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Isolation of Compounds with Antioxidative Activity from Quickly Fermented Soy-Based Foods

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Abstract A bacterial strain, initially identified as B1-3, was isolated from *cheonggukjang*, a traditional Korean dish made from fermented soybeans. Using the Biolog system and 16S rRNA sequence analysis, we identified B1-3 as *Bacillus mojavensis*. We manufactured a quickly fermented soybean (QFS) food product using the *B. mojavensis*, and guided by their 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging ability. We isolated substances with antioxidative activity from it. Using mass spectrometry (MS) and nuclear magnetic resonance (NMR) techniques, we isolated 4 compounds from the ethyl acetate (EtOAc)-soluble neutral fraction of methyl alcohol (MeOH) extracts of the QFS food product (genistein, daidzein, 3*R*,4*R*-3-methyl-3,4-dihydroxy-2-pentanone, and 3*S*,4*R*-3-methyl-3,4-dihydroxy-2-pentanone) and 3 compounds from its acidic fraction (4-hydroxyphenylacetic acid, genistin, and daidzein). Two compounds from the neutral fraction (3*R*,4*R*-3-methyl-3,4-dihydroxy-2-pentanone and 3*S*,4*R*-3-methyl-3,4-dihydroxy-2-pentanone) were not detected in nonfermented soybeans (NFS) or in the filtrate of the LB broth used to culture *B. mojavensis*. However, they were detected in the filtrate of the same broth when it contained 2% glucose. These results suggest that these 2 compounds were derived from glucose (or other saccharides) in the soybean during fermentation. One compound that was found in the acidic fraction (4-hydroxyphenylacetic acid) was readily detected in NFS, but not in the culture broth. This suggests that 4-hydroxyphenylacetic acid was derived from NFS. We concluded that the antioxidative activity of *cheonggukjang* is a result of the interactions between soybean components and the microorganisms used in the fermentation of *cheonggukjang*.

Key words: *cheonggukjang*, quickly fermented soybean, antioxidative activity, *Bacillus mojavensis*

Introduction

Cheonggukjang is a traditional Korean food made from soybeans fermented with microorganisms that grow in rice straw. It has a unique odor, taste, and nutritional value that are acquired during fermentation with those microorganisms (1). It is one of the most important sources of protein among traditional fermented Korean foods (2). In recent research on the physiological function of soybeans, fermented soybean-based foods were investigated for their antihypertensive (3-6), antimutagenic (7), antioxidative (8-14), and antitumor activity (15) based on their release of isoflavones, saponins, phenolic acids, and phytic acid (1). A fibrinolytic enzyme that was reportedly a product of the *Bacillus* spp. was isolated from *cheonggukjang* (16, 17). In a previous paper, we reported that an antimicrobial substance, phenylacetic acid, was released from soybeans fermented with *Bacillus licheniformis* to make *cheonggukjang*. It has been confirmed that the phenylacetic acid identified in our study originated from *B. licheniniformis* (18). The physiologically active substances identified in *cheonggukjang* originated from the soybeans, the microorganisms, or the fermentation of soybean with microorganisms. These substances, particularly those resulting from the fermentation process during the manufacture of *cheonggukjang*, have not yet been fully investigated.

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In this study, our attention was focused on compounds with antioxidative activity in *cheonggukjang*. We attempted to isolate and identify the antioxidative substances in a quickly fermented soybean (QFS) food made with a microbial strain seen in *cheonggukjang* fermented with rice straw. We also determined the origin of the antioxidants isolated from our QFS food product.

Materials and Methods

Screening and isolation of microorganisms from *cheonggukjang* made by traditional methods *Cheonggukjang* was prepared using a traditional method (1). Briefly, soybeans (500 g) were soaked in water (1 L) at room temperature for 12 hr and sterilized at 121°C for 30 min. The cooked soybeans were then fermented with rice straw (Jeonnam, Hwasun, Korea, 2001) at 42°C for 3 days. The result was *cheonggukjang* (10 g), which was mixed with 90 mL of a saline solution (0.85% NaCl) and incubated at 42°C for 30 min. The culture solution was diluted serially 10-fold with the saline solution and spread over a nutrient agar medium. The plate was incubated at 42°C for 3 days, and a bacterial strain was then isolated from colonies growing on the medium.

Identification of the microorganism Preliminary identification of the microorganism isolated from *cheonggukjang*, tentatively labeled B1-3, was carried out using the Biolog system (Biolog Inc., Hayward, CA, USA), which is based on profiles of the use of 95 carbon sources and the findings of a 16S ribosomal RNA (rRNA) sequence

analysis. To determine the 16S rRNA sequence, genomic DNA was isolated using the CTAB/NaCl method developed by Ausubel *et al.* (19) The DNA manipulations for the cloning, transformation, plasmid isolation, ligation, and electrophoresis were carried out as described by Sambrook *et al.* (20) Polymerase chain reaction (PCR) was carried out to amplify a partial 16S rRNA fragment in the B1-3 strain using the universal primers (Y1:5'-TGGCTCAGAACGCTGGCGGC-3' and Y2:5'-CCCAC TGCCTCCCGTAAGGAGT-3') (21) using the GeneAmp Thermal Cycler (model 2400; Perkin-Elmer, Norwalk, CT, USA). The amplified PCR product was purified on agarose gels using a gel elution kit (Bioneer Inc., Daejeon, Korea) and was ligated into the pGEM-T vector (Promega Co., Madison, WI, USA) according to the manufacturer's instructions. The PCR products were then sequenced using an Applied Biosystem (ABI373) DNA automated sequencer (PE Applied Biosystem, Foster City, CA, USA). The 16S rRNA sequence of the B1-3 strain was aligned with reference sequences obtained from the GenBank databases (National Center for Biotechnology Information, NCBI, Bethesda, MD, USA).

Preparation of soybeans for fermentation with microbial strain B1-3 Soybeans (1.3 kg) were soaked in water (2.6 L) at room temperature for 12 hr and then steamed at 121°C for 30 min. The steamed soybeans were drained and cooled to 50°C then inoculated with a suspension of the B1-3 strain of bacteria and incubated at 42°C for 3 days.

Extraction of fermented soybeans The fermented soybeans (≥1.2 kg) were extracted with methanol (2.6 L) for 24 hr at room temperature. The extract was concentrated in vacuo at 37°C. The methanol extract (43.2 g) was partitioned between 3.0 L ethyl acetate (EtOAc) and 2.5 L of a buffer solution (0.2 M glycine-0.2 M HCl at pH 3.0). The organic phase was refractionated with 2.5 L of another buffer solution (0.2 M NaH_2PO_4-0.2 M Na_2HPO_4 at pH 8.0). The aqueous layer (pH 8.0) was adjusted to pH 3.0 using 1.0 N HCl and extracted with EtOAc (3 L). The EtOAc-soluble neutral and acidic fractions were then concentrated in vacuo at 37°C.

Isolation of antioxidants from the EtOAc-soluble neutral fraction The EtOAc-soluble neutral fraction (6.4 g) was subjected to silica gel (Kieselgel 60) adsorption column chromatography (70-230 mesh, 100 g, 3×53 cm; Merck, Darmstadt, Germany) and eluted step-wise with an increasing concentration of EtOAc in *n*-hexane. Two active fractions (I and II) were purified by Sephadex LH-20 column chromatography (25-100 mesh, 2.1×88 cm, 250 mL; Pharmacia Fine Chemicals, Uppsala, Sweden) using a methyl alcohol/chloroform (MeOH/$CHCl_3$) (4:1, v/v) solvent system as the mobile phase. The active fraction (I-1) obtained from the Sephadex LH-20 column chromatography of fraction I was purified further by high-performance liquid chromatography (HPLC) using an octadecyl silane (ODS) column (μBondapak, 7.8×300 mm, 10 μm; Waters, Millipore, Mass, USA) with an ultraviolet (UV) detector (Dual UV-VIS absorbance detector, 210 and 260 nm; Waters, Millipore, MA, USA). The mobile phase was eluted with a water/MeOH (6:4, v/v; solvent A) and MeOH (solvent B). It was held on solvent A for 1 min, then solvent B was increased from 0 to 100% over 30 minutes; when solvent B was the only solvent, the mobile phase was held on it for 10 min at a flow rate of 4 mL/min to produce Compound **1** (245.8 mg).

Active fraction II was also subjected to Sephadex LH-20 column chromatography under the same conditions as active fraction I. The active fractions II-1 and II-2, which were separated from the Sephadex LH-20 column chromatography of fraction II, were also purified by HPLC. Active fraction II-1 was purified under the same conditions as that for isolation of Compound **1** to yield Compound **2** (111.4 mg). Active fraction II-2 was successively purified by ODS (YMC-GEL, 70-230 mesh, 17 g, 1.6×29 cm; YMC Co, Kyoto, Japan) column chromatography, beginning with a 30% MeOH solution that was increased to 100% MeOH in a step-wise elution of 34 mL for each step, and with preparative HPLC (μBondapak, 7.8×300 mm, 10 μm, 25% MeOH, v/v) to yield a mixture of Compounds **3** and **4** (4.9 mg).

Isolation of antioxidants from the EtOAc-soluble acidic fraction The EtOAc-soluble acidic fraction underwent silica gel adsorption column chromatography (Kieselgel 60, 70-230 mesh, Merck, 20 g, 1.6×35 cm) and was eluted in a step-wise fashion with increasing concentrations of MeOH in EtOAc. Active fractions III and IV were divided after chromatography and purified by different methods: fraction III by Sephadex LH-20 column chromatography [MeOH/$CHCl_3$ (4:1, v/v), 2.1×88 cm, 250 mL] and fraction IV by HPLC using an ODS column (μBondapak, 7.8×300 mm, 10 μm; Waters) with a UV detector (Dual UV/VIS absorbance detector, Waters, 254 and 280 nm). The active fraction (Compound **5**) was identified by means of gas chromatography-mass spectrometry (GC-MS), as described in the following section. The mobile phase in HPLC was eluted with 40% MeOH at a flow rate of 4 mL/min to yield Compounds **6** (9.0 mg) and **7** (2.3 mg).

Instrumental analyses of isolated compounds Liquid chromatography-electron impact MS (LC-EI-MS) analysis was performed using LC (2690 Separation Module; Waters, Millipore) and MS (LC-MS; Waters Integrity^{TM} System, Millipore) under the following conditions: ion source temperature, 200°C; ionizing voltage, 70 eV; scanning mass range, 50 to 500 *m/z*. ^{1}H-nuclear magnetic resonance (^{1}H-NMR) 500 MHz, ^{13}C NMR 125 MHz, and 2-dimensional NMR (2D NMR) spectra were recorded on a ^{unit}INOVA 500 spectrometer (500 MHz; Varian, Walnut Creek, CA, USA) in CD_3OD as the solvent and tetramethylsilane (TMS) as the internal standard.

GC-MS was performed using gas chromatography (Saturn 3; Varian) under the following GC (Star 3400CX; Varian) conditions: column, Rtx-1 capillary (0.32 mm × 30 m; Varian Instruments 2700); carrier gas, helium; injection port temperature, 240°C; split ratio, 20:1; and flow rate, 1 mL/min. The column oven temperature was held at 100°C for 2 min and increased to 240°C at 5°C/min, then maintained at 240°C for 5 min. Trime-thylsilylation was carried out by heating the samples with 20 μL of pyridine/

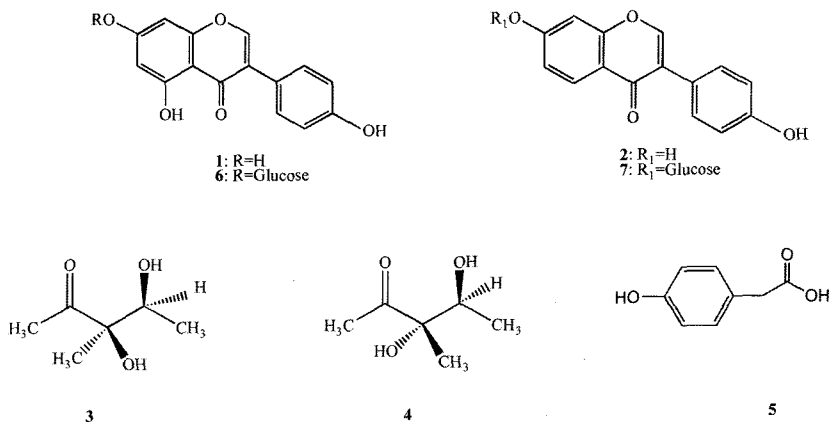

Fig. 1. Structure of the antioxidants identified in the MeOH extract of quickly fermented soybeans.

N,O-bis(trimethylsilyl) acetamide/trimethyl-chlorosilane (10: 5:1, v/v/v) at 60°C for 30 min.

Spectral data for isolated compounds Compound **1** (genistein): white powder; LC-EI-MS, *m/z* 270 [M^+]; ^{1}H NMR (CD_3OD, 500 MHz) δ 6.22 (1H, d, *J*=2.0 Hz, H-6), 6.33 (1H, d, *J*=2.0 Hz, H-8), 6.84 (2H, dd, *J*=2.0, 10.5 Hz, H-3′, 5′), 7.37 (2H, dd, *J*=2.0, 10.5 Hz, H-2, 6), 8.04 (1H, s, H-2); ^{13}C NMR (CD_3OD, 125 MHz) δ 94.89 (C-8), 100.23 (C-6), 106.40 (C-10), 116.37 (C-3′, 5′), 123.43 (C-1′), 124.85 (C-3), 131.48 (C-2′, 6′), 154.88 (C-2), 158.92 (C-4′), 159.83 (C-9), 163.97 (C-5), 166.06 (C-7), 182.36 (C-4).

Compound **2** (daidzein): white powder; LC-EI-MS, *m/z* 254 [M^+]; ^{1}H NMR (CD_3OD, 500 MHz) δ 6.85 (3H, m, H-3′, 5′, 8), 6.94 (1H, dd, *J*=2.5, 9.0 Hz, H-6), 7.37 (2H, d, *J*=9.0 Hz, H-2′, 6′), 8.05 (1H, d, *J*=9.0 Hz, H-5), 8.13 (1H, s, H-2); ^{13}C NMR (CD_3OD, 125 MHz) δ 102.07 (C-8), 115.04 (C-3,5), 115.33 (C-6), 116.97 (C-10), 123.15 (C-1′), 124.77 (C-3), 127.32 (C-5), 130.24 (C-2, 6), 153.46 (C-2), 157.51 (C-4′), 158.65 (C-9), 163.61 (C-7), 177.01 (C-4).

Compound **3** (3*R*,4*R*-methyl-3,4-dihydroxy-2-pentanone): colorless; ^{1}H NMR (CD_3OD, 500 MHz) δ 3.96 (1H, m, H-4), 2.25 (3H, s, H-1) 1.20 (3H, s, H-6), 1.17 (3H, d, *J*=6.5 Hz, H-5); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.03 (1H, m, H-4), 2.29 (3H, s, H-1), 1.28 (3H, d, *J*=6.0 Hz, H-5), 1.26 (3H, s, H-6); ^{13}C NMR (CD_3OD, 125 MHz) δ 15.71 (C-5), 20.69 (C-6), 24.47 (C-1), 71.23 (C-4), 81.64 (C-3), 213.94 (C-2).

Compound **4** (3*S*,4*R*-methyl-3,4-dihydroxy-2-pentanone): colorless; ^{1}H NMR (CD_3OD, 500 MHz) δ 3.81 (1H, m, H-4), 2.23 (3H, s, H-1) 1.27 (3H, s, H-6), 1.09 (3H, d, *J*=6.5 Hz, H-5); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.86 (1H, m, H-4), 2.24 (3H, s, H-1), 1.43 (3H, s, H-5), 1.08 (3H, d, *J*=7.0 Hz, H-6); ^{13}C NMR (CD_3OD, 125 MHz) δ 16.44 (C-5), 20.05 (C-6), 25.19 (C-1), 71.28 (C-4), 81.40 (C-3), 214.00 (C-2).

Compound **5** (*p*-hydroxyphenylacetic acid): white powder; GC-MS, *m/z* 296 ($C_{14}H_{24}O_3Si_2$, [M^+], 9.9%), 281 ([M-CH_3]$^+$, 40.9), 252 ([M-(CH_3)$_3$]$^+$, 47.9), 179 ([M-2OSi(CH_3)$_3$]$^+$, 22.5).

Compound **6** (genistin): white powder; ^{1}H NMR (CD_3OD, 500 MHz) δ 8.14 (1H, s, H-2), 7.40 (2H, d, *J*=8.5 Hz, H-3′,5′), 6.85 (2H, d, *J*=8.5 Hz, H-2′, 6′), 6.71 (1H, d, *J*=2.5 Hz, H-8), 6.53 (1H, d, *J*=2.5 Hz, H-6), 5.05 (1H, d, *J*=7.5 Hz, H-1″), 3.40-3.55 (4H, m, H-2-H-5), 3.92 (1H, dd, *J*=2.5, 12.25 Hz, H-6″a), 3.72 (1H, dd, *J*=6.0, 12.0 Hz, H-6″b); ^{13}C NMR (CD_3OD, 125 MHz) δ 182.64 (C-4′), 164.91 (C-7), 163.68 (C-5), 159.39 (C-9), 159.07 (C-4), 155.42 (C-2), 131.49 (C-2′, 6′), 125.18 (C-3), 123.18 (C-1′), 116.41 (C-3′, 5′), 108.15 (C-10), 101.75 (C-1″), 101.21 (C-6), 95.97 (C-8), 78.50 (C-5″), 77.96 (C-3″), 74.81 (C-2″), 71.32 (C-4″), 62.52 (C-6″).

Compound **7** (daidzin): white powder; ^{1}H NMR (CD_3OD, 500 MHz) δ 8.21 (1H, s, H-2), 8.15 (1H, d, *J*=6.5 Hz, H-5), 7.39 (2H, d, *J*=8.5 Hz, H-2, 6), 7.26 (1H, d, *J*=2.5 Hz, H-8), 7.22 (1H, dd, *J*=2.0, 9.0 Hz, H-6), 6.86 (2H, d, *J*=8.5 Hz, H-3′, 5′), 5.11 (1H, d, *J*=2.5 Hz, H-1″), 3.40-4.00 (4H, m, H-2″- H-5″), 3.93 (1H, dd, *J*=2.5, 12.25 Hz, H-6″a), 3.71 (1H, dd, *J*=6.0, 12.0 Hz, H-6″b); ^{13}C NMR (CD_3OD, 125 MHz) δ 178.20 (C-4), 163.62 (C-7), 159.36 (C-9), 158.91 (C-4), 155.16 (C-2), 131.51 (C-2′, 6′), 128.41 (C-5), 126.35 (C-3), 124.19 (C-1′), 120.36 (C-10), 117.15 (C-6), 116.37 (C-3′, 5′), 105.08 (C-8), 101.95 (C-1″), 78.55 (C-5″), 77.99 (C-3″), 74.86 (C-2″), 71.38 (C-4″), 62.57 (C-6″).

Determination of antioxidative activity The assay used to purify the antioxidants consisted of spraying the 200 μM 1,1-diphenyl-2-picrylhydrazyl (DPPH; Sigma Chemical Co., St. Louis, MO, USA) ethanol (EtOH) solution on a silica gel thin-layer chromatograph (TLC; silica gel 60 F_{254}, 0.25 mm thickness; Merck) (22). Each fraction was spotted on the plate and developed using a suitable solvent. After the DPPH solution was sprayed, the decolorized spots were regarded as representing the fractions possessing antioxidative activity.

Confirmation of the origin of compounds 3, 4, and 5 We carried out analyses of Compounds **3**, **4**, and **5** in 3 kinds of samples: nonfermented soybeans, a B1-3 strain culture solution, and the B1-3 broth cultured with glucose.

Nonfermented soybeans (200 g) that had not been inoculated with the B1-3 strain were homogenized and extracted using MeOH (500 mL). The extracts were

filtered through No. 2 filter paper (Whatman, Maidstone, England) and evaporated. The concentrate (7.7 g) was then solvent-fractionated by the method used to extract soybeans fermented with the B1-3 strain. In solvent fractionation, the volume of buffers and solvents was in proportion to the weight of the concentrate. After solvent fractionation, the EtOAc-soluble acidic fraction was subjected to GC-MS analysis under the following conditions: Rtx-1 capillary column (0.32×30 mm; Varian); carrier gas, helium; injection port temperature, 250°C; injection interface, 250°C; and split ratio, 20:1. The column oven temperature was held at 100°C for 2 min, then increased to 250°C at 5°C/min, and then maintained at 250°C for 5 min. The EtOAc-soluble neutral fraction was analyzed after solvent-fractionation by GC-MS under different conditions: VB-Wax capillary column (0.25×60 mm; Shimadzu, Kyoto, Japan); carrier gas, helium; injection port temperature, 200°C; injection interface, 230°C; and split ratio, 20:1. The column oven temperature was held at 80°C for 2 min, then increased to 160°C at 5°C/min, and then maintained at 160°C for 5 min.

Second, the filtrate of the B1-3 strain solution (975 mL) was incubated in LB broth (Luria-Bertani, Detroit, MI, USA) at 35°C for 72 hr with the pH adjusted to 3.0 using 1.0 N HCl and extracted with EtOAc. The EtOAc phase was also solvent-fractionated by the method used to extract soybeans fermented with B1-3 strain bacteria. The EtOAc-soluble neutral fraction was subjected to GC-MS analysis under the same conditions that were used for the EtOAc-soluble neutral fraction from nonfermented soybeans.

Last, the 2% glucose broth containing the B1-3 bacterial strain was incubated under the same conditions that were used to culture these organisms, as described above. The culture broth (973 mL) was successively partitioned with EtOAc and buffer solutions using the same method that was used to culture these organisms in broth. The EtOAc-soluble neutral fraction was analyzed by GC-MS after partitioning under the same conditions that were used to analyze the EtOAc-soluble neutral fraction of the nonfermented soybean MeOH extracts.

Results and Discussion

Isolation of microorganisms from *cheonggukjang* and identification of the B1-3 strain The B1-3 strain was isolated from *cheonggukjang* because it was the most active strain cultured from this food on nutrient agar. To identify the organism represented by this strain, we examined its morphological and biochemical characteristics by carrying out a Biolog™ analysis. These organisms utilized sucrose, glucose, arabinose, mannitol, fructose, galactose, and maltose, but not lactose. They were identified as *B. mojavensis*, a gram-positive, rod-shaped organism with a similarity index of 0.86 and a 99% probability that the B1-3 strain is *B. mojavensis*, based on 95 carbon source utilization profiles. To confirm this identify more accurately, we amplified a portion of the 16S rRNA gene for the B1-3 strain using PCR and then compared with the reference sequence of other bacterial 16S rRNA genes using a Blast search at NCBI. The partial bp sequence for the16S rRNA of the B1-3 strain showed a 99% match with that of *B. mojavensis*. Consequently, the B1-3 strain was identified as *B. mojavensis*.

Antioxidative activity in the MeOH extract of QFS MeOH extracts of the QFS food made with *B. mojavensis* and the nonfermented soybeans (NFS) were subjected to TLC analysis (benzene/acetone/acetic acid = 6.0:4.0:0.1, v/v). The TLC pattern was compared by spraying the DPPH EtOH solution (data not shown). The MeOH extract of QFS was more variable than that of the NFS in terms of the pattern of compounds with antioxidative activity. It was suggested that the QFS contains *B. mojavensis* excretions, products of soybean fermentation, or both.

Isolation of antioxidants from the QFS MeOH extract and elucidation of their structures Compounds **1**, **2**, **3**, and **4** were isolated from the EtOAc-soluble neutral fraction of MeOH extracts of QFS by means of silica gel column chromatography, Sephadex LH-20 column chromatography, and HPLC.

In the EI-MS spectra of Compounds **1** and **2**, prominent ion peaks were detected at *m/z* 270 and 254, indicating that their molecular weights are 270 and 254, respectively. The singlet proton signals at δ 8.04 (1H) and δ 8.13 (1H) in the ^{1}H NMR spectra of Compounds **1** and **2** comprise the diagnostic signal of the H-2 resonance of isoflavones (23). The structures of Compounds **1** and **2** were identified as genistein and daidzein, respectively, by comparing their spectral data with those reported for these compounds in the literature (24-27).

The identification of Compounds **3** and **4** was carried out by means of 1D and 2D NMR analyses. The ^{13}C NMR spectrum showed 12 carbon signals that included 2 carbonyl carbons, 6 methyl carbons, 2 methine carbons, and 2 quaternary carbons. Interestingly, the ^{1}H and ^{13}C NMR spectra showed evidence of a mixture of the 2 compounds, because they were observed with an unequal intensity of proton and carbon signals in a 3:1 Compound **3**:Compound **4** ratio. Moreover, the proton and carbon signals of Compounds **3** and **4** showed similar patterns, except for a difference in chemical shifts, which strongly suggested that Compounds **3** and **4** are stereoisomers of each other. The ^{1}H and ^{13}C NMR, as well as the ^{1}H-^{1}H correlated spectroscopy, total correlation spectroscopy, and heteronuclear multiple-bond correlation MS experiments on the mixture of Compounds **3** and **4** allowed us to assign of all the ^{1}H and ^{13}C signals to 3-methyl-3,4-dihydroxy-2-pentanone as one of a pair of stereoisomers. Moreover, the complete absolute configuration was established by direct comparison with their ^{1}H NMR spectral data (3*R*,4*R*-3-methyl-3,4-dihydroxy-2-pentanone ($CDCl_3$, 500 MHz): δ 4.02 (1H, s, H-4), 2.30 (3H, s, H-1) 1.28 (3H, s, H-5), 1.26 (3H, s, H-6); 3*S*,4*R*-3-methyl-3,4-dihydroxy-2-pentanone ($CDCl_3$, 500 MHz): δ 3.87 (1H, s, H-4), 2.25 (3H, s, H-1) 1.44 (3H, s, H-5), 1.08 (3H, s, H-6), previously isolated from culture broth of *B. cereus* YUF-4 (28).

In addition, the EtOAc-soluble acidic fraction (2.6 g) of the MeOH extracts of QFS was subjected to silica gel column chromatography (EtOAc-MeOH) using a step-wise elution. Compounds with antioxidative activity were eluted separately in fractions of 0% (fraction III, 434 mg) and 10% (fraction IV, 180 mg) concentrations of MeOH in

EtOAc. Fraction III was purified further by Sephadex LH-20 column chromatography (MeOH/$CHCl_3$ = 4:1, v/v) to produce a fraction with antioxidative activity (Ve/Vt 0.92-0.96; Compound **5**, 5.6 mg) that was identified by GC-MS analysis after trimethylsilylation.

The molecular weight of 296 for Compound 5 was determined based on its GC-EI-MS spectrum, with *m/z* 296 ($C_{14}H_{24}O_3Si_2$, $[M]^+$), 281 ($[M\text{-}CH_3]^+$), 252 ($[M\text{-}(CH_3)_3]^+$), and 179 ($[M\text{-}2OSi(CH_3)_3]^+$) corresponding to the trimethylsilylated 4-hydroxyphenylacetic acid. In addition, the fragment ion signals were in agreement compared with those of the Wiley library data. The GC chromatogram of trimethylsilylated Compound **5** showed a peak in retention time (t_R) at 11.59 min; it was then compared with that of the authentic 4-hydroxyphenylacetic acid (Sigma Chemical Co.). The t_R, (11.59 min) was identical to that obtained from the trimethylsilylated derivative of the authentic 4-hydroxyphenylacetic acid. Therefore, the structure of Compound **5** was unambiguously concluded to be 4-hydroxyphenylacetic acid.

Compounds **6** and **7** were isolated by means of HPLC from the active fraction IV yielded by silica gel column chromatography of the EtOAc-soluble acidic fraction of the MeOH extract of QFS. Identification of Compounds **6** and **7** was carried out by means of NMR analysis. First, Compound **6** showed 21 carbons signals in the ^{13}C NMR spectrum, including 15 carbons for aglycone and 6 carbons for a monosaccharide. The ^1H NMR spectrum of Compound **6** suggested that part of its structure is the same as Compound **1**. A sequential *trans*-1,2-diaxial relationship of H-1"-H-5" (J > 6.0 Hz) suggested further that Compound **6** is a genistein glucoside (genistin). The ^1H and ^{13}C NMR spectral data were identical with those in the literature (24). Therefore, we concluded that the structure of Compound **1** is genistein 7-*O*-β-D-glucopyronoside (genistin).

The structure of Compound **7** was identified in a similar manner to that used to identify Compound **6**. The ^1H and ^{13}C NMR spectral data showed that Compound **7** contained Compound **2** (aglycone) and glucose superimposed on the data for daidzein 7-*O*-β-D-glucopyronoside (daidzin) isolated from soybean (24).

Confirmation of the origin of compounds 3, 4, and 5 in QFS Compounds **1**, **2**, **6**, and **7** had already been reported as having antioxidative activity in soybeans and fermented soybeans. However, Compounds **3**, **4**, and **5**, to our knowledge, had not been identified in soybeans or fermented soybean-based food products, even though they are not novel compounds. Therefore, we became interested in the origin of these compounds.

The analyses of Compounds **3**, **4**, and **5** were carried out using EtOAc-soluble neutral fractions (in the case of Compounds **3** and **4**) and acidic fractions (in case of Compound **5**) obtained after solvent fractionation of the NFS MeOH extract. Trimethylsilyated Compound **5** was detected by GC and GC-MS analysis of the EtOAc-soluble acidic fraction. However, Compounds **3** and **4** were not detected by these methods in the EtOAc-soluble neutral fraction of the extract. Therefore, it was suggested that Compounds **3** and **4** originated from the B1-3 strain or were produced by fermentation of soybean with *B. mojavensis*. To confirm these possibilities, the bacterium was incubated in LB broth at 35°C without soybean. The incubated solution was also treated using the same method that was used to isolate antioxidants from QFS. The EtOAc-soluble neutral fraction was then subjected to GC-MS analysis, but Compounds **3** and **4** were not detected. This result strongly suggested that Compounds **3** and **4** are produced during the fermentation of soybean with *B. cereus*. According to Ui *et al.* (28), Compounds **3** and **4** are produced when *B. cereus* YUF-4 is cultured with glucose. The organisms used this study are also *Bacillus* spp. Therefore, we thought that Compounds **3** and **4** might be produced in a *B. cereus* culture to which glucose had been added, as was reported by Ui *et al.* (28). The medium used to confirm their culture broth was free from glucose. For this reason, 2% glucose was added to the medium under the same conditions and it was used to culture *B. cereus*. The EtOAc-soluble neutral fraction of the MeOH extracts of the culture broth was subjected to GC-MS analysis. This time Compounds **3** and **4** were detected. Based on these results, we concluded that Compounds **3** and **4** derived from QFS MeOH extracts originated from the glucose (or saccharides) in soybean during fermentation with the B1-3 strain.

Compounds **1**, **2**, **6**, and **7** had already been reported as antioxidants in soybean and fermented soybean-based food products (29-32). It is particularly well known that the concentration of the aglycones (Compounds **1** and **2**) in fermented soybean foods (e.g. *cheonggukjang*, *miso*, and *natto*) is higher than that of the glucosides (Compounds **6** and **7**) (29-31), even though the concentration of glucosides in soybeans before fermentation was higher than that of the aglycones. Murakami *et al.* (33) also identified isoflavones and their glucosides in *tempeh* (a fermented soybean product) and found that the isoflavones had been liberated from the glucosides during fermentation. They also stated that the main components responsible for antioxidant activity in tempeh might be genistein and daidzein, rather than the glucosides.

As we have observed throughout this study, it is likely that antioxidative activity can be attributed to interactions among native compounds in soybeans, compounds produced by interaction of soybean components and microorganisms during fermentation, and metabolic products secreted by the microorganisms.

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