

Note

Structural Analysis of Novel Low-Digestible Sucrose Isomers Synthesized from D-Glucose and D-Fructose by Thermal Treatment

(Received August 22, 2016; Accepted November 21, 2016)

(J-STAGE Advance Published Date: December 16, 2016)

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Abstract: The synthesis of the saccharide β -D-fructopyranosyl-(2 \rightarrow 6)-D-glucopyranose, which was isolated from Super Ohtaka[®], has recently been reported. During the synthesis of this saccharide, the formation of two novel saccharides from D-glucose and D-fructose was observed. The present study aimed to confirm the structures of the two disaccharides synthesized from D-glucose and D-fructose by thermal treatment. Furthermore, various properties of the saccharides were investigated. Both saccharides were isolated from the reaction mixture by carbon-Celite column chromatography and an HPLC system and were determined to be novel sucrose-isomers, β -D-fructopyranosyl-(2 \leftrightarrow 1)- β -D-glucopyranoside (**1**) and β -D-fructofuranosyl-(2 \leftrightarrow 1)- β -D-glucopyranoside (**2**), by MALDI-TOF MS and NMR analyses. Both saccharides showed low digestibility *in vitro*, and the sweetness of saccharide **2** was 0.45 times that of sucrose.

Key words: sucrose-isomer, disaccharide, thermal treatment, fermented beverage of plant extract

We previously reported the structural analysis of the oligosaccharides β -D-fructopyranosyl-(2 \rightarrow 6)-D-glucopyranose,¹⁾ α -D-fructofuranosyl-(2 \rightarrow 6)-D-glucopyranose,²⁾ β -D-fructopyranosyl-(2 \rightarrow 6)- β -D-glucopyranosyl-(1 \rightarrow 3)-D-glucopyranose,³⁾ β -D-fructopyranosyl-(2 \rightarrow 6)-[β -D-glucopyranosyl-(1 \rightarrow 3)]-D-glucopyranose,³⁾ β -D-fructopyranosyl-(2 \rightarrow 1)- β -D-fructofuranosyl-(2 \leftrightarrow 1)- α -D-glucopyranoside,⁴⁾ and β -D-fructopyranosyl-(2 \rightarrow 6)- α -D-glucopyranosyl-(1 \leftrightarrow 2)- β -D-fructofuranoside⁴⁾ derived from Super Ohtaka[®], which is produced by fermenting the extracts from 50 types of fruits and vegetables. The extract is obtained after sucrose-osmotic pressure treatment in a cedar barrel for 7 days and fermentation by lactic acid bacteria and yeast at 37 °C for 180 days. This beverage primarily includes glucose and fructose, but it also contains various oligosaccharides. A previous study has shown that β -D-fructopyranosyl-(2 \rightarrow 6)-D-glucopyranose has non-cariogenic qualities and low digestibility.⁵⁾ This saccharide was selectively used by the beneficial bacteria *Bifidobacterium adolescentis* and *B. longum*, but it was not used by the harmful bacteria *Clostridium perfringens*, *Escherichia coli*, and *Enterococcus faecalis*, which produce mutagenic substances.⁵⁾ Furthermore, we synthesized β -D-fructopyranosyl-(2 \rightarrow 6)-D-glucopyranose⁶⁾ and α -D-fructofuranosyl-(2 \rightarrow 6)-D-glucopyranose⁷⁾

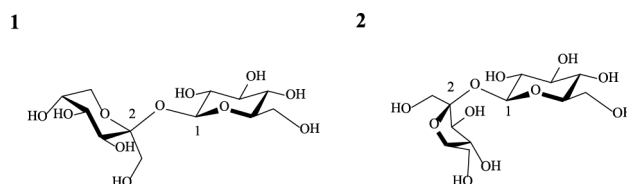


Fig. 1. Structure of β -D-fructopyranosyl-(2 \leftrightarrow 1)- β -D-glucopyranoside (**1**) and β -D-fructofuranosyl-(2 \leftrightarrow 1)- β -D-glucopyranoside (**2**).

from D-glucose and D-fructose by a thermal treatment. In the present study, we describe the synthesis of two novel saccharides (sucrose isomers), β -D-fructopyranosyl-(2 \leftrightarrow 1)- β -D-glucopyranoside and β -D-fructofuranosyl-(2 \leftrightarrow 1)- β -D-glucopyranoside (Fig. 1), from D-glucose and D-fructose by caramelization with thermal treatment, which is the first step for potential applications in food industry. Furthermore, we investigated various properties of these two sucrose isomers.

A powder mixture of 180 g each of D-glucose and D-fructose was carefully heated at 150 °C for 60 min in an electric furnace. The saccharide melt was then dissolved in 1,500 mL distilled water. The saccharide solution (1.86 L) was loaded onto an 8.1 \times 72-cm carbon-Celite column (1:1; charcoal: Celite-535) and successively eluted with water (27 L). Almost all of the D-glucose and D-fructose was eluted first within the first 2.5 L, and saccharides **1** and **2** were subsequently eluted with water (19 L). The water

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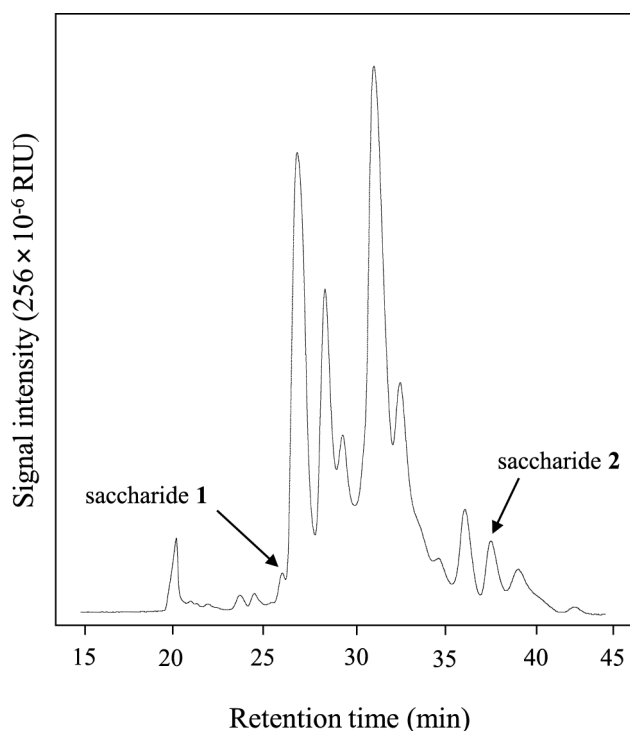


Fig. 2. Preparative-HPLC of the disaccharide fractions eluted by carbon-Celite column chromatography.

The following operating conditions for HPLC were used: column, ODS-100V (20×250 mm; Tosoh Corporation); column temperature, 20 °C; eluent, distilled water; flow rate, 3.0 mL/min; detection, by refractive index.

fraction containing saccharides **1** and **2** was concentrated to 80.0 mL and frozen for storage. Part of this fraction (7.5 mL) was diluted with distilled water of 12.5 mL, and repeatedly purified with preparative-HPLC (Fig. 2). Purified saccharides **1** (14 mg) and **2** (35 mg) were finally obtained as a white powder and confirmed as homogeneous by HPAEC⁽⁹⁾⁽¹⁰⁾ with retention times of 1.88 and 3.66 min and relative retention times of 0.46 and 0.90 (the retention time of sucrose being 1.0). The degrees of polymerization of saccharides **1** and **2** were established as 2 by $[M+Na]^+$ (m/z 365) MALDI-TOF MS measurement. The complete hydrolysis of saccharides **1** and **2** was investigated using analytical-HPLC, where saccharides **1** and **2** (3.0 mg) were dissolved in 0.1 N HCl (0.2 mL) and hydrolyzed by heating at 100 °C for 30 min. As a result, glucose and fructose were detected in equal amounts.

The structural confirmations of the saccharides **1** and **2** were provided by ¹H- and ¹³C-NMR analyses (¹H at 500 MHz and ¹³C at 126 MHz), and the subsequent complete assignments of ¹H- and ¹³C-NMR signals were carried out using 2D-NMR techniques, including COSY,⁽¹¹⁾⁽¹²⁾ E-HSQC, HSQC-TOCSY,⁽¹³⁾⁽¹⁴⁾ and HMBC.⁽¹⁵⁾⁽¹⁶⁾ The 1D ¹H- and ¹³C-NMR spectra of saccharides **1** and **2** are shown in Figs. 3A and 3B. Saccharides **1** and **2** were not an anomeric mixture based on the 1D ¹H- and ¹³C-NMR spectra. The disaccharide consisted of one anomeric proton, three methylenes, and one quaternary carbon based on the E-HSQC spectra. First, the HSQC-TOCSY spectrum of saccharide **1** revealed the ¹H and ¹³C signals of Glc and Fru residues: from C-1 (δ_C 95.40 ppm, δ_H 4.71 ppm, d, 8.0 Hz) to C-6 of Glc and

from C-3 to C-6 in Fru. The COSY spectrum assigned the spin system of Glc from H-1 to H-6. The corresponding ¹³C signals were assigned from the E-HSQC spectrum. β -Glc was assigned by the J value and chemical shift. The quaternary carbon (δ_C 102.96 ppm) and the isolated methylene carbon (δ_C 62.04 ppm) were assigned as C-2 and C-1 in Fru, respectively. The HMBC correlation between C-2 and H-1 confirmed these assignments. The other methylene carbon (δ_C 62.96 ppm) was assigned as C-6 in Fru by the HSQC-TOCSY spectrum. The H-5 in Fru was assigned from H-6 in Fru by the COSY correlations. The HMBC correlations of C-3/H-1 in Fru confirmed the assignment of these signals. The other methine (δ_C 74.52 ppm) was assigned as C-4 in Fru. The β -fructopyranose was assigned from the HMBC correlations of C-Fru 2/H-Glc 6 and its chemical shift. The C-2 of Fru showed HMBC correlations to H-1 of Glc. These results indicated the Fru_p-(2 \leftrightarrow 1)-Glc linkage (Fig. 4A), and all ¹H- and ¹³C-NMR signals were assigned as shown in Table 1. The COSY spectrum of saccharide **2** was used to assign the spin system of Glc residues from the anomeric proton to H-6. The corresponding ¹³C signals were assigned from the E-HSQC spectrum. The β form of Glc was assigned from the chemical shift of C-1 (δ_C 95.38 ppm) and the J (H-1/H-2) value in Glc (8.1 Hz). The methine proton (δ_H 4.28 ppm, d, 8.7 Hz) was assigned as H-3 in Fru. The COSY spectrum assigned the spin system from H-3 to H-6 in Fru. The corresponding ¹³C signals were assigned from the E-HSQC spectrum. The isolated methylene carbon was assigned as C-1 in Fru. The corresponding C-1 in Fru was assigned from the E-HSQC. The HMBC correlations of C-2/H-1 in Fru confirmed the assignment of the signals. The inter-residual HMBC correlations between C-2 in Fru and H-1 in Glc indicated the Fru_p-(2 \leftrightarrow 1)-Glc linkage (Fig. 4B). In addition, the β form of Fru was assigned from the chemical shift. All ¹H- and ¹³C-NMR signals were assigned as shown in Table 1. Based on the results, saccharides **1** and **2** were confirmed to be new saccharides, β -D-fructopyranosyl-(2 \leftrightarrow 1)- β -D-glucopyranoside and β -D-fructofuranosyl-(2 \leftrightarrow 1)- β -D-glucopyranoside, respectively. The purity of saccharide **1** and **2** were 95 and 98 %, and the yields of the saccharides were about 0.1 and 0.3 %, respectively.

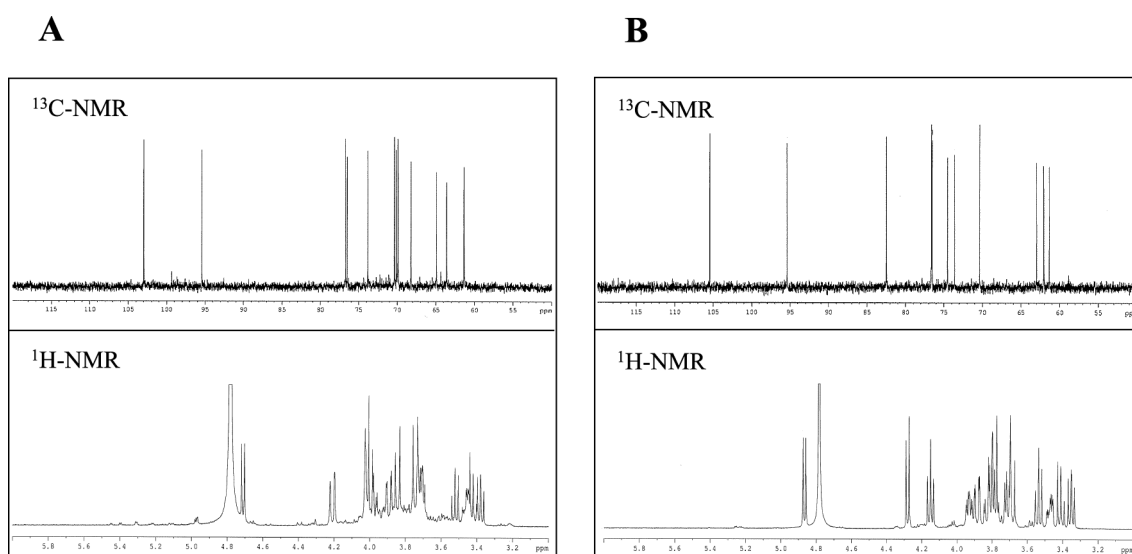
The optimal conditions for saccharide **2** synthesis were investigated. The saccharide could be synthesized by heating for 60 min at 130 to 190 °C, with a maximum yield at 160 °C. Furthermore, the saccharide was efficiently synthesized at 160 °C for 45 min as well as at 170 °C for 30 min. Stabilities during heating was investigated as follows. A 50-mM aliquot of Britton-Robinson buffer (pH 3.0, 5.0, 7.0, and 9.0) containing 5 % saccharide [sucrose, saccharide **1** or saccharide **2**] was heated in a tube at 100 °C for 15, 30, 45, and 60 min in a dry thermal bath heater. Saccharides **1** and **2** were less stable than sucrose for all pH values tested.

The digestibility of saccharides **1** and **2** by human saliva, pig pancreatic amylase, rat intestinal enzyme, and artificial gastric juice were investigated as previously described by Okada *et al.*⁽¹⁷⁾ Human saliva was examined as previously described by Miyamura.⁽¹⁸⁾ Saliva was collected from 3 in-

Table 1. ^1H - and ^{13}C -NMR spectra data (δ^a in ppm, J in Hz) of saccharides 1 and 2.

			δ_{C}	δ_{H}	$J_{\text{H,H}}$	
Saccharide 1	βGlc_p	1	95.40	4.71	d	8.0
		2	73.84	3.38	dd	9.4, 8.0
		3	76.72	3.52	dd	9.4, 8.7
		4	70.37	3.42	dd	9.8, 8.7
		5	76.51	3.46	ddd	9.8, 5.5, 2.3
		6	61.34	3.89	dd	12.5, 2.3
	βFru_p	1	63.59	3.84	d	12.4
				3.74	d	12.4
		2	102.96			
		3	68.22	4.02	d	10.7
		4	70.11	3.97	dd	10.7, 3.4
		6	64.91	4.21	dd	12.2, 1.2
			3.72	dd	12.2, 2.0	
Saccharide 2	βGlc_p	1	95.38	4.87	d	8.1
		2	73.61	3.35	dd	9.3, 8.1
		3	76.58	3.54	dd	9.3, 9.0
		4	70.35	3.41	dd	9.9, 9.0
		5	76.50	3.47	ddd	9.9, 5.6, 2.3
		6	61.33	3.89	dd	12.4, 2.3
	βFru_f	1	62.04	3.79	d	12.7
				3.68	d	12.7
		2	105.42			
		3	76.63	4.28	d	8.7
		4	74.52	4.15	dd	8.7, 8.4
		6	62.96	3.93	ddd	8.4, 6.6, 3.0
			3.83	dd	12.5, 3.0	
			3.78	dd	12.5, 6.6	

^a The chemical shifts of ^1H (δ_{H}) and ^{13}C (δ_{C}) in ppm were respectively determined relative to the external standard of sodium [2,2,3,3- $^2\text{H}_4$]-3-(trimethylsilyl) propanoate in D_2O (δ_{H} 0.00 ppm) and 1,4-dioxane (δ_{C} 67.40) in D_2O . ^b m, multiplet. * NMR data of other oligosaccharides containing D-fructofuranosyl or D-fructopyranosyl residue were referred.²¹⁾²²⁾

**Fig. 3.** 1D ^1H - and ^{13}C -NMR spectra of saccharides 1 (A) and 2 (B).

dividuals, 2 h after a meal. The oral cavity of each subject was rinsed with tap water, and then, the subject was requested to gargle with distilled water. The naturally secreted sal-

iva (2 mL) was collected and shaken well at room temperature. A 100- μL aliquot of human saliva (43 U/mL) was added to 100 μL of 50 mM Bis-Tris buffer (pH 6.0) con-

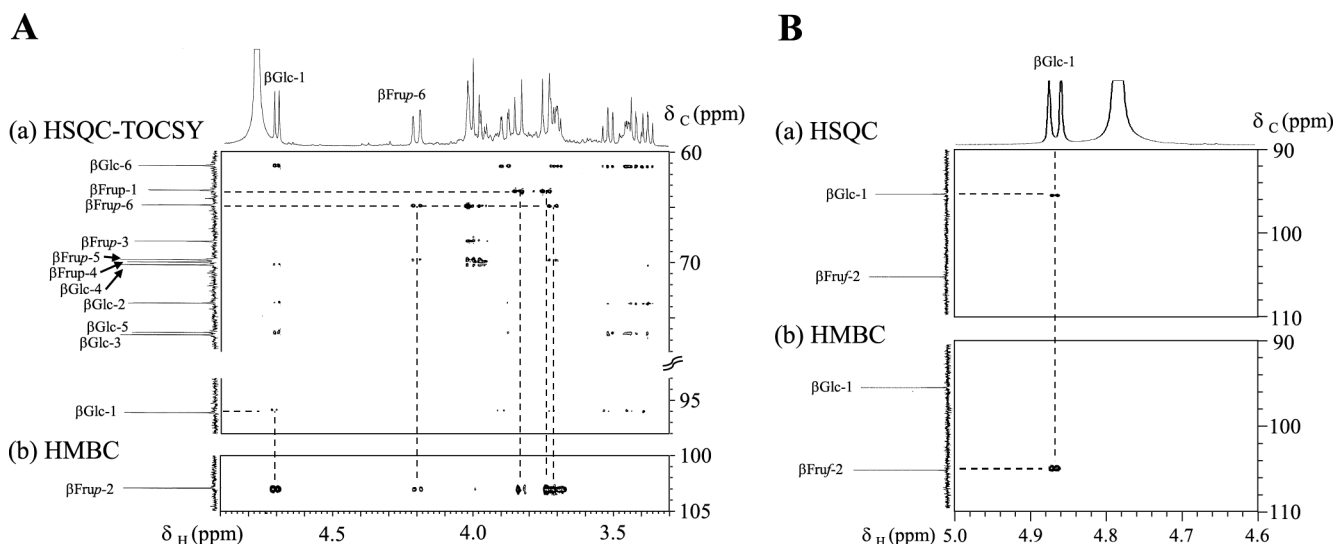


Fig. 4. Portions of the HSQC-TOCSY (Aa), HSQC (Ba), and HMBC (Ab and Bb) spectra of saccharides **1** (A) and **2** (B).

taining 1 mM calcium chloride, and 10 % saccharides **1** and **2**. Digestion was performed at 37 °C for 0, 1, 2, 3, 4, 5, and 6 h, and the reaction was terminated by heating in a dry thermal bath heater at 100 °C for 10 min. Glucose and fructose formed from saccharides **1** and **2** were assayed by HPAEC. Pig pancreatic amylase was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). A 100- μ L aliquot of pig pancreatic amylase suspension (4.0 U/mL) was added to 100 μ L of 50 mM Bis-Tris buffer (pH 6.6) containing 1 mM calcium chloride and 20 mM saccharides **1** and **2**. Digestion was performed at 37 °C for 1, 2, 3, 4, 5, and 6 h, and the enzyme reaction was terminated by heating in a dry thermal bath heater at 100 °C for 10 min. Glucose and fructose formed from saccharides **1** and **2** were assayed by HPAEC. Rat intestinal enzyme was prepared from intestinal acetone powder (Sigma Chemical Co., St. Louis, USA). A suspension of 300 mg rat intestinal acetone powder in 2.7 mL of 10 mM phosphate buffer (pH 6.8)¹⁹ was homogenized for 5 min using a glass homogenizer and then centrifuged at $12,070 \times G$ for 15 min to obtain the intestinal enzyme solution in the supernatant. A 100- μ L aliquot of rat intestinal enzyme solution (4.0 U/mL) was added to 100 μ L of 10 mM phosphate buffer (pH 6.8) containing 20 mM of saccharides **1** and **2**. Digestion was performed at 37 °C for 0, 15, 30, 60, and 120 min, and the reaction was stopped by heating in a dry thermal bath heater at 100 °C for 10 min. Glucose and fructose formed from saccharides **1** and **2** were assayed by HPAEC. Artificial gastric juice solution (pH 2.0) was prepared from 0.9 mM CaCl_2 , 50 mM hydrochloric acid, and 50 mM potassium chloride. A 50- μ L aliquot of this solution was added to 100 μ L of 20 mM saccharides **1** and **2**, and digestion was performed at 37 °C for 0, 15, 30, 60, and 120 min. Digestion was terminated by adding 50 μ L of 10 mM sodium hydroxide. Digestibility was determined as the amount of saccharides **1** and **2** in the digestive solution using analytical-HPLC.

The enzyme activities and units were defined as follows. The activities of the saliva and pig pancreatic amylase were assayed by the Somogyi-Nelson method, and 1 U of activity was defined as the amount of enzyme required to provide

reducing power equivalent to that of 1.0 μ mol glucose from 0.1 % soluble-starch per min at 37 °C and pH 6.0. The activity of intestinal enzymes was assayed by analytical-HPLC, and 1 U of activity was defined as the amount of enzyme required to liberate 2 μ mol glucose from 200 mM maltose per min at 37 °C and pH 6.8. Saccharide **1** and **2** were not hydrolyzed by human saliva (Digestion rate; saccharide **1**, 0 %, saccharide **2**, 0 %) and rat intestinal enzyme (saccharide **1**, 0 %, saccharide **2**, 0 %) but were slightly hydrolyzed by pig pancreatic amylases (saccharide **1**, 0 %, saccharide **2**, 3.0 %), or artificial gastric juice (saccharide **1**, 1.2 %, saccharide **2**, 3.5 %). These results indicate that both saccharides have low digestibility. The degree of sweetness was measured as previously described by Takenaka *et al.*²⁰ We determined the sucrose concentration corresponding to the sweetness of 10 % saccharide **2**, and 1 volunteer chose 3.0 % sucrose, 3 chose 4.0 %, and 6 chose 5.0 %. Therefore, the sweetness of saccharide **2** was approximately 0.45 times that of sucrose.

In this study, sucrose isomers were produced D-glucose and D-fructose by thermal treatment. Both saccharides showed low digestibility. These saccharides could be useful as a novel material for manufacture of foods and chemicals.

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