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Isolation and Identification of Select Oligosaccharides from Commercially Produced Total Invert Sugar with a Proposed Mechanism for Their Formation

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Three disaccharides were isolated and purified from a commercial total invert sugar (TIS). The structures of these compounds were determined by a combination of acid and enzymatic hydrolysis studies, chromatographic comparison to standards, and nuclear magnetic resonance spectroscopy. These carbohydrates were identified as *O*-α-D-glucopyranosyl-(1→3)-D-fructose (IS1), *O*-β-D-fructofuranosyl-(2→6)-D-glucose (IS2), and *O*-α-D-glucopyranosyl-β-D-glucopyranoside (IS3). On the basis of these structures a mechanism for the hydrochloric acid catalyzed hydrolysis of sucrose is proposed: protonation of the glycosidic oxygen of sucrose leading to the formation of glucopyranosyl and fructofuranosyl oxonium ions of D-glucose or D-fructose, respectively, followed by nucleophilic attack of these ions by D-glucose or D-fructose at either the α- or β-face.

KEYWORDS: Carbohydrates; authenticity; acid reversion; sucrose hydrolysis

INTRODUCTION

Invert sugar (IS) is the hydrolysate product resulting from the reaction of sucrose with acid or invertase. The majority of invert syrup used as a sweetener in the food industry is produced by acid hydrolysis that involves treatment of sucrose (60−70 °Brix) with hydrochloric acid (final concentration of 0.05% w/w; pH ∼2.0), at temperatures between 45 and 70 °C for 2−4 h, depending on the extent of hydrolysis desired for the final glucose/fructose/sucrose ratio of the invert sugar. Hydrolysis is terminated by the addition of sodium hydroxide to a final pH of ∼4.5, and the syrup is immediately cooled to 38 °C. Invert sugar can be produced at different degrees of inversion, rates, and scales by employing various acid and temperature−time conditions depending on the desired final sugar composition and food application (*1*). Total invert sugar (TIS) and medium invert sugar (MIS) are defined as solutions containing a 1:1 ratio of glucose to fructose and low levels of sucrose (∼0.8−10%), and a 1:1:2 ratio of glucose, fructose, and sucrose, respectively (*2*).

It has been shown that oligosaccharides are synthesized during the acid-catalyzed hydrolysis of sucrose, and this process is known as reversion (*3*, *4*). Formation of reversion products during hydrolysis has been shown to occur under conditions of weak acid (<0.1% HCl) (*5*) and high sucrose concentration (65 °Brix) (*4*).

Although a number of oligosaccharides have been shown to be synthesized during the acid-catalyzed hydrolysis of sucrose, two of these compounds, identified in the literature as IS1 and IS2 on the basis of their retention times by capillary gas chromatography (*6*), have been shown to be of significant importance for the detection of TIS adulteration of foods (*2*, *7*).

Analytical methods employing high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) (*8*) and capillary gas chromatography with flame ionization detection (CGC-FID) (*6*) have been developed for the detection of TIS adulteration of foods by monitoring the presence and concentrations of IS1 and IS2. Application of these methods to the detection of low levels (5−10%) of TIS adulteration in fruit juices (*6*), maple syrup (*9*), and honey (*8*, *10*) have been successful. To date, the isolation and structural identification of IS1 and IS2 from TIS have not been achieved, nor has the mechanism of oligosaccharide formation in this material been conclusively elucidated. Therefore, the objectives of this study were to isolate and structurally identify IS1 and IS2 plus other oligosaccharides in total invert sugar and, from these structures, to postulate the mechanism of formation of oligosaccharides produced during the acid-catalyzed hydrolysis of sucrose.

MATERIALS AND METHODS

The commercial TIS sample used in the study was obtained from Lantic Sugar Limited, Toronto, ON, Canada. The total soluble solids content of this sample was found to be 69.8 ± 0.1 °Brix using a Leica Auto Abbe refractometer.

Bulk Isolation of Oligosaccharides from TIS. Monosaccharides were removed from TIS using the modified charcoal/Celite method developed by Swallow and Low (*11*). Briefly, 12.5 (± 0.1) g of TIS (69.8 °Brix) was dissolved in 25 mL of ddH_2O (distilled and deionized water) with a Milli-Q water system (Millipore, Milford, MA), and to

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this solution was added 150 (± 1) g of charcoal (Fisher Scientific Ltd., St-Jean-Sur-Richelieu, QC, Canada) in 250 mL of ddH_2O. The resulting mixture was stirred for ~20 h at 4 ± 1 °C. This mixture was loaded onto a 40 × 4.5 cm glass column containing 100 (± 1) g of a 50:50 (w/w) mixture of charcoal and Celite (Fisher Scientific Ltd., Fair Lawn, NJ) fitted with a 1000 mL mobile phase reservoir.

The column was washed with 6.25 L of 0.1% ethanol (v/v) at a flow rate of 5 mL/min to remove monosaccharides. The column eluate was monitored by HPAE-PAD (Waters Chromatography, Milford, MA) to ensure that glucose and fructose were removed without appreciable loss of oligosaccharides (e.g., sucrose). Fructose, glucose, and sucrose were separated on a 250 × 4 mm i.d. CarboPac PA1 pellicular anion-exchange column in series with a 25 × 3 mm i.d. guard column (Dionex, Sunnyvale, CA). Carbohydrates were detected using a Waters model 464 PAD with a dual gold electrode at a sensitivity of 50 μA maintained at the following potentials and durations: $E_1 = 0.05$ V ($t_1 = 0.299$ s); $E_2 = 0.60$ V ($t_2 = 0.299$ s); $E_3 = -0.80$ V ($t_3 = 0.499$ s) (*11*). The mobile phase was 80 mM sodium hydroxide (50% w/w) at a flow rate of 1.0 mL/min, and the injection volume was 50 μL (LC method 1). Oligosaccharides were eluted from the column by washing with 6.25 L of warm (60 °C) 75% ethanol (v/v) at a flow rate of 5 mL/min. The monosaccharide-free TIS (MFTIS) was concentrated to ~3−4 mL (final °Brix of 21.9) by rotary evaporation at 35 ± 2 °C and stored at −20 °C until required for analysis.

Isolation of Individual Oligosaccharides from TIS by HPAE-PAD. Three oligosaccharides, IS1, IS2, and IS3, were isolated from MFTIS via HPAE-PAD. Oligosaccharide separation of 50 μL of a 3.5 (± 0.1) °Brix solution of MFTIS was afforded by HPAE-PAD (Waters Chromatography) using two 250 × 4 mm i.d. CarboPac PA1 pellicular anion-exchange columns connected in series with a 25 × 3 mm i.d. CarboPac PA1 guard column. Oligosaccharides were detected using a Waters model 464 PAD with a dual gold electrode at a sensitivity of 50 μA maintained at the following potential and durations: $E_1 = 0.05$ V ($t_1 = 0.299$ s); $E_2 = 0.60$ V ($t_2 = 0.299$ s); $E_3 = -0.80$ V ($t_3 = 0.499$ s) (*11*). Data acquisition and processing were carried out using Millenium[32] LC Processing software (Waters Corp., Milford, MA). Oligosaccharides were separated employing the following gradient program: initial 100% A for 4 min, followed by a linear gradient to 3% B at 20 min, followed by a linear gradient to 100% B at 50 min, hold at 100% B for 10 min, followed by a change to 100% C at 60.1 min, hold at 100% C for 60 min, followed by a change to 100% A at 120.1 min, hold at 100% A for 55 min, where A = 100 mM NaOH, B = 100 mM NaOH/100 mM sodium acetate (NaOAc), and C = 300 mM NaOH. The mobile phase flow rate was 0.70 mL/min (LC method 2).

Individual 0.7 mL fractions were collected in 10 × 100 mm glass test tubes. Fractions containing IS1, IS3, and IS2 had approximate retention times of 15−16, 16−17, and 20−21 min, respectively, and were immediately pH adjusted to 6.5 ± 0.1 with 100 mM acetic acid. The pH adjusted fractions were then stored at −20 °C.

Neutralized fractions containing IS1, IS2, and IS3 were individually desalted by passage through a 8.5 × 1.5 cm column containing ~5.0 mL of cation-exchange resin (100−200 mesh; hydrogen form) (Bio-Rad Laboratories, Richmond, CA) followed by washing with 5 × 2 mL aliquots of ddH_2O at 4 ± 1 °C. The eluent was evaporated to a final volume of ~0.5 mL using a rotary evaporator at a temperature of 35 ± 2 °C. The remaining water and acetic acid were removed by freeze-drying.

Purity Determination of IS1, IS2, and IS3 by HPAE-PAD and CGC. The purity of IS1, IS2, and IS3 fractions obtained from analytical HPAE-PAD analysis of MFTIS was monitored by using LC method 2.

Desalted fractions containing IS1, IS2, and IS3 were also analyzed by CGC for purity. Freeze-dried fractions containing IS1 or IS2 or IS3 (~0.2 mg) were diluted with 200 μL of ddH_2O, and 100 μL of each fraction was transferred to a 1.5 mL glass vial and freeze-dried for 1 h. To the resulting foam/syrup was added 500 μL of Sylon TP [*N*-trimethylsilylimidazole (TMSI)/pyridine], 1:4; (Supelco, Bellefonte, PA) using a gastight glass syringe. The vials were capped immediately and placed in a block heater maintained at 70 ± 2 °C for 1 h with shaking every 10−15 min (*6*, *12*).

The derivatized samples were analyzed by CGC with a model 6890 instrument (Hewlett-Packard, Wilmington, DE) equipped with an autosampler. Sample introduction was in the splitless mode and oligosaccharides were separated on a DB-5 30 m × 0.25 mm i.d., 0.25 μm DB-5 open tubular fused-silica capillary column [95% dimethyl-(5%)-diphenyl-polysiloxane] (J&W Scientific, Folsom, CA). The carrier gas was ultrapure (99.999%) hydrogen at a constant flow rate of 1.4 mL/min. Ultrapure (99.999%) nitrogen at a flow rate of 30 mL/min was used as the makeup gas. The injection port temperature was 250 °C, and the flame ionization detector (FID) was maintained at 300 °C. The injection volume was 3.0 μL, and the following temperature program was used for oligosaccharide separation: 210 °C for 10 min, 1 °C/min from 210 to 248 °C, 248 °C for 1 min, 20 °C/min from 248 to 295 °C, 295 °C for 15 min (total run time of 66.35 min) (*6*). Data acquisition and processing were carried out using HP Chem Station rev. A.06.03 (509) software (Hewlett-Packard, Wilmington, DE).

The purity of IS1, IS2, and IS3 following a single chromatographic separation was monitored by GC, and each fraction was found to require further purification due to the presence of multiple peaks with appreciable peak heights/ratios. Rechromatography (LC method 2) of highly purified fractions containing IS1, IS2, and IS3 resulted in concentrated fractions of IS1, IS2, and IS3 (>95% peak purity based on peak area measurement by GC).

Structural Determination. *Sodium Borohydride Reduction.* Approximately 0.05 mg of IS1 or IS2 was dissolved in 500 μL of ddH_2O. To this solution was added ~100 equiv (w/w) of sodium borohydride (Eastman, Rochester, NY), and the resulting solution was mixed at 200 rpm with a Vortex Genie 2 (Fisher Scientific) at room temperature (23 ± 1 °C) for 1 h. The reaction was pH adjusted (6.0 ± 0.5) by the dropwise addition of 10% (v/v) glacial acetic acid. The resulting solution was passed through a 8.5 × 1.5 cm column containing ~5.0 mL of cation-exchange resin (100−200 mesh; hydrogen form) (Bio-Rad Laboratories), followed by washing (10 × 2 mL) with ddH_2O at 4 °C. The eluent was evaporated in vacuo and coevaporated with methanol (5 × 5 mL) to a volume of ~200 μL (*13*) and quantitatively transferred to a 1.5 mL glass vial, freeze-dried, derivatized, and analyzed by GC.

Total Acid Hydrolysis. Individual 300 μL solutions of IS1, IS2, and IS3 (~0.1 mg/mL) were separately treated with 14 μL of 1 M HCl for 1 h at 75 °C. Controls consisting of 300 μL of a 0.1 mg/mL sucrose solution (positive control) and 14 μL of ddH_2O (negative control) were run under the same reaction conditions. Acidic reactions were pH adjusted to 5.0 ± 0.2 by the addition of 14 μL of 1 M NaOH. Carbohydrate analysis of each sample was afforded by HPAE-PAD (LC methods 1 and 2).

Enzymatic Hydrolysis. Three hundred microliter aliquots of purified IS1 (~0.1 mg/mL) were individually placed in four separate 0.5 mL Eppendorf tubes (VWR Canlab, Mississauga, ON, Canada). To tube 1 was added 50 μL of ddH_2O, to tube 2 was added 50 μL of β-glucosidase (2 units) (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada), to tube 3 was added 50 μL of α-glucosidase (2 units) (Sigma-Aldrich Canada Ltd.), and to tube 4 was added 50 μL of β-fructosidase (2 units) (Sigma-Aldrich Canada Ltd.). The samples were gently agitated at room temperature for 25 h (200 rpm) with a Vortex Genie 2. Enzymatic reactions were terminated by placing the tubes in boiling water for 5 min. Positive controls of sucrose and cellobiose (~0.1 mg/mL) were run with β-fructosidase and α-glucosidase and with β-glucosidase, respectively, using the same conditions described above. Negative controls of sucrose and cellobiose (~0.1 mg/mL) were run without enzyme using the same reaction conditions. All enzyme reactions were carried out in ddH_2O (pH 6.8). All samples were filtered through 13 mm diameter, 0.2 μm pore size, nylon syringe filters (Chromatographic Specialties Inc., Brockville, ON, Canada) prior to analysis by HPAE-PAD (LC methods 1 and 2). This protocol was also used for purified IS2 and IS3.

^{13}C Nuclear Magnetic Resonance (NMR) Spectroscopy. HPAE-PAD-purified IS1, IS2, IS3, and carbohydrate standards (~5 mg) were individually dissolved in D_2O (Sigma-Aldrich, Milwaukee, WI) and quantitatively transferred (total volume of 0.5 mL) to individual 175 × 5 mm NMR tubes. The ^{13}C NMR spectrum of each sample was obtained at 125.795 MHz, with a Bruker Avance 500 NMR spectrometer (Bruker Instruments Inc., Billerica, MA) at 300.0 K. The sweep

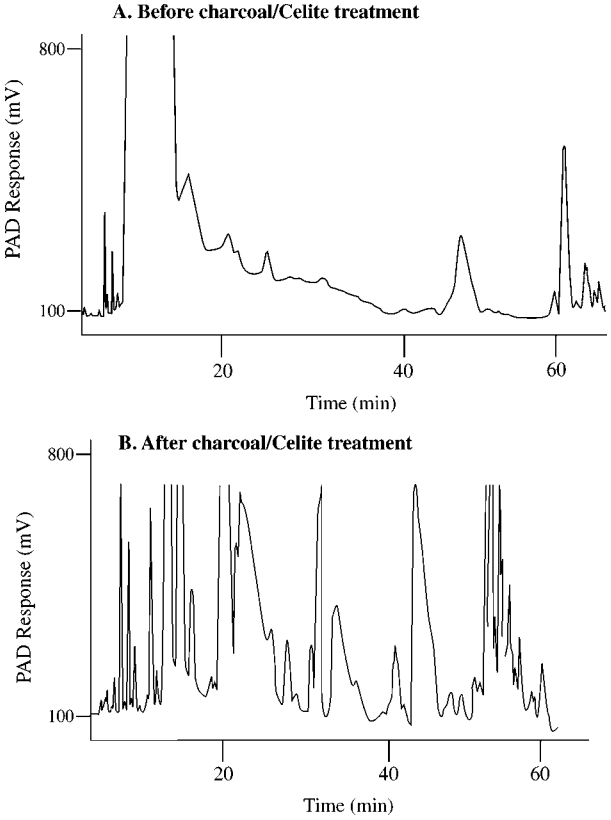


Figure 1. HPAE-PAD (LC method 2) chromatograms of TIS (**A**) before and (**B**) after charcoal/Celite treatment.

width was 30030.0 Hz with a pulse angle of 30°. Repetition time was 4.09 s, consisting of 1.09 s acquisition and 3.00 s relaxation times. Between 2000 and 19000 scans were required for each ^{13}C spectrum.

RESULTS AND DISCUSSION

Chromatographic results (LC method 1) showed that glucose and fructose accounted for ~98% of the total soluble solids in TIS. The first step in oligosaccharide isolation was to remove the majority of the glucose and fructose from TIS, thereby enabling bulk isolation of the compounds of interest. To achieve this goal, TIS was subjected to charcoal/Celite chromatography, which enables effective bulk separation of different classes of carbohydrates (mono-, di-, trisaccharides) (*14*). A modified charcoal/Celite chromatography method developed by Swallow and Low (*11*) was used in this work to produce MFTIS. This modified method was found to be effective for monosaccharide removal from TIS with final glucose and fructose levels in MFTIS of 1.0 and 3.5%, respectively. On the basis of these results, ~94% of the monosaccharides in TIS were removed without appreciable loss of oligosaccharides (e.g., sucrose).

A representative HPAE-PAD chromatogram (LC method 2) of TIS, before and after charcoal/Celite treatment, is shown in **Figure 1**. The column eluate was monitored by HPAE-PAD (LC method 1), to ensure that the majority of glucose and fructose were removed without significant oligosaccharide loss. Oligosaccharides IS1 and IS3 and oligosaccharide IS2 eluted in the HPAE-PAD chromatographic regions of 15−20 and 20−25 min, respectively. The approximate retention times for glucose, fructose, and sucrose under these chromatographic conditions were 14, 15, and 28 min, respectively.

Relative Retention Times of Chromatographically Purified IS1, IS2, and IS3 in TIS by HPAE-PAD. The relative retention times (RRT) of purified IS1, IS3, and IS2 by HPAE-PAD were found to be 15.5, 16.5, and 20.5 min, respectively. On the basis of these retention times and those of standard oligosaccharides (maltose and maltotriose were found to have HPAE RRTs of 30.1 and 45.1 min, respectively), each of these isolated oligosaccharides was considered to be a disaccharide.

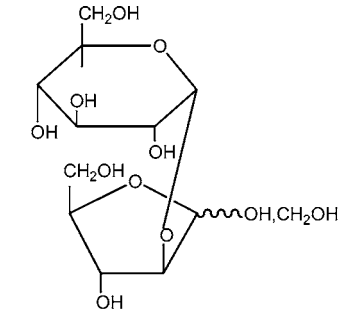
Acid Hydrolysis. Hydrochloric acid treatment of individual samples of IS1, IS2, and IS3 resulted in complete hydrolysis to glucose or to glucose and fructose as determined by HPAE-PAD retention time comparison to standards (LC method 1). Acid hydrolysis of IS1 and IS2 showed a 1:1 ratio of glucose to fructose for each by HPAE-PAD. These results confirmed the hypothesis that these compounds were disaccharides as other possibilities for their basic structure would be polymers of these units (e.g., tetraose) that would have exhibited much longer retention times than those observed for IS1 and IS2 by HPAE-PAD and GC. Acid hydrolysis of IS3 resulted in the production of glucose, indicating a disaccharide composed only of glucose.

A positive control of sucrose (~0.10 mg) was hydrolyzed under the same acid reaction conditions and resulted in the expected production of a 1:1 ratio of glucose to fructose. No other carbohydrates were detected under the reaction conditions employed (LC methods 1 and 2), showing that monosaccharide and/or oligosaccharide formation was not occurring under these reaction conditions.

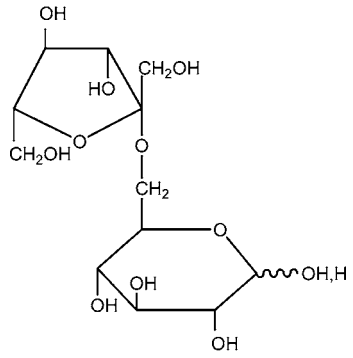
Enzymatic Hydrolysis. Enzymatic hydrolysis was conducted on individual purified samples of IS1, IS2, and IS3 to determine the type of linkage between monosaccharides for each disaccharide. The three enzymes employed in this study were α-glucosidase, β-glucosidase, and β-fructosidase. α-Glucosidase hydrolyzes terminal, nonreducing α-linked D-glucose residues of oligo- and polysaccharides, whereas β-glucosidase hydrolyzes terminal, nonreducing β-linked D-glucose residues. β-Fructofuranosidase hydrolyzes terminal, nonreducing β-linked D-fructose residues of oligo- and polysaccharides (*15*). The positive controls used for each of these enzyme reactions were maltose (α-glucosidase), cellobiose (β-glucosidase), and sucrose (β-fructosidase).

Positive controls of cellobiose, maltose, and sucrose, a negative control (ddH_2O), IS1, IS2, and IS3 were separately incubated with and without α-glucosidase, β-glucosidase, and β-fructosidase, and the resulting carbohydrate profiles were determined by HPAE-PAD (LC methods 1 and 2). The percent hydrolysis of each sample was estimated by peak area comparison before and after enzyme incubation.

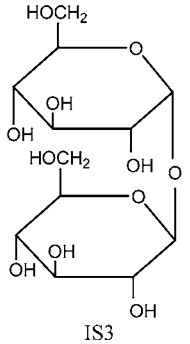
The positive controls, maltose, cellobiose, and sucrose were hydrolyzed (>95%) by α-glucosidase, β-glucosidase, and β-fructosidase, respectively. The negative control (ddH_2O) and IS1, IS2, IS3, maltose, cellobiose, and sucrose without enzyme treatment showed no hydrolysis. IS1 was hydrolyzed (>95%) by α-glucosidase with no hydrolysis observed by either β-glucosidase or β-fructosidase. These results suggested that D-glucose is α-linked through the anomeric carbon (C1) to fructose in IS1. IS2 was hydrolyzed (>90%) by β-fructosidase and showed no hydrolysis by α- or β-glucosidase, suggesting a D-fructose moiety β-linked to glucose in IS2. IS3 was hydrolyzed (>95%) by both α-glucosidase and β-glucosidase and showed no hydrolysis when treated with β-fructosidase. These results reveal the overall structure of IS3 as the two D-glucose units must be covalently linked through their anomeric centers. Therefore, the linkages between monosaccharides were determined to be as follows: IS1, D-glucose α-linked to fructose; IS2, D-fructose β-linked to glucose; and IS3, two D-glucose molecules α- and β-linked.



IS1



IS2



IS3

Figure 2. Stuctures of IS1, *O*-α-D-glucopyranosyl-(1→3)-D-fructose; IS2, *O*-β-D-fructofuranosyl-(2→6)-D-glucose; and IS3, *O*-α-D-glucopyranosyl-β-D-glucopyranoside.

Sodium Borohydride Reduction. Reducing carbohydrates can exist in six different tautomeric forms in solution, including two pyranoses (α and β), two furanoses (α and β), and the acyclic form and its hydrate (*16*). Each of these forms is a distinct compound, and, depending on the carbohydrate, most can be observed by GC. Treatment of a reducing carbohydrate with sodium borohydride to yield the corresponding alditol provides structural information and can be used as an indicator of carbohydrate purity (*17*). Before and after sodium borohydride reduction, IS3 showed a single peak by GC, and it was assumed that this compound was a nonreducing oligosaccharide. The remaining two oligosaccharides (IS1 and IS2) isolated and purified from MFTIS by HPAE-PAD were subjected to sodium borohydride reduction and GC analysis. Sodium borohydride reduction of IS1 and IS2 followed by GC analysis showed a single peak for each compound with retention times of 27.9 ± 0.1 and 34.9 ± 0.1 min, respectively. These results indicated that IS1 and IS2 are reducing carbohydrates. A standard of turanose (~0.05 mg) reduced under the same reaction conditions gave a single peak with a retention time of 27.9 ± 0.1 min, which matched that of reduced IS1. The results of sodium borohydride reduction of IS1 and IS2 confirmed that the isolated compounds were reducing carbohydrates and strongly suggested that IS1 was *O*-α-D-glucopyranosyl-(1→3)-D-fructose (turanose) (**Figure 2**).

Table 1. ^{13}C NMR Chemical Shifts and Carbon Assignments for IS1 and Experimental and Literature (*18*) Values for Turanose

^{13}C chem shifts for IS1 (ppm)	^{13}C chem shifts for turanose (ppm)		C assignment
	exptl	lit.	
104.6	104.5	103.7	1′
102.0	102.0		
101.3	101.2		
98.8	98.8		
98.0	98.0		
97.2	97.2		
85.1	85.1		
81.8	81.8		
81.2	81.1		
80.8	80.8		
77.0	77.0		
74.8	74.8	75.5	5′
74.7	74.6	75.5	3′
73.2	73.2	74.2	2′
73.1	73.1		
73.0	73.0		
72.6	72.6		
72.3	72.3	72.2	4′
71.7	71.7		
71.5	71.5		
70.6	70.6		
69.6	69.6		
69.4	69.4		
64.4	64.3		
63.7	63.7		
63.2	63.1	63.2	6′
62.6	62.6		
61.3	61.3		
60.8	60.7		
60.6	60.6		

^{13}C Nuclear Magnetic Resonance Spectroscopic Analysis of IS1, IS2, and IS3. Carbon chemical shifts values for IS1 and turanose and turanose literature values for this compound (*18*) are presented in **Table 1**. The experimental results for IS1 and turanose were virtually identical and confirmed the structure of IS1. The assignment of fructosyl carbon atoms of IS1/turanose was not possible due to multiple tautomeric forms in solution (*18*, *19*).

The chemical shifts and carbon assignments for IS2, sucrose, and isomaltose standards are presented in **Table 2**. The chemical shifts of the anomeric carbon (C1′) of the glucose moiety in IS2 were assigned as 96.2 and 92.4 ppm for C1′β and C1′α, respectively. These assignments were based on similar experimental carbon chemical shifts observed for an isomaltose standard of 96.4 and 92.5 ppm, respectively and those reported in the literature (*20*) of 97.7 and 93.8 ppm. Glycosylation at C6 of glucose has been shown to result in a ~8 ppm downfield shift when compared to that observed for glucose (*21*). This chemical shift was observed for C6 in isomaltose and IS2 showed a similar chemical shift at 67.9 ppm. The ^{13}C NMR results support the hypothesis that the fructose moiety of IS2 is linked to the C6′ position of glucose.

The chemical shift for C2 of fructose in IS2 was assigned as 101.0 ppm; although this value is significantly different from that observed for this carbon atom in sucrose (104.4), it is similar to that observed for C2 in methyl α-D-fructofuranoside of 102.6 ppm (*20*). On the basis of these results the structure for IS2 was determined to be *O*-β-D-fructofuranosyl-(2→6)-D-glucose (**Figure 2**).

Table 2. Experimentally Determined ^{13}C Chemical Shifts and Carbon Assignments for Sucrose, Isomaltose, and IS2

sucrose		α-isomaltose		β-isomaltose		IS2	
chem shift (ppm)	C no.	chem shift (ppm)	C no.	chem shift (ppm)	C no.	chem shift (ppm)	C no.
104.4	2′						
						101.0	2′
		98.2	1′	98.3	1′		
				96.4	1	96.2	1′
92.9	1						
		92.5	1			92.4	1′
82.2	5′					81.0	5′
77.4	3′	74.6	3′	76.3	3′	76.1	3′
75.0	4′			74.3	2′	75.4	4′
73.6	3			73.4	5′	74.5	3
73.3	5	73.3	3	73.3	3		
72.0	2	72.1	2′				
		71.8	5	71.8	5		
		71.7	2	71.7	2		
70.2	4	70.3	5′			71.5	5
		69.8	4′	69.8	4′	70.0	2
		69.7	4	69.7	4	69.5	4
		66.0	6′	66.0	6′	67.9	6
63.4	6′					64.3	6′
63.3	1′					63.7	1′
61.1	6	60.7	6	60.7	6		

Carbon assignments and chemical shifts for IS3 and neotrehalose and literature (*20*) values for this compound are shown in **Table 3**. The ^{13}C NMR data for IS3 match those of neotrehalose, confirming the *O*-α-D-glucopyranosyl-β-D-glucopyranoside structure (**Figure 2**).

Table 3. ^{13}C NMR Chemical Shifts and Carbon Assignments for IS3 and Experimental and Literature (*20*) Values for Neotrehalose

^{13}C chem shifts for IS3 (ppm)	^{13}C chem shifts for neotrehalose (ppm)		C no.
	exptl	lit.	
103.4	103.3	103.6	1′
100.6	100.6	100.9	1
76.5	76.5	77.0	5′
75.7	75.7	76.4	3′
73.5	73.4	74.1	2′
73.1	73.1	73.8	3
73.0	73.0	73.6	5
71.8	71.7	72.4	2
69.7	69.7	70.4	4,4′
61.0	60.9	62.0	6′
60.8	60.7	61.6	6

Mechanism of Invert Sugar Disaccharide (IS1, IS2, and IS3) Formation in Acid-Catalyzed Sucrose Hydrolysis. Elucidation of the mechanism of formation of oligosaccharides in TIS produced via acid hydrolysis was one of the objectives of this research. Results from the structural determination of IS1, IS2, and IS3 were used to confirm the mechanism of formation proposed by Swallow and Low (*11*) for oligosaccharide formation in beet medium invert sugar.

Two mechanisms have been proposed for oligosaccharide formation during the acid-catalyzed hydrolysis of sucrose. Szejtli et al. (*3*) proposed that protonation occurs at the ring oxygen of fructose that results in the formation of a fructofuranosyl cation and D-glucose. Nucleophilic attack of this cation by water results in D-fructose formation. The basis of this mechanism was the observed rates of hydrolysis of raffinose and melibiose

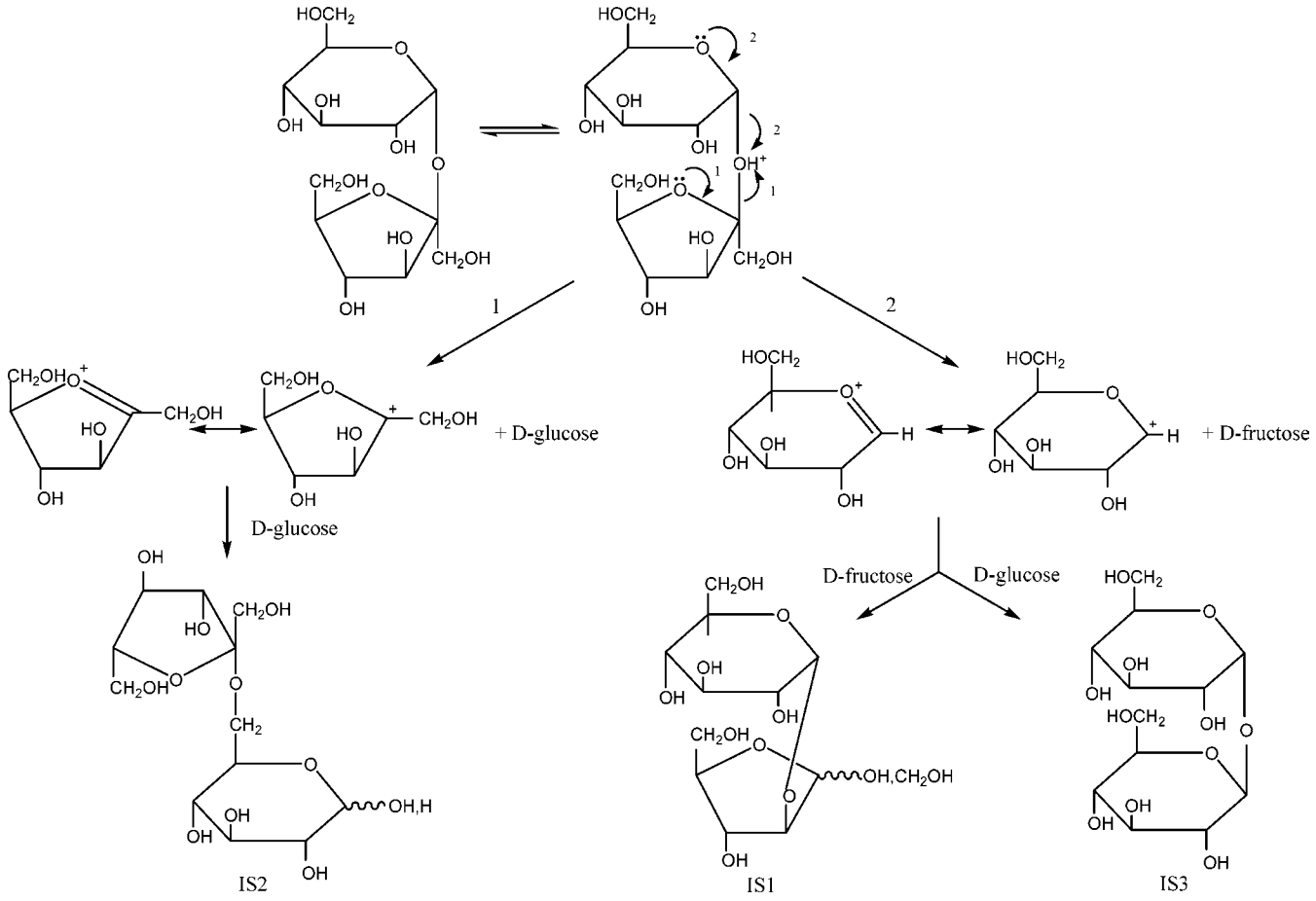


Figure 3. Proposed mechanism of IS1, IS2, and IS3 formation in TIS during the acid-catalyzed hydrolysis of sucrose.

with respect to fructose and glucose release, respectively. The authors observed that the release of fructose from raffinose was ~1000 times faster than the release of glucose from melibiose. They attributed this difference in reaction rate to a slight positive entropy change for ketofuranosides.

The mechanism (**Figure 3**) proposed by Swallow and Low (*11*) is based on initial protonation of the glycosidic oxygen followed by formation of either a fructofuranosyl oxonium ion and D-glucose or a glucopyranosyl oxonium ion and D-fructose. The authors suggested that the fructofuranosyl cation is more stable and that formation of the cyclopentyl (fructofuranosyl) cation occurs more readily than does that of the cyclohexyl cation due to the more planar character of the furanose ring. In addition, the stability of the tertiary fructofuranosyl carbonium ion is greater than that of the secondary glucopyranosyl carbonium ion. Therefore, the probability is much higher that this intermediate (fructofuranosyl oxonium ion) will be present in solution to react with carbohydrate nucleophiles.

The oligosaccharides isolated, purified, and identified in this research support the latter mechanism as IS1 and IS3 are formed from fructose and glucose addition, respectively, to a glucopyranosyl intermediate (**Figure 3**). The structure of IS2 can also be explained by mechanism proposed by Swallow and Low (*11*) via glucose addition to a fructofuranosyl oxonium ion intermediate (**Figure 3**). The confirmed structures of IS1 and IS3 cannot be readily explained by the mechanism proposed by Szejtli et al. (*3*).

On the basis of GC peak height measurements, the concentration of IS3 in TIS was found to be lower than that of IS1 and IS2. This may be explained by the greater stability of the fructofuranosyl oxonium ion and/or the fact that IS3 formation is balanced by its hydrolysis under commercial TIS production conditions.

Nucleophilic attack on the fructofuranosyl or glucopyranosyl carbonium ion by carbohydrate (i.e., glucose and fructose in TIS) leads to oligosaccharide formation. Nucleophilic attack could occur at either the α- or β-face of these carbonium ion intermediates leading to both α- and β-fructofuranosyl/glucopyranosyl oligosaccharides. Such nucleophilic transglycosylations occur preferentially at the primary hydroxyl group of any accompanying alcohols. For example, the 1- and 6-positions of fructose and the 6-position of glucose would be favored because of accessibility to these hydroxyl groups (*11*).

Nucleophilic attack by the least sterically hindered hydroxyl group (i.e., C6, primary hydroxyl) of glucopyranose to the β-face of the fructofuranosyl oxonium ion, as observed for IS1, supports this observation. However, peak height results by GC also suggested that the IS2 concentration in TIS was greater than that of IS1. The apparent lower concentration of IS1 by GC may be due to the single anomer observed with a relative retention time of 28.2 min, with the other more prominent anomer possibly coeluting with sucrose, as no other significant increase in the height of a peak in the chromatogram was observed.

The structures of IS1, IS2, and IS3 and their mechanism of formation are illustrated in **Figure 3**. The mechanism proposed by Szejtli et al. (*3*) based on initial protonation of the ring oxygen of fructofuranose followed by nucleophilic attack (S_N2-like) of the resulting carbonium ion supports disaccharides containing a nonreducing fructose moiety (i.e., IS2) only. The mechanism proposed by Swallow and Low (*11*) based on the formation of glucopyranosyl and fructofuranosyl intermediates followed by nucleophilic attack by carbohydrates (glucose and fructose) supports the structures of the three disaccharides isolated and purified from TIS. However, it is possible that oligosaccharides are formed in TIS from reactions involving D-glucose and/or D-fructose without the sucrose hydrolysis intermediate oxocarbonium ions.

During the acid-catalyzed hydrolysis of sucrose to produce TIS, oligosaccharides are synthesized via acid reversion. Three of these oligosaccharides have been isolated and identified from a commercial TIS with two, IS1 and IS2, having been shown to be of significant importance for detecting the presence of the undeclared addition (adulteration) of this sweetener to foods. On the basis of HPAE-PAD isolation and purification from commercial TIS, total acid and enzymatic hydrolysis studies, and NMR spectroscopy, the structures of these three compounds were determined to be *O*-α-D-glucopyranosyl-(1→3)-D-fructose (IS1), *O*-β-D-fructofuranosyl-(2→6)-D-glucose (IS2), and *O*-α-D-glucopyranosyl-β-D-glucopyranoside (IS3) (**Figure 2**).

On the basis of the structural identification of IS1, IS2, and IS3 the mechanism of oligosaccharide formation in TIS was postulated. It was concluded that acid-catalyzed hydrolysis of sucrose involves initial protonation of the glycosidic oxygen of sucrose, leading to the formation of either glucopyranosyl or fructofuranosyl oxonium ions and D-fructose or D-glucose, respectively. Nucleophilic attack on these ions by glucose or fructose at either the α- or β-face leads to IS1, IS2, and IS3 formation.

NOTE ADDED AFTER ASAP PUBLICATION

The original posting of February 28, 2006, contained an incorrect version of Figure 3. The correct version is shown in the posting of March 8, 2006.

LITERATURE CITED

(1) Pancoast, H. M.; Junk, W. R. *Handbook of Sugars*; AVI Publishing: Westport, CT, 1980.
(2) Low, N. H. Oligosaccharide analysis. In *Analytical Methods of Food Authentication*; Ashurst, P. R., Dennis, M. J., Eds.; Blackie Academic and Professional: New York, 1998; pp 97−136.
(3) Szejtli, J.; Henriques, R. D.; Castineira, M. Mechanism of the acid hydrolysis of saccharose and raffinose. *Acta Chim. Acad. Sci. Hung.* **1970**, *66*, 213−227.
(4) Krol, B. Side reactions of acid hydrolysis of sucrose. *Acta Aliment. Pol.* **1978**, *6*, 373−380.
(5) Thompson, A.; Anno, K.; Wolfrom, M. L.; Inatome, M. Acid reversion products from D-glucose. *J. Am. Chem. Soc.* **1954**, *76*, 1309−1311.
(6) Low, N. H. Determination of fruit juice authenticity by capillary gas chromatography with flame ionization detection. *J. AOAC Int.* **1996**, *79*, 724−737.
(7) Prodolliet, J.; Hischenhuber, C. Food authentication by carbohydrate chromatography. *Z. Lebensm. Unters. Forsch. A* **1998**, *207*, 1−12.
(8) Swallow, K.; Low, N. H. Analysis and quantification of the carbohydrates in honey by high performance liquid chromatography. *J. Agric. Food Chem.* **1990**, *38*, 1828−1832.
(9) Stuckel, J.; Low, N. H. Maple syrup analysis by anion-exchange liquid chromatography with pulsed amperometric detection. *J. Agric. Food Chem.* **1995**, *43*, 3046−3051.
(10) Low, N. H.; South, W. Determination of honey authenticity by capillary gas chromatography. *J. Assoc. Off. Anal. Chem.* **1994**, *78*, 1106−1113.
(11) Swallow, K.; Low, N. H. Isolation and identification of oligosaccharides in a commercial beet medium invert syrup. *J. Agric. Food Chem.* **1993**, *41*, 1587−1592.
(12) Sweely, C. C.; Bentley, R.; Makita, M.; Wells, W. W. Gas−liquid chromatography of trimethylsilyl derivatives of sugars and related substances. *J. Am. Chem. Soc.* **1963**, *85*, 2497−2507.

(13) Low, N. H.; Sporns, P. Analysis and quantitation of minor di- and trisaccharides in honey, using capillary gas chromatography. *J. Food Sci*. **1988**, *53*, 558−561.
(14) Whistler, R. L.; Durso, D. F. Chromatographic separation of sugars on charcoal. *J. Am. Chem. Soc*. **1950**, *72*, 677−679.
(15) Webb, E. C. *Enzyme Nomenclature. Recommendation (1992) of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology*; Academic Press: San Diego, CA, 1992.
(16) Angyal, S. J. The composition of reducing sugars in solution. *Adv. Carbohydr. Chem. Biochem*. **1984**, *42*, 15−68.
(17) Low, N. H. Carbohydrate analysis. In *Introduction to the Chemical Analysis of Foods*; Nielsen, S. S., Ed.; Jones and Bartlett Publishers: Boston, MA, 1994; pp 137−167.
(18) Thompson, J.; Robrish, S. A.; Pikis, A.; Brust, A. Phosphorylation and metabolism of sucrose and its five linkage-isomeric α-D-glucosyl-D-frucotses by *Klebsiella pneumoniae*. *Carbohydr. Res*. **2001**, *331*, 149−161.
(19) Lichtenthaler, F. W.; Ronninger, S. α-D-Glucopyranosyl-D-fructose: distribution of furanoid and pyranoid tautomers in water, dimethyl sulphoxide, and pyridine. Studies on ketoses. Part 4. *J. Chem. Soc., Perkin Trans. 2* **1990**, *8*, 1489−1497.
(20) Bock, K.; Thogersen, H. Nuclear magnetic resonance spectroscopy of mono- and oligosaccharides. *Annu. Rep. NMR Spectrosc*. **1982**, *13*, 1−57.
(21) Agrawal, P. K. NMR spectroscopy in the structural elucidation of oligosaccharides and glycosides. *Phytochemistry* **1992**, *31*, 3307−3330.

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