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Structural confirmation of novel oligosaccharides isolated from sugar beet molasses

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ABSTRACT

Eleven oligosaccharides were isolated from sugar beet molasses using carbon–Celite column chromatography and HPLC. The constituent sugars and linkage positions were determined using methylation analysis, MALDI–TOF-MS, and NMR measurements. The configurations of isolated oligosaccharides were confirmed based on detailed NMR analysis. Based on our results, three of the 11 oligosaccharides were novel.

1. Introduction

Molasses is a viscous by-product in beet and cane sugar processing, and it is used in the animal feed, yeast, citric acid, alcohol, and pharmaceutical industries. Sugar beet molasses contains relatively large amounts of raffinose, a typical trisaccharide with a well-established structure. Raffinose has characteristics of a prebiotic compound similar to other non-digestible oligosaccharides, such as fructo-oligosaccharides and galacto-oligosaccharides (Benno, Endo, Shiragami, Sayama, & Mitsuoka, 1987; Hidaka, Tashiro, & Eida, 1991; Sako, Matsumoto, & Tanaka, 1999). In addition to raffinose, several other oligosaccharides have been reported in beet and cane sugar processing. For example, isokestose (1-kestose), kestose (6-kestose), and neokestose are present in beet refinery molasses (Binkley, 1964; Schaffler & Juckes, 1971; Tsang, Cargel, & Clarke, 1991). These oligosaccharides are composed of sucrose and D-fructose. In a recent preliminary study, we isolated four oligosaccharides composed of sucrose and D-glucose or D-galactose from sugar beet molasses and confirmed their structures (Abe et al., 2012). We also demonstrated that many types of oligosaccharides are present in molasses (Abe et al., 2012).

In this study, we isolated 11 oligosaccharides from sugar beet molasses and confirmed their structures. Some of these oligosaccharides were composed of sucrose and D-fructose, D-mannose, or D-xylose.

2. Materials and methods

2.1. Materials

Sugar beet molasses was produced by Nippon Beet Sugar Mfg. Co., Ltd., Hokkaido, Japan. Isokestose (1-kestose), neokestose, nystose, and fructosyl nystose were isolated from asparagus roots (Shiomi, Yamada, & Izawa, 1976) and 6-kestose was provided by Dr. M. Iizuka, Osaka City University. Fructosylxyloside (β-D-fructofuranosyl-α-D-xylopyranoside) was obtained from sucrose and xylose using *Scopulariopsis brevicaulis* enzyme (Takeda & Kinoshita, 1995). Methyl 2,3,4,6-tetra-*O*-methyl-α/β-D-glucoside and methyl 2,3,4,6-tetra-

Abbreviations: COSY, correlation spectroscopy; GC–MS, gas chromatography–mass spectrometry; GC–FID, gas chromatography–flame ionisation detector; HMBC, heteronuclear multiple bond correlation; HPAEC, high-performance anion-exchange chromatography; HPLC, high-performance liquid chromatography; HSQC, heteronuclear single quantum coherence; H2BC, heteronuclear 2 bond correlation; MALDI–TOF-MS, matrix assisted laser desorption ionisation/time of flight mass spectrometry; NMR, nuclear magnetic resonance; SPT, selective population transfer; TOCSY, total correlation spectroscopy.

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O-methyl-α/β-D-galactoside were prepared from methyl-α/β-D-glucoside and methyl-α/β-D-galactoside, respectively. Methyl-α/β-D-glucoside, methyl-α/β-D-galactoside, kojibiose, lactulose, maltose, maltotriose, nigerose, raffinose, and levan were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other materials used in this study were of analytical grade.

2.2. Quantitative determination of sugar

Total sugars were determined using the anthrone method (Morris, 1948). Reducing sugars were quantified using the methods described by Somogyi and Nelson (Somogyi, 1945, 1952; Nelson, 1944).

2.3. High-performance anion-exchange chromatography (HPAEC)

Oligosaccharides were analysed using a Dionex Bio LC Series apparatus equipped with an HPLC carbohydrate column (CarboPac PA1, inert styrene divinyl benzene polymer) and pulsed amperometric detection (PAD) (Johnson, 1986; Rocklin & Pohl, 1983). Eluent A (150 mM aqueous NaOH) and eluent B (500 mM sodium acetate in 150 mM aqueous NaOH) were used as the mobile phase 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; at 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 respectively, and the output range was 1 μC (Shiomi, Onodera, Chatterton, & Harrison, 1991). Sucrose was used as a standard sugar. For complete hydrolysis, saccharide (1 mg) was dissolved in 0.5 N hydrochloric acid (0.5 ml) and hydrolysed at 100 °C for 30 min.

2.4. Isolation of saccharides

Freeze-dried sugar beet molasses (13 g dry weight) was diluted with 100 ml water, and the diluted molasses was loaded onto a carbon–Celite [charcoal (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and Celite-535 (Nakarai Chemical Industries, Ltd., Osaka, Japan); 1:1] column (5.5 × 47 cm). The carbon–Celite column was successively eluted with water (7.0 L), 5% v/v ethanol (21.4 L), 10% v/v ethanol (5.1 L), and 20% v/v ethanol (3.0 L). Each oligosaccharide fraction from sugar beet molasses was concentrated *in vacuo* and freeze-dried. A total of 31 different powdered fractions were obtained as shown in Table S1. Fraction R5-10 (747 mg dry weight) was dissolved in water (40 ml) and an aliquot of the solution (0.5 ml) was repeatedly applied to a preparative 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 a flow rate of 3.0 ml/min. Purified **S I** (t_R = 21.0–23.0 min. 85.7 mg) and **S II** (t_R = 25.0–27.0 min. 27.8 mg) were obtained from this fraction as white powders. Using the same HPLC conditions as above, **S III** (t_R = 22.0–23.0 min. 38.0 mg), **S IV** (t_R = 26.0–27.0 min. 30.5 mg), and **S V** (t_R = 27.5–28.5 min. 13.4 mg) were purified from fraction R5-15, and **S VI** (t_R = 29.5–31.0 min. 15.3 mg) and **S VII** (t_R = 29.5–30.5 min. 28.0 mg) were purified from fraction R5-20 (342 mg) and R10-3 (310 mg), respectively, as white powders. Using the same approach, **S VIII** (t_R = 30.5–31.5 min. 55.0 mg), **S IX** (t_R = 35.0–37.0 min. 5.4 mg), **S X** (t_R = 41.0–42.5 min. 20.7 mg), and **S XI** (t_R = 49.5–51.0 min. 10.7 mg) were purified from fraction R20 (380 mg) as white powders.

2.5. Methylation and methanolysis

Methylation of the oligosaccharides was performed using the Hakomori method (Hakomori, 1964). The permethylated saccharides were methanolysed by heating at 96 °C for 10–30 min with 1.5% methanolic hydrochloric acid. 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 methanol and analysed using GC–FID or GC–MS.

2.6. Gas chromatography flame ionisation detector (GC–FID)

For the analysis of methanolysate, GC–FID was performed using a Shimadzu GC-8A 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. The flow rate of the nitrogen carrier gas was 80 ml/min.

2.7. Gas chromatography mass spectrometry (GC–MS)

GC–MS analysis was performed using a JMS-T100GCV mass spectrometer (JEOL, Japan) with a VF-23 ms (30 m × 0.25 mm I. D., 0.25 μm film) capillary column (Agilent, USA). The injection temperature was 250 °C. Helium was used as carrier gas with a ramped flow rate. The flow was initially constant at 1.4 ml/min for 6 min and then ramped to 2 ml/min at 6 ml/min/min. The oven temperature program was as follows: an initial temperature of 100 °C (1 min) followed by increasing temperature at a rates of 35 °C/min to 170 °C, 10 °C/min to 210 °C, 40 °C/min to 250 °C, and 2.5 °C/min to 260 °C. Mass spectra were obtained by field ionisation. The interface was heated to 250 °C and the ion source to 80 °C.

2.8. Matrix assisted laser desorption ionisation/time of flight mass spectrometry (MALDI–TOF-MS)

MALDI–TOF-MS spectra were obtained using a Shimadzu-Kratos mass spectrometer (KOMPACT Probe) in positive ion mode with 2.5%-dihydroxybenzoic acid as a matrix. Ions were formed by a pulsed UV laser beam (nitrogen laser, 337 nm). Calibration was performed using 1-kestose as an external standard.

2.9. Nuclear magnetic resonance (NMR) measurements

Saccharides **I**–**XI** (1–6 mg) were each dissolved separately in 0.5 ml or 0.06 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 or 2.5-mm diameter C/H dual probe (1D spectra) and a TXI triple probe (2D spectra). Chemical shifts in ppm for ^{1}H (δ_H) and ^{13}C (δ_C) spectra were determined relative to an external standard of sodium [2,2,3,3-$^2H_4$]-3-(trimethylsilyl)-propionate 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 (Aue, Bartholdi, & Ernst, 1976; Von Kienlin, Moonen, Von der Toorn, & Van Zijl, 1991), H2BC (Nyberg, Duus, & Sørensen, 2005; Petersen et al., 2006), E-HSQC (Davis, 1991; Fukushi, Onodera, Yamamori, Shiomi & Kawabata, 2000; Willker, Leibfritz, Kerssebaum, & Bermel, 1993), HSQC-TOCSY (Domke, 1991; Willker et al., 1993), and HMBC (Bax & Summers, 1986; Hurd & John, 1991) spectra were obtained using gradient selected pulse sequences. The TOCSY mixing time (0.17 s) was according to the decoupling in the presence of scalar interactions (DIPSI)-2 method.

3. Results and discussion

Sugar beet molasses was loaded onto a carbon–Celite chromatography column and eluted with water and 5%, 10%, and 20% v/v ethanol aqueous solutions in a stepwise manner to yield 31 fractions: R5-1 to R5-27, R10-1 to R10-3, and R20 (Table S1).