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Production of a High Value-Added Soybean Containing Bioactive Mevinolins and Isoflavones

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Abstract

The production of mevinolin, a potent hypocholesterolemic drug, and the bioconversion of isoflavones were investigated in soybeans fermented with *Monascus pilosus* KFRI-1140. The highest yields of 2.94 mg mevinolins and 1.13 mg isoflavone aglycones per g dry weight of soybean were obtained after 20 days of fermentation. Mevinolin was present in the fermentation substrate predominantly in the hydroxycarboxylate form (open lactone, 94.8∼96.7%), which is currently being used as an hypocholesterolemic agent. The significant ($p<0.01$) bio-conversion (96.6%) of the glucoside isoflavones (daidzin, glycitin, genistin) present in the soybean to the bioactive aglycones (daidzein, glycitein, genistein), with a 15.8-fold increase of aglycones was observed. The results suggest that *Monascus*-fermented soybean has potential as a novel medicinal food or multifunctional food supplement.

Key words: hypocholesterolemic agent, isoflavones, medicinal food, mevinolin, *Monascus*-fermented soybean

INTRODUCTION

Functional foods, also known as nutraceuticals and medicinal foods, have received increased attention in recent years. The genus *Monascus* has been utilized as food coloring and antimicrobial agents, and in pharmaceuticals for thousands of years in East Asia (1,2). Since *Monascus* is used in foods, its ability to produce mevinolin (also known as lovastatin, monacolin K, Mevacor or $C_{24}H_{36}O_5$) is well characterized (2). Mevinolin inhibits cholesterol synthesis by inhibiting the rate limiting step in cholesterol biosynthesis, namely the conversion of 3-hydroxy-methyl-3-glutaryl-coenzyme A (HMG-CoA) into mevalonate, catalyzed by HMG-CoA reductase (3,4). Mevinolins are a group of compounds existing in both β-hydroxy lactone and the active form, β-hydroxy acid (5,6). This compound can be produced through secondary metabolism of fungi, such as *Aspergillus terreus* (7) or *Monascus rubber* (8) by submerged fermentation. However, only a submerged fermentation with *Aspergillus terreus* was developed to manufacture lovastatin on a large scale (9). In recent years, researchers have shown an increasing interest in solid state fermentation (SSF) as a potential alternative to submerged fermentation, because it uses economical substrates (agricultural residues) and offers higher yields of bioavailable secondary metabolites (10). Traditionally, steamed rice is the common medium used for *Monascus* solid-state fermentation. Although some studies have attempted to add various nitrogen sources to the rice medium in an effort to improve mevinolin productivity (11), no other substrate has been reported as an alternative medium.

Soybeans and soy products are the main source of isoflavones, a well-studied group of phytoestrogens with numerous biological effects such as improving bone density (12,13) and cardiovascular health (14), cancer prevention (15,16) and modulating menopausal symptoms (17). The primary soy isoflavones are genistein, daidzein and much lower amounts of glycitein and their respective β-glucosides, genistin, daidzin and glycitin (15). Human isoflavone bioavailability depends upon the relative ability of gut microflora to degrade these compounds (18, 19). β-Glucosidases of intestinal microflora in the lower bowel can hydrolyze the glucoside isoflavones to aglycones and promote their absorption (19,20). Therefore, microbes with β-glucosidase activity are potentially important in the production of isoflavone compounds with higher estrogenicity and better absorption (21). This research is focused on the production of mevinolin and isoflavone aglycones in soybeans fermented with *Monascus* sp. to obtain bioactive multi-ingredients-containing soy products. The production of mevinolins and isoflavone aglycones from *Monascus*-fermented soybean were identified and quantified by LC/ESI-MS and HPLC, respectively.

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MATERIALS AND METHODS

Chemicals

Authentic standards of daidzein, genistein, mevinolin, *p*-nitrophenol-β-D-glucopyranoside (*p*NPG), and *p*-nitrophenol (*p*NP) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Daidzin, genistin, glycitin, and glycitein standards were obtained from Funakoshi Chemical Co. (Tokyo, Japan). All other reagents were of the highest grade available unless otherwise indicated. To obtain the mevinolinic acid, mevinolin (the standard lactone) was converted to the β-hydroxy acid form according to the method of Friedrich et al. (5) with minor modification (22). Stock solutions of mevinolin and mevinolinic acid were prepared in distilled water and appropriate dilutions were made with water.

Fungal strain and fermentation

Monascus pilosus KFRI-1140, which was previously determined to be the best strain for mevinolin production without citrinin (23), a toxic fungal secondary metabolite, was obtained from Korea Food Research Institutes (KFRI, Seongnam-City, Korea). During the *Monascus*-fermentation, some strains also produce an additional unwanted co-metabolite, citrinin, a nephrotoxin. Thus, screening the strain of *Monascus* with non-producing/ low-producing citrinin is very important. A two-stage-fermentation process was used to produce mevinolin, which included a seed culture stage (liquid culture) and a metabolite production stage (SSF). The fungal strain-was maintained on petri dishes of potato dextrose agar (PDA, Difco, MI, USA) and incubated at 30°C for 7 days. After cultivation, colonies of spores that appeared on the plates were transferred (~1 cm; 1.0×10^{6} CFU/mL) and inoculated into 100 mL of nutrient broth (22,23) and incubated at 30°C for 4 days with shaking at 150 rpm for seed culture. Preparation of the soybean medium for SSF proceeded as follows: whole soybeans (from Pyungchang, Korea, 2004) were washed and soaked overnight in distilled water. After decanting the water, the soaked soybeans (moisture contents, 55~60%) were weighed to 100 g in an Erlenmeyer flask with a baffle and autoclaved (121°C) for 30 min. After cooling, the substrate was inoculated with 10 mL (10%, v/w) of liquid seed (pH 6.0) and incubated at 30°C for 30 days. At 5-days intervals, samples were asceptically collected from each flask to analyze mevinolins and isoflavones.

Sample preparation

Fermented soybeans were lyophilized and powdered. Approximately 0.1 g of soy powder, accurately weighed, was extracted with 1 mL of 70% ethanol for 50 min with sonication. After centrifugation for 10 min at 5,000 ×*g* and filtering through a 0.45 μm membrane (Millipore Co., Bedford, MA, USA), the filtrate was directly analyzed for isoflavones and mevinolins.

β-Glucosidase activity

The β-glucosidase activity was assayed by determining the rate of hydrolysis of *p*NPG (24,25). *Monascus*-fermented soybean was powdered (0.5 g) and mixed with 15 mL distilled water, vortex-mixed for 1 min, and centrifuged at 6,000×*g* at 4°C for 10 min using a refrigerated centrifuge. The supernatant was collected and filtered through a 0.45 μm filter before analysis. One unit of β-glucosidase activity in soybean was defined as the amount of enzymes that released 1 mol of *p*NP from the substrate per min.

HPLC analysis of isoflavones

Reversed phase HPLC analysis was carried out with a JASCO system (Tokyo, Japan), using a YMC AM 303 ODS-A column (4.6×250 mm, Kyoto, Japan). For the analysis of isoflavones (25), the mobile phase was composed of 0.1% phosphoric acid in acetonitrile (solvent A) and 0.1% phosphoric acid in water (solvent B). Following the injection of 20 μL of sample, solvent A was increased from 15% to 35% over 50 min, and then held at 35% for 10 min. The solvent flow rate was 1 mL/min and the eluted isoflavones were detected at 254 nm. Quantitative data for β-glucosides and their aglycones isoflavone were obtained by comparison to known standards.

HPLC/ESI-MS analysis for mevinolins

The β-hydroxy acid (mevinolinic acid) and the β-hydroxy lactone (mevinolin) were determined using LC/ ESI-MS (6). LC-MS analyses were accomplished on a liquid chromatograph (NANOSPACE SI-2, Shisceido, Japan) directly coupled with a Finnigan (LCQ DECA XP Bremen, Germany) mass spectrometer equipped with an ESI source. The separation at 238 nm was completed on a narrow-bore reversed-phase Luna C_{18} HPLC column (1 mm×150 mm i.d. 5 m, NANOSPACE SI-2, Shisceido, Japan) with a gradient elution consisting of 5% acetonitle (0.1% formic acid, eluent A) and 95% acetonitrile (0.1% formic acid, eluent B) at a flow rate of 50 μL per minute and a 5 μL injection volume. Linear gradient elution from 30 to 90% B in 50 min and keeping 90% B from 50 to 60 min was applied. In the single ion monitoring (SIM) experiments, the ion for mevinolin was $[M+H]^{+}$ (*m/z*=405.1) and that for mevinolinic acid was $[M-H]^{-}$ (*m/z*=421.4), both ions had a dwell time of 500 ms ion^{-1}. The concentrations of mevinolinic acid and mevinolin were determined from the single ion response (*m/z*=421.4 and 405.1) peak height at *t*R=33.06 and *t*R=38.79 min, repectively.

Statistical analysis

Results are presented as mean value ± standard deviation. Statistical analysis between experimental results was based on one-way analysis of variance using SAS software. Significant difference was considered at the level of $p<0.01$ or $p<0.05$.

RESULTS AND DISCUSSION

Production and identification of mevinolins

Mevinolin and mevinolinic acid in 70% ethanol extracts from *Monascus*-fermented soybean were identified by comparison of their retention time (*t*R) and mass spectra data with those of standards. As shown in Fig. 1, the molecular ion of the predominant peak at *t*R=33.06 min was 421.4 (M-1), identified as the hydroxy acid form of mevinolin, which was confirmed by injection of mevinolinic acid standard. The second strong peak (*t*R=38.79 min) displayed the molecular ion 405.1 (M+1) belonging to the mevinolin of β-hydroxy lactone. It has been reported that mevinolins are the secondary metabolites of biosynthesis during fermentation, and that they are biogenetically related to each other (2). Mevinolin, an HMG-CoA reductase inhibitor produced by the fungus *Monascus,* is composed of two polyketide chains, C-18 and C-14 synthesized from incorporation of acetate and methionine (26). One is a nonaketide that undergoes cyclization to a hexahydronaphthalene ring system and the other is a simple diketide, 2-methylbutyrate. Thus, all mevinolins contains a methylbutyric side-chain and a β-hydroxylactone, the latter being present as the corresponding β-hydroxy acid in the pharmaceutically active drug (26,27) (Fig. 1). As expected, negative-ion mode with $[M-H]^-$ for mevinolinic acid was more sensitive than positive-ion mode with $[M+H]^+$ as a precursor ion for LC/ESI-MS. The data in Table 1 reveal that mevinolinic acid was the main component, contributing 94.8 to 96.7% of the total, of mevinolin in the fermented soybean, which is similar to the results of Li et al. (6), who showed that monacolin K hydroxy acid (mevinolinic acid) was the main component in the red yeast rice powder. The composition ratios and productivity of mevinolins can be influenced by the *Monascus* strain, medium composition, and fermentation conditions in which they are produced (7,9). Therefore, many studies have attempted to enhance the production of *Monascus* mevinolin through strain improvement (26), carbon source change (11), and improvement of culture conditions (7,9). Selection and composition optimization of a suitable medium is therefore important for establishing a process for producing mevinolin. The maximum yield of mevinolin (2.94 mg/g dry weight) was reached

Fig. 1. Negative ion spectra (top: SIM at m/z 421.4) and positive ion spectra (bottom: SIC at m/z 405.1) of the extract from *Monascus*-fermented soybean. The assays were performed as described in Materials and Methods.

Table 1. Mevinolin concentrations in aqueous alcoholic extracts from soybean fermented with *M. pilosus* KFRI-1140

Fermentation (days)	Mevinolins (mg/g dry weight)	
	Hydroxy acid form (%)	Lactone form
5	0.42 ± 0.01^{a} (95.2)	0.02 ± 0.001
10	1.12 ± 0.03^{b} (94.8)	0.06 ± 0.002
15	1.84 ± 0.05^{c} (95.2)	0.09 ± 0.001
20	2.83 ± 0.02^{d} (95.7)	0.11 ± 0.002
25	2.68 ± 0.01^{d} (96.7)	0.12 ± 0.003

All assays were performed as described in Materials and Methods. All data are expressed as means (n=3).
Different letters indicate significantly different values ($p<0.05$) (vertical comparison on each day).

at 20 days, this then decreased slightly, but the production of mevinolins had not ceased at the time of termination of fermentation ($p<0.05$, Table 1).

There have been many reports on the mevinolin production by *Monascus* sp. with submerged fermentation (7-9). However, the production yield of mevinolin by submerged fermentation is extremely low (less than 20~131 mg /L). In comparison, this study demonstrated that the mevinolin yield (2.94 mg/g) increased significantly

with the use of the solid state fermentation technique. Pandy et al. (10) previously reported that SSF has become a very attractive alternative to submerged fermentation for specific applications due to its simplicity and lower cost. These facts indicate that the production of mevinolin from soybean fermentation using *M. pilosus* KFRI-1140 might be advantageous with saving in capital costs, because the extraction and purification steps might not be necessary.

Production of isoflavone aglycones

The HPLC results for isomeric isoflavones in the 70% aqueous alcoholic extracts from soybean fermented with *M. pilosus* KFRI-1140 are shown in Fig. 2 and the quantitative analyses for isomeric isoflavones are shown in Table 2. Table 2 shows that fermentation time (days) significantly ($p<0.01$) affected the isoflavone composition. Non-fermented soybeans had mostly the β-glucoside (daidzin+glycitin+genistin) isoflavones (94.6%), but the concentration of bioactive aglycones (daidzein+glycitein+genistein) in soybean fermented with the fungal strain increased dramatically with fermentation time until 20 days of incubation and comprised 96.6% of the total isoflavones (Table 2, Fig. 3). These results are in agreement with those of Park et al. (28), who demonstrated

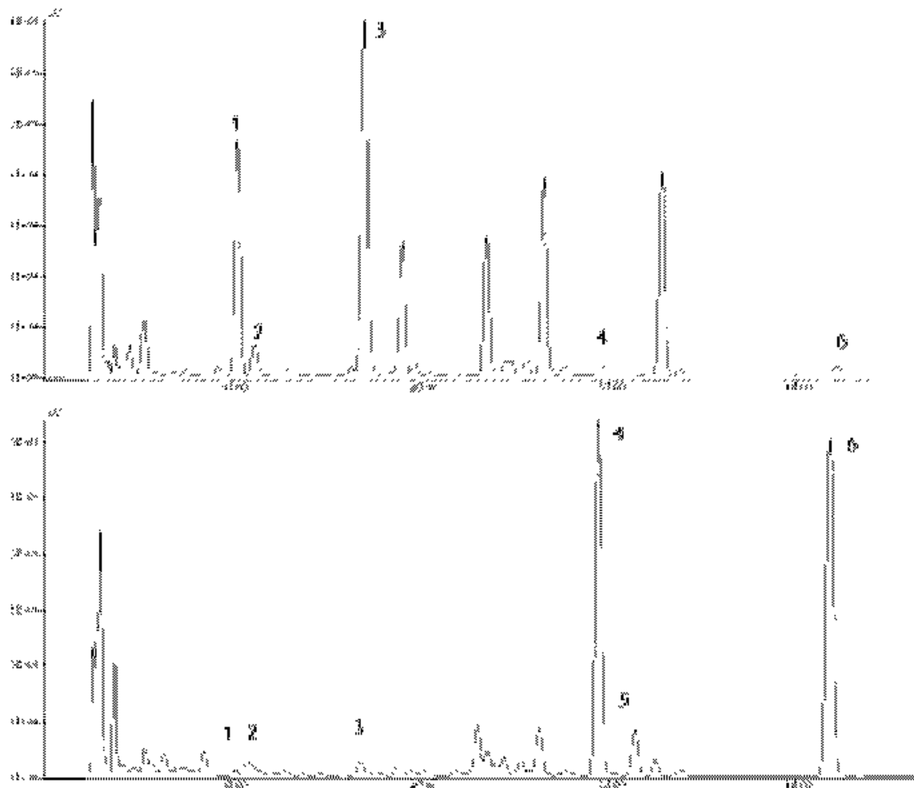

Fig. 2. HPLC chromatogram of isomeric isoflavones in aqueous alcoholic extracts from soybean fermented without (top) and with *M. pilosus* KFRI-1140 (bottom) for 20 days at 30°C (1, daidzin; 2, glycitin; 3, genistin; 4, daidzein; 5, glycitein; 6, genistein). The assays were performed as described in Materials and Methods.

Table 2. Isoflavone concentrations (μg/g dry weight) in aqueous alcoholic extracts from soybean fermented with *M. pilosus* KFRI 1140

Fermentation (days)	β-Glucosides			Aglycones		
	Daidzin	Glycitin	Genistin	Daidzein	Glycitein	Genistein
0	441.5±9.2^{h}	155.4±8.9^{h}	656.8±8.3^{h}	21.4±3.6^{h}	17.9±2.3^{h}	32.2±3.4^{h}
5	301.8±8.6^{i}	127.4±6.7^{h}	501.9±9.1^{i}	115.8±9.8^{i}	52.5±3.6^{i}	138.5±7.8^{i}
10	138.4±8.8^{j}	67.9±5.8^{i}	347.4±6.2^{j}	278.6±7.5^{j}	81.8±5.1^{j}	299.5±6.3^{j}
15	55.5±7.4^{k}	18.7±7.5^{k}	68.4±7.6^{k}	468.2±9.6^{k}	80.6±6.2^{j}	511.2±8.8^{k}
20	15.9±8.4^{l}	4.4±0.9^{l}	18.5±3.3^{l}	482.8±8.3^{l}	91.4±6.5^{j}	558.6±9.2^{l}
25	20.5±6.1^{l}	ND	1.8±6.9^{l}	439.7±9.4^{n}	67.6±5.9^{j}	447.4±8.5^{n}

All assays were performed as described in Materials and Methods.
All data are expressed as means (n=3). ND: not detected.
Different letters indicate significantly different values ($p<0.01$) (vertical comparison on each day).

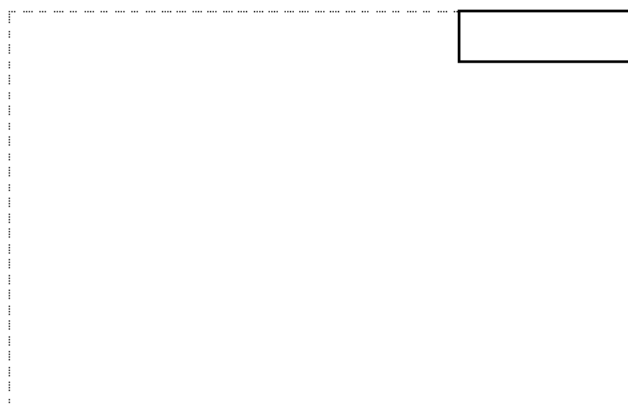

Fig. 3. Changes in isoflavone isomers during soybean fermentation by *Monascus pilosus* KFRI-1140.
The assays were performed as described in Materials and Methods. All data are expressed as means (n=3).

that the glucoside isoflavones in soybean fermented with *Aspergillus oryzae* RIB 430 (ATCC 22786) were efficiently transformed into aglycones. It has been reported that the isoflavone β-glucosides are the predominant isomeric forms in soybean and isoflavone glucosides require microbes-induced hydrolytic deconjugation for bioconversion into their responding aglycone form (25, 28,29). Our data showed that *M. pilosus* KFRI-1140 produced β-glucosidase which hydrolyzed isoflavone glucosides to aglycones. As shown in Fig. 4, a linear correlation (r=0.829) between β-glucosidase activity of the strain used and bioconversion (%) of isoflavone glucosides to bioactive aglycones was observed. For example, soybeans fermented with *M. pilosus* KFRI-1140 exhibited the highest bioconversion of glucosides (96.6%)

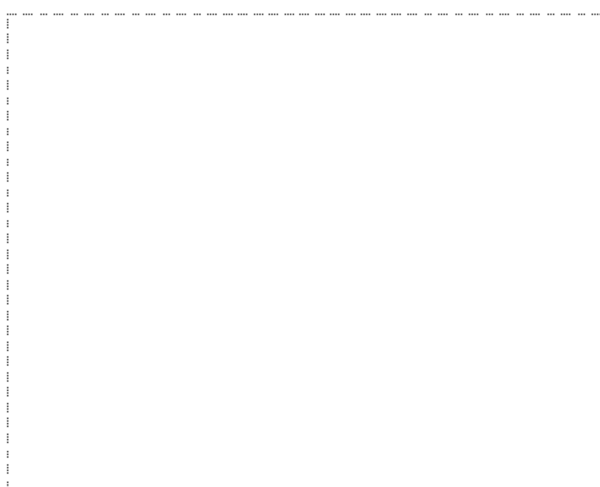

Fig. 4. Relationship (r=0.829) between % bioconversion of isoflavone β-glucosides to aglycones and β-glucosidase activity (mU/g dry weight) in extract from soybean fermented with *Monascus pilosus* KFRI-1140 at 30°C.
The assays were performed as described in Materials and Methods. All data are expressed as means (n=3).
% Bioconversion={1 − (β-glucosides/total isoflavons)} × 100.

after 20 days of fermentation, and this strain also showed the highest β-glucosidase activity (56.0 mU/g dry weight). The significant bioconversion ($p<0.01$) of the glucoside isoflavones into their responding aglycones during soybean fermentation was observed (Table 2, Fig. 3). Thus, the reduction in the contents of β-glucosides, and the increase in the contents of their respective aglycones may be due to the hydrolytic reaction catalyzed by β-glucosidase produced by *M. pilosus* KFRI-1140.

In summary, *M. pilosus* KFRI-1140 used in this study efficiently hydrolyzed the conjugated isoflavones in the soybean to the respective unconjugated isoflavones, resulting in a good production of mevinolin. The results suggest that *Monascus*-fermented soybean may be regarded as a natural multifunctional food additive, which is abundant in bioactive mevinolin and isoflavones. This study is the first report on a simultaneous production of bioactive mevinolin and isoflavones from soybeans fermented with *Monascus* sp.

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