Isolation and Identification of Oligosaccharides in a Commercial Beet Medium Invert Syrup

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Research was conducted to isolate and identify oligosaccharides in a commercial beet medium invert syrup (BMIS). Isolation of two BMIS oligosaccharides was accomplished using charcoal/Celite chromatography, preparative high-performance liquid chromatography, and gel permeation chromatography. Partial and total acid hydrolysis, spray reagents for reducing and ketose-containing carbohydrates, selective enzymatic hydrolysis, and ^{13}C nuclear magnetic resonance spectroscopy were used to identify these oligosaccharides. 6-Kestose (*O*-β-D-fructofuranosyl-(2→6)-β-D-fructofuranosyl-α-D-glucopyranoside) and the previously unidentified carbohydrate, kelose (*O*-α-D-fructofuranosyl-(2→6)-β-D-fructofuranosyl-α-D-glucopyranoside), were identified as two of the oligosaccharides in a commercial BMIS.

INTRODUCTION

Hydrolysates from beet and cane sucrose can be added to pure foods for economic gain by unscrupulous producers and/or processors. Controlled acid and/or enzymatic hydrolysis of sucrose can be used to produce syrups with a specific glucose/fructose/sucrose ratio. These syrups can be used to adulterate foods such as honey and citrus juices. During sweetener production minor oligosaccharides are synthesized which are either present in very low concentrations or not present in pure foods as analyzed by anion-exchange high-performance liquid chromatography (HPLC) with pulsed amperometric detection (Swallow and Low, 1993; Swallow et al., 1991; Low and Swallow, 1991).

Acid hydrolysis of sucrose is the main commercial method employed for the production of beet medium invert syrup (BMIS) (Alberta Sugar Co., personal communication). Research has shown that oligosaccharides are synthesized during acid-catalyzed hydrolysis of sucrose (Szejtli et al., 1970; Krol, 1978; Low and Wudrich, 1993). The production of complex oligosaccharides by this mechanism is known as reversion. Formation of reversion products during hydrolysis has been shown to occur in weak acid (<0.1% HCl) (Thompson et al., 1954) and high sucrose concentration (65 °Brix) (Krol, 1978). Reversion reactions can result in the formation of oligosaccharides that are not stereospecific at the anomeric carbon (Thompson et al., 1954). The majority of oligosaccharides formed during the acid hydrolysis of sucrose would be comprised of either fructofuranose or glucopyranose α- or β-linked to sucrose. This is due to the fact that sucrose is the only carbohydrate in the original solution (75.7 °Brix).

The mechanism of the reversion reaction involves initial protonation of the glycosidic linkage. The glycosidic bond is broken, resulting in the formation of either glucopyranose and a charged fructofuranose moiety or fructofuranose and a charged glucopyranose moiety (Szejtli et al., 1970). The glucose or fructose intermediate carrying the positive charge (oxonium ion) is partially planar due to the partial sp^2 nature of the bond. The 5-membered furanose ring is more planar than the 6-membered pyranose ring. Therefore, formation of the cyclopentyl (i.e., fructofuranosyl) cation occurs more readily than does that of the cyclohexyl cation (Szejtli et al., 1970). Since the fructofuranosyl oxonium ion intermediate is more readily formed than the glucopyranosyl oxonium ion, the probability is much higher that this molecule will react with other carbohydrates in solution.

Certain structures would be favored in the reversion reactions involving sucrose because of the primary structure and/or steric hindrance of certain hydroxyl groups. Thompson et al. (1954) and Wolfrom et al. (1958) showed that certain linkages such as 1→2, 1→4, and 1→6 were favored in glucose reversion reactions. Although there has not been a great deal of research conducted on the identification of oligosaccharides formed during acid-catalyzed sucrose hydrolysis, Krol (1978) hypothesized that kestoses (i.e., fructose linked to sucrose) were formed during the acid-catalyzed hydrolysis of sucrose. Szejtli et al. (1970) suggested that linkages at the 1- and 6-positions of fructose and the 6-position of glucose would be favored because of the ease of accessibility of these hydroxyl groups.

Fingerprint oligosaccharides present in BMIS have been used to determine authenticity of foods such as orange juice (Swallow et al., 1991; Low and Swallow, 1991). One purpose for the identification of these fingerprint oligosaccharides in a commercial BMIS was to gain some insight into the mechanism of acid-catalyzed synthesis of oligosaccharides during sucrose hydrolysis. Second, structural identification could be used as the first step in the development of a rapid and sensitive BMIS adulteration detection test based on a single fingerprint oligosaccharide.

MATERIALS AND METHODS

Dr. N. Shiomi (Rakuno Gakuen University, Japan) donated 1-kestose (*O*-β-D-fructofuranosyl-(2→1)-β-D-fructofuranosyl α-D-glucopyranoside), neokestose (*O*-β-D-fructofuranosyl-(2→6)-α-D-glucopyranosyl β-D-fructofuranoside), and 6-kestose (*O*-β-D-fructofuranosyl-(2→6)-β-D-fructofuranosyl α-D-glucopyranoside); Dr. A. Cairns (AFRC Institute of Grassland and Environmental Research, Wales) donated 1-kestose, 6-kestose, and nystose (*O*-β-D-fructofuranosyl-(2→1)-β-D-fructofuranosyl-(2→1)-β-D-fructofuranosyl α-D-glucopyranoside); Dr. H. Hidaka (Meiji Seika Kaisha Ltd., Japan) donated 1-kestose and nystose; Dr. N. Chatterton (Utah State University) donated neokestose, 6-kestose, and 1-kestose. The BMIS sample used in the study was obtained from the Alberta Sugar Co. and contained 24.5% H_2O as determined by Karl–Fisher titration.

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Table I. Gradient Elution Used To Isolate the BMIS Oligosaccharides (Method 1)

time, min	% A[a]	% B[a]
0	100	0
4.0	100	0
20.0	97	3
70.0	20	80
70.1	100	0
125.0	100	0

[a] % A = 100 mM NaOH; % B = 100 mM NaOH and 100 mM sodium acetate (NaOAc).

Bulk Oligosaccharide Isolation from BMIS. A modification of the charcoal/Celite chromatography method developed by Whistler and Durso (1950) was used to remove the majority of glucose and fructose from the BMIS. BMIS (97.0 g) at 75.7 °Brix was dissolved in 200 mL of water and stirred with 129.0 g of activated charcoal for 17 h at 4 °C. A 4.5 × 40 cm glass column equipped with a 1000-mL reservoir was previously packed with 150.0 g of a 50:50 (w/w) mixture of charcoal/Celite and washed with 4 L of water. The slurry containing the mixture of BMIS and activated charcoal was placed on the column and washed with 14 L of water, 14 L of 1.0% EtOH (v/v), and 45 L of 3.0% EtOH at a flow rate of 2 mL/min. The column eluate was monitored by HPLC to ensure that glucose and fructose were removed without loss of oligosaccharides. Oligosaccharides were eluted from the charcoal/Celite column by washing with 4 L of 50.0% ethanol at 60 °C. The eluate was concentrated to approximately 30 mL (11.1 °Brix) by rotary evaporation at 35 °C (Buchi, Model KRV 65). Analysis of the concentrated 50% ethanol wash by HPLC revealed that >95% of the monosaccharides were removed.

Individual Oligosaccharide Isolation. Isolation of two oligosaccharides from the charcoal/Celite-treated BMIS was accomplished using a Dionex Bio LC 4000 gradient HPLC equipped with a Dionex 10-μm preparatory Carbo Pac PA1 pellicular anion-exchange column (9 × 250 mm). The flow rate was 2.0 mL/min, sample loop was 800 μL, and the carbohydrates were detected by a pulsed amperometric detector (PAD) with a gold electrode and triple pulsed amperometry at a sensitivity of 30 K. The electrode was maintained at the following potentials and durations: E_1 = 0.05 V (t_1 = 120 ms); E_2 = 0.80 V (t_2 = 120 ms); E_3 = −0.60 V (t_3 = 420 ms). The HPLC program used to isolate the oligosaccharides is described in Table I. The carbohydrates eluting from the column were plotted by a Spectra Physics Model 4290 integrator. The BMIS solution isolated by charcoal/Celite chromatography was diluted from 11.1 to 3.0 °Brix and loaded on the preparatory HPLC column for each injection.

The BMIS oligosaccharides eluting from the column were manually collected in beakers (A–E), but only fractions B and E were used in this study. To minimize cross-contamination of oligosaccharides, only the initial and final eluates from peaks that were not baseline resolved were collected. After collection, fractions B and E were neutralized by addition of 0.1 M acetic acid to pH 5 and stored at −20 °C. After sufficient eluate was collected (225 mL), each fraction was passed through a 10-cm³ Luer Lok syringe containing 10 cm³ of AG 50W-X8 cation-exchange resin (Bio-Rad Laboratories) at 4 °C to remove sodium ions. The solutions were evaporated at 35 °C and stored at −20 °C.

Determination of Oligosaccharide Purity by GLC and HPLC. Purity of the two BMIS oligosaccharide fractions was determined by gas–liquid chromatography (GLC) and HPLC. In preparation for GLC analysis, each solution containing approximately 0.02 mg of purified BMIS oligosaccharide was placed in a separate glass autosampler vial (32 × 11 mm i.d.) and frozen at −70 °C. The samples were lyophilized in a LabConCo freeze-dryer (Freeze Dry 5) at 25 °C for 6 h. To the resulting off-white powder was added 0.5 mL of Tri-Sil Z (Chromatographic Specialties Inc.). The vials were capped, and the solutions were heated at 80 °C for 1 h. The trimethylsilylated carbohydrates were analyzed by gas–liquid chromatography (Hewlett-Packard, Model 5890A) equipped with an autosampler (Model 7673A). The gas (UHP Helium) flow rate was 0.75 mL/min, and 5 μL of sample was injected into the column with a split ratio of 4.9:1.

Table II. Gradient Elution Used To Achieve Separation of the Carbohydrate Standards and BMIS Oligosaccharides (Method 2)

time, min	% A[a]	% B[a]	% C[a]
0	100	0	0
4.0	100	0	0
20.0	97	3	0
50.0	0	100	0
60.0	0	100	0
60.1	0	0	100
120.0	0	0	100
120.1	100	0	0
175.0	100	0	0

[a] % A = 100 mM NaOH; % B = 100 mM NaOH and 100 mM NaOAc; % C = 300 mM NaOH.

The injector temperature was maintained at 300 °C, and the capillary column used was a 0.25 mm × 30 m DB-5 (J&W Scientific). The program used to separate the carbohydrates was as follows: 210 °C for 15 min followed by a 1 °C/min gradient to a temperature of 290 °C; this temperature was held for 20 min. The derivatized carbohydrates were detected with a flame ionization detector (FID) maintained at 300 °C. The carbohydrates eluting from the column were plotted with a Hewlett-Packard Model 3396A integrator.

Determination of sample purity and comparison of retention times of the two BMIS oligosaccharides with the oligosaccharide standards was carried out on a Waters 625 LC system with two Dionex 10-μm Carbo Pac PA1 pellicular anion-exchange columns (4 × 250 mm) connected in series at a flow rate of 0.70 mL/min. The carbohydrates were detected by a PAD (Waters Model 464) with a dual gold electrode at a sensitivity of 50 μA. The working electrode was maintained at the following potentials and durations during operation: 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). Using the HPLC program (method 2) listed in Table II, the retention times of the BMIS oligosaccharides were compared to the retention times of the trisaccharide standards. A postcolumn delivery system of 300 mM sodium hydroxide (NaOH) at a flow rate of 0.80 mL/min (Scientific Systems Inc., Model 350) was used to minimize baseline drift. The carbohydrates eluting from the columns were plotted by a Maxima 820 chromatography work station (Millipore).

Analysis of fraction E by HPLC revealed that there was trace contamination of this sample with glucose, fructose, and sucrose. Purification of this sample was achieved using gel permeation chromatography (GPC). This method of chromatography has been used by a number of researchers for the bulk separation of monosaccharides, disaccharides, and trisaccharides (Sloan et al., 1984; Kennedy et al., 1989). A Fast Protein liquid chromatography (FPLC) system (Pharmacia) equipped with two P-500 pumps and a LCC-500 Plus liquid chromatography controller was used. A 2.5 × 100 cm glass column packed with 123 g of Bio-Gel P-2 resin, 200–400 mesh (Bio-Rad Laboratories), was used to remove the trace glucose, fructose, and sucrose contaminants. Approximately 3.5 mg of the BMIS oligosaccharide and carbohydrate contaminants was dissolved in 0.5 mL of water and placed on the column with a Pasteur pipet for each run. Water was used as the mobile phase at a flow rate of 0.30 mL/min, and the carbohydrates started eluting after 960 min. The carbohydrates were detected using a refractive index detector (Waters, Model R401). Ten-minute fractions were collected (Pharmacia, Model Frac-100), and carbohydrates eluting from the column were plotted with a two-channel recorder (Pharmacia, Model REC-482).

Partial Acid Hydrolysis of Oligosaccharides. Partial acid hydrolysis of the two BMIS oligosaccharides was achieved by passing 5.0 mL of each sample (approximately 0.1 mg/mL) through 10 cm³ of AG 50W-X8 cation-exchange resin (Bio-Rad Laboratories), 100–200 mesh, at a flow rate of 0.5 mL/min. Each carbohydrate had a residence time in the column of about 10 min. Hydrolysis of the BMIS oligosaccharides was monitored by HPLC (method 2).

Total Acid Hydrolysis of Oligosaccharides. A solution containing 150 μL of each of the two BMIS oligosaccharides (approximately 0.1 mg/mL) was treated with 7 μL of 1 M HCl

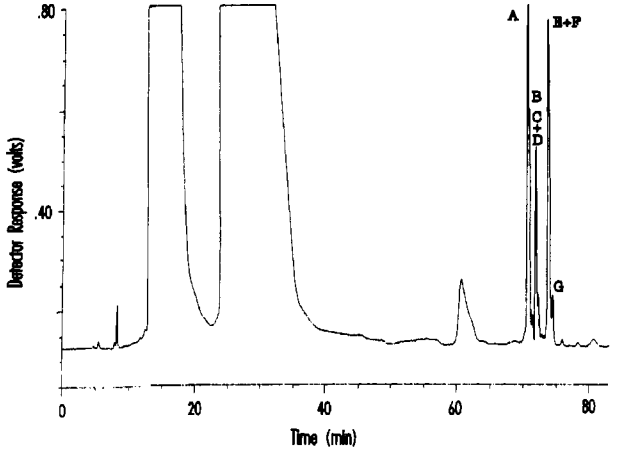

Figure 1. HPLC chromatogram of the carbohydrates in commercial BMIS (Alberta Sugar Co.). A–G represent the fingerprint peaks.

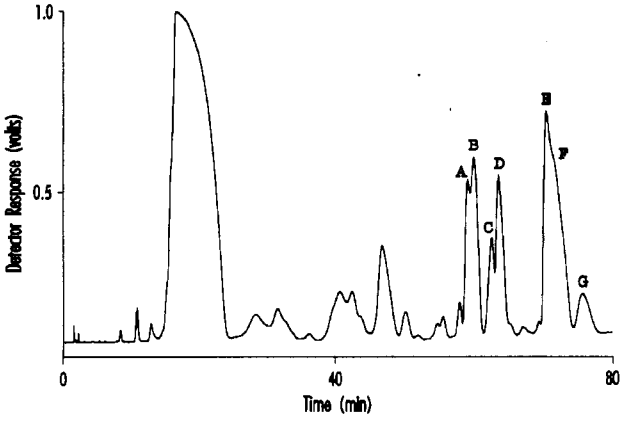

Figure 2. HPLC chromatogram of carbohydrates in BMIS (Alberta Sugar Co.) after charcoal/Celite chromatography using the preparatory column. Peaks B and E contain the two fingerprint oligosaccharides.

for 1 h at 75 °C. A blank was run with each sample in which 7 μL of water was substituted for the acid. The reaction was neutralized by the addition of 1 M NaOH to a final pH of 5. Quantitation of the resulting carbohydrates was determined by HPLC (method 2).

Reducing Sugar Determination. The BMIS oligosaccharide solutions (10 μL), glucose, fructose, sucrose, maltotriose, and raffinose, were spotted on two separate Whatman No. 3 papers (23 × 25 cm). The concentration of each carbohydrate was approximately 0.1 mg/mL. The paper chromatograms were developed in 2-propanol/1-butanol/water (140:20:40) (Menzies and Seakins, 1969) for 8 h using ascending chromatography. After development, each paper chromatogram was dipped in either anthrone or dinitrosalicylic acid reagent (Chemical Rubber Co., 1972). The carbohydrate standards were run as controls to confirm the specificity of these spray reagents.

Enzymatic Hydrolysis. Solutions of compounds **B** and **E**, raffinose, sucrose, erlose, 1-kestose, and 6-kestose (150 μL at approximately 0.1 mg/mL) were placed in four separate test tubes (13 × 100 mm). Water (25 μL) was added to tube 1, 25 μL (1 unit) of β-glucosidase was added to tube 2, 25 μL (1 unit) of α-glucosidase was added to tube 3, and 25 μL (1 unit) of β-fructosidase was added to tube 4 (all enzymes were obtained from Sigma Chemical Co.). The samples were kept at 25°C, pH 5, for 28 h on an orbital shaker (Model G2, New Brunswick Scientific Co. Inc.) at 200 rpm. Each tube was then placed in boiling water for 5 min to denature the enzyme. All samples were filtered (0.22-μm Millex-GV filter, Millipore) prior to analysis by HPLC (method 2). The carbohydrate standards were included to confirm the specificity of the enzymes.

^{13}C Nuclear Magnetic Resonance Spectroscopy. Each of the isolated BMIS oligosaccharides (**B** and **E**) were dissolved in 10 mL of absolute ethanol and re-evaporated to dryness in preparation for ^{13}C nuclear magnetic resonance (NMR) spectroscopic analysis. After drying, each fraction contained between 10 and 12 mg of carbohydrate. Each of the BMIS oligosaccharide samples and carbohydrate standards was dissolved in 0.6 mL of D_2O and quantitatively transferred to a 5 × 175 mm NMR tube. A ^{13}C NMR spectrum of each sample was obtained using a 75.481 MHz (Bruker, Model AM 300) NMR spectrometer. The sweep width was 18 518.5 Hz with a pulse angle of 33°. Repetition time was 4 s, made up of a 2-s acquisition and a 2-s relaxation. Between 2000 and 4000 scans were required for each spectrum. The protons were decoupled at 300.13 MHz; offset was 514 Hz.

RESULTS AND DISCUSSION

Figure 1 is an HPLC chromatogram of the carbohydrates in BMIS (Alberta Sugar Co.). The fingerprint oligosaccharides in this material which we have used to detect the presence of BMIS in orange juice eluted between 70 and 75 min (Swallow et al., 1991; Low and Swallow, 1991). It was estimated by HPLC that the oligosaccharide concentration in the commercial BMIS was <1% by comparison of the area of these peaks to the area of the major carbohydrate peaks.

A modification of the charcoal/Celite chromatographic method developed by Whistler and Durso (1950) was utilized to remove the bulk of glucose and fructose from the BMIS. Removal of these monosaccharides, which make up approximately 50% of the total carbohydrate content in BMIS, facilitated the separation of the fingerprint oligosaccharides by preparatory HPLC. Analysis of the carbohydrates in the 50% ethanol wash by HPLC revealed that the concentration of glucose and fructose was reduced to <5% of the initial concentration.

Using preparatory anion-exchange HPLC with a pulsed amperometric detector (PAD), fractions **B** and **E** were collected from the charcoal/Celite-treated BMIS mixture (Figure 2). These fractions were analyzed by GLC and HPLC and determined to be >90% pure. In total, 11 mg of compound **B** and 12 mg of compound **E** were isolated. Compounds **B** and **E** were isolated from 97.0 g of BMIS, resulting in a total yield of 0.02%.

Preliminary analysis of the two BMIS oligosaccharides and standard carbohydrates by HPLC revealed that compound **B** had the same retention time as 6-kestose. Analysis by HPLC of compound **B** spiked with 6-kestose resulted in a chromatogram with one peak. These results warranted the further analysis of this compound to positively ascertain its chemical structure. No other oligosaccharide standards in our possession had retention times similar to those of the two BMIS oligosaccharides isolated by HPLC. Further analysis of compound **E** was also carried out, because it was one of the two oligosaccharides in BMIS that comprised the largest fingerprint peak.

Structural Determination of Compound B. *Partial Acid Hydrolysis.* Passage of compound **B** through a strong cation-exchange resin resulted in the partial hydrolysis of this compound. Analysis of the partial acid digest by HPLC showed that this oligosaccharide was comprised of glucose, fructose, and sucrose. This experiment proved that compound **B** had either a glucose or a fructose monomer linked to a sucrose backbone due to the carbohydrate pattern obtained upon hydrolysis.

Total Acid Hydrolysis. Total acid hydrolysis of compound **B** resulted in complete sample breakdown, yielding only glucose and fructose in a ratio of 1:2 as determined by HPLC. These results, coupled with the partial acid hydrolysis, revealed that this oligosaccharide consisted of fructose linked to sucrose.

Reducing Sugar Determination. The partial and total acid hydrolysis experiments with compound **B** not only showed that this BMIS oligosaccharide contained a fructose linked to sucrose but also indicated that it was a nonreducing carbohydrate. To corroborate this evidence, compound **B** was sprayed with indicator reagents for reducing sugars and ketose-containing carbohydrates. Compound **B** was spotted on two separate pieces of chromatographic paper and developed by ascending chromatography using 2-propanol/1-butanol/water to remove residual glucose, fructose, and sucrose which might have interfered with these experiments. After development, the papers were dipped in dinitrosalicylic acid (DA) (paper 1) and anthrone reagents (An) (paper 2). These reagents have been reported to be specific for reducing sugars (DA), ketoses, and oligosaccharides containing a ketose moiety (An) (Chemical Rubber Co., 1972). Compound **B** yielded a negative response to the DA reagent and therefore was a nonreducing oligosaccharide. In addition, this BMIS oligosaccharide tested positive with anthrone reagent, which confirmed the presence of at least one ketose monomer.

Enzymatic Hydrolysis. The results from the acid hydrolysis experiments indicated that the fingerprint oligosaccharide B contained a fructose (F″) which was α- or β-linked to either the glucose (G) or the fructose (F′) moiety of sucrose (see Figure 3). Experiments were conducted employing hydrolase enzymes to obtain additional structural information for this BMIS oligosaccharide. The three hydrolase enzymes used for these experiments were α-glucosidase, β-fructosidase, and β-glucosidase. Following incubation of compound **B** with each commercial hydrolase enzyme, the resulting carbohydrate profile was analyzed by HPLC. The extent of hydrolysis was estimated by HPLC by comparison of the area of the parent oligosaccharide peak following incubation with the enzyme to the area of the peak in the control.

Incubation of β-glucosidase resulted in little or no hydrolysis of compound **B**. These results were expected as all experiments conducted indicated that this compound did not contain a β-linked glucose moiety. Incubation of compound **B** with β-fructosidase resulted in 29% hydrolysis. There was also a concurrent increase in the fructose concentration following hydrolysis. This increase in fructose was due to the cleavage of either F″ (if β-linked to sucrose) or F′ of sucrose (if it was at the end of the oligosaccharide). Incubation of compound **B** with α-glucosidase resulted in 7% hydrolysis of the parent peak and an increase in the glucose concentration. The ability of α-glucosidase to hydrolyze compound **B** demonstrated that F″ was β-linked to the F′ of sucrose.

HPLC and ^{13}C NMR Analysis. Compound **B** had the same retention time as 6-kestose as determined by HPLC, which suggested that these two carbohydrates could be identical. ^{13}C nuclear magnetic resonance spectroscopy (^{13}C NMR) was used to investigate this possibility. Table III lists the chemical shifts of compound **B** and 6-kestose obtained with the 300-MHz spectrometer. Also listed in Table III are the carbon assignments of 6-kestose made by Liu et al. (1991). The HPLC chromatogram suggested that compound **B** could be 6-kestose, while the ^{13}C NMR experiment definitively proved that it was.

Structural Determination of Compound E. *Partial Acid Hydrolysis.* Partial acid hydrolysis of compound **E** yielded results similar to those obtained with compound **B**. Glucose, fructose, and sucrose were released during hydrolysis of this BMIS oligosaccharide.

Total Acid Hydrolysis. Total acid hydrolysis of compound **E** yielded glucose and fructose in a 1:2 ratio as determined by HPLC. The partial and total acid hydrolysis experiments determined that this oligosaccharide consisted of fructose linked to sucrose.

Reducing Sugar Determination. The reaction of compound **E** with the spray reagents described for the identification of compound **B** confirmed that this oligosaccharide was a nonreducing sugar containing at least one ketose monomer.

Enzymatic Hydrolysis. Following incubation of compound **E** with each commercial hydrolase enzyme, the resulting carbohydrate profile was analyzed by HPLC. Incubation of β-glucosidase resulted in little or no hydrolysis of compound **E**. The inability of β-fructosidase to hydrolyze compound **E**, <3% hydrolysis, demonstrated that F″ was α-linked to F′ of sucrose, although F″ could have been β-linked to sucrose with a slow rate of hydrolysis. Treatment with α-glucosidase resulted in 93% hydrolysis of compound E, a concurrent appearance of a new peak with a retention time approximately 4 min shorter than that of the parent peak, and a large increase in free glucose. This new peak was most likely a difructan; however, the structure of this oligosaccharide was not determined. These results indicated that the structure of compound **E** was F″ α-linked to F′ of sucrose.

^{13}C NMR Analysis. Carbon assignments and glycosidic linkages for compound **E** were made with the aid of ^{13}C NMR spectra of α- and β-fructofuranose, sucrose, 1-kestose, and 6-kestose. Literature values for the ^{13}C chemical shifts and carbon assignments of α- and β-fructofuranose (Angyal and Bethell, 1976) and sucrose (Pfeffer et al., 1979) are shown in Table IV. Experimental ^{13}C chemical shifts obtained for sucrose and β-fructofuranose and for 1-kestose and 6-kestose are listed in Tables V and VI, respectively. These experimental values were shifted 1.2–1.5 ppm

Table III. ^{13}C NMR Chemical Shifts of Compound B and 6-Kestose and Carbon Assignments of 6-Kestose (Liu et al., 1991)

compound **B** chemical shift, ppm	6-kestose chemical shift, ppm	6-kestose C[a]
106.1	106.1	2″
106.1	106.1	2′
94.5	94.5	1
83.5	83.5	5″
82.7	82.7	5′
78.8	78.7	3″
78.4	78.5	3′
77.0	77.0	4″
76.7	76.7	4′
74.9	74.9	3
74.7	74.8	5
73.4	73.4	2
71.6	71.6	4
65.3	65.3	6′
64.9	64.9	6″
63.5	63.5	1′
62.6	62.6	6
62.0	62.1	1″

[a] Unprimed carbons are glucose, primed carbons are the fructose moiety of sucrose, and double-primed carbons correspond to the fructose linked to sucrose (Figure 3).

Table IV. ^{13}C Chemical Shifts of α- and β-Fructofuranose (Angyal and Bethell, 1976) and Sucrose (Pfeffer et al., 1979)

compound	group	chemical shift, ppm C1	C2	C3	C4	C5	C6
β-fructofuranose		63.6	102.6	76.4	75.4	81.6	63.2
α-fructofuranose		63.8	105.5	82.9	77.0	82.2	61.9
sucrose	F′	62.2	104.5	77.3	74.8	82.2	63.2
	G	92.9	71.9	73.4	70.0	73.2	61.0

Table V. Experimental ^{13}C Chemical Shifts of β-Fructofuranose and Sucrose and Calculated Chemical Shifts of α-Fructofuranose

compound	group	chemical shift, ppm C1	C2	C3	C4	C5	C6
β-fructofuranose		65.0	103.8	77.7	76.8	83.0	64.7
α-fructofuranose		65.2	106.7	84.2	78.4	83.6	63.4
sucrose	F′	63.6	106.0	78.7	76.3	83.7	64.6
	G	94.5	73.4	74.9	71.5	74.7	62.4

Table VI. Experimental ^{13}C Chemical Shifts for Sucrose, 6-Kestose, Compound E, and 1-Kestose and Carbon Assignments for Sucrose (Pfeffer et al., 1979), 6-Kestose (Liu et al., 1991), 1-Kestose (Calub et al., 1990), and (Partial) Compound E

sucrose		6-kestose		compound E		1-kestose	
chemical shift, ppm	C	chemical shift, ppm	C	chemical shift, ppm	C	chemical shift, ppm	C
106.0	2′	106.1	2′	110.7	2″	106.1	2″
		106.1	2″	106.0	2′	105.6	2′
94.5	1	94.5	1	94.4	1	94.8	1
		83.5	5″	85.7	3″	83.5	5′
83.7	5′	82.7	5′	82.5	5′?	83.5	5″
		78.8	3″	82.0	5″?	78.9	3″
78.7	3′	78.5	3′	79.7	3′?	78.9	3′
		77.0	4″	78.3	4″?	76.8	4″
76.3	4′	76.7	4′	76.6	4′?	76.2	4′
74.9	3	74.9	3	74.9	3	74.9	3
74.7	5	74.8	5	74.6	5	74.7	5
73.4	2	73.4	2	73.4	2	73.5	2
71.5	4	71.6	4	71.7	4	71.5	4
64.6	6′	65.3	6′	64.8	6′?	64.7	6″
		64.9	6″	63.6	6″?	64.5	6′
63.6	1′	63.5	1′	63.6	1′	63.2	1′
62.4	6	62.6	6	62.8	6	62.7	1″
		62.1	1″	60.6	1″	62.4	6

downfield when compared to literature values due to spectrometer differences.

The ^{13}C chemical shifts for α-fructofuranose were calculated by (1) comparison of our experimental values for β-fructofuranose and literature values for the same compound (Angyal and Bethell, 1976) and (2) chemical shift calculation based on literature values (Angyal and Bethell, 1976) and our spectrometer differences.

Figure 3 illustrates the structures of sucrose, 1-kestose, 6-kestos, and compound **E** (kelose). Chemical shift and carbon assignments for compound **E** are shown in Table VI. A number of these shifts were readily assigned by comparison to the previously mentioned literature values (and our experimentally determined values on these standards). Unfortunately, not all assignments could be made in this manner, and a number of carbon assignments required in-depth analysis of the experimental data.

For compound **E** the chemical shift of 110.7 ppm was assigned to the C2 of F″. This assignment was based on the 2.2 ppm shift observed for C2 of F′ when α-fructofuranose was bonded to the C1 of glucose (Table V). Assuming that this same downfield shift would occur when a fructose moiety was α-linked to the β-fructose moiety of sucrose, the expected chemical shift for C2 of F″ would be 108.9 ppm, which is close to the observed shift of 110.7 ppm. This assignment establishes the glycosidic linkages of compound **E** to be fructose α-linked to the β-fructofuranose moiety of sucrose.

The C3 chemical shift for F′ and those for C5 (F′) and C5 (F″) of compound **E** were assigned at 85.7, 82.5, and 82.0 ppm, respectively. These assignments were based on the chemical shifts observed for fructofuranoses in trisaccharides containing sucrose (Pfeffer et al., 1979; Calub et al., 1990; Liu et al., 1991).

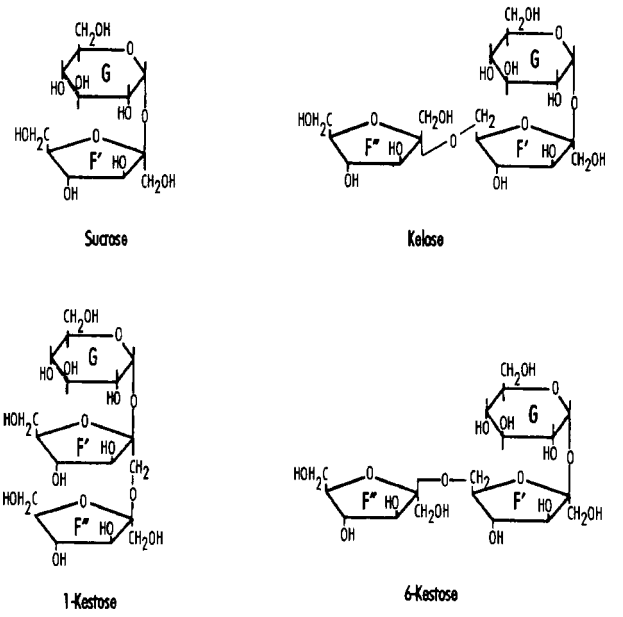

Figure 3. Structures of sucrose, 1-kestose, 6-kestose, and compound **E** (kelose).

Two literature examples [Munksgaard, 1981, as found in Bock et al. (1984)] that illustrate the dramatic downfield shift which occurs when a glycosidic bond is formed between a fructose (or glucose) monomer and another carbohydrate are (1) the 7.7 ppm shift observed for the glucose moiety of sucrose when α-glucose was attached to the C4 position and (2) the 4.7 ppm shift observed for the C3 of β-fructofuranose after glucose attachment. Compound **E** showed no dramatic downfield shifts for either C3 or C4 of F′. This observation clearly indicated that F″ was not linked to either of these carbon atoms. These results reduced the structural possibilities of compound **E** to F″ being α-linked to either C1 or C6 of the F′ of sucrose.

The ^{13}C NMR chemical shift data (Table VI) revealed three important similarities between 6-kestose (6-K) and compound E which favored assignment of the α-6 over the α-1 linkage. The first of these similarities occurs in the region of the spectra from 60.6 to 65.3 ppm, where the chemical shifts of C6(G), C1(F′), and C6(F″) occurred. The 60.6 ppm shift for compound **E** was further upfield than any of these standards but was numerically close to that observed for the C1(F″) of 6-K. The second similarity exists with the chemical shifts for C2(F′) of 6-K (106.1 ppm) and the virtually identical shift (106.0 ppm) which was assigned to C2(F′) for compound **E**. The most conclusive evidence was the chemical shift data for C5′ of sucrose, 6-K, 1-kestose (1-K), and compound **E**. The C5′ of sucrose (83.7 ppm) shifted upfield for 6-K (82.7 ppm) and had no appreciable shift for 1-K (83.5 ppm). Compound **E** had a chemical shift of 82.7 ppm, which closely matched that of 6-K.

The ^{13}C NMR data favored the structural assignment of compound **E** as *O*-α-D-fructofuranosyl-(2→6)-β-D-fructofuranosyl α-D-glucopyranoside. As this is the first "natural" appearance of this oligosaccharide, we have assigned the common name kelose to this compound.

Conclusion. During the acid-catalyzed hydrolysis of sucrose, oligosaccharides are synthesized which are different from the oligosaccharides naturally present in pure foods. Identification of compounds such as kelose in foods could be the first step in the development of a rapid and sensitive analytical test for determining the undeclared

addition of sucrose hydrolysates to foods such as honey, maple syrup, and citrus juices.

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