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Identification of Oligosaccharides Formed during Stachyose Hydrolysis by Pectinex Ultra SP-L

ANTONIA MONTILLA,[‡] NIEVES CORZO,*[,‡] AGUSTIN OLANO,[‡] AND MARÍA LUISA JIMENO[§]

[‡]Instituto de Fermentaciones Industriales and [§]Centro Química Orgánica Lora Tamayo, CSIC, Juan de la Cierva 3, 28006 Madrid, Spain

The commercial enzyme preparation Pectinex Ultra SP-L containing fructosyltransferase activity was used to hydrolyze stachyose. During this reaction, besides the formation of mono-, di-, and trisaccharides (DP_3), the presence of one pentasaccharide (DP_5) and a new oligosaccharide (DP_6) has been detected by gas chromatography. DP_5 and DP_6 oligosaccharides were isolated and fully characterized for the first time by an extensive nuclear magnetic resonance (NMR) study. Complete structure elucidation and full proton and carbon assignments were carried out using 1D (^1H, ^{13}C) and 2D (gCOSY, multiplicity-edited gHSQC, gHSQC-TOCSY, and gHMBC) NMR experiments. The two oligosaccharides were shown to be stachyose-based structures; the pentasaccharide has a fructose unit linked to the C-1 of the fructose end of stachyose, and the hexasaccharide has a fructose unit linked to the C-1 of the fructose end of the pentasaccharide. The fructosyltransferase activity present in Pectinex Ultra SP-L allows new uses of this commercial enzyme preparation in the synthesis of oligosaccharides derived from α-galactosides.

KEYWORDS: Galactosides; stachyose; Pectinex-Ultra SP-L; NMR

INTRODUCTION

α-Galactosides are oligosaccharides abundant in grain legumes such as soy, lupin, and chickpea. Although their consumption is associated with the production of flatulence, they might be used as a prebiotic growth substrate for intestinal bacteria because they pass undigested to the colon due to the absence of α-galactosidase among human endogenous enzymes, and they are therefore available for a few bacteria that are known to have high α-galactosidase activity (*1*−*5*). Several studies provide convincing evidence that α-galactosides have beneficial effects on the survival of different bifidobacteria strains (*6*−*9*). In vitro assays have shown the ability of *Lactobacillus reuteri* (*4*) and *Bifidobacterium adolescentis* (*5*) to grow when α-galactosides were used as the sole carbon source.

Prebiotic carbohydrates may be fermented in different parts of the digestive tract. Rapidly fermented prebiotics stimulate the production of bifidobacteria in the proximal colon, whereas slowly fermented prebiotics can maintain fermentation all the way to the distal end of the large intestine. In addition to the health benefits in the colon, other parts of the gastrointestinal tract are considered to be potential prebiotic targets, and new prebiotics could be designed to promote the health of the oral cavity and of the small intestine. Also, the urogenital tract can be considered to be a new potential target for prebiotics as it harbors a diverse microbiota susceptible to disturbance (*10*). The rate at which oligosaccharides are fermented in the gut depends on, among other factors, the monosaccharide composition, chain length, and linkage types. Therefore, the synthesis of new oligosaccharides can contribute to the development of prebiotic compounds with complementary properties. Enzyme-catalyzed transglycosylation has been widely used to increase the prebiotic oligosaccharide groups mainly through the synthesis of galactooligosaccharides (GOS) by transgalactosylation during lactose hydrolysis using *β*-galactosidases from different sources (*11*−*13*) and fructooligosaccharides from sucrose by transfructosylation (*14*−*16*).

The tetrasaccharide stachyose and the trisaccharide raffinose are the main α-galactosides in legume seeds. They are derived from sucrose by successive condensations of α-D-galactose units attached by α-1,6 linkages to the glucose moiety. Because these carbohydrates contain a sucrose residue, they may be used as both donor and acceptor substrates for transfructosylation. Purified fructosyltransferase from *Aspergillus niger* has been used to catalyze the synthesis of new oligosaccharides from raffinose (*17*). Glycosidases usually cleave glycosidic bonds, but can also be used in a reverse mode for the formation of glycosidic bonds; they are inexpensive compared to transglycosidases and do not need complex expensive substrates, like sugar nucleotides (*18*). The relatively inexpensive commercial enzyme preparation Pectinex Ultra SP-L, produced by *Aspergillus aculeatus* and commonly used in fruit juice processing to reduce viscosity, was shown to contain high fructosyltransferase activity (*14*, *19*, *20*); therefore, it could be used as a catalyst in the large-scale production of prebiotic oligosaccharides. The present study was carried out to examine the possibility of using the commercial preparation Pectinex-Ultra SP-L for the synthesis of fructooligosaccharides derived from stachyose.

*Author to whom correspondence should be addressed (telephone: + 34 91 258 74 85; fax: + 34 91 564 48 53; e-mail: ncorzo@ifi.csic.es).

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MATERIALS AND METHODS

Materials. D-Galactose, D-glucose, D-fructose, sucrose, melibiose, raffinose, stachyose, and phenyl-β-glucoside were purchased from Sigma (St. Louis, MO). The commercial enzyme preparation Pectinex Ultra SP-L, a soluble preparation of fructosyltransferase produced by *A. aculeatus*, was a generous gift from Novozymes (Dittingen, Switzerland) and used as received without further purification.

Enzyme Characterization. The fructosidase and fructosyltransferase activities of Pectinex Ultra SP-L were measured using sucrose as substrate. The hydrolysis of sucrose (300 g/L) was assayed at 60 °C in sodium acetate solution (0.1 M, pH 5.5). Samples of 0.1 mL were withdrawn at different times. The reaction was stopped by adding 10 μL of 0.1 N acetic acid. The amounts of fructose and glucose formed were determined by GC using the method described below. The fructosidase activity of Pectinex Ultra SP-L was expressed as the amount of free glucose, and its fructosyltransferase activity was expressed as the difference between the amount of released glucose and fructose; this indicates the amount of fructose involved in the transfructosylation reaction. Enzyme activity measurements were repeated three times, and the experimental error was < 5%.

Pectinex Ultra SP-L expressed a fructosidase activity of 450 units, where 1 unit is defined as the amount of enzyme releasing 1 μmol of glucose per minute per milliliter under the assayed conditions. The fructosyltransferase activity was 400 units, where 1 unit is defined as the amount of enzyme joining 1 μmol of fructose per minute per milliliter at other molecules under the assayed conditions.

The protein concentration in the enzyme preparation was determined using the bicinchoninic acid (BCA) assay (*21*). Bovine serum albumin (BSA) was used as standard. Specific activity (units/mg) of the enzyme was calculated from the relationship of the activity units (units/mL) over protein concentration (mg/mL). The protein content in the enzyme preparation was 82 mg/mL. Therefore, the enzyme expressed a fructosidase specific activity of 5.5 units/mg and a fructosyltransferase specific activity of 4.9 units/mg.

Enzymatic Synthesis of α-Galactosides. Enzymatic syntheses of modified α-galactosides from stachyose (600 g/L) using 34 units/mL of commercial enzyme preparations Pectinex Ultra-SP-L in 0.1 M sodium acetate at pH 5.5 and a temperature of 60 °C were carried out following the method of Ghazi et al. (*20*). Samples were withdrawn from the reaction mixture at different times (0.5, 1, 3, 6, and 24 h). Reactions were carried out in duplicate with individual tubes of 2 mL, incubated in an orbital shaker at 300 rpm, and stopped by heating at 100 °C for 5 min. The samples were stored at −18 °C for subsequent analysis.

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

α-Galactosides Purification. Purification was performed following the method described by Morales et al. (*22*). In brief, a total of 2.5 mL of reaction mixture containing 1.5 g of carbohydrates was dissolved in 300 mL of water and stirred for 30 min with 9 g of activated charcoal Darco G60, 100 mesh (Sigma) to remove mono- and disaccharides. This mixture was filtered through Whatman no. 1 filter paper under vacuum, and the activated charcoal was washed with 50 mL of water. The oligosaccharides adsorbed onto the activated charcoal were then extracted by stirring the mixture with 300 mL of a 50:50 (v/v) ethanol/water solution. Activated charcoal was eliminated by filtering, and the solution was evaporated under vacuum at 30 °C. The solid residue was dissolved in 5 mL of deionized water, filtered through 0.22 μm filters (Millipore Corp., Bedford, MA), and purified by semipreparative high-performance liquid chromatography (HPLC-RI) using a refractive index detector RID-10 (Shimadzu) and a Kromasil 100 NH_2 5 μm 25 × 1.0 cm, column (Tecknocroma, Spain). The mobile phase was acetonitrile/water (70:30, v/v) at a flow rate of 3 mL/min. Fractions corresponding to the main peaks were pooled and freeze-dried for mass spectrometry (MS) and NMR analysis.

Gas Chromatographic (GC) Analysis. Trimethylsilylated oximes (TMS-oxime) of mono-, di-, and α-galactosides were analyzed by GC following the method of Montilla et al. (*23*). Chromatographic analysis was performed on a Varian 3800G (Harbor City, CA) gas chromatograph equipped with a flame ionization detector (FID). Separation was carried out in 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). The carrier gas (nitrogen) flow rate was 1.1 mL/min. The injector and detector temperatures were 280 and 340 °C, respectively. The oven temperature was programmed from 150 to 165 °C at 3 °C/min and then at 5 °C/min to 340 °C, keeping this temperature for 10 min. Injections were made in the split mode (1:10). Data acquisition and integration were done using HP ChemStation software (Hewlett-Packard, Wilmington, DE).

The TMS-oximes were formed following the method of Brobst and Lott (*24*). First, a quantity of sample corresponding to approximately 4 mg of sugars was added to 0.4 mL of internal standard (I.S.) solution (0.5 mg/mL phenyl-β-glucoside). Afterward, the mixture was dried at 38−40 °C in a rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland). TMS-sugar oxime derivatives were formed by adding 200 μL of hydroxylamine chloride (2.5%) in pyridine and heating the mixture at 75 °C for 30 min and then silylated with hexamethyldisilazane (200 μL) and trifluoroacetic acid (20 μL) and kept at 45 °C for 30 min. Reaction mixtures were centrifuged at 7000*g* for 5 min at 5 °C (*25*). Supernatants were injected in the GC or stored at 4 °C prior to analysis.

Stock standard solutions of each carbohydrate (galactose, glucose, fructose, sucrose, melibiose, raffinose, and stachyose) were prepared by dissolution in water/methanol (50:50, v/v) of an appropriate amount of carbohydrate. Then, several working standard solutions were prepared at different concentrations by various mixtures of stock standard solutions. Response factors (RF) were calculated using phenyl-β-glucoside as internal standard. Analysis of solutions was performed in triplicate.

The repeatability of the GC method was calculated using a standard solution prepared, derivatized, and analyzed five times on the same day. Reproducibility of the analytical method was calculated using a standard solution prepared, derivatized, and analyzed one time on five days. The relative standard deviation (RSD) was < 8%.

Structural Characterization. *Acid Hydrolysis.* To identify the monosaccharide residues that constitute the isolated oligosaccharides, 1 mg of each sample was dissolved in 1 mL of 1 N HCl and then heated in a hot-air oven in sealed tubes at 100 °C for 1 h (*26*). The reaction product was dried at 38−40 °C in a rotary evaporator and then converted to the TMS derivatives for GC analysis.

Mass Spectrometry (MS) Analysis. The molecular mass of the purified compounds was determined by MS using a quadrupole HP 1100 mass detector in the electrospray positive mode (API-ES). The mass spectrometer was operating with a 4000 V needle potential, 330 °C gas temperature drying gas flow of 10 mL/min, and 40 psi nebulizer pressure. Scan *m*/*z* was from 100 to 1500.

Nuclear Magnetic Resonance (NMR) Analysis. NMR spectra were recorded at 303 K, using D_2O as the solvent, on a Varian System 500 NMR spectrometer equipped with a 5 mm HCN cold probe. ^1H chemical shifts were referenced to the residual D_2O signal at δ_H 4.72 relative to TMS. ^{13}C chemical shifts were referenced using dioxane as an internal reference (δ_C 66.6 relative to external TMS in water). ^1H spectra were obtained using presaturation solvent suppression. gCOSY spectra were carried out with the following parameters: a delay time of 1.3 s, a spectral width of 1822.2 Hz in both dimensions, 4096 complex points in t2 and 4 transients for each of 256 time increments, and linear prediction to 512. The data were zero-filled to 4096 × 4096 real points. 2D [$^1H-^{13}C$] NMR experiments (multiplicity-edited gHSQC, gHSQC-TOCSY, and gHMBC) used the same ^1H spectral window, a ^{13}C spectral window of 7353 Hz, 1 s of relaxation delay, 1024 data points, and 256 time increments, with linear prediction to 512. The data were zero-filled to 2048 × 1024 real points. Typical numbers of transients per increment were 4, 8, and 16, respectively. For gHSQC-TOCSY spectra a mixing time of 80 ms was used. Multiplicity-edited gHSQC and gHMBC spectra were optimized for coupling constants of 145 and 8 Hz, respectively.

RESULTS AND DISCUSSION

Figure 1 shows the GC profiles of TMS-oxime derivatives of carbohydrates formed during stachyose hydrolysis by Pectinex Ultra SP-L after 3 h of reaction at pH 5.5, 60 °C, 600 mg/mL of stachyose, and 34 units/mL of enzyme. Besides stachyose (peak 6), compounds with retention times of fructose (peak 1), glucose (peak 2), galactose (peak 3), melibiose (peak 4), and

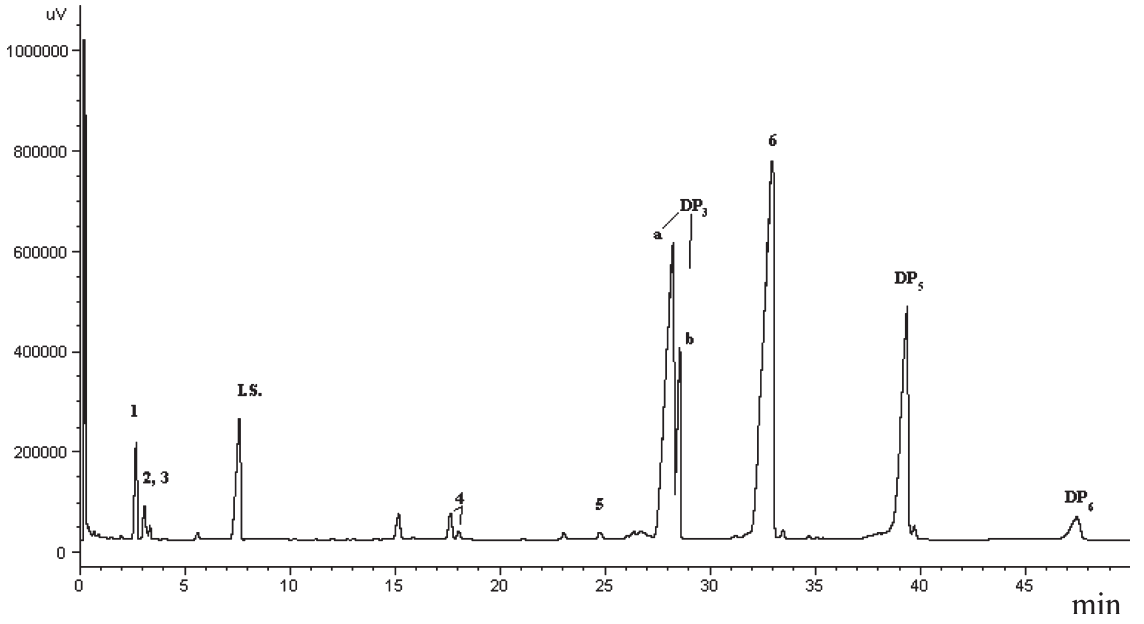

Figure 1. Gas chromatographic profile of the TMS oximes of oligosaccharides formed during enzymatic hydrolysis of stachyose (600 mg/mL) with Pectinex Ultra SP-L (34 units/mL) after 3 h at 60 °C in acetate buffer (0.1 M, pH 5.5). Peaks: 1, fructose; 2, glucose; 3, galactose; 4, melibiose; 5, raffinose; DP_3 (a and b); 6, stachyose; DP_5; DP_6; internal standard (I.S., phenyl-β-glucoside).

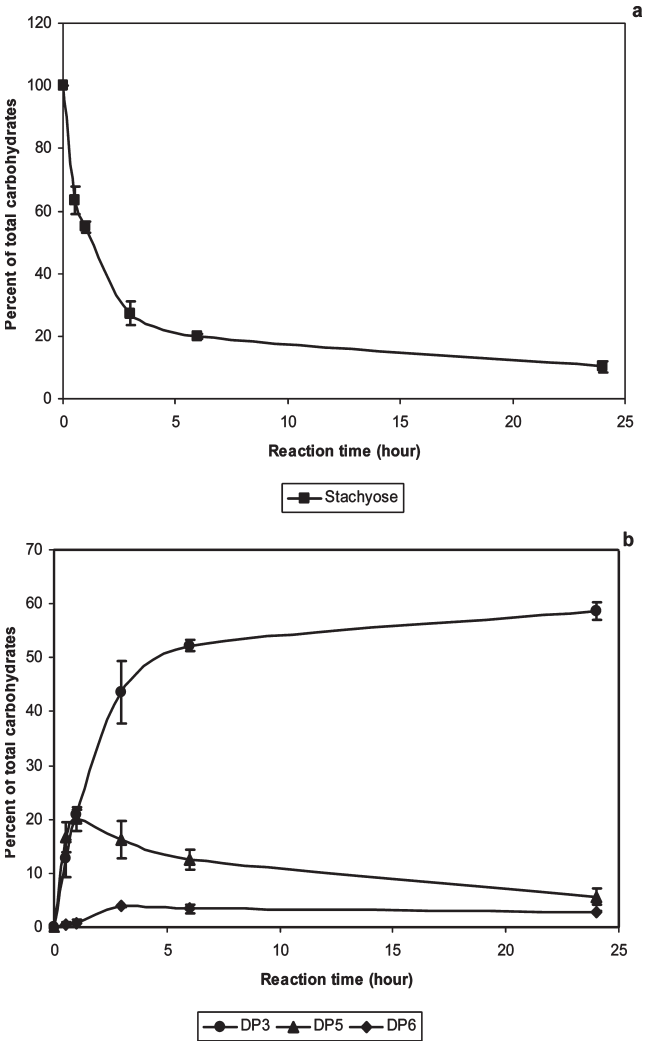

Figure 2. Remaining stachyose content (**a**) and DP_3, DP_5, and DP_6 formation (**b**) during the time course of the reaction performed at 60 °C, initial stachyose concentration of 600 mg/mL, 34 units/mL of enzyme, and pH 5.5.

raffinose (peak 5), indicators of fructosidase and galactosidase activities, were observed. Oligosaccharides with retention times corresponding to trisaccharides, DP_3 (a and b), and higher than tetrasaccharides, DP_5 and DP_6, were also detected. Peak DP_3 was tentatively assigned as 6′ galactosyl melibiose (α-D-Galp-(1→6)-α-D-Galp-(1→6)-α-D-Glcp) [isomers of the TMS oximes syn (a) and anti (b)]; peak DP_5 was assigned as 1^F fructofuranosyl stachyose, the presence of which has been previously observed in stachyose hydrolysates (*17*), and DP_6 corresponded to a unknown peak.

Mass spectrometry analysis of pure oligosaccharides gave two peaks at m/z 527.5 (M + Na) and 543.3 (M + K) for DP_3, corresponding to trisaccharide, a peak at m/z 853.5 (M + Na), corresponding to DP_5 and a peak at m/z 1015.8 (M + Na) corresponding to a DP_6. Besides, acid hydrolysis of DP_3, DP_5, and DP_6 yielded glucose and galactose (1:2); fructose, glucose, and galactose (2:1:2); and fructose, glucose, and galactose (3:1:2), respectively.

The hydrolysis of stachyose and formation of oligosaccharides DP_3, DP_5, and DP_6 are shown in **Figure 2**. Under the assay conditions used, 80% of initial stachyose was hydrolyzed after 6 h. The formation of DP_3 increased with time, reaching a maximum of 58.68% (based on total carbohydrates) after 24 h. At this time 90.76% of stachyose was hydrolyzed. With regard to DP_5 the maximum formation (20.05%) was observed after 1 h at 45.18% stachyose hydrolysis. At prolonged reaction times a decrease of DP_5 was observed, probably contributing to the DP_3 formation. DP_6 content increased at a slower rate, reaching a maximum of 3.83% after 3 h and 72.74% of stachyose of hydrolysis.

Uhm et al. (*17*) proposed the formation of DP_2, DP_4, and DP_5 and DP_3 and DP_5 during enzymatic hydrolysis of raffinose and stachyose, respectively, catalyzed by fructosyltransferase from *A. niger*. However, they did not found oligosaccharides with a polymerization degree of higher than 5, and only a DP_4 compound was characterized by NMR. They suggested that the maximum chain length of five could fill the active sites of the enzyme. Because Pectinex Ultra SP-L is an enzyme preparation obtained from *A. aculeatus*, the observed differences could be due

Table 1. ^{1}H and ^{13}C NMR Chemical Shift Values for Oligosaccharides Stachyose, DP_3, DP_5, and DP_6

		terminal galactose		internal galactose		glucose		fructose					
oligosaccharide	position	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
stachyose[29]	1	4.99	100.82	4.98	101.15	5.42	94.88	3.66	64.23				
								3.66					
	2	3.82	71.06	3.83	71.21	3.56	73.72		106.58				
	3	3.85	72.29	3.90	72.15	3.75	75.50	4.22	79.16				
	4	3.98	72.01	4.03	72.11	3.52	72.27	4.06	76.79				
	5	4.01	73.76	4.13	71.58	4.05	74.06	3.88	84.12				
	6	3.74	63.91	3.73	69.27	3.65	68.66	3.76	65.23				
		3.74		3.87		4.03		3.81					
DP_3	1	4.98	100.82	4.98	100.88	5.23[a]	94.95						
		4.98	100.77	4.98	100.81	4.66[b]	98.84						
	2	3.82	71.04	3.83	71.21	3.54	74.20						
					71.19	3.25	76.83						
	3	3.84	72.25	3.88	72.19	3.71	75.78						
				3.90	72.19	3.48	78.74						
	4	3.97	72.01	4.02	72.14	3.48	72.48						
					72.11	3.49	72.31						
	5	3.99	73.75	4.15	71.59	4.00	72.70						
				4.17	71.53	3.64	76.95						
	6	3.74	63.93	3.72	69.35	3.69	68.62						
		3.74		3.86		4.00							
				3.72	69.24	3.74	68.51						
				3.86		3.96							
DP_5	1	4.99	100.82	4.98	101.15	5.44	95.16	3.70	63.78	3.67	63.24		
								3.81		3.73			
	2	3.82	71.06	3.83	71.22	3.55	73.76		106.14		106.49		
	3	3.84	72.28	3.90	72.15	3.74	75.53	4.27	79.39	4.19	79.43		
	4	3.98	72.01	4.03	72.12	3.53	72.24	4.05	76.65	4.08	77.27		
	5	3.99	73.76	4.13	71.58	4.04	74.09	3.88	84.00	3.86	83.90		
	6	3.74	63.91	3.72	69.28	3.66	68.57	3.74	65.05	3.76	65.15		
		3.74		3.86		4.04		3.76		3.82			
DP_6	1	4.98	100.83	4.98	101.16	5.44	95.16	3.72	63.91	3.71	63.66	3.67	63.19
								3.82		3.85		3.74	
	2	3.82	71.06	3.83	71.23		73.80		106.10		105.83		106.43
	3	3.84	72.28	3.90	72.16	3.74	75.53	4.27	79.46	4.21	80.26	4.27	79.54
	4	3.97	72.01	4.03	72.12	3.54	72.22	4.05	76.66	4.06	77.25	4.09	77.13
	5	4.01	73.76	4.13	71.59	4.04	74.11	3.87	84.02	3.85	83.85	3.85	83.85
	6	3.73	63.91	3.72	69.29	3.65	68.56	3.75	65.06	3.75	65.02	3.75	65.01
		3.73		3.86		4.04		3.81		3.83		3.83	

[a] J(H1,H2) = 3.8 Hz. [b] J(H1,H2) = 8.1 Hz.

not only to operating conditions but also to the specificity of the enzymes. Recently, Ghazi et al. (*27*) purified and characterized a fructosyltransferase present in Pectinex Ultra SP-L and observed that stachyose was not a substrate of the enzyme. They indicated that the specific activity diminished with longer oligosaccharides and suggested limitations for binding of large molecules.

Structural Characterization. Unequivocal structural elucidation of purified products was carried out by the combined use of 1D and 2D [^{1}H, ^{1}H] and [$^{1}H-^{13}C$] NMR experiments (gCOSY, gHSQC-TOCSY, multiplicity-edited gHSQC and gHMBC). ^{1}H and ^{13}C NMR assignments for stachyose, DP_3, DP_5, and DP_6 are given in **Table 1**. A full set of spectra are collected in the Supporting Information.

The ^{1}H NMR spectrum of tetrasaccharide stachyose showed three doublets in the anomeric region (see Figure S7 of the Supporting Information). Besides, in the anomeric region of the ^{13}C spectrum, four signals at 100.82, 101.15, 94.88, and 106.58 ppm were observed. A multiplicity-edited gHSQC spectrum was used to link the carbon signals to the corresponding proton resonances. From this experiment, a quaternary carbon (C-2 of the fructose residue at 106.58 ppm), 18 methine carbons, and 5 methylene carbons (69.27, 68.66, 65.23, 64.23, and 63.91 ppm) were identified. Taking anomeric protons and carbons as a starting point, gCOSY and gHSQC-TOCSY experiments allowed us to assign the signals belonging to each sugar residue. Finally, for sequential assignment of the sugar residues, a gHMBC experiment was used. Correlations between the anomeric proton of the terminal galactose (4.99 ppm) and the C6 carbon of the internal galactose (69.27 ppm), between the anomeric carbon of the internal galactose (101.15 ppm) and the methylene protons of glucose (4.03 and 3.65 ppm), and finally between the C-2 of the fructose residue (106.58 ppm) and the anomeric proton of the glucose unit (5.42 ppm), completed the full assignment (see **Figures 3** and **5B**). Carbon and proton chemical shifts values found were very similar to those reported by McIntyre and Vogel (*28*).

For DP_3 two sets of resonances were observed in the NMR spectra, indicating the presence of two compounds. For the major component, inspection of the anomeric regions of both ^{1}H and ^{13}C 1D spectra showed the presence of three anomers.

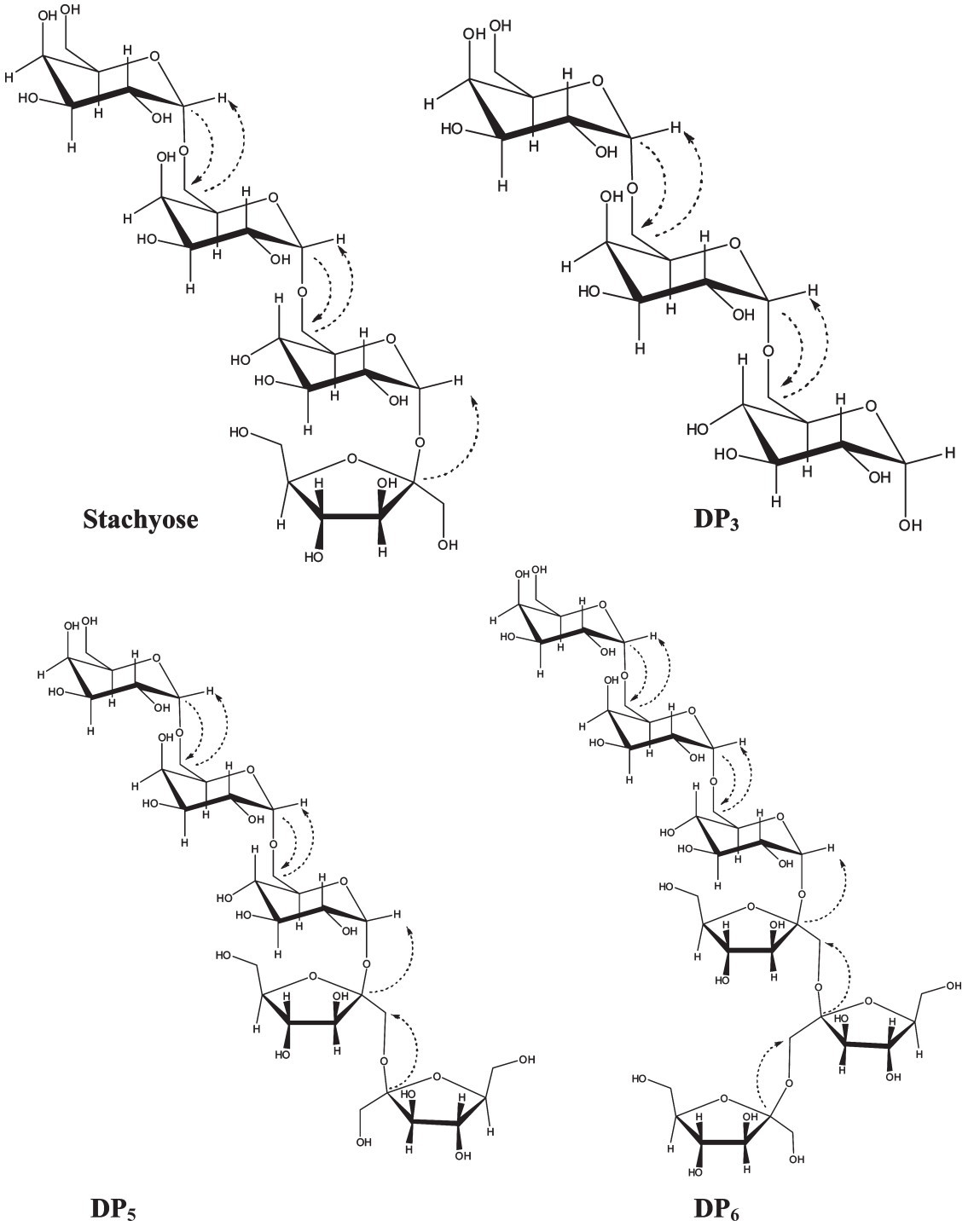

Figure 3. Structures of compounds stachyose, DP_3, DP_5, and DP_6. Relevant interglycosidic gHMBC NMR correlations are indicated by arrows.

Therefore, taking the anomeric protons as starting point, two residues of α-galactose and one residue of α-glucose [J(H1,H2) = 3.8 Hz] were identified from gHSQC-TOCSY and gCOSY spectra. Finally, the observed gHMBC correlations between the anomeric proton of the terminal galactose (4.98 ppm) and the C6 carbon of the internal galactose (69.35 ppm) and between the anomeric carbon of the internal galactose (100.88 ppm) and the methylene protons of glucose (4.00 and 3.69 ppm) completed the full assignment (see **Figures 3** and **5A**), so the major compound was identified as galactosyl melibiose (α-D-Galp-(1→6)-α-D-Galp-(1→6)-α-D-Glcp). By using the same procedure for the minor component, two residues of α-galactose and one residue of β-glucose [J(H1,H2) = 8.1 Hz] were determined. This compound was identified as α-D-Galp-(1→6)-α-D-Galp-(1→6)-β-D-Glcp.

The ^{1}H NMR spectrum of DP_5 showed three doublets in the anomeric region. Besides, in the anomeric region of the ^{13}C spectrum, five resonances at 106.49, 106.14, 101.15, 100.82, and 94.88 were observed. From multiplicity-edited gHSQC, 2 quaternary (106.49 and 106.14 ppm), 21 methine, and 7 methylene carbons (69.28, 68.57, 65.15, 65.05, 63.91, 63.78, and 63.24 ppm) were found. The combined use of gCOSY, gHSQC-TOCSY, and multiplicity-edited gHSQC and gHMBC spectra revealed the presence of two galactose rings, a glucose ring, and two fructose residues. Therefore, all signals belonging to each sugar residue resonances of pentasaccharide were assigned. Interglycosidic

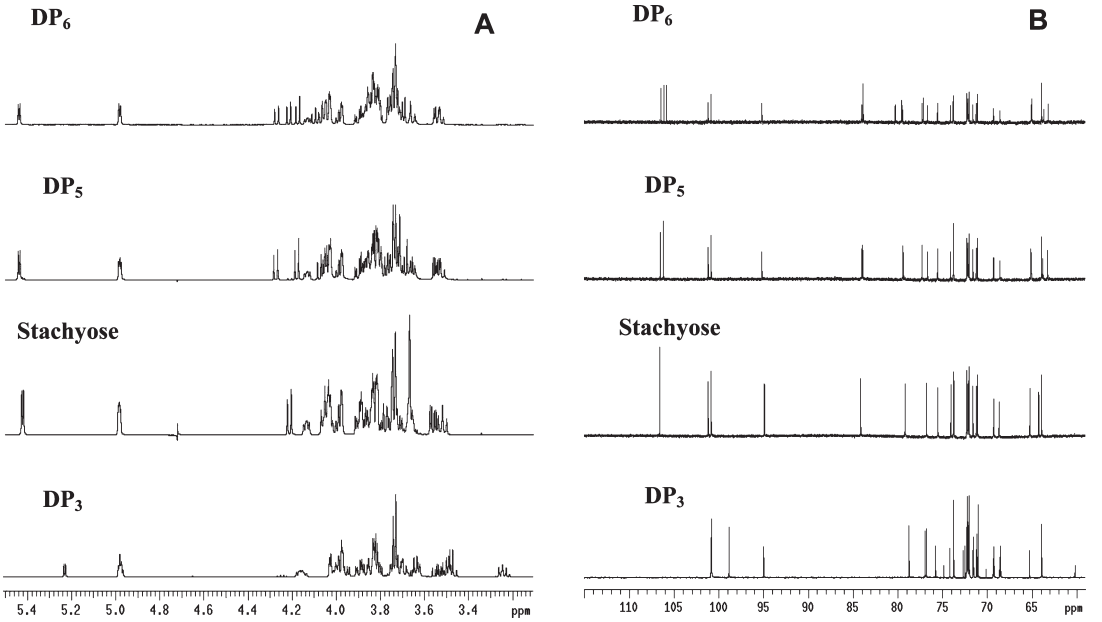

Figure 4. NMR spectra of DP_3, stachyose, DP_5, and DP_6 oligosaccharides: (**A**) 500 MHz ^1H spectra; (**B**) 125 MHz proton-decoupled ^{13}C spectra.

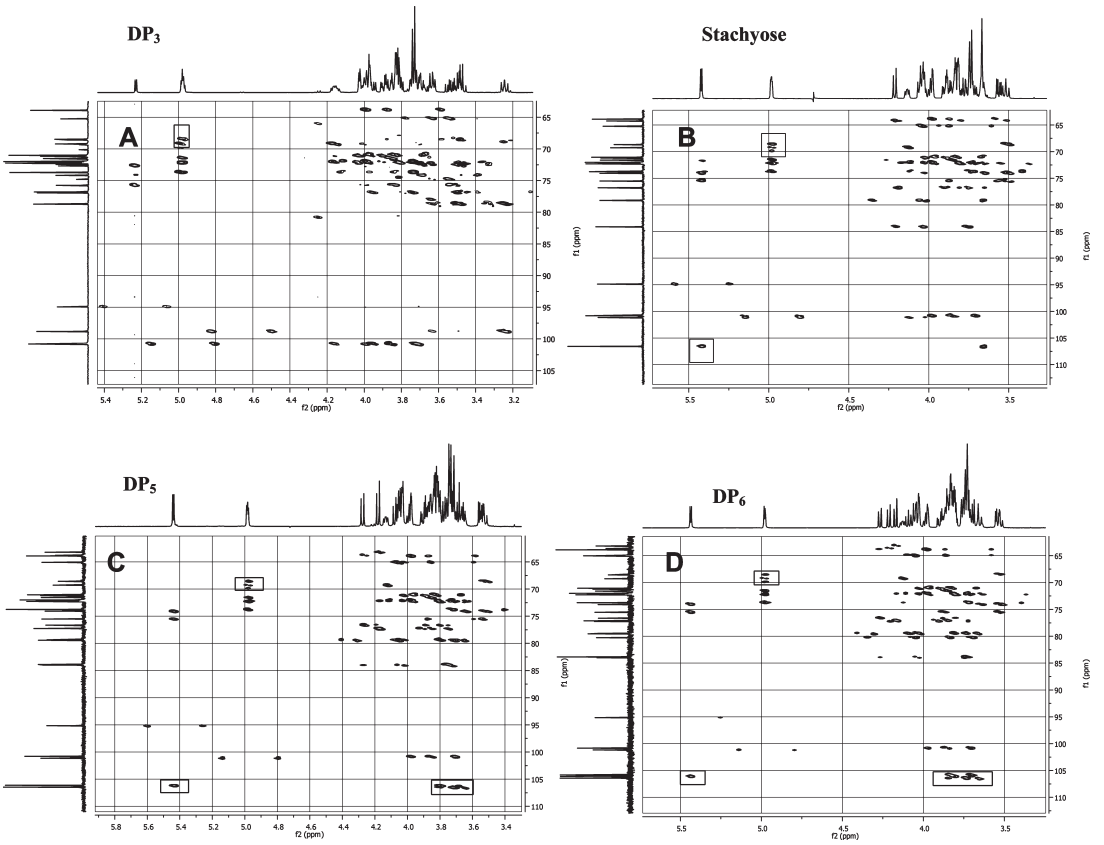

Figure 5. gHMBC NMR spectra of (**A**) DP_3, (**B**) stachyose, (**C**) DP_5, and (**D**) DP_6. Boxed cross-peaks are due to relevant interglycosidic correlations.

linkages were established from the gHMBC spectrum and by comparison with the completely assigned ^1H and ^{13}C spectrum of stachyose. Interresidual correlations were observed between the anomeric proton of the terminal galactose (4.99 ppm) and the C6 carbon of the internal galactose (69.28 ppm), between the anomeric carbon of the internal galactose (101.15 ppm) and the methylene protons of glucose (4.04 and 3.66 ppm), between the C-2 of the internal fructose (106.14 ppm) and the glucose anomeric proton (5.44 ppm), and finally between the C-2 of the terminal fructose residue (106.49 ppm) and methylene C1 protons of the internal fructose (3.81 and 3.70 ppm) (see **Figures 3** and **5C**). From these results and by comparison with stachyose spectra (see **Figure 4**), the structure of DP_5 was established as (α-D-Galp-(1→6)-α-D-Galp-(1→6)-α-D-Glcp-(1→2)-β-D-Fruf-(1→2)-β-D-Fruf).

The ^1H NMR spectrum of DP_6 showed only three doublets in the anomeric region, whereas six resonances at 106.43, 106.10, 105.83, 101.16, 100.83, and 95.16 ppm were observed in the anomeric region of the corresponding ^{13}C spectrum. From the multiplicity-edited gHSQC experiment, 3 quaternary (106.43, 106.10, and 105.83 ppm), 24 methine, and 9 methylene carbons

were identified. 2D gCOSY, gHSQC-TOCSY, multiplicity-edited gHSQC and gHMBC NMR spectra revealed the existence of two galactose rings, a glucose ring, and three fructose residues. Finally, by the observation of relevant gHMBC correlations (see **Figures 3** and **5D**) and comparison with ^{1}H and ^{13}C NMR spectra of DP_5 (see **Figures 4** and **5D**), the structure of DP_6 was established as the hexasaccharide (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).

In this work, the formation and characterization of a new oligosaccharide (DP_6) 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 well as for the first time characterization of pentasaccharide (DP_5) α-D-Galp-(1→6)-α-D-Galp-(1→6)-α-D-Glcp-(1→2)-β-D-Fruf-(1→2)-β-D-Fruf, found during stachyose hydrolysis by fructosyltransferase activity from Pectinex Ultra SP-L, have been described. These results provide confirmatory evidence of the fructosyltransferase activity of Pectinex Ultra SP-L toward stachyose and therefore show that α-galactosides seems to be a promising raw material for the synthesis of new oligosaccharides by the use this commercial enzyme preparation.

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Supporting Information Available: Additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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