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Note

Characteristics of *O*-β-D-Fructopyranosyl-(2→6)-D-glucopyranose Isolated from Fermented Beverage of Plant Extract

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Abstract: Fermented beverage of plant extract was prepared from 55 kinds of fruits and vegetables. Natural fermentation was conducted by lactic acid bacteria (*Leuconostoc* spp.) and yeast (*Zygosaccharomyces* spp. and *Pichia* spp.). We have previously found that the fermented beverage contained the novel saccharide, *O*-β-D-fructopyranosyl-(2→6)-D-glucopyranose, which is produced by the fermentation process. The characteristics of *O*-β-D-fructopyranosyl-(2→6)-D-glucopyranose were investigated. The saccharide showed 0.2 times the sweetness of sucrose, non-cariogenicity and low digestibility. Furthermore, the unfavorable bacteria, *Clostridium perfringens*, *Escherichia coli* and *Enterococcus faecalis* that produce mutagenic substances did not use the saccharide. Therefore, it was thought that saccharide 1 could be a new material for foods.

Key words: *O*-β-D-fructopyranosyl-(2→6)-D-glucopyranose, fermented beverage of plant extract, *Bifidobacterium*

The extract from fifty kinds of fruits and vegetables was fermented to produce a new beverage. 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 rats.[1)]

We have previously examined the production of novel saccharides in a fermented beverage of plant extract, such as *O*-β-D-fructopyranosyl-(2→6)-D-glucopyranose,[2)] β-D-fructopyranosyl-(2→6)-β-D-glucopyranosyl-(1→3)-D-glucopyranose and β-D-fructopyranosyl-(2→6)-[β-D-glucopyranosyl-(1→3)]-D-glucopyranose.[3)] Furthermore, these saccharides have been confirmed to be produced by fermentation.

Palatinose is known as a disaccharide composed of glucose and fructose as well as *O*-β-D-fructopyranosyl-(2→6)-D-glucopyranose. Palatinose is about half as sweet as sucrose, and is hydrolyzed by isomaltase.[4)] In the present work, we investigated some characteristics of *O*-β-D-fructopyranosyl-(2→6)-D-glucopyranose (saccharide **1**) from a fermented beverage of plant extract to compare with those of palatinose.

Preparation of the beverage was described previously.[1)] In brief, plant juice extracted by sucrose was incubated for 7 days at 37°C in darkness by using yeast and lactic acid bacteria. Then, the fermented extract was kept at 37 °C during 6 to 10 months for additional maturation and aging.

The time course of the formation of saccharide **1** by fermentation was measured (Fig. 1). An amount of the saccharide was determined by HPLC with *p*-aminobenzoic acid ethyl ester (ABEE) conversion method.[5,6)] The formation of the saccharide was significantly slow at the beginning of fermentation. The production of the saccharide reached its maximum after 48 days' fermentation. Then, the amount of the saccharide gradually decreased. Saccharide **1** was considered to be used as the substrate for production of other saccharides, which have higher degrees of polymerization.

Isolation of saccharide **1** was carried out according to the method of a previous paper.[2)] The plant extract (100 g) was loaded onto a carbon-Celite column. These chromatographic procedures were carried out ten times. Subsequently, the saccharide was successfully purified repeatedly using a preparative HPLC system (Tosoh, Tokyo, Japan) equipped with an Amide-80 column (Fig. 2) and ODS-80Ts column. Finally, purified saccharide **1** (980 mg) was obtained as a white powder. The structure of the isolated saccharide was confirmed by MALDI-TOF-MS and NMR measurements (data not shown). The yield and purity of saccharide **1** were 49 and 98%, respectively.

The degree of sweetness was measured according to the method of Takenaka *et al*.[7)] The degree of sweetness of sucrose solutions ranging from 1.0 to 2.5% (w/v) concentration at intervals of 0.5% was compared at room temperature with that of 10% saccharide **1** by 8 panelists.

At the degree of sweetness corresponding to 10% (w/v) saccharide **1**, two panelists chose 1.5% sucrose, four panelists chose 2.0% sucrose and two panelists chose 2.5% sucrose. Therefore, saccharide **1** had about 0.2 times the sweetness of sucrose.

Stability during heating was investigated as follows: a 50 mM acetate buffer (pH 3, 4 and 5) containing 4% (w/

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v) of a saccharide (saccharide **1** or sucrose) was enclosed in a tube and then heated at 100°C for 30 and 60 min. Stability during heating of saccharide **1** was similar to that of sucrose (Fig. 3).

The cariogenicity was examined according to the method of Miyamura.[11] The oral cavity of a subject was rinsed with tap water and then the subject gargled with distilled water. The saliva naturally secreted was collected and well shaken at room temperature. A 0.5 mL aliquot of 1% (w/v) saccharide **1**, palatinose, glucose or distilled water was then added to a mixture of 1.5 mL of this fresh saliva and 0.5 mL of a brain heart infusion broth. These mixtures were incubated at 37°C to show the time-dependent pH variation as the cariogenicity. The same experiment was done with 1.5 mL of culture of *Streptococcus mutans* JCM 5705 (1.6×10^6 CFU/mL) instead of sa-

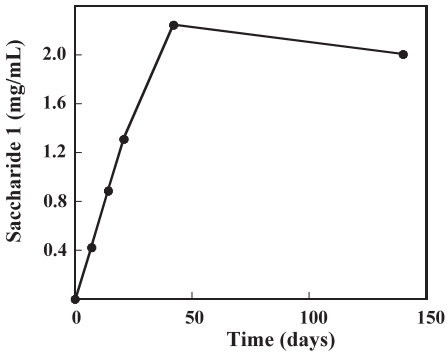

Fig. 1. Time course of the formation of saccharide **1** during fermentation.

Saccharide **1** produced during the fermentation was assessed by HPLC with the ABEE conversion method.[4,5] A 10 µL of plant extract 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. ABEE-converted saccharide was detected by UV at 305 nm.

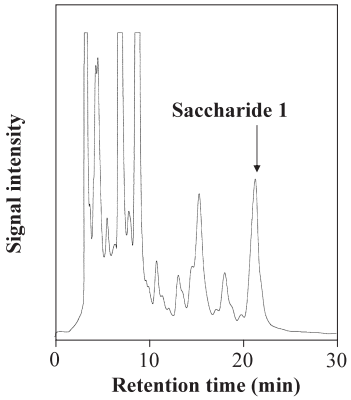

Fig. 2. Preparative HPLC of fermentation products.

The water fraction containing saccharide **1** (0.05 mL, 0.1 mg of total saccharide) was injected. Operating conditions of HPLC were as follows: column, Amide-80 column (ϕ7.8 mm×30 cm, Tosoh); column temperature, 80°C; eluate, 80% (v/v) acetonitrile; flow rate, 1.0 mL/min; detection, refractive index detection.

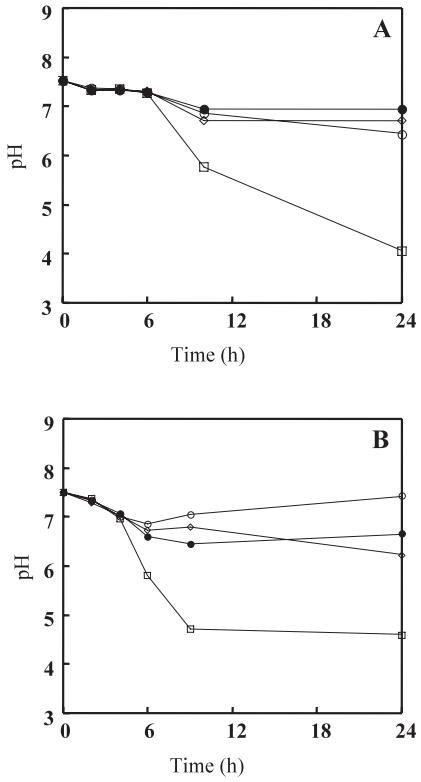

Fig. 4. Cariogenicity of saccharide **1**.

A 0.5 mL aliquot of 1% (w/v) saccharide **1**, palatinose, glucose or distilled water was added to a mixture of 1.5 mL of fresh saliva or culture of *S. mutans*, and 0.5 mL of a brain heart infusion broth. A, *Streptococcus mutans* JCM 5705; B, human saliva. Symbols: ○, blank; □, sucrose; ◇, palatinose; ●, saccharide **1**.

Table 1. Digestion of saccharide **1** *in vitro*.

	Digestibility (%)
Human saliva	0
Artificial gastric juice	0
Porcine pancreas	3.5
Rat intestinal acetone powder	12.2

The digestibility was investigated according to the method of Okada *et al*.[12] Details were described in the text.

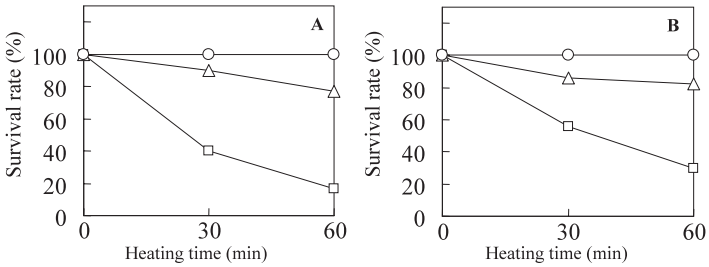

Fig. 3. pH stability of saccharide **1** and sucrose during heating.

A 50 mM acetate buffer (pH 3, 4 and 5) containing 4% (w/v) of a saccharide (saccharide **1** or sucrose) was heated at 100°C for 30 and 60 min. Hydrolysates of saccharides were measured by HPAEC.[8–10] A, saccharide **1**; B, sucrose. Symbols: ○, pH 5.0; △, pH 4.0; □, pH 3.0.

Table 2. Utilization of saccharide **1** and several other saccharides by some human intestinal bacteria.

Bacterial species	Saccharide **1**	1-Kestose	Raffinose	Turanose	Palatinose	Meribiose	Lactose	Sucrose	Glucose
Bifidobacterium adolescentis 2793	+	+++	+++	−	+++	+++	+++	+++	+++
B. bifidum 2777	±	+++	+++	++	++	+++	+++	+++	+++
B. breve 2776	−	+++	+++	++	+	+++	+++	+++	+++
B. infantis 2775	±	+++	+++	++	++	+++	+++	+++	+++
B. longum 2778	+	+++	+++	+++	+++	+++	+++	++	+++
Lactobacillus acidophilus 2243	−	+++	+	−	+++	+++	+++	+++	+++
L. casei 2036	−	+	++	−	±	+++	+++	++	+++
L. fermentum 2046	−	±	++	−	−	+++	+++	++	+++
Enterobacter cloacae 1180	−	−	++	−	±	+++	++	++	+++
Escherichia coli 1099	−	−	−	−	−	±	+	++	++
Enterococcus faecalis 2048	−	−	−	−	±	++	++	++	+++
Clostridium perfringens 1211	−	+	±	−	−	++	++	++	++

liva.

As shown in Fig. 4, the saccharide **1** was a non-cariogenic sugar because *S. mutans* and oral bacteria produce no acid.

The digestibility was investigated according to the method of Okada *et al*.[12] The hydrochloric acid-potassium chloride buffer (50 mM, pH 2.0) was used as an artificial gastric juice. To 0.5 mL of 2.0% (w/v) saccharide **1**, 0.25 mL of this artificial buffer was added, and digestion was performed at 37°C for 100 min. The digestion was stopped by adding 0.31 mL of 10 mM sodium hydroxide.

To one mL of a 50 mM Bis-Tris buffer (pH 6.6) containing 1 mM calcium chloride and 1.0% saccharide **1**, 0.1 mL of pig pancreatic amylase suspension (4 U/mL) was added. The digestion was also performed at 37°C for 6 h, and the reaction of this intestinal enzyme was stopped by heating at 100°C for 5 min. A suspension of 300 mg of rat intestinal acetone powder in 2.7 mL of 10 mM phosphate buffer (pH 7.0) was homogenized for 5 min and then centrifuged at 10,000 rpm for 15 min to obtain the supernatant as an intestinal enzyme solution. To one mL of a 10 mM phosphate buffer (pH 6.8) containing 1.0% saccharide **1**, 0.2 mL of a rat intestinal enzyme solution (1.5 U/mL) was added. The digestion was performed at 37°C for 3 h, and the reaction was stopped by heating at 100°C for 5 min.

The saccharide was slowly hydrolyzed by pig pancreatin and rat intestinal enzymes (Table 1). In those conditions, soluble starch and sucrose were almost completely hydrolyzed by pig pancreatin and rat intestinal enzyme, respectively. Those results showed that saccharide **1** had low digestibility.

LB broth[13] was used as basal medium for testing utilization of the saccharides by intestinal bacteria. D-Glucose, sucrose, palatinose, 1-kestose, or saccharide **1** was added to the LB medium at a final concentration of 0.5% (w/v). After incubation at 37°C for 72 h under anaerobic condition, bacterial growth was measured by pH analysis of the medium. The symbols "+++", "++", "+", "±" and "−" show the medium[14] pH of <4.5, 4.5−5.0, 5.0−5.5, 5.5−6.0, >6.0, respectively.

Bifidobacteria and *Lactobacilli* are beneficial to both the nutrition and health of humans and animals, while some intestinal bacteria such as *Escherichia coli*, *Enterobactor cloaceae* and *Enterococcus faecalis* are detrimental intestinal bacteria.

Under the normal conditions of *Bifidobacteria* growth, the pH of the medium supplemented with non saccharide (control), saccharide **1**, 1-kestose, raffinose, turanose, palatinose, meribiose, lactose, sucrose or glucose was 6.51−6.68, 5.47−6.14, 4.08−4.40, 4.03−4.18, 4.13−6.05, 4.22−5.12, 4.00−4.16, 4.02−4.50, 4.12−4.94 or 4.00−4.40, respectively. Saccharide **1** as well as 1-kestose and turanose was not fermented by *Enterobacter cloacae*, *Escherichia coli* or *Enterococcus faecalis*. Moreover, saccharide **1** was selectively used by the four beneficial bacteria strains of *Bifidobacteria* (Table 2).

In this study, we found *O*-β-D-fructopyranosyl-(2→6)-D-glucopyranose from the fermented beverage of plant extract. The saccharide showed 0.2 times the sweetness of sucrose, non-cariogenicity and low digestibility. Furthermore, the unfavorable bacteria that produce mutagenic substances did not use the saccharide. Therefore, it was thought that *O*-β-D-fructopyranosyl-(2→6)-D-glucopyranose could be a new material for foods.

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植物エキス発酵飲料から分離した *O*-β-D-Fructopyranosyl-(2→6)-D-glucopyranose の特性

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植物エキス発酵飲料は約 50 種類の植物を原料として，ショ糖の浸透圧を利用してエキスを抽出し，おもに酵母 (*Zygosaccharomyces* spp. and *Pichia* spp.)，乳酸菌 (*Leuconostoc* spp.) の発酵によって製造される．この飲料から *O*-β-D-fructopyranosyl-(2→6)-D-glucopyranose (糖 1) が見出され，発酵によって生成される．糖 1 は，ショ糖と比較し 0.2 倍の甘さであり，酸に対する安定性はショ糖とほぼ同等であった．また，低う蝕性，難消化性糖であることが認められ，さらに，腸内細菌による資化性を調査したところ，この糖質はクロストリディウム属菌などに利用されず，ビフィドバクテリウム属菌によってのみ資化された．