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Note

Two Novel Oligosaccharides Formed by 1^F-Fructosyltransferase Purified from Roots of Asparagus (*Asparagus officinalis* L.)

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Two novel oligosaccharides, tetra-and penta-saccharides were synthesized by fructosyl transfer from 1-kestose to 4^G-β-D-galactopyranosylsucrose with a purified 1^F-fructosyltransferase of asparagus roots and identified as 1^F-β-D-fructofuranosyl-4^G-β-D-galactopyranosylsucrose, *O*-β-D-fructofuranosyl-(2→1)-β-D-fructofuranosyl-*O*-[β-D-galactopyranosyl-(1→4)]-α-D-glucopyranoside and 1^F(1-β-D-fructofuranosyl)$_2$-4^G-β-D-galactopyranosylsucrose, [*O*-β-D-fructofuranosyl-(2→1)]$_2$-β-D-fructofuranosyl-*O*-[β-D-galactopyranosyl-(1→4)]-α-D-glucopyranoside, respectively. Both oligosaccharides were scarcely hydrolyzed by carbohydrase from rat small intestine.

Human intestinal bacterial growth by 1^F-β-D-fructofuranosyl-4^G-β-D-galactopyranosylsucrose was compared with that by the tetrasaccharides, stachyose and nystose. *Bifidobacteria* utilized 1^F-β-D-fructofuranosyl-4^G-β-D-galactopyranosylsucrose to the same extent as stachyose or nystose. On the other hand, the unfavorable bacteria, *Clostridium perfringens, Escherichia coli* and *Enterococcus faecalis*, that produce mutagenic substances did not use the synthetic oligosaccharide.

Key words: fructosyloligosaccharide; indigestible oligosaccharide; 1^F-fructosyltransferase; *Bifidobacterium*

We have recently investigated the enzymatic production of non-digestible oligosaccharides having activities as ''tertiary functional ingredients'' of foods. Fructo-oligosaccharides synthesized from sucrose by *Eurotium repense* fructosyltransferase[1] had no elevating effect on the blood glucose and insulin concentrations in rats.[1] Inulo-oligosaccharides produced from inulin with *Penicillium purpurogenum* inulinase[2,3] reduced the serum cholesterol level in rats.[1] Fructosylxyloside[4] formed from sucrose and xylose by the fructosyltransferase action of *Scopulariopsis brevicaulis cells* suppressed the serum glucose and insulin responses[5] and/or promoted the absorption of calcium and magnesium ions[5] in rats administered with sucrose. These oligosaccharides also selectively stimulated the growth of *Bifidobacterium (B.) longum, B. adolescentis* and other strains of *Bifidobacteria*.[1] We have also studied the purification and characterization of several fructosyltransferases, sucrose:sucrose 1-fructosyltransferase (SST), 1^F-fructosyltransferase (1^F-FT) and the new enzyme, 6^G-fructosyltransferase (6^G-FT),[6] from asparagus roots. We have previously found that asparagus 1^F-FT catalyzed fructosyl transfer from 1-kestose to the non-reducing fructosyl residue terminating in some kinds of oligosaccharides.[7,8]

We now report the syntheses of new functional oligosaccharides elongated with one or two additional fructose units by fructosyl transfer from 1-kestose to 4^G-β-D-galactopyranosylsucrose by using asparagus 1^F-FT,[8] and then test utilization of the saccharide synthesized by *Bifidobacteria* and other intestinal bacteria.

The synthesis of saccharides A and B from mixture of 1-kestose and 4^G-β-D-galactopyranosylsucrose was investigated by using asparagus 1^F-FT purified according to the improved method reported in the previous paper.[8] A mixture of asparagus 1^F-FT (0.02 U), 0.1 M 1-kestose and 0.1 M 4^G-β-D-galactosylsucrose in a McIlvaine buffer (pH 5.5, 0.1 ml) was incubated at 30°C for 0, 2, 5, 10, 20 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 the reaction mixture was subjected to HPAEC.[9–11] As shown in Fig. 1, saccharides A, B and nystose accompanied with sucrose were produced from 1-kestose and 4^G-β-D-galactopyranosylsucrose with the 48-h reaction. The syntheses of saccharide A and nystose reached the maximum level of 9.7 mg and 6.5 mg per ml of the reaction mixture after 48 h and proceeded at a much higher rate than the synthesis of saccharide B. A reaction mixture (25 ml, 0.5 U of enzyme) with the same components was incubated at 30°C for 20 hours. After the reaction had been terminated by heating in a boiling water bath for 5 min, a 0.2-ml aliquot of the mixture was subjected to repeated preparative HPLC equipped with ODS column (Tosoh, TSKgel ODS-80Ts, 20 mm × 25 cm). Each eluate corresponding to saccharide A

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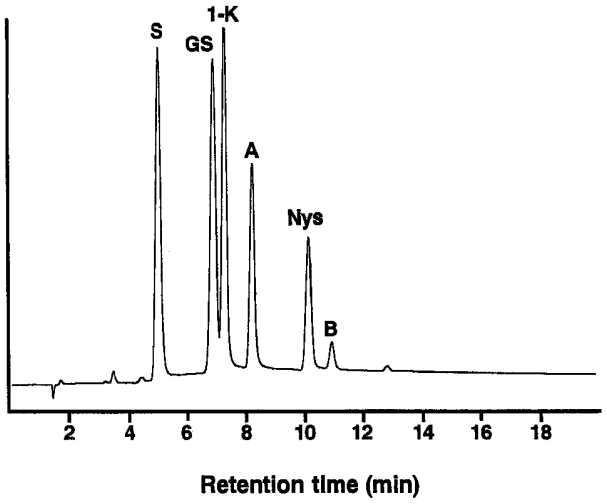


Fig. 1. HPAEC of Saccharides Produced from 1-Kestose and 4^G-β-D-Galactopyranosylsucrose by Asparagus 1^F-FT.

The enzymatic incubation was carried out for 48 h with 0.1 M 1-kestose and 4^G-β-D-galactosylsucrose in the mixture. The reaction mixture was diluted 100 times with distilled water, and an aliquot (25 μl) was subjected to HPAEC (see the text for details).

A, saccharide A; B, saccharide B; S, sucrose; GS, 4^G-β-D-galactopyranosylsucrose; 1-K, 1-kestose; Nys, nystose

and saccharide B was collected, evaporated at 35°C, and rechromatographed in the same manner. The two eluates were separately concentrated *in vacuo* and lyophilized to give white powder of saccharide A (120 mg, 9.5% yield from the donor saccharide) and saccharide B (19 mg, 1.5% yield from the donor saccharide).

Saccharides A ($[a]_D^{22}+32.5$) and B ($[a]_D^{22}+21.7$) were found to be homogeneous by PC[12] ($R_{sucrose}$, 0.230 and 0.160), TLC[12] ($R_{sucrose}$, 0.330 and 0.266, pre-coated plate), HPAEC ($t_{Rsucrose}$, 1.72 and 2.28) and HPLC (Tosoh, TSKgel ODS-80Ts, 4×25 cm; $t_{Rsucrose}$, 2.98 and 4.37). A and B were respectively found to be a tetrasaccharide and pentasaccharide made up of fructose (A, 2 mol; B, 3 mol), glucose (A and B, 1 mol) and galactose (A and B, 1 mol) by measuring the $[M+Na]^+$ ions (m/z: A, 689 and B, 851) by TOF-MS and molar ratios (A, 2.1:1.0:1.0; B, 3.2:1.0:1.0) of D-fructose, D-glucose and D-galactose in enzymatic hydrolysates (β-fructofuranosidase + β-galactosidase) of the saccharides by HPLC equipped with Sugar-Pak column (Ca-type, 7.8 mm×30 cm, Nihon Waters K. K., Japan).

To clarify the bond structures of the component sugars, saccharides A and B were permethylated by the Hakomori method.[13] The permethylated saccharides were methanolyzed with 1.5% methanolic hydrogen chloride and subjected to GLC as described in the previous paper.[12]

The methanolyzate of permethylated saccharide A gave seven peaks corresponding to methyl 1,3,4,6-tetra-*O*-methyl-D-fructoside, methyl 3,4,6-tri-*O*-methyl-D-fructoside, methyl 2,3,6-tri-*O*-methyl-D-glucoside and methyl 2,3,4,6-tetra-*O*-methyl-*D*-galactoside. The two peaks corresponding to methyl 3,4,6-tri-*O*-methyl-D-fructoside observed in the methanolysate of permethylated saccharide B were larger than those peaks in that of permethylated saccharide A.

Saccharides A and B were thus proved to be 1^F-β-D-fructosyl-4^G-β-D-galactosylsucrose and 1^F(1-β-D-fructosyl)$_2$-4^G-β-D-galactosylsucrose.

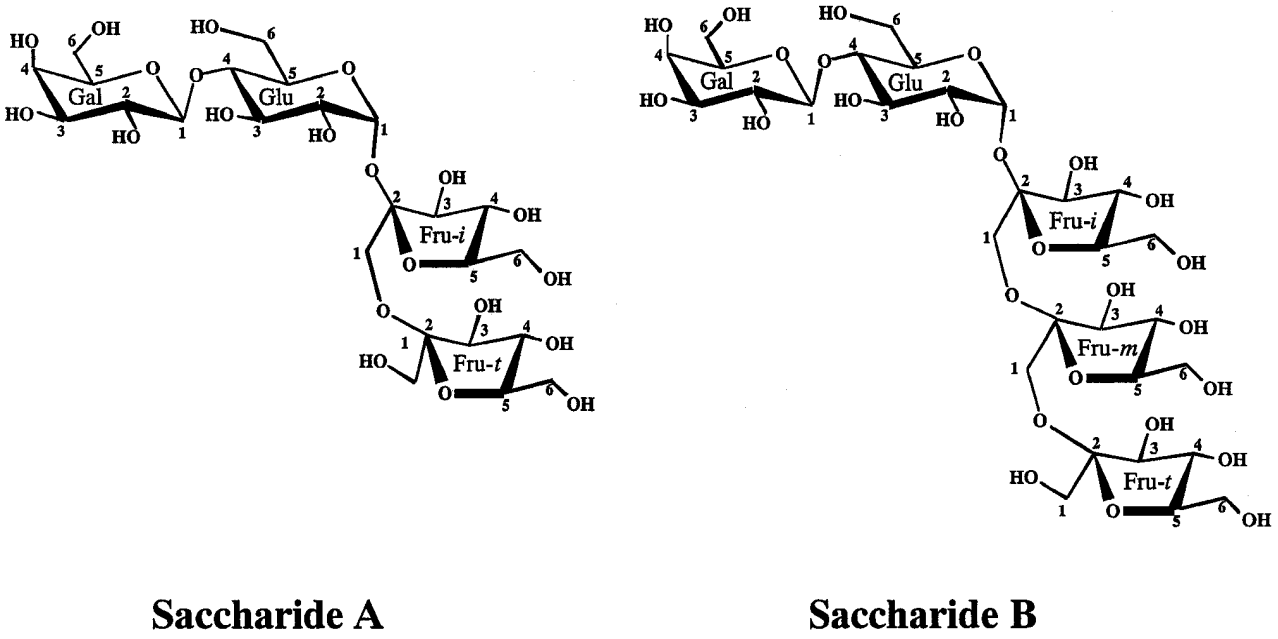
The structures of saccharides A and B were confirmed by a ^{13}C-NMR analysis according to the data shown in Table 1. The general assignment of resonances in the spectrum of saccharide A in D_2O was tentatively made by comparing the observed chemical shifts with the data for 1-kestose,[14] nystose[14] and 4^G-β-D-galactopyranosylsucrose.

All of ^{13}C-NMR data are in accord with the clarified structures of saccharides A and B shown in Fig. 2.

Table 1. ^{13}C-NMR Chemical Shifts of Saccharides A and B Formed by Asparagus 1^F-FT

Carbon atom	Saccharide A	Saccharide B
Terminal fructose		
C-1	61.30	61.24
C-2	104.57	104.50
C-3	77.43	77.58
C-4	75.32	75.17
C-5	81.96	81.91
C-6	63.15	63.08
Middle fructose		
C-1		61.74
C-2		103.88
C-3		78.34
C-4		75.29
C-5		81.91
C-6		63.08
Inner fructose		
C-1	61.69	61.88
C-2	104.12	104.09
C-3	77.50	77.58
C-4	74.69	74.71
C-5	82.08	82.10
C-6	62.99	63.00
Glucose		
C-1	93.07	93.08
C-2	71.65[a]	71.69[b]
C-3	72.08	72.08
C-4	78.93	78.93
C-5	71.92[a]	71.94[b]
C-6	60.37	60.38
Galactose		
C-1	103.75	103.75
C-2	71.83[a]	71.83[b]
C-3	73.39	73.39
C-4	69.41	69.41
C-5	76.21	76.21
C-6	61.88	61.88

Chemical shifts are expressed in ppm downfield from the signal for TMS, relative to which the 1, 4 dioxane signal appears at δ 67.40. The assigments of the resonances marked with a or b may be exchangeable.



Saccharide A **Saccharide B**

Fig. 2. Structures of Saccharides A and B Formed by Asparagus 1^F-FT.

Table 2. Utilization of SaccharideA and Several Other Saccharides by Some Human Intestinal Bacteria

Bacterial species	Saccharide A	Sta	Nys	LS	1-K	Suc	Lac	Glc
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 cloaceae 1180	—	—	—	—	—	+	+	+
Escherichia coli 1099	—	—	—	—	—	##	##	##
Enterococcus faecalis 2048	—	—	—	—	—	##	—	##
Clostridium perfringens 1211	—	—	—	—	—	±	—	±

* Sta, stachyose; Nys, nystose; LS, 4^G-β-D-galactopyranosylsucrose; 1-K, 1-kestose; Suc, sucrose; Lac, lactose; Glc, glucose

Saccharides A and B formed by fructosyl transfer to 4^G-β-D-galactopyranosylsucrose from 1-kestose with asparagus 1^F-FT were confirmed to be two new saccharides, 1^F-β-D-fructofuranosyl-4^G-β-D-galactopyranosylsucrose, *O*-β-D-fructofuranosyl-(2→1)-β-D-fructofuranosyl-*O*- [β-D-galactopyranosyl-(1→4)]-α-D-glucopyranoside and 1^F(1-β-D-fructofuranosyl)$_2$-4^G-β-D-galactopyranosylsucrose, [*O*-β-D-fructofuranosyl-(2→1)]$_2$-β-D-fructofuranosyl-*O*- [β-D-galactopyranosyl-(1→4)]-α-D-glucopyranoside.

The indigestibility of saccharides A and B was examined by using rat intestinal disaccharidase.[15] Neither saccharide A nor B was hydrolyzed by the action of α-glucosidases in a crude enzyme preparation from rat small intestinal mucosa, although each saccharide was slightly degraded by the action of β-galactosidase in the enzyme preparation to release a small amount of galactose and 1-kestose or nystose. 1-Kestose and nystose were also not hydrolyzed by the crude enzyme preparation. Therefore, saccharides A and B were indigestible.

The utilization of saccharide A by human intestinal bacteria was studied. *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, Enterococcus faecalis* and *Clostridium perfringens* are detrimental intestinal bacteria. The *in vitro* utilization of several saccharides, especially tetrasaccharides, by human intestinal bacteria is shown in Table 2. The bacterial growth was measured by analysing the pH value of the medium.[16] The symbols "###", "##", "+", "±" and "−" respectively show pH<4.5, 4.5–5.0, 5.0–5.5, 5.5–6.0, >6.0 of the medium. *Bifidobacterium adolescentis* 2793, *B. bifidum* 2777, *B. breve* 2776, *B. infantis* 2775, *B. lon-*

gum 2778 utilized saccharide A to the same extent as stachyose and nystose, which are respectively known to be one of the main components of soy oligosaccharide and fructooligosaccharide, although 4^G-β-D-galactopyranosylsucrose and 1-kestose were more effectively utilized by *B. breve*.

Under the normal conditions for *Bifidobacterium* growth, the pH value of the medium without saccharide (control), and with saccharide A, stachyose, nystose, 4^G-β-D-galactopyranosylsucrose, 1-kestose, lactose, sucrose or glucose added for *Bifidobacteria* growth was respectively 6.77–6.89, 4.59–4.66, 4.52–4.63, 4.65–5.11,4.39–5.83, 4.49–4.83, 4.57–4.64, 4.44–4.66 and 4.48–4.65. On the other hand, neither saccharide A nor stachyose, nystose, 4^G-β-D-galactopyranosylsucrose or 1-kestose was used by *E. coli, Enterobacter cloaceae, Enterococcus faecalis* and *C. perfringens*. Saccharide A was selectively used by five strains of the beneficial bacteria, *Bifidobacteria* and *Lactobacillus acidophilus*.

Saccharide A was more indigestible than 4^G-β-D-galactopyranosylsucrose and had lower osmotic pressure in a solution than 4^G-β-D-galactopyranosylsucrose or 1-kestose, making it better for intestinal conditions.

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