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

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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)-D-glucopyranose (F*p*2-6G) were investigated, and included non-cariogenicity and low digestibility. Furthermore, the unfavorable bacteria, *Clostridum perfringens*, *Escherichia coli* and *Enterococcus faecalis*, that produce mutagenic substances did not use the saccharide. The F*p*2-6G synthesis activity of crude enzyme of the 5 yeast strains isolated from plant extract was examined using the ABEE-converting method. F*p*2-6G 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-picrylhydrazyl (DPPH) radical, and reduced significantly the ethanol-induced damage to gastric mucosa in rats.[1] Analysis by high performance anion exchange chromatography (HPAEC) showed that this beverage contained high levels of saccharides, estimated between 550 and 590 gL^{-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.[3–5] 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.[1] 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.[6,7] As shown in Fig. 2, various kinds of saccha-

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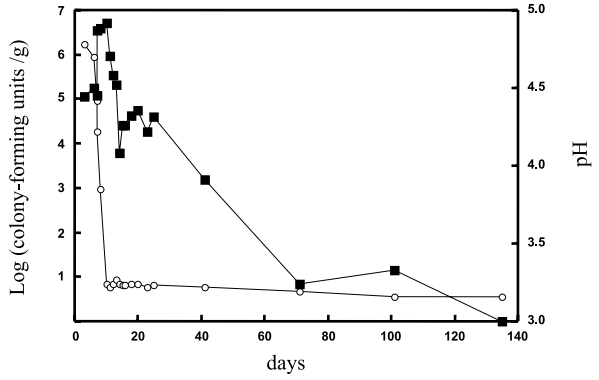

Fig. 1. Fermentation process of plant extracts.

○, pH; ■, colony-forming U/g.

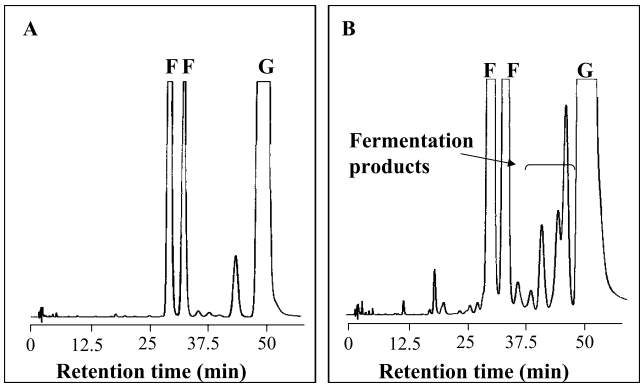

Fig. 2. High performance liquid chromatogram of fermentation products.

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.

rides 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.

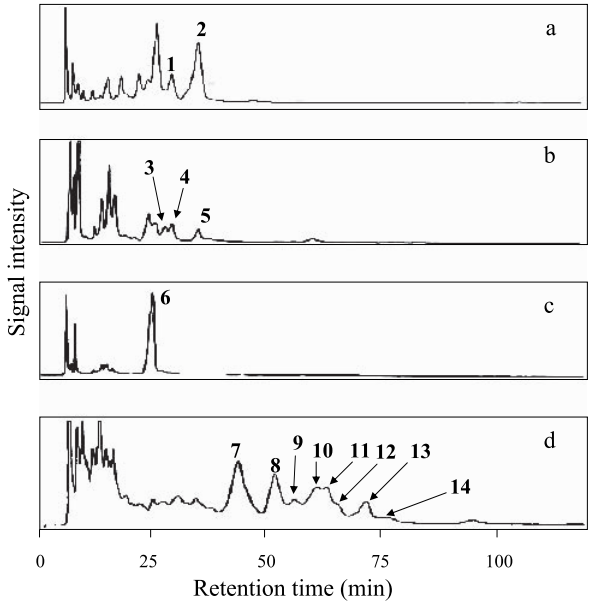

Fig. 3. Isolation of saccharides from plant extract by Amide-80 column chromatography.

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),[8,9] 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.[10−12] *O*-β-D-Glucopyranosyl-(1→6)-*O*-β-D-glucopyranosyl-(1→3)-D-glucopyranose was also reported to be synthesized by β-glucosidase.[13] 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 $t_{R,sucrose}$ 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 [t_R (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-

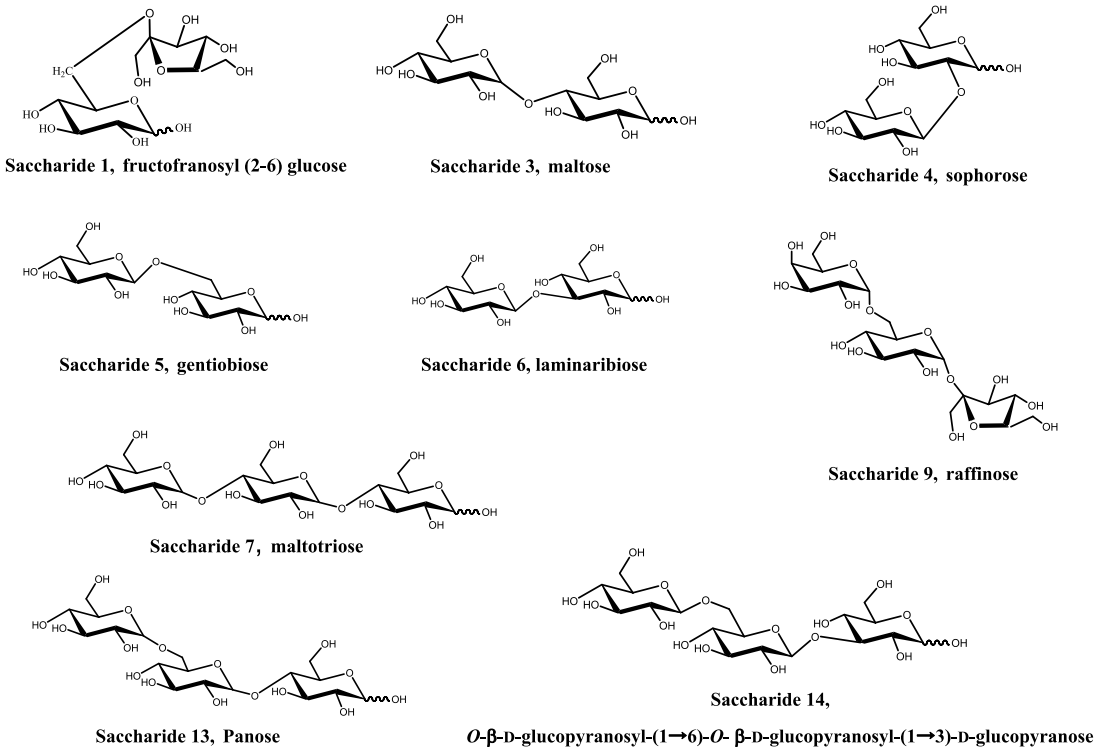

Fig. 4. Structures of saccharides isolated from fermented beverage of plant extract.

sponded to methyl 2,3,4-tri-*O*-methyl-D-glucoside (t_R, 2.58 and 3.56). From the finding, saccharide **2** was presumed to be fructosyl (2→6) glucose. However, other 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 peaks were estimated to 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 (9.14 and 10.57 min), 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[15] spectrum revealed the ^{1}H and ^{13}C signals of each β-Glc, α-Glc and Fru residues. The COSY[16,17] 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[18] 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[19,20]

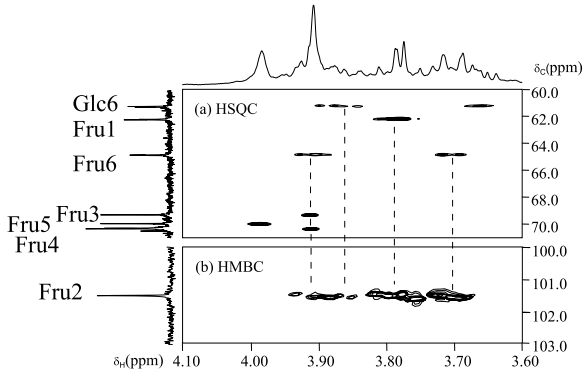

Fig. 5. Part of HSQC (a) and HMBC (b) spectra of saccharide **2**.

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*p*2→6Glc linkage. The coupling patterns of overlapped ^{1}H 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, and 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 hydrolysates of saccharides **8** and **12** were liberated to glu-

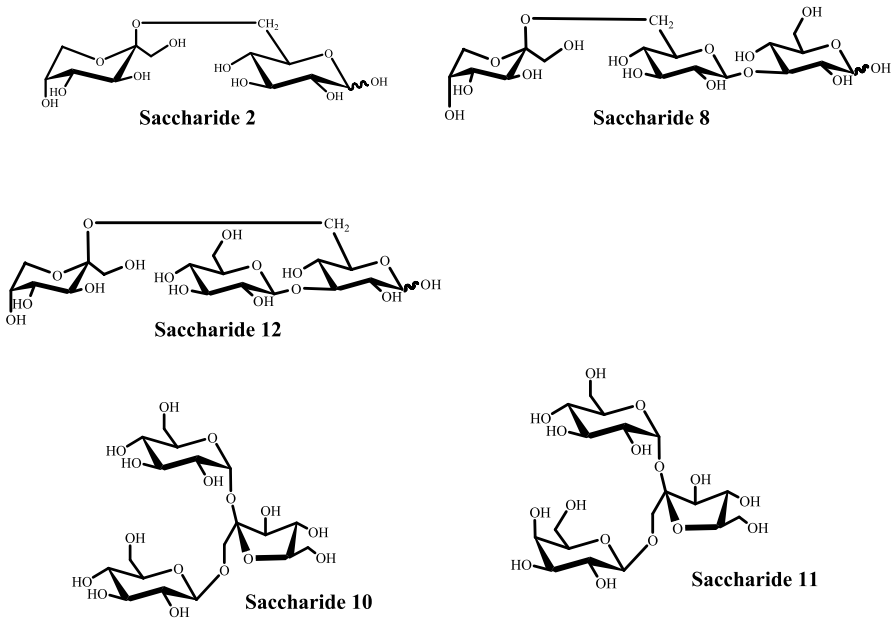

Fig. 6. Structures of novel saccharides isolated from fermented beverage of plant extract.

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

Bacterial species	Saccharide **2**	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	−	+	±	−	−	++	++	++	++

cose, fructose and laminaribiose. Saccharide **8** and **12** also have a fructose residue of the pyranose form. The saccharides were identified as new saccharides, *O*-β-D-fructopyranosyl-(2→6)-*O*-β-D-glucopyranosyl-(1→3)-D-glucopyranose and *O*-β-D-fructopyranosyl-(2→6)-[*O*-β-D-glucopyranosyl-(1→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 glucose, fructose and sucrose, and saccharide **11** was liberated to galactose, glucose, fructose and sucrose. From the results of methylation analysis and NMR measurement, saccharides **10** and **11** were *O*-β-D-glucopyranosyl-(1→1)-β-D-fructofuranosyl-(2↔1)-α-D-glucopyranoside; 1^F-β-D-glucopyranosylsucrose and *O*-β-D-galactopyranosyl-(1→1)-β-D-fructofuranosyl-(2↔1)-α-D-glucopyranoside; 1^F-β-D-galactopyranosylsucrose, respectively (Fig. 6). Melezitose, 1^F-α-galactosylsucrose and 6^F-β-galactosylsucrose, has already been reported by Courtois *et al*.[24] and Pazur *et al*.[25] However, 1^F-β-D-glucopyranosylsucrose and 1^F-β-D-galactopyranosylsucrose 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, O-β-D-fructopyranosyl-(2→6)-D-glucopyranose (Fp2-6G).

Time course of formation of F*p*2-6G by fermentation was measured. The formation of F*p*2-6G was significantly slow at the beginning of fermentation. The maximum production of F*p*2-6G was reached after 48 days' fermentation.

The characteristics of F*p*2-6G were investigated; sweetness was 0.2-fold compared with that of sucrose. F*p*2-6G was a non-cariogenic sugar because *S. mutans* and oral bacteria did not produce acid from F*p*2-6G, and F*p*2-6G 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 F*p*2-6G had low digestibility.

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

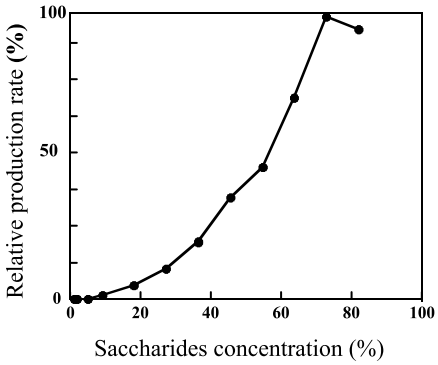

Fig. 7. Effect of glucose and fructose concentrations on production of saccharide **2** by intracellular enzyme from yeast.

Under the normal conditions of *Bifidobacteria* growth, the pH of the medium supplemented with no saccharide (control), F*p*2-6G, 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. On the other hand, F*p*2-6G as well as 1-kestose and turanose were not fermented by *Enterobacter cloacae*, *Escherichia coli* or *Enterococcus faecalis*. Moreover, F*p*2-6G 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 of the YPD (yeast extract, peptone and dextrose) broth at 30°C for 72 hours. F*p*2-6G synthesis activity of crude enzyme from the yeast 5 strains was examined using the ABEE-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 F*p*2-6G at the same retention time. Structural analysis of the saccharide isolated by various chromatographies from the reaction mixture of the Y-1 strain was done and the saccharide was confirmed to be F*p*2-6G. The Y-1 strain was identified as *Pichia* spp. Though the production of F*p*2-6G 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-Fructopyranosyl-(2→6)-D-glucopyranose, β-D-fructopyranosyl-(2→6)-β-D-glucopyranosyl-(1→3)-D-glucopyranose, β-D-fructopyranosyl-(2→6)-[β-D-glucopyranosyl-(1→3)]-D-glucopyranose, *O*-β-D-glucopyranosyl-(1→1)-β-D-fructofuranosyl-(2↔1)-α-D-glucopyranoside [1^F-β-D-glucosylsucrose] and *O*-β-D-galactopyranosyl-(1→1)-β-D-fructofuranosyl-(2↔1)-α-D-glucopyranoside [1^F-β-D-galactosylsucrose] 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 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.

The characteristics of *O*-β-D-fructopyranosyl-(2→6)-D-glucopyranose were non-cariogenicity and low digestibility. Furthermore, the unfavorable bacteria, *Clostridum perfringens*, *Escherichia coli* and *Enterococcus faecalis* that produce mutagenic substances did not use the saccharide.

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REFERENCES

1) H. Okada, K. Kudoh, E. Fukushi, S. Onodera, J. Kawabata and N. Shiomi: Antioxdative activity and protective effect of fermented plant extract on ethanol-induced damage to rat gastric mucosa. *J. Jpn. Soc. Nutr. Food Sci.*, **58**, 209−215 (2005).
2) H. Okada, E. Fukushi, A. Yamamori, N. Kawazoe, S. Onodera, J. Kawabata and N. Shiomi: Structural analysis of a novel saccharide isolated from fermented beverage of plant extract. *Carbohydr. Res.*, **341**, 925−929 (2006).
3) T. Kohmoto, F. Fukui, H. Takaku, Y. Machida, M. Arai and T. Mitsuoka: Effect of isomalto-oligosaccharides on human fecal flora. *Bifidobact. Microflora*, **7**, 61−69 (1988).
4) S. Murosaki, K. Muroyama, Y. Yamamoto, H. Kusaka, T. Liu and Y. Yoshikai: Immunopotentiating activity of nigerooligosaccharides for the T helper 1-like immune response in mice. *Biosci. Biotechnol. Biochem.*, **63**, 373−378 (1999).
5) S. Murosaki, K. Muroyama, Y. Yamamoto, T. Liu and Y. Yoshikai: Nigerooligosaccharides augments natural killer activity of hepatic mononuclear cells in mice. *Int. Immunopharmacol.*, **2**, 151−159 (2002).
6) S. Yasuno, T. Murata, K. Kokubo, T. Yamaguchi and M. Kamei: Two-mode analysis by high-performance liquid chromatography of *p*-aminobenzoic ethyl ester-derivatized monosaccharides. *Biosci. Biotechnol. Biochem.*, **61**, 1944−1946 (1997).
7) I. Kobayashi, S. Yasuno, H. Hashimoto, K. Ikura, H. Nakano and S. Kitahata: Analysis of positional isomers of α-linked glucooligosaccharides by *p*-aminobenzoic ethyl ester-conversion method. *J. Appl. Glycosci.*, **49**, 461−468 (2002).
8) N. Shiomi, S. Onodera, N.J. Chatterton and P.A. Harrison: Separation of fructooligosaccharide isomers by anion-exchange chromatography. *Agric. Biol. Chem.*, **55**, 1427−1428 (1991).
9) H. Okada, E. Fukushi, S. Onodera, T. Nishimoto, J. Kawabata, M. Kikuchi and N. Shiomi: Synthesis and structural analysis of five novel oligosaccharides prepared by glucosyltransfer from β-D-glucose 1-phosphate to isokestose and nystose using *Thermoanaerobacter brockii* kojibiose phosphorylase. *Carbohydr. Res.*, **338**, 879−885 (2003).
10) H. Fujimoto, H. Nishida and K. Ajisaka: Enzymatic syntheses of glucobioses by a condensation reaction with α-glucosidase, β-glucosidases and glucoamylase. *Agric. Biol. Chem.*, **52**, 1345−1351 (1988).
11) R.A. Rastall, S.F. Pikett, M.W. Adlard and C. Bucke: Synthesis of oligosaccharides by reversal of a fungal β-glucanase. *Biotechnol. Lett.*, **14**, 373−378 (1992).
12) T.G. Villa and H.J. Phaff: A method for preparing laminaribiose by using yeast endo-(1→3)-β-D-glucanase. *Carbohydr. Res.*, **74**, 369−370 (1979).
13) S. Kawamura, T. Watanabe, N. Abe and K. Matsuda: Transglucosylation products from β-linked glucobiose with crude glucoamylase from *endomyces* sp. *J. Ferment. Bioeng.*, **49**, 725−732 (1971).
14) I. Ciucanu and C.E. Costello: Elimination of oxidative degradation during the per-*O*-methylation of carbohydrates. *J. Am. Chem. Soc.*, **125**, 16213−16219 (2003).
15) W. Willker, D. Leibfritz, R. Kerssebaum and W. Bermel: Gradient selection in inverse heteronuclear correlation spectros-

copy. *Magn. Reson. Chem.*, **31**, 287–292 (1993).
16) I. Brereton, S. Crozier, J. Field and D.M. Doddrell: Quadrature detection in F_1 induced by pulsed field gradients. *J. Magn. Reson.*, **93**, 54–62 (1991).
17) M. Kienlin, C.W. Moonen, A. Toorn and P.M. Zijl: Rapid recording of solvent-suppressed 2D COSY spectra with inherent quadrature detection using pulsed field gradients. *J. Magn. Reson.*, **93**, 423–429 (1993).
18) Y.-C. Li and G.T. Montelione: Solvent Saturation-Transfer effects in pulsed-field-gradient heteronuclear single-quantum-coherence (PFG-HSQC) spectra of polypeptides and proteins. *J. Magn. Reson.*, **B101**, 315–319 (1993).
19) A. Bax and M.F. Summers: ^{1}H and ^{13}C assignments from sensitivity-enhanced detection of heteronuclear multiple quantum NMR. *J. Am. Chem. Soc.*, **108**, 2093–2094 (1986).
20) R.E. Hurd and B.K. John: Gradient-enhanced proton-detected heteronuclear multiple-quantum coherence spectroscopy. *J. Magn. Reson.*, **91**, 648–653 (1991).
21) K.G.R. Pachler and P.L. Wessels: Selective Population Inversion (SPI). A pulsed double resonance method in FT NMR spectroscopy equivalent to INDOR. *J. Magn. Reson.*, **12**, 337–339 (1973).
22) J. Uzawa and S. Yoshida: A new selective population transfer experiment using a double pulsed field gradient spin-echo. *Magn. Reson. Chem.*, **42**, 1046–1048 (2004).
23) H.B. Sinclaer: Two methyl anhydro-D-fructopyranosides prepared from D-mannitol *Carbohydr. Res.*, **181**, 115–123 (1988).
24) J.E. Courtois and U. Ariyoshi: Saccharose galactosides from the roots of *Cucubalus baccifer* (Caryophyllaceae). Study of their structure. *Bull. Soc. Chim.*, **42**, 737–751 (1960).
25) J.H. Pazur, J.M. Marsh and C.L. Tipton: Cosubstrate specificity of the transgalactosylase of *Saccharomyces fragilis*. *J. Biol. Chem.*, **233**, 277–279 (1958).

植物エキス発酵飲料中の新規オリゴ糖とその生成

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植物エキス発酵飲料 (以下 FPE) は数十種類の野菜，果物を原料とし，sucrose の浸透圧を利用してエキスを抽出し約半年間，酵母および乳酸菌によって自然発酵させた褐色，粘ちょう性の飲料である．この飲料の投与によりラットの寿命延長効果が示唆されており，アルコール性胃粘膜障害抑制効果を有することや β-(1,3)glucan が存在することをすでに報告している．FPE 中に含まれる glucose，fructose 以外の糖質の検索は，60% 近い糖濃度の飲料であるため困難であったが，高感度に検出する方法を用いることにより微量に含まれるオリゴ糖類の検出が可能となり，その結果，数種の新規糖質および希少糖が見いだされた．FPE から活性炭クロマトグラフィー，amide-80 カラムおよび ODS-80Ts カラムを用いた HPLC により十数種の糖類を単離した．これら糖質について酸加水分解による構成糖分析，MALDI-TOF-MS 分析，完全メチル化メタノリゼートの GC 分析，COSY，HSQC，HSQC-TOCSY，HMBC 等の 2 次元 NMR 分析等により構造解析を行った．この結果，5 種類の新規糖質を見いだし，それぞれ *O*-β-D-fructopyranosyl-(2→6)-D-glucopyranose (F*p* 2-6 G)，*O*-β-D-fructopyranosyl-(2→6)-*O*-β-D-glucopyranosyl-(1→3)-D-glucopyranose，*O*-β-D-fructopyranosyl-(2→6)-*O*-[β-D-glucopyranosyl-(1→3)]-D-glucopyranose，1^{F}-β-D-glucopyranosylsucrose，1^{F}-β-D-galactopyranosylsucrose と決定した．これらの糖質は，発酵熟成中に生成された．新規糖質合成株のスクリーニングを行ったところ植物エキス発酵飲料の発酵熟成中の液から酵母 5 株 (Y-1，-2，-3，-4，-5) を分離した．この株から調製した粗酵素液 10 mL を pH 5.0 の緩衝液に 30% 濃度になるよう溶解した glucose，fructose 混液 100 mL に加え，37°C で加温した．5 株について F*p*2-6G 合成活性を調べたところ，Y-1 株粗酵素反応液中に F*p*2-6G と同じ保持時間の糖が検出された．この反応液について各種クロマトグラフィーを行い糖を単離し，構造解析したところ F*p*2-6G であることが確認された．Y-1 株粗酵素による F*p*2-6G の生成量は，上記反応系では非常に少なかったが，反応 168 時間後においても増加していた．また，基質濃度 (glucose:fructose=1:1) の増加にともなって生成量が増加したが，70% 濃度が上限であった．

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〔**質問**〕　㈱林原生物化学研究所　西本

1）Y-1 株粗酵素による F*p*2-6G の合成は，縮合反応によるものと理解してよろしいでしょうか．

2）植物エキス発酵飲料の機能性と含まれる糖質の関係について，何か知見は得られていますか．

〔**答**〕

1）縮合反応であると推測しています．

2）含量は少量ですが，ほとんど難消化性の糖質であることから整腸作用等が期待できると考えております．