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Note

Structural analysis of a novel saccharide isolated from fermented beverage of plant extract

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Abstract—Fermented beverage of plant extract was prepared from about 50 kinds of vegetables and fruits. Natural fermentation was carried out mainly by lactic acid bacteria (*Leuconostoc* spp.) and yeast (*Zygosaccharomyces* spp. and *Pichia* spp.). Three kinds of saccharides have been found in this beverage and produced by fermentation. The saccharides isolated from the beverage using carbon-Celite column chromatography and preparative HPLC, were identified as a new saccharide, β-D-fructopyranosyl-(2→6)-D-glucopyranose, laminaribiose and maltose by examination of constituted sugars, GLC and GC–MS analyses of methyl derivatives and MALDI-TOF-MS and NMR measurements of the saccharides.
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The extract from 50 kinds of fruits and vegetables was fermented to produce a new beverage. The juices were extracted using sucrose-osmotic pressure in a cedar barrel for one week and was fermented by lactic acid bacteria (*Leuconostoc* spp.) and yeast (*Zygosaccharomyces* spp. and *Pichia* spp.). The fermented beverage showed scavenging activity against 1,1′-diphenyl-2-picrylhydrazyl (DPPH) radical, and reduced significantly the ethanol-induced damage of gastric mucosa in rat.[1] Previously, we showed that this beverage contained high levels of saccharides, estimated between 550 and 590 g L^{-1}, mainly glucose and fructose, and a small amount of disaccharides. In this paper, we confirmed structures of the saccharides, laminaribiose, maltose and a novel saccharide; β-D-fructopyranosyl-(2→6)-D-glucopyranose (Fig. 1) isolated from the fermented beverage using GC–MS, MALDI-TOF-MS and NMR measurements.

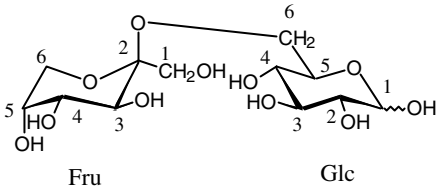

Figure 1. Structure of β-D-fructopyranosyl-(2→6)-D-glucopyranose.

Synthesis of saccharides by the fermentation of plant extract was investigated using HPLC with *p*-aminobenzoic acid ethyl ester (ABEE)-conversion method.[2,3] The plant extract was fermented at 37 °C for 180 days in a dark place. As shown in Figure 2, the saccharides **1**, **2** and **3** were produced during fermentation. These saccharides **1**, **2** and **3** were isolated by carbon-Celite column chromatography and preparative HPLC, and were shown to be homogeneous using anion exchange HPLC ($t_{R,sucrose}$ (relative retention time; retention time

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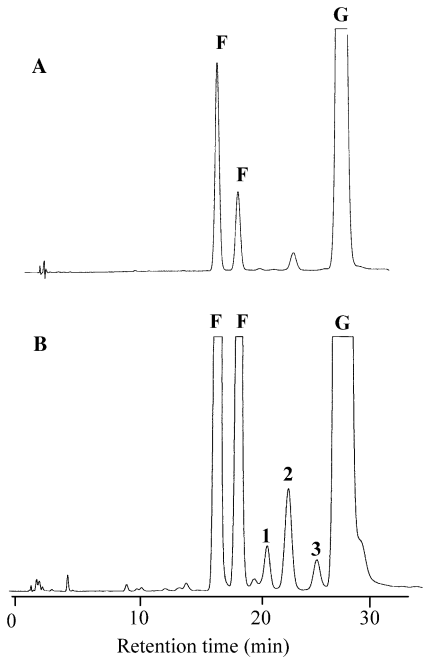

Figure 2. High performance liquid chromatogram of fermentation products. HPLC analysis of saccharides produced during fermentation was done by ABEE conversion method. A: plants extract was fermented for 0 days. B: plants extract was fermented for 180 days. G: glucose; F: fructose.

of sucrose = 1.0): 1.33, 0.83 and 1.43). The retention time of saccharide **2** did not correspond to that of any authentic saccharides, although $t_{R,sucrose}$ of saccharides **1** and **3** corresponded to those of laminaribiose and maltose, respectively. The degree of polymerization of saccharide **2** was established as 2 by measurements of $[M+Na]^+$ ions (*m*/*z*: 365) using TOF-MS, and analysis of the molar ratios (1.0) of D-glucose to D-fructose in the acid hydrolysate, although yeast β-fructofuranosidase could not hydrolyze saccharide **2**.

From the GC analysis, relative retention times of the methanolysate of the permethylated saccharide were investigated [t_R (relative retention time; retention time of methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucoside = 1.0; retention time, 8.70 min)]. The methanolysate of permethylated saccharide **2** exhibited four peaks (Table 1) and two of them corresponded to methyl 2,3,4-tri-*O*-methyl-D-glucoside (t_R, 2.58 and 3.56). From the finding, saccharide **2** was presumed as fructosyl (2→6) glucose. However, other two peaks (t_R, 1.06 and 1.48) did not correspond to methyl 1,3,4,6-tetra-*O*-methyl-D-fructoside (t_R, 1.07 and 1.28) from permethylated raffinose. Because the two peaks were estimated to correspond to methyl 1,3,4,5-tetra-*O*-methyl-D-fructopyranoside, the methyl fructopyranoside was tried to prepare from D-fructose. Fructose, dissolved in water and freeze-dried, was permethylated. The permethylated fructose and methanolysate of permethylated saccharide **2** were analyzed by GC–MS. The permethylated fructose gave four peaks that were confirmed to be methyl 1,3,4,6-tetra-*O*-methyl-β- and α-D-fructofuranoside (retention time, 8.34 and 8.56 min) and methyl 1,3,4,5-tetra-*O*-methyl-β- and α-D-fructopyranoside (9.14 and 10.57 min), respectively, from the retention time and pattern of fragmentation.[4] The methanolysate from permethylated saccharide **2** exhibited two peaks (9.14 and 10.63 min) corresponding to methyl 1,3,4,5-tetra-*O*-methyl-D-fructopyranoside. No peaks corresponding to methyl 1,3,4,5-tetra-*O*-methyl-D-fructopyranoside were detected from the methanolysate of permethylated raffinose.

From these findings as above, saccharide **2** was proved to be D-fructopyranosyl (2→6)-D-glucose. Saccharides **1** and **3** were confirmed to be laminaribiose and maltose, respectively, in the same manners as above, and the ^1H and ^{13}C NMR spectra agreed with those of standard samples.

The NMR spectra of **2** showed that it was anomer mixture of fructosylglucose. The β anomer was predominant. Some signals of α anomer were separated and could be assigned. The HSQC–TOCSY[5] spectrum revealed the ^1H and ^{13}C signals of each β-Glc, α-Glc and Fru residues. The COSY[6,7] spectrum assigned the spin systems of these residues; from H-1 to H-6 in β-Glc, from H-1 to H-5 of α-Glc and from H-4 to H-6 in Fru. The corresponding ^{13}C signals were assigned by HSQC[8] spectrum. The assignment of resting signals, the position of glycosidic linkage, and pyranoside form of Fru were analyzed as follows. There was one methylene carbon not assigned yet, which was estimated as C-1 of Fru (Fig. 3a). Its protons showed HMBC[9,10] correlations to methine carbon at δ_C 69.29 and the only one quaternary carbon (δ_C 101.52) (Fig. 3b), which was assigned as C-3 and C-2 of Fru, respectively. The C-2 of Fru also correlated to H-6 of Glc and H-6 of Fru (Fig. 3b). These results revealed **2** had fructopyranoside residue and Fru*p*(2→6)Glc linkage, and all ^1H and ^{13}C NMR signals were assigned as shown in Table 2. The coupling patterns of overlapped ^1H were analyzed by

Table 1. Gas-liquid chromatographic analysis of methanolysates of permethylated saccharide **2**

Methanolysate origin	Relative retention time[a]						
Saccharide **2**	1.06			1.48		2.58	3.56
Fructose	0.99	1.12	1.36	1.49			
Raffinose		1.07	1.28		1.73	2.50	3.55
Methyl-2,3,4,6,-tetra-*O*-methyl-β-D-glucoside		1.00					

[a] Retention time of methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucoside = 1.0.

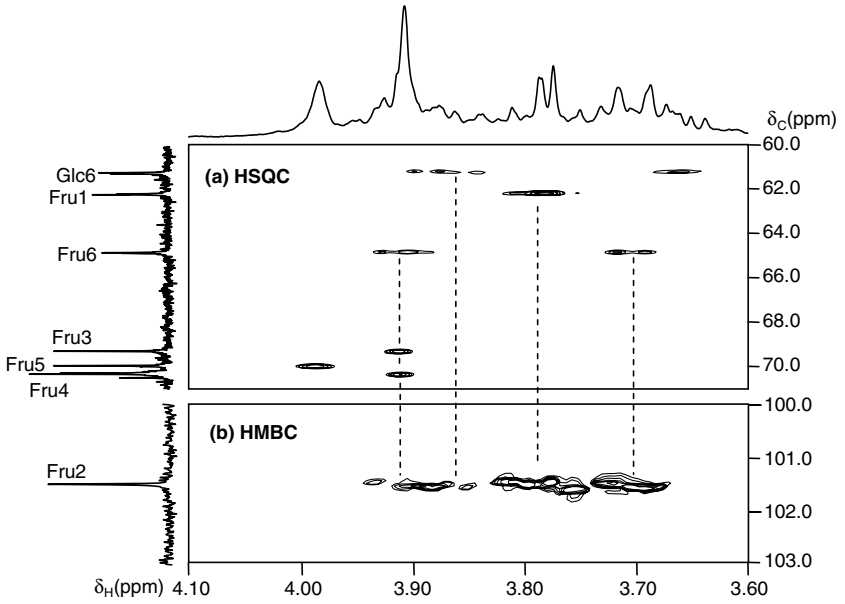

Figure 3. Part of HSQC (a) and HMBC (b) spectra of saccharide **2**.

Table 2. ^{1}H and ^{13}C NMR spectral data (δ^{a} in ppm, J in Hz) of saccharide **2**

		δ_C	δ_H	J_{HH}
Fru	1	62.19	3.76 d	11.7
			3.79 d	11.7
	2	101.52		
	3	69.29	3.91 m	
	4	70.34	3.91 m	
	5	70.00	3.89 br s	
	6	64.83	3.91 m	
			3.70 m	
β-Glc	1	96.72	4.61 d	7.7
	2	74.94	3.22 dd	9.4, 7.7
	3	76.31	3.45 m	
	4	70.34	3.45 m	
	5	75.88	3.56 dd	9.3, 5.7
	6	61.20	3.65 dd	10.8, 5.7
			3.88 d	10.8
α-Glc	1	92.87	5.21 d	3.7
	2	72.24	3.52 dd	9.3, 3.7
	3	73.39	3.68 dd	9.8, 9.3
	4	70.51	3.39 dd	9.8, 9.3
	5	71.62	3.92 m	
	6	61.24	3.67 m	
			3.85 m	

[a] Chemical shifts of ^{1}H (δ_H) and ^{13}C (δ_C) in parts per million were determined relatively to the external standard of sodium [2,2,3,3-$^{2}H_4$]-3-(trimethylsilyl) propanoate in D_2O (δ_H 0.00 ppm) and 1, 4-dioxane (δ_C 67.40 ppm) in D_2O, respectively.

SPT method. Due to strong coupling between H-3 and H-4 in β-Glc, H-3 and H-4 in Fru, H-5 and H-6 in Fru and H-5 and H-6 in α-Glc, these couplings could not be analyzed in first order. The δ_C values of Fru indicated its β anomer form, by comparing with those of α and β form of methyl-D-fructopyranoside.[11]

From these findings, fructose residue of non-reducing terminal of this saccharide was in pyranose form, and saccharide **2** found out from fermented beverage of plant extracts was confirmed to be a new saccharide, β-D-fructopyranosyl-(2→6)-D-glucopyranose (Fig. 1). Furthermore, this novel saccharide was confirmed to be produced by fermentation.

No saccharides containing fructopyranoside residues were found in natural resources excepting the saccharide in the fermented beverage of plant extract.

1. Experimental

1.1. Preparation of fermented beverage of plant extract

For preparation of the initial juice, 50 kinds of fruits and vegetables were used to produce the final extract as shown in a previous paper.[1] The 50 fruits and vegetables 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, then barrels were left during one week at room temperature. The juice exudates was then separated without compression from solids and used for fermentation. The fermented beverage was obtained by incubation of the juice 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 in a closed enameled tank at 37 °C for six months for additional maturation and ageing, obtaining finally a brown and slightly sticky liquid.

1.2. *p*-Aminobenzoic acid ethyl ester (ABEE) conversion method

Conversion of the saccharides at the reducing end with ABEE was carried out according to the method of Yasuno et al.[2,3] Ten microlitres of standard saccharide solution was added to an ABEE reagent solution (40 μL). The mixture was incubated at 80 °C for 1 h. Distilled water (0.2 mL) and chloroform (0.2 mL) were added and the mixture was centrifuged at 10,000 rpm for 1 min, aqueous layer was diluted 100-fold with water and subjected to HPLC analysis. ABEE-converted saccharide was detected by UV at 305 nm.

1.3. High performance anion-exchange chromatography (HPAEC)

The oligosaccharides were analyzed using a Dionex Bio LC Series apparatus equipped with an HPLC carbohydrate column (Carbo Pack PA1, inert styrene divinyl benzene polymer) and a pulsed amperometric detection (PAD)[12,13] in the same way as described in a previous paper.[14,15]

1.4. Isolation of saccharides

Fermented beverage of plant extract (100 g) was loaded onto a carbon-Celite [1:1; charcoal (Wako Pure Chemical Industries, Ltd, Osaka, Japan) and Celite-535 (Nakarai Chemical Industries, Ltd, Osaka, Japan)] column (4.5 × 35 cm) and successively eluted with water (14 L) and 5% ethanol (30 L). Almost all of glucose and fructose were eluted with water (4 L) and then saccharide **2** was eluted with water (5–6 L). Saccharide **2** fraction was concentrated in vacuo and freeze-dried to give 428 mg.

Saccharide **1** accompanied with saccharide **3** were eluted with 5% ethanol (1–2 L). Saccharides **1** and **3** fractions were also concentrated in vacuo and freeze-dried to give 199 mg. Subsequently, saccharide **2** fraction, and saccharides **1** and **3** fractions were successfully purified repeatedly using a HPLC system (Tosoh, Tokyo, Japan) equipped with an Amide-80 column (4.6 mm × 25 cm, Tosoh, Tokyo, Japan) at 80 °C, and eluted with 80% acetonitrile at 1.0 mL/min, and using refractive index detection. Purified saccharides **1** (139 mg), **2** (98 mg) and **3** (21 mg) were obtained as white powders.

1.5. Methylation and methanolysis

Methylation of the oligosaccharides was carried out by the method of Hakomori.[16]

The permethylated saccharides were methanolyzed by heating with 1.5% methanolic hydrochloric acid at 96 °C for 10 or 180 min. The reaction mixture was treated with Amberlite IRA-410 (OH^-) to remove hydrochloric acid, and evaporated in vacuo to dryness. The resulting methanolysate was dissolved in a small volume of methanol and analyzed using gas chromatography.

1.6. Gas liquid chromatography (GC)

For the analysis of the methanolysate, GC was carried out using a Shimadzu GC8A gas chromatograph equipped with a glass column (2.6 mm × 2 m) packed with 15% butane 1,4-diol succinate polyester on acid-washed Celite at 175 °C. Flow rate of the nitrogen gas carrier was 40 mL/min.

1.7. GC–MS analysis

GC–MS analysis was performed using JMS-AX500 mass spectrometer (JEOL, Japan) using a DB-17HT capillary column (30 m × 0.25 mm I.D., J&W Scientific, USA). Injection temperature was 200 °C. The column temperature was kept at 50 °C for 2 min after sample injection, increased to 150 °C at 50 °C/min, kept 150 °C for 1 min, then increased to 250 °C at 4 °C/min. The mass spectra were recorded in the positive ion electron ionization (EI) mode.

1.8. 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 Probe).

1.9. NMR measurements

The saccharide ca. 6 mg was dissolved in 0.5 mL D_2O. NMR spectra were recorded at 27 °C with a Bruker AMX 500 spectrometer (^1H 500 MHz, ^{13}C 125 MHz) equipped with a 5 mm diameter C/H dual (1D spectra) and TXI prove (2D spectra). Chemical shifts of ^1H (δ_H) and ^{13}C (δ_C) in parts per million were determined relatively to the external standard of sodium [2,2,3,3-$^2H_4$]-3-(trimethylsilyl) propanoate in D_2O (δ_H 0.00 ppm) and 1,4-dioxane (δ_C 67.40 ppm) in D_2O, respectively. ^1H–^1H COSY,[6,7] HSQC[8] and HMBC[9,10] spectra were obtained using gradient selected pulse sequences. The phase sensitive HSQC–TOCSY spectra were determined with the sequence including inversion of direct resonance (IDR).[5] The TOCSY mixing time (108 ms) was composed of MLEV-17 composite pulses guarded by trim pulse (2.5 ms).

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