Structural Analysis and Synthesis of Oligosaccharides Isolated from Fermented Beverage of Plant Extract

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Hideki Okada,¹,* Naoki Kawazoe,¹ Akira Yamamori,¹ Shuichi Onodera² and Norio Shiomi²

¹Ohtakakohso Co., Ltd. (1–22–10, Sakura, Otaru 047–0193, Japan)
²Department of Food and Nutrition Sciences, Graduate School of Dairy Science Research, Rakuno Gakuen University (582–1, Bankyoudai-Midorimachi, Ebetsu 069–8501, Japan)

Abstract: Fermented beverage of plant extract was prepared from about fifty kinds of fruits and vegetables. Natural fermentation was conducted by lactic acid bacteria (Leuconostoc spp.) and yeast (Zygosaccharomyces spp. and Pichia spp.). Fourteen kinds of oligosaccharides have been isolated from this beverage; their structures were confirmed by methylation analysis, MALDI-TOF-MS and NMR measurements. In these saccharides, five novel oligosaccharides have been found to be constructed by fructose residue of pyranose form, and fructosyl residues of sucrose bond with the β-D-galactose and β-D-glucose. The characteristics of one of the novel saccharides, O-β-D-fructopyranosyl(2→6)-β-D-glucopyranose (Fp2-6G) were investigated, and included 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. The Fp2-6G synthesis activity of crude enzyme of the 5 yeast strains isolated from plant extract was examined using the ABEE-converting method. Fp2-6G synthetic activity was observed only in a reaction mixture using crude enzyme from the Y-1 strain. The Y-1 strain was identified as Pichia spp.

Key words: fermented beverage of plant extract, oligosaccharide, natural fructopyranoside, β-D-fructopyranoside

The extract from 50 kinds of fruits and vegetables was fermented to produce a new beverage.¹,² The juices were extracted using sucrose-osmotic pressure in a cedar barrel for one week and were 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-pirclyhydrazyl (DPPH) radical, and reduced significantly the ethanol-induced damage to gastric mucosa in rats.³ Analysis by high performance anion exchange chromatography (HPAEC) 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 undetermined oligosaccharides. Recently, it was reported that different positions of glycosidic linkage of oligosaccharide isomers affected physiological properties as well as physical properties.⁴,⁵ Development of HPLC analyses with high sensitivity and separation ability enables the detection and isolation of oligosaccharides in the fermented beverage.

In this paper, we reported isolation and structure confirmation of the saccharides from fermented beverage of plant extract, and screening of yeast synthesizing novel saccharides was done.

Preparation of fermented beverage of plant extract.

For preparation of initial juice, fifty kinds of fruits and vegetables were used to produce the final extract as shown in a previous paper.⁶ Fruits and vegetables, obtained from the local markets of the Hokkaido region, were sorted for absence of defects or attacks, washed and immediately processed for juice extraction. The fifty fruits and vegetables were cut, sliced or diced in small pieces, mixed and put in cedar barrels. Afterwards, an equivalent weight of sucrose was added to samples, mixed well to allow high contact between samples and sucrose, and then barrels were left during one week at room temperature. Sucrose was almost completely hydrolyzed to glucose and fructose during fermentation. 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 the 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 during six to ten months for additional maturation and aging, obtaining finally a brown and slightly sticky liquid.

The pH of plant extract was about 3.2, reached after 7 days of fermentation (Fig. 1).

Isolation of saccharides from fermented beverage of plant extract.

Synthesis of saccharides by the fermentation of plant extract was investigated using the HPLC with p-aminobenzoic acid ethyl ester (ABEE)-conversion method.⁷ As shown in Fig. 2, various kinds of saccha-
Saccharides were produced during fermentation. Saccharides from the plant extract were isolated as follows:

Fermented beverage of plant extract (5 kg) was loaded onto a carbon-Celite [1:1; charcoal (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and Celite-535 (Nacalai Chemical Industries, Ltd., Osaka, Japan)] column (4.5×35 cm) and successively eluted with water (14 L), 5% ethanol (30 L) and 30% ethanol (Fig. 3). Almost all of the glucose and fructose were eluted with water (4 L) and then saccharides 1 and 2 were eluted with water (5~6 L). Fractions including saccharides 1 and 2 were concentrated in vacuo and freeze-dried to give 1.25 g. Finally, the yield and purity of saccharide 2 were 49 and 98%, respectively. Saccharides 3, 4, 5 were eluted with 1 L of 5% ethanol. Saccharide 6 was obtained by additional elution (1 L) of 5% ethanol. The 5% ethanol fractions were also concentrated in vacuo and freeze-dried to give 0.97 g and 1.70 g. Saccharides 7~14 were eluted with 30% ethanol (1~2 L, 0.84 g). Subsequently, those fractions were successfully purified repeatedly using a HPLC system (Tosoh, Tokyo, Japan) equipped with an Amide-80 column (7.8 mm×30 cm, Tosoh, Tokyo, Japan) at 80°C, and eluted with 80% acetonitrile at 2.0 mL/min, and using refractive index detection. Furthermore, those saccharides were purified using HPLC with an ODS-80Ts column (4.6 mm×25 cm, Tosoh, Tokyo, Japan) at room temperature.

**Fig. 1.** Fermentation process of plant extracts.
- ○, pH; ■, colony-forming U/g.

**Fig. 2.** High performance liquid chromatogram of fermentation products.

HPLC analysis of saccharides produced during fermentation was done by the ABEE conversion method. (A) plant extract was fermented for 0 days. (B) plant extract was fermented for 180 days. G, glucose; F, fructose.

**Fig. 3.** Isolation of saccharides from plant extract by Amide-80 column chromatography.

Fermented beverage of plant extract was loaded onto a carbon-Celite column and successively eluted with water (a), 5% ethanol (b, c) and 30% ethanol (d).

**Structure analysis of saccharides.**

The saccharides isolated from the fermented beverage were identified using high performance anion exchange chromatography (HPAEC), MALDI-TOF-MS and NMR.

Saccharides 1, 3, 4, 5, 6, 7, 9, 13 and 14 corresponded to authentic saccharides fructofranosyl-(2→6)-glucose, maltose, sophorose, gentiobiose, laminaribiose, maltotriose, raffinose, panose and O-β-D-glucopyranosyl-(1→6)-O-β-D-glucopyranosyl-(1→3)-D-glucopyranose, respectively (Fig. 4). Sophorose, gentiobiose and laminaribiose were already known to be synthesized enzymatically under highly concentrated solutions of glucose and the presence of yeast and Penicillium β-glucosidase. The saccharides isolated from the fermented beverage of plant extract were not detected before fermentation. Therefore, these saccharides were estimated to be synthesized by action of yeast β-glucosidase during fermentation. Though fructofranosyl-(2→6)-glucose was obtained from partial hydrolysate of neokestose, it was presumed to be a reverse-reaction of invertase of yeast.

The $R_{sucrose}$ values of HPAEC of saccharides 2, 8, 10, 11 and 12 did not correspond to those of any authentic saccharides.

The degree of polymerization (DP) of saccharide 2 was established as 2 by measurements of [M+Na]$^+$ ions (m/z: 365) using TOF-MS and similar analyses of saccharides 8, 10, 11 and 12 established their DP as 3 (m/z: 527). From the GC analysis, relative retention times of the methanolysate of the permethylated saccharide were investigated ($R_{rel}$ (relative retention time; retention time of methyl 2,3,4,6-tetra-O-methyl-β-D-glucoside=1.0; retention time, 9.60 min]). The methanolysate of permethylated saccharide 2 exhibited four peaks and two of them corre-
sponded to methyl 2,3,4-tri-O-methyl-D-glucoside (tR, 2.58 and 3.56). From the finding, saccharide 2 was presumed to be fructosyl (2→6) glucose. However, other peaks (tR, 1.06 and 1.48) did not correspond to methyl 1,3,4,5-tetra-O-methyl-D-fructopyranoside, the preparation of methyl fructopyranoside from D-fructose was attempted. 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, respectively from the retention time and pattern of fragmentation.14 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.

Subsequently, NMR analysis was done. The HSQC-TOCSY spectrum revealed the 1H and 13C signals of each β-Glc, α-Glc and Fru residues. The COSY 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 13C signals were assigned by HSQC spectrum. The assignment of resting signals, the position of glycosidic linkage, and pyranoside form of fructose were analyzed as follows. There was one methylene carbon not assigned yet, which was estimated as C-1 of Fru (Fig. 5(a)). Its protons showed HMBC correlations to methine carbon at δC 69.29 and the only quaternary carbon (δC 101.52) (Fig. 5(b)), 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. 5(b)). These results revealed saccharide 2 had a fructopyranoside residue and Fru 2→6Glc linkage. The coupling patterns of overlapped 1H were analyzed by the SPT method.21,22 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, H-5 and H-6 in α-Glc, these couplings could not be analyzed in the first order. The δC values of Fru indicated its β anomer form, by comparing those of α and β form of methyl-D-fructopyranoside.23 From these findings, the fructose residue of the non-reducing terminal of this saccharide was pyranose form, and saccharide 2 found from the fermented beverage of plant extracts was confirmed to be a novel saccharide, β-D-fructopyranosyl(2→6)-D-glucopyranose (Fig. 6).

Saccharides 8 and 12 were similarly analyzed by methylation analysis and NMR measurement. Partial acid hydrolyses of saccharides 8 and 12 were liberated to glu-
cose, fructose and laminaribiose. Saccharide 8 and 12 also have a fructose residue of the pyranose form. The saccharides were identified as new saccharides, \( \alpha \)-\( \beta \)-D-fructofuranosyl-(2 \( \rightarrow \) 6)-\( \beta \)-D-glucopyranosyl(1 \( \rightarrow \) 3)-D-glucopyranose and \( \alpha \)-\( \beta \)-D-fructofuranosyl(2 \( \rightarrow \) 6)[\( \alpha \)-\( \beta \)-D-glucopyranosyl(1 \( \rightarrow \) 3)]-D-glucopyranose, respectively (Fig. 6). Furthermore, these novel saccharides were 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.

The partial acid hydrolysate of saccharide 10 was liberated to galactose, glucose, fructose and sucrose. From the results of methylation analysis and NMR measurement, saccharides 10 and 11 were \( \alpha \)-\( \beta \)-D-glucopyranosyl- (1 \( \rightarrow \) 1 )-\( \beta \)-D-fructofuranosyl(2 \( \rightarrow \) 1 )-\( \alpha \)-D-glucopyranoside; \( \alpha \)-\( \beta \)-D-glucopyranosylfructofuranosyl and \( \alpha \)-\( \beta \)-D-galactopyranosyl(1\( \rightarrow \)1)-\( \beta \)-D-fructosefructofuranosyl(2 \( \rightarrow \) 1 )-\( \alpha \)-D-glucopyranoside; \( \alpha \)-\( \beta \)-D-galactopyranosylfructofuranosyl, respectively (Fig. 6). Melezitose, \( \alpha \)-\( \alpha \)-galactosylfructofuranosyl and \( \beta \)-\( \beta \)-galactopyranosylfructofuranosyl, has already been reported by Courtois et al.\(^{26}\) and Pazur et al.\(^{25}\) However, \( \alpha \)-\( \beta \)-D-glucopyranosylfructofuranosyl and \( \alpha \)-\( \beta \)-D-galactopyranosylfructofuranosyl were not found in natural resources excepting the saccharides of the fermented beverage of plant extract. Synthesis of saccharides by fermentation of plant extract was investigated using HPAEC. Saccharides 2, 8, 10, 11 and 12 were confirmed to be produced during fermentation.

**The characteristics of saccharide 2, \( \alpha \)-\( \beta \)-D-fructofuranosyl-(2 \( \rightarrow \) 6)-D-glucopyranose (Fp2-6G).**

Time course of formation of Fp2-6G by fermentation was measured. The formation of Fp2-6G was significantly slow at the beginning of fermentation. The maximum production of Fp2-6G was reached after 48 days’ fermentation.

The characteristics of Fp2-6G were investigated; sweetness was 0.2-fold compared with that of sucrose. Fp2-6G was a non-cariogenic sugar because S. mutans and oral bacteria did not produce acid from Fp2-6G, and Fp2-6G was hydrolyzed by pig pancreatin and rat intestinal enzymes 3.5% and 12.2% compared with starch and sucrose, respectively, and the results showed that Fp2-6G had low digestibility.

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

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

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Saccharide 2</th>
<th>1-Kestose</th>
<th>Raffinose</th>
<th>Turanose</th>
<th>Palatinose</th>
<th>Meribiose</th>
<th>Lactose</th>
<th>Sucrose</th>
<th>Glucose</th>
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<tr>
<td>Bifidobacterium adolescentis 2793</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>Lactobacillus acidophilus 2243</td>
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<td>Enterobacter cloaceae 1180</td>
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<tr>
<td>Escherichia coli 1099</td>
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<td>Enterococcus faecalis 2048</td>
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<tr>
<td>Clostridium perfringens 1211</td>
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Under the normal conditions of *Bifidobacteria* growth, the pH of the medium supplemented with no saccharide (control), Fp2-6G, l-kestose, raffinose, turanose, palatinose, melibiose, 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. On the other hand, Fp2-6G as well as l-kestose and turanose were not fermented by *Enterobacter cloacae*, *Escherichia coli* or *Enterococcus faecalis*. Moreover, Fp2-6G was selectively used by the four beneficial bacteria strains of *Bifidobacteria* (Table 1).

**Screening of yeast synthesizing Fp2-6G.**

The 5 yeast strains were isolated during the fermentation of plant extract. The cultivation was done on the YPD broth at 30°C for 72 hours. Fp2-6G synthesis activity of crude enzyme from the yeast 5 strains was examined using the ABBEC-converting method. The substrate concentration was 30% glucose and 30% fructose. A disaccharide in the reaction mixture containing crude enzyme from the Y-1 strain was detected as Fp2-6G at the same retention time. Structural analysis of the saccharide isolated by various chromatographic methods from the reaction mixture of the Y-1 strain was identified as Pichia spp. Though the production of Fp2-6G with Y-1 strain crude enzyme was very slow, it increased over 168 h of reaction. The amount of production of the saccharide increased as the substrate concentration increased (glucose : fructose=1:1). However, 70% concentration was the maximum (Fig. 7).

**Conclusions.**

We confirmed structures of novel saccharides such as O-β-D-fructofuranosyl-(2 → 6)-β-D-glucopyranose, β-D-fructofuranosyl-(2 → 6)-β-D-glucopyranosyl-(1 → 3)-β-D-glucopyranose, β-D-fructofuranosyl-(2 → 6)-[β-D-galactopyranosyl-(1 → 3)]-β-D-glucopyranose, and O-β-D-galactopyranosyl-(1 → 2)-β-D-fructofuranosyl-(2 → 1)-α-D-glucopyranoside [1′-β-D-glucosyglucose] and O-β-D-galactopyranosyl-(1 → 2)-β-D-fructofuranosyl-(2 → 1)-α-D-glucopyranoside [1′-β-D-galactosylglucose] isolated from the fermented beverage using methylation analysis, MALDI-TOF-MS and NMR measurements. Those novel saccharides were confirmed to be produced by fermentation. Furthermore, we also isolated fructofuranosyl-(2 → 6)-glucose, maltose, sophorose, gentiobiose, laminaribiose, maltotriose, raffinose, panose and O-β-D-glucopyranosyl-(1 → 6)-O-β-D-glucopyranosyl-(1 → 3)-D-glucopyranose.

The characteristics of O-β-D-fructofuranosyl-(2 → 6)-p-glucopyranose were 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.

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