

Note

Structural Analysis of a Novel Oligosaccharide Isolated from Fermented Beverage of Plant Extracts

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Abstract: A fermented beverage of plant extracts (Super Ohtaka[®]) was prepared from about 50 kinds of fruits and vegetables. This natural fermentation was performed by yeast (*Zygosaccharomyces* spp. and *Pichia* spp.) and lactic acid bacteria (*Leuconostoc* spp.) and resulted in the production of a novel fructopyranose-containing saccharide, which was subsequently isolated using carbon-Celite column chromatography and preparative-HPLC. The structure of the saccharide was determined using MALDI-TOF MS and NMR, and the saccharide was identified as β -D-fructopyranosyl-(2 \rightarrow 6)- β -D-fructofuranosyl-(2 \leftrightarrow 1)- α -D-glucopyranoside. This is the first description of this novel saccharide and its isolation from a natural source.

Key words: pyrano-6-kestose, fructopyranosyl sucrose, fructopyranose, trisaccharide, fermented beverage, structural analysis

Super Ohtaka[®] (fermented beverage of plant extracts¹⁾) displayed scavenging activity against the radical 1,1'-diphenyl-2-picrylhydrazyl and significantly reduced ethanol-induced damage of the gastric mucosa in rats.¹⁾ This beverage contained primarily glucose and fructose, but also various oligosaccharides as well. We have previously analyzed the structures of some of these oligosaccharides, including β -D-fructopyranosyl-(2 \rightarrow 6)-D-glucopyranose (β -Fp2 \rightarrow 6G),²⁾ α -D-fructofuranosyl-(2 \rightarrow 6)-D-glucopyranose (α -Ff2 \rightarrow 6G),²⁾ β -D-fructopyranosyl-(2 \rightarrow 6)- β -D-glucopyranosyl-(1 \rightarrow 3)-D-glucopyranose,³⁾ β -D-fructopyranosyl-(2 \rightarrow 6)-D-fructofuranose,⁴⁾ and β -D-fructopyranosyl-(2 \rightarrow 1)- β -D-fructofuranosyl-(2 \leftrightarrow 1)- α -D-glucopyranoside.⁴⁾ Two of the above saccharides, β -Fp2 \rightarrow 6G and α -Ff2 \rightarrow 6G, can be synthesized from D-glucose and D-fructose using a thermal melting treatment. Several characteristics of these saccharides have been reported.^{5,6)} In this paper, the structure of a novel saccharide, β -D-fructopyranosyl-(2 \rightarrow 6)- β -D-fructofuranosyl-(2 \leftrightarrow 1)- α -D-glucopyranoside, isolated from the fermented beverage was determined using MALDI-TOF MS and NMR.

Super Ohtaka[®] (1 kg, Brix: 59 %) was passed through a carbon-Celite column (4.5 \times 35 cm) and successively eluted in water (14 L) and 5 % ethanol (30 L). Almost all of glucose and fructose were eluted with water (0–4 L), while the fraction containing the target saccharide named saccharide **1** was eluted in 5 % ethanol fraction (1–3 L). Then, the fraction containing saccharide **1** was concentrated and purified using HPLC systems with an Amide-80 column (Fig. 1) and with an ODS-100V column. Purified saccharide **1** (1.8 mg) was obtained as a white powder. It was determined to be homogeneous based on HPAEC,^{7,8,9)} where the retention time was 5.51 min and the relative retention time was 1.10 (the retention time of sucrose is 1.0) (chromatogram not shown). The retention time of saccharide **1** did not correspond with any saccharides examined ($t_{R\text{sucrose}}$; maltose, 1.50; sophorose, 1.26; gentiobiose, 1.59; laminaribiose, 1.33; maltotriose, 2.59; raffinose, 1.23; panose, 1.89; gentiotriose, 1.90; 1-kestose, 1.52; nystose, 2.28). The degree of polymerization (DP) of saccharide **1** was 3 based on measurements of $[M+Na]^+$ ions (m/z : 527) using MALDI-TOF MS. The complete hydrolysis of saccharide **1** was investigated using HPAEC, where saccharide **1** (1.0 mg) was dissolved in 0.1 N HCl (0.2 mL) and hydrolyzed by heating at 100 °C for 30 min. As result, glucose and fructose were detected in molar ratio (0.5:1.0).

NMR analysis for saccharide **1** was performed as follows. ¹H- and ¹³C-NMR signals of saccharide **1** were all assigned using 2D-NMR techniques, including COSY,^{10,11)} E-HSQC,^{12,13)} HSQC-TOCSY,^{12,14)} HMBC,^{15,16)} and HR-

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Abbreviations: MALDI-TOF MS, matrix-assisted laser desorption ionization time of flight mass spectrometry; HPAEC, high performance anion-exchange chromatography; COSY, correlation spectroscopy; E-HSQC, editing heteronuclear single quantum coherence; HSQC-TOCSY, HSQC-total correlation spectroscopy; HMBC, heteronuclear multiple-bond correlation; HR-HMBC, high resolution-HMBC.

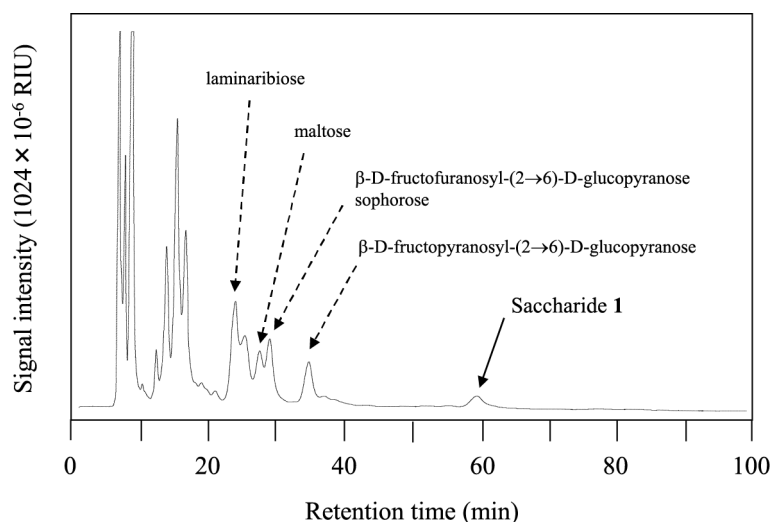


Fig. 1. HPLC of 5 % ethanol fraction separated from fermented beverage of plant extracts by carbon-Celite column chromatography. Fermented beverage of plant extracts was loaded onto a carbon-Celite column and successively eluted with 5 % Ethanol. The following operating conditions for HPLC were used: column, Amide-80 (7.8 × 300 mm; Tosoh Corporation); column temperature, 80 °C; eluent, 80 % acetonitrile; flow rate, 2.0 mL/min; detection, by refractive index.

HMBC.¹⁷ The HSQC-TOCSY spectrum of saccharide **1** revealed ¹H and ¹³C signals of C-1 (93.05 ppm) to C-6 belonging to Glc and signals of C-3 to C-6 belonging to Fru residues. The COSY spectrum assigned the spin system of Glc from H-1 to H-6, Fru_f from H-3 to H-6, and Fru_p from H-6 to H-5. The corresponding ¹³C signals were assigned from the E-HSQC spectrum. The E-HSQC spectrum gives CH₂ signals in opposite phase to CH and CH₃ signals, like DEPT 135. This spectrum of **1** discriminated methylene carbons and protons. The HMBC correlations for C-Fru_f-3/H-Fru_f-1 and C-Fru_f-2/H-Fru_f-1 facilitated assignment of H-Fru_f-1 and C-Fru_f-2. β-Fructofuranose was assigned based on chemical shifts of C-2 and large value of *J*(H-3/H-4) and *J*(H-4/H-5).¹⁸ The HMBC correlations for C-Fru_p-2/H-Fru_p-1, C-Fru_p-2/H-Fru_p-6, and C-Fru_p-3/H-Fru_p-1 confirmed signal assignment. β-Fructopyranose was assigned based on the HMBC correlations of C-Fru_p-2/H-Fru_p-6 and chemical shifts of C-3, 4, and 5.¹⁹ C-Fru_f-2 (δ_C 104.66 ppm) had an inter-residual HMBC correlation for H-Glc-1 (δ_H 5.39 ppm). The E-HSQC spectrum, shown in Fig. 2A, revealed H-Fru_p-1 and H-Fru_f-6 had similar chemical shifts. Both the intra-residual HMBC correlation peak of C-Fru_p-2/H-Fru_p-1 and the inter-residual correlation peak HMBC of C-Fru_p-2/H-Fru_f-6 should appear in the same position in the 2D spectrum, presented in Fig. 2B. Discrimination of these two correlations was performed based on the characteristic multiplet pattern of correlation peaks in HR-HMBC spectrum. In the HR-HMBC spectrum, splitting of the cross-peaks due to the long range *J*_{CH} and *J*_{HH} were scaled by scaling factor *N* and *N*+1 along the *F*₁ dimension, respectively.¹⁷ A typical multiplet pattern was shown at the C-Glc-4/H-Glc-3 in the HR-HMBC spectrum of saccharide **1** (Fig. 3). This correlation peak consisted of 6 signals; two sets of three signals aligned in parallel downward-sloping lines. The same pattern was shown at C-Glc-2/H-Glc-3. The distance of three signals were corresponding to the proton peak pattern (triplet-like *dd*). For the two correlation peaks between C-Fru_f-3 and methylene pro-

tons of Fru_f-1, each signal intensity was weighted by the strong-coupling of methylene protons. Further artificial signals were induced by strong coupling.²⁰ The correlation peak of C-Fru_p-3/H-Fru_p-1 in HR-HMBC spectrum showed as a doublet. That is because two protons of H-Fru_p-1 had the same chemical shifts indicated in E-HSQC (Fig. 2A), the correlation peak did not split by *J*_{HH}, therefore, the peak was split only by *NJ*_{CH}. The same doublet pattern was found at C-Fru_p-2/H-Fru_p-1. An additional multiplet pattern can be observed at the same position. This multiplet was considered H-Fru_f-6 because methylene protons Fru_f-6 were the strongly coupled each other (Fig. 2A), they gave the center-weighted multiplet pattern. All ¹H- and ¹³C-NMR signals were assigned as shown in Table 1. Based on these results, saccharide **1** was confirmed to be the novel saccharide β-D-fructopyranosyl-(2→6)-β-D-fructofuranosyl-(2↔1)-α-D-glucopyranoside. This saccharide, which is derived from the substitution of the 6^F-β-D-fructopyranosyl residue for the 6^F-β-D-fructofuranosyl residue in 6-kestose was named pyrano-6-kestose. The purity and yield of saccharide **1** were 98 and 0.18 %, respectively.

In this study, the structure of the novel saccharide β-D-fructopyranosyl-(2→6)-β-D-fructofuranosyl-(2↔1)-α-D-glucopyranoside was clarified. In addition, this saccharide was confirmed to be produced during fermentation.

EXPERIMENTAL

Preparation of fermented beverage of plant extracts (Super Ohtaka®). For preparation of “fermented beverage of plant extracts” (Super Ohtaka® ; Ohtakakohso Co., Ltd., Otaru, Japan), 50 kinds of fruits and vegetables were used to produce the final extract as shown in previous paper.¹ The 50 fruits and vegetable were cut, sliced or diced into small pieces, mixed and put in cedar barrels. Afterwards, equivalent weight of sucrose was added to samples, mixed well to allow high contact samples-sucrose, and then barrels were left during one week at room temperature. The

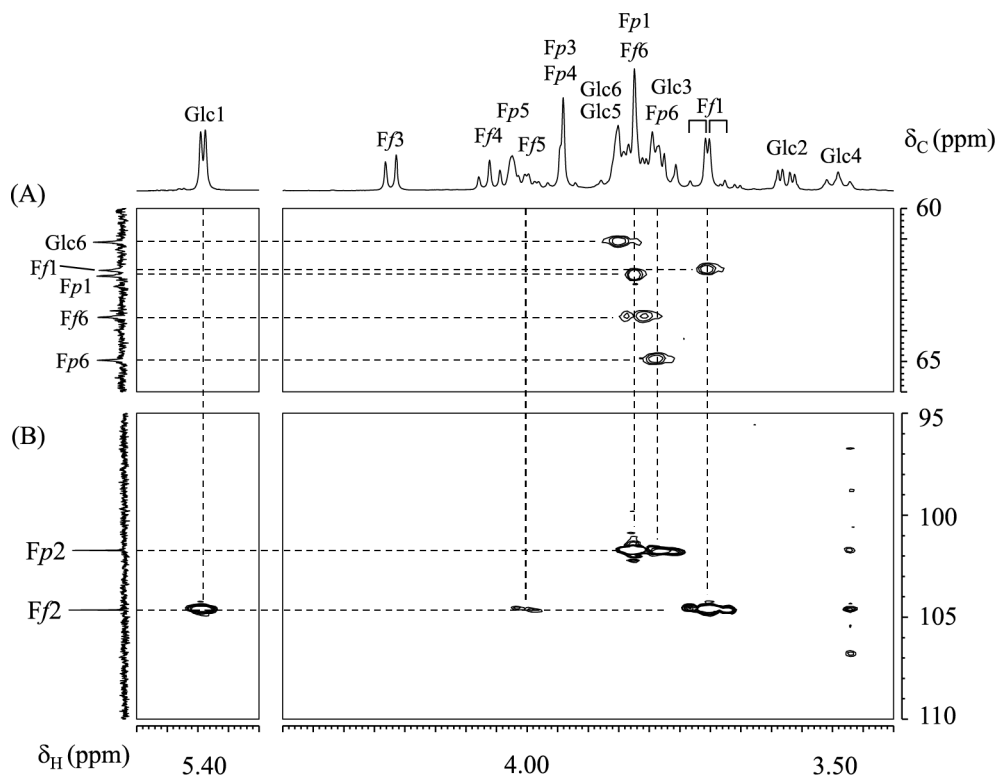


Fig. 2. Portions of E-HSQC (A) and HMBC (B) spectra of saccharide 1.

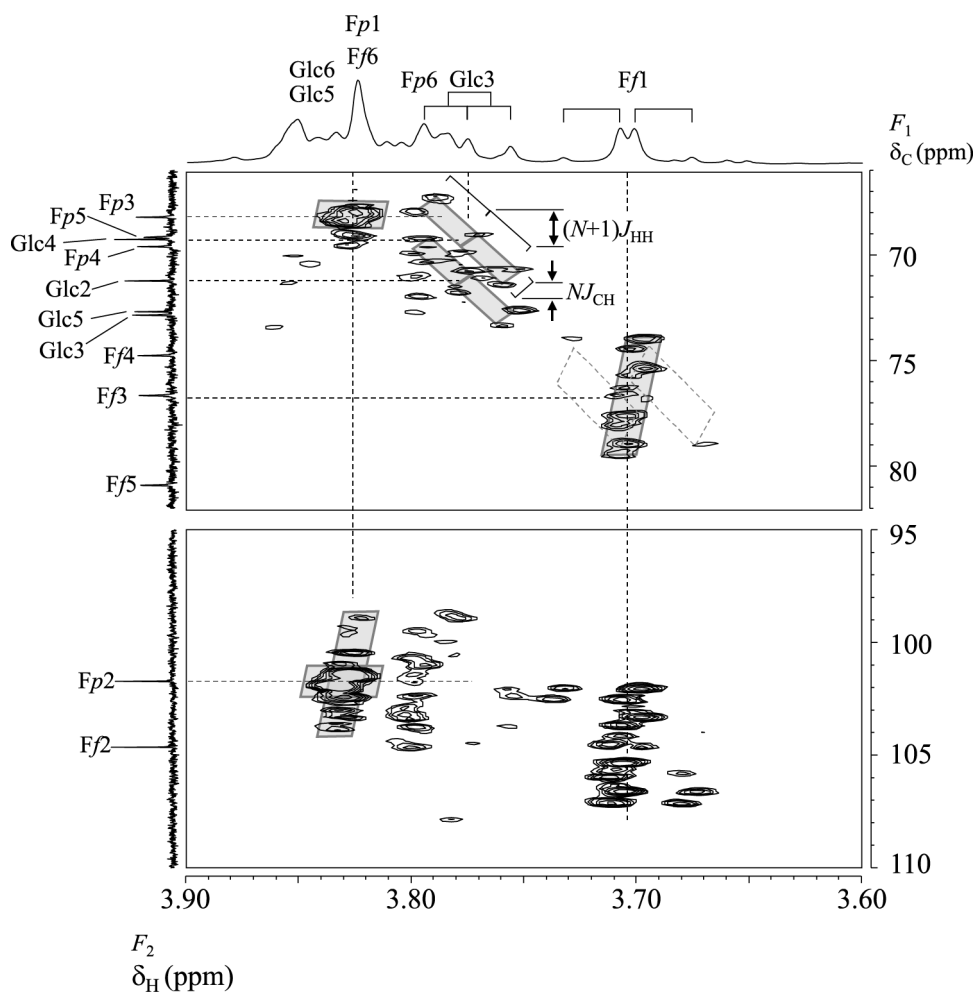


Fig. 3. Portions of HR-HMBC spectrum of saccharide 1.

Table 1. ^1H and ^{13}C NMR spectral data (δ^a in ppm, J in Hz) of saccharide **1**.

		δ_{C}	δ_{H}		$J_{\text{H,H}}$
Fru <i>f</i>	1	62.04	3.74	d	13.0
			3.69	d	13.0
	2	104.66			
	3	77.00	4.22	d	8.7
	4	75.23	4.06	dd	8.7, 8.5
	5	80.98	4.00	ddd	8.5, 8.0, 2.9
Fru <i>p</i>	1	62.22	3.82	s	
	2	101.74			
	3	69.09	3.94	m	
	4	70.39	3.94	m	
	5	69.99	4.02	br s ^c	
	6	64.97	3.79	m	
Glc	1	93.05	5.39	d	3.9
	2	71.91	3.58	dd	10.3, 3.9
	3	73.43	3.78	dd	10.3, 8.7
	4	70.08	3.49	dd	9.5, 8.7
	5	73.28	3.84	m	
	6	61.12	3.85	m	

^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. ^c br s, broad singlet. * NMR data of other oligosaccharides containing D-fructofuranosyl or D-fructopyranosyl residue were referred.²¹⁾²²⁾²³⁾

juice exudates was then separated without compression from solids and used for fermentation. The fermentation beverage was obtained by incubation of the extract at 37 °C in dark by natural fermentation using yeast (*Zygosaccharomyces* spp. and *Pichia* spp.) and lactic acid bacteria (*Leuconostoc* spp.). After 7 days, the fermented beverage was kept additional maturation and ageing, obtaining finally a brown and slightly sticky liquid.

Isolation of saccharide. Super Ohtaka[®] (1 kg, Brix: 59 %) was loaded onto a 4.5 × 35 cm carbon-Celite column consisting of 1:1 charcoal/Celite-535 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and successively eluted in water (14 L) and 5 % ethanol (30 L). Almost all of glucose and fructose were eluted with water (0–4 L), while the fraction containing the target saccharide named saccharide **1** was eluted in 5 % ethanol fraction (1–3 L). Then, the fraction (2 L) containing the saccharide was concentrated to 10 mL. Subsequently, the concentrated fraction was repeatedly applied to preparative-HPLC (Tosoh Corporation, Tokyo, Japan) equipped with an Amide-80 column (7.8 mm × 30 cm, Tosoh Corporation) at 80 °C and eluted with 80 % acetonitrile at 2 mL/min using refractive index detection (Fig. 1). Furthermore, saccharide **1** was purified at room temperature using an HPLC system (Tosoh Corporation) equipped with an ODS-100V column (4.6 mm × 25 cm, Tosoh Corporation) and eluted with distilled water at 0.5 mL/min using refractive index detection. The purified saccharide **1** solution was freeze-dried to give a white powder (1.8 mg).

High performance anion-exchange chromatography (HPAEC). The saccharide was analyzed using Dionex Bio

LC Series (Dionex Corp., Sunnyvale, USA) apparatus equipped with an HPLC carbohydrate column (Carbo Pack PAI, inert styrenedivinylbenzene polymer) and pulsed amperometric detection (PAD).⁷⁾⁸⁾ The mobile phase⁹⁾ consisted of eluent A (150 mM NaOH) with eluent B (500 mM sodium acetate in 150 mM NaOH) with a sodium acetate gradient as follows: 0–1 min, 25 mM; 1–2 min, 25–50 mM; 2–20 min, 50–200 mM; 20–22 min, 500 mM; 22–30 min, 25 mM at a flow rate of 1 mL/min. The applied PAD potentials for E1 (500 ms), E2 (100 ms), and E3 (50 ms) were 0.1, 0.6 and –0.60 V, respectively, and the output range was 1 μC .

Matrix assisted laser desorption/ionization/time of flight mass spectrometry (MALDI-TOF MS). MALDI-TOF MS spectra were measured using a Shimadzu-Kratos mass spectrometer (KOMPACT Prove; Shimadzu Corp., Kyoto, Japan) in positive ion mode with 10 % 2,5-dihydroxybenzoic acid as the matrix. Ions were formed by a pulsed UV laser beam (nitrogen laser, 337 nm). Calibration was conducted using 1-kestose as the external standard.

Nuclear magnetic resonance (NMR) measurements. Saccharide (1.8 mg) was dissolved in 60 μL D_2O . NMR spectra were recorded at 27 °C with a Bruker AMX-500 spectrometer (^1H 500 MHz, ^{13}C 126 MHz; Bruker BioSpin GmbH, Karlsruhe, Germany) equipped with a 2.5-mm diameter C/H dual probe (1D spectra) and a TXI triple probe (2D spectra). Chemical shifts in ppm for ^1H (δ_{H}) and ^{13}C (δ_{C}) spectra were determined relative to an 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 ppm) in D_2O , respectively. COSY,¹⁰⁾¹¹⁾ E-HSQC,¹²⁾ HSQC–TOCSY,¹³⁾¹⁴⁾ HMBC,¹⁵⁾¹⁶⁾ and HR-HMBC¹⁷⁾ spectra were obtained using gradient-selected pulse sequences. The TOCSY mixing time (0.17 s) was determined using the decoupling in the presence of scalar interactions (DIPSI)-2 methods.

Full NMR spectra are provided in Supplementary material.

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