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Structural analysis of six novel oligosaccharides synthesized by glucosyl transfer from β-D-glucose 1-phosphate to raffinose and stachyose using *Thermoanaerobacter brockii* kojibiose phosphorylase

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Abstract—Novel oligosaccharides (one tetra-, two penta-, two hexa- and one hepta-saccharides) were synthesized by glucosyl transfer from β-D-glucose 1-phosphate (β-D-G1P) to raffinose or stachyose using *Thermoanaerobacter brockii* kojibiose phosphorylase. Gas liquid chromatography analysis of methyl derivatives, MALDI-TOF-MS and NMR measurements were used for structural confirmation. The ^{1}H and ^{13}C NMR signals of each saccharide were assigned using 2D-NMR including COSY, HSQC, HSQC-TOCSY, HMBC and CH_2-selected E-HSQC techniques. These oligosaccharides were identified as 2-α-D-glucopyranosyl-raffinose, 2^{G}(2-α-D-glucopyranosyl)$_2$-raffinose, 2^{G}(2-α-D-glucopyranosyl)$_3$-raffinose, 2-α-D-glucopyranosyl-stachyose, 2^{G}(2-α-D-glucopyranosyl)$_2$-stachyose and 2^{G}(2-α-D-glucopyranosyl)$_3$-stachyose.

1. Introduction

Soybean oligosaccharides, one group of non-digestible oligosaccharides, are composed of galactooligosaccharide such as raffinose and stachyose, sucrose and monosaccharides. They are used as sweeteners of confectionery and drinks, and currently, are valued as functional foods, especially raffinose and stachyose, which are expected to be used more as food ingredients. Furthermore, these are known to stimulate selectively the growth of beneficial bacteria such as bifidobacteria,[1] to enhance immune responses,[2] and to promote the absorption of calcium and magnesium.[3]

Previously, we have studied the production of non-digestible oligosaccharides as nutritional and functional ingredients such as inulo-oligosaccharide,[4,5] fructooligosaccharide,[6] fructosylxyloside[7] and fructosyllactosucrose[8] using *Penicillium purpurogenum* inulinase,[4,5] *Scopulariopsis brevicaulis* fructosyltransferase[6,7] and asparagus 1^{F}-fructosyltransferase,[8–10] respectively. These oligosaccharides were shown to suppress the rise of serum glucose and cholesterol, insulin responses in rats[11] and having prebiotic effects.[12]

Recently, we examined the synthesis of novel oligosaccharides elongated with one, two or three additional glucose units by glucosyl transfer from β-D-glucose 1-phosphate to isokestose or nystose using *Thermoanaerobacter brockii* kojibiose phosphorylase.[13]

Herein, we report the structural analysis of six novel oligosaccharides synthesized from β-D-glucose 1-phosphate to raffinose or stachyose using kojibiose phosphorylase. These oligosaccharides have a higher degree of polymerization than fructosylxyloside, lactosucrose, 1-kestose and raffinose, thus they are expected to be more utilized by bifidobacteria and could have a better effect on the body by keeping a lower osmotic pressure than di- and tri-saccharides. The structure of

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these oligosaccharides were determined by methylation analysis, MALDI-TOF-MS measurement and assignment using 2D-NMR techniques such as COSY, HSQC, HSQC-TOCSY, HMBC and CH_2-selected E-HSQC.

2. Results and discussion

Saccharides **1**, **2** and **3** were produced from raffinose and β-D-glucose 1-phosphate using kojibiose phosphorylase. Investigation of the reaction revealed that **1**, **2** and **3** were produced after 120 h reaction as shown in Figure 1a. The maximum production of **1** was reached at a reaction time of 120 h, and then gradually decreased. From the reaction mixture, saccharides **1**, **2** and **3** were isolated by successive chromatographic procedures using carbon-Celite and ODS columns, and finally obtained as white powder.

Saccharides **4**, **5** and **6** were synthesized in a same manner. They were produced from stachyose and β-D-G1P after 168 h reaction as shown in Figure 1b, and were isolated as white powder by similar chromatographic procedures as described above.

Saccharide **1** ($[\alpha]_D^{20} = +138.4$), **2** ($[\alpha]_D^{20} = +145.7$), **3** ($[\alpha]_D^{20} = +151.0$), **4** ($[\alpha]_D^{20} = +157.4$), **5** ($[\alpha]_D^{20} = +146.4$) and **6** ($[\alpha]_D^{20} = +169.4$) were shown to be homogenous using HPAEC (t_R, retention time of sucrose = 1.00, 1.13, 1.35, 1.50, 1.09, 1.35 and 1.52). The degrees of polymerization were established as 4 **1**, 5 **2** and **4**, 6 **3** and **5**, 7 **6**, as shown by measurements of $[M+Na]^+$ ions (*m*/*z*: 689, **1**; 851, **2** and **4**; 1013, **3** and **5**; 1175, **6**) using TOF-MS, and analysis of the molar ratios of D-glucose, D-galactose and D-fructose in the acid hydrolysates of the oligosaccharides.

From the GC analysis, relative retention time of the methanolysates of the permethylated saccharides were investigated [t_R retention times of methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucoside = 1.0 (retention time, 9.05 min)]. The methanolysate of permethylated **1** exhibited five peaks corresponding to methyl 2,3,4,6-tetra-*O*-methyl-D-glucoside (t_R, 1.09 and 1.46), methyl 1,3,4,6-tetra-*O*-methyl-D-fructoside (t_R, 1.09 and 1.31), methyl 2,3,4,6-tetra-*O*-methyl-D-galactoside (t_R, 1.77) and methyl 3,4-di-*O*-methyl-D-glucoside (t_R, 2.61). The methanolysate of permethylated **2**, **3**, **4**, **5** and **6** also exhibited five peaks, which corresponded to the same methyl glycosides as those from **1**. Furthermore, the methanolysate of permethylated **4**, **5** and **6** gave a peak corresponding to methyl 2,3,4-tri-*O*-methyl-D-galactoside (t_R, 7.07). The methanolysate of permethylated saccharides **2**, **3**, **5** and **6** gave two peaks corresponding to methyl 3,4,6-tri-*O*-methyl-D-glucoside (t_R, 2.96 and 3.53), the peaks of the methanolysates of the saccharides **3** and **6** were larger than those of permethylated **2** and **5**. Peak of methyl 3,4,6-tri-*O*-methyl-D-glucoside indicating 1→2 glucosyl linkage of each saccharide were increased by additional units of glucose.

From these findings, **1**, **2**, **3**, **4**, **5** and **6** were proved to be 2-α-D-glucopyranosyl-raffinose, 2^G(2-α-D-glucopyranosyl)$_2$-raffinose, 2^G(2-α-D-glucopyranosyl)$_3$-raffinose, 2-α-D-glucopyranosyl-stachyose, 2^G(2-α-D-glucopyranosyl)$_2$-stachyose and 2^G(2-α-D-glucopyranosyl)$_3$-stachyose, respectively.

The structural confirmation of the saccharides **1**, **2**, **3**, **4**, **5** and **6** according to ^1H, ^{13}C NMR analyses and the subsequent complete assignment of ^1H, ^{13}C NMR signals were carried out using 2D-NMR techniques, including COSY,[15,16] HSQC,[17] HSQC-TOCSY,[18] CH_2-selected E-HSQC[19] and HMBC.[20,21] The NMR spectral analysis was started from several anomeric proton and carbon signals of aldose units, galactose and glucose, since they showed separate characteristic signals in ^1H and ^{13}C NMR spectra. After assignment of the ^1H and ^{13}C signals of units of aldose, they were assigned as galactose and glucose using proton–proton coupling constants. The HMBC spectrum revealed a galactose residue attached to the C-6 of galactose or glucose, a fructose residue attached to C-1 of glucose and a glucose residue attached to the C-2 of glucose. In this way, we identified the structure of these oligosaccharides.

Galactose, glucose and fructose residues of these saccharides are represented as Gal, Gal′, Glc, Glc′, Glc″, Glc‴ and Fru as shown in Figure 2. The proton and carbon positions in a particular residue are represented by H-1-Gal, H-1-Glc and C-1-Fru, respectively.

First, the NMR spectra of **1** were analyzed. From three anomeric protons (δ_H 4.98 ppm, d, 3.8 Hz, δ_H 5.67 ppm, d, 3.5 Hz and δ_H 5.11 ppm, d, 3.8 Hz) and carbons (δ_C 99.11 ppm, δ_C 90.36 ppm and δ_C 97.20 ppm) in **1**, one

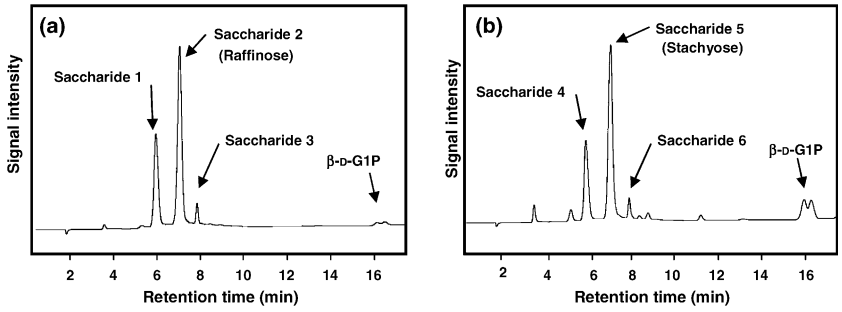

Figure 1. (a) HPAEC of saccharides produced from raffinose and β-D-G1P by kojibiose phosphorylase. (b) HPAEC of saccharides produced from stachyose and β-D-G1P by kojibiose phosphorylase.

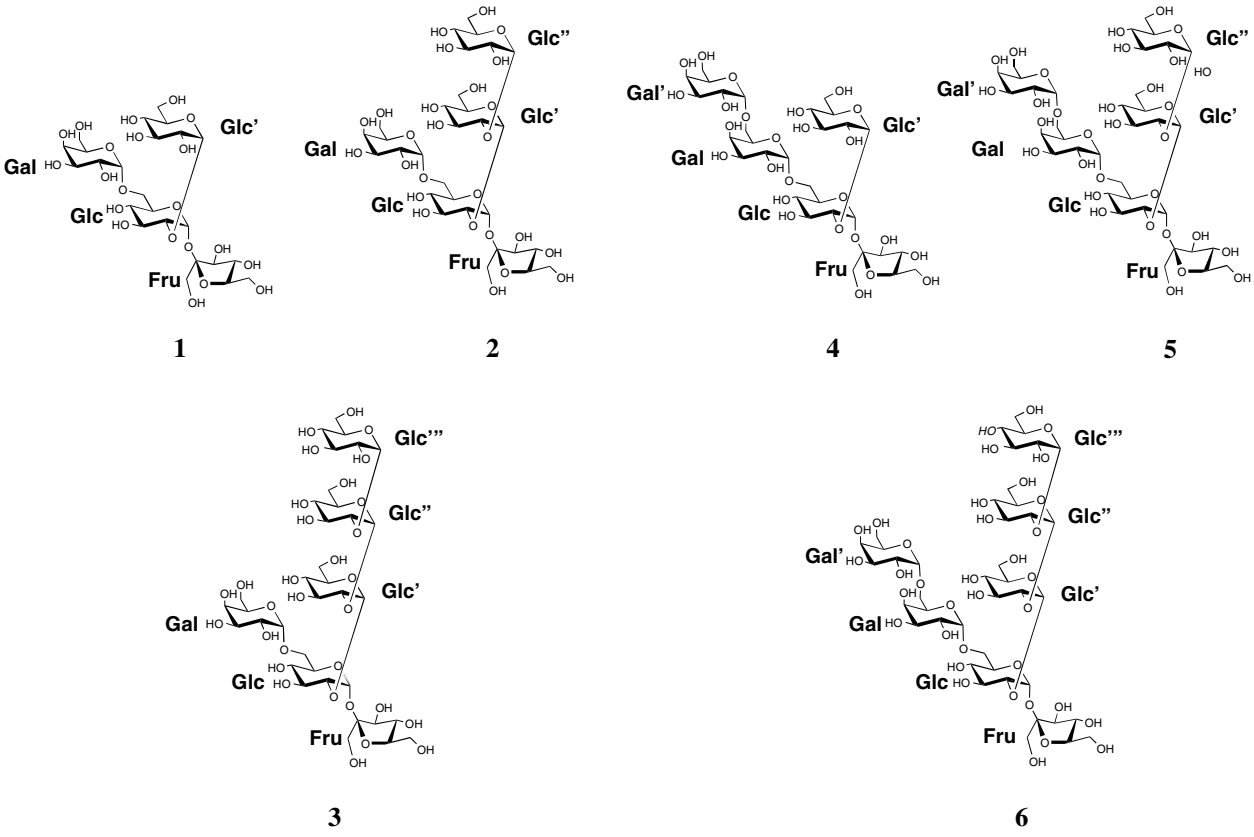

Figure 2. Structures of saccharides **1**, **2**, **3**, **4**, **5** and **6** formed by kojibiose phosphorylase.

galactosyl residue and two glucosyl residues were assigned by ^{1}H–^{1}H COSY and J_{HH}. The inter residual HMBC correlation between one of the anomeric proton (δ_H 5.67 ppm) and one of the quaternary carbon (δ_C 105.12 ppm) assigned the proton and carbon to H-1-Glc and C-2-Fru, respectively. The HMBC correlations between C-2-Fru and H-1-Fru (δ_H 3.37 ppm) and between C-1-Fru (δ_C 62.59 ppm) and H-3-Fru (δ_H 4.22 ppm, d, 8.8 Hz) as well as ^{1}H–^{1}H-COSY correlations enabled the assignments of fructose residue. The connectivity of Gal (1→6) Glc and Glc′ (1→2) Glc were also deduced from HMBC correlations between C-6-Gal (δ_C 99.11 ppm) and H-1-Glc (δ_H 4.00 and 5.67 ppm) and between C-2-Glc (δ_C 76.23 ppm) and H-1-Glc′ (δ_H 5.11 ppm). The characteristic J (H-1, H-2) values of the Glc (J = 3.6 Hz) and Glc′ (J = 4.0 Hz) determined both glucosyl bounds were α forms as shown in sucrose, respectively.

The tetrasaccharide unit of **1** in pentasaccharide **2** and hexasaccharide **3** was determined in the same manner as in **1**. As shown in Figure 3a, further glucosyl linkages Glc″ (1→2) Glc′ in **2** and Glc‴ (1→2) Glc″ (1→2) Glc′ in **3**, were determined by additional HMBC correlation

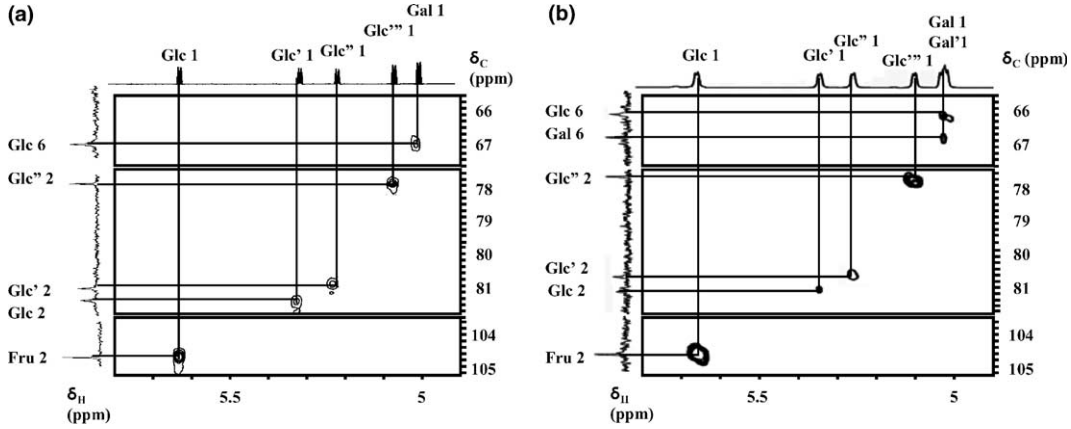

Figure 3. (a) Part of the HMBC spectra of **3**. Connectivities of Glc→Glc′→Glc″→Glc‴ and assignment of these free glucose residues were completed. (b) Part of the HMBC spectra of **6**. Connectivities of Glc→Glc′→Glc″→Glc‴ and assignment of these free glucose residues were completed.

between H-1-Glc″ (δ_H 5.08 ppm, d, 3.7 Hz) and C-2-Glc′ (δ_C 77.83 ppm) in **2** and between H-1-Glc‴ (δ_H 5.07 ppm, d, 3.7 Hz) and C-2-Glc″ (δ_C 77.81 ppm) in **3**, respectively. The tetrasaccharide unit of **1** in saccharide **4** and the pentasaccharide unit of **2** in saccharide **5** and the hexasaccharide unit of **3** in saccharide **6** were determined in the same manner as in **1**, **2** and **3**, respectively. As shown in Figure 3b, further galactosyl linkages, Gal′ (6→1) Glc in **4**, **5** and **6**, were determined by additional HMBC correlation between H-1-Gal′ (δ_H 4.99 ppm in **4**, **5** and **6**) and C-6-Gal (δ_C 67.17 ppm in **4**, 67.19 ppm in **5**, 67.27 ppm in **6**), respectively. These δ_C and δ_H values agreed well with those of the kojibiose (Glc′α1→2Glc),[22] raffinose (Galα1→6Glcα1→2Fru) and stachyose (Gal′α1→6Galα1→6Glcα1→2Fru). Methylene signals that overlapped in the narrow region were separated by limiting the F_1 spectral width using CH_2-selected E-HSQC[23] spectra. Moreover, separated HMBC correlation nearer part of chemical shifts of carbon by ct-HMBC[19] of sharp line shape in F_1 (^{13}C). The

Table 1. ^{13}C and ^{1}H NMR chemical shift of saccharides **1**, **2** and **3**

		1				**2**				**3**			
		δ_C	δ_H		J_{HH}	δ_C	δ_H		J_{HH}	δ_C	δ_H		J_{HH}
Gal′	1												
	2												
	3												
	4												
	5												
	6												
Gal	1	99.11	4.98	d	3.8	99.29	5.00	d	3.7	99.38	5.00	d	3.7
	2	69.25	3.81	dd	10.6, 3.8	69.28	3.84	dd	10.5, 3.7	69.26	3.84	dd	10.3, 3.7
	3	70.22	3.88	dd	10.6, 3.3	70.26	3.91	dd	10.5, 3.2	70.20	3.91	dd	10.3, 3.5
	4	69.97	3.98	dd	3.3, 1.0	70.04	4.10	d	3.2	70.00	4.00	d	3.5
	5	71.80	3.94	ddd	6.4, 6.4, 1.0	71.85	3.95	br d	6.3	71.82	3.97	dd	6.3, 6.3
	6	61.87	3.73	d	6.4	61.92	3.75	d	6.4	61.97	3.75	d	6.3
Glc	1	90.36	5.67	d	3.5	91.68	5.71	d	3.7	91.88	5.64	d	3.5
	2	76.23	3.65	dd	10.0, 3.5	81.77	3.63	dd	9.9, 8.9	81.64	3.77	dd	9.1, 3.5
	3	72.04	3.86	dd	10.0, 9.1	72.85	3.91	dd	9.9, 8.9	72.86	3.88	dd	10.1, 9.1
	4	70.22	3.56	dd	10.0, 9.1	70.17	3.57	dd	10.1, 8.9	70.41	3.54	dd	10.1, 9.5
	5	71.80	4.12	ddd	10.0, 4.3, 2.0	71.93	4.11	ddd	10.1, 3.9, 2.0	71.95	4.12	ddd	9.5, 4.3, 1.9
	6	66.65	4.00	dd	11.2, 4.3	66.80	4.04	dd	11.3, 3.9	67.19	4.02	dd	11.3, 4.3
			3.70	d	11.2, 20		3.71	dd	11.3, 2.11		3.72	dd	11.3, 1.9
Glc′	1	97.20	5.11	d	3.8	98.70	5.25	d	3.7	99.83	5.32	d	3.6
	2	72.04	3.56	dd	9.8, 3.8	77.83	3.67	dd	10.1, 3.7	81.26	3.62	dd	10.3, 3.6
	3	73.65	3.73	dd	9.8, 9.1	72.33	3.84	dd	10.1, 9.1	72.76	3.82	dd	10.3, 8.7
	4	70.09	3.44	dd	10.2, 9.1	70.34	3.49	dd	10.1, 9.1	70.55	3.47	dd	9.5, 8.7
	5	72.63	3.90	ddd	10.2, 4.7, 2.3	73.29	3.88	ddd	10.0, 5.7, 2.0	73.05	3.87	ddd	9.5, 4.7, 2.2
	6	61.10	3.82	dd	10.3, 4.7	61.24	3.88	m		61.15	3.84	m	
			3.76	dd	10.3, 2.3		3.75	m			3.76	m	
Glc″	1					98.30	5.08	d	3.7	98.60	5.22	d	3.5
	2					71.99	3.61	dd	10.3, 3.7	77.81	3.61	dd	10.4, 3.5
	3					73.48	3.82	dd	9.0, 10.3	72.10	3.97	dd	10.4, 9.0
	4					70.17	3.48	dd	10.2, 9.0	70.23	3.53	dd	10.2, 9.0
	5					72.73	3.98	ddd	10.2, 4.2, 2.2	72.79	3.97	ddd	10.2, 5.4, 2.0
	6					61.15	3.86	m		60.99	3.81	m	
Glc‴	1						3.79						
	2									98.5	5.07	d	3.7
	3									71.97	3.6	dd	10.1, 3.7
	4									73.52	3.82	dd	10.1, 8.9
	5									70.09	3.48	dd	10.0, 8.9
	6									72.69	3.98	ddd	10.0, 3.9, 2.5
										61.03	3.81	m	
Fru	1												
	2	62.59	3.73	s		62.20	3.99	d	11.6	62.10	3.64	d	12.6
	3						3.64	d	11.6		3.88	d	12.6
	4	105.12			8.8	105.04				104.79			
	5	76.82	4.22	d	8.8, 8.6	76.34	4.25	d	8.8	76.10	4.26	d	8.9
	6	74.41	4.10	dd		74.64	4.06	dd	8.8, 8.6	74.56	4.05	dd	8.9, 9.1
		81.90	3.84	m		82.26	3.90	m		82.34	3.92	m	9.1, 6.2, 3.9

Chemical shifts (δ) in ppm were determined relatively to the external standard sodium [2,2,3,3-$^2H_4$]-3-(trimethylsilyl)propanoate (δ_H 0.00 ppm) and 1,4-dioxane (δ_C 67.40 ppm) in D_2O.

assignments of all ^{1}H and ^{13}C signals of these saccharides **1**–**6** are shown in Tables 1 and 2.

The six saccharides formed by glucosyltransfer from β-D-G1P to raffinose and stachyose using *T. brockii* kojibiose phosphorylase were confirmed to be new oligosaccharides, 2^{G}(2-α-D-glucopyranosyl)$_m$-raffinose: $m = 1$ **1**, 2 **2** and 3 **3**, and 2^{G}(2-α-D-glucopyranosyl)$_n$-stachyose: $n = 1$ **4**, 2 **5** and 3 **6**.

3. Experimental

3.1. Saccharides

Crystalline raffinose (*O*-α-D-galactopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside) and stachyose (*O*-α-D-galactopyranosyl-(1→6)-*O*-α-D-galactopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside) were purchased from Wako Pure

Table 2. ^{13}C and ^{1}H NMR chemical shift of saccharides **4**, **5** and **6**

		4				**5**				**6**			
		δ_C	δ_H		J_{HH}	δ_C	δ_H		J_{HH}	δ_C	δ_H		J_{HH}
Gal′	1	98.71	4.99	d	3.7	98.70	4.99	d	3.5	98.89	4.99	d	3.7
	2	70.30	3.84	m		69.07	3.82	m		69.08	3.82	m	
	3	69.07	3.82	m		70.28	3.84	m		70.26	3.86	m	
	4	70.03	3.98	s		70.01	3.98	m		70.04	3.98	s	
	5	71.78	4.00	m		71.78	3.99	m		71.78	4.11	ddd	
	6	61.94	3.74	s		61.93	3.47	m		61.96	3.74	m	
Gal	1	98.97	4.99	d	3.7	99.13	4.98	d	3.2	99.22	5.00	d	3.8
	2	69.22	3.83	dd	10.8, 3.7	69.19	3.81	m	10.5, 3.8	69.25	3.84	dd	10.6, 3.8
	3	70.17	3.91	d	10.8	70.15	3.91	d	11.0	70.10	3.93	m	
	4	70.11	4.04	s		70.11	4.03	d	3.0	70.12	4.04	d	2.9
	5	69.55	4.15	m		69.54	4.14	dd	7.7, 4.6	69.62	4.73	dd	7.3, 4.7
	6	67.17	3.87	dd	10.8, 8.1	67.19	3.85	d	7.7	67.27	3.87	m	
			3.73	d	10.8		3.70	d	4.6		3.72	m	
Glc	1	90.41	5.69	d	3.7	91.63	5.70	d	3.5	91.91	5.64	d	3.3
	2	76.27	3.66	dd	10.5, 2.7	81.75	3.60	dd	10.1, 3.5	81.42	3.74	dd	10.3, 3.3
	3	72.13	3.87	dd	10.5, 8.8	72.84	3.89	dd	10.1, 8.9	72.98	3.87	dd	10.3, 9.0
	4	70.32	3.55	dd	9.7, 8.8	70.19	3.53	dd	10.5, 8.9	70.19	3.58	dd	10.0, 9.0
	5	71.67	4.14	d	9.7	71.78	4.10	ddd	10.5, 4.2, 2.0	71.78	4.10	m	
	6	66.62	4.01	dd	11.1, 3.9	66.72	4.03	d	11.0, 4.2	66.57	3.69	m	
			3.70	d	3.9		3.68	d	10.0, 2.0		4.08	m	
Glc′	1	97.24	5.12	d	3.9	98.66	5.24	d	3.6	99.55	5.32	d	3.2
	2	72.06	3.58	dd	10.6, 3.9	77.73	3.64	dd	10.2, 3.6	80.93	3.63	dd	10.5, 3.2
	3	73.68	3.75	dd	10.6, 8.9	72.28	3.82	dd	10.2, 8.9	72.74	3.82	dd	10.5, 8.8
	4	70.11	3.44	dd	9.8, 8.9	70.28	3.48	dd	9.8, 8.9	70.51	3.47	dd	10.0, 8.8
	5	72.64	3.92	m		73.21	3.86	dd	9.8, 3.7	73.05	3.87	m	
	6	61.11	3.85	d	10.8	61.19	3.86	d	12.2	61.13	3.85	m	
			3.79	m				dd	12.2, 3.7		3.76	m	
Glc″	1					98.24	5.07	d	4.0	98.33	5.23	d	3.1
	2					71.95	3.58	dd	10.2, 4.0	77.71	3.61	dd	9.1, 3.1
	3					73.43	3.80	dd	10.2, 8.8	72.08	3.95	dd	9.1, 8.8
	4					70.11	3.46	dd	9.7, 8.8	70.21	3.93	dd	10.1, 8.8
	5					72.67	3.96	ddd	9.7, 1.7, 1.0	72.80	3.96	dd	10.1, 4.7
	6					61.07	3.82	m		60.99	3.81	m	
Glc‴	1									98.40	5.23	d	3.4
	2									71.97	3.60	dd	10.0, 8.6
	3									73.49	3.82	dd	10.0, 8.6
	4									70.04	3.60	dd	9.7, 3.4
	5									72.74	3.60	dd	9.7, 3.4
	6									61.03	3.97	dd	10.5, 8.6
											3.81	m	
Fru	1	62.59	3.57	s		62.13	3.97	d	12.2	62.07	3.87	d	12.2
							3.64	d	12.2		3.64	d	12.2
	2	105.16		d	8.8	105.14				104.82			
	3	76.90	4.23	dd	8.8, 8.6	76.27	4.24	d	9.0	76.05	4.26	d	8.8
	4	74.43	4.11	m		74.58	4.04	dd	9.0, 8.3	74.52	4.04	dd	8.8, 8.7
	5	81.94	3.85	m		82.24	3.88	ddd	8.3, 3.2, 0.8	82.33	3.92	m	
	6	62.86	3.86			63.27	3.83	m		63.42	3.85	m	
			3.78										

Chemical shifts (δ) in ppm were determined relatively to the external standard sodium [2,2,3,3-$^{2}H_4$]-3-(trimethylsilyl)propanoate (δ_H 0.00 ppm) and 1,4-dioxane (δ_C 67.40 ppm) in D_2O.

Chemical Industries, Ltd, Osaka, Japan. Kojibiose and β-D-glucose 1-phosphate (β-D-G1P) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

3.2. Enzyme

Kojibiose phosphorylase was purified from a cell-free extract of *T. brockii ATCC 35047*.[14]

3.3. High performance anion-exchange chromatography (HPAEC)

The oligosaccharides was analyzed using a Dionex Bio LC Series apparatus equipped with an HPLC carbohydrate column (Carbo Pack PA-1, inert styrene divinylbenzene polymer) and a pulsed amperometric detection (PAD).[24,25] The mobile phase consisted of eluent A (150 mM NaOH) with a sodium acetate gradient as follows: 0–1 min, 25 mM; 1–2 min, 25–50 mM; 2–20 min, 50–200 mM; 20–22 min, 500 mM; 22–30 min, 25 mM; using a flow rate of 1.0 mL/min. The applied PAD potentials for E_1 (500 ms), E_2 (100 ms) and E_3 (50 ms) were 0.1, 0.6 and −0.6 V,[26] respectively, and the output range was 1 μC.

3.4. Isolation of the oligosaccharide synthesized from raffinose and β-D-G1P by kojibiose phosphorylase

Reaction mixture (24.6 mL), which contains kojibiose phosphorylase (5.76 units), raffinose (500 mg), β-D-G1P (273 mg) and acetate buffer (0.05 M, pH 5.5), was incubated at 50 °C for 120 h. After terminating the reaction by heating in a boiling water bath for 5 min, reaction mixture 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 (3.5 × 29 cm) and successively eluted with water, 5% EtOH, 10% EtOH and 13% EtOH. The 10% EtOH fraction, which contained a mixture of **2** and **3**, was concentrated and purified using preparative HPLC. A portion of the saccharides **2** and **3** mixture was purified using an HPLC system (JASCO GULLIVER, Tokyo, Japan) equipped with an ODS column (TSKgel ODS-80Ts, 20 mm × 25 cm, Tosoh, Tokyo, Japan) at 35 °C, and eluted with water at 3.5 mL/min, using refractive index detection. Saccharides **1** (96.0 mg), **2** (47.5 mg) and **3** (29.4 mg) were obtained by repeated HPLC purification.

3.5. Isolation of the oligosaccharide synthesized from stachyose and β-D-G1P by kojibiose phosphorylase

The reaction mixture (24.6 mL), which contains kojibiose phosphorylase (5.76 units), stachyose (500 mg), β-D-G1P (273 mg) and acetate buffer (0.05 M, pH 5.5), was incubated at 50 °C for 168 h. After terminating the reaction by heating in a boiling water bath for 5 min, reaction mixture was loaded onto a carbon-Celite column (3.5 × 29 cm) and successively eluted with water, 5% EtOH, 8% EtOH, 10% EtOH and 13% EtOH. Saccharide **4** (eluted with 10% EtOH), saccharide **5** (eluted with 13% EtOH) and saccharide **6** (eluted with 13% EtOH) were purified using HPLC under similar conditions described above. Saccharides **4** (78.3 mg), **5** (34.5 mg) and **6** (16.1 mg) were obtained by repeated HPLC purification.

3.6. Methylation and methanolysis

Methylation of the oligosaccharides was carried out by the method of Hakomori.[27] 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 MeOH and analyzed using gas chromatography.

3.7. 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.

3.8. 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).

3.9. NMR measurements

Each oligosaccharide **1**, **2**, **3**, **4**, **5** and **6** (10, 10, 10, 10, 5 and 5 mg, respectively) was dissolved in 0.5 mL D_2O. NMR spectra were recorded at room temperature 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 ppm 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–^{1} COSY,[15,16] HSQC[17] and CH_2-selected E-HSQC[19] 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).[18] The TOCSY mixing time (264 ms) was composed of MLEV-17 composite pulses guarded by trim pulse (2.5 ms). HMBC spectra were obtained using the pulse sequences of ct-HMBC2 proposed by Furihata and Seto,[20] and its slightly modified version without gradient pulses, and the conventional HMBC pulses sequences.[21]

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