 1100 mass detector in the electrospray positive mode (API-ES) scanning from *m*/*z* 100 to 1500. The working conditions for the ESI source were as follows: 4000 V needle potential, 330 °C gas temperature, drying gas flow of 10 L/min, and 40 psi nebulizer pressure. The chemical structure was identified by ^{13}C NMR and ^{1}H NMR. 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

The chromatographic profiles of oligosaccharides formed during enzymatic hydrolysis of lactulose by Pectinex Ultra SP-L are shown in **Figure 1**. The reaction was performed at 60 °C using solutions of lactulose (650 g/L) in 0.1 M phosphate buffer, pH 6.5, 16 units/mL of enzyme, and monitored during 24 h. As can be observed, after 1 h of reaction, lactulose (peak 4) gave rise to galactose (peak 1), fructose (peak 2), and other oligosaccharides (peaks 3 and 5–7), the concentrations of which increased with the progress of the reaction (7 h).

Using an authentic standard and with the standard-addition technique, peak 3 was identified as the disaccharide Gal-β(1,6)-Gal. Peak 5, the major oligosaccharide formed, was isolated using HPLC-RI and then analyzed by MS, giving an intense *m*/*z* 504 ion corresponding to a trisaccharide. ^{1}H NMR and ^{13}C NMR analyses revealed that chemical shifts were coincident with those of β-D-Gal-(1→6)-β-D-Gal-(1→4)-β-D-Fru (6′-galactosyl lactulose) reported previously by Martinez-Villaluenga et al. (*3*).

Peak 6 (**Figure 1**) was also identified as a trisaccharide, β-D-Gal-(1→4)-β-D-Fru-(1→1)-β-D-Gal on the basis of the previously reported retention time of this compound detected in higher concentrations in reaction mixtures of lactulose and β-galactosidase from Lactozym 3000L HP G by Martínez-Villaluenga et al. (*3*). Peaks marked with the number 7 could correspond to oligosaccharides with a degree of polymerization (DP) ≥ 3 and have been named high retention time oligosaccharides (HRTOS).

Optimization of Lactulose-Derived Oligosaccharide Formation. *Effect of Temperature.* The effect of the reaction temperature on the oligosaccharides formation was studied at 40, 50, and 60 °C using lactulose solutions of 650 g/L at pH 6.5 and 16 units/mL of enzyme (**Figure 2**). The rate of lactulose hydrolysis increased with temperature (**Figure 2a**) as well as the formation of transgalactosylation products (**Figures 2c**,**d**). The amount of galactose (**Figure 2b**) present in the reaction mixture was lower than that of fructose in all studied samples, which agrees with the observed formation of OS. Disaccharide formation (**Figure 2c**) was maximal at 50 °C, reaching levels up to 10% after 24 h of reaction. The disaccharide formation and the release of galactose indicate that the hydrolysis of lactulose is favored at 50 °C.

At 60 °C, the formation of 6′-galactosyl-lactulose (**Figure 2d**) was optimal, reaching values near 15% after 7 h and then decreased progressively up to 10% at the end of the reaction (24 h). This indicates that the trisaccharide is susceptible to hydrolysis by Pectinex Ultra SP-L. These results are in agreement with those of Van Casteren et al. (*10*) and Cardelle-Cobas et al. (*12*), who found the optimal temperature for β-galactosidase activity of Pectinex Ultra SP-L, using lactose as substrate, to be in the range of 55–60 °C. Similar yields of HRTOS were obtained at the three temperatures assayed after 7 h of reaction, and a slight increase was observed at the end of reaction (24 h).

Effect of pH. It is well-known that the effect of pH on the production of GOS may vary with the source of β-galactosidase (*16*). In some cases, the pH values dramatically affect the ratio of transgalactosylation over hydrolysis reactions (*16, 17*) and, in other examples, hardly any effects are detected (*18, 19*). Thus, assays were performed at different pH values (4.5, 5.5, and 6.5) and 60 °C using 650 g/L of lactulose and 16 units/mL of enzyme.

Panels **a** and **b** of **Figure 3** show the increase of the rate of lactulose hydrolysis as the pH of the medium decreased. Moreover, the formation of disaccharides increased as the pH decreased (**Figure 3c**), whereas the content of 6′-galactosyl-lactulose in the mixture decreased with pH (**Figure 3d**). At pH 4.5 and 5.5 the optimal amount of disaccharides was reached

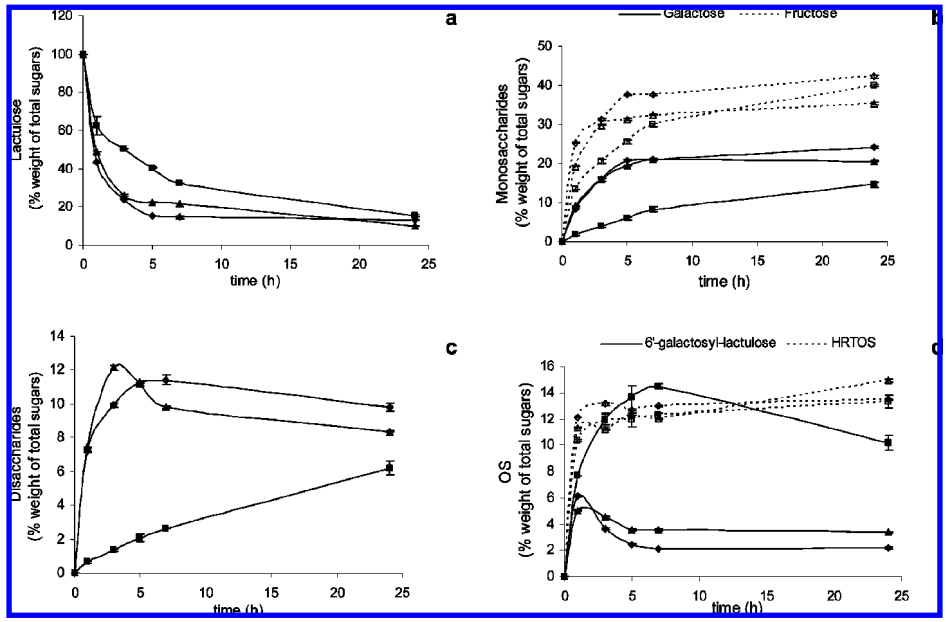

Figure 3. (a–d) Effect of pH on oligosaccharide production during the enzymatic hydrolysis of lactulose (650 g/L) with Pectinex Ultra SP-L (16 units/mL) at 60 °C: (◆) pH 4.5; (▲) pH 5.5; (■) pH 6.5. HRTOS, high retention time oligosaccharides; OS, oligosaccharides.

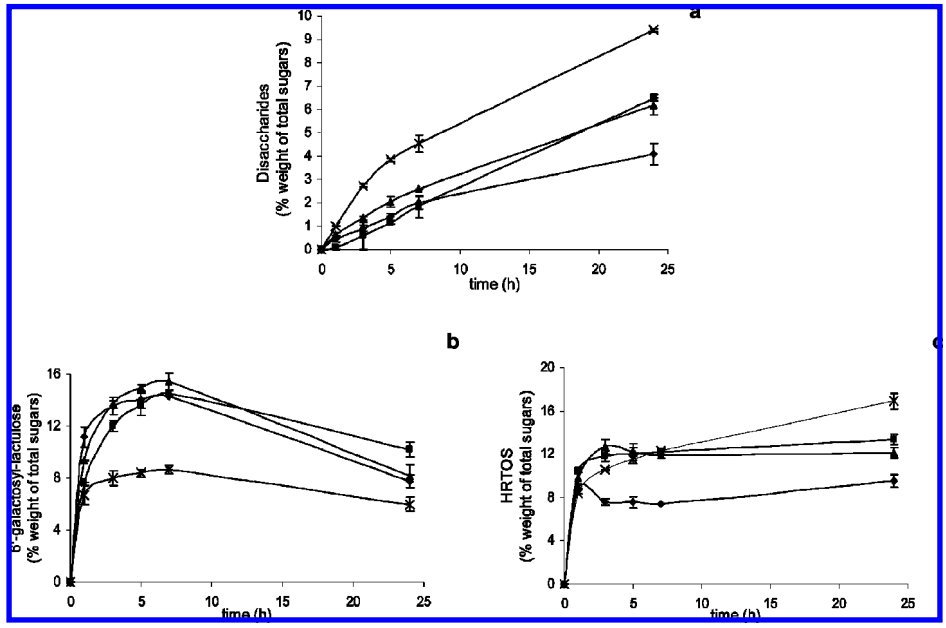

Figure 4. (a–c) Effect of lactulose concentration on oligosaccharide production during the enzymatic hydrolysis of lactulose with Pectinex Ultra SP-L (16 units/mL) at 60 °C and 0.1 M buffer phosphate, pH 6.5: (◆) 250 g/L; (■) 450 g/L; (▲) 650 g/L; (×) 850 g/L. HRTOS, high retention time oligosaccharides.

after 5 h and then progressively decreased. At the three pH values studied, HRTOS increased quickly at the first hour of reaction (**Figure 3d**) without important changes during the 24 h of reaction.

Higher values of pH were not studied because several studies had previously demonstrated that the enzymatic activity is out of its optimal range (*10–20*). Similar trends were observed when we synthesized GOS using lactose and β-galactosidase from Pectinex-Ultra SP-L (*12*).

Effect of Lactulose Concentration. One of the most influential factors on yields of transgalactosylation is the concentration of substrate, and an increase in this parameter as high as possible could shift the equilibrium toward the transgalactosylation reaction (*16, 21*). Thus, a study on the influence of lactulose concentration was performed to select the optimal conditions for the formation of disaccharides, 6′-galactosyl-lactulose, and HRTOS (**Figure 4**). The percentages of galactose and fructose found were similar at all studied lactulose concentrations (data not shown), but disaccharides and HRTOS increased with lactulose concentration (**Figure 4a**,**c**). No effect on the 6′-galactosyl-lactulose formation was observed in the lactulose range of 250–650 g/L, whereas the lowest formation was observed at the higher lactulose concentration assayed (**Figure 4b**). The effect of lactulose concentration on the formation of OS may be due to a faster hydrolysis of the trisaccharide at the higher lactulose concentration assayed.

Effect of Enzyme Concentration. Using different concentrations of enzyme, 8, 16, and 24 units/mL, a study on the influence of enzyme concentration on oligosaccharide formation from lactulose and Pectinex Ultra SP-L was carried out at 60 °C, pH

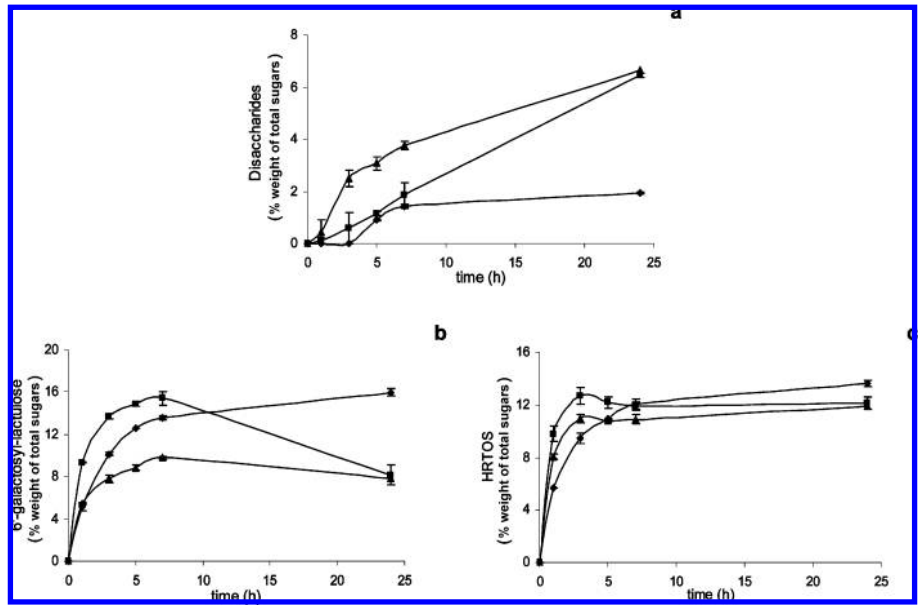

Figure 5. (a–c) Effect of enzyme concentration on oligosaccharide production during the enzymatic hydrolysis of lactulose (450 g/L) with Pectinex Ultra SP-L at 60 °C and 0.1 M buffer phosphate, pH 6.5: (◆) 8 units/mL; (■) 16 units/mL; (▲) 24 units/mL. HRTOS, high retention time oligosaccharides.

6.5, and 450 g/L of lactulose (**Figure 5**). As expected, an increase of hydrolysis rate with enzyme concentration was observed. Formation of disaccharides increased with enzyme concentration (**Figure 5a**), whereas the lowest formation of 6′-galactosyl-lactulose was observed using 24 units/mL of enzyme (**Figure 5b**). For enzyme concentrations of 16 and 24 units/mL, after 7 h, the maximum amount of 6′-galactosyl-lactulose was reached and then decreased, whereas the use of 8 units/mL led to a constant increase during the studied period.

The formation of HRTOS is shown in **Figure 5c**; the higher content was obtained using 16 units/mL after 3 h of reaction. The lower enzyme concentration (8 units/mL) produced a gradual increase of HRTOS content, reaching 13% after 24 h.

Product Inhibition. As is known, the hydrolysis products of lactose (galactose and glucose) can exert an inhibition effect during the transgalactosylation reaction (*22*). With the aim to investigate this effect in the case of lactulose, a study on the possible inhibition of galactose and fructose, derived from its hydrolysis, was carried out. As was observed in **Table 1**, after 6 h of reaction, the amount of lactulose consumed decreased when galactose was added initially. Nevertheless, hydrolysis inhibition by fructose was not observed. With regard to 6′-galactosyl-lactulose formation, galactose addition resulted in stronger inhibition than fructose, when they were present in equal amounts. It should be noted that, for HRTOS formation, only galactose produced inhibition. These results are in agreement with those obtained in the studies of monosaccharides inhibition on GOS formation from lactose (*11, 22*) in which galactose was a competitive inhibitor for the enzyme active sites.

In this work a characterization and synthesis optimization of novel oligosaccharides derived from hydrolysis of lactulose by β-galactosidase from Pectinex Ultra SP-L has been carried out. The enzyme has proved to be selective for the production of di- or trisaccharides depending on the operating conditions. The trisaccharide 6′-galactosyl-lactulose has been identified, characterized, and quantified as the major oligosaccharide formed in the reaction mixture. Also, another new trisaccharide, detected at very low concentration, was identified as β-Gal-(1,4)-β-Fru-(1,1)-β-Gal. The composition of the resulting mixture was strongly affected by the reaction conditions. The optimal conditions for the synthesis of trisaccharide 6′-galactosyl-lactulose and for the formation of other oligosaccharides with ≥DP were 60 °C, pH 6.5, 450 g/L of lactulose, 16 units/mL of enzyme, and 7 h of reaction, reaching yields of 15%. The best conditions for the selective formation of disaccharides were the same with the exception of the pH because, in this case, 4.5 was the value that gave rise to the highest disaccharide yield (12%). These results were similar to those obtained for GOS derivatives from lactose and Pectinex-Ultra SP-L.

Because oligosaccharides derived from lactulose may present two or more molecules of galactose linked to one molecule of fructose, an extension of GOS definition should be made. More studies using different enzyme and operating conditions are necessary to know the structure and linkages of oligosaccharides derived from lactulose, as well as to evaluate their prebiotic potential.

Table 1. Effect of Monosaccharide Addition on Oligosaccharide Production and Lactulose Consumption during Reaction at 60 °C for 6 h of Mixtures of Lactulose (250 g/L) and 16 Units/mL Enzyme in 0.1 M Phosphate Buffer, pH 6.5

		content in the reaction mixture (% of total carbohydrates)			
added monosaccharide	g/L	remaining lactulose	disaccharides	6′-galactosyl-lactulose	HRTOS[a]
none	0	34.1	1.7	14.2	7.5
galactose	50	55.9	3.0	3.0	6.3
	100	53.5	3.2	2.1	5.5
	150	52.2	2.6	0.3	5.5
fructose	50	33.1	2.3	9.8	7.3
	100	32.8	3.3	6.9	7.3
	150	30.8	4.0	3.9	7.3

[a] HRTOS, oligosaccharides with retention time higher than that of 6′-galactosyl-lactulose.

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