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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

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Abstract

Five novel oligosaccharides (tetra-, penta- and hexa-saccharides) were synthesized by glucosyltransfer from β-D-glucose fructofuranosyl- $(2 \rightarrow 1)$ -O- β -D-fructofuranosyl- $(2 \rightarrow 1)$ -O- β -D-fructofuranosyl- $(2 \rightarrow 1)$ - α -D-glucopyranoside) using Thermoanaerobacter brockii kojibiose phosphorylase. The oligosaccharides were identified as 2(2-α-D-glucopyranosyl), isokestose; $[O-\alpha-D-glucopyranosyl-(1\to 2)]_m-O-[\beta-D-fructofuranosyl-(2\to 1)]_2-\alpha-D-glucopyranoside: m=1, 2, and 3, and 2(2-\alpha-D-glucopyranosyl-(2\to 1)]_2-\alpha-D-glucopyranosyl-(2\to 1)]_2-\alpha-D-glucopyranosyl-(2$ osyl), nystose; $[O-\alpha-D-\text{glucopyranosyl-}(1\rightarrow 2)]_n-O-[\beta-D-\text{fructofuranosyl-}(2\rightarrow 1)]_3-\alpha-D-\text{glucopyranoside}$: 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. 1H, 13C NMR signals of each saccharide were assigned using 2D-NMR techniques, including COSY, HSQC, HSQC-TOCSY, HMBC, CH₂-selected E-HSQC, and CH₂-selected E-HSQC-TOCSY. © 2003 Elsevier Science Ltd. All rights reserved.

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

1. Introduction

We have previously examined the formation of oligosaccharides as nutritional and functional food ingredients, such as inulo-oligosaccharides, 1,2 fructooligosaccharides,3 fructosyl xylosides,4 and fructosyl lactosucroses⁵ by using *Penicillium purpurogenum* inulinase, 1,2 Scopulariopsis brevicaulis fructosyltransferase, 3,4 and asparagus 1F-fructosyltransferase, 5-7 respectively. Among these oligosaccharides, fructosyl xyloside suppressed serum glucose and insulin responses,8 and pro-

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moted 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-Dglucose 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-

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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,

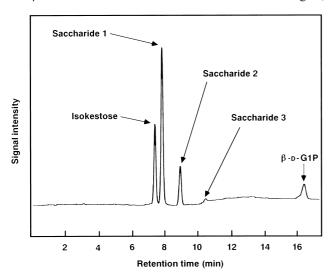


Fig. 1. HPAEC of saccharides produced from isokestose and $\beta\text{-D-G1P}$ by kojibiose phosphorylase. The enzyme incubation was carried out with 40 $\mu mol/mL$ isokestose and 38.5 $\mu mol/mL$ $\beta\text{-D-G1P}$ in the mixture for 48 h.

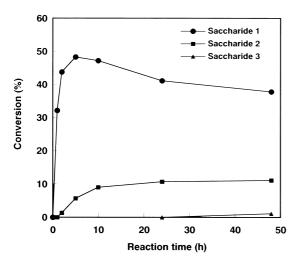


Fig. 2. Time course of formation of saccharides 1, 2, and 3 from isokestose and β -D-G1P by kojibiose phosphorylase. The conversion (%) was shown as the ratio (w/w) of the Saccharide 1, 2, or 3 to the isokestose.

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 ($t_{R \text{ sucrose}}$: 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.

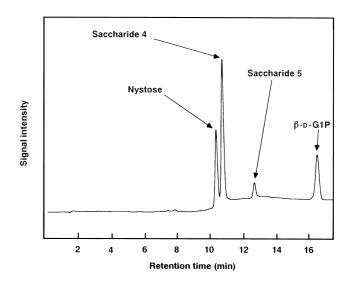


Fig. 3. HPAEC of saccharides produced from nystose and β -D-G1P by kojibiose phosphorylase. The enzyme incubation was carried out with 30 μ mol/mL nystose and 38.5 μ mol/mL β -D-G1P in the mixture for 48 h.

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)₂isokestose, 2(2- α -D-glucosyl)₃isokestose, 2- α -D-glucosyl nystose, and 2(2- α -D-glucosyl)₂nystose, respectively.

The structural confirmation of the saccharides 1, 2, 3, 4, and 5 according to ¹H, ¹³C NMR analyses and the subsequent complete assignment of ¹H, ¹³C 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} CH₂-selected E-HSQC, ¹⁷ and CH₂-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

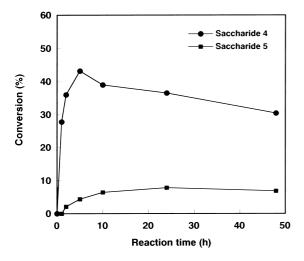


Fig. 4. Time course of formation of saccharides 4 and 5 from nystose and β -D-G1P by kojibiose phosphorylase. The conversion (%) was shown as the ratio (w/w) of the Saccharide 4 or 5 to the nystose.

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 ($\delta_{\rm H}$ 5.71 ppm, d, 3.6 Hz and $\delta_{\rm H}$ 5.13 ppm, d, 4.0 Hz) and carbons ($\delta_{\rm C}$ 90.57 ppm and $\delta_{\rm C}$ 96.77 ppm) in 1, two glucose residues were assigned by ¹H-¹H COSY, HSQC, and HSQC-TOCSY spectra. The inter residual HMBC correlation between one of the anomeric proton ($\delta_{\rm H}$ 5.71 ppm) and one of the quaternary carbon ($\delta_{\rm C}$ 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 $(\delta_{\rm H}$ 3.88 ppm, d, 9.5 Hz and $\delta_{\rm H}$ 3.80 ppm, d, 9.5 Hz) and between C-1-Fru ($\delta_{\rm C}$ 62.01 ppm) and H-3-Fru ($\delta_{\rm H}$ 4.30 ppm, d, 8.8 Hz) as well as ¹H-¹H COSY correlations enabled the assignments of Fru residue. The connectivity of Glc' $(1 \rightarrow 2)$ Glc and Fru' $(2 \rightarrow 1)$ Fru were also deduced from HMBC correlations between C-2-Glc ($\delta_{\rm C}$ 75.85 ppm) and H-1-Glc' ($\delta_{\rm H}$ 5.13 ppm) and between C-2-Fru' ($\delta_{\rm C}$ 104.50 ppm) and H-1-Fru ($\delta_{\rm H}$ 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 CH₂-selected E-HSQC. Moreover, ¹H−¹H coupling patterns of overlapping ¹H 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" $(1 \rightarrow 2)$ Glc' in 2 and Glc" $(1 \rightarrow 2)$ Glc' $(1 \rightarrow 2)$ Glc' in 3, were determined by additional HMBC correlation between H-1-Glc" ($\delta_{\rm H}$ 5.08 ppm, d, 3.7 Hz) and C-2-Glc' ($\delta_{\rm C}$ 76.81 ppm) in 2 and between H-1-Glc" ($\delta_{\rm H}$ 5.27 ppm, d, 3.5 Hz) and C-2-Glc' ($\delta_{\rm C}$ 78.67 ppm) and between H-1-Glc'' ($\delta_{\rm H}$ 5.09 ppm, d, 3.7 Hz) and C-2-Glc" ($\delta_{\rm C}$ 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' $(2 \rightarrow 1)$ Fru' in 4 and 5, were determined by additional HMBC correlation between H-1-Fru' ($\delta_{\rm H}$ 3.87 ppm, d, 10.3 Hz in 4 and $\delta_{\rm H}$ 3.85 ppm, d, 10.9 Hz in **5**) and C-2-Fru" ($\delta_{\rm C}$ 104.65 ppm in 4 and $\delta_{\rm C}$ 104.54 ppm in 4), respectively. The assignments of all ¹H and ¹³C 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 $^{1}\mathrm{H}$ and $^{13}\mathrm{C}$ spectral data (δ in ppm, J in Hz) for 1–5

| $_{ m H}_{ m Q}$ | 3.05 | 3.84 d 10.8 | 3.84 d
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Chemical shifts of ¹H ($\delta_{\rm H}$) and ¹³C in ppm were determined relatively to the external standard of sodium [2,2,3,3,-2H₃]-3-(trimethylsilyl) propanoate in D₂O ($\delta_{\rm H}$ 0.00 ppm) and 1,4-dioxane (δ 67.40, ¹³C) in D₂O, 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, $2(2-\alpha-D-\text{glucopyranosyl})_m$ isokestose; $[O-\alpha-D-\text{glucopyranosyl-}(1\to 2)]_m-O-[\beta-D-\text{fructofuranosyl-}(2\to 1)]_2-\alpha-D-\text{glucopyranoside:}$ m=1 (1), 2 (2), and 3(3), and $2(2-\alpha-D-\text{glucopyranosyl})_n$ nystose; $[O-\alpha-D-\text{glucopyranosyl-}(1\to 2)]_n-O-[\beta-D-\text{fructofuranosyl-}(2\to 1)]_3-\alpha-D-\text{glucopyranoside:}$ 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-($2 \rightarrow 1$)-O- β -D-fructofuranosyl-($2 \rightarrow 1$)-O-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 μ mol/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). 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, 25-30 mM; 20-20 min, 25-30 mM; 20-20 mM; 20-

3.4. Enzymatic synthesis of oligosaccharides

A mixture (1.0 mL) of kojibiose phosphorylase (0.1 units), isokestose (40 $\mu mol)$ or nystose (30 $\mu mol)$, $\beta\text{-D-G1P}$ (38.5 $\mu mol)$, 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 $\beta\text{-}\mathrm{D}\text{-}G1P$ 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 $(3.5 \times 32 \text{ cm})$ 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 \times 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 D₂O. NMR spectra were recorded at 27 °C with a

Bruker AMX 500 spectrometer (1H 500 MHz, 13C 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 [2,2,3,3-2H₄]-3-(trimethylsilyl) propanoate in D_2O (δ_H 0.00 ppm) and 1, 4-dioxane ($\delta_{\rm C}$ 67.40 ppm) in D₂O, respectively. $^{1}{\rm H}-$ ¹H COSY, ^{11,12} HSQC¹³ 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 CH₂-selected HSQC¹⁷ spectra were measured using the pulse sequence reported previously.

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