JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Study of Influential Factors on Oligosaccharide Formation by Fructosyltransferase Activity during Stachyose Hydrolysis by Pectinex Ultra SP-L

Antonia Montilla,[†] Agustín Olano,[†] Cristina Martínez-Villaluenga,[‡] and Nieves Corzo*[,†]

[†]Instituto de Investigación en Ciencias de la Alimentación CIAL, CSIC-UAM, C/Nicolás Cabrera 9, Campus de la Universidad Autónoma de Madrid, 28049 Madrid, Spain

[‡]Instituto de Ciencia y Tecnología de Alimentos y Nutrición ICTAN, CSIC, C/Juan de la Cierva 3, 28006 Madrid, Spain

ABSTRACT: The influence of reaction conditions for oligosaccharide synthesis from stachyose using a commercial enzymatic preparation from *Aspergillus aculeatus* (Pectinex Ultra SP-L) was studied. Oligosaccharides were analyzed by gas chromatography with flame ionization detection (GC-FID) and matrix-assisted laser desorption/ionization−time-of-flight−mass spectrometry (MALDI-TOF-MS). Galactosyl-melibiose (DP_3) was synthesized as a result of fructosidase activity, whereas fructosyl-stachyose (DP_5) and difructosyl-stachyose (DP_6) were formed as a consequence of the fructosyltransferase activity of Pectinex Ultra SP-L. The optimal reaction conditions for the synthesis of penta- and hexasaccharides were 60 °C, pH 5.5, 600 mg/mL stachyose, and 34 U/mL enzyme. Reaction time played an important role in oligosaccharide mixture composition constituted by 20% DP_5, 0.7% DP_6, 55% stachyose, 21% galactosyl-melibiose, and 1% monosaccharides after 1 h and 16% DP_5, 4% DP_6, 27% stachyose, 44% galactosyl-melibiose, and 2% monosaccharides after 3 h. In conclusion, stachyose could be used as a substrate for the enzymatic synthesis of new oligosaccharides that may open new opportunities in the development of future prebiotics.

KEYWORDS: α-galactosides, stachyose, Pectinex Ultra SP-L, transfructosylation, oligosaccharide synthesis

INTRODUCTION

The consumption of prebiotic carbohydrates has gained increased interest due to their recognition as agents inducing beneficial physiological effects in the colon and in extraintestinal compartments or reducing the risk of associated intestinal and systemic pathologies.[1] Prebiotic carbohydrates may be fermented in different parts of the digestive tract. Rapidly fermented prebiotics stimulate the growth and activity of beneficial bacteria in the proximal colon, whereas slowly fermented prebiotics may reach the distal colon, where most chronic diseases (e.g., colorectal cancer and ulcerative colitis) take place.[2,3] Consequently, there is greater interest in finding prebiotics that can persist to more distal regions of the colon.[4]

Currently, the development of future prebiotics targets the modulation of microbial fermentation along the gastrointestinal tract. Future prebiotics could be mixtures of oligosaccharides that have different effects in different parts of the gastrointestinal tract. Besides the colon, the oral cavity and the small intestine are also considered to be potential prebiotic targets.[5]

α-Galactosides (raffinose, stachyose, verbascose, and ajugose) are sucrose-based oligosaccharides found more abundantly in grain legumes.[6] These carbohydrates derive from sucrose and contain 1−4 units of galactose linked by α-(1,6) linkages to the glucose moiety. These oligosaccharides are resistant to digestion, promoting the growth of beneficial bacteria in the colon.[7]

In vitro assays have shown the ability of *Bifidobacterium* and *Lactobacillus* strains to grow using α-galactosides as carbon source, due to the production of α-galactosidase.[8−10] Today, α-galactosides are obtained on an industrial scale as a byproduct from the production of soy protein isolates and soy protein concentrates.[6] The recovery of α-galactosides from soy byproduct could be an interesting alternative for their utilization as raw material in the manufacture of future prebiotics.

Transglycosylation reactions catalyzed by enzymes are being used for the production of oligosaccharide mixtures with a specific composition. Production of galactooligosaccharides mixtures from lactose or lactulose using galactosyltransferases from different sources has been carried out.[11−15] In addition, fructooligosaccharide mixtures were produced from sucrose by fructosyltransferases from bacteria or molds.[16−19] Because α-galactosides contain a fructosyl end, fructosyltransferases may transfer the fructosyl moiety to a suitable acceptor to give rise to new oligosaccharides. Purified fructosyltransferase from *Aspergillus niger* has been used to catalyze the synthesis of new oligosaccharides from raffinose.[20] The relatively inexpensive commercial enzyme preparation Pectinex Ultra SP-L (Pectinex), produced by *A. aculeatus*, was shown to contain fructosyltransferase activity;[16,19,21,22] therefore, it could be used as a catalyst in the large-scale production of new oligosaccharides. The objective of this work has been to investigate in more detail the fructosyltransferase activity from *A. aculeatus* of the commercial enzymatic preparation Pectinex over stachyose hydrolysis.

MATERIALS AND METHODS

Chemicals. D-Galactose, D-glucose, D-fructose, melibiose, raffinose, stachyose, and phenyl-β-glucoside were purchased from Sigma

Received: June 21, 2011
Revised: August 31, 2011
Accepted: September 1, 2011
Published: September 01, 2011

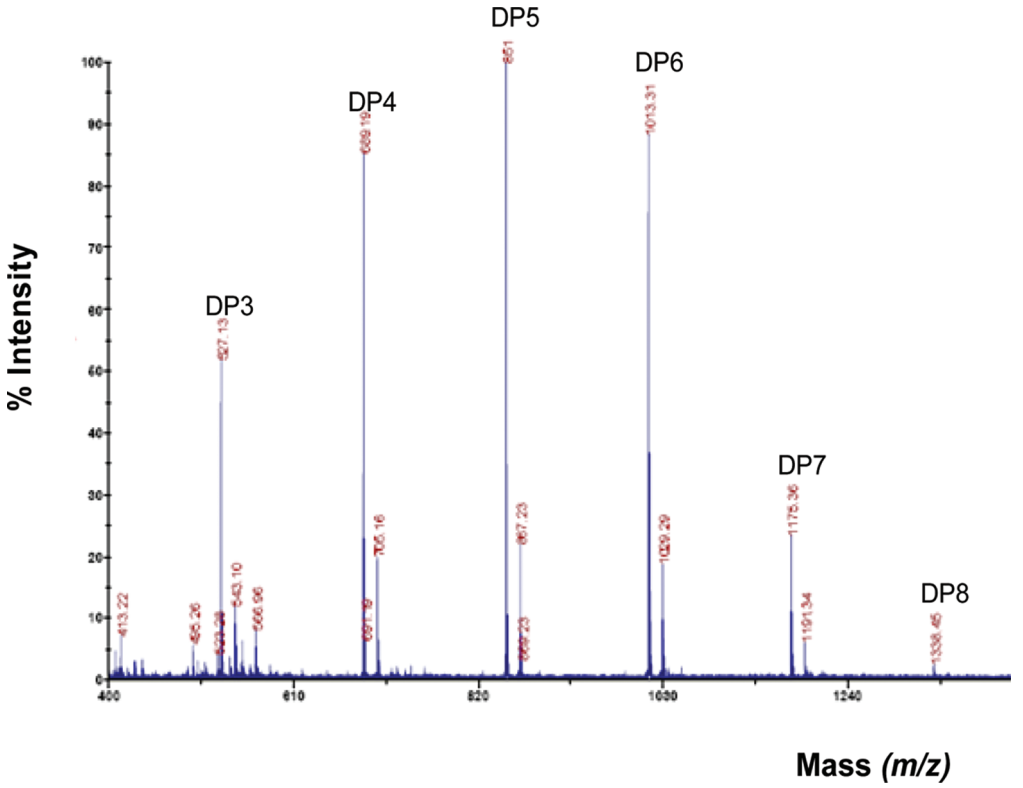

Figure 1. MALDI-TOF-MS profile of the purified fraction of oligosaccharides formed during enzymatic hydrolysis of stachyose with Pectinex Ultra SP-L (DP, degree of polymerization).

(St. Louis, MO). The commercial enzyme preparation from *A. aculeatus*, Pectinex Ultra SP-L, containing fructosyltransferase activity was a generous gift from Novozymes (Dittingen, Switzerland).

Determination of Enzymatic Activity. The fructosyltransferase activity of Pectinex was measured using sucrose as substrate. The activity was assayed at 60 °C using sucrose at 300 g/L in 0.1 M sodium acetate buffer (pH 5.5). Aliquots (0.1 mL) were withdrawn at different times. The reaction was stopped by adding 10 μL of 0.1 N acetic acid. The amount of fructose and glucose released was determined by gas chromatography (GC-FID) using the method described below. Fructosyltransferase activity was calculated as the difference between the amount of free glucose and fructose, which indicated the amount of fructose involved in the transfructosylation reaction. The fructosyltransferase activity was 400 units (U), where 1 U is defined as the amount of enzyme transferring 1 μmol of fructose per minute per milliliter under assayed conditions. Enzyme activity measurements were performed in triplicate, and the experimental error (RSD relative standard deviation) was <5%.

The soluble protein concentration in the enzymatic preparation was determined using the bicinchoninic acid (BCA) assay.[23] Bovine serum albumin (BSA) was used as standard. Specific activity was defined as units per milligram of protein. The soluble protein concentration in the enzyme preparation was 82 mg/mL. Therefore, the enzyme expressed a fructosyltransferase specific activity of 4.9 U/mg soluble protein.

Enzymatic Synthesis of Oligosaccharides. Enzymatic synthesis of oligosaccharides from stachyose using Pectinex was carried out under different reaction conditions such as temperature (50, 60, and 70 °C), pH (3.5, 4.5, 5.5, 6.5, and 7.5), stachyose concentration (100, 300, and 600 g/L), enzyme concentration (17, 34, and 78 U/mL), and time (0.5, 1, 3, 6, and 24 h). Reactions were performed at a final volume of 1.5 mL in microtubes incubated in an orbital shaker at 300 rpm. Aliquots (120 μL) were withdrawn from the reaction mixture at the different times and immediately immersed in boiling water for 5 min to inactivate the enzyme. Samples were stored at −18 °C for subsequent analysis. Experiments were carried out in duplicate.

The amount of remaining stachyose and the yield of oligosaccharides in the reaction mixtures were expressed as weight percentage of total carbohydrate content.

Purification and Characterization of Reaction Mixtures. Purification was performed following the method described by Morales et al.[24] Briefly, a total of 2.5 mL of reaction mixture, containing 1.5 g of carbohydrates, was dissolved in 300 mL of a 1% aqueous solution of ethanol and stirred for 30 min with 9 g of activated charcoal Darco G60, 100 mesh (Sigma, St. Louis, MO), to remove mono- and disaccharides. This mixture was vacuum-filtered through Whatman no. 1 filter paper, and activated charcoal was washed with 50 mL of water. The oligosaccharides adsorbed onto the activated charcoal were extracted by stirring for 30 min with 300 mL of ethanol/water solution (1:1, v/v) and then vacuum-filtered. The ethanol/water solution was evaporated under vacuum at 30 °C. The sample was dissolved in 5 mL of deionized water and filtered through 0.22 μm filters (Millipore Corp., Bedford, MA) for further characterization by mass spectrometry. This methodology allowed very good recoveries to be obtained for high molecular weight oligosaccharides (48% trisaccharides, 91% tetrasaccharides, and 100% of DP_5 and DP_6).

The enriched fraction containing the main synthesis products was characterized by matrix-assisted laser desorption/ionization−time-of-flight−mass spectrometry (MALDI-TOF-MS) on a Voyager DE-PRO mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a pulsed nitrogen laser (λ = 337 nm, 3 ns pulse width, and 3 Hz frequency) and a delayed extraction ion source. Ions generated by laser desorption were introduced into a time-of-flight analyzer (1.3 m flight path) with an acceleration voltage of 20 kV, 74% grid voltage, 0.001% ion guide wire voltage, and delay time of 300 ns in the reflector positive ion

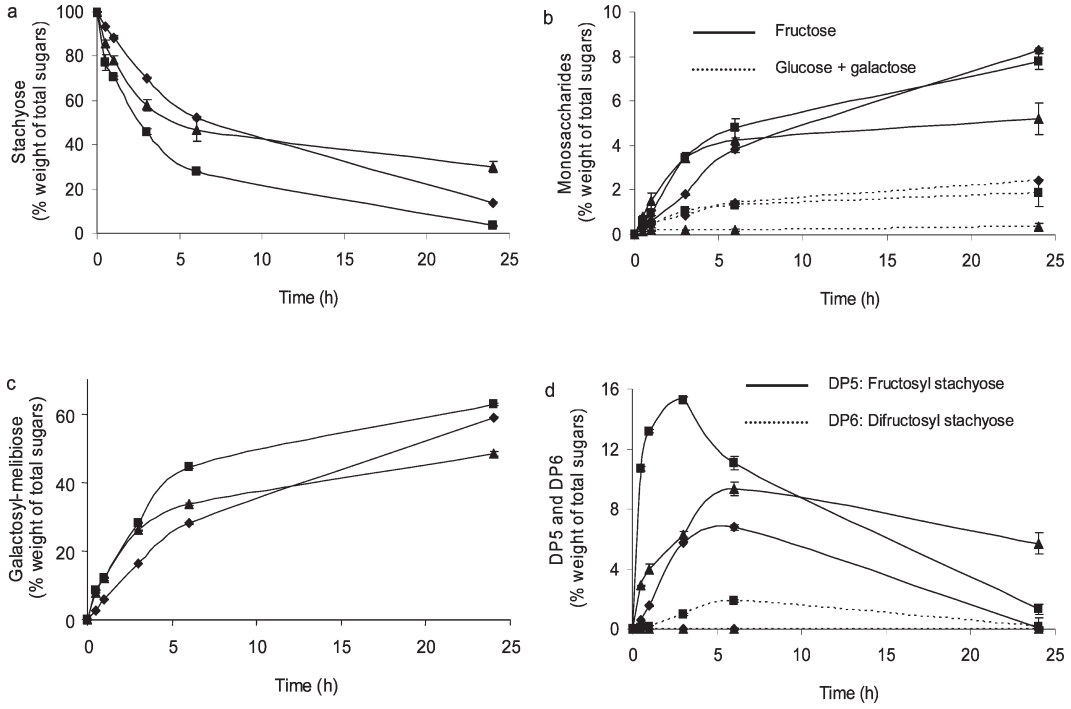

Figure 2. Effect of temperature on stachyose hydrolysis and oligosaccharide production during the enzymatic treatment of stachyose (300 mg/mL) with Pectinex Ultra SP-L (34 U/mL) in 0.1 M sodium acetate buffer (pH 5.5): (◆) 50 °C; (■) 60 °C; (▲) 70 °C.

mode. Mass spectra were obtained over the m/z range 100−1500. External mass calibration was applied using the monoisotopic $[M + H]^+$ values of des-Arg[1] bradykinin and angiotensin I of the Calibration Mixture 1, Sequazyme Peptide Mass Standards Kit, Applied Biosystems. 2,5-Dihydroxybenzoic acid (>98%, Fluka) at 10 mg/mL in water was used as matrix. Sample was diluted 100 times in water and mixed with the matrix at a ratio of 1:4 (v/v). One microliter of this solution was spotted onto a flat stainless steel sample plate and dried in air before analysis.

Carbohydrate Analysis by Gas Chromatography (GC-FID). Trimethylsilylated oximes (TMS-oxime) of mono-, di-, and oligosaccharides were analyzed by GC following the method of Montilla et al.[25] GC analysis was performed with a gas chromatograph (3800GC, Varian, Palo Alto, CA) equipped with a flame ionization detector (FID). The trimethylsilyl oximes were separated using a 8 m × 0.25 mm × 0.25 μm film fused silica capillary column coated with CP-SIL 5CB (methyl silicone from Chrompack, Middelburg, The Netherlands), giving rise to two peaks, corresponding to the syn (*E*) and anti (*Z*) isomers. The carrier gas (nitrogen) flow rate was 1.1 mL/min. The injector temperature was 280 °C, the detector temperature was 340 °C, and the oven temperature was programmed from 150 to 165 °C at 3 °C/min, then at 5 °C/min to 340 °C, and held at this temperature for 10 min. Injections were made in the split mode (1:10). Data acquisition and integration was done using HP ChemStation software (Hewlett-Packard, Wilmington, DE).

The oximes were formed following the method of Brobst and Lott.[26] A volume of 7−40 μL of sample constituted of approximately 4 mg of sugars was added to 0.4 mL of internal standard (IS) solution containing 0.5 mg/mL phenyl-β-glucoside. The mixture was dried at 38−40 °C in a rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland). Sugar oximes were formed by adding 200 μL of hydroxylamine chloride (2.5%) in pyridine and heating the mixture at 70 °C for 30 min. Subsequently, the oximes obtained in this step were silylated with hexamethyldisilazane (200 μL) and trifluoroacetic acid (20 μL) and kept at 50 °C for 30 min. Reaction mixtures were centrifuged at 7000*g* for 5 min at 5 °C. Supernatants were injected in the GC or stored at 4 °C prior to analysis.

Quantitative analysis was carried out by the internal standard method. Response factors were calculated using a triplicate analysis of seven standard solutions (galactose, glucose, fructose, sucrose, melibiose, raffinose, and stachyose) at a concentration ranging from 0.06 to 2 mg/mL (from 0.06 to 4 mg/mL for stachyose).

RESULTS AND DISCUSSION

The fructosidase, galactosidase, and fructosyltransferase activities of Pectinex resulted in the production of melibiose (α-D-Galp-(1→6)-α-D-Glcp), raffinose (α-D-Galp-(1→6)-α-D-Glcp-(1→2)-β-D-Fruf), 6′-galactosyl melibiose (α-D-Galp-(1→6)-α-D-Galp-(1→6)-α-D-Glcp), fructosyl stachyose (DP_5) (1^F fructofuranosyl stachyose, α-D-Galp-(1→6)-α-D-Galp-(1→6)-α-D-Glcp-(1→2)-β-D-Fruf-(1→2)-β-D-Fruf), and difructosyl stachyose (DP_6) (1^F (fructofuranosyl)$_2$ stachyose (α-D-Galp-(1→6)-α-D-Galp-(1→6)-α-D-Glcp-(1→2)-β-D-Fruf-(1→2)-β-D-Fruf-(1→2)-β-D-Fruf) as has been previously reported.[22] Besides, MALTI-TOF-MS analysis of oligosaccharide-enriched fractions allowed the detection of oligosaccharides with DP_7 (m/z 1175.4 and 1191.3) and DP_8 (m/z 1338.5) (Figure 1), whereas previous studies have reported only the formation of oligosaccharides DP_3 and DP_5 from stachyose by fructosyltransferase from *A. niger*.[20]

Several papers have demonstrated that glycosidic linkages and degree of polymerization of oligosaccharides contribute toward the selectivity of fermentation by beneficial bacteria.[2,3,27] In addition, a wide molecular weight range could give rise to modulation of microbial fermentation. Higher molecular oligosaccharides may be slowly fermented, exhibiting higher colonic persistence than low molecular weight carbohydrates, reaching the most distal regions where most intestinal disorders are encountered.[3] On the basis of this information, the production of α-galactoside mixtures from stachyose composed of carbohydrates with DP from 3 to 8 may represent an opportunity in the development of future prebiotics.

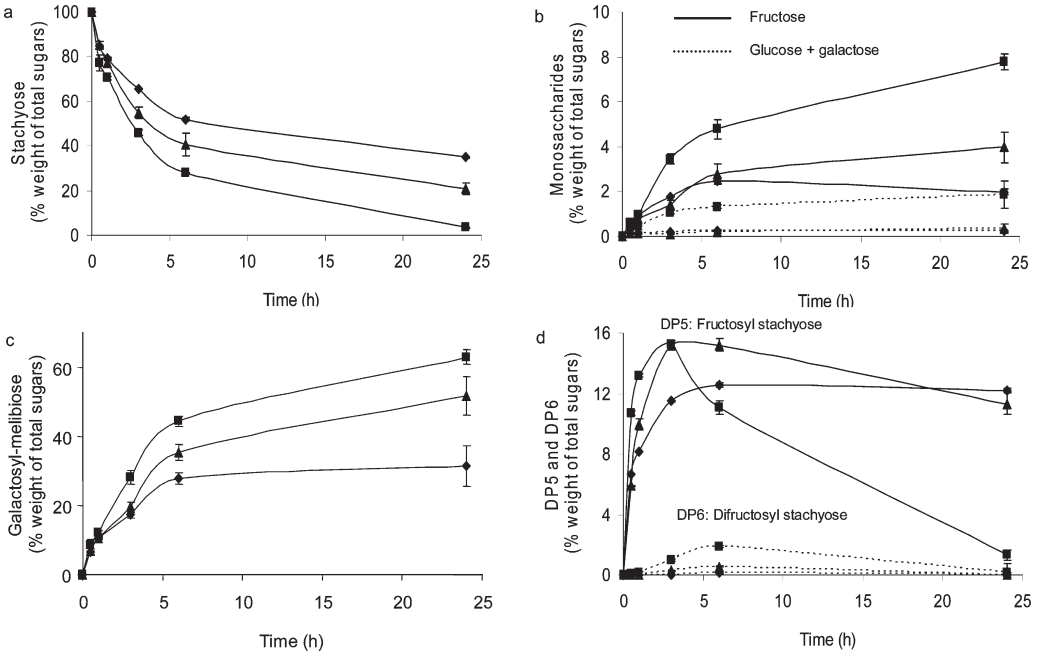

Figure 3. Effect of pH on stachyose hydrolysis and oligosaccharide production during the enzymatic treatment of stachyose (300 mg/mL) with Pectinex Ultra SP-L (34 U/mL) at 60 °C in 0.1 M sodium acetate buffer: (◆) pH 4.5; (■) pH 5.5; (▲) pH 6.5.

Study of the Formation of Oligosaccharides from Hydrolysis of Stachyose. A series of experiments were performed in which temperature, pH, and substrate and enzyme concentrations were varied to select the optimum conditions for tri- and oligosaccharide synthesis.

Effect of Temperature. To determine the influence of temperature on oligosaccharide synthesis, reaction was performed in a temperature range of 50−70 °C, 300 mg/mL stachyose, 34 U/mL enzyme, and pH 5.5, for 24 h (Figure 2). The loss of stachyose (Figure 2a) increased with temperature from 50 to 60 °C but declined with further increase of temperature to 70 °C. This fact could be attributed to a thermal denaturation of enzyme at 70 °C.

During the first 6 h of the reaction, fructose release (Figure 2b) was favored at 60 °C; however, after 24 h, when most of the stachyose was consumed, the amount of fructose reached at 50 °C was similar to that observed at 60 °C. Galactosyl-melibiose production (Figure 2c) reached maximum yields after 24 h either at 50 or at 60 °C (59 and 63%, respectively).

The transferase/hydrolase ratio of the enzyme was optimal at 60 °C, the highest DP_5 oligosaccharide yield (15%) being observed after 3 h (Figure 2d). There is a sharp decrease of this pentasaccharide at 50 and 60 °C after reaching maximum yield, whereas larger amounts of this α-galactoside remained after 24 h at 70 °C. DP_6 oligosaccharide production was possible only at 60 °C, reaching the highest concentration (2%) after 6 h. Our results are in agreement with previous works which reported that the optimal temperature for fructooligosaccharide production from sucrose by fructosyltransferase activity of Pectinex was around 60 °C.[21,28]

Effect of pH. Different experiments were carried out at pH values from 3.5 to 7.5 at 60 °C using reaction mixtures of stachyose (300 mg/mL) and 34 U/mL enzyme. In assays carried out at pH 3.5 and 7.5 hardly any stachyose hydrolysis was observed (data not shown). The highest rates of stachyose decrease (Figure 3a), fructose release (Figure 3b), and galactosyl-melibiose formation (Figure 3c) were attained at pH 5.5. At pH 4.5 the lowest fructosidase activity was observed.

The maximum formation of DP_5 (Figure 3d) was observed at pH 5.5 and 6.5 at 3 h of reaction, reaching a yield of 15%, and then slowly decreased to 11% after 24 h. However, a marked decrease to 1% was observed at pH 5.5, probably due to a higher fructosidase activity of the enzyme under these conditions.

The highest amount of DP_6 formed was 2%, at pH 5.5 after 6 h of reaction, whereas yields at pH 4.5 and 6.5 were negligible. These results are consistent with previous studies of Ghazi et al.[28] showing that the optimal activity/stability of purified fructosyltransferase from Pectinex was in the pH range of 5.0−6.0, similar to those of other fructosyltransferases derived from fungi.[29,30]

Effect of Enzyme Concentration. The effect of enzyme concentration (17, 34, and 68 U/mL) was studied using reaction mixtures containing 300 mg/mL stachyose at pH 5.5 and 60 °C (Figure 4). Loss of stachyose and formation of galactosyl-melibiose noticeably increased when enzyme concentration was raised to 34 U/mL, whereas a further increase of the enzyme concentration did not result in a significant improve of stachyose conversion and trisaccharide yield (Figure 4a,c). With regard to DP_5 formation, enzyme concentration did not exert any influence on maximum yields attained, although the formation rate of this pentasaccharide increased with enzyme concentration (Figure 4d). DP_6 yields decreased with enzyme concentration, the highest yield (2%) being observed at an enzyme concentration of 17 U/mL after 24 h (Figure 4d). With stachyose, the most important effect observed was an increase of hydrolysis rate with enzyme concentration. Also, the hydrolysis rate of the DP_5 and DP_6 formed during the first 3−6 h increased with the enzyme concentration (Figure 4d). This was consistent with the monosaccharide concentrations observed (Figure 4b). The highest enzyme concentration tested (68 U/mL) gave rise to the highest fructose (13%) and galactose + glucose (5%) concentrations after 24 h of reaction. However, in all conditions it was observed

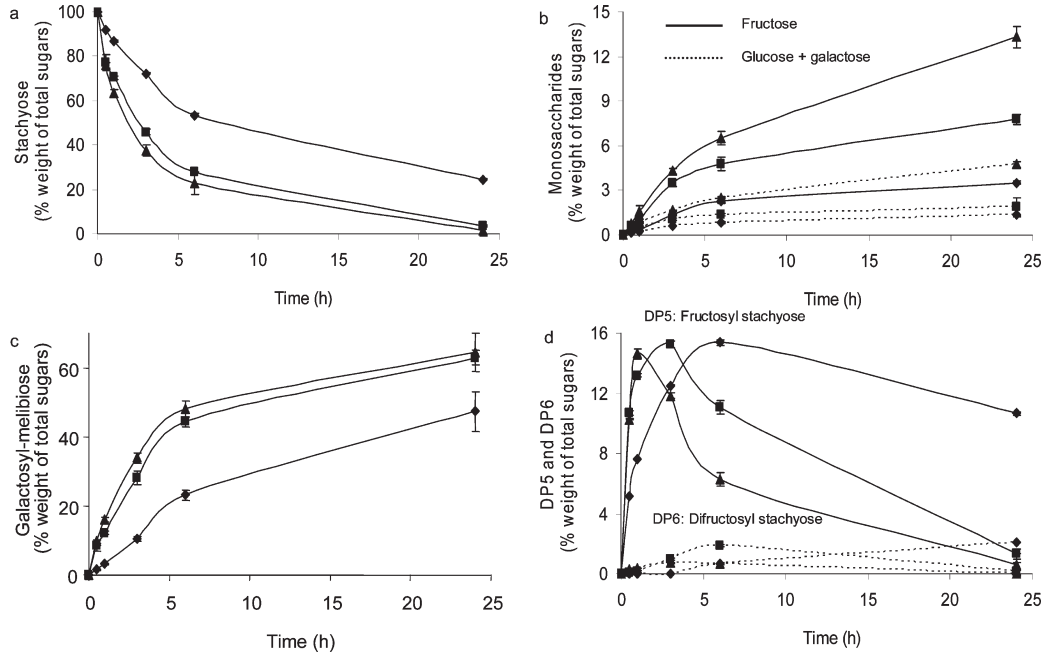

Figure 4. Effect of enzyme concentration on stachyose hydrolysis and oligosaccharide production during the enzymatic treatment of stachyose (300 mg/mL) at 60 °C and pH 5.5 with Pectinex Ultra SP-L: (◆) 17 U/mL; (■) 34 U/mL; (▲) 68 U/mL.

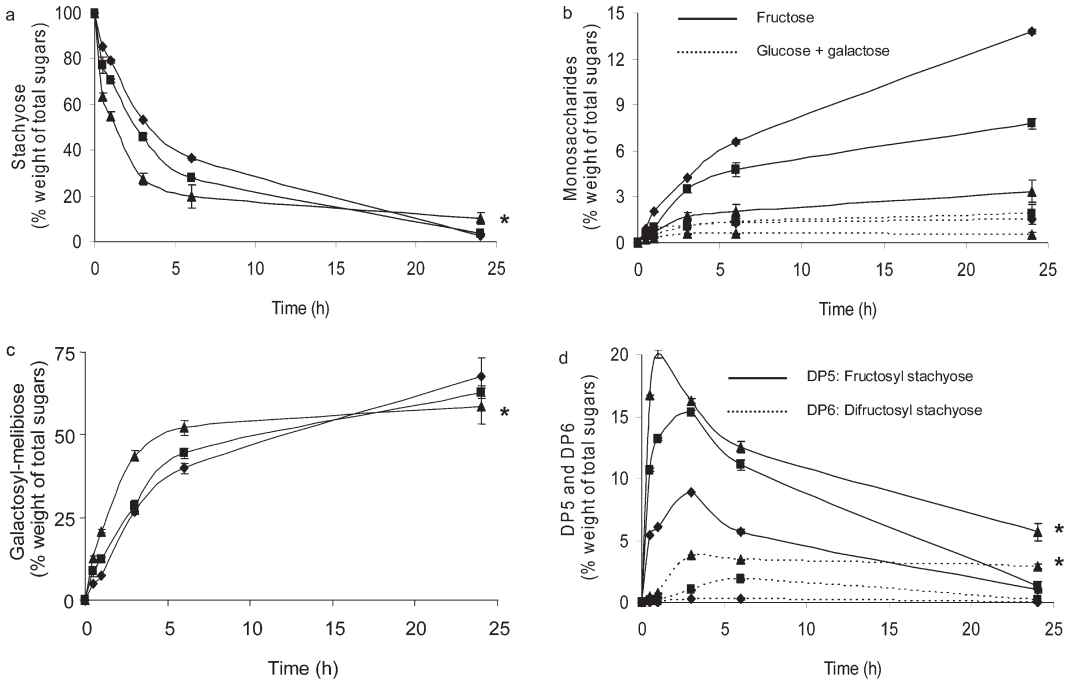

Figure 5. Effect of stachyose concentration on hydrolysis and oligosaccharide production from reaction mixtures of stachyose [(◆) 100 mg/mL; (■) 300 mg/mL; (▲) 600 mg/mL] and Pectinex Ultra SP-L (34 U/mL) at pH 5.5 and 60 °C. (∗) Data from Montilla et al.[22]

that galactosyl-melibiose was stable to hydrolysis; the highest melibiose formation was only 3% after 24 h with 68 U/mL enzyme concentration.

Effect of Initial Stachyose Concentration. To investigate the influence of substrate concentration on the composition of synthesis mixtures of oligosaccharides derived from stachyose, different assays were carried out in a range from 100 to 600 mg/mL (Figure 5). Reactions were performed at 60 °C, pH 5.5, and 34 U/mL enzyme for 24 h. During the first 6 h of reaction, stachyose decreased rapidly (80% of hydrolysis), especially at the highest concentration tested, 600 mg/mL (Figure 5a). At 24 h of reaction, similar levels of stachyose were found at three studied concentrations. The fructosidase activity of Pectinex was favored with low stachyose concentrations (100 mg/mL) and longer reaction time; thus, increasing amounts of free fructose were released, reaching 14% after 24 h of reaction (Figure 5b).

The rate of galactosyl-melibiose formation increased with initial stachyose concentration during the first 6 h of reaction, reaching levels of 313 mg/mL (corresponding to a yield of 52%) (Figure 5b). However, the highest yield of galactosyl-melibiose (67%) was achieved when the low stachyose amount was assayed (100 mg/mL). The galactosyl-melibiose yield obtained using β-galactosidase from *A. aculeatus* (Pectinex) and stachyose as substrate was much higher than that obtained by Van Laere et al.,[31] who achieved 33% using α-galactosidase from *Bifidobacterium adolescentis* and 103 mg/mL melibiose. Tzortzis et al.[32] and Goulas et al.[33] obtained 26 and 21% of galactosyl melibiose, respectively. The former used α-galactosidase from *Lactobacillus reuteri* and 230 mg/mL melibiose and the latter, α-galactosidase from *B. bifidus* and 400 mg/mL melibiose.

Galactosyl-melibiose is an interesting compound with prebiotic and symbiotic properties when used along with *L. acidophilus* and *L. reuteri*. This has been proven using galactosyl-melibiose mixtures, which produced higher increases in bifidobacterium and lactobacillus populations and higher decreases of clostridia and *Escherichia coli* count than carbohydrates such as FOS, melibiose, and raffinose used as reference.[34]

Transfructosylation reaction mainly occurred at reaction times of <3 h and increased with initial substrate concentrations to 600 mg/mL (Figure 5d). The highest yields of transfructosylation products, 20% for DP_5 (120 mg/mL) and 4% DP_6 (23 mg/mL), were found at the highest initial stachyose concentration (600 mg/mL) after 1 and 3 h, respectively. Our research group used previously this concentration to obtain and characterize DP_5 and DP_6 compounds.[22] These results are consistent with previous studies showing that a high substrate concentration exerts a noticeable influence on the formation of stachyose-derived oligosaccharides by fructosyltransferases due to an increase in transferase/hydrolysis ratio.[13,35] Ghazi et al.[28] reported that the fructosyltransferase activity in a purified enzyme from *A. aculeatus* is approximately 20-fold higher than hydrolysis activity at sucrose concentrations >342 g/L.

These results show the noticeable effect of reaction conditions on oligosaccharide formation during the enzymatic treatment of stachyose with Pectinex. The optimal reaction conditions for the synthesis of DP_5 and DP_6 were 60 °C, pH 5.5, 600 mg/mL stachyose, and 34 U/mL enzyme. Under these conditions the time of reaction may noticeably influence the composition of α-galactoside mixtures; thus, 0.7% DP_6, 20% DP_5, 55% stachyose, 21% galactosyl-melibiose, and 1% monosaccharides were found after 1 h of reaction and 4% DP_6, 16% DP_5, 27% stachyose, 44% galactosyl-melibiose, and 2% monosaccharides after 3 h of reaction. However, to obtain the maximum yield of galactosyl-melibiose (67%), the assays should be carried out at 60 °C and pH 5.5, using 100 mg/mL stachyose and 34 U/mL enzyme during 24 h.

α-Galactosides, which are industrially available in large amounts as a byproduct from the production of soy protein isolate, seem to be a promising raw material for the production of new oligosaccharides through hydrolysis and transglycosylation using a GRAS commercial enzyme preparation. Further research is warranted to evaluate the prebiotic properties of these new α-galactosides mixtures that may be used to influence the microbial composition in the distal colon, where most gut disorders occur.

AUTHOR INFORMATION

Corresponding Author

*Phone: + 34 91 0017 954. Fax: + 34 91 0017 905. E-mail: nieves.corzo@csic.es.

Funding Sources

This work has been financed under a R+D program of the Spanish Ministry of Science and Innovation Science, Projects AGL-2008-00941/ALI and Consolider Ingenio 2010 (FUN-C-FOOD) CSD 2007-00063; a R+D program of the Comunidad de Madrid, Project ALIBIRD P2009/AGR-1469; and as a R+D program of the Comunidad de Castilla-La Mancha, POII10-0178-4685.

ACKNOWLEDGMENT

We are grateful to Ramiro Martinez (Novozymes A/S, Spain) for providing us with Pectinex Ultra SP-L and to Plácido Galindo for his help in acquiring the MALDI-TOF-MS spectral data.

REFERENCES

(1) Roberfroid, M.; Gibson, G. R.; Hoyles, L.; McCartney, A. L.; Rastall, R.; Rowland, I.; Wolvers, D.; Watzl, B.; Szajewska, H.; Stahl, B.; Guarner, F.; Respondek, F.; Whelan, K.; Coxam., V.; Davicco, M. J.; Leotoing, L.; Wittrant, Y.; Delzenne, N. M.; Cani, P. D.; Neyrinck, A. M.; Meheust, A. Prebiotic effects: metabolic and health benefits. *Br. J. Nutr.* **2010**, *104*, S1–S63.

(2) Sanz, M. L.; Gibson, G. R.; Rastall, R. A. Influence of disaccharide structure on prebiotic selectivity in vitro. *J. Agric. Food Chem* **2005**, *53*, 5192–5199.

(3) Sanz, M. L.; Cote, G. L.; Gibson, G. R.; Rastall, R. A. Influence of glycosidic linkages and molecular weight on the fermentation of maltose-bases oligosaccharides by human gut bacteria. *J. Agric. Food Chem.* **2006**, *54*, 9779–9784.

(4) Hughes, S. A.; Shewry, P. R.; Li, L.; Gibson, G. R.; Sanz, M. L.; Rastall, R. A. In vitro fermentation by human fecal microflora of wheat arabinoxylans. *J. Agric. Food Chem.* **2007**, *55*, 4589–4595.

(5) Ouwehand, A. C.; Derrien, M.; de Vos, W.; Tiihonen, K.; Rautonen, N. Prebiotics and other microbial substrates for gut functionality. *Curr. Opin. Biotechnol.* **2005**, *16*, 212–217.

(6) Martinez-Villaluenga, C.; Frias, J.; Vidal-Valverde, C. α-Galactosides: antinutritional factors or functional ingredients? *Crit. Rev. Food Sci. Nutr.* **2008**, *48*, 301–316.

(7) Van den Broek, L. A. M.; Hinz, S. W. A.; Beldman, G.; Doeswijk-Voragen, C. H. L.; Vincken, J. P.; Voragen, A. G. J. Glycosyl hydrolases from *Bifidobacterium adolescentis* DSM20083. An overview. *Lait* **2005**, *85*, 125–133.

(8) Trojanova, I.; Vlkova, E.; Rada, V.; Marcunek, M. Different utilization of glucose and raffinose in *Bifidobacterium breve* and *Bifidobacterium animalis*. *Folia Microbiol.* **2006**, *51*, 320–324.

(9) Farnworth, E. R.; Mainville, I.; Desjardins, M. P.; Gardner, N.; Fliss, I.; Champagne, C. Grow of probiotic bacteria and bifidobacteria in a soy yogurt formulation. *Int. J. Food Microbiol.* **2007**, *116*, 174–181.

(10) Martinez-Villaluenga, C.; Gomez, R. Characterization of bifidobacteria as starters in fermented milk containing raffinose family of oligosaccharides from lupin as prebiotic. *Int. Dairy J.* **2007**, *17*, 116–122.

(11) Chockchaisawasdee, S.; Athanasopoulos, V. I.; Niranjan, K.; Rastall, R. A. Synthesis of galacto-oligosaccharide from lactose using β-galactosidase from *Kluyveromyces lactis*: studies on batch and continuous UF membrane-fitted bioreactors. *Biotechnol. Bioeng.* **2005**, *89*, 434–443.

(12) Cardelle-Cobas, A.; Martinez Villaluenga, C.; Villamiel, M.; Olano, A.; Corzo, N. Synthesis of oligosaccharides derived from lactulose and Pectinex Ultra SP-L. *J. Agric. Food Chem.* **2008**, *56*, 3328–3333.

(13) Cardelle-Cobas, A.; Villamiel, M.; Olano, A.; Corzo, N. Study of galactooligosaccharides formation from lactose using Pectinex Ultra SP-L. *J. Sci. Food Agric.* **2008**, *88* (6), 954–961.

(14) Martínez-Villaluenga, C.; Cardelle-Cobas, A.; Corzo, N.; Olano, A.; Villamiel, M. Optimization of conditions for galactooligosaccharide synthesis during lactose hydrolysis by a β-galactosidase from *Kluyveromyces lactis* (Lactozym 3000 L HP G). *Food Chem.* **2008**, *107*, 258–264.

(15) Martínez-Villaluenga, C.; Cardelle-Cobas, A.; Olano, A.; Corzo, N.; Villamiel, M.; Jimeno, M. L. Enzymatic synthesis and identification of two trisaccharides produced from lactulose by transgalactosylation. *J. Agric. Food Chem.* **2008**, *56*, 557–563.

(16) Hang, Y. D.; Woodams, E. E. Fructosylransferase activity of commercial enzyme preparations used in fruit processing. *Biotechnol. Lett.* **1995**, *17*, 741–744.

(17) Yun, J. W. Fructooligosaccharides — occurrence, preparation, and application. *Enzyme Microb. Technol.* **1996**, *19*, 107–117.

(18) Sangeetha, P. T.; Ramesh, M. N.; Prapulla, S. G. Recent trends in the microbial production, analysis and application of Fructooligosaccharides. *Trends Food Sci. Technol.* **2005**, *16*, 442–457.

(19) Ghazi, I.; Fernandez-Arrojo, L.; Gomez de Segura, A.; Alcalde, M.; Plou, F. J.; Ballesteros, A. Beet sugar syrup and molasses as low-cost feedstock for the enzymatic production of fructo-oligosaccharides. *J. Agric. Food Chem.* **2006**, *54*, 2964–2968.

(20) Uhm, T. B.; Baek, N. I.; Jhon, D. Y.; Kim, D. M.; Hwang, K. T.; Ryu, E. J. Synthesis the new oligosaccharides from raffinose by *Aspergillus niger* fructosyltransferase. *Biotechnol. Tech.* **1999**, *13*, 169–171.

(21) Tanriseven, A.; Aslan, Y. Immobilization of Pectinex Ultra SP-L to produce fructooligosaccharides. *Enzyme Microb. Technol.* **2005**, *36*, 550–554.

(22) Montilla, A.; Corzo, N.; Olano, A.; Jimeno, M. L. Identification of oligosaccharides formed during stachyose hydrolysis by Pectinex Ultra SP-L. *J. Agric. Food Chem.* **2009**, *57*, 5007–5013.

(23) Smith, P. K.; Krohn, R. I.; Hermanson, G. T.; Mallia, A. K.; Gartner, F. H.; Provenzano, M. D.; Fujimoto, E. K.; Goeke, N. M.; Olson, B. J.; Klenk, D. C. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **1985**, *150*, 76–85.

(24) Morales, V.; Sanz, M. L.; Olano, A.; Corzo, N. Rapid separation on activated charcoal of high oligosaccharides in honey. *Chromatographia* **2006**, *64*, 233–238.

(25) Montilla, A.; van de Lagemaat, J.; Olano, A.; del Castillo, M. D. Determination of oligosaccharides by conventional high-resolution gas chromatography. *Chromatographia* **2006**, *63*, 453–458.

(26) Brobst, K. M.; Lott, C. E. Determination of some components in corn syrup by gas-liquid chromatography of trimethylsilyl derivatives. *Cereal Chem.* **1996**, *43*, 35–43.

(27) Rowland, I. R.; Tanaka, R. The effects of transgalactosylated oligosaccharides on gut flora metabolism in rats associated with a human fecal microflora. *J. Appl. Bacteriol.* **1993**, *74*, 667–674.

(28) Ghazi, I.; Fernandez-Arrojo, L.; Garcia-Arellano, H.; Ferrer, M.; Ballesteros, A.; Plou, F. J. Purification and kinetic characterization of a fructosyltransferase from *Aspergillus aculeatus*. *J. Biotechnol.* **2007**, *128*, 204–211.

(29) Hang, Y. D.; Woodams, E. E. Optimization of enzyme production of fructo-oligosaccharides from sucrose. *LWT—Food Sci. Technol.* **1996**, *29*, 578–580.

(30) Rezende, S. T.; Felix, C. R. Raffinose-hydrolyzing activity of *Aspergillus fumigatus*. *Biotechnol. Lett.* **1997**, *19*, 217–220.

(31) Van Laere, K. M. J.; Hartemink, R.; Beldman, G.; Pitson, S.; Dijkema, C.; Schols, H. A.; Voragen, A. G. J. Transglycosidase activity of *Bifidobacterium adolescentis* DSM 20083 α-galactosidase. *Appl. Microbiol. Biotechnol.* **1999**, *52*, 681–688.

(32) Tzortzis, G.; Jay, A. J.; Baillon, M. L. A.; Gibson, G. R.; Rastall, R. A. Synthesis of α-galactooligosaccharides with α-galactosidase from *Lactobacillus reuteri* of canine origin. *Appl. Microbiol. Biotechnol.* **2003**, *63*, 286–292.

(33) Goulas, T.; Goulas, A.; Tzortzis, G.; Gibson, G. R. A novel α-galactosidase from *Bifidobacterium bifidum* with transgalactosylating properties: gene molecular cloning and heterologous expression. *Appl. Microbiol. Biotechnol.* **2009**, *82*, 471–477.

(34) Tzortzis, G.; Goulas, A. K.; Baillon, M. L. A.; Gibson, G. R.; Rastall, R. A. In vitro evaluation of the fermentation properties of galactooligosaccharides synthesised by α-galactosidase from *Lactobacillus reuteri*. *Appl. Microbiol. Biotechnol.* **2004**, *64*, 106–111.

(35) Mahoney, R. R. Galactosyl-oligosaccharide formation during lactose hydrolysis: a review. *Food Chem.* **1998**, *6*, 147–154.