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ARTICLE

Anti-hyperglycemic activity of polyphenols isolated from barnyard millet (*Echinochloa utilis* L.) and their role inhibiting α-glucosidase

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Abstract The extracts and chemical compounds isolated from barnyard millet (*Echinochloa utilis*) grains were investigated as part of a search for naturally derived anti-hyperglycemic medicinal plants. Among the five different solvent extracts, the ethyl acetate extract showed the lowest IC_{50} value against *Saccharomyces cerevisiae* α-glucosidase (70.2 μg/mL). Chromatography yielded eight phenolic compounds that may have been responsible for this effect. Among them, *N*-*p*-coumaroyl serotonin (**1**, CS), feruloyl serotonin (**2**, FS), and luteolin (**5**) potently inhibited α-glucosidase with IC_{50} values of 1.3–17.8 μM compared with those of deoxynojirimycin (DNJ, $IC_{50} = 2.5 \pm 0.1$ μM) and acarbose ($IC_{50} = 255.1 \pm 15.6$ μM). Additionally, for the first time, we found that CS and FS were significantly inhibited mammalian rat intestinal sucrase (IC_{50} of 3.0 and 8.2 μM) and reduced glucose content (73 and 52 % at 0.1 mg/wells) in Caco-2 (human intestinal epithelial) cells. Furthermore, oral glucose tolerance test revealed improved glucose tolerance following treatment with the barnyard millet grains extract by retarding the postprandial rise in blood glucose in vivo. These results suggest that barnyard millet grain can be used as a natural functional medicine to prevent and alleviate type-2 diabetes.

Keywords α-Glucosidase · Anti-hyperglycemic · Barnyard millet grains · Caco-2 cells · Serotonin

Introduction

A modern lifestyle has resulted in a rise in metabolic syndrome diseases including obesity, hyperlipidemia, heart disease, and diabetes. Diabetes will affect 440 million people worldwide by 2030, with 70 % of cases occurring in low-middle income countries (Zhang et al. 2011). In particular, diabetes mellitus is characterized by chronic hyperglycemia, which is associated with an increased risk for cardiovascular diseases (Jenkins et al. 2002). Postprandial hyperglycemia results from abnormal insulin secretion by β-cells in response to a meal, impaired hepatic glucose production, and defective glucose uptake by insulin-sensitive peripheral tissues (Leahy 2005). Thus, postprandial hyperglycemia plays a critical role in the development of type II diabetes and complications associated with micro and macrovascular diseases (Lebovitz 1998).

One of the most effective therapeutic approaches to control postprandial blood glucose level is to inhibit intestinal α-glucosidase; therefore, a number of inhibitors of this enzyme have been developed to target postprandial hyperglycemia (Moller 2001). The glycosidases, a large family of enzymes that process complex carbohydrates, have been singled out as a significant therapeutic target (Bertozzi and Kiessling 2001). Additionally, glycosidase

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inhibitors can be used to treat various diseases such as cancer (Walker et al. 1993), human immunodeficiency virus (HIV) (Gloster and Davies 2010), and diabetes mellitus type II (Mitrakou et al. 1998). Among them, the α-glucosidase (EC 3.2.1.20, α-D-glucoside glucohydrolase) is a key enzyme required to cleave maltose and sucrose into glucose for absorption into the blood in the small intestine. This enzyme is widely distributed in microorganisms, plants, and animals, although substrate specificity of α-glucosidase varies greatly depending on the source (Kim et al. 2008; Prajapati and Patel 2012). Most α-glucosidase inhibitors mimic the pyranosyl moiety of this enzyme such as acarbose and deoxynojirimycin (1-DNJ). These compounds currently used to orally treat diabetes inhibit α-glucosidase. Previous methods aimed at developing new α-glucosidase inhibitors have focused on sugar mimics such as carbasugars, iminosugars, and thiosugars (Melo et al. 2006). However, there are numerous drawbacks to this method including low activity and complicated stereochemistry, which makes them difficult to approach synthetically. Therefore, more recent studies have investigated nonsugar natural product derivatives, particularly polyphenols, which have received great attention as α-glucosidase inhibitors because of their high natural abundance, safety, and biological potential (Yang et al. 2001; Benalla et al. 2010).

Barnyard millet (*Echinochloa utilis*) is widely distributed in Korea, Japan, and East Asia. This plant has good agricultural characteristics, including excellent nutrition, ease of cultivation, cold tolerance, drought resistance, and salt tolerance (Kim et al. 2011). Furthermore, barnyard millet grain extracts (BME) have been reported and used as traditional medicine for patients with allergic diseases, atopic dermatitis, and hyperlipidemia (Nishizawa et al. 2009). Although Korean native barnyard millet has excellent agricultural characteristics, knowledge of its biological activities such as an anti-hyperglycemic effect is limited.

In this study, we investigated α-glucosidase inhibitory activities of BME. Eight isolated compounds found to exhibit significant inhibitory activities against microbial and mammalian α-glucosidase sources in vitro and in vivo for the first time.

Materials and methods

Plant material

Barnyard millet (*Echinochloa* spp.) was bred and harvested in September 2010 at the experimental field of the National Institute of Crop Science, Rural Development Administration, Miryang, Republic of Korea. The IT number (K141285) of the barnyard millet was provided by Dr. Myung Chul Lee, and all seeds were supported by the RDA-Genebank Information Center, Republic of Korea.

Instruments and reagents

^{1}H-NMR (nuclear magnetic resonance) and ^{13}C-NMR 500 and 125 MHz spectral data were collected on a Bruker AM 500 spectrometer (Bruker-Franzen Analytik GmbH Bremen, Germany) in MeOH, DMSO-d_6, and $CDCl_3$ with TMS as the internal standard (Cambridge Isotope Laboratories, CIL, Tewksbury, MA, USA). The electron ionization mass spectrum (EIMS) and high-resolution electron ionization mass spectrum (HREIMS) were obtained on a JEOL JMS-700 mass spectrometer (JEOL JMS-700, Tokyo, Japan). Thin-layer chromatography (TLC) was conducted on precoated Kieselgel 60F_{254} plates (Art. 5715; Merck), and the spots were detected either by examining the plates under a ultraviolet lamp or treating the plates with a 10 % ethanol solution of phosphomolybdic acid (Wako Pure Chemical Industries, Osaka, Japan) followed by heating at 110 °C. Analytical grade acetonitrile, methanol, and acetic acid for ultra performance liquid chromatography (UPLC) were purchased from J. T. Baker (Phillipsburg, NJ, USA). A Waters ACQUITY TQD UPLC/MS system coupled online with a photo diode array detector (PDA 2996, Waters, Milford, MA, USA) and a TQD Tandem MS detector (Waters) were used. The analytes were separated on a Waters ACQUITY BEH C18 column (1.7 μm, 2.1 × 100 mm). Optical rotation was measured on a Perkin-Elmer 343 polarimeter (Waltham, MA, USA). Column chromatography was carried out using silica gel (230–400 mesh, Merck, Darmstadt, Germany), RP-18 (ODS-A, 12 nm, S-150 mM, YMC), and Sephadex LH-20 (Amersham Biosciences, Piscataway, NJ, USA). α-Glucosidase (EC 3.2.1.20, from *Saccharomyces cerevisiae*, *p*NPG (*p*-nitrophenyl-α-D-glucopyranoside), and rat intestinal acetone powder used for the bioassay were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Glucose oxidase/peroxidase assay kit was purchased from Invitrogen Co. (Grand Island, NY, USA). Dulbecco's modified minimum essential medium (DMEM), fetal bovine serum (FBS), streptomycin, and phosphate buffer saline (PBS) were obtained from Gibco (Grand Island, NY, USA).

Extraction and isolation

Samples were obtained by removing and polishing the barnyard millet grains. Five gram of powder was extracted with 20 mL of organic solvent with shaking at 30 °C for 24 h. The five different solvent extraction systems were used, including ethyl acetate, ethanol, 50 % ethanol, methanol, and distilled water. Suitable filtered aliquots

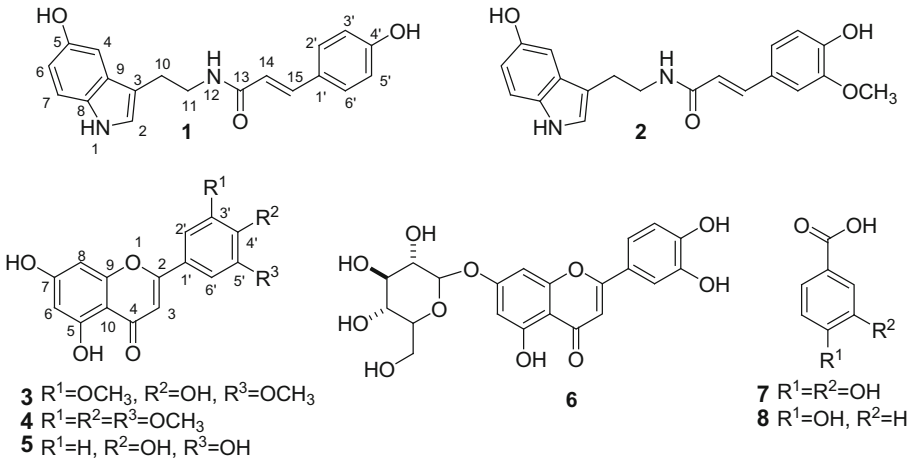

Fig. 1 Chemical structure of the isolated compounds (**1**–**8**) from BME

were then used for enzymatic assays and HPLC analyses. Briefly, dried barnyard millet grain powder (2 kg) was extracted with 5 L of ethyl acetate in a shaking incubator at room temperature for 1 week. The solvent was evaporated under reduced pressure at 50 °C. This extract was fractionated by silica gel column chromatography (10 × 75 cm, 230–400 mesh, 800 g) eluted with *n*-hexane–ethyl acetate with a gradual increase in the ethyl acetate content (90:10 → 80:20 → 70:30 → 60:40 → 50:50 → 40:60 → 20:80 → 0:100, each 1000 mL), and eight fractions (A–H) were collected. Fractions B (7.8 g) and E (10.3 g) were pooled and rechromatographed over a silica gel column (4 × 60 cm, 230–400 mesh, 450 g) using an *n*-hexane–ethyl acetate solvent system (15:1 → 1:1) and then purified with a second flash silica gel column (3 × 50 cm, 230–400 mesh, 150 g) using a gradient of *n*-hexane–acetone [15:1 (500 mL), 12:1 (350 mL), 8:1 (350 mL), 5:1 (350 mL), 3:1 (350 mL), 1:1 (350 mL)] to yield compounds **2** (52 mg) and **4** (68 mg). Fraction G (14.6 g) was separated by silica gel column chromatography (4.5 × 70 cm, 650 g) (230–400 mesh) with *n*-hexane–ethyl acetate mixtures of increasing polarity (12:1 → 1:1) to afford 26 subfractions, based on a comparison with the thin-layer chromatography profile. Subfractions 17–22 were subjected to silica gel column chromatography (3.0 × 50 cm, 130 g) (230–400 mesh) with *n*-hexane–acetone (12:1 → 3:1) gradients, and the pale yellow fractions were concentrated. The combined mixtures were chromatographed on a Sephadex LH-20 column (2.5 × 25, 80 g) with methanol, and 12 fractions of 20 mL each were collected. The collected fractions (310 mg) were chromatographed on a second Sephadex LH-20 column (1.5 × 20 cm, 35 g) with methanol as the elution solvent to yield compounds **1** (19 mg), **8** (35 mg), and **3** (27 mg). Further purification of the mixture of **5**, **6**, and **7** on Sephadex LH-20 with methanol as the eluant yielded compounds **5** (84 mg), **6** (45 mg), and **7** (98 mg) (Fig. 1).

N-p-coumaroyl serotonin (1)

Amorphous powder, ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.87 (1H, s, NH), 7.45 (2H, d, $J = 8.5$ Hz, H-2′ and 6′), 7.38 (1H, d, $J = 15.7$ Hz, H-7′), 7.15 (1H, d, $J = 8.5$ Hz, H-7), 7.02 (1H, d, $J = 2.1$ Hz, H-2), 6.96 (1H, d, $J = 2.5$ Hz, H-4), 6.76 (2H, d, $J = 8.5$ Hz, H-3′ and 5′), 6.67 (1H, dd, $J = 8.5$, 2.0 Hz, H-6), 6.38 (1H, d, $J = 15.7$ Hz, H-8′), 3.56 (2H, t, H-11), 2.92 (2H, t, H-10); ^{13}C NMR (75 MHz, $CDCl_3$) 169.7 (C-9′), 160.8 (C-4′), 151.5 (C-5), 142.1 (C-7′), 133.5 (C-8), 130.9 (C-2′ and C-6′), 129.8 (C-9), 128.2 (C-1′), 124.7 (C-2), 119.0 (C-8′), 117.1 (C-3′ and C-5′), 113.1 (C-7), 112.8 (C-6), 103.9 (C-3), 79.8 (C-4), 41.9 (C-11), 26.8 (C-10); EIMS, $m/z = 322$ $[M]^+$, HREIMS, m/z 322.1315 (calcd for $C_{19}H_{18}N_2O_3$ 322.1308).

Feruloyl serotonin (2)

Amorphous powder, ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.06 (1H, s, NH), 7.33 (1H, d, $J = 16.0$ Hz, H-7′), 7.13 (1H, d, $J = 8.6$ Hz, H-7), 7.14 (1H, d, $J = 2.1$ Hz, H-6′), 7.06 (1H, d, $J = 2.0$ Hz, H-2), 7.00 (1H, dd, $J = 8.0$, 2.1 Hz, H-2′), 6.85 (1H, d, $J = 2.0$ Hz, H-4), 6.80 (1H, d, 8.0 Hz, H-3′), 6.60 (1H, dd, $J = 8.5$, 2.5 Hz, H-6), 6.46 (1H, d, $J = 16.0$ Hz, H-8′), 3.81 (3H, s, 5′-OMe), 3.17 (2H, t, H-11), 2.78 (2H, t, H-10); ^{13}C NMR (75 MHz, $CDCl_3$) 165.6 (C-9′), 150.5 (C-5), 148.5 (C-4′), 148.1 (C-5′), 139.1 (C-7′), 131.1 (C-8), 128.2 (C-9), 126.8 (C-1′), 123.4 (C-2), 121.8 (C-2′), 119.5 (C-8′), 115.9 (C-3′), 112.0 (C-7), 111.6 (C-6), 111.1 (C-3), 111.0 (C-6′), 102.5 (C-4), 55.8 (C-3′), 48.9 (C-11), 25.7 (C-10); EIMS, $m/z = 352$ $[M]^+$; HREIMS, m/z 352.1423 (calcd for $C_{20}H_{20}N_2O_4$ 352.1422).

5,7-Dihydroxy-2-(4-hydroxy-3,5-dimethoxy-phenyl)-chromen-4-one (Tricin) (3)

Amorphous powder, ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.32 (2H, s, H-2′, 6′), 6.98 (1H, s, H-3), 6.56 (1H, s, H-8), 6.20

(1H, s, H-6), 3.90 (6H, s, 3′ and 5′OCH_3); ^{13}C NMR (75 MHz, $CDCl_3$) 182.1 (C-4), 164.4 (C-2), 164.0 (C-7), 161.7 (C-9), 157.7 (C-5), 148.5 (C-3′ and C-5′), 140.1 (C-1′), 120.7 (C-4′), 104.6 (C-2′ and C-6′), 104.1 (C-10), 104.0 (C-6), 99.2 (C-8), 94.6 (C-3), 56.7 (3′-OMe and 5′-OMe); EIMS, *m/z* = 330 $[M]^+$; HREIMS, *m/z* 330.0742 (calcd for $C_{17}H_{14}O_7$ 330.0740).

5,7-Dihydroxy-2-(3,4,5-trimethoxy-phenyl)-chromen-4-one (Tricin methyl ester) (4)

Amorphous powder, ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.33 (2H, s, H-2′, 6′), 7.07 (1H, s, H-3), 6.57 (1H, s, H-8), 6.22 (1H, s, H-6), 3.90 (6H, s, 3′ and 5′OCH_3), 3.75 (3H, s, 4′OCH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ 182.2 (C-4), 164.8 (C-2), 163.3 (C-7), 161.7 (C-9), 157.7 (C-5), 153.5 (C-5′), 141.1 (C-4′), 126.4 (C-1′), 105.4 (C-10), 104.5 (C-2′), 99.4 (C-8), 94.7 (C-3), 60.6 (4′-OMe), 56.7 (3′-OMe and 5′-OMe); EIMS, *m/z* = 344$[M]^+$; HREIMS, *m/z* 344.0894 (calcd for $C_{18}H_{16}O_7$ 344.0889).

2-(3,4-Dihydroxy-phenyl)-5,7-dihydroxy-chromen-4-one (Luteolin) (5)

Amorphous powder, ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.43 (1H, d, *J* = 2.1 Hz, H-6′), 7.40 (1H, dd, *J* = 8.1, 2.1 Hz, H-2′), 6.88 (1H, d, *J* = 8.1 Hz, H-3′), 6.67 (1H, s, H-3), 6.44 (1H, d, *J* = 1.5 Hz, H-8), 6.19 (1H, d, *J* = 1.5 Hz, H-6).; ^{13}C NMR (75 MHz, $CDCl_3$) 182.1 (C-4), 164.6 (C-2), 164.3 (C-7), 161.9 (C-9), 157.7 (C-5), 150.1 (C-3′), 146.1 (C-4′), 121.9 (C-1′), 119.4 (C-6′), 116.4 (C-5′), 113.8 (C-2′), 104.1 (C-10), 103.3 (C-6), 99.2 (C-8), 94.2 (C-3); EIMS, *m/z* = 286$[M]^+$; HREIMS, *m/z* 286.0475 (calcd for $C_{15}H_{10}O_6$ 286.0469).

Luteolin-7-glucoside (6)

Amorphous powder, ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.46 (1H, dd, *J* = 8.1, 2.0 Hz, H-2′), 7.42 (1H, d, *J* = 2.1 Hz, H-6′), 6.90 (1H, d, *J* = 8.1 Hz, H-3′), 6.79 (1H, d, *J* = 2.1 Hz, H-8), 6.75 (1H, s, H-3), 6.45(1H, d, *J* = 1.5 Hz, H-6), 5.08 (1H, d, *J* = 7.2 Hz, H-1″), 3.68 (1H, m, H-2″), 3.42 (4H, m, 3″,4″,5″ and 6″); ^{13}C NMR (75 MHz, $CDCl_3$) 182.3 (C-4), 164.9 (C-2), 163.3 (C-7), 161.5 (C-9), 157.4 (C-5), 151.9 (C-5′), 150.3 (C-4′), 146.2 (C-1′), 141.1 (C-2′), 121.8 (C-3′), 119.6 (C-6′),116.4 (C-10), 114.0 (C-1″), 105.7 (C-6), 103.6 (C-8), 99.9 (C-3), 77.9 (C-2″), 77.6 (C-5″), 76.8 (C-3″), 73.5 (C-4″), 69.9 (C-6″); EIMS, *m/z* = 448$[M]^+$; HREIMS, *m/z* 448.1042 (calcd for $C_{21}H_{20}O_{11}$ 448.1038).

3,4-Dihydroxy benzoic acid (7)

Amorphous powder, ^{1}H NMR (300 MHz, DMSO) δ 7.32 (1H, d, *J* = 2.1 Hz, H-2), 7.27 (1H, dd, *J* = 8.1, 1.8 Hz, H-6), 6.76 (1H, d, *J* = 8.1 Hz, H-5); ^{13}C NMR (75 MHz, DMSO) δ 168.4 (–COOH), 152.1 (C-4), 147.8 (C-3), 126.3 (C-6), 122.8 (C-1), 115.2 (C-2), 112.4 (C-5); EIMS, *m/z* = 154$[M]^+$; HREIMS, *m/z* 154.0258 (calcd for $C_7H_6O_4$ 154.0266).

4-Hydroxybenzoic acid (8)

Amorphous powder, ^{1}H NMR (300 MHz, DMSO) δ 7.78 (2H, d, *J* = 8.7 Hz, H-2,6), 6.81 (2H, d, *J* = 8.7 Hz, H-3,5); ^{13}C NMR (75 MHz, DMSO) 167.2 (–COOH), 161.2 (C-4), 131.5 (C-3,5), 120.8 (C-1). 114.7 (C-2,6); EIMS, *m/z* = 138$[M]^+$; HREIMS, *m/z* 138.0317 (calcd for $C_7H_6O_3$ 138.0311).

Inhibitory activities of *S. cerevisiae* and rat intestinal α-glucosidase (sucrase and maltase)

The *S. cerevisiae* α-glucosidase inhibitory activity was assayed according to standard procedures by following the hydrolysis of nitrophenyl glycosides (Ryu et al. 2011). Briefly, the reaction mixture consisted of the enzyme solution (0.02 units α-glucosidase, 50 μL), substrate (1 mM *p*-nitrophenyl-α-D-glucopyranoside, 50 μL) in 50 mM potassium phosphate buffer (pH 6.8), and test compound in 5 % DMSO (10 μL). The reaction mixture was incubated in a 96-well plate at 37 °C, and *p*-nitrophenol release was monitored spectrophotometrically at 405 nm every minute for 30 min. α-Glucosidase inhibitory activity was determined by measuring the area under the curve (0–30 min) for each compound and comparing it with that of the control. Compounds showing the highest inhibitory activity were further characterized by determining the concentration required to inhibit 50 % of the α-glucosidase activity under the assay conditions (defined as the IC_{50} value). Kinetic parameters were determined using the Dixon plot and Lineweaver–Burk double reciprocal plot method at increasing concentration of inhibitors and substrates. DNJ and acarbose were used in the assays for comparison as positive control. We prepared the mammalian intestinal α-glucosidases from rat intestinal acetone powder. Commercial rat intestine acetone powder (10 mg) was dissolved in 10 mL of 0.1 M potassium phosphate buffer (pH 7.0) (buffer A), sonicated at 14 °C for 30 min, and then centrifuged at 13,000 rpm and 4 °C for 15 min to obtain the supernatant. The supernatant was dialyzed using buffer A. Maltase and sucrase inhibitory activities of the crude enzyme solution were 0.21 and 0.58 U/mg protein, respectively. Maltase and sucrase inhibitory activities were

measured according to Kimura A et al. (Kimura et al. 2004) using 28 mM maltose and 28 mM sucrose solution in 0.1 M potassium phosphate buffer (pH 7.0) as substrates for maltase and sucrase inhibition determination, respectively. The reaction mixtures for sucrase inhibitory activity determination consisted of 0.4 mL crude enzyme solution, 0.4 mL substrate solution, and 0.2 mL samples in 50 % DMSO, whereas the reaction mixtures for maltase inhibitory activity determination consisted of 0.1 mL crude enzyme solution, 0.7 mL substrate solution, and 0.2 mL samples in 50 % DMSO. The glucose release in the solution was measured using a glucose assay kit based on the glucose oxidase/peroxidase method in 96-wells microplate at 37 °C for 30 min. The OD (optical density) of the wells was measured at 490 nm.

Cell culture and α-glucosidase inhibitory activity in Caco-2 cells

Caco-2 cells (a human intestinal epithelial cell line) were obtained from the Korean Cell Line Bank (Seoul, Korea) and were grown in DMEM supplemented with 10 % FBS, glutamine (2.5 mM), and antibiotics [penicillin (50 U/mL) and streptomycin (50 g/mL)] at 37 °C in a 5 % CO_2 humidified atmosphere. The α-glucosidase inhibitory experiments were performed following a previous method (Huang et al. 2010). Briefly, Caco-2 cells were fed on polyethylene terephthalate membranes (Falcon, pore size: 0.4 μm, pore density 1.6×10^6 pore/cm^2, diameter: 23.1 mm) in a 6-well plate. After 23 days, the culture medium was removed, and both the apical and basal chambers were washed three times with 5 mL PBS (phosphate buffered saline). The culture medium in the apical chamber was replaced with a reaction mixture consisting of each isolated compound (0.1 mL) with 28 mM sucrose in PBS (0.8 mL) and 28 mM maltose solution in PBS (0.8 mL) as substrates for determining sucrase and maltase inhibitory activity, respectively. After incubating the Caco-2 cells, 50 μL of bathing solution on the apical side was transferred to a microtiter plate, and the glucose content liberated was measured using the glucose oxidase method (Toda et al. 2000). The commercial α-glucosidase inhibitor, acarbose, was used as a positive control to verify the culture system.

In vivo oral glucose tolerance test

Ten-week-old C57BL/6J male mice were purchased from Samtako Bio Korea (Seoul, Korea). After 1 week of acclimation, mice were randomly assigned to two groups fed the AIN-93G-based normal chow. The oral glucose tolerance test (OGTT) was performed 1 week after initiating the chow diet followed by a 10 h overnight fast. Vehicle (PBS) and sample (ethanol crude extracts, 5 mg/kg) were orally administered to mice 30 min prior to the glucose solution gavage (1.5 g/kg body weight). Blood glucose concentrations were measured at 0, 10, 20, 40, 60, 90, and 120 min with an Accu-Check glucometer (Roche, Indianapolis, IN, USA).

Table 1 Comparison of extraction yield and α-glucosidase inhibition of BME using different solvents

Extraction solvent	Extraction yield[a] (%)	α-glucosidase	
		IC_{50} (μg/mL)	Inhibition[b] (%)
Ethyl acetate	2.0 ± 0.2	70.2 ± 0.3	94.8 ± 4.2
Ethanol	4.1 ± 0.5	168.4 ± 3.5	88.7 ± 3.5
50 % ethanol	5.3 ± 0.2	199.6 ± 4.3	50.4 ± 4.5
Methanol	4.5 ± 0.7	182.5 ± 2.8	56.4 ± 2.2
Water	10.4 ± 1.1	NT	NT

All extracts were examined in a set of experiments repeated three times

NT not tested

[a] Extraction yields are given as g/2 g dry weight

[b] Sample concentration was 0.2 mg/mL

Statistical analysis

All measurements were repeated three times, and results are shown as mean ± SD of three experiments. Statistical analysis was carried out using ANOVA (analysis of variance). A $p < 0.05$ was accepted as statistically significant. Curve fitting was performed using Sigma Plot 2001 (Systat Software Inc., Chicago, IL, USA).

Results and discussion

α-Glucosidase inhibitory potential of the extracts

The BME were obtained using five different organic solvents to confirm the inhibitory effect of each fraction against *S. cerevisiae* α-glucosidase. As shown in Table 1, all extracts, except the water extract, significantly inhibited α-glucosidase ($IC_{50} < 200$ μg/mL). In particular, the ethyl acetate extract exhibited the best inhibitory activity against α-glucosidase ($IC_{50} = 70.2$ μg/mL). The high potency of α-glucosidase inhibition in the ethyl acetate extract encouraged us to identify the compounds responsible for this effect.

Evaluation of *S. cerevisiae* α-glucosidase inhibitory activity and kinetic analysis

The initial screening for α-glucosidase inhibitory activities used *S. cerevisiae* as the source, which is readily available

Table 2 Inhibitory effects of compounds **1–8** on bacterial α-glucosidase

Compounds	α-glucosidase (*S. cerevisae*)	
	IC_{50}^{a} (μM)	Kinetic mode (K_i, μM)[b]
1	1.3 ± 0.1	Noncompetitive (1.9 ± 0.4)
2	14.1 ± 1.2	Noncompetitive (2.3 ± 1.1)
3	74.8 ± 2.3	Noncompetitive (77.8 ± 1.8)
4	>100	NT[c]
5	17.8 ± 1.3	Noncompetitive (26.8 ± 1.3)
6	>100	NT
7	21.6 ± 1.8	Noncompetitive (18.2 ± 0.9)
8	>100	NT
Acarbose	255.1 ± 15.6	NT
DNJ	2.5 ± 0.1	NT

NT is not tested, *DNJ* deoxynojirimycin

[a] All compounds were examined in a set of experiments repeated three times; IC_{50} values of compounds represent the concentration that caused 50 % enzyme activity loss

[b] Values of inhibition constant

in a pure form and has been widely using for nutraceutical and medicinal investigations as a model for screening potential inhibitors (Kim et al. 2010; Li et al. 2010). All isolated compounds (**1–8**) were investigated for their inhibitory activity against *S. cerevisiae* α-glucosidase and were compared with that of DNJ and acarbose. As shown in Table 2, almost all compounds inhibited α-glucosidase (IC_{50} 1.3–74.8 μM), except compounds **4**, **6,** and **8**. The results are shown in Fig. 2. Two serotonin derivatives (CS and FS) showed potent inhibitory activity. In particular, CS revealed the highest inhibitory activity (IC_{50} = 1.3 ± 0.1 μM) that was higher than that of DNJ (2.5 ± 0.1 μM) and acarbose (255.1 ± 15.6 μM), respectively. In contrast, FS, a methoxy derivative of CS, had reduced α-glucosidase inhibitory activity compared with that of CS. Takahashi et al. investigated the inhibitory activity of serotonins against yeast α-glucosidase (Takahashi and Miyazawa 2012). That report suggested that α-glucosidase was inhibited to a great extent when the compound contained a fused-aromatic ring and a heteroaromatic component. Thus, it appears that better inhibition is observed when there are free hydroxyl groups at the 5-position in the serotonin C ring. Therefore, CS, the presence of indole ring moiety and a hydroxyl group at the 5-position on the C ring, appears to be essential for inhibitory activity.

The inhibition of *S. cerevisiae* α-glucosidase by CS is illustrated in (Fig. 2A). The enzyme inhibition properties of these derivatives were modeled using double reciprocal

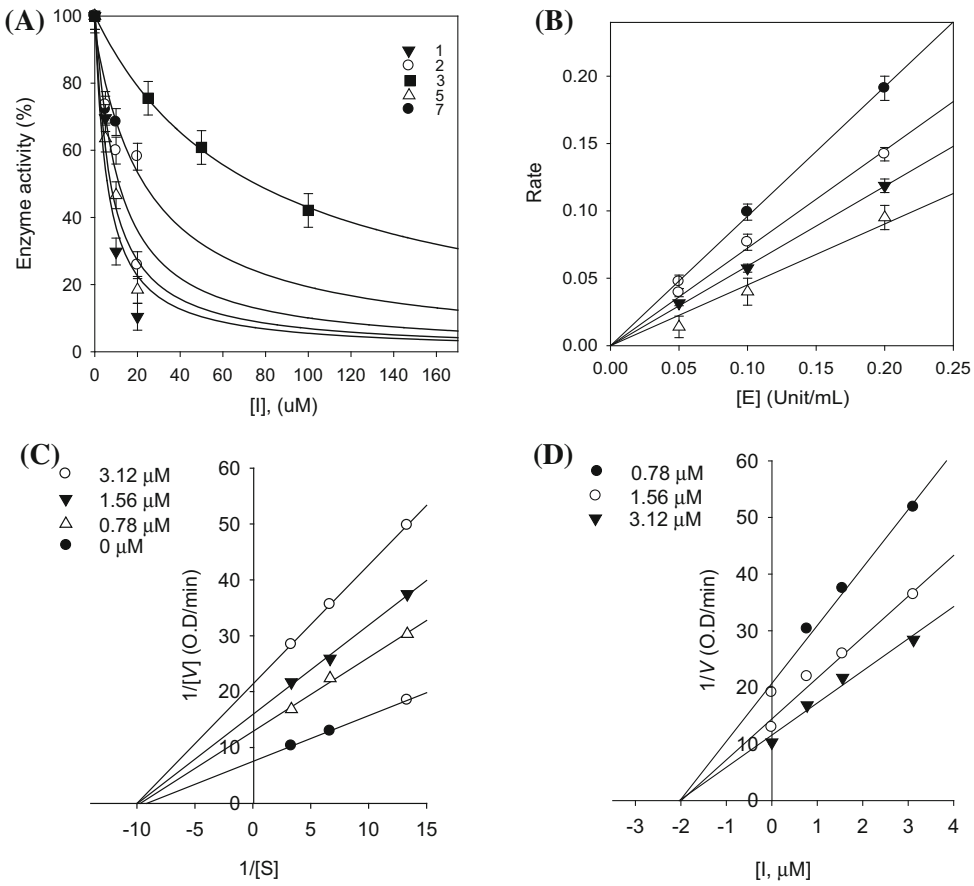

Fig. 2 **A** Effect of compounds **1** (*filled inverted triangle*), **2** (*open circle*), **3** (*filled square*), **5** (*open triangle*), and **7** (*filled circle*) on α-glucosidase activities and hydrolysis of *p*-nitrophenyl-α-D-glucopyranoside. **B** Catalytic activity of α-glucosidase as a function of enzyme concentration at different concentrations of CS (*filled circle* 0 μM; *open circle* 5 μM; *filled inverted triangle* 10 μM; *open triangle* 20 μM). **C** Lineweaver–Burk plots for inhibition by CS. **D** Dixon plot for inhibition by CS

Table 3 Inhibitory effects of CS and FS on rat intestinal α-glucosidase activities

Compound	IC_{50}^{a}	
	Rat intestinal sucrase (μM)	Rat intestinal maltase (μM)
CS	3.1 ± 0.1	>200
FS	8.1 ± 0.1	>200
Acarbose	0.9 ± 0.2	0.08 ± 0.01

[a] All compounds were examined in a set of experiments repeated three times; IC_{50} values of compounds represent the concentration that caused 50 % enzyme activity loss

plots (Lineweaver–Burk). Such an analysis of CS and FS revealed that V_{max} (1/*y*-intercept) decreased, whereas the K_m value (−1/*x*-intercept) remained constant as inhibitor concentration increased. The enzyme inhibition mode and kinetic analyses of the inhibitors showed curves typical of reversible, noncompetitive-type inhibitors (Dixon plot). (Fig. 2B–D).

Inhibitory activity against rat intestinal α-glucosidase (sucrase and maltase)

The mammalian rat intestinal α-glucosidase consists of three individual complex enzymes, namely sucrase, maltase, and isomaltase (Adachi et al. 2003). The inhibitory activities of BME compounds against mammalian rat intestinal sucrase and maltase were also compared with that of acarbose (Table 3). A previous report had indicated that CS and FS of alkaloid compounds from safflower possessed yeast α-glucosidase inhibitory activities (Takahashi and Miyazawa 2012). However, CS and FS from BME did not evaluate formammalian intestinal α-glucosidase inhibitory activities. The IC_{50} values of CS and FS were 3.1 ± 0.1 and 8.1 ± 0.1 μM against sucrase and >200 μM against maltase, respectively. CS was a more effective inhibitor than FS. The IC_{50} value of acarbose, a commercial α-glucosidase inhibitor, was 0.9 μM against sucrase and 0.08 μM against maltase, respectively.

Kim et al. reported most mammalian α-glucosidase inhibitors did not effectively inhibit microbial α-glucosidases, whereas catechin, an inhibitor of *S. cerevisiae* α-glucosidase, did not inhibit mammalian α-glucosidases (Kim et al. 2008). However, we found that the CS and FS were potent inhibitory activities against both microbial and mammalian α-glucosidases. Therefore, the α-glucosidase inhibitory activity of acarbose is likely due to the substrate specificities that depend on the source of α-glucosidase. Whereas serotonin derivatives (CS and FS) might be less specific to the enzyme, because they could bind various α-glucosidases such as microbial and mammalian. As a result, the serotonin compounds isolated from BME can potentially be developed as nutraceuticals because of their high inhibitory activity against α-glucosidase.

Caco-2 cells test

This experiment was based on the presumption that sucrose, which was added to the Caco-2 cells, can be hydrolyzed and yield glucose from the mammalian α-glucosidase in Caco-2 cells. The inhibition of carbohydrate degradation ability can be determined by measuring the liberated extracellular glucose concentration on the apical side of the Caco-2 cells monolayer compared with acarbose (Hansawasdi and Kawabata 2006). Thus, Caco-2 cells were treated with 0.1 mg ethyl acetate extract/wells to investigate α-glucosidase inhibitory activity of BME, CS, and FS in Caco-2 cells (Fig. 3). After 6 h incubation with the extract or CS and FS, α-glucosidase activity for sucrose hydrolysis decreased, resulting in a 45 % inhibition compared to that in the saline-treated control. At the same concentration, CS and FS showed 73 and 52 % inhibitory activity, respectively. Acarbose showed 92 % inhibitory activity against sucrose hydrolysis of α-glucosidase in Caco-2 cells at the same concentration. These results indicate that the serotonin compounds and BME exert potent inhibition effects against sucrase in Caco-2 cells and could be used as a functional anti-diabetic plant material.

Hypoglycemic effect of BME in the OGTT

Compared with controls, the BME showed delayed glucose appearance as assessed by the OGTT; thus, glucose concentrations from 60 to 90 min were significantly lower than those in the vehicle-fed control group (Fig. 4). This result suggests that the effect of the BME on intestinal glucose uptake may become significant 1 h after glucose intake. The area under the curve values showed borderline significance ($p = 0.057$) in the sample group with an 8 % reduction compared with that in the control group (Fig. 4). Considering that postprandial glucose metabolism in normoglycemic mice is usually well-controlled, the improved glucose removal 1 h after glucose administration indicates meaningful biological role of the BME, and the effect may be amplified in hyperglycemic animal models.

In conclusion, we determined the inhibitory effects of isolated compounds from barnyard millet grain on various α-glucosidase activities assay in vitro for the first time. Our isolation studies yielded eight compounds, which were noncompetitive inhibitors against *S. cerevisiae* α-glucosidase. The most potent compounds, serotonin derivatives (CS and FS), had 1.3–14.1 μM IC_{50} values. Takahashi's group has suggested that serotonin derivatives such as CS

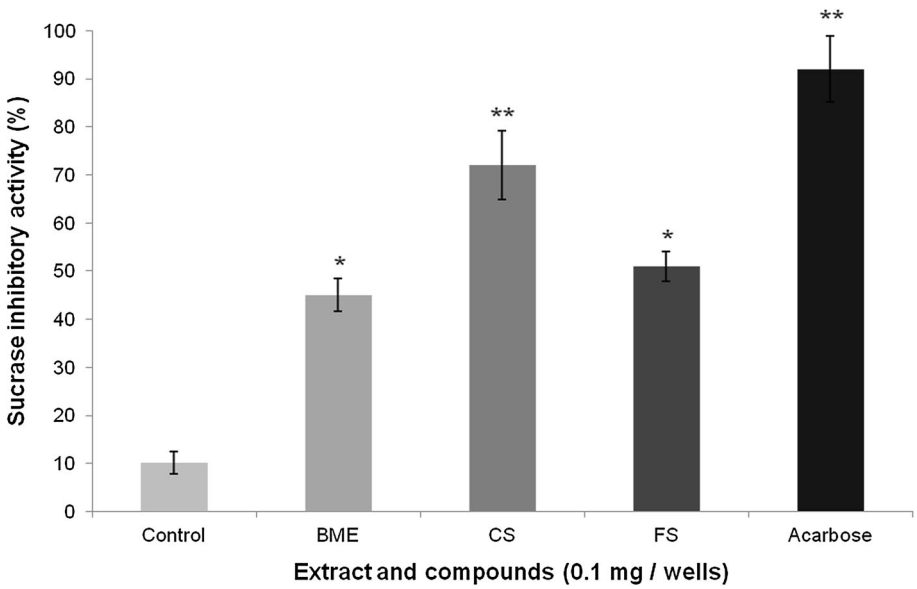

Fig. 3 Effect of the BME and serotonin compounds (CS and FS) on sucrase inhibitory activity in Caco-2 cells. Each *bar* represents the mean and SD ($n = 3$) $*p < 0.05$, $**p < 0.01$ versus control

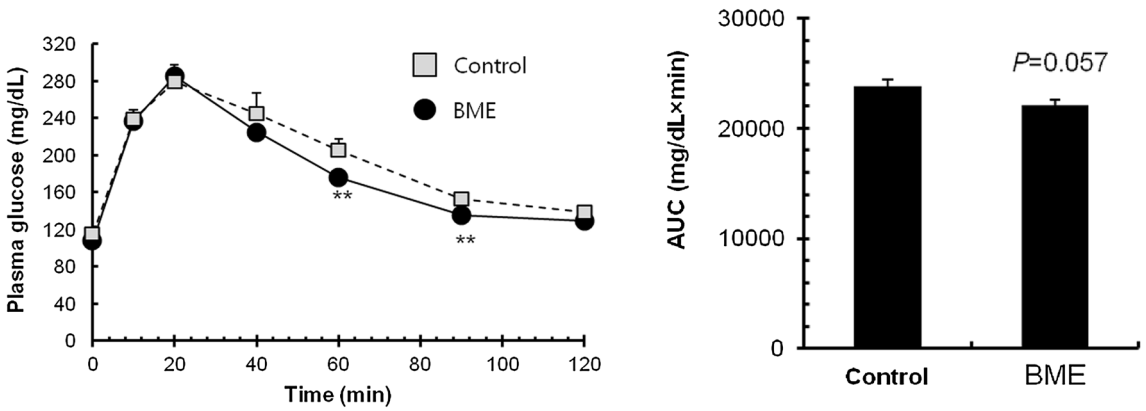

Fig. 4 Inhibition of postprandial glycemic response by the BME using OGTT (the oral glucose tolerance test)

($IC_{50} = 47.2$ μM) and FS ($IC_{50} = 99.8$ μM) have yeast α-glucosidase inhibitory activities, respectively. However, these compounds were not previously known to inhibit the mammalian rat intestinal and Caco-2 cells α-glucosidase systems. Mammalian α-glucosidase inhibitors have become exciting candidates to slow digestion of carbohydrates and alleviate postprandial hyperglycemic excursions. Recent studies have shown that polyphenols such as flavonoids and anthocyanins have significant mammalian α-glucosidase inhibitory activities and contribute to suppressing postprandial hyperglycemia. The mechanism of anti hyperglycemia by polyphenols was to reduce glucose absorption rate via inhibition of the carbohydrate hydrolyzing enzymes such as α-glucosidase in the digestive tract (Rabasa-Lhoret and Chiasson 2004). These studies led us to a further investigate compounds from BME from their anti-diabetic biological activity. The BME dramatically inhibited α-glucosidase activity for sucrose hydrolysis at a concentration of 0.1 mg/well in Caco-2 cells. Further study is needed to investigate the utility of this extract in hyperglycemic rats for the first time. Compared with control mice, the extract sample group showed accelerated removal of plasma glucose as assessed by OGTT; thus, glucose concentrations from 60 to 90 min were significantly lower than those in the vehicle-fed control group. As a result, we believe that barnyard millet grain could be a useful functional crop to prevent or improve human diabetes.

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