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Effect of *Aspergillus oryzae*-Challenged Germination on Soybean Isoflavone Content and Antioxidant Activity

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ABSTRACT: Application of microbial stress to soybean during germination induces the accumulation of phytoalexins, which have many health benefits. In this study, the effects of stress induced by *Aspergillus oryzae* on the phytochemical composition of germinating soybeans were investigated, and their radical scavenging activity was compared with those of ungerminated (US) and germinated (GS) soybeans. Additionally, the antioxidant activity of coumestrol, a soybean phytoalexin, against hydrogen peroxide-induced reactive oxygen species (ROS) was investigated in HepG2 cells. *A. oryzae* exposure significantly decreased the total isoflavone content and induced coumestrol and glyceollin I. *A. oryzae*-challenged germinated soybeans exhibited the highest radical scavenging activity (IC_{50} = 0.55 mg/mL) as compared to US and GS. Coumestrol exhibited significantly higher radical scavenging activity than daidzein and genistein. Furthermore, coumestrol significantly prevented hydrogen peroxide-induced ROS production and lipid peroxidation and inhibited decreases in cell viability, intracellular glutathione (GSH) levels, and superoxide dismutase (SOD) activity. These results indicate that using food-grade *A. oryzae* to elicit the biosynthesis of phytoalexins alters the secondary metabolite profiles of the soybeans and offers enhanced bioactivity of soybean as a functional food ingredient.

KEYWORDS: *soybean, germination, Aspergillus oryzae challenge, coumestrol, antioxidant*

INTRODUCTION

Soybeans [*Glycine max* (L.) Merrill] and other processed soybean products have long been used as a food in Asia.[1] Soybean is widely grown for its edible bean, which possesses numerous health-promoting properties.[2] Soybeans are high in protein and contain beneficial phytochemicals, such as isoflavones.[3] Isoflavone, a functional compound in soybeans, has been intensively studied for its pharmacological properties, such as antioxidative, anti-inflammatory, neuroprotective, and anticarcinogenic effects, and protective effects against bone loss, hormone-dependent and -independent cancers, cardiovascular diseases, and autoimmune diseases.[4−7]

The isoflavone content and composition of soybeans are affected by various processing conditions, such as germination, fermentation, heat treatments, and chemical and enzymatic hydrolysis.[8] The germination process modifies the phytochemical composition of soybeans and results in a substantial increase in some biologically active compounds, such as lecithin, phytoesterols, and saponins.[9] Fermentation or microbial infection has been demonstrated to produce new compounds derived from isoflavones, soy proteins, and dietary fibers.[10] Furthermore, inoculation of germinating soybeans has been demonstrated to further modify their phytochemical composition and elicit the formation of phytoalexins.[11] Challenging soybeans with *Rhizopus* microspore during the germination process markedly induces phytoalexins, alters isoflavones composition, and enhances the estrogenic potential of soybeans.[12]

Phytoalexins are low molecular weight antimicrobial compounds that are synthesized and accumulated in plants in response to pathogens or chemical stimuli.[13] Phytoalexins are typically toxic to microbes but have been proven to have health benefits in humans, which has attracted increasing research interest in recent years.[14] The most well-known phytoalexin is resveratrol found in grape skin. Resveratrol has antioxidant, anti-inflammatory, and anticancer activities.[15] Glyceollins and coumestrol are two of the major inducible phytoalexins generated by soybeans under fungal, chemical, and environmental stress.[16] Although the pharmacological properties of several soybean phytoalexins have been described, much of the research on plant defense and human health has focused on the isoflavonoid phytoalexin compounds glyceollin I, II, and III.[17]

Coumestrol is an estrogen agonist that has been shown to be effective in reducing bone loss.[18] Coumestrol has stronger binding affinities for both estrogen receptor (ER) α and ER β than isoflavone, and the binding affinities are comparable to those of 17β-estradiol. Coumestrol has also been shown to be a safe neuroprotective agent and α-glucosidase inhibitor.[19,20] Although coumestrol is expected to have pharmacological properties similar to those of other phytoalexins, biological activities of coumestrol have not been studied extensively because its distribution in human foods is more restricted than that of other isoflavones.[21,22]

In this study, soybeans were germinated in the presence of the food-grade fungus *Aspergillus oryzae*, and the phytochemical composition and radical scavenging activity were assessed in comparison to ungerminated (US) and germinated (GS) soybeans. Additionally, the effect of coumestrol on the antioxidant defense system of HepG2 cells was assessed by measuring oxidative stress biomarkers.

Reactive oxygen species (ROS) are a major cause of damage to biological molecules leading to cellular aging and age-related degenerative diseases, such as cancer, brain dysfunction, and

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coronary heart disease.[23] Scavenging of ROS may prevent oxidative stress-related diseases.[24] Effects of coumestrol on oxidative stress chemically induced by a potent pro-oxidant, hydrogen peroxide (H_2O_2), were investigated in cultures of human hepatoma HepG2 cells. HepG2, a well differentiated transformed cell line, is a well-characterized and reliable model widely used for biochemical and nutritional studies.[25] Cell viability and several markers of oxidative damage were evaluated to estimate the effect of coumestrol on HepG2 cell survival and antioxidant defense response.

To our knowledge, changes in phytochemical composition of soybeans induced by *A. oryzae*-challenged germination and the concomitant changes in antioxidant activity have not been quantified in any published study until date. Furthermore, although some biological activities and pharmacological functions of coumestrol have been investigated, no previous cell culture study has been conducted to evaluate the effect of coumestrol on antioxidant defense responses to an oxidative insult.

MATERIALS AND METHODS

A. oryzae-Challenged Germination of Soybeans. Soybeans, *G. max* (L.) Merrill, were provided by Jeju Agricultural Research and Extension Services (Jeju, Korea); the specimens were harvested in 2010 and used for research in 2011. Seed germinations and fungal inoculations were carried out according to the method described previously.[26] In brief, soybeans were surface sterilized with 70% ethanol for 3 min and then rinsed with sterile water before they were soaked in sterile water for 24 h. For fungal inoculation, *A. oryzae* culture powder (1×10^9 conidia/g, 0.2 g; Mediogen, Seoul, Korea) was suspended in 20 mL of sterilized water for 24 h at 25 °C in an incubator to obtain a spore suspension. The soaked soybeans were placed in a sterile plastic container (30 cm × 15 cm). The soybeans were then inoculated with the sporangiospore suspension (0.5 mL/g) by gently distributing the suspension manually onto the soaked soybeans. The containers were placed on a clean bench at room temperature [25 °C (2 °C)] in the dark for 5 days, and sterile water was sprayed 5 times a day. GS soybeans without fungal inoculation were also prepared using identical procedures.

All of the US, GS, and *A. oryzae*-challenged soybeans (AO-GS) were dehydrated in a drying oven at 60 °C for 24 h. Three replicates were performed for each sample. Ten grams of each soybean sample was homogenized in 80% ethanol (7 mL/g of seeds) and then heated at 50 °C for 3 h in a water bath with sonication. After the samples cooled down, the mixtures were centrifuged at 10000*g* for 10 min. The supernatant was collected and filtered with a polytetrafluoroethylene (PTFE) membrane (0.45 μM) and evaporated. Finally, the extract solution was lyophilized to obtain a powder of soybean sample extracts. Extract yields were 12.3%.

High-Performance Liquid Chromatography (HPLC) Analysis. The total isoflavone, coumestrol, and glyceollin I contents in soybean sample extracts were determined using the HPLC-UV method. Total isoflavones were calculated as the sum of the 11 forms, including malonyldaidzin, malonylgenistin, acetyldaidzin, acetylglycitin, acetylgenistin, daidzin, glycitin, genistin, daidzein, glycitein, and genistein. The authentic standards daidzein, daidzin, genistein, genistin, glycitin, and coumestrol were purchased from Sigma (St. Louis, MO), and acetylgenistin, malonylgenistin, acetyldaidzin, malonyldaidzin, acetylglycitin, and glycitein were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Glyceollin I was kindly provided by Prof. B. S. Yun, Chonbuk National University, Korea. The concentrations of prepared authentic standards solutions ranged from 0.01 to 1 μg/mL. About 400 mg of each sample extract was dissolved in 20 mL of 50% methanol in water and were ultrasonicated for 10 min. Samples were filtered, and the 10 μL of resultant filtrates was directly injected into HPLC. Chromatographic separations were achieved using an XTerra RP18 HPLC column (3.9 mm × 150 mm, 5 μm particle size; Waters, Milford, MA), and UV detection was performed at 260 nm. HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific (Fair Lawn, NJ), and trifluoroacetic acid was obtained from Sigma. The water used was ultrapure deionized water (18.2 MΩ cm) produced from Millipore Milli-Q Gradient system (Millipore, Bedford, MA). The eluents used were 5% (v/v) methanol containing 0.04% (v/v) trifluoroacetic acid (eluent A) and 100% methanol containing 0.04% (v/v) trifluoroacetic acid (eluent B). The initial composition was 90% A and 10% B, and gradient elution was as follows: 0−2 min, 90% A; 2−33 min, 0% A; 33−36 min, 0% A; 36−39 min, 90% A; and 39−40 min, 90% A. The flow rate was 1 mL/min, and the injection volume was 10 μL.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) Assay. The DPPH radical scavenging activity of each sample was evaluated as previously described with some modifications[27] Briefly, DPPH solution was prepared by dissolving 4 mg of DPPH in 100 mL of ethanol. Sample or pure isoflavone solution (20 μL) was added to 200 μL of freshly made DPPH radical solution. Ethanol was used as a blank solution. After 30 min of incubation at room temperature, the absorbance was measured at 515 nm. The synthetic antioxidant reagent Trolox was used as a positive control, and all tests were carried out in triplicate. All of the reagents used in DPPH assay were purchased from Sigma. The results are expressed as IC_{50} (mg/mL) and TEAC (Trolox equivalent antioxidant capacity) values. TEAC of samples and pure isoflavones are presented as μmol Trolox equivalent (TE)/g of dry weight and μM TE, respectively.

Ferric-Reducing Antioxidant Power (FRAP) Assay. The FRAP of the pure isoflavone was determined as described previously.[28] Briefly, to prepare the FRAP solution, 25 mL of 300 mM acetate buffer, adjusted to pH 3.6 by the addition of acetic acid, was mixed with 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$ dissolved in distilled water and 2.5 mL of 10 mM TPTZ (2,4,6-tripyridyl-*s*-triazine) dissolved in 40 mM HCl. The pure isoflavone solution (100 μL) was mixed with 3 mL of working FRAP reagent, and absorbance (593 nm) was measured at 0 min after vortexing. Thereafter, samples were placed in a 37 °C water bath, and absorbance was again measured after 4 min. All of the reagents used in FRAP assay were purchased from Sigma. FRAP values of samples were calculated as follows:

$$\begin{aligned}&\text{FRAP value of sample}\\&= (\text{change in absorbance of sample from 0 to 4 min}\\&\quad /\text{change in absorbance of Trolox from 0 to 4 min})\\&\quad \times \text{FRAP value of Trolox}(1000\ \mu\text{M})\end{aligned}$$

Results are expressed in μM TE.

Cell Culture. HepG2 cells were purchased from ATCC (Manassas, VA) and were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in 5% CO_2 and 95% air. Cells were grown in transparent or black 96-well plates or 6- or 24-well plates for the assessment of coumestrol antioxidant activity. Cells were grown to 50% confluence before pretreatment.

Cell Viability Assays. HepG2 cells were plated at a density of 1×10^4 cells in 96-well plates for 24 h. Cells were then treated with coumestrol (0.01, 0.1, or 1 μM) or 10 μM Trolox for 8 h and subsequently with 1 mM H_2O_2 for 24 h. At the end of the incubation, cell viability was evaluated using cell counting kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) assay. Cell viability was calculated as % vehicle-treated control.

Measurement of Intracellular ROS. The production of intracellular ROS was measured by 2′,7′-dichlorofluorescin diacetate (DCFH-DA) assay. DCFH-DA is a cell-permeable fluorescent dye, which is oxidized inside the cells to form fluorescent dichlorofluorescein in the presence of ROS.[28] HepG2 cells (1×10^4 cells per well) were incubated in a black 96-well plate for 24 h. Cells were then treated with samples as described above. After treatment, cells were washed with phosphate-buffered saline (PBS) and incubated with 10 μM DCFH for 30 min. The cells were then washed with 100 μL of PBS to remove excess DCFH, and 1 mM H_2O_2 was added. Fluorescence was measured at 485 and 530 nm excitation and emission, respectively, over

30 min in a Tecan Infinite M200 Pro instrument (Tecan Group Ltd., Männedorf, Switzerland) at 37 °C. The percentage increase in fluorescence was calculated using the formula $[(F_{30\ min} - F_{0\ min})/F_{0\ min}] \times 100$, where $F_{30\ min}$ = fluorescence at 30 min, and $F_{0\ min}$ = fluorescence at 0 min.

Lipid Peroxidation Assay. To measure lipid peroxidation, HepG2 cells were plated at 0.5×10^6 cells/well of a 6-well plate. Cells were treated with coumestrol (0.01, 0.1, or 1 μM) or 10 μM Trolox for 8 h and exposed to 1 mM H_2O_2 for 24 h. The cells were washed with PBS and scraped into 0.1 mL of lysis buffer containing protease inhibitors (Cell Signaling Co., Danvers, MA). The cell lysate was centrifuged at 10000*g* for 5 min, and the supernatant was used for the in lipid peroxidation assay. Proteins were quantified with a BCA protein assay kit (Pierce, Rockford, IL). The extent of lipid peroxidation was estimated by the levels of malondialdehyde (MDA). MDA is one of many low molecular weight end products of lipid hydroperoxide decomposition and is the one most often measured as an index of lipid peroxidation.[29] For MDA determination, a MDA assay kit (Northwest Life Science Specialties, LLC., WA, Canada) was used. The assay principle is based on the reaction of MDA with thiobarbituric acid (TBA), forming an MDA-TBA_2 adduct that exhibits strong absorption at 532 nm. The results, normalized to the cell protein content, are expressed in nmol MDA/mg protein.

Determination of GSH Level and SOD Activity. To measure total intracellular gluthathione (GSH) content and superoxide dismutase (SOD) activity, HepG2 cells were plated at 0.5×10^6 cells/well of a 6-well plate. Cells were treated with coumestrol (0.01, 0.1, or 1 μM) or 10 μM Trolox for 8 h and exposed to 1 mM H_2O_2. The cells were harvested, and the cell lysate supernatant was used in the analysis. Proteins were quantified as described above.

Total intracellular GSH was measured using the NWLSS Glutathione assay kit (Northwest Life Science Specialties), according to the manufacturer's protocol. The assay is based on the spectrophotometric measurement of 5-thio-2-nitrobenzoate (TNB), formed from 5,5′-dithiobis-2-nitrobenzoate (DTNB) by GSH. The total GSH content is proportional to the increase in absorbance. TNB was measured by detecting absorbance at 412 nm using a microplate reader, and the concentration of total GSH in the samples was determined against a GSH standard calibration curve. The GSH levels, normalized to the cell protein content, are presented as μmol/mg of protein.

The SOD activity was measured using the SOD assay kit-WST (Dojindo Laboratories, Kumamoto, Japan), according to manufacturer's instructions. In the SOD assay, the highly water-soluble tetrazolium salt, WST-1 [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium], is used to produce a water-soluble formazan dye upon reduction with a superoxide anion. The rate of reduction with a superoxide anion is linearly related to xanthine oxidase activity and is inhibited by SOD. Thus, the inhibition rate of xanthine oxidase activity determined by a colorimetric method was used to measure SOD levels. The absorbance of WST-1-formazan was measured at 450 nm using a spectrophotometric microplate reader. The SOD activity was calculated according to the following formula:

$$\text{SOD activity} = [(A_{\text{blank1}} - A_{\text{blank3}}) - (A_{\text{sample}} - A_{\text{blank2}})] / (A_{\text{blank1}} - A_{\text{blank3}}) \times 100$$

where A_{blank1}, A_{blank2}, A_{blank3}, and A_{sample} indicate coloring without sample, sample blank, reagent blank, and sample, respectively. The SOD activity, normalized to the cell protein content, is presented as U/mg of protein.

Statistical Analysis. Results are expressed as means (SDs). Statistical analysis was performed using the SPSS 12.0 software (SPSS Inc., Chicago, IL). A one-way analysis of variance with Duncan's LSD test was used to compare mean values. Values of $p < 0.01$ were considered statistically significant.

RESULTS

HPLC Analysis. Detection limits were less than 5.0 ng/mL for all standards. The correlation coefficient (r) of each standard curve was greater than 0.99 (data not shown). Changes in total isoflavone levels and glyceollin I and coumestrol content in US, GS, and AO-GS were analyzed and compared. A representative HPLC of the three samples is shown in Figure 1. The changes in

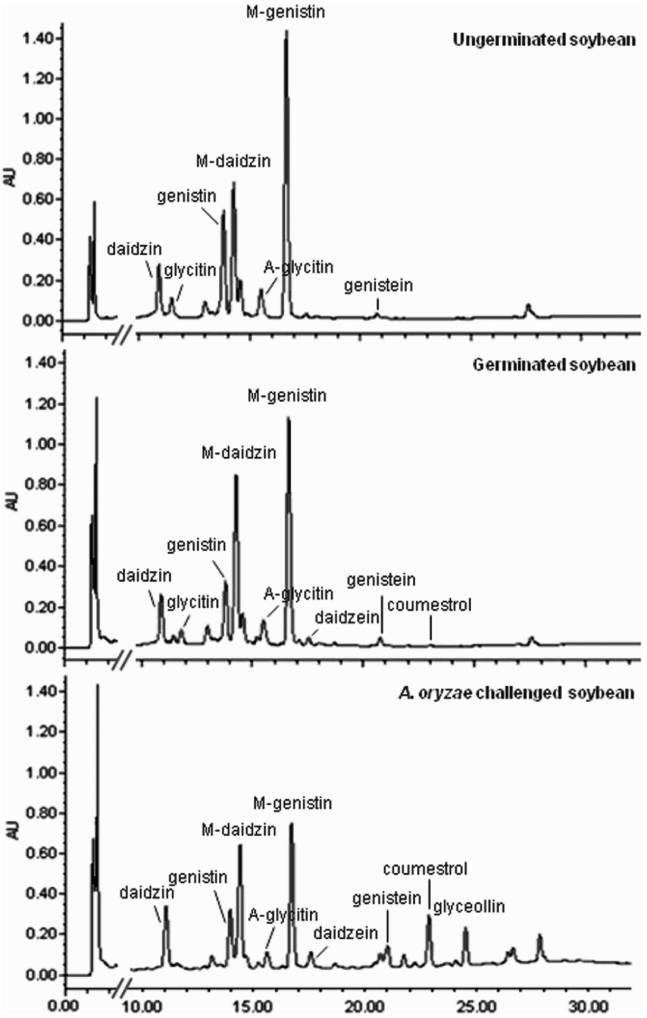

Figure 1. HPLC chromatograms (260 nm) of US, GS, and AO-GS. M-daidzin, malonyldaidzin; M-genistin, malonylgenistin; and A-glycitin, acetylglycitin.

the amount of 11 individual isoflavones were monitored and quantified based on each peak area of the HPLC chromatogram (Table 1). Only five (US) or seven (GS and AO-GS) isoflavones were found at concentrations above the detection limit. The total isoflavonoid level was significantly lower in the GS soybeans (38.88 mg/g extract) than in US soybeans (46.74 mg/g extract). The percentage of total aglycones (daidzein and genistein) relative to total isoflavones was slightly increased from 0.1 to 2.2%, and a minor amount of coumestrol (0.06 mg/g extract) was induced by the germination process. Although a significant decrease in the total isoflavone content was observed, germination without the *A. oryzae* stress did not lead to major changes in the HPLC profile as compared to the US soybeans. In AO-GS, the total isoflavone content further decreased to 31.90 mg/g extract, and the total aglycones proportion markedly increased to 22.9%. Not only coumestrol but glyceollin I was also induced, and the concentrations were 2.48 and 2.70 mg/g extract, respectively.

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Table 1. Phytochemical Content and IC_{50} and TEAC Values of DPPH Scavenging Antioxidant Activities of US, GS, and AO-GS[a]

	phytochemical content (mg/g extract)			DPPH[b] assay	
sample	total isoflavone	coumestrol	glyceollin I	IC_{50} (mg/mL)	TEAC[c] (TE μmol/g)
US	46.74 (0.13) a	ND[d]	ND	2.01	19.4 (0.4) a
GS	38.88 (1.07) a	0.06 (0.01) a	ND	1.03	71.6 (5.8) a
AO-GS	31.90 (0.56) b	2.48 (0.09) b	2.70 (0.28)	0.55	147.1 (1.7) b

[a]Data are presented as means (SDs) of three independent experiments. Total isoflavone and phytoalexins (coumestrol and glyceollin I) contents are expressed as mg/g extract. Total isoflavones are calculated as the sum of the 11 forms: malonyldaidzin, malonylgenistin, acetyldaidzin, acetylglycitin, acetylgenistin, daidzin, glycitin, genistin, daidzein, glycitein, and genistein. Within a column, values sharing the same letters are not significantly different ($p > 0.01$). Values with different letters are significantly different ($p < 0.01$). [b]DPPH, 2,2-diphenyl-1-picrylhydrazyl. [c]TEAC, Trolox equivalent antioxidant capacity. [d]ND, not detected.

Results of DPPH and FRAP Assays. The radical scavenging activities of the US, GS, and AO-GS were evaluated using the DPPH assay. As shown in Table 1, AO-GS exhibited the highest radical scavenging activity among the three samples. The IC_{50} of the AO-GS for DPPH scavenging activity was 0.55 mg/mL, which represents about 2-fold higher activity than that of GS soybeans (IC_{50} = 1.03 mg/mL). US soybeans exhibited the lowest radical scavenging activity with IC_{50} of 2.01 mg/mL.

The radical scavenging activity of the representative soybean isoflavones daidzein and genistein and *A. oryzae*-induced phytoalexins coumestrol and glyceollin I were evaluated with the DPPH and FRAP assays. The results are expressed as TEAC with reference to the standard antioxidant Trolox. Coumestrol and glyceollin I exhibited significantly higher radical scavenging activity than daidzein and genistein. In the DPPH assay, the TEAC value was highest for glyceollin I (427.5 μM) followed by coumestrol, genistein, and daidzein. However, in the FRAP assay, coumestrol exhibited the highest FRAP value, 418.6 μM, followed by glyceollin I, genistein, and daidzein (Table 2).

Table 2. Antioxidant Activities of Daidzein, Genistein, Coumestrol, and Glyceollin I in DPPH and FRAP Assays[a]

	TEAC[b] (TE μM)	
	DPPH[c] assay	FRAP[d] assay
daidzein	15.7 (0.1) a	67.1 (0.5) a
genistein	58.0 (7.6) a	92.4 (5.4) b
coumestrol	325.5 (3.1) b	418.6 (1.4) c
glyceollin I	427.5 (19.4) c	300.0 (6.4) d

[a]Data are means (SDs) of three independent experiments. Within a column, values sharing the same letter are not significantly different ($p > 0.01$). Values with different letters are significantly different ($p < 0.01$). [b]TEAC, Trolox equivalent antioxidant capacity. [c]DPPH, 2,2-diphenyl-1-picrylhydrazyl. [d]FRAP, ferric-reducing antioxidant power.

Effect of Coumestrol on H_2O_2-Induced Cellular Damage in HepG2 Cells. As shown in Figure 2, an 8 h pretreatment with coumestrol protected HepG2 cells from H_2O_2-induced cellular damage and increased cell viability in a dose-dependent manner. H_2O_2 at 1 mM decreased cell viability (58.2%), and 0.01, 0.1, 1 μM coumestrol significantly prevented the cell death and increased cell viability up to 64.3, 69.2, and 72.3%, respectively. The protective effect of 0.1 μM coumestrol was similar, and the effect of 1 μM coumestrol was 10% higher than that of 10 μM Trolox.

Effect of Coumestrol on H_2O_2-Induced ROS Production in HepG2 Cells. To investigate the mechanisms by which coumestrol protected against H_2O_2, the effect of coumestrol on intracellular ROS levels was measured. HepG2 cells were pretreated with different doses of coumestrol for 8 h and then exposed to 1 mM H_2O_2 for 30 min. ROS levels were measured using DCFH fluorescence. The results showed that H_2O_2 exposure induced greater than 2-fold increase in intracellular ROS levels as compared to the unexposed control (Figure 3).

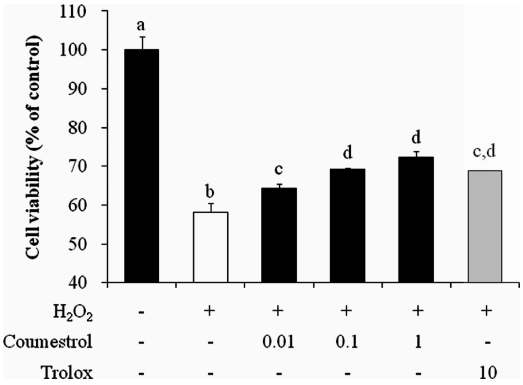

Figure 2. Effect of coumestrol on H_2O_2-induced cellular damage. Cells were pretreated with coumestrol (0.01, 0.1, or 1 μM) or 10 μM Trolox for 8 h and exposed to 1 mM H_2O_2 for 24 h. Cell viability was measured using cell counting kit (CCK-8) assay and expressed as percentage untreated cells at the same time point. Bars represent means (SDs) of triplicate experiments. Values not sharing the same letter are significantly different ($p < 0.01$).

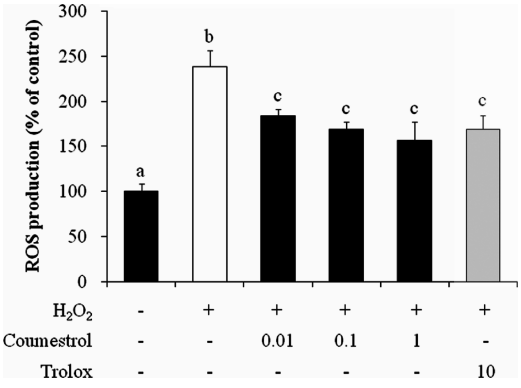

Figure 3. Effect of coumestrol on H_2O_2-induced intracellular ROS production. After pretreatment with coumestrol (0.01, 0.1, or 1 μM) or 10 μM Trolox for 8 h, the cells were incubated with 20 μM dichlorofluorescin diacetate (DCFH) for 20 min. Cells were washed and exposed to 1 mM H_2O_2 for 30 min, and the ROS level was measured with a fluorescence microplate reader. Bars represent means (SDs) of triplicate experiments. Values not sharing the same letter are significantly different ($p < 0.01$).

Coumestrol significantly inhibited the increase in intracellular ROS levels, and its effects were comparable to that of Trolox.

Effect of Coumestrol on H_2O_2-Induced Lipid Peroxidation in HepG2 Cells. MDA was determined as a marker of lipid peroxidation. The lipid peroxidation inhibition effect of coumestrol is shown in Figure 4. Incubation of HepG2 cells

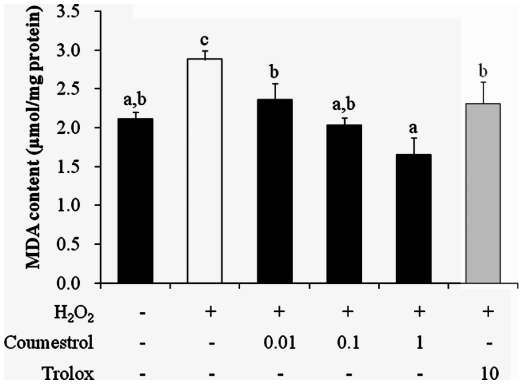

Figure 4. Effect of coumestrol on H_2O_2-induced lipid peroxidation. The MDA content was determined as an indicator of lipid peroxidation. Proteins were quantified before analytical processing. Bars represent means (SDs) of triplicate experiments. Values not sharing the same letter are significantly different ($p < 0.01$).

with 1 mM H_2O_2 for 24 h increased lipid peroxidation by 36.7%. Coumestrol significantly reduced the accumulation of MDA ($p < 0.01$), and the effect of 0.01 μM coumestrol was similar to that of 10 μM Trolox. The inhibitory effect of coumestrol increased in a concentration-dependent manner.

Effect of Coumestrol on Antioxidant Enzymes in HepG2 Cells. Exposure of HepG2 cells to 1 mM H_2O_2 led to a noticeable decrease in GSH levels to one-third of unexposed control (Figure 5A). Although there were slight increases in the mean intracellular GSH levels in 0.1 and 1 μM coumestrol pretreated group, the increases were not statistically significant with $p = 0.011$ and $p = 0.037$, respectively. On the other hand, pretreatment of 1 μM coumestrol for 8 h prior to H_2O_2 exposure significantly increased the intracellular GSH level ($p < 0.01$, Figure 5A).

Coumestrol also attenuated the H_2O_2-induced decrease in SOD activity. H_2O_2 significantly decreased SOD activity by 82%, and coumestrol treatment alleviated the decrease in a dose-dependent manner (Figure 5B). Pretreatment with 0.1 and 1 μM coumestrol restored SOD activity to control levels, indicating that coumestrol completely counteracted the adverse effects of H_2O_2 on SOD.

DISCUSSION

This study was performed to quantify the changes in phytochemical composition of soybeans and concomitant changes in antioxidant activity by applying microbial stress to soybean during germination. We used *A. oryzae* as a biotic elicitor because *Aspergillus* species are recognized as the most widely distributed fungi in foodstuffs, soils, and other materials. *A. oryzae* is widely used in Asian traditional fermentation industries, including soy sauce, miso, alcoholic beverages, and vinegar.[30]

We observed a 17% decrease in total isoflavone content during the 5 day germination process, and inoculation of *A. oryzae* during germination further decreased the total isoflavone content by 68.3% of US soybeans. However, the total aglycones content was increased during germination from 0.34 to 0.85 mg/g extract, and the increase was more marked

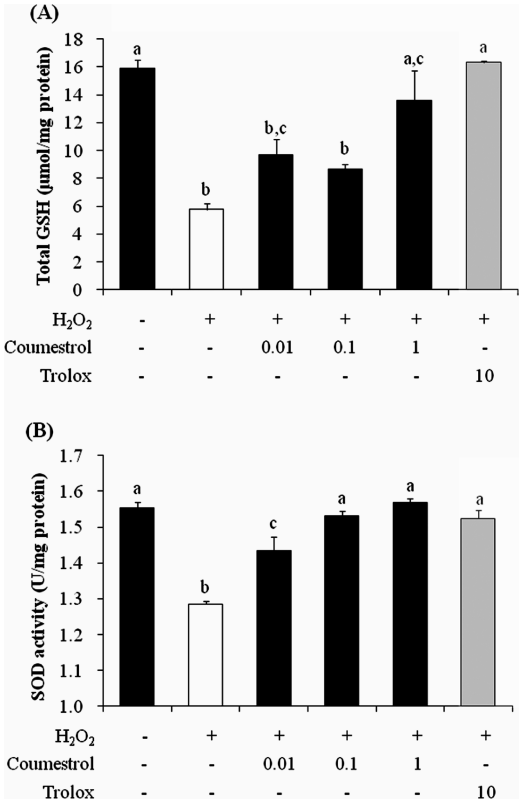

Figure 5. Effect of coumestrol on H_2O_2-induced decrease in total glutathione (GSH) content and superoxide dismutase (SOD) activity. Following pretreatment with coumestrol (0.01, 0.1, or 1 μM) or 10 μM Trolox for 8 h, the cells were exposed to 1 mM H_2O_2 for 1 h. The cells were harvested, and total GSH levels (A) and SOD activity (B) were measured. Proteins were quantified before analytical processing. Bars represent mean (SD) of triplicate experiments. Values not sharing the same letter are significantly different ($p < 0.01$).

following the *A. oryzae* challenge (7.28 mg/g extract). These results are comparable to those of a previous study, which reported that the total isoflavone content increases during the initial stage (6−24 h) of germination and gradually decreases thereafter.[31] It has also been reported that fermentation with *A. oryzae* for 5 days decreases the total content of the six isoflavones daidzein, genistein, glycitein, daidzin, genistin, and glycitin from 176.05 to 62.81 μg/g dry weight of soybean.[32] Furthermore, isoflavones are known to be metabolized from glycosides to aglycone by β-glucosidase during germination or fermentation of soybean.[33,34] The results of the current study indicate that the combination of germination and microbial inoculation stimulates isoflavone modification more than the individual treatments. Graham et al. reported that the preexisting conjugates of both daidzein and genistein are rapidly hydrolyzed in cotyledon tissues infected by a fungal pathogen, whereby large quantities of free daidzein and genistein are released.[35] Germinating soybeans are prone to colonized by *A. oryzae* since the germinating condition is generally under high temperature and moisture.[36] This might be a reason for the synergistic effect of the combined treatment.

In the case of phytoalexins, germination induced trace amounts of coumestrol but not glyceollin I, whereas *A. oryzae* challenge markedly induced both coumestrol and glyceollin I. A previous study reported a coumestrol concentration of 0.75−3.94 μg/g dry weight in response to germination.[37] With an approximate extraction yield of 12−15%, the coumestrol

level would be 0.005−0.03 mg/g extract, whereas a concentration of 0.06 mg/g extract was found in our study. Potential reasons for the difference include the germination condition and inherent varietal differences in coumestrol biosynthesis. *A. oryzae* inoculation more drastically induced coumestrol and glyceollin I synthesis than germination. It has been extensively demonstrated that coumestrol and glyceollins are synthesized de novo and accumulated when soybeans are exposed to microbes. The soybean plant under microbial stress produces a mixture of glyceollin isomers, glyceollin I, II, and III.[38] Previous studies have reported that glyceollin levels range from 0.03 to 7 mg/g dry weight in fungus-challenged soy seedlings.[39,40] In our study, only glyceollin I was detected at a concentration of 2.7 mg/g extract, which is consistent with the finding from a previous study that glyceollin I is the predominant isomer induced in cotyledon tissue by *A. sojae*.[41] However, our results differ from those of a prior study in which the induction of glyceollins was 33−43-fold higher than that of coumestrol. It is known that different genera and species of microbes have significantly different abilities to induce phytoalexins.[42,43] The conditions and soybean varieties used in this study may also contribute to the difference.

Germination and *A. oryzae* challenge modified the antioxidant activity of soybean, measured as free radical scavenging activity. AO-GS exhibited the highest antioxidant activity, followed by GS and US soybeans. The results suggest that germination and microbial inoculation modify the phenolic composition of soybeans, which may be responsible for the significant increase in DPPH values. Higher levels of isoflavone aglycones (77.5%) have been observed in fermented soybean foods as compared to that of unfermented soybeans (19.3%), which contribute to higher antioxidant activities.[44] Therefore, the increase in aglycone ratio in response to germination and *A. oryzae* challenge may contribute to the higher antioxidant activity. Furthermore, it can be postulated that the increase in coumestrol and glyceollin I content may also contribute to the increased antioxidant activity given the fact that coumestrol and glyceollin I exhibited 5−20-fold higher antioxidant activity than daidzein and genistein, which are the representative isoflavone algycones in soybeans. It is noteworthy that the antioxidant activities of coumestrol and glyceollin I were comparably strong, although their antioxidant values measured in the DPPH and FRAP assays were slightly different. It is not unusual that different methods produce different antioxidant values for the same compound because chemically distinct methods are based on different reaction mechanisms.[45]

Kim et al. have measured the potent antioxidant activity of glyceollins using various assay systems.[46] However, the antioxidant activity of coumestrol has not been extensively investigated. This study clearly demonstrated that coumestrol has strong antioxidant potential.

To examine the effect of coumestrol in HepG2 cells, the cells were incubated with different concentrations of coumestrol (up to 100 μM) for 24 h in serum-free medium. Because concentrations over 10 μM were slightly cytotoxic (data not shown), the test concentrations were determined as 0.01, 0.1, and 1 μM. Coumestrol protected HepG2 cells from H_2O_2-induced oxidative damage and significantly decreased ROS production and lipid peroxidation. A decrease in ROS production may be explained by the radical scavenging activity of coumestrol, and the decreased ROS production may reduce lipid peroxidation. Coumestrol also recuperated H_2O_2-induced decrease of intracellular GSH levels. This may be caused by decreased export of glutathione or the enhanced expression and/or activity of key enzymes involved in glutathione biosynthesis.[47] Furthermore, coumestrol increased the SOD activity in a dose-dependent manner. Induction of the cellular antioxidant enzyme system plays a crucial role in the antioxidant defense response, and changes in antioxidant enzyme activities can be considered as biomarkers of the antioxidant response.[48] Among the antioxidant systems, SOD is a major scavenger of ROS. Mild oxidative stress should induce antioxidant enzymes as a positive-feedback response to fight the oxidative stress. On the other hands, high doses are detrimental due to irreversible inactivation of antioxidant defense system result in decreased antioxidant enzyme activity.[49] The response of the antioxidant defense enzymes may depend on the experimental model and the types of pro-oxidants. In this study, 1 mM H_2O_2 significantly decreased the SOD activity, and coumestrol markedly alleviated the decrease. These results indicate that a relatively low and nontoxic concentration of coumestrol exhibits a powerful antioxidant activity by scavenging reactive radicals, inhibiting lipid peroxidation and increasing protective enzyme activity.

In summary, *A. oryzae* challenge during soybean germination greatly altered metabolite profiles, including a much higher antioxidant activity. AO-GS exhibited higher isoflavone aglycone ratio and phytoalexins (coumestrol and glyceollin I) content and a higher antioxidant activity in DPPH assay. This higher antioxidant activity may be partly due to the increased content of the phytoalexin coumestrol, which exhibited strong antioxidant activity in this study. Using food-grade *A. oryzae* to elicit the biosynthesis of phytoalexins alters the secondary metabolite profiles of the soybeans and offers higher potency nutraceutical candidates for the prevention of oxidative stress-related chronic diseases. Although metabolomic analysis of microbe-challenged GS soybeans is required to comprehensively characterize phytochemicals other than coumestrol, our results form a basis for further study to examine the beneficial effects of bioprocessing for producing novel and higher potency soybean products.

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Notes

The authors declare no competing financial interest.

REFERENCES

(1) Choi, Y. S.; Lee, B. H.; Kim, J. H.; Kim, N. S. Concentration of phytoestrogens in soybeans and soybean products in Korea. *J. Sci. Food Agric.* **2000**, *80*, 1709−1712.

(2) Kim, S. H.; Jung, W. S.; Ahn, J. K.; Chung, I. M. Analysis of isoflavone concentration and composition in soybean [*Glycine max* (L.)] seeds between the cropping year and storage for 3 years. *Eur. Food Res. Technol.* **2005**, *220*, 207−214.

(3) Sirtori, C. R.; Gianazza, E.; Manzoni, C.; Lovati, M. R.; Murphy, P. A. Role of isoflavones in the cholesterol reduction by soy proteins in the clinic. *Am. J. Clin. Nutr.* **1997**, *65*, 166−167.

(4) Kim, J. A.; Chung, I. M. Change in isoflavone concentration of soybean (*Glycine max* L.) seeds at different growth stages. *J. Sci. Food Agric.* **2007**, *87*, 496−503.

(5) Barnes, S. Evolution of the health benefits of soy isoflavones. *Proc. Soc. Exp. Biol. Med.* **1998**, *217*, 386−392.

(6) Kramer, F.; Jensen, P. S.; Vinggaard, A. M.; Larsen, E. H.; Breinholt, V. M. Effect of in utero-administered coumestrol, equol, and organic selenium on biomarkers for phase 2 enzyme capacity and redox status. *Nutr. Cancer* **2003**, *46*, 73−81.

(7) Shimoda, K.; Hamada, H. Synthesis of beta-maltooligosaccharides of glycitein and daidzein and their anti-oxidant and anti-allergic activities. *Molecules* **2010**, *15*, 5153−5161.
(8) Kim, J. A.; Hong, S. B.; Jung, W. S.; Yu, C. Y.; Ma, J. G.; Chung, I. M. Comparison of isoflavones composition in seed, embryo, cotyledon and seed coat of cooked-with-rice and vegetable soybean (*Glycine max* L.) varieties. *Food Chem.* **2007**, *102*, 738−744.
(9) Bau, H. M.; Villaume, C.; Méjean, L. Effects of soybean (*Glycine max*) germination on biologically active components, nutritional values of seeds, and biological characteristics in rats. *Nahrung* **2000**, *44*, 2−6.
(10) Lee, M. R.; Kim, J. Y.; Chun, J.; Park, S.; Kim, H. J.; Kim, J. S.; Jeong, J. I.; Kim, J. H. Induction of glyceollins by fungal infection in varieties of Korean soybean. *J. Microbiol. Biotechnol.* **2010**, *20*, 1226−1229.
(11) Wu, Z.; Song, L.; Huang, D. Food grade fungal stress on germinating peanut seeds induced phytoalexins and enhanced polyphenolic antioxidants. *J. Agric. Food Chem.* **2011**, *59*, 5993−6003.
(12) Simons, R.; Vincken, J. P.; Roidos, N.; Bovee, T. F.; Iersel, M.; Verbruggen, M. A.; Gruppen, H. Increasing soy isoflavonoid content and diversity by simultaneous malting and challenging by a fungus to modulate estrogenicity. *J. Agric. Food Chem.* **2011**, *59*, 6748−6758.
(13) Ebel, J. Phytoalexin synthesis: The biochemical analysis of the induction process. *Annu. Rev. Phytopathol.* **1986**, *24*, 235−264.
(14) Boue, S. M.; Cleveland, T. E.; Carter-Wientjes, C.; Shih, B. Y.; Bhatnagar, D.; McLachlan, J. M.; Burow, M. E. Phytoalexin-enriched functional foods. *J. Agric. Food Chem.* **2009**, *57*, 2614−2622.
(15) Baur, J. A.; Jang, M.; Cai, L.; Udeani, G. O.; Slowing, K. V.; Thomas, C. F.; Beecher, C. W. W.; Fong, H. H. S.; Farnsworth, N. R.; Kinghorn, A. D.; Mehta, R. G.; Moon, R. C.; Pezzuto, J. M. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* **1997**, *275*, 218−220.
(16) Liu, S.; Norris, D. M.; Hartwig, E. E.; Xu, M. Inducible phytoalexins in juvenile soybean genotypes predict soybean looper resistance in the fully developed plants. *Plant Physiol.* **1992**, *100*, 1479−1485.
(17) Lyon, G. D.; Albersheim, P. Host-pathogen interactions. XXI. Extraction of a heat-labile elicitor of phytoalexin accumulation from frozen soybean stems. *Plant Physiol.* **1982**, *70*, 406−409.
(18) Al-Maharik, N.; Botting, N. P. A new short synthesis of coumestrol and its application for the synthesis of [6,6a,11a-$^{13}C_3$]-coumestrol. *Tetrahedron* **2004**, *60*, 1637−1642.
(19) Liu, M. H.; Tsuang, F. Y.; Sheu, S. Y.; Sun, J. S.; Shih, C. M. The protective effects of coumestrol against amyloid-beta peptide- and lipopolysaccharide-induced toxicity on mice astrocytes. *Neurol. Res.* **2011**, *33*, 663−672.
(20) Yuk, H. J.; Lee, J. H.; Curtis-Long, M. J.; Lee, J. W.; Kim, Y. S.; Ryu, H. W.; Park, C. G.; Jeon, T. S.; Park, K. H. The most abundant polyphenol of soy leaves, coumestrol, displays potent α-glucosidase inhibitory activity. *Food Chem.* **2011**, *126*, 1057−1063.
(21) Moon, H. J.; Seok, J. H.; Kim, S. S.; Rhee, G. S.; Lee, R. D.; Yang, J. Y.; Chae, S. Y.; Kim, S. H.; Kim, J. Y.; Chung, J. Y.; Kim, J. M.; Chung, S. Y. Lactational coumestrol exposure increases ovarian apoptosis in adult rats. *Arch. Toxicol.* **2009**, *83*, 601−608.
(22) Whitten, P. L.; Patisaul, H. B.; Young, L. J. Neurobehavioral actions of coumestrol and related isoflavonoids in rodents. *Neurotoxicol. Teratol.* **2002**, *24*, 47−54.
(23) Martín, M. A.; Ramos, S.; Mateos, R.; Izquierdo-Pulido, M.; Bravo, L.; Goya, L. Protection of human HepG2 cells against oxidative stress by the flavonoid epicatechin. *Phytother. Res.* **2010**, *24*, 503−509.
(24) Droge, W. Free radicals in the physiological control of cell function. *Physiol. Rev.* **2001**, *82*, 47−95.
(25) Alía, M.; Ramos, S.; Mateos, R.; Bravo, L.; Goya, L. Response of the antioxidant defense system to tert-butyl hydroperoxide and hydrogen peroxide in a human hepatoma cell line (HepG2). *J. Biochem. Mol. Toxicol.* **2005**, *19*, 119−128.
(26) Feng, S.; Saw, C. L.; Lee, Y. K.; Huang, D. Fungal-stressed germination of black soybeans leads to generation of oxooctadecadienoic acids in addition to glyceollins. *J. Agric. Food Chem.* **2007**, *55*, 8589−8595.
(27) Katsube, T.; Tabata, H.; Ohta, Y.; Yamasaki, Y.; Anuurad, E.; Shiwaku, K.; Yamane, Y. Screening for antioxidant activity in edible plant products: Comparison of low-density lipoprotein oxidation assay, DPPH radical scavenging assay, and Folin-Ciocalteu assay. *J. Agric. Food Chem.* **2004**, *52*, 2391−2396.
(28) Benzie, I. F. F.; Strain, J. J. The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power. *Anal. Biochem.* **1996**, *239*, 70−76.
(29) Mateos, R.; Goya, L.; Bravo, L. Determination of malondialdehyde by liquid chromatography as the 2,4-dinitrophenylhydrazone derivative: A marker for oxidative stress in cell cultures of human hepatoma HepG2. *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.* **2004**, *805*, 33−39.
(30) Luh, B. S. Industrial production of soy sauce. *J. Ind. Microbiol.* **1995**, *14*, 467−471.
(31) Kim, W. J.; Lee, H. Y.; Won, M. H.; Yoo, S. H. Germination effect of soybean on its contents of isoflavones and oligosaccharides. *Food Sci. Biotechnol.* **2005**, *14*, 498−502.
(32) Chaiyasut, C.; Kumar, T.; Tipduangta, P.; Rungseevijitprapa, W. Isoflavone content and antioxidant activity of Thai fermented soybean and its capsule formulation. *Afr. J. Biotechnol.* **2010**, *9*, 4120−4126.
(33) Ha, E. Y. W.; Morr, C. V.; Seo, A. Isoflavone aglycones and volatile organic compounds in soybeans: Effects of soaking treatments. *J. Food Sci.* **1992**, *57*, 414−417.
(34) Kim, N. Y.; Song, E. J.; Kwon, D. Y.; Kim, H. P.; Heo, M. Y. Antioxidant and antigenotoxic activities of Korean fermented soybean. *Food Chem. Toxicol.* **2008**, *46*, 1184−1189.
(35) Wu, Z.; Song, L.; Huang, D. Food grade fungal stress on germinating peanut seeds induced phytoalexins and enhanced polyphenolic antioxidants. *J. Agric. Food Chem.* **2011**, *59*, 5993−6003.
(36) Graham, T. L.; Kim, J. E.; Graham, M. Y. Role of constitutive isoflavone conjugates in the accumulation of glyceollin in soybean infected with Phytophthora megasperma. *Mol. Plant-Microbe Interact.* **1990**, *3*, 157−166.
(37) Lookhart, G. L.; Finney, R. L.; Finney, K. F. Note on coumestrol in soybeans and fractions at various germination times. *Cereal Chem.* **1979**, *56*, 495−496.
(38) Tilghman, S. L.; Boué, S. M.; Burow, M. E. Glyceollins, a novel class of antiestrogenic phytoalexins. *Mol. Cell Pharmacol.* **2010**, *2*, 155−160.
(39) Bhattacharyya, M. K.; Ward, E. W. B. Resistence, susceptibility and accumulation of glyceollins I-III in soybean organs inoculated with *Phytophthora megasperma* f. sp. glycinea. *Physiol. Mol. Plant Pathol.* **1986**, *29*, 227−237.
(40) Shi, H.; Nam, P. K.; Ma, Y. Comprehensive profiling of isoflavones, phytosterols, tocopherols, minerals, crude protein, lipid, and sugar during soybean (*Glycine max*) germination. *J. Agric. Food Chem.* **2010**, *58*, 4970−4976.
(41) Boué, S. M.; Carter, C. H.; Ehrlich, K. C.; Cleveland, T. E. Induction of the soybean phytoalexins coumestrol and glyceollin by *Aspergillus*. *J. Agric. Food Chem.* **2000**, *48*, 2167−2172.
(42) Albersheim, P.; Valent, B. S. Host-pathogen interactions in plants. Plants, when exposed to oligosaccharides of fungal origin, defend themselves by accumulating antibiotics. *J. Cell. Biol.* **1978**, *78*, 627−643.
(43) Darvill, A. G.; Albersheim, P. Phytoalexins and their elicitors: A defense against microbial infection in plants. *Annu. Rev. Plant Physiol.* **1984**, *35*, 243−275.
(44) Kwak, C. S.; Lee, M. S.; Park, S. C. Higher antioxidant properties of chungkookjang, a fermented soybean paste, may be due to increased aglycone and malonylaglycone isoflavone during fermentation. *Nutr. Res. (N.Y.)* **2007**, *27*, 719−727.
(45) Ou, B.; Huang, D.; Hampsch-Woodill, M.; Flanagan, J. A.; Deemer, E. K. Analysis of antioxidant activities of common vegetables employing oxygen radical absorbance capacity (ORAC) and ferricreducing antioxidant power (FRAP) assays: A comparative study. *J. Agric. Food Chem.* **2002**, *50*, 3122−3128.

(46) Kim, H. J.; Suh, H. J.; Kim, J. H.; Park, S.; Joo, Y. C.; Kim, J. S. Antioxidant activity of glyceollins derived from soybean elicited with *Aspergillus sojae*. *J. Agric. Food Chem.* **2010**, *58*, 11633−11638.
(47) Lu, S. C. Regulation of glutathione synthesis. *Mol. Aspects Med.* **2009**, *30*, 42−59.
(48) Hayes, J. D.; Ellis, E. M.; Neal, G. E.; Harrison, D. J.; Manson, M. M. Cellular response to cancer chemopreventive agents: Contribution of the antioxidant responsive element to the adaptive response to oxidative and chemical stress. *Biochem. Soc. Symp.* **1999**, *64*, 141−168.
(49) Lien, A. P.; Hua, H.; Chuong, P. Free radicals, antioxidants in disease and health. *Int. J. Biomed. Sci.* **2008**, *4*, 89−96.