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Effect of aromatic glucosides isolated from black sugar on intestinal absorption of glucose

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Abstract

Three aromatic glucosides were isolated from non-sugar fraction of crude black sugar prepared from sugar-cane. These compounds were determined to be 3,4-dimethoxyphenyl-β-D-glucoside, 3-methoxy-4-hydroxyphenyl-β-D-glucopyranoside (tachioside), 4-hydroxyphenyl-β-D-glucopyranoside (arbutin). While we reported in the previous paper[1] that 3,4-dimethoxyphenyl-β-D-glucoside was obtained from black sugar as BS-1 which was a mixture with 3,4,6-trimethoxyphenyl-D-glucoside, it was isolated as a pure form in the present works. These three compounds were found to inhibit glucose uptake in brush border membrane vesicles prepared from rat jejunum.

Key words black sugar, 3,4-dimethoxyphenyl-β-D-glucoside, tachioside, arbutin, intestinal absorption, brush border membrane, glucose uptake.

Introduction

In the previous paper,[2] we reported that non-sugar fraction of crude black sugar prepared from sugar-cane reduced plasma insulin without elevating plasma glucose in glucose tolerance test and its mechanism was supposed to be due to its inhibitory action on intestinal absorption of glucose. The active principle, named BS-1 which inhibited glucose absorption from small intestine were found to be a mixture of 3,4-dimethoxyphenyl-D-glucoside and 3,4,6-trimethoxyphenyl-D-glucoside.[1] In the course of this experiments, we found that there existed other active principles in non-sugar fraction. In the present paper, we have carried out the experiments on isolation of the other active principles and determination of their chemical structure.

Materials and Methods

Materials : Black sugar produced in Okinawa was kindly donated by Manda Co., Ltd. (Japan). D-[U-^{14}C]-glucose (304.7 mCi/mmol) was purchased from New England Nuclear. Phloridzin was obtained from Sigma (St. Louis, MO). Arbutin was obtained from Tokyo Kasei (Tokyo, Japan). Column chromatography was carried out using charcoal (Wako Pure Chemical Industry, Ltd., Osaka, Japan) and silica gel 60 (70-230 mesh, ASTM, Merck Co.). TLC was carried out using silica gel 60 (0.25 mm in thickness, Merck Co.). Reversed phase HPLC was carried out using a TSK gel ODS-120T column (TOSOH Co., Ltd., Japan). Other chemicals were of reagent grade.

Chemical analysis : EI and CI mass spectra were recorded on a Shimadzu QP-1000 mass spectrometer at 70 eV and 200 eV, respectively, using a direct insertion probe. ^{1}H- and ^{13}C-NMR spectra were recorded at 270 MHz and 67.8 MHz, respectively, on a JEOL GSX-270 spectrometer in CD_3OD or DMSO-d_6. Chemical shifts were expressed in δ ppm relative to internal CD_3OD (^{1}H = δ3.30, ^{13}C = δ49.80) or DMSO-d_6 (^{13}C = δ39.50).

Detection of sugar in eluent from charcoal

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column : Eluent (0.3 ml) from charcoal column was mixed with equal volume of 5% phenol. Then 1.5 ml of conc. H_2SO_4 was added to the mixture and stirred. After 20 min, absorbance at 490 nm was measured.

Isolation of aromatic glucosides : Crude black sugar (1 kg) was dissolved in water (1 l). then 2.5 l of ethanol was added to the solution to precipitate sucrose. Insoluble materials were removed by centrifugation at 16,000×*g* for 10 min. The supernatant fraction was concentrated by a rotary evaporator at 40℃ under reduced pressure. The non-sugar fraction was chromatographed on a charcoal column (160 g) with H_2O (1 l), 5% EtOH (2 l), 10% EtOH (1 l), 20% EtOH (1 l), 30% EtOH (1 l), 40% EtOH (3 l), 100% EtOH (6 l), successively (Fig. 1). The fraction eluted with 20−100% EtOH was divided into five fractions. Fraction 3 (Fr-3), fraction 4 (Fr-4) and fraction 5 (Fr-5) were combined and chromatographed on a silica gel column with $CHCl_3$-MeOH and reversed phase HPLC with 0.1% TFA-CH_3CN to give three aromatic glucosides (**1**, **2**, and **3**).

Compound **1**, 3,4-dimethoxyphenyl-β-D-glucoside, BS-1e[1] : colourless needles ; mp 169−170℃ ; ^{1}H-NMR (270 MHz, CD_3OD) : δ3.30−3.50 (4H, m), 3.68 (1H, dd, J=5.34, 11.90 Hz), 3.77, 3.80 (each3H, s), 3.89 (1H, dd, J=1.83, 11.90 Hz), 4.77 (1H, d, J=7.32 Hz) 6.66 (1H, dd, J=2.75, 8.55 Hz), 6.80 (1H, d, J=2.75 Hz), 6.84 (1H, d, J=8.55 Hz) ; EIMS m/z (%) : 316 (M^+, 3), 154 (100), 139 (89) ; CIMS (i-butane) m/z : 317 (M^++1).

Compound **2**, 4-hydroxy-3-methoxyphenyl-β-D-glucopyranoside, tachioside[3] : colourless needles ; mp 212−213℃ ; $[\alpha]_D$=−26.67℃ (c=0.3, MeOH) ; ^{1}H-NMR (270 MHz, CD_3OD) : δ3.30−3.50 (4H, m), 3.67 (1H, dd, J=2.14, 11.90 Hz), 3.82 (3H, s), 3.89 (1H, dd, J=2.14, 11.90 Hz). 4.73 (1H, d, J=7.63 Hz), 6.57 (1H, dd, J=2.75, 8.54 Hz), 6.68 (1H, d, J=8.54 Hz), 6.79 (1H, d, J=2.75 Hz) ; ^{13}C-NMR (67.8 MHz, DMSO-d_6, DEPT) : δ55.50 (OMe), 60.87 (C6'), 69.97 (C4'), 73.37 (C2'), 76.73 (C5'), 77.08 (C3'), 101.72 (C1'), 102.51 (C2), 107.94 (C6), 115.20 (C5), 141.33 (C4), 147.81 (C3), 150.76 (C1) ; EIMS m/z (%) : 302 (M^+, 2), 140 (100), 126 (65) ; CIMS (i-butane) m/z : 303 (M^++1).

Compound **3**, 4-hydroxyphenyl-β-D-glucopyranoside, arbutin : colourless needles ; mp 206-207℃ ; $[\alpha]_D$=−38.46℃ (c=0.26, H_2O) ; ^{1}H-NMR (270 MHz, CD_3OD) : δ3.30−3.50 (4H, m), 3.68 (1H, m), 3.87 (1H, d, J=11.30 Hz), 4.72 (1H, d, J=7.63 Hz), 6.67 (2H, d-like, J=8.85 Hz), 6.95 (2H, d-like, J=8.85 Hz) ; EIMS m/z (%) : 272 (M^+, 0.2), 110 (100) ; CIMS (i-butane) m/z : 273 (M^++1).

Preparation of brush border membrane vesicles : Vesicles were prepared by the method of Kessler *et al*.[4] as follows. Mucosal scrapings from jejunum of male Wistar-King strain rats were suspended in ice-cold 50 mM mannitol plus 2 mM Tris/chloride buffer, pH 7.1, and homogenized. Then solid $CaCl_2$ was added to the homogenate, at a final concentration of 10 mM. After standing in the cold for 15 min, the suspension was spun down at 3,000×*g* for 15 min. The supernatant was then spun down at 27,000×*g* for 30 min. The resulting supernatant was discard-

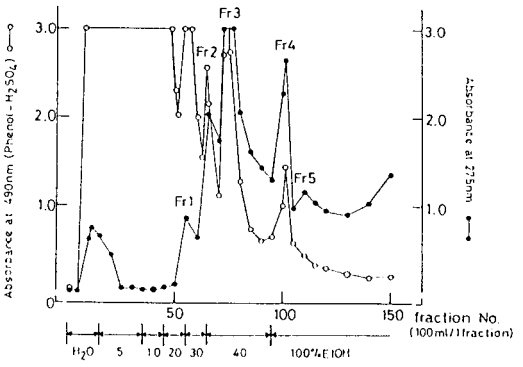


Fig. 1 chromatogram of non-sugar fraction on charcoal column.

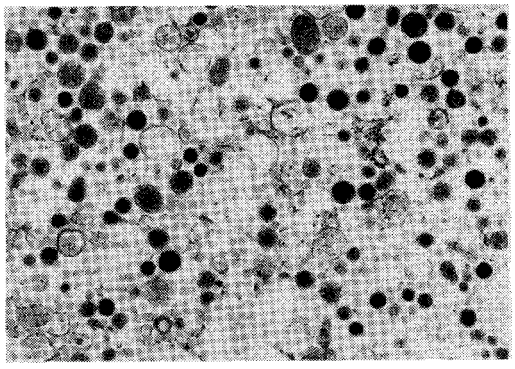


Fig. 2 Electron microscopic appearance of the final vesicle preparation (×46,000).

ed. The pellet was resuspended in 50 mM mannitol plus 10 mM HEPES/Tris buffer, pH 7.5 and spun down once more at 27,000×*g* for 30 min. This pellet formed vesicles as shown in Fig. 2 and was used for the measurement of glucose uptake.

Electron microscopy : A pellet of bruch border membrane vesicles was fixed in 0.1 M cacodylate buffer containing 1% glutaraldehyde and 0.5% OsO_4, pH 7.2 for 2 hr at 4℃. After dehydration it was embedded in Epon. The section was stained with uranyl acetate and lead citrate.

Estimation of the effect on glucose uptake : Lyophilized test compounds were dissolved in buffer containing 100 mM mannitol and 10 mM Tris/HEPES, pH 7.5 at concentration shown in Fig. 3 and Fig. 4. Then the solution of 10 μl were added to 20 μl of membrane vesicles (2−9 mg protein/ml) suspended in the same buffer. After the mixtures were preincubated for 3 min at 20℃, 30 μl of 2 mM D-[^{14}C]-glusoce solution containing

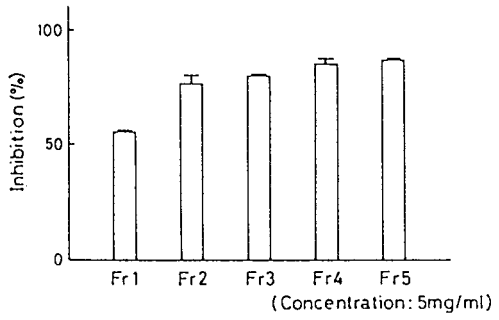


Fig. 3 Inhibition of glucose uptake by non-sugar fraction in brush border membrane vesicles.

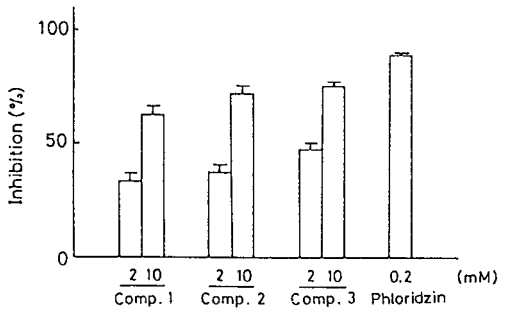


Fig. 4 Inhibition of glucose uptake by aromatic glucoside in bruch border membrane vesicles. IC_{50} : compound **1** = 4.58 mM, compound **2** = 5.06 mM, compound **3** = 2.53 mM, phloridzin = 0.03 mM.

100 mM NaSCN, 100 mM mannitol and 10 mM Tris/HEPES, pH 7.5 was added to them and incubated for 15 sec at 20℃. The uptake of glucose was terminated by dilution with 1 ml of ice-cold 150 mM NaCl solution containing 0.2 mM phloridzin and 1 mM Tris/chloride, pH 7.5. The vesicles were immediately collected on a cellulose nitrate filter (0.2 μm pore size) and washed with 4 ml of the above NaCl solution. Then radioactivity of the vesicles on the filter was counted. Inhibitory effect of the compounds was calculated by the following formula :

$$\text{Inhibition (\%)} = \frac{\text{control cpm} - \text{experimental cpm}}{\text{control cpm} - \text{background cpm}} \times 100$$

Where control cpm = the radioactivity incorporated into the vesicles preincubated without any test compounds, experimental cpm = the radioactivity incorporated into the vesicles preincubated with test compounds, background cpm = the radioactivity incorporated into the vesicle sprein-cubated without any test compoundsand with termination solution preceding D-[^{14}C]-glucose solution. Test compounds assayed in this experiment at 10 mM had no effect on background.

Results and Discussion

Isolation of the inhibiting substances toward glucose uptake

As shown in Fig. 1, non-sugar fraction of black sugar was chromatographed on a charcoal column to remove sugar completely. Most of the sugar was found to be eluted in non-absorbed fractions. The absorbed fractions eluted with 20−100% EtOH showed five peaks (Fr 1, 2, 3, 4 and 5) of absorption at 275 nm. Then, each peak was subjected to estimation of inhibitory activity toward glucose uptake in brush border membrane vesicles. It was found that these five peaks showed inhibitory activity (Fig. 3). TLC analysis showed Fr 3, 4 and 5 contained the same component (Fig. 5), so they were combined, and subjected to chromatography to afford the compounds of Rf 0.19 (compound **1**), Rf 0.10 (compound **2**) and Rf 0.06 (compound **3**). Compound **1** was supposed to be BS-1[1] which was a mixture of 3,4-dimethoxy-

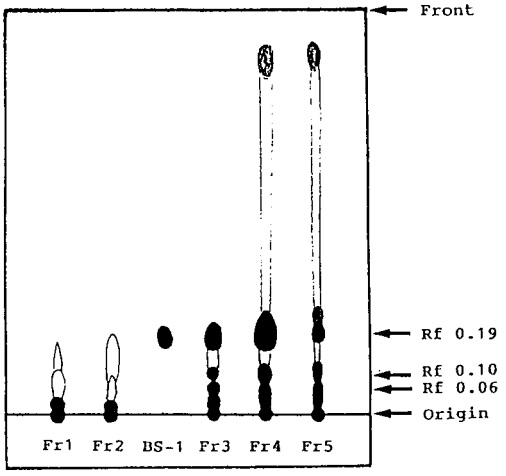


Fig. 5 TLC of non-sugar fraction eluted from charcoal column. Developer ; chloroform : methanol=9 : 1, Detection ; sprayed with 80% H_2SO_4 and heated at 200℃.

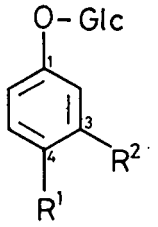
phenyl-D-glucoside (BS-1e) and 3,4,6-trimethoxyphenyl-D-glucoside (BS-1f) by identical Rf, and further spectral data showed that it was pure BS-1e.

Determination of chemical structure

Compound **1** ; The ^{1}H-NMR spectrum of compound **1** showed signals due to trisubstituted aromatic ring, two methoxyl groups and one glucosyl moiety. The EIMS showed a molecular ion m/z 316 ($C_{13}H_{18}O_8$), and peak at m/z 154 resulting from elimination of glucosyl moiety. these spectral data were identical with those of authentic sample BS-1e.

Compound **2** ; The ^{1}H-NMR spectrum of compound **2** was very similar to compound **1** and showed absence of one of two methoxyl groups in comparison with compound **1**. EIMS also suggested lack of a methyl group in compound **2** by a molecular ion m/z 302. Therefore compound **2** was supposed to be 3-hydroxy-4-methozyphenylglucoside or 4-hydroxy-3-methoxyphenylglucoside. The location of the methyl group was defined by 2D-NMR experiments (NOESY). NOE was observed between methyl group and C2-H, leading to a 4-hydroxy-3-methoxypehnylglucoside structure for compound **2** (data not shown). 4-Hydroxy-3-methoxyphenyl-β-D-glucopyranoside has been isolated as tachioside from *Berchemia racemosa* Sieb. et Zucc.[3] The reported ^{13}C-NMR spectral data of tachioside are identical with those of compound **2**. Thus compound **2** was identified with 4-hydroxy-3-methoxyphenyl-β-D-glucopyranoside.

Compound **3** ; The ^{1}H-NMR spectrum of compound **3** was also similar to compounds **1** and **2**, showing absence of any methoxyl group and presence of a para-disubstituted aromatic ring and a glucosyl moiety. These data suggested that compound **3** was arbutin, 4-hydroxyphenyl-β-D-glucopyranoside. Their physicochemical properties were identical with those of authentic sample of arbutin (from Tokyo Kasei). The chemical structure of these compounds is shown in Fig. 6.



1 $R^1 = R^2 = OCH_3$

2 $R^1 = OH$, $R^2 = OCH_3$

3 $R^1 = OH$, $R^2 = H$

Fig. 6 Chemical structure of aromatic glucosides.

Effects of three aromatic glucosides on glucose uptake

as shown in Fig. 4, compounds **1**, **2** and **3** inhibited glucose uptake in brush border membrane vesicles to similar extent, although their inhibitory activity was weaker than phloridzin. In 1984, Kimura *et al.* reported that non-sugar fraction containing compounds **1**, **2** and **3** reduced plasma insulin in rats by oral glucose tolerance test and inhibited the absorption of glucose from the small intestine of rats.[2] These results and the present experimental results suggest that compounds **1**, **2** and **3** may inhibit intestinal absorption of glucose *in vivo*.

Acknowledgement

The authors wish to thank Mr.H. Tani (the Advanced Instrumentation Center for Chemical Analysis, Ehime University) for measurement of ^{1}H- and ^{13}C-NMR spectra.

和文抄録

黒砂糖の非蔗糖分画から3種の芳香族配糖体を単離した。これらの化合物は3,4-ジメトキシフェニル-β-D-グルコシド，3-メトキシ-4-ヒドロキシフェニル-β-D-グルコピラノシド（タキオシド），4-ヒドロキシフェニル-β-D-グルコピラノシド（アルブチン）と同定した。3,4-ジメトキシフェニル-β-D-グルコシドは，3,4,6-トリメトキシフェニル-D-グルコシドとの混合物であるBS-1として黒砂糖中より得られたことを前報中で報告しているが，今回，単一な形で単離した。これら3種の化合物はいずれもラット空腸から調製した刷子縁膜小胞におけるグルコースの取り込みを阻害することを明らかとした。

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