J. Appl. Glycosci., **64**, 15–19 (2017)
doi: 10.5458/jag.jag.JAG-2016_011

Note

Structural Analysis of Novel Low-Digestible Sucrose Isomers Synthesized from D-Glucose and D-Fructose by Thermal Treatment

(Received August 22, 2016; Accepted November 21, 2016)
(J-STAGE Advance Published Date: December 16, 2016)

Akira Yamamori,[1,†] Yusuke Takata,[2] Eri Fukushi,[2] Jun Kawabata,[2] Hideki Okada,[1] Naoki Kawazoe,[1] Keiji Ueno,[3] Shuichi Onodera,[3] and Norio Shiomi[3]

[1]*General Institute of Ohtakakohso Co., Ltd.*
(*1–22–10 Sakura, Otaru 047–0193, Japan*)
[2] *Graduate School of Agriculture, Hokkaido University*
(*Kita 8, Nishi 5, Kita-ku, Sapporo 060–8589, Japan*)
[3]*Department of Food and Nutrition Sciences, Graduate School of Dairy Science Research, Rakuno Gakuen University*
(*582–1 Bunkyodai-Midorimachi Ebetsu 069–8501, Japan)*)

Abstract: The synthesis of the saccharide β-D-fructopyranosyl-(2→6)-D-glucopyranose, which was isolated from Super Ohtaka®, has recently been reported. During the synthesis of this saccharide, the formation of two novel saccharides from D-glucose and D-fructose was observed. The present study aimed to confirm the structures of the two disaccharides synthesized from D-glucose and D-fructose by thermal treatment. Furthermore, various properties of the saccharides were investigated. Both saccharides were isolated from the reaction mixture by carbon-Celite column chromatography and an HPLC system and were determined to be novel sucrose-isomers, β-D-fructopyranosyl-(2↔1)-β-D-glucopyranoside (1) and β-D-fructofuranosyl-(2↔1)-β-D-glucopyranoside (2), by MALDI-TOF MS and NMR analyses. Both saccharides showed low digestibility *in vitro*, and the sweetness of saccharide 2 was 0.45 times that of sucrose.

Key words: sucrose-isomer, disaccharide, thermal treatment, fermented beverage of plant extract

We previously reported the structural analysis of the oligosaccharides β-D-fructopyranosyl-(2→6)-D-glucopyranose,[1)] α-D-fructofuranosyl-(2→6)-D-glucopyranose,[2)] β-D-fructopyranosyl-(2→6)-β-D-glucopyranosyl-(1→3)-D-glucopyranose,[3)] β-D-fructopyranosyl-(2→6)-[β-D-glucopyranosyl-(1→3)]-D-glucopyranose,[3)] β-D-fructopyranosyl-(2→1)-β-D-fructofuranosyl-(2↔1)-α-D-glucopyranoside,[4)] and β-D-fructopyranosyl-(2→6)-α-D-glucopyranosyl-(1↔2)-β-D-fructofuranoside[4)] derived from Super Ohtaka®, which is produced by fermenting the extracts from 50 types of fruits and vegetables. The extract is obtained after sucrose-osmotic pressure treatment in a cedar barrel for 7 days and fermentation by lactic acid bacteria and yeast at 37 °C for 180 days. This beverage primarily includes glucose and fructose, but it also contains various oligosaccharides. A previous study has shown that β-D-fructopyranosyl-(2→6)-D-glucopyranose has non-cariogenic qualities and low digestibility.[5)] This saccharide was selectively used by the beneficial bacteria *Bifidobacterium adolescentis* and *B. longum*, but it was not used by the harmful bacteria *Clostridium perfringens*, *Escherichia coli*, and *Enterococcus faecalis*, which produce mutagenic substances.[5)] Furthermore, we synthesized β-D-fructopyranosyl-(2→6)-D-glucopyranose[6)] and α-D-fructofuranosyl-(2→6)-D-glucopyranose[7)] from D-glucose and D-fructose by a thermal treatment. In the present study, we describe the synthesis of two novel saccharides (sucrose isomers), β-D-fructopyranosyl-(2↔1)-β-D-glucopyranoside and β-D-fructofuranosyl-(2↔1)-β-D-glucopyranoside (Fig. 1), from D-glucose and D-fructose by caramelization with thermal treatment, which is the first step for potential applications in food industry. Furthermore, we investigated various properties of these two sucrose isomers.

1 **2**

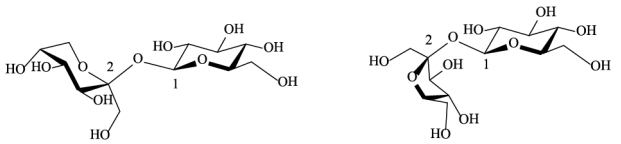

Fig. 1. Structure of β-D-fructopyranosyl-(2↔1)-β-D-glucopyranoside (**1**) and β-D-fructofuranosyl-(2↔1)-β-D-glucopyranoside (**2**).

A powder mixture of 180 g each of D-glucose and D-fructose was carefully heated at 150 °C for 60 min in an electric furnace. The saccharide melt was then dissolved in 1,500 mL distilled water. The saccharide solution (1.86 L) was loaded onto an 8.1×72-cm carbon-Celite column (1:1; charcoal: Celite-535) and successively eluted with water (27 L). Almost all of the D-glucose and D-fructose was eluted first within the first 2.5 L, and saccharides **1** and **2** were subsequently eluted with water (19 L). The water

† Corresponding author (Tel. +81–134–54–7311, Fax. +81–134–52–2610, E-mail: oelab-akira@ohtakakohso.co.jp).

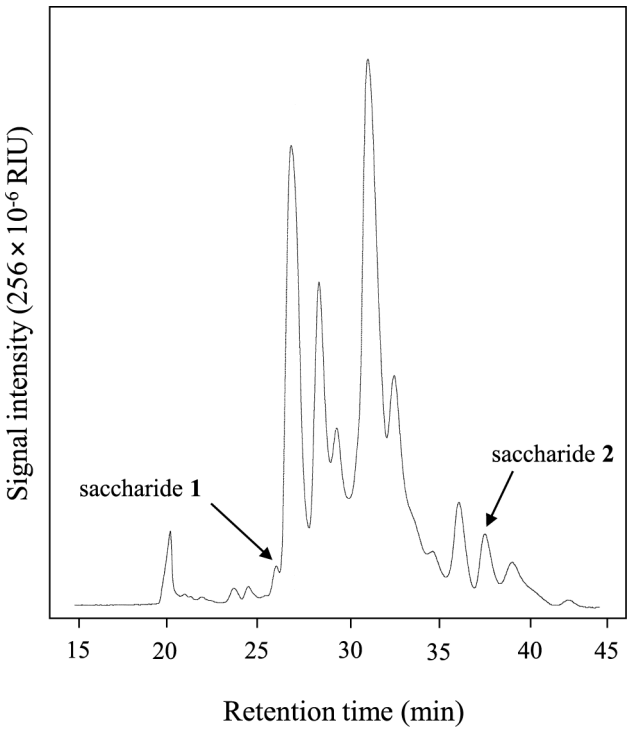

Fig. 2. Preparative-HPLC of the disaccharide fractions eluted by carbon-Celite column chromatography.

The following operating conditions for HPLC were used: column, ODS-100V (20×250 mm; Tosoh Corporation); column temperature, 20 °C; eluent, distilled water; flow rate, 3.0 mL/min; detection, by refractive index.

fraction containing saccharides **1** and **2** was concentrated to 80.0 mL and frozen for storage. Part of this fraction (7.5 mL) was diluted with distilled water of 12.5 mL, and repeatedly purified with preparative-HPLC (Fig. 2). Purified saccharides **1** (14 mg) and **2** (35 mg) were finally obtained as a white powder and confirmed as homogeneous by HPAEC[8)9)10)] with retention times of 1.88 and 3.66 min and relative retention times of 0.46 and 0.90 (the retention time of sucrose being 1.0). The degrees of polymerization of saccharides **1** and **2** were established as 2 by $[M+Na]^+$ (*m/z* 365) MALDI-TOF MS measurement. The complete hydrolysis of saccharides **1** and **2** was investigated using analytical-HPLC, where saccharides **1** and **2** (3.0 mg) were dissolved in 0.1 N HCl (0.2 mL) and hydrolyzed by heating at 100 °C for 30 min. As a result, glucose and fructose were detected in equal amounts.

The structural confirmations of the saccharides **1** and **2** were provided by ^{1}H- and ^{13}C-NMR analyses (^{1}H at 500 MHz and ^{13}C at 126 MHz), and the subsequent complete assignments of ^{1}H- and ^{13}C-NMR signals were carried out using 2D-NMR techniques, including COSY,[11)12)] E-HSQC, HSQC-TOCSY,[13)14)] and HMBC.[15)16)] The 1D ^{1}H- and ^{13}C-NMR spectra of saccharides **1** and **2** are shown in Figs. 3A and 3B. Saccharides **1** and **2** were not an anomeric mixture based on the 1D ^{1}H- and ^{13}C-NMR spectra. The disaccharide consisted of one anomeric proton, three methylenes, and one quaternary carbon based on the E-HSQC spectra. First, the HSQC-TOCSY spectrum of saccharide **1** revealed the ^{1}H and ^{13}C signals of Glc and Fru residues: from C-1 (δ_C 95.40 ppm, δ_H 4.71 ppm, d, 8.0 Hz) to C-6 of Glc and from C-3 to C-6 in Fru. The COSY spectrum assigned the spin system of Glc from H-1 to H-6. The corresponding ^{13}C signals were assigned from the E-HSQC spectrum. β-Glc was assigned by the *J* value and chemical shift. The quaternary carbon (δ_C 102.96 ppm) and the isolated methylene carbon (δ_C 62.04 ppm) were assigned as C-2 and C-1 in Fru, respectively. The HMBC correlation between C-2 and H-1 confirmed these assignments. The other methylene carbon (δ_C 62.96 ppm) was assigned as C-6 in Fru by the HSQC-TOCSY spectrum. The H-5 in Fru was assigned from H-6 in Fru by the COSY correlations. The HMBC correlations of C-3/H-1 in Fru confirmed the assignment of these signals. The other methine (δ_C 74.52 ppm) was assigned as C-4 in Fru. The β-fructopyranose was assigned from the HMBC correlations of C-Fru 2/H-Glc 6 and its chemical shift. The C-2 of Fru showed HMBC correlations to H-1 of Glc. These results indicated the Fru$_p$-(2↔1)-Glc linkage (Fig. 4A), and all ^{1}H- and ^{13}C-NMR signals were assigned as shown in Table 1. The COSY spectrum of saccharide **2** was used to assign the spin system of Glc residues from the anomeric proton to H-6. The corresponding ^{13}C signals were assigned from the E-HSQC spectrum. The β form of Glc was assigned from the chemical shift of C-1 (δ_C 95.38 ppm) and the *J* (H-1/H-2) value in Glc (8.1 Hz). The methine proton (δ_H 4.28 ppm, d, 8.7 Hz) was assigned as H-3 in Fru. The COSY spectrum assigned the spin system from H-3 to H-6 in Fru. The corresponding ^{13}C signals were assigned from the E-HSQC spectrum. The isolated methylene carbon was assigned as C-1 in Fru. The corresponding C-1 in Fru was assigned from the E-HSQC. The HMBC correlations of C-2/H-1 in Fru confirmed the assignment of the signals. The inter-residual HMBC correlations between C-2 in Fru and H-1 in Glc indicated the Fru$_f$-(2↔1)-Glc linkage (Fig. 4B). In addition, the β form of Fru was assigned from the chemical shift. All ^{1}H- and ^{13}C-NMR signals were assigned as shown in Table 1. Based on the results, saccharides **1** and **2** were confirmed to be new saccharides, β-D-fructopyranosyl-(2↔1)-β-D-glucopyranoside and β-D-fructofuranosyl-(2↔1)-β-D-glucopyranoside, respectively. The purity of saccharide **1** and **2** were 95 and 98 %, and the yields of the saccharides were about 0.1 and 0.3 %, respectively.

The optimal conditions for saccharide **2** synthesis were investigated. The saccharide could be synthesized by heating for 60 min at 130 to 190 °C, with a maximum yield at 160 °C. Furthermore, the saccharide was efficiently synthesized at 160 °C for 45 min as well as at 170 °C for 30 min. Stabilities during heating was investigated as follows. A 50-mM aliquot of Britton-Robinson buffer (pH 3.0, 5.0, 7.0, and 9.0) containing 5 % saccharide [sucrose, saccharide **1** or saccharide **2**] was heated in a tube at 100 °C for 15, 30, 45, and 60 min in a dry thermal bath heater. Saccharides **1** and **2** were less stable than sucrose for all pH values tested.

The digestibility of saccharides **1** and **2** by human saliva, pig pancreatic amylase, rat intestinal enzyme, and artificial gastric juice were investigated as previously described by Okada *et al.*[17)] Human saliva was examined as previously described by Miyamura.[18)] Saliva was collected from 3 in-

Table 1. ^{1}H- and ^{13}C-NMR spectra data (δ^{a} in ppm, J in Hz) of saccharides 1 and 2.

			δ_C	δ_H		$J_{H,H}$
Saccharide **1**	βGlc_p	1	95.40	4.71	d	8.0
		2	73.84	3.38	dd	9.4, 8.0
		3	76.72	3.52	dd	9.4, 8.7
		4	70.37	3.42	dd	9.8, 8.7
		5	76.51	3.46	ddd	9.8, 5.5, 2.3
		6	61.34	3.89	dd	12.5, 2.3
				3.71	dd	12.4, 5.5
	βFru_p	1	63.59	3.84	d	12.4
				3.74	d	12.4
		2	102.96			
		3	68.22	4.02	d	10.7
		4	70.11	3.97	dd	10.7, 3.4
		5	69.90	4.02	m[b]	
		6	64.91	4.21	dd	12.2, 1.2
				3.72	dd	12.2, 2.0
Saccharide **2**	βGlc_p	1	95.38	4.87	d	8.1
		2	73.61	3.35	dd	9.3, 8.1
		3	76.58	3.54	dd	9.3, 9.0
		4	70.35	3.41	dd	9.9, 9.0
		5	76.50	3.47	ddd	9.9, 5.6, 2.3
		6	61.33	3.89	dd	12.4, 2.3
				3.71	dd	12.4, 5.6
	βFru_f	1	62.04	3.79	d	12.7
				3.68	d	12.7
		2	105.42			
		3	76.63	4.28	d	8.7
		4	74.52	4.15	dd	8.7, 8.4
		5	82.46	3.93	ddd	8.4, 6.6, 3.0
		6	62.96	3.83	dd	12.5, 3.0
				3.78	dd	12.5, 6.6

[a] The chemical shifts of ^{1}H (δ_H) and ^{13}C (δ_C) in ppm were respectively determined relative to the external standard of sodium [2,2,3,3-2H4]-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. * NMR data of other oligosaccharides containing D-fructofuranosyl or D-fructopyranosyl residue were referred.[21)22)]

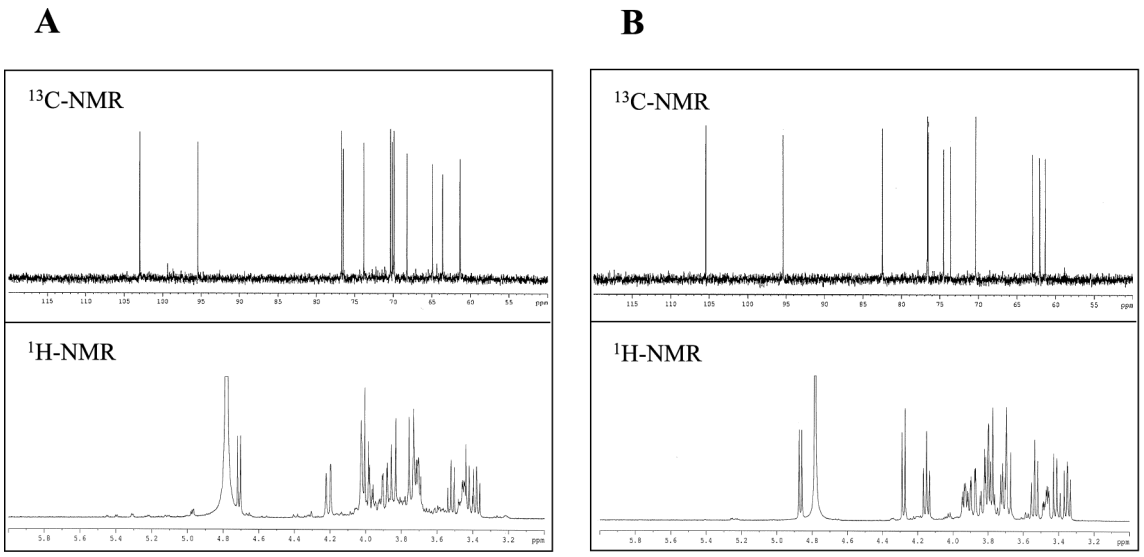

Fig. 3. 1D ^{1}H- and ^{13}C-NMR spectra of saccharides **1** (A) and **2** (B).

dividuals, 2 h after a meal. The oral cavity of each subject was rinsed with tap water, and then, the subject was requested to gargle with distilled water. The naturally secreted saliva (2 mL) was collected and shaken well at room temperature. A 100-µL aliquot of human saliva (43 U/mL) was added to 100 µL of 50 mM Bis-Tris buffer (pH 6.0) con-

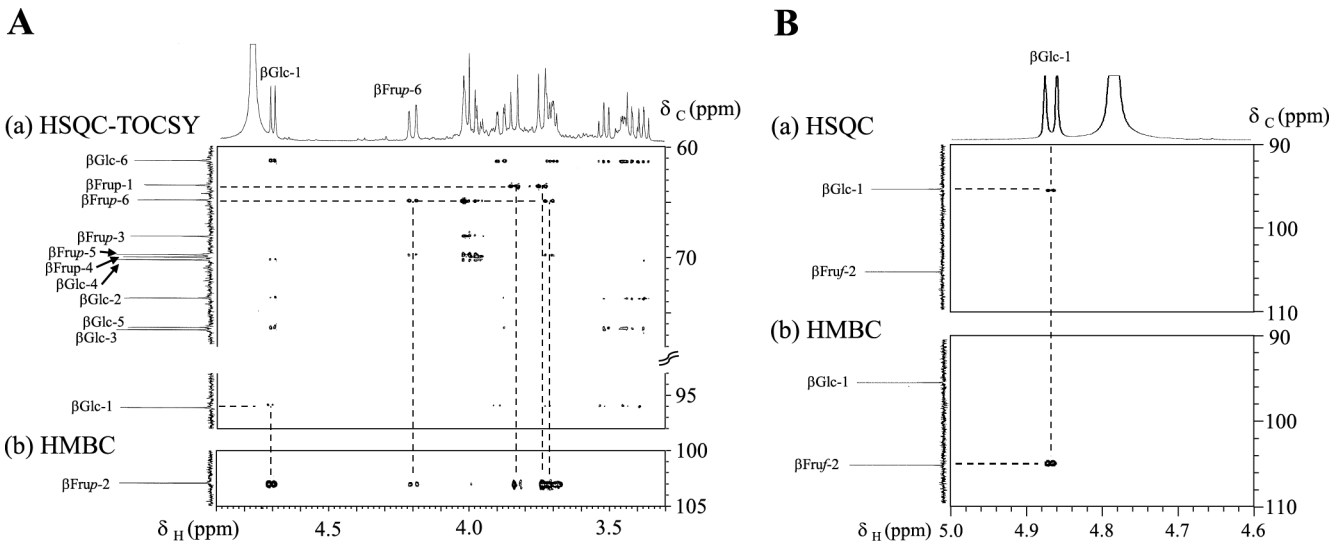

Fig. 4. Portions of the HSQC-TOCSY (Aa), HSQC (Ba), and HMBC (Ab and Bb) spectra of saccharides **1** (A) and **2** (B).

taining 1 mM calcium chloride, and 10 % saccharides **1** and **2**. Digestion was performed at 37 °C for 0, 1, 2, 3, 4, 5, and 6 h, and the reaction was terminated by heating in a dry thermal bath heater at 100 °C for 10 min. Glucose and fructose formed from saccharides **1** and **2** were assayed by HPAEC. Pig pancreatic amylase was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). A 100-μL aliquot of pig pancreatic amylase suspension (4.0 U/mL) was added to 100 μL of 50 mM Bis-Tris buffer (pH 6.6) containing 1 mM calcium chloride and 20 mM saccharides **1** and **2**. Digestion was performed at 37 °C for 1, 2, 3, 4, 5, and 6 h, and the enzyme reaction was terminated by heating in a dry thermal bath heater at 100 °C for 10 min. Glucose and fructose formed from saccharides **1** and **2** were assayed by HPAEC. Rat intestinal enzyme was prepared from intestinal acetone powder (Sigma Chemical Co., St. Louis, USA). A suspension of 300 mg rat intestinal acetone powder in 2.7 mL of 10 mM phosphate buffer (pH 6.8)[19] was homogenized for 5 min using a glass homogenizer and then centrifuged at 12,070 × *G* for 15 min to obtain the intestinal enzyme solution in the supernatant. A 100-μL aliquot of rat intestinal enzyme solution (4.0 U/mL) was added to 100 μL of 10 mM phosphate buffer (pH 6.8) containing 20 mM of saccharides **1** and **2**. Digestion was performed at 37 °C for 0, 15, 30, 60, and 120 min, and the reaction was stopped by heating in a dry thermal bath heater at 100 °C for 10 min. Glucose and fructose formed from saccharides **1** and **2** were assayed by HPAEC. Artificial gastric juice solution (pH 2.0) was prepared from 0.9 mM $CaCl_2$, 50 mM hydrochloric acid, and 50 mM potassium chloride. A 50-μL aliquot of this solution was added to 100 μL of 20 mM saccharides **1** and **2**, and digestion was performed at 37 °C for 0, 15, 30, 60, and 120 min. Digestion was terminated by adding 50 μL of 10 mM sodium hydroxide. Digestibility was determined as the amount of saccharides **1** and **2** in the digestive solution using analytical-HPLC.

The enzyme activities and units were defined as follows. The activities of the saliva and pig pancreatic amylase were assayed by the Somogyi-Nelson method, and 1 U of activity was defined as the amount of enzyme required to provide reducing power equivalent to that of 1.0 μmol glucose from 0.1 % soluble-starch per min at 37 °C and pH 6.0. The activity of intestinal enzymes was assayed by analytical-HPLC, and 1 U of activity was defined as the amount of enzyme required to liberate 2 μmol glucose from 200 mM maltose per min at 37 °C and pH 6.8. Saccharide **1** and **2** were not hydrolyzed by human saliva (Digestion rate; saccharide **1**, 0 %, saccharide **2**, 0 %) and rat intestinal enzyme (saccharide **1**, 0 %, saccharide **2**, 0 %) but were slightly hydrolyzed by pig pancreatic amylases (saccharide **1**, 0 %, saccharide **2**, 3.0 %), or artificial gastric juice (saccharide **1**, 1.2 %, saccharide **2**, 3.5 %). These results indicate that both saccharides have low digestibility. The degree of sweetness was measured as previously described by Takenaka *et al.*[20] We determined the sucrose concentration corresponding to the sweetness of 10 % saccharide **2**, and 1 volunteer chose 3.0 % sucrose, 3 chose 4.0 %, and 6 chose 5.0 %. Therefore, the sweetness of saccharide **2** was approximately 0.45 times that of sucrose.

In this study, sucrose isomers were produced D-glucose and D-fructose by thermal treatment. Both saccharides showed low digestibility. These saccharides could be useful as a novel material for manufacture of foods and chemicals.

REFERENCES

1) 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).
2) H. Okada, E. Fukushi, A. Yamamori, N. Kawazoe, S. Onodera, J. Kawabata, and N. Shiomi: Isolation and structural confirmation of the oligosaccharides containing α-D-fructofuranoside linkage isolated from fermented beverage of plant extracts. *Carbohydr. Res*., **346**, 2633–2637 (2011).
3) N. Kawazoe, H. Okada, E. Fukushi, A. Yamamori, S. Onodera, J. Kawabata, and N. Shiomi: Two novel saccharides isolated from fermented beverage of plant extract. *Carbohydr. Res*., **343**, 549–554 (2008).

4) H. Okada, E. Fukushi, A. Yamamori, N. Kawazoe, S. Onodera, J. Kawabata, and N. Shiomi: Novel fructopyranose oligosaccharides isolated from fermented beverage of plant extract. *Carbohydr*. *Res*., **345**, 414−418 (2010).
5) H. Okada, N. Kawazoe, A. Yamamori, S. Onodera, M. Kikuchi, and N. Shiomi: Characteristics of *O*-β-D-fructopyranosyl-(2→6)-D-glucopyranose isolated from fermented beverage of plant extract. *J*. *Appl*. *Glycosci*., **55**, 179−182 (2008).
6) A. Yamamori, H. Okada, N. Kawazoe, S. Onodera, and N. Shiomi: Synthesis of β-D-fructopyranosyl-(2→6)-D-glucopyranose from D-glucose and D-fructose by thermal treatment. *Biosci*. *Biotechnol*. *Biochem*., **74**, 2130−2132 (2010).
7) A. Yamamori, H. Okada, N. Kawazoe, , K. Muramatsu, S. Onodera, and N. Shiomi: Characteristics of α-D-fructofuranosyl-(2→6)-D-glucose synthesized from D-glucose and D-fructose by thermal treatment. *J*. *Appl*. *Glycosci*., **61**, 99−104 (2014).
8) R. D. Rocklin and C. A. Pohl: Determination of carbohydrate by anion exchange chromatography with pulse amperometric detection. *J*. *Liq*. *Chromatogr*., **6**, 1577−1590 (1983).
9) D. C. Johnson: Carbohydrate detection gains potential. *Nature*, **321**, 451-452 (1986).
10) N. Shiomi, S. Onodera, N. J. Chatterton, and P. A. Harrison: Separation of fructooligosaccharide isomer by anion-exchange chromatography. *Agric*. *Biol*. *Chem*., **55**, 1427−1428 (1991).
11) W. P. Aue, E. Batrholdi, and R. R. Ernst: Two-dimensional spectroscopy. Application to nuclear magnetic resonance. *J*. *Chem*. *Phys*., **64**, 2229−2246 (1975).
12) M. von Kienlin, C. T. W Moonen, A. von der Toorn, and P. C. M. van Zijl: Rapid recording of solvent-supressed 2D COSY spectra with inherent quadrature detection using pulsed fieldgradients. *J*. *Magn*. *Reson*. **93**, 423−429 (1991).
13) W. Willker, D. Leibfritz, R. Kerssebaum, and W. Bermel: Gradient selection in inverse heteronuclear correlation spectroscopy. *Magn*. *Reson*. *Chem*., **31**, 287−292 (1993).
14) T. Domke: A new method to distinguish between direct and remote signals in proton-relayed X, H correlations. *J*. *Magn*. *Reson*. **95**, 174−177 (1991).
15) A. Bax and M. F. Summers: ^{1}H and ^{13}C assignments from sensitivity-enhanced detection of heteronuclear multiple-bond connectivity by 2D multiple quantum NMR. *J*. *Am*. *Chem*. *Soc*., **108**, 2093−2094 (1986).
16) R. E. Hurd and B. K. John: Gradient-enhanced proton-detected heteronuclear multiple-quantum coherence spectroscopy. *J*. *Magn*. *Reson*. **91**, 648−653 (1991).
17) K. Okada, M. Yoneyama, T. Mandai, H. Aga, S. Sakai, and T. Ichikawa: Digestion and fermentation of pullulan. *J*. *Jpn*. *Soc*. *Nutr*. *Food Sci*., **43**, 23−29 (1990). (in Japanese)
18) E. Miyamura: pH Analysis of the bacterial fermentation of sugar alcohol maltitol and lactitol, in human whole saliva. *Shigaku*, **60**, 717−731 (1973). (in Japanese)
19) Y. Tajima: Jikken doubutsu no seibutsugakuteki tokusei deta (Biological reference date book on experimental animals). Soft Science Inc., Tokyo, pp. 23 (1989). (in Japanese)
20) F. Takenaka and H. Uchiyama: Synthesis of α-glucosylglycerol by α-glucosidase and some of its characteristics. *Biosci*. *Biotechnol*. *Biochem*., **64**, 1821−1826 (2000).
21) H. Okada, N. Kawazoe, A. Yamamori, S. Onodera, and N. Shiomi: Structural analysis and characteristics of oligosaccharides isolated from fermented beverage of plant extract. *Dyn*. *Biochem*. *Process Biotechnol*. *Mol*. *Biol*., **1**, 1−9 (2009).
22) N. Shiomi, T. Abe, H. Kikuchi, Y. Takata, E. Fukushi, Y. Fukushi, J. Kawabata, K. Ueno, and S. Onodera: Structural analysis of novel kestose isomers isolated from sugar beet molasses. *Carbohydr*. *Res*., **424**, 1−7 (2016).