Monascus-Mediated Fermentation Improves the Nutricosmetic Potentials of Soybeans

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Abstract The potential nutricosmetic activities and compositional changes of 80% ethanol extracts of white soybean (MFWS) and black soybean (MFBS) fermented with *Monascus pilosus* KCCM 60084 at 30°C for 30 days were investigated. *Monascus*-fermented soybean extracts (MFSEs) showed stronger nutricosmetic potentials in terms of antioxidant as well as tyrosinase and elastase inhibitory activities compared to those of unfermented soybean extracts ($p < 0.05$). Extracts (50 mg/mL) from MFBS inhibited the α -melanocyte stimulating hormone (α -MSH)-induced melanogenesis in B16F10 cells more potently than arbutin. HPLC/MS analysis demonstrated that aglycone isoflavones and coenzyme Q10 (CoQ10) levels increased about 33.4- and 3.0-fold in the MFSEs after 20 days of fermentation, respectively. A linear correlation ($r^2 = 0.67$ to 0.99) between nutricosmetic activity and concentrations of CoQ10, genistein, and daidzein, which are commonly associated with nutraceutical effects, was observed. Results indicate that *Monascus*-mediated fermentation can be an efficient strategy to improve nutricosmetic functionality of soybeans.

Keywords: *Monascus*-fermented soybean, antioxidant, anti-tyrosinase, anti-elastase, anti-melanogenic activity

Introduction

Nutricosmetics are nutritional supplements which purposes to support the function and the structure of the skin (1). Many micronutrients such as vitamin C, carotenes, CoQ10, omega-3 fatty acids, and flavonoids have this effect. These micronutrients protect the skin from the damaging effects of ultraviolet light exposure as well as free radicals in the skin, which may lead to accelerated skin aging and wrinkle formation (2). Since consumers nowadays are more aware of nutritional products that contribute to both skin health and disease prevention (3), the market for nutricosmetics shows a significant annual growth.

Aging of skin is characterized by progressive loss of skin tissue as well as changes in macromolecules of the extracellular matrix of the dermis (3,4). Elastase and tyrosinase are two key enzymes involved in skin deterioration. Elastase is an enzyme capable of degrading elastin, an extracellular protein that provides elasticity to skin connective tissue (5). Tyrosinase is a key enzyme for melanin biosynthesis in plants, microorganisms, and mammalian cells (6). Melanin is responsible for skin pigmentation and plays an important role in protection from the harmful effects of solar ultraviolet radiation (7). In addition, α -MSH increases melanin synthesis and deposition, resulting in skin pigmentation (6). Over-activity of tyrosinase causes several dermatological and

cosmetic problems such as melanoma and age spots (8). Recently, plant-derived polyphenolic substances as anti-aging ingredients have been found to be effective in reducing the rate and intensity of wrinkle formation (1,9).

Soybeans possess a wide range of potential benefits for health and nutrition including dermatological effects (10,11). In particular, soy isoflavones have multiple beneficial health effects due to their estrogen-like activity, which is largely attributed to their aglycone forms such as daidzein and genistein. These aglycones help balance estrogen levels and keep skin healthy (12). It was previously demonstrated that the β -glycosyl bond in isoflavone glucosides from fermented soybeans is cleaved by microbes during fermentation to produce these aglycones (13,14).

The filamentous fungus genus *Monascus* has been used in solid-state fermentation (SSF) in Asia for centuries in order to produce various types of products. Studies have demonstrated that products subjected to SSF with *Monascus* possess a number of functional secondary metabolites including pigments and mevinolin (natural statins) (15,16). We recently reported that *Monascus*-fermented products contain high amounts of mevinolin and CoQ10 (17). CoQ10 has been widely used in cosmetic products for its anti-oxidative and anti-aging effects (18). However, information on the nutricosmetic activities of *Monascus*-fermented soybeans is not yet available. Thus,

changes in the contents of bioactive constituents (total phenols, total carotenes, proanthocyanidins, isoflavones, and CoQ10) and nutricosmetic potentials of soybeans during *Monascus*-mediated fermentation at 30°C for 30 days were examined. Furthermore, the contribution of each identified component to the observed nutricosmetic properties of *Monascus*-fermented soybean extracts was investigated.

Materials and Methods

Materials and chemicals White and black soybeans [*Glycine max* (L.) Merr.] were purchased from a local market. Lyophilized culture of *Monascus pilosus* KCCM 60084 was obtained from the Korean Culture Center of Microorganisms (KCCM, Korean Federation of Culture Collections, Seoul, Korea). The stock culture was grown on potato dextrose agar (PDA) and maintained at 25°C. Mushroom tyrosinase, porcine pancreatic elastase (PPE), α -MSH, arbutin, Bradford Reagent, theophylline, N-succinyl-L-alanine-4-nitroanilide, tris-HCl buffer, L-tyrosine, L-DOPA, dimethyl sulfoxide (DMSO), kojic acid, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), melanin, 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), catechin, epigallocatechingallate (EGCG), and CoQ10 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The materials used for cell culture, including Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and antibiotic antimycotics, were purchased from Gibco BRL (Grand Island, NY, USA). HPLC-grade acetonitrile, methanol, hexane, propanol, and acetic acid were supplied by J. T. Baker (Phillipsburg, NJ, USA). Standards for 12 isoflavones (daidzein, genistein, glycitein, daidzin, genistin, glycitin, acetyl daidzin, acetylgenistin, acetylglycitin, malonyldaidzin, malonylgenistin, and malonylglycitin) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). General aerobic medium were purchased from Difco Co. (Detroit, MI, USA). All other reagents were of the highest grade available unless otherwise indicated.

Preparation of *Monascus*-fermented soybean *Monascus*-fermented soybeans were produced as described elsewhere (17). A two-stage-fermentation process including a seed culture stage (liquid culture) and metabolite production stage (SSF), was used to produce bioactive compounds. In brief, soybeans were washed, soaked, and autoclaved. After cooling, the substrate was inoculated with nutrient broth including *Monascus pilosus* KCCM 60084, and incubated at 30°C for 30 days. *Monascus*-fermented soybeans were collected, lyophilized, and powdered. A subsample was then extracted with 80% ethanol and filtered. Combined extracts were then rotary evaporated (DU-650; Beckman Coulter, Brea, CA, USA) at 40°C and lyophilized. The dried extract was used directly for the study.

Antioxidant activity Antioxidant activity was measured based on

DPPH radical scavenging activity and ferric reducing antioxidant power (FRAP) assay. The DPPH free radical scavenging activity of the MFSEs was measured using the method of Brand-Williams *et al.* (19) with some modifications. Briefly, 75 mM DPPH solution in methanol was prepared, and 2.5 mL of this solution was added to 0.5 mL of the test sample (50 mg/mL). After 20 min of incubation at ambient temperature, the absorbance at 517 nm was measured. Results were expressed in mM trolox equivalents per g of dry weight (mM TE/g DW).

FRAP assay was carried out according to Benzie and Strain (20) with some modifications. Stock solutions consisted of 300 mM acetate buffer (3.1 g of $C_2H_3NaO_2 \cdot 3H_2O$ and 16 mL of $C_2H_4O_2$), pH 3.6, 10 mM TPTZ solution in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ solution. Fresh working solution was prepared by mixing 25 mL of acetate buffer, 2.5 mL of TPTZ solution, and 2.5 mL of $FeCl_3 \cdot 6H_2O$ solution, followed by warming at 37°C before use. MFSEs (50 mg/mL) were allowed to react with 1.5 mL of FRAP solution for 30 min in the dark. Readings of the colored product [ferrous tripyridyltriazine complex] were then taken at 593 nm. Results were expressed in mM TE/g DW.

Anti-tyrosinase activity Anti-tyrosinase assay was performed according to Wu *et al.* (8) with some modifications. MFSEs were tested at different concentrations after dissolving microparticles in aqueous reaction solution (final concentration; 1, 10, 30, and 50 mg/mL). Mushroom tyrosinase was solubilized in phosphate buffer (pH 6.8) to obtain a final concentration of 100 units/mL. After MFSE samples were pre-incubated with 125 μ L of tyrosinase solution for 10 min, 250 μ L of L-tyrosine (2 mM) was added. Final volume of mixture was 2.5 mL, and reaction kinetics were evaluated at a wavelength of 480 nm using a UV-vis spectrophotometer. Reaction mixture without samples was used as a negative control, whereas kojic acid (10 mg/mL) was used as a positive control. Results are reported as the mean $\pm$ standard deviation of the inhibition percentage according to the following formula: % Inhibition = $[(A-B)/A] \times 100$, where A is the absorbance of the negative control and B is the absorbance of the test solution.

Anti-elastase activity To evaluate inhibitory effect of MFSEs on elastase enzyme activity, the method described by James *et al.* (5) was employed with some modifications. Aqueous MFSE solutions at different concentrations (1, 10, 30, and 50 mg/mL) were tested. PPE was diluted in phosphate buffer at pH 6.8 at a concentration of 1.2 units/mL. The substrate N-succinyl-L-alanine-4-nitroanilide was diluted in tris buffer at pH 8 in order to obtain a final concentration of 0.6 mM. The sample was pre-incubated with substrate for 20 min after which 125 μ L of PPE was added. Reaction kinetics were evaluated at a wavelength of 410 nm using a UV-vis spectro-photometer. The reaction mixture without sample was evaluated as a negative control, whereas EGCG was used as a positive control at a concentration of 10 mg/mL. Relative anti-elastase activity (%) was evaluated using the

following formula: % activity=(A-B)/A×100, where A is the absorbance of the negative control and B is the absorbance of the test solution.

Total phenols Total phenolic content was determined by the Folin-Ciocalteu method (21). MFSEs (50 mg/mL) were mixed with 1 mL of Folin-Ciocalteu reagent, 2 mL of 20% sodium carbonate solution, and 2 mL of distilled water. Readings were made by using a spectrophotometer at 750 nm using a gallic acid standard curve. The results were expressed as mg gallic acid equivalents (mg GAE/g DW).

Total carotenoids Total carotenes were extracted from 50 mg of sample in 10 mL of solvent (*n*-hexane:acetone:ethanol, v/v/v; 50:25:25) (22). All extraction experiments were conducted under subdued light, and the extraction tubes were wrapped with aluminum foil to avoid sample degradation by photooxidation. The supernatant was measured at 450 nm using a spectrophotometer. Total carotenoid content was expressed as μg β-carotene equivalents (μg BCE/g DW).

Proanthocyanidins Total content of proanthocyanidins (PAs) was measured by the vanillin-sulfuric acid method (23) with minor modification. Each extract (50 mg/mL) was mixed in 10 mL of methanol/1 N H₂SO₄ (85:15) and 4% vanillin in methanol (w/v). For the sample control, vanillin solution was substituted with 100% methanol. The mixture was incubated for 15 min in a 30°C water bath, and the absorbance was determined at 500 nm against a reagent blank. The difference in absorbance between the sample and sample control was used to determine the total PA concentration, which was expressed as mg (+)-catechin equivalents (mg CE/g DW).

HPLC/MS analysis of Coenzyme Q10 Quantification of CoQ10 was performed as described in our previous work (17). Briefly, ubiquinone extraction was based on saponification before solvent extraction. Analyses for ubiquinones were performed by reversed-phase column with LC, followed by electrospray ionization in positive mode and MS detection (Agilent 1100; Agilent Technologies, Santa Clara, CA, USA). Zorbax eclipse plus C18 column (50 mm×3.0 mm; 1.8 μm particle size; Agilent Technologies) was used for chromatographic separation. For quantitative analysis of CoQ10, a mixture of 2-propanol:methanol (50:50; v/v) containing ammonium formate (5 mmol/L) was used as the mobile phase at a flow rate of 0.16 mL/min. Temperatures of the column and autosampler were maintained at 30 and 10°C, respectively. Aliquots (1 μL) were injected automatically into the HPLC system. Concentrations of CoQ10 (*M*⁺ 881.7 *m/z* 863.7 *m/z*) in sample extracts were determined from single ion responses (SIRs) for each extract. The limit of detection (LOD) for CoQ10 was 0.01 μg/g.

HPLC analysis of isoflavones Determination of isoflavones in MFSEs was conducted according to previously described procedures

(24). Isoflavones were analyzed using an HPLC system (Agilent 1100; Agilent Technologies) equipped with a UV detector at 254 nm and hydrosphere C18 reverse-phase column (50×4.6 mm, YMC, Kyoto, Japan) with a guard cartridge column (23×4.0 mm, YMC). The mobile phase consisted of 0.1% glacial acetic acid in distilled water (solvent A) and 0.1% glacial acetic acid in ACN (solvent B). Solvent B was increased from 15 to 35% for 50 min and then held at 35% for 10 min. The solvent flow rate was 1 mL/min, and the injection volume was 20 μL. The isoflavone standards were dissolved in DMSO at several concentrations (25, 50, 100, and 250 μg/mL), and high linearity (*r*²>0.996) was obtained for each compound. The 12 isoflavones were identified based on their retention times, and their concentrations were calculated by comparing peak areas of samples with those of the standards.

Cell culture The B16F10 mouse melanoma cells were obtained from Korean Cell Line Bank (Seoul, Korea) and were maintained in DMEM containing 10% FBS, penicillin, and streptomycin (100 μg/mL). The cells were placed in an incubator at 37°C under 5% CO₂ and 95% humidity. When confluent, the cells were detached with 0.05% (w/v) trypsin and 0.02% (w/v) EDTA in PBS and resuspended in an appropriate medium for use in subsequent procedures.

Cell viability The cell viability due to treatment with a test sample was examined by an MTT assay as previously reported (25). Briefly, the B16F10 cells were seeded in 6-well plates at 1×10⁵ cells/mL and cultured for 24 h with or without the test sample. The formazan precipitates were dissolved by DMSO and the concentrations were measured at 540 nm in a microplate reader with a reference wavelength of 655 nm. Cell viability was calculated using the following formula: cell viability (%)=(A sample/A control)×100, where A sample and A control are the absorbances from the mixture with, or without the addition of test sample, respectively.

Melanin contents Melanin content was measured as described previously (26) with slight modifications. The B16F10 melanoma cells were seeded in 6-well plates at 1×10⁵ cells/well and incubated overnight to allow cells to adhere. The cells were exposed to various concentrations (10, 30 and 50 mg/mL) of the sample extracts or arbutin (100 μg/mL) for 72 h in the presence or absence of 2 μM α-MSH. The absorbance at 405 nm was measured by using a microplate reader. The melanin content was determined from a standard curve prepared from an authentic standard of synthetic melanin.

Statistical analysis Mean values and standard deviations were calculated from the data obtained from triplicate experiments. Means were analyzed with unpaired two-tailed Student's *t*-test and compared using Duncan's multiple range test method in SAS, version 8 (SAS Institute, Cary, NC, USA). Significant difference was considered at the level of *p*<0.05.

Results and Discussion

Total phenols, total carotenoids, and proanthocyanidins (PAs)

Changes in total phenol, total carotenoid, and PA contents in white soybean (MFWS) and black soybean (MFBS) during *Monascus*-mediated fermentation at 30°C for 30 days are shown in Table 1. The total phenolic contents of unfermented white and black soybeans (day 0) were 2.88 mg and 3.21 mg of gallic acid equivalents per g dried weight (mg GAE/g DW), whereas MFWS and MFBS showed phenolic contents of 5.25 and 6.71 mg on day 30 of fermentation, respectively. Both MFWS and MFBS contained 1.8- and 2.1-fold higher total phenolic contents compared to those of unfermented samples, respectively ($p < 0.05$). The total phenolic contents of these samples tended to decrease after 30 days of fermentation (data not shown). These results are similar to a report of Lim *et al.* (27), who showed that *Monascus*-fermented soybeans contain 2-fold higher total phenolic content compared to non-fermented soybeans. In general, the fermentation-induced structural breakdown of cell wall may occur, leading to the liberation of various bioactive compounds (23). Thus, catalytic release of total phenolic compounds from the soybean substrate during fermentation may lead to increased levels of these compounds (24). Total phenolic content of MFBS was significantly higher ($p < 0.05$) than that of MFWS. In a report by Xu and Chang (28), the total phenolic content of black soybeans was 3.23-fold higher than the average value of yellow soybean genotypes.

Carotenoids are widespread natural pigments found in all kingdoms of the living world (22). Among them, β -carotene is an orange-yellow pigment that can be used as a coloring agent, a source of provitamin A, and as an antioxidant agent. β -Carotene is used commercially in the food, feed, cosmetic, and pharmaceutical industries (29). In the present study, the highest total carotenoid content of MFBS was 24.13 ± 0.16 μ g β -carotene equivalents after 20 days of fermentation, after which total carotenoids tended to decrease as shown in Table 1. Similar to the results, total carotenoids of rice bran fermented with *Rhodotorula glutinous* increased after 6 days of incubation, reaching a maximum content of 1.65 mg/kg rice bran, and declined thereafter (30). Total carotenoids of soybean extracts increased during fungal growth and reached a maximum in late log phase (22). In general, reduction of total carotenoids is attributed to lipid oxidation. Carotenoids

possess potent antioxidant activity and are degraded to avoid lipid oxidation (29). For instance, the antioxidant effect of β -carotene against lipid peroxidation was reported to be accompanied by degradation of the pigment and loss of color (31). The total carotenoid content of MFBS was on 3.3 times higher than the corresponding value of MFWS during the fermentation period ($p < 0.05$).

PAs are a major group of polyphenols. Complex polyphenols containing the same polymeric building block form tannins (31). As displayed in Table 1, PAs contents of unfermented white and black soybeans (day 0) were 3.21 and 7.32 mg, whereas *Monascus*-fermented samples showed contents of 6.73 and 10.72 mg after 20 days of fermentation, respectively. Thus, contents of PAs in white and black soybeans increased by 1.5- and 2.1-fold during *Monascus*-mediated fermentation ($p < 0.05$). As shown in Table 1, total PA content of MFBS was significantly higher ($p < 0.05$) than the corresponding value of MFWS during the fermentation period. Another report similarly observed that seed coats of black soybeans have a higher content of polyphenols, which are predominantly anthocyanins and PAs (28,32). PAs are widely distributed in tissues of various plant foods and have antioxidant, anti-cancer, and anti-cardiovascular disease effects (23,32).

Coenzyme Q10 CoQ10 (ubiquinone) is a well-known antioxidant that is used in many skincare products for anti-aging purposes (33). As intracellular levels of CoQ10 decrease with age, supplementation of dietary or topical CoQ10 has been proposed to decelerate the skin aging process (3). Recently, Zhang *et al.* (18) reported that CoQ10 presents with anti-aging benefits against intrinsic aging as well as photooxidative damage. CoQ10 contents of MFSEs are presented in Table 1. Maximum yields of CoQ10 were obtained after 20 days of fermentation and decreased thereafter. Highest contents of CoQ10 in MFWS and MFBS were 165.59 ± 6.34 and 172.78 ± 5.12 μ g, respectively, and these levels increased by 2.7- to 3.0-fold compared to those of unfermented products. Several types of microorganisms, including bacteria, fungi, and yeasts, have been reported as CoQ10 producers (34). As expected, CoQ10 content of MFBS was significantly higher ($p < 0.05$) than that of MFWS.

Isoflavones Changes in total isoflavone, β -glucoside, malonyl-

Table 1. Changes in total phenols, total carotenoids, proanthocyanidins, and CoQ10 contents of white and black soybean extracts during *Monascus*-mediated fermentation at 30°C for 30 days

Fermentation days	Total phenols (mg gallic acid/g)		Total carotenoids (μ g β -carotene/g)		Proanthocyanidins (mg catechin/g)		CoQ10 (μ g/g)	
	white	black	white	black	white	black	white	black
0	2.88 $\pm$ 0.06a ¹⁾	3.21 $\pm$ 0.05a*	6.24 $\pm$ 0.27a	20.56 $\pm$ 0.44a	3.21 $\pm$ 0.15a	7.32 $\pm$ 0.17a	60.25 $\pm$ 4.54a*	57.41 $\pm$ 3.12a*
10	3.41 $\pm$ 0.03b	4.27 $\pm$ 0.08b	7.11 $\pm$ 0.32c	21.89 $\pm$ 0.14b	5.91 $\pm$ 0.12b	8.78 $\pm$ 0.09b	124.75 $\pm$ 4.46b	150.24 $\pm$ 5.01b
20	4.63 $\pm$ 0.05c	5.59 $\pm$ 0.07c	7.58 $\pm$ 0.11d	24.13 $\pm$ 0.16d	6.73 $\pm$ 0.22d	10.72 $\pm$ 0.21d	165.59 $\pm$ 6.34d	172.78 $\pm$ 5.12d
30	5.25 $\pm$ 0.16d	6.71 $\pm$ 0.11d	6.79 $\pm$ 0.31b	22.74 $\pm$ 0.21c	6.21 $\pm$ 0.17c	9.03 $\pm$ 0.11c	161.46 $\pm$ 4.03c	168.13 $\pm$ 6.22c

¹⁾Each value is mean $\pm$ SD ($n=3$).

Different letters in the same column indicate significant difference at $p < 0.05$.

The superscripts (*) in the same row are not significantly different at $p < 0.05$.

Table 2. Changes in isoflavone-conjugated profiles of white and black soybean extracts during *Monascus*-mediated fermentation at 30°C for 30 days

Isoflavones (µg/g dry weight)	Fermentation days							
	0		10		20		30	
	white	black	white	black	white	black	white	black
Daidzin	1667.93±13.45a ¹⁾	1720.28±19.51a	255.75±8.56b*	258.22±7.12b*	323.61±4.99c*	313.7±6.58c*	472.8±6.89d	315.27±7.36c
Glycitin	394.48±8.56a	230.84±4.78a	48.56±2.45b	30.73±1.12b	41.86±2.04b	19.49±1.56c	76.65±3.78c	28.51±2.23b
Genistin	2206.83±11.34a	2241.31±15.78a	96.83±7.23b*	94.75±8.55b*	47.33±2.15c	13.98±1.22c	26.62±1.25d	21.64±1.43c
Malonyl daidzin	300.43±7.22a	197.04±5.12a	109.59±7.32b*	103.98±2.87b*	90.84±4.78b	38.96±1.96c	101.47±6.92b	50.81±3.58d
Malonyl genistin	87.85±5.12a	35.53±1.59	55.83±4.13b	41.89±6.82	75.40±3.67c	35.57±5.43	67.05±4.35bc	31.58±7.16
Acetyl glycitin	295.28±5.23a	171.99±2.44a	77.98±4.22b	113.73±1.58b	17.73±1.66c	13.26±1.43c	22.74±2.02c	17.23±1.11c
Acetyl genistin	62.79±3.89a	44.53±5.01a	23.33±1.22b	14.89±1.76b	22.05±1.24b	10.32±3.22b	47.88±2.02c	18.58±1.58b
Acetyl daidzin	378.61±6.01a	306.86±4.77a	56.93±3.36b	74.62±4.89b	30.96±1.59c	39.78±1.52c	12.98±0.78d	17.22±1.46d
Daidzein	247.88±4.66a	210.37±5.78a	71.97±5.35b*	62.92±6.61b*	71.38±4.19b	39.82±5.14c	94.57±5.68c	78.56±6.77b
Glycitein	47.87±1.89a	58.56±2.11a	1194.07±16.74b	1463.96±11.56b	1536.49±17.66c	1719.94±15.67c	844.73±12.31d	1642.44±14.24d
Genistein	17.74±0.67a	7.77±1.03a	246.01±4.07b	172.86±3.51b	330.62±5.67c	183.67±6.03b	185.19±2.03d	161.02±4.21c
Totals (µg/g)	91.32±4.01a	103.68±4.16a	2333.56±19.34b	3144.92±16.47b	2853.51±14.14c	3817.72±11.04c	1042.89±8.03d	3045.89±9.97d
	5799.01±18.77a	5328.76±16.51a	4570.41±18.24b	5577.47±11.46b	5441.78±15.14c	6246.26±13.18c	2995.64±14.07d	5428.75±10.24d

¹⁾Each value is mean±SD (n=3).

Different letters in the same column indicate significant difference at $p < 0.05$.

The superscripts (*) in the same row are not significantly different at $p < 0.05$.

Fig. 1. Changes in isoflavone isomers of white (A) and black soybean (B) extracts during *Monascus*-mediated fermentation at 30°C for 30 days.

glucoside, acetyl-glucoside, and aglycone contents of soybeans during *Monascus*-mediated fermentation are shown in Table 2. Levels of aglycones significantly increased while corresponding levels of glucosides decreased ($p < 0.05$). Unfermented soybean extracts contained 5563.89 ± 17.64 µg/g of total isoflavones including β-glucosides, malonyl glucosides, acetyl glucosides, and aglycones with percentages of 76.04, 9.78, 11.24, and 2.94%, respectively (Fig. 1). However, after 20 days of fermentation, aglycones in MFWS and MFBS extracts accounted for 86.7 and 91.6% of total isoflavones, respectively. At the same time, concentrations of malonyl- (1.4–3.4%), acetyl- (1.4–2.3%), and β-glucosides isoflavones (5.6–7.6%) were significantly reduced (Fig. 1 and Table 2). Among isoflavone-conjugated profiles, the major isoflavones of *Monascus*-fermented soybean were daidzein and genistein, whereas the predominant isoflavones in unfermented soybeans were daidzin and genistin (84.9 and 70.4% of total isoflavones, respectively) (Fig. 1A). Significant bioconversion of glucoside isoflavones into aglycones has been reported in soybeans fermented with β-glucosidase-producing microorganisms (14,35). Generally, fungi may mediate enzymatic breakdown of plant cell walls to release esterified and insoluble-bound nutrients, leading to increased content of isoflavone aglycones (35). As shown in Table 2, isoflavone aglycone content of MFBS was 1.2-fold higher than that of MFWS ($p < 0.05$). Among isoflavone aglycones, genistein was shown to substantially inhibit skin carcinogenesis and cutaneous aging induced by UV light in mice as well as photooxidative damage in humans (11). Specifically, genistein mediated these effects by protection of photooxidative-damaged DNA, down-regulation of UV-B-activated signal transduction cascades, and antioxidant activities (12).

Antioxidant activity Antioxidants are often needed to protect against lipid oxidation, which causes rancidity and undesirable changes in texture, appearance, and quality of cosmetic products (3). Antioxidant activities of *Monascus*-fermented soybean samples were tested by two *in vitro* methods at concentrations of 50 mg/mL (Table 3). In the DPPH radical scavenging activity test, unfermented MFWS and MFBS extracts (day 0) displayed 0.95 ± 0.01 and 1.25 ± 0.03 mg of trolox equivalents per g of dried weight (mg TE/g DW), whereas *Monascus*-fermented samples showed values of 1.52 ± 0.08 and 2.17 ± 0.07 mg after 20 days of fermentation, respectively. The DPPH radical scavenging effects of MFBS after 20 days of fermentation were significantly higher ($p < 0.05$) than those of MFWS. Previous studies have reported similar observations using *Monascus* spp. (16,27). It was shown that *Monascus*-fermented products are scavengers of free radicals, particularly peroxy radicals, which are major propagators of fat oxidation (33).

MFSEs exhibited a significantly higher ($p < 0.05$) ferric reducing power than extracts of unfermented soybeans (Table 3). FRAP assay directly measures the reducing potential of an antioxidant reacting with a Fe^{3+} -TPTZ complex, resulting in a colored Fe^{2+} -TPTZ (20). The highest reducing powers of MFWS (2.07 ± 0.02 mg TE/g) and MFBS (2.56 ± 0.01 mg TE/g) were evaluated after 30 and 20 days of fermentation, respectively. The maximum reducing powers of MFWS and MFBS increased by 36.2 to 66.2% compared with that of unfermented soybeans, respectively ($p < 0.05$). These findings suggest that the enhanced antioxidant activity of fermented soybeans can be attributed to the substrate and mycelia during *Monascus*-mediated fermentation. The reducing power of MFBS during fermentation was significantly higher ($p < 0.05$) than that of MFWS (Table 3). The increased antioxidant

Table 3. Antioxidant, anti-tyrosinase, and anti-elastase activities of white and black soybean extracts during *Monascus*-mediated fermentation at 30°C for 30 days

Fermentation days	DPPH (mg trolox/g) ¹⁾		FRAP (mg trolox/g)		Anti-tyrosinase (%)		Anti-elastase (%)	
	white	black	white	black	white	black	white	black
0	0.95±0.01a ³⁾	1.25±0.03a	1.52±0.01a*	1.54±0.03a*	43.12±0.21a	42.31±0.16a	37.46±0.21a	41.22±0.13a
10	1.26±0.06b	1.72±0.05b	1.74±0.04b	2.03±0.05b	71.23±0.65b	75.34±0.22b	46.78±0.14b	55.21±0.25b
20	1.52±0.08c	2.17±0.07d	1.95±0.05c	2.56±0.01d	82.55±0.78d	90.63±0.71d	56.22±0.22c	71.28±0.41d
30	1.47±0.04c	1.98±0.04c	2.07±0.02d	2.29±0.02c	78.53±0.54c	84.97±0.41c	58.67±0.19d	65.34±0.33c
Kojic acid ²⁾					90.23±0.33e	93.23±0.55e		
EGCG ²⁾							87.23±0.15e	89.23±0.11e

¹⁾Expressed as mg trolox equivalent per g of dry mass

²⁾Positive control

³⁾Each value is mean±SD (n=3).

Different letters in the same column indicate significant difference at $p<0.05$.

The superscripts (*) in the same row are not significantly different at $p<0.05$.

activity of black soybeans was consistent with the results of previous reports (24,28). These studies demonstrated that seed coats of black soybeans had higher antioxidant properties due to increased contents of polyphenols, including anthocyanins and proanthocyanidins, compared to regular soybeans (white or yellow soybeans).

Tyrosinase and elastase inhibitory activities The tyrosinase and elastase inhibitory activities of *Monascus*-fermented soybeans at 50 mg/mL are shown in Table 3. Anti-tyrosinase and anti-elastase activities in MFWS and MFBS increased by 1.6–1.9- and 1.7–2.1-fold compared to those of unfermented samples, respectively ($p<0.05$). In particular, inhibition of tyrosinase activity was abrogated by MFBS after 20 days of fermentation (90.6%), which was similar to the results of treatment with 10 mg/mL of kojic acid (93.2%) as a positive control (Table 3). This result suggests that the observed tyrosinase inhibitory activity may be due to other active metabolites generated during the *Monascus*-mediated fermentation process. Extracts from MFBS also showed dose-dependent inhibition of tyrosinase and elastase activities with increasing concentrations (data not shown), whereas elastase inhibitory activity was significantly lower ($p<0.05$) than EGCG as a positive control. Elastin is an extracellular matrix protein that affects skin elasticity while elastase is a proteinase enzyme capable of elastin degradation, which can lead to aging (5).

Based on these results, the nutricosmetic activity of the extracts of black soybean (MFBSEs) after 20 days of fermentation, which exhibits the highest antioxidant and tyrosinase inhibition activities compared to other samples, was expected to be high even at low concentration. Thus, we performed assays to determine inhibition of melanin synthesis using the MFBSEs.

Anti-melanogenic activity The inhibitory effects of MFBSEs after 20 days of fermentation, at concentrations from 10 to 50 mg/mL, on melanin production in α -MSH stimulated B16 melanoma cells were assessed. Melanoma cells not stimulated with α -MSH were used as the negative control group, and arbutin (100 μ g/mL) treated cells were used as the positive control group. As shown in Fig. 2, extracts

Fig. 2. Melanin inhibition activity of B16F10 melanocyte treated with extracts of *Monascus*-fermented black soybean (MFBS: 10, 30, 50 mg/mL). Arbutin, 100 μ g/mL; α -MSH, 3.3 μ g/mL; Each value was expressed as the mean±SD; Different letters show a significant difference at $p<0.05$.

from MFBS significantly ($p<0.05$) inhibited in a dose dependent manner compared with control cells without any cytotoxicity (data not shown) within the concentration used. The extracts from MFBS, at concentrations of 10, 30, and 50 mg/mL, reduced the melanin content of α -MSH-stimulated B16 melanoma cells by 40.18, 27.46, and 16.34 μ g, respectively (Fig. 2). Furthermore, the extracts at 50 mg/mL exhibited a strong inhibitory effect on melanin synthesis, similar to that observed with the positive control, arbutin (17.91 μ g). The tyrosinase catalyzes the oxidation of L-Dopa to *ortho*-dopaquinone, and antioxidants may prevent the oxidation step by tyrosinase reaction in melanogenesis (26). Extracts from MFBS which exhibited a higher of antioxidant and anti-tyrosinase capacities than other samples seems to have inhibition effect of melanin synthesis. Thus, the anti-melanogenic effect of MFBSEs probably originates from both the tyrosinase inhibitory effect and the antioxidant activity. However, it is necessary to additional study how individual antioxidants such as genistein, daidzein, and CoQ10 and another compounds of

Fig. 3. Contribution of identified components to the nutraceutical capacities of extract of *Monascus*-fermented black soybean.

MFBSes affect on melanin inhibition activity.

Correlation between nutraceutical activities and bioactive constituents

The *in vitro* nutraceutical activities (DPPH, FRAP, anti-tyrosinase, and anti-elastase activities) showed a positive correlation with the bioactive compounds (CoQ10, genistein, and daidzein) identified in this study (data not shown). Especially, there were significant correlations ($p < 0.05$) between CoQ10 content as well as antioxidant (average, $r^2 = 0.9547$), anti-tyrosinase ($r^2 = 0.9998$), and anti-elastase ($r^2 = 0.9316$) activities. The results indicate that the strong nutraceutical capacities of the MFSEs as compared to unfermented samples may be due to the high contents of CoQ10 in those samples. To better understand the contribution of each CoQ10 compound and the two major isoflavone aglycones to nutraceuticals, we evaluated the antioxidant and inhibitory activities against tyrosinase and elastase of each standard compound (genistein, daidzein, and CoQ10) at the same concentration in samples and expressed results as % contribution of each compound (Fig. 3). CoQ10 showed higher antioxidant activity (DPPH 27.2%, FRAP 25.4%) as well as anti-tyrosinase (30.2%) and anti-elastase (33.3%) activities than genistein and daidzein. Genistein was less effective in inhibiting tyrosinase (10.2%) and elastase (9.3%) than daidzein (19.2, 21.6%) but displayed stronger antioxidant activity (17.3%) compared to daidzein (8.7%). These results are in agreement with that of Xiao (10) who demonstrated that daidzein had significantly higher mushroom tyrosinase inhibitory activity than genistein. However,

these sample components were not responsible for all of the nutraceutical capacities of MFSEs. The results suggest that compounds other than CoQ10, genistein, and daidzein in the MFSEs possess strong nutraceutical activities. For instance, other substances such as ascorbic acid, tocopherols, carotenoids, saponins, and phenolics may be responsible for the significant nutraceutical capacity of *Monascus*-fermented soybeans.

In conclusion, significant elevation ($p < 0.05$) of the bioactive constituents and nutraceutical capacity during *Monascus*-mediated fermentation was evaluated in soybean extracts. Black soybean genotype (MFBS) exhibited average nutraceutical activities ($p < 0.05$), which was significantly higher than those of white soybean genotype (MFWS). Extracts from MFBS after 20 days of fermentation also inhibited melanin synthesis using the B16F10 mouse cell line. The potentially significant nutraceutical properties of MFSEs were found to be associated with CoQ10, genistein, and daidzein contents, suggesting that these compounds were the dominant antioxidant, anti-tyrosinase, and anti-elastase components in the MFSEs. It is suggested that *Monascus*-fermented soybeans may be used as natural and potent nutraceuticals or nutraceuticals due to their effective melanin formation inhibitors as well as antioxidant, anti-tyrosinase, and anti-elastase activities.

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