Available online at www.sciencedirect.com
SCIENCE DIRECT®
Carbohydrate Research 338 (2003) 879–885
CARBOHYDRATE RESEARCH
www.elsevier.com/locate/carres

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

Hideki Okada,[a] Eri Fukushi,[b] Shuichi Onodera,[a] Tomoyuki Nishimoto,[c] Jun Kawabata,[b] Masanori Kikuchi,[a] Norio Shiomi[a,*]

[a]*Department of Food Production and Utility Development, Graduate School of Dairy Science Research, Rakuno Gakuen University, Ebetsu 069-8501, Japan*
[b]*Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan*
[c]*Hayashibara Biochemical Laboratories, Inc., Okayama 700-0834, Japan*

Received 3 September 2002; received in revised form 3 December 2002; accepted 3 January 2003

Abstract

Five novel oligosaccharides (tetra-, penta- and hexa-saccharides) were synthesized by glucosyltransfer from β-D-glucose 1-phosphate to isokestose (*O*-β-D-fructofuranosyl-(2→1)-*O*-β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside) or nystose (*O*-β-D-fructofuranosyl-(2→1)-*O*-β-D-fructofuranosyl-(2→1)-*O*-β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside) using *Thermoanaerobacter brockii* kojibiose phosphorylase. The oligosaccharides were identified as 2(2-α-D-glucopyranosyl)$_m$isokestose; [*O*-α-D-glucopyranosyl-(1→2)]$_m$-*O*-[β-D-fructofuranosyl-(2→1)]$_2$-α-D-glucopyranoside: $m = 1$, 2, and 3, and 2(2-α-D-glucopyranosyl)$_n$nystose; [*O*-α-D-glucopyranosyl-(1→2)]$_n$-*O*-[β-D-fructofuranosyl-(2→1)]$_3$-α-D-glucopyranoside: $n = 1$ and 2 using gas liquid chromatography analysis of the methyl derivatives, and MALDI-TOF-MS and NMR measurements of the newly formed oligosaccharides. ^{1}H, ^{13}C NMR signals of each saccharide were assigned using 2D-NMR techniques, including COSY, HSQC, HSQC–TOCSY, HMBC, CH_2-selected E-HSQC, and CH_2-selected E-HSQC–TOCSY. © 2003 Elsevier Science Ltd. All rights reserved.

Keywords: Kojibiose phosphorylase; Oligosaccharide; Glucosylated isokestose; Glucosylated nystose; ^{1}H NMR; ^{13}C NMR

1. Introduction

We have previously examined the formation of oligosaccharides as nutritional and functional food ingredients, such as inulo-oligosaccharides,[1,2] fructo-oligosaccharides,[3] fructosyl xylosides,[4] and fructosyl lactosucroses[5] by using *Penicillium purpurogenum* inulinase,[1,2] *Scopulariopsis brevicaulis* fructosyltransferase,[3,4] and asparagus 1^F-fructosyltransferase,[5–7] respectively. Among these oligosaccharides, fructosyl xyloside suppressed serum glucose and insulin responses,[8] and promoted the absorption of calcium and magnesium in rats.[8] Both fructosyl lactosucrose and fructo-oligosaccharide were shown to selectively stimulate the growth of *Bifidobacteria*.[5,9]

Recently, our studies have involved the isolation, purification, and characterization of kojibiose phosphorylase from *Thermoanaerobacter brockii*, which has been shown to catalyze the glucosyltransfer from β-D-glucose 1-phosphate to glucosyl residue of several oligosaccharides.[10]

Now, we report on the synthesis of five new oligosaccharides; one tetra-, two penta-, and two hexa-saccharides. These have lower osmotic pressure than di- and tri-saccharides such as fructosyl xyloside, isokestose, raffinose and lactosucrose making them superior for intestinal conditions. The oligosaccharides were synthe-

* Corresponding author. Tel.: +81-11-3884754; fax: +81-11-3875848
E-mail address: n-shiomi@rakuno.ac.jp (N. Shiomi).

0008-6215/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0008-6215(03)00020-X

sised by glucosyltransfer from β-D-glucose 1-phosphate to isokestose and nystose using the kojibiose phosphorylase. Subsequent structure confirmation was provided by methylation analysis, and MALDI-TOF-MS and NMR measurements.

2. Results and discussion

The course of the reaction for the syntheses of saccharides **1**, **2**, and **3** from a mixture of isokestose and β-D-glucose 1-phosphate (β-D-G1P) using kojibiose phosphorylase was investigated. As shown in Fig. 1, saccharides **1**, **2**, and **3** were produced from isokestose and β-D-G1P after 48 h's reaction. As shown in Fig. 2,

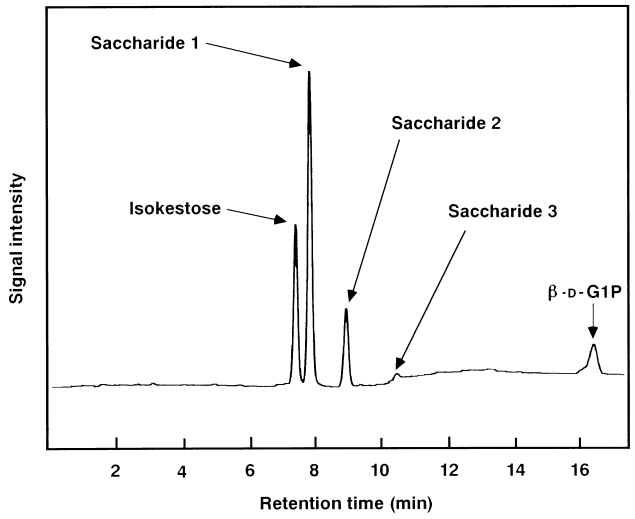

Fig. 1. HPAEC of saccharides produced from isokestose and β-D-G1P by kojibiose phosphorylase. The enzyme incubation was carried out with 40 μmol/mL isokestose and 38.5 μmol/mL β-D-G1P in the mixture for 48 h.

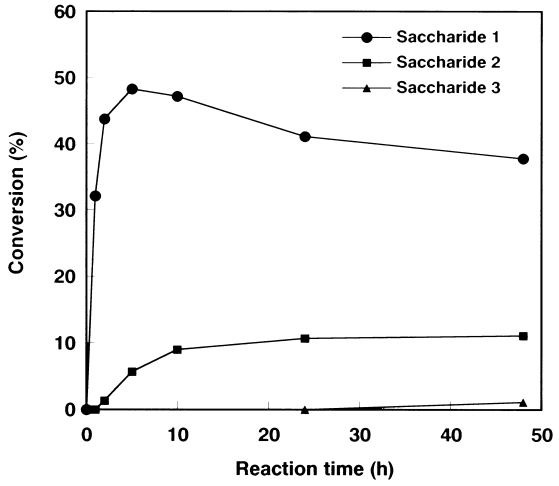

Fig. 2. Time course of formation of saccharides **1**, **2**, and **3** from isokestose and β-D-G1P by kojibiose phosphorylase. The conversion (%) was shown as the ratio (w/w) of the Saccharide **1**, **2**, or **3** to the isokestose.

the formation of saccharide **1** proceeded significantly faster than those of saccharides **2** and **3**, furthermore, formation of saccharide **3** was slow, even after 24 h. The maximum production of saccharide **1** was reached at reaction time of 5 h, which then gradually decreased. Saccharide **1** was considered to be used as the precursor for production of saccharides **2** and **3**, which have higher degrees of polymerization. Saccharides **1**, **2**, and **3** were isolated from the reaction mixture A by successive chromatographic procedures using carbon-Celite, gel filtration and ODS columns. A high yield of saccharide **1** (36.9%(w/w)) in relation to the amount of substrates (the sum of the donor and the acceptor saccharides) was finally obtained as powder.

The syntheses of saccharides **4** and **5** from a mixture of nystose and β-D-G1P using kojibiose phosphorylase was also examined. As shown in Fig. 3, saccharides **4** and **5** were produced from nystose and β-D-G1P at 48 h's reaction. As shown in Fig. 4, the course of the reaction for the formation of saccharides **4** and **5** was similar to that of saccharides **1** and **2**. Saccharides **4** and **5** were isolated from reaction mixture B following similar chromatographic procedures as described above, and similarly, a high yield of saccharide **4** (21.9%(w/w)) was obtained finally.

Saccharides **1** ($[\alpha]_D^{20}$ + 65.18), **2** ($[\alpha]_D^{20}$ + 89.9), **3** ($[\alpha]_D^{20}$ not determined), **4** ($[\alpha]_D^{20}$ + 48.58), and **5** ($[\alpha]_D^{20}$ + 66.36) were shown to be homogeneous using HPAEC ($t_{R\ sucrose}$: 1.57, 1.79, 2.09, 2.17, 2.63). The degrees of polymerization were established as 4 (saccharide **1**), 5 (saccharides **2** and **4**), and 6 (saccharides **3** and **5**), as shown by measurements of $[M + Na]^+$ ions (m/z: **1**, 689; **2** and **4**, 851; **3** and **5**, 1013) using TOF-MS, and analysis of the molar ratios of D-glucose and D-fructose in the acid hydrolysates of the oligosaccharides.

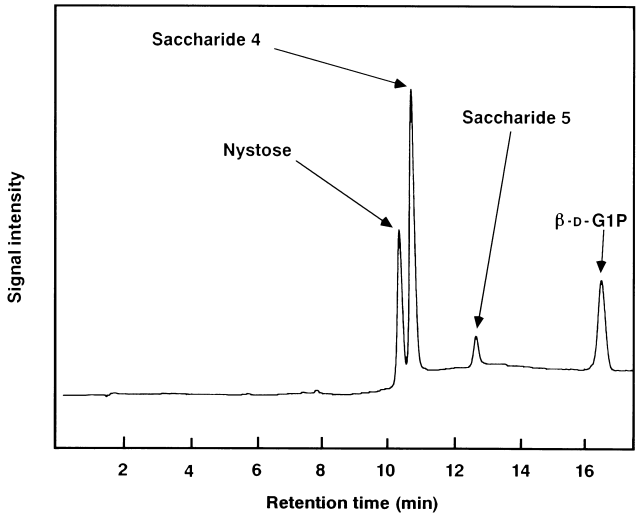

Fig. 3. HPAEC of saccharides produced from nystose and β-D-G1P by kojibiose phosphorylase. The enzyme incubation was carried out with 30 μmol/mL nystose and 38.5 μmol/mL β-D-G1P in the mixture for 48 h.

From the GC analysis, relative retention times of the methanolysates of the permethylated saccharides were investigated [t_R, retention time of methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucoside = 1.0 (retention time, 8.70 min)]. The methanolysate of permethylated saccharide **1** exhibited seven peaks corresponding to methyl 2,3,4,6-tetra-*O*-methyl-D-glucoside (t_R, 1.07 and 1.42), methyl 1,3,4,6-tetra-*O*-methyl-D-fructoside (t_R, 1.07 and 1.27), methyl 3,4,6-tri-*O*-methyl-D-fructoside (t_R, 2.66 and 3.99), and methyl 3,4,6-tri-*O*-methyl-D-glucoside (t_R, 2.96 and 3.54). The methanolysates of permethylated saccharides **2**, **3**, **4**, and **5** also exhibited seven peaks, which corresponded to the same methyl glycosides as those from saccharide **1**. The two peaks that correspond to methyl 3,4,6-tri-*O*-methyl-D-glucoside of the methanolysate of the permethylated saccharides **2**, **3**, and **5** were larger than those of permethylated saccharides **1** and **4**. Peaks of methyl 3,4,6-tri-*O*-methyl-D-glucoside indicating 1,2-bond of each saccharide were increased by additional units of glucose.

From these findings, saccharide **1**, **2**, **3**, **4**, and **5** were proved to be 2-α-D-glucosyl isokestose, 2(2-α-D-glucosyl)$_2$isokestose, 2(2-α-D-glucosyl)$_3$isokestose, 2-α-D-glucosyl nystose, and 2(2-α-D-glucosyl)$_2$nystose, respectively.

The structural confirmation of the saccharides **1**, **2**, **3**, **4**, and **5** according to ^{1}H, ^{13}C NMR analyses and the subsequent complete assignment of ^{1}H, ^{13}C NMR signals of the five saccharides were carried out using 2D-NMR techniques, including COSY,[11,12] HSQC,[13] HSQC–TOCSY,[14] HMBC,[15,16] CH$_2$-selected E-HSQC,[17] and CH$_2$-selected E-HSQC–TOCSY.[17] Each glucose and fructose residue of saccharides **1**, **2**, **3**, **4**, and **5** is represented as Glc, Glc′, Glc″, Glc‴, Fru, Fru′, and Fru″ as indicated in Fig. 5. The proton and carbon

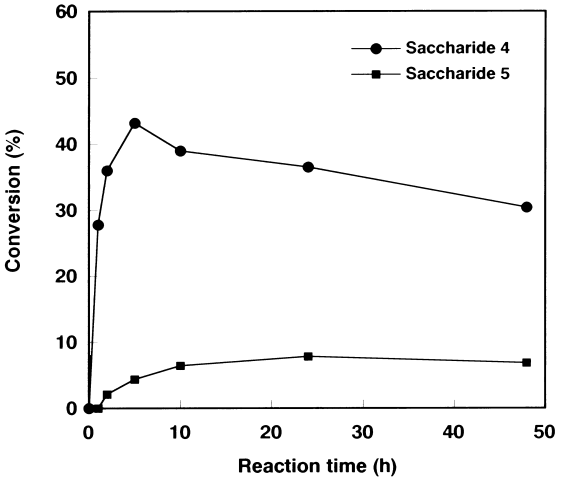

Fig. 4. Time course of formation of saccharides **4** and **5** from nystose and β-D-G1P by kojibiose phosphorylase. The conversion (%) was shown as the ratio (w/w) of the Saccharide **4** or **5** to the nystose.

positions in a particular residue are represented by, e.g. H-1-Glc and C-1-Fru, respectively.

First, the NMR spectra of **1** were analyzed. From two anomeric protons (δ_H 5.71 ppm, d, 3.6 Hz and δ_H 5.13 ppm, d, 4.0 Hz) and carbons (δ_C 90.57 ppm and δ_C 96.77 ppm) in **1**, two glucose residues were assigned by ^{1}H–^{1}H COSY, HSQC, and HSQC–TOCSY spectra. The inter residual HMBC correlation between one of the anomeric proton (δ_H 5.71 ppm) and one of the quaternary carbon (δ_C 104.53 ppm) assigned these 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 3.88 ppm, d, 9.5 Hz and δ_H 3.80 ppm, d, 9.5 Hz) and between C-1-Fru (δ_C 62.01 ppm) and H-3-Fru (δ_H 4.30 ppm, d, 8.8 Hz) as well as ^{1}H–^{1}H COSY correlations enabled the assignments of Fru residue. The connectivity of Glc′ (1→2) Glc and Fru′ (2→1) Fru were also deduced from HMBC correlations between C-2-Glc (δ_C 75.85 ppm) and H-1-Glc′ (δ_H 5.13 ppm) and between C-2-Fru′ (δ_C 104.50 ppm) and H-1-Fru (δ_H 3.88 ppm). The characteristic *J* (H-1, H-2) values of the Glc ($J = 3.6$ Hz) and Glc′ ($J = 4.0$ Hz) determined the glucosyl bonds were α forms as shown in sucrose, respectively. Six methylene signals that overlapped in the narrow region were separated by limiting the F_1 spectral width using CH$_2$-selected E-HSQC. Moreover, ^{1}H–^{1}H coupling patterns of overlapping ^{1}H signals were extracted from SPT difference spectra.[18] This technique, however, could not utilized for H-6 of glucose residues because H-5 of glucose residues were also in the crowded region. The *J* (H-5, H-6) values of fructose residues could not be obtained by a first-order analysis because of their strong coupling to each other.

The tetrasaccharide unit of **1** in saccharides **2** and **3** was determined in the same manner as in **1**. 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 between H-1-Glc″ (δ_H 5.08 ppm, d, 3.7 Hz) and C-2-Glc′ (δ_C 76.81 ppm) in **2** and between H-1-Glc″ (δ_H 5.27 ppm, d, 3.5 Hz) and C-2-Glc′ (δ_C 78.67 ppm) and between H-1-Glc‴ (δ_H 5.09 ppm, d, 3.7 Hz) and C-2-Glc″ (δ_C 77.66 ppm) in **3**, respectively. The tetrasaccharide unit of **1** in saccharide **4** and the pentasaccharide unit of **2** in saccharide **5** were determined in the same manner as in **1** and **2**, respectively. Further fructosyl linkages, Fru″ (2→1) Fru′ in **4** and **5**, were determined by additional HMBC correlation between H-1-Fru′ (δ_H 3.87 ppm, d, 10.3 Hz in **4** and δ_H 3.85 ppm, d, 10.9 Hz in **5**) and C-2-Fru″ (δ_C 104.65 ppm in **4** and δ_C 104.54 ppm in **4**), respectively. The assignments of all ^{1}H and ^{13}C signals of these saccharides **1**–**5** are shown in Table 1. These values agreed well with those of the kojibiose,[19] isokestose,[20,21] and nystose.[21]

The five saccharides formed by glucosyltransfer from β-D-G1P to isokestose and nystose using *Thermoanaer-*

Table 1
^{1}H and ^{13}C spectral data (δ in ppm, J in Hz) for **1**–**5**

Saccharide **1**						Saccharide **2**						Saccharide **3**						Saccharide **4**						Saccharide **5**					
		δ_C	δ_H		J (H,H)			δ_C	δ_H		J (H,H)			δ_C	δ_H		J (H,H)			δ_C	δ_H		J (H,H)			δ_C	δ_H		J (H,H)
Fru	1	62.01	3.88	d	9.5	Fru	1	61.79	3.98	d	11.1	Fru	1	61.83	3.89	d	8.7	Fru	1	62.15	3.89	d	9.5	Fru	1	62.13	3.95	d	10.8
			3.80	d	9.5				3.73	d	11.1				3.72	d	8.7				3.82	d	9.5				3.84	d	10.8
	2	104.53					2	104.53					2	104.32					2	104.63					2	104.69			
	3	77.54	4.30	d	8.8		3	76.74	4.34	d	8.5		3	76.59	4.32	d	8.8		3	77.83	4.27	d	8.7		3	72.24	4.31	d	8.6
	4	74.54	4.09	dd	8.8,8.6		4	74.48	4.04	dd	8.5,8.1		4	74.29	4.02	dd	9.0,8.8		4	74.70	4.08	dd	8.7,9.8		4	74.45	4.04	dd	8.6,8.1
	5	81.77	3.81	m			5	82.10					5	82.13	3.83	m			5	81.86	3.79	ddd	9.8,6.9,3.5		5	82.11	3.82	m	
	6	62.73	3.84	m			6	62.98	3.08	m			6	63.10	3.80	m			6	62.85	3.82	dd	12.7,3.5		6	62.80	3.81	m	
			3.77	m																	3.75	dd	12.7,6.9				3.77	m	
Fru′	1	61.11	3.76	d	12.2	Fru′	1	61.50	3.79	d	14.3	Fru′	1	61.49	3.65	d	11.2	Fru′	1	61.52	3.87	d	10.3	Fru′	1	62.50	3.85	d	10.9
			3.69	d	12.2				3.70	dd	14.3				3.74	d	11.2				3.72	d	10.3				3.75	d	10.9
	2	104.50					2	104.71					2	104.76					2	103.97					2	104.20			
	3	77.58	4.18	d	8.7		3	77.33	4.20	d	8.5		3	77.09	4.20	d	8.5		3	78.25	4.21	d	8.6		3	78.05	4.26	d	8.6
	4	75.33	4.09	dd	8.7,7.2		4	75.14	4.08	dd	8.5,7.9		4	75.19	4.06	dd	8.5,8.4		4	75.34	4.06	dd	8.6,9.4		4	74.85	4.11	dd	8.6,8.1
	5	81.99	3.87	m			5	81.93					5	81.79	3.83	m			5	82.06	3.85	m			5	81.56	3.81	m	
	6	63.29	3.88	m			6	63.14	3.84	m			6	63.04	3.84	m			6	63.32	3.82	m			6	62.38	3.85	m	
			3.83	m					3.81	m					3.82	m					3.75	m					3.75	m	
Glc	1	90.57	5.71	d	3.6	Glc	1	91.82	5.67	d	3.4	Glc	1	92.04	5.61	d	3.5	Fru″	1	61.46	3.75	d	11.9	Fru″	1	61.47	3.74	d	12.0
	2	75.85	3.66	dd	10.3,3.6		2	80.19	3.61	dd	9.9,3.4		2	79.51	3.83	dd	9.6,3.5				3.68	d	11.9				3.68	d	12.0
	3	72.01	3.85	dd	10.3,9.2		3	72.74	3.90	dd	9.9,8.8		3	72.79	3.87	dd	10.1,9.6		2	104.65					2	104.54			
	4	70.06	3.50	dd	10.1,9.2		4	69.78	3.49	dd	9.6,8.8		4	69.48	3.54	dd	10.1,9.1		3	77.60	4.18	d	8.3		3	77.53	4.18	d	8.6
	5	73.04	3.94	ddd	10.1,3.4,2.4		5	72.95	3.90	m			5	72.96	3.90	m			4	75.21	4.10	dd	11.1,8.3		4	75.12	4.09	dd	8.6,8.1
	6	61.07	3.83	m			6	60.97	3.81	m			6	60.78	3.81	m			5	82.02	3.85	m			5	81.79	3.84	m	
			3.78	m											3.79	m			6	63.13	3.82	m			6	62.92	3.83	m	
																					3.75	m					3.75	m	
Glc′	1	96.77	5.13	d	4.0	Glc′	1	96.96	5.31	d	3.6	Glc′	1	97.17	5.36	d	3.6	Glc	1	90.71	5.71	d	3.7	Glc	1	91.52	5.69	d	3.7
	2	72.13	3.57	dd	10.3,4.0		2	76.81	3.69	dd	10.1,3.6		2	78.67	3.64	dd	10.3,3.6		2	75.82	3.65	dd	10.2,3.7		2	78.39	3.62	dd	10.1,3.7
	3	73.73	3.76	dd	10.3,9.2		3	72.38	3.83	dd	10.1,9.0		3	72.68	3.82	dd	10.3,9.1		3	72.12	3.85	dd	10.2,9.0		3	72.52	3.88	dd	10.1,8.9
	4	70.19	3.44	dd	10.7,9.2		4	70.52	3.47	dd	9.6,9.0		4	70.55	3.44	dd	9.3,9.1		4	70.19	3.48	dd	10.1,9.0		4	69.96	3.48	dd	9.6,8.9
	5	72.65	3.91	ddd	10.7,4.4,2.2		5	73.33	3.83	m			5	73.36	3.80	ddd	9.3,3.0,2.5		5	73.16	3.94	ddd	10.1,6.1,2.5		5	73.00	3.94	m	
	6	61.19	3.84	m			6	61.29	3.88	m			6	61.24	3.85	d	12.1,2.5		6	61.20	4.04	dd	9.1,6.1		6	61.01	3.79	m	
			3.77	m					3.75	m					3.74	d	12.1,3.0				3.80	dd	9.1,2.5						
						Glc″	1	97.56	5.08	d	3.7	Glc″	1	96.78	5.27	d	3.5	Glc	1	96.77	5.12	d	3.7	Glc′	1	95.72	5.36	d	3.6
							2	72.03	3.59	dd	10.2,3.7		2	77.66	3.59	dd	10.1,3.5		2	72.28	3.54	dd	9.2,3.7		2	76.39	3.67	dd	10.3,3.6
							3	73.38	3.91	dd	10.2,9.1		3	71.82	4.03	dd	10.1,8.7		3	73.83	3.76	dd	10.0,9.2		3	72.18	3.87	dd	10.3,8.8
							4	70.27	3.45	dd	9.6,9.1		4	70.36	3.48	dd	9.8,8.7		4	70.28	3.43	dd	10.0,9.9		4	70.13	3.48	dd	9.6,8.8
							5	72.63	3.96	m			5	72.59	3.98	ddd	9.8,3.5,3.5		5	72.74	3.90	ddd	9.9,5.6,2.2		5	73.33	3.79	m	
							6	61.20	3.84	m			6	61.05	3.79	d	3.5		6	61.30	3.83	dd	11.1,2.2		6	61.24	3.86	m	
									3.78	m											3.77	dd	11.1,5.6				3.75	m	
												Glc‴	1	98.28	5.09	d	3.7							Glc″	1	97.05	5.10	d	3.7
													2	72.08	3.57	dd	9.8,3.7								2	72.08	3.57	dd	9.3,3.7
													3	73.53	3.78	dd	9.8,9.4								3	73.43	3.81	dd	10.2,9.3
													4	70.13	3.45	dd	10.1,9.4								4	70.22	3.44	dd	10.2,9.3
													5	72.62	3.92	ddd	10.1,3.3,3.3								5	72.65	3.92	m	
													6	61.00	3.80	d	3.3								6	61.19	3.84	m	
																											3.79	m	

Chemical shifts of ^{1}H (δ_H) and ^{13}C in ppm were determined relatively to the external standard of sodium [2,2,3,3-$^{2}H_4$]-3-(trimethylsilyl) propanoate in D_2O (δ_H 0.00 ppm) and 1,4-dioxane (δ 67.40, ^{13}C) in D_2O, respectively.

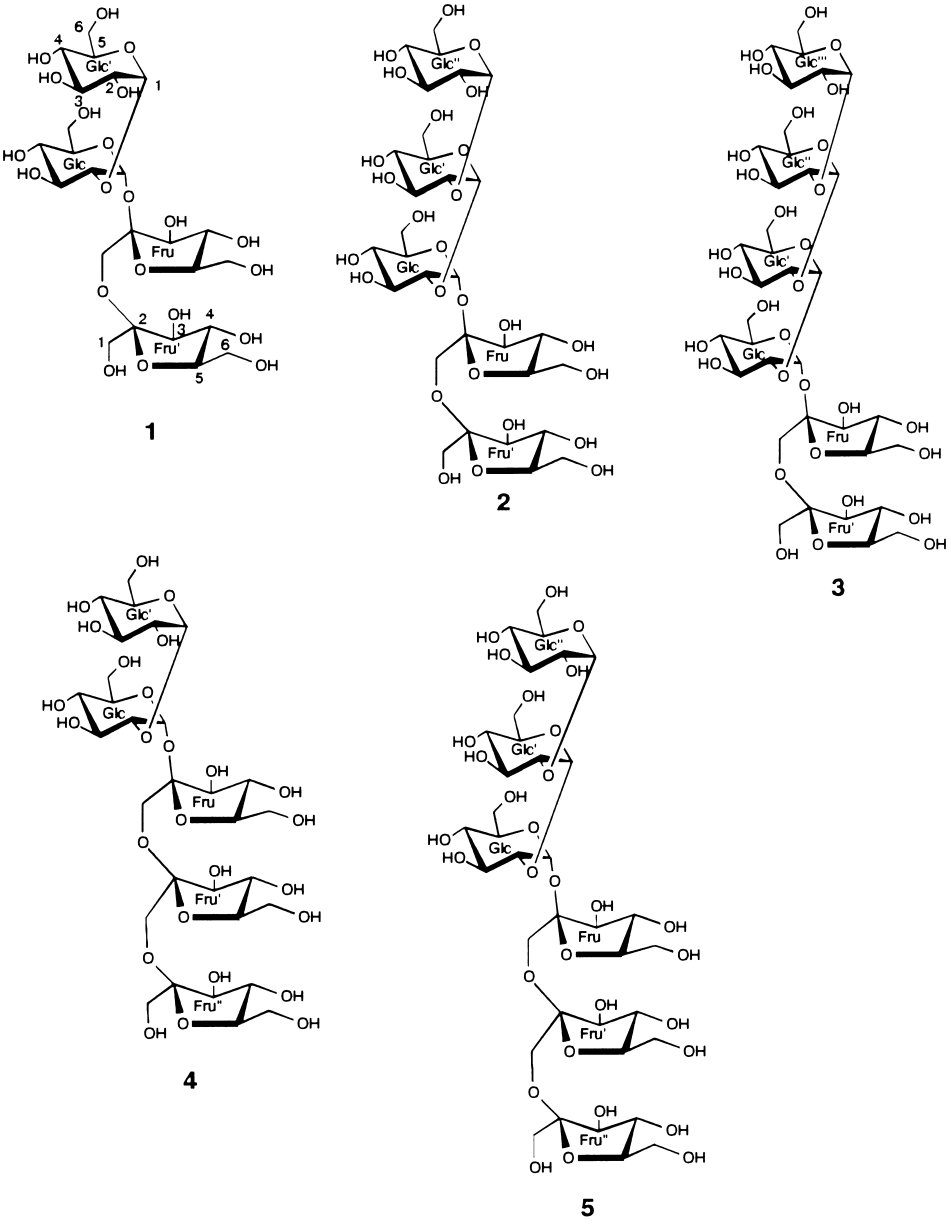

Fig. 5. Structures of saccharides **1**, **2**, **3**, **4**, and **5** formed by kojibiose phosphorylase.

obacter brockii kojibiose phosphorylase were confirmed to be new saccharides, 2(2-α-D-glucopyranosyl)$_m$isokestose; [*O*-α-D-glucopyranosyl-(1→2)]$_m$-*O*-[β-D-fructofuranosyl-(2→1)]$_2$-α-D-glucopyranoside: $m=1$ (**1**), 2 (**2**), and 3(**3**), and 2(2-α-D-glucopyranosyl)$_n$nystose; [*O*-α-D-glucopyranosyl-(1→2)]$_n$-*O*-[β-D-fructofuranosyl-(2→1)]$_3$-α-D-glucopyranoside: $n=1$ (**4**) and 2 (**5**).

3. Experimental

3.1. Materials

Kojibiose phosphorylase was purified from a cell-free extract of *T. brockii* ATCC 35047.[10] β-D-Glucose 1-phosphate (β-D-G1P) and kojibiose were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Crystalline isokestose (*O*-β-D-fructofuranosyl-(2→1)-*O*-β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside) and nystose (*O*-β-D-fructofuranosyl-(2→1)-*O*-β-D-fructofuranosyl-(2→1)-*O*-β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside) were prepared from sucrose using a *Scopulariopsis brevicaulis* enzyme.[3]

3.2. Enzyme assay

The reaction mixture for kojibiose phosphorolysis contained 6.4 μmol kojibiose, McIlvaine buffer (pH 5.5, Pi concentration; 102 mM), and the enzyme in a total

volume of 2.2 mL. After incubation at 60 °C for 30 min, the reaction was stopped by boiling for 10 min. One unit of enzyme activity was defined as the amount of the enzyme that liberates glucose at 1 μmol/min under the above-mentioned conditions.[10]

3.3. High performance anion-exchange chromatography (HPAEC)

The oligosaccharides were analyzed using a Dionex Bio LC Series apparatus equipped with an HPLC carbohydrate column (Carbo Pack PA1, inert styrene divinyl benzene polymer) and a pulsed amperometric detection (PAD).[22,23] The mobile phase consisted of eluent A (150 mM NaOH) and eluent B (500 mM sodium acetate in 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 E1 (500 ms), E2 (100 ms), and E3 (50 ms) were 0.1, 0.6, and -0.6 V,[24] respectively, and the output range was 1 μC.

3.4. Enzymatic synthesis of oligosaccharides

A mixture (1.0 mL) of kojibiose phosphorylase (0.1 units), isokestose (40 μmol) or nystose (30 μmol), β-D-G1P (38.5 μmol), and acetate buffer (0.1 M, pH 5.5) was incubated at 50 °C for 0, 1, 2, 5, 10, 24, and 48 h in the presence of a small amount of toluene. The reaction was terminated by heating in a boiling water bath for 5 min, and subsequently, the resulting mixture was subjected to HPAEC.

3.5. Isolation of the oligosaccharides synthesized from isokestose or nystose and β-D-G1P by kojibiose phosphorylase

Reaction mixture A (243 mL) which contains kojibiose phosphorylase (27 units), isokestose (4.68 g), β-D-G1P (2.34 g), and acetate buffer (0.1 M, pH 5.5), was incubated at 50 °C for 48 h. After terminating the reaction by heating in a boiling water bath for 5 min, the reaction mixture was concentrated to 30 mL. Concentrated reaction mixture A (10 mL) 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 (3.5×32 cm) and successively eluted with water (2 L), 5% EtOH (8 L), and 10% EtOH (6 L). These chromatographic procedures were carried out three times for each sample. Subsequently, the 5% EtOH fraction was successfully purified using gel filtration chromatography (Toyopearl HW-40S, 4.0×150 cm, Tosoh, Tokyo, Japan) with water as the solvent at a flow rate of 25 mL/h to yield saccharide **1** (2.59 g). The 10% EtOH fraction, which contained a mixture of saccharides **2** and **3**, was concentrated and purified using preparative HPLC. A portion of the saccharides **2** and **3** mixture (15 mg) 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 5 mL/min, and using refractive index detection. Saccharides **2** (306.3 mg) and **3** (18.8 mg) were obtained by repeated HPLC purification.

Reaction mixture B (243 mL), which contained kojibiose phosphorylase (27 units), nystose (4.68 g), β-D-G1P (2.34 g), and acetate buffer (0.1 M, pH 5.5) was incubated at 50 °C for 48 h. After terminating the reaction by heating in a boiling water bath for 5 min, the reaction mixture was concentrated to 30 mL. Concentrated reaction mixture B (5 mL) was loaded onto a carbon-Celite column (3.5×32 cm) and successively eluted with water (2 L), 10% EtOH (6 L), and 13% EtOH (4 L). Saccharide **4** (eluted with 10% EtOH) and saccharide **5** (eluted with 13% EtOH) were purified using HPLC under similar conditions described above. Saccharides **4** (1.54 g) and **5** (252.0 mg) were obtained by repeating the carbon-Celite column chromatography and the HPLC purifications.

3.6. Methylation and methanolysis

Methylation of the oligosaccharides was carried out by the method of Hakomori.[25] The permethylated saccharides were methanolysed 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 ca. 10 mg was dissolved in 0.5 mL D_2O. NMR spectra were recorded at 27 °C with a

Bruker AMX 500 spectrometer (^{1}H 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 ^{1}H (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. ^{1}H–^{1}H COSY,[11,12] HSQC[13] 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).[14] 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,[15] in a slightly modified version of it without gradient pulses, and the conventional pulse sequence.[16] CH_2-selected HSQC[17] spectra were measured using the pulse sequence reported previously.

Acknowledgements

This work was supported in part by Grant-aid for the promotion of high-technology centered projects from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (2001) and for Cooperative Research from Rakuno Gakuen University (2002) for which the authors express their appreciation.

References

1. Onodera, S.; Shiomi, N. *Agric. Biol. Chem.* **1988**, *52*, 2569–2576.
2. Onodera, S.; Murakami, T.; Ito, H.; Matsui, H.; Chiba, S.; Shiomi, N. *Biosci. Biotechnol. Biochem.* **1996**, *60*, 1780–1785.
3. Takeda, H.; Sato, K.; Kinoshita, S.; Sasaki, H. *J. Ferment. Bioeng.* **1994**, *77*, 386–389.
4. Takeda, H.; Kinoshita, S. *J. Ferment. Bioeng.* **1995**, *79*, 242–246.
5. Yamamori, A.; Onodera, S.; Kikuchi, M.; Shiomi, N. *Biosci. Biotechnol. Biochem* **2002**, *66*, 1419–1422.
6. Shiomi, N. *Carbohydr. Res.* **1982**, *99*, 157–169.
7. Yamamori, A.; Fukushi, E.; Onodera, S.; Kawabata, J.; Shiomi, N. *Magn. Reson. Chem.* **2002**, *40*, 541–544.
8. Fukumori, Y.; Onodera, S.; Shiomi, N. *J. Appl. Glycosci.* **2001**, *48*, 205–213.
9. Mitsuoka, T. *Bifidobacteria Microflora* **1982**, *1*, 3–24.
10. Chaen, H.; Yamamoto, T.; Nishimoto, T.; Nakada, T.; Fukuda, S.; Sugimoto, T.; Kurimoto, M.; Tsujisaka, Y. *J. Appl. Glycosci.* **1999**, *46*, 423–429.
11. Brereton, I.; Crozier, S.; Field, J.; Doddrell, D. M. *J. Magn. Reson.* **1991**, *93*, 54–62.
12. Kienlin, M.; Woonen, C. W.; Toorn, A.; Zijl, P. M. *J. Magn. Reson.* **1991**, *93*, 423–429.
13. Li, Y.-C.; Monterione, G. T. *J. Magn. Reson.* **1993**, *B101*, 315–319.
14. Willker, W.; Leibfrits, D.; Kerssebaum, R.; Bermel, W. *Magn. Reson. Chem.* **1993**, *31*, 287–292.
15. Furihata, K.; Seto, H. *Tetrahedron Lett.* **1998**, *39*, 7337–7340.
16. Bax, A.; Summers, M. F. *J. Am. Chem. Soc.* **1986**, *108*, 2093–2094.
17. Fukushi, E.; Tanabe, S.; Watanabe, M.; Kawabata, J. *Magn. Reson. Chem.* **1998**, *36*, 741–746.
18. Pachler, K. G. R.; Wessels, P. L. *J. Magn. Reson.* **1973**, *12*, 337–339.
19. Duus, J. O.; Gotfredsen, C. J.; Bock, K. *Chem. Rev.* **2000**, *100*, 4589–4614.
20. Calub, T. M.; Waterhouse, A. L.; Catterton, N. J. *Carbohydr. Res.* **1990**, *199*, 11–17.
21. Fukushi, E.; Onodera, S.; Yamamori, A.; Shiomi, N.; Kawabata, J. *Magn. Reson. Chem.* **2000**, *38*, 1005–1011.
22. Rocklin, R. D.; Pohl, C. A. *J. Liq. Chromatogr.* **1983**, *6*, 1577–1590.
23. Johnson, D. C. *Nature* **1986**, *321*, 451–452.
24. Shiomi, N.; Onodera, S.; Chatterton, N. J.; Harrison, P. A. *Agric. Biol. Chem.* **1991**, *55*, 1427–1428.
25. Hakomori, S. *J. Biochem.* **1964**, *55*, 208.