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Antioxidant Activity of Glyceollins Derived from Soybean Elicited with *Aspergillus sojae*

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The extract of soybean exposed to biotic elicitors such as food-grade fungus is known to have antioxidant activity. Glyceollins were major bioactive compounds present in soybean elicited by fungi and shown to have antifungal and anticancer activities. The purpose of present study was to evaluate the antioxidant activities of glyceollins by measuring ferric reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, singlet oxygen quenching, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging, hydroxyl radical scavenging activity, and lipid peroxidation inhibition. In addition, the antioxidant potential of glyceollins were measured by a fluorescent probe, 2′,7′-dichlorofluorescin diacetate (DCFDA), and dihydroethidium (DHE) in mouse hepatoma hepa1c1c7 cells in which they were insulted with H_2O_2 to generate reactive oxygen species (ROS). Glyceollins showed a strong reducing power and inhibited lipid peroxidation, with significant scavenging activities of radicals including singlet oxygen, superoxide anion, ABTS, and DPPH. We also found that glyceollins significantly suppressed H_2O_2-induced ROS production in hepa1c1c7 cells. Therefore, glyceollins deserve further study as natural antioxidants and nutraceuticals.

KEYWORDS: Glyceollins; soybean; antioxidant activity

INTRODUCTION

Phytoalexins may be beneficial to human health as they are shown to have antioxidant, antiinflammatory, and anticancer activities (*1*). Glyceollins are a class of phytoalexins which is derived from soybean in response to various stress signals such as fungal infection (*2*, *3*). As glyceollin isomers (I, II, and III) (**Figure 1**) have similar structure to other soybean isoflavonoids, there is a good possibility that they share common physiological properties with isoflavones including antioxidant, estrogen-like, or antiestrogenic activities. In fact, glyceollins have been reported to have inhibitory effects on growth of the human androgen-responsive prostate cancer cells and proliferation of breast and ovarian carcinoma cells implanted in ovariectomized athymic mice (*4*, *5*). In addition, our study confirmed the estrogenic and antidiabetic activities of the glyceollins (*6*, *7*).

Oxidative stress is the term referring to the imbalance between the generation of reactive oxygen species (ROS) and the activity of the antioxidant defenses. Severe oxidative stress has been implicated in chronic diseases such as cancer and heart disease due to the damage of biological molecules (*8*, *9*). For instance, damage in DNA as modification of DNA bases and DNA strand breakage induced by ROS may lead to the development of cancer under condition of diminished DNA repair capacity. A scavenger of these ROS may be used as a preventive tool to control oxidative stress-related diseases. Many polyphenolic compounds from plant materials have shown the antioxidant activity against ROS (*10*, *11*).

Although some biological activities and pharmacological functions of glyceollins have been reported as mentioned above, the antioxidant activity of glyceollins has not been studied. In this study the antioxidative effect of glyceollins was evaluated using *in vitro* and cell-based assays.

MATERIALS AND METHODS

Preparation of Glyceollin Mixture. Soybeans (Aga No. 3), which have an exceedingly high level of isoflavones (~10 mg/g) (*12*), were obtained from Kyungpook National University Soyventure Co., Ltd. (Daegu, South Korea) and were subjected to elicitation by *Aspergillus sojae* (*A. sojae*), an edible fungus, for de novo biosynthesis of glyceollins (*4*−*6*) of which molecular structures are shown in **Figure 1**. *A. sojae* cultures were grown at 25 °C in the dark on potato dextrose agar media. Inocula were prepared by harvesting fungi after 5 day incubation. Soybeans were surface-sterilized for 3 min in 70% ethanol, followed by a quick rinse with deionized water. Seeds were presoaked in sterile deionized water for 4−5 h before placing into treatment chambers. Each compartment was moistened with distilled water. Soybean seeds were minced in several pieces by a food homogenizer (Hanil, Bucheon, S. Korea). *A. sojae* spore suspension (10 μL) was applied to the cut surface of each seed. Soybean seeds were stored in a chamber at 26 °C in the dark for 3 days and then stored at −20 °C. Soybean seeds (5 g) exposed to

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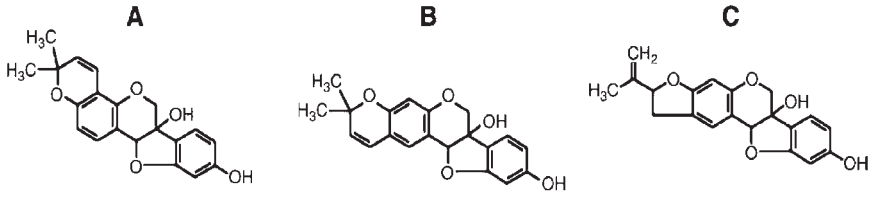

Figure 1. Molecular structures of glyceollin isomers I (**A**), II (**B**), and III (**C**).

A. sojae were extracted with 15 mL of 80% (v/v) ethanol and heated at 50 °C for 1 h, cooled, and then centrifuged at 14000*g* for 10 min. Extracts were filtered through a syringe sterile filter with 0.45 μm pore size (Sartorius Biotech GmbH, Goettingen, Germany). Each crude extract was freeze-dried and dissolved in dimethyl sulfoxide at a concentration of 100 mg/mL prior to use.

The freeze-dried crude extract was suspended in deionized water and then successively partitioned with hexane and ethyl acetate (EtOAc) to distinguish hexane-soluble and EtOAc-soluble fractions. The EtOAc-soluble fraction was subjected to silica gel column chromatography using EtOAc and MeOH mixtures (1:1) of increasing polarity (50:1 to 5:1) to yield five fractions (F1−F5). Fraction F2 was subjected to TLC and HPLC to determine the purity of the glyceollin-rich fraction, and then the fraction was evaporated under reduced pressure (*13*). The concentration of glyceollin isomers in the fraction (thereafter called "glyceollins") was ∼90% as assayed by HPLC. The relative ratio of glyceollins I, II, and III in the mixture was 12:1.3:1.

FRAP Assay. The ferric reducing antioxidant power (FRAP) of the sample was determined as described previously (*14*). Briefly, 30 μL of H_2O, 30 μL of ferrous sulfate as standard, or samples were incubated at room temperature with 1 mL of FRAP reagent (containing 300 mmol/L acetate buffer (pH 6.3), 10 mmol/L TPTZ (2,4,6-tri(2-pyridyl)-1,3,5-triazine) solution, 20 mmol/L $FeCl_3$ solution, and H_2O), and the absorbance was recorded after 4 min. FRAP values of unknowns were calculated on the basis of standard curves established using standard at 0.1−1.0 mmol/L.

DPPH Radical Scavenging Assay. The DPPH radical scavenging activity of glyceollins was evaluated as previously described (*15*, *16*). Briefly, 50 μL of glyceollin solution (or DMSO) was added to 200 μL of 200 μM DPPH radical solution, which was freshly made. After 30 min of incubation at room temperature, the absorbance at 515 nm was measured. Synthetic antioxidant reagent, L-ascorbic acid (AA), was used as a positive control, and all tests were carried out in triplicate.

$ABTS^+$ Radical Cation Decolorization Assay. ABTS was prepared by reacting 5 mL of 7 mM ABTS and 80 μL of 2.45 mM potassium persulfate, and the mixture was shaken in the dark at room temperature for 12 h before use (*17*). It was diluted with ethanol so that its absorbance was adjusted to 0.7 ± 0.02 at 734 nm. ABTS (1 mL) was added to glass test tubes containing 50 μL of samples, mixed by a vortex mixer for 30 s. Absorbance was measured at 734 nm after 5 min. The percentage of radical scavenging activity was calculated by comparing the absorbance values of control without glyceollins. All determinations were performed three times.

Hydroxyl Radical Scavenging Activity. The hydroxyl radical scavenging activity was assayed by using a model hydroxyl radical-generating system where the OH radical was generated using ascorbic acid iron−EDTA (*20*). The standard reaction mixture consisted of 100 mM phosphate buffer, pH 7.4, 167 μM iron−EDTA complex, 0.1 mM EDTA, 2 mM ascorbic acid, and 33 mM DMSO in a final volume of 3 mL. Iron-catalyzed oxidation of ascorbic acid at 37 °C for 30 min was used to generate formaldehyde from DMSO. Appropriate controls, reaction mixtures without ascorbic acid, were maintained. For preparing the test sample mixture, glyceollins were added to obtain final concentrations ranging from 50 to 400 μg/mL. The reaction was stopped by the addition of 1 mL of ice-cooled TCA (17.5% w/v) and 2 mL of Nash reagent and then left at room temperature for 30 min. The intensity of color was measured at 412 nm spectrophotometrically.

Singlet Oxygen Quenching Activity. In order to assess the ability of the sample to scavenge singlet oxygen ($^1O_2$), the imidazole−RNO method was employed in which $^1O_2$ was photogenerated from rose bengal (RB, well-known type II photosensitizer) (*21*). The $^1O_2$-mediated bleaching of RNO via imidazole oxidation was monitored spectrophotometrically at 440 nm in reaction mixtures of RB (5 μM), imidazole (8 mM), and RNO (5 μM) in 20 mM Tris−succinate buffer (pH 6.5). The sample mixtures were allowed to pass through a 6 mm Plexiglas and UV-35 filter from a 150 W Halogen lamp (Osram, Augsburg, Germany) (white light ($\lambda > 350$ nm)) for 5 min at 25 °C. L-Ascorbic acid was used as a positive control.

Lipid Peroxidation Inhibition Assay. Assay of lipid peroxidation was performed according to the method described previously (*18*). Rats were anesthetized with diethyl ether and sacrificed by exsanguination. The perfused liver was isolated and homogenized with 9 parts of isotonic phosphate buffer saline using a homogenizer at 4 °C. The homogenate was centrifuged at 10000*g* for 5 min, and the supernatant was used for the *in vitro* lipid peroxidation assay. Briefly, various concentrations of sample were mixed with 1 mL of 0.15 M potassium chloride and 0.5 mL of rat liver homogenate. Peroxidation was initiated by adding 100 μL of 100 mM H_2O_2. After incubation at 37 °C for 30 min, the reaction was stopped by adding 2 mL of ice-cold 0.25 M HCl containing 15% TCA, 0.38% TBA, and 0.5% BHT. The reaction mixture was heated at 90 °C for 30 min, cooled, and centrifuged at 10000*g* for 5 min, and the supernatant was read at 532 nm. A control without added sample was also run simultaneously. The percentage of lipid peroxidation inhibitory activity was calculated as follows; lipid peroxidation rate = 1 − (sample OD/blank OD) × 100. Rat brain and kidney were homogenized with a Polytron in 10 mL of ice-cold Tris-HCl buffer (20 mM, pH 7.4) (*19*). The homogenate was centrifuged at 24795*g* for 15 min. The supernatants (1 mL) were incubated with different concentrations of sample in the presence of 10 mM H_2O_2 at 37 °C for 1 h. The reaction was terminated by addition of 1.0 mL of TCA (28%) and 1.5 mL of TBA (1%). The reaction solution was heated at 100 °C for 15 min, cooled to room temperature, and centrifuged at 4696*g* for 15 min, and the color of the MDA−TBA complex in the supernatant was read at 532 nm using a spectrophotometer. BHA was used as a positive control. The inhibition ratio (%) was calculated using the following formula: inhibition ratio (%) = $(A - A_1)/A \times 100$, where A is the absorbance of the control and A_1 is the absorbance of the test sample.

DCF Assay. Oxidative stress was quantified in cells by 2,7-dichlorofluorescein (DCF) assay according to Wang and Joseph (*22*), with slight modifications. A mouse hepatoma cell line (hepa1c1c7) was obtained from ATCC (Manassas, VA, USA). For routine maintenance, cells were grown in α-MEM (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37 °C in an atmosphere of 5% CO_2/95% air under saturating humidity and passaged every other day (1:4 split ratio) by trypsinization with 0.25% trypsin/0.02% EDTA sodium salt solution (Thermo Fisher Scientific Inc., Waltham, MA, USA).

The cells (100000 cells per well) were seeded into a black-bottom 96-well plate and cultivated for 24 h. After cell attachment, plates were washed with PBS and incubated with H_2O_2 (1 mM) for 2 h prior to treatment with increasing concentrations of each generation of sample prepared in 10% FBS-containing media for 12 h. The stimulated cells were washed with PBS and incubated for 30 min with dichlorfluorescein diacetate (DCFDA) dissolved in DMSO (final concentration 50 μM). Fluorescence was measured at 0 and 40 min using an excitation of 485 nm and emission of 535 nm, in a fluorescence microplate reader (Infinite 200; Tecan, Grodig, Austria). Most of the steps including incubation of the reaction mixture containing dye and oxidant, washing, and fluorometric determination were performed in the dark. The intensity of fluorescence was calculated as $[(F_{40\text{min}} - F_{0\text{min}})/F_{0\text{min}}] \times 100$ as described elsewhere (*22*). Results are expressed as relative intensity of fluorescence (in percent of H_2O_2 control). Intracellular fluorescence was visualized using a fluorescent microscope (Nikon Eclipse 80i, Japan).

Dihydroethidium Assay. The production of superoxide anion in cell culture was measured using the oxidation-sensitive dye dihydroethidium

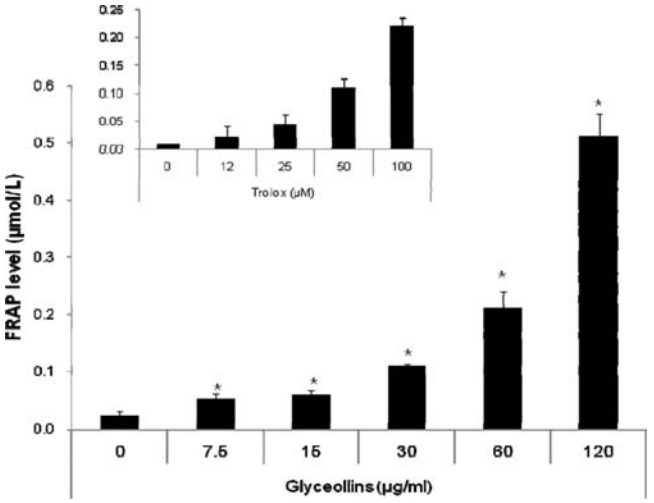

Figure 2. Ferric-reducing antioxidant power (FRAP) of glyceollins. Trolox was used as positive control. The results are expressed as means ± SD of four separate experiments. *, $p < 0.05$, compared to the control.

(DHE) (*23*). Hepa1c1c7 cells were stained by incubation for 30 min at 37 °C with 10 μM DHE (Sigma). After staining, the cultures were washed briefly with PBS and mounted. The stained cells were imaged using a Nikon Eclipse 80i fluorescence microscope and a Nikon Digital Sight DS-Fi1c camera.

Statistical Analysis. Statistical differences between mean values were determined by Student's *t* test. The significance level was set at $p < 0.05$.

RESULTS

Reducing Power of Glyceollins. The ability of glyceollins to reduce ferric (Fe^{3+}) to ferrous ion (Fe^{2+}) was assayed with the reference compound Trolox. As shown in **Figure 2**, the glyceollin-rich fraction exhibited high FRAP values at the concentration of 30 μg/mL or higher. For instance, FRAP values at 30, 60, and 120 μg/mL were 0.11, 0.21, and 0.51 μmol/L, respectively, although the compounds did not show noteworthy increased FRAP reducing activity at the concentrations of 7.5 and 15 μg/mL.

DPPH Radical Scavenging Activity of Glyceollins. As shown in **Figure 3**, glyceollins were found to have significantly strong antioxidant activities as assayed by DPPH scavenging activities. At a concentration of 20 μg/mL, the DPPH radical scavenging activity of glyceollins reached 39%. The IC_{50} value of glyceollins for DPPH scavenging activity was found to be 27.7 μg/mL, which represents about 2-fold lower activity than ascorbic acid, a positive control (IC_{50} 12.0 μg/mL).

$ABTS^+$ Radical Scavenging Activity of Glyceollins. In the superoxide and $ABTS^+$ scavenging assay, the glyceollin mixture inhibited radical formation in a dose-dependent manner, and the efficacy was just a little lower than Trolox, a well-known antioxidant (**Figure 4**). The IC_{50} value of the glyceollin mixture for $ABTS^+$ scavenging was found to be 18.7 μg/mL. This high $ABTS^+$ scavenging activity of the glyceollin sample may ascribe to minor contaminated compounds as well as glyceollins in the glyceollin-rich fraction.

Effect of Glyceollins on Lipid Peroxidation. The lipid peroxidation suppressing activity of glyceollins was shown in **Figure 5**. The glyceollins significantly reduced the accumulation of lipid peroxides in a dose-dependent manner. As observed in model systems, glyceollins also showed an inhibitory effect on lipid peroxidation in the model system using tissue extracts of brain (48.9%), kidney (33.9%), and liver (31.8%) at the concentration of 150 μg/mL (**Figure 5**), and the effect was also found to be dose-dependent. The data showed that the inhibitory activity of glyceollins on lipid peroxidation in the kidney was similar to that in the brain.

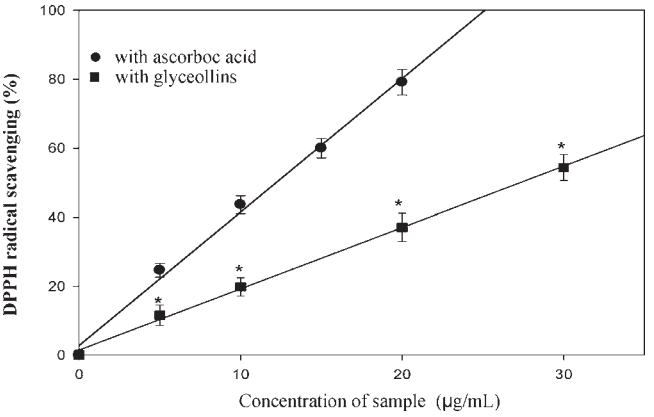

Figure 3. Dose-dependent DPPH radical scavenging activity of glyceollins. DPPH in methanol was incubated with glyceollins (■) or ascorbic acid (●) at 25 °C for 30 min, and absorbance was measured at 515 nm. *, $p < 0.05$, compared to the control.

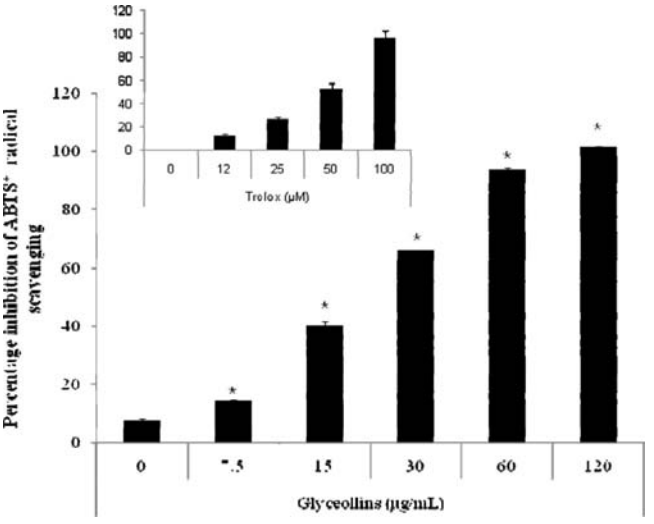

Figure 4. $ABTS^+$ scavenging activity of glyceollins. As positive control, trolox was used. The results are expressed as means ± SD of four separate experiments. *, $p < 0.05$, compared to the control.

Hydroxyl Radical Scavenging Activity of Glyceollins. The scavenging activity by glyceollins of the hydroxyl radical generated in the Fe^{3+}/ascorbic acid system was examined. As shown in **Figure 6**, glyceollins exhibited limited hydroxyl radical scavenging activities, with 11.3% and 16.0% at at 150 and 300 μg/mL, respectively.

Singlet Oxygen Quenching Activity of Glyceollins. The $^1O_2$ quenching effects of various extracts on RNO−imidazole bleaching as a result of the reaction of imidazole with $^1O_2$ produced by RB in irradiation are shown in **Figure 7**. The RNO-mediated bleaching was decreased in inverse proportion to the glyceollin concentrations in the photolysis system. The ratio of decrease on RNO bleaching was expressed as quenching efficacy (IC_{50} = 25 μg/mL). These results suggest that glyceollins are potent singlet oxygen quenchers at very low concentration in an aqueous system, even more effective than ascorbic acid (IC_{50} = 129 μg/mL).

Suppression of Intracellular ROS Generation by Glyceollins. In order to further elucidate the protective effect of glyceollins in the mammalian cells, we determined the effect on ROS production using the oxidant-sensitive fluorescent dye DCFDA and DHE. As visualized by DCFDA staining, intracellular ROS generation induced by H_2O_2 treatment was significantly suppressed by

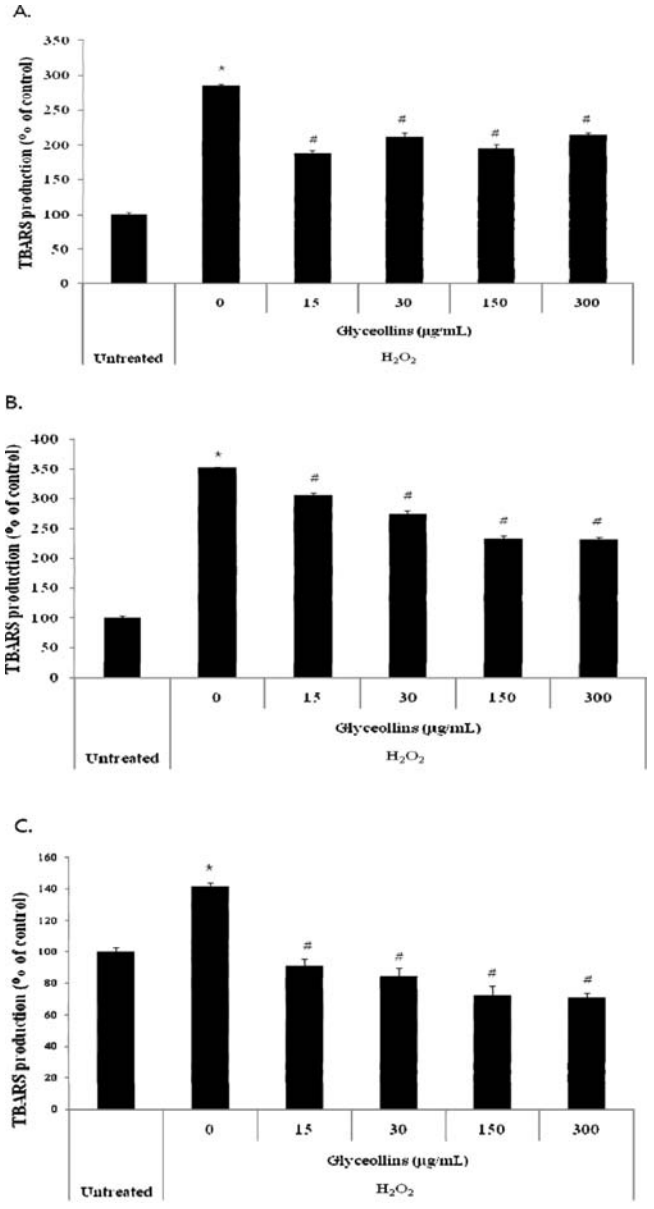

Figure 5. Lipid peroxidation inhibition by glyceollins in liver (**A**), kidney (**B**), and brain (**C**) tissue extracts. The results are expressed as means ± SD of four separate experiments. *, $p < 0.05$, compared to the untreated as control; #, $p < 0.05$, compared to the H_2O_2 treatment.

addition of the glyceollins (**Figure 8B**). Treatment with glyceollins alone showed little ROS production while hydrogen peroxide significantly stimulated ROS generation. Inhibition of superoxide anion formation is also observed in the H_2O_2-treated cells by coexistence of glyceollins at the concentration of 3 μg/mL in the reaction (**Figure 8B**, d). Contrary to the cells treated with H_2O_2 that showed strong red fluorescence by DHE staining (**Figure 8A**), the cells treated with H_2O_2 plus glyceollins (3 μg/mL) exhibited weak fluorescence by DHE staining, again confirming the antioxidant activity of glyceollins in the cell-based system.

DISCUSSION

Oxidative stress has been implicated in numerous chronic diseases such as cancer, heart disease, diabetes, and others. Natural antioxidants, therefore, have been considered as valuable food components in preventing chronic diseases. For instance, phenolic compounds in wine have been reported to protect LDL

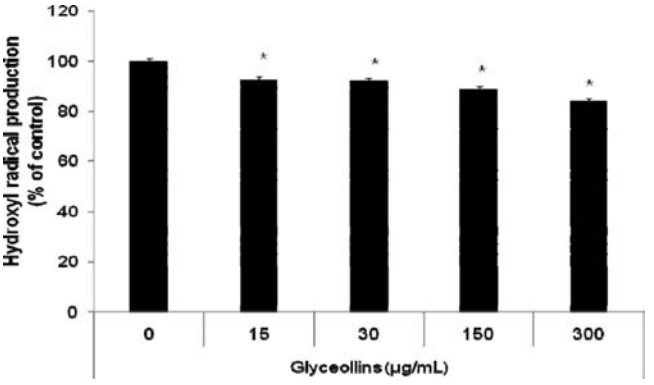

Figure 6. Hydroxyl radical scavenging activity of glyceollins. The results are expressed as means ± SD of four separate experiments. *, $p < 0.05$, compared to the control.

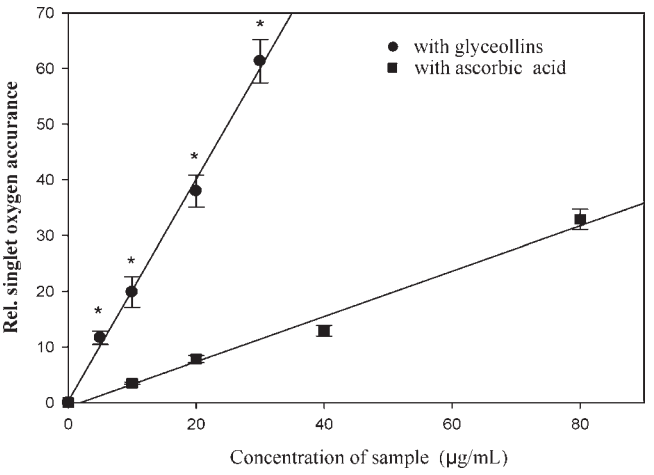

Figure 7. Kinetics of singlet oxygen quenching capacity of glyceollins (●) and ascorbic acid (■). Data are representative of four independent experiments as means ± SD. *, $p < 0.05$, compared to the control.

oxidation and thereby contribute to prevention of cardiovascular disease in wine drinkers (*24*). The term antioxidant can be defined in multiple ways depending on the methods employed to measure antioxidative activity, and therefore, the hierarchy of antioxidant activity will change depending upon assay methods (*25*). In this study, we evaluated the antioxidant activity of the mixture of glyceollin isomers I, II, and III (glyceollins) using as many methods as possible to improve the reliability of antioxidant activity of the sample.

Glyceollins have been reported to be de novo synthesized in raw soybean exposed to some biotic elicitors such as fungi. The compounds were known to show antifungal and antidiabetic effects as well as antiestrogenic and anticancer activities (*4*−*7*). As soybean isoflavones were reported to have antioxidant activity, there is a good possibility that glyceollins, prenylated isoflavones derived from daidzein, also show antioxidant effect. This study clearly demonstrated that the mixture of glyceollin isomers had strong antioxidant potential in several assay systems such as radical (ABTS and DPPH) scavenging activity, ferric ion (Fe^{3+}) reduction potential to ferrous ion (Fe^{2+}) (FRAP), inhibitory activity against singlet oxygen formation, and inhibitory activity against TBARS production in animal tissue challenged by hydrogen peroxide. We also speculate that glyceollin I is mainly responsible for the antioxidant activity of the glyceollin mixture because it accounts for 84% of the mixture. Recent studies also suggested that glyceollin isomer I had stronger physiological

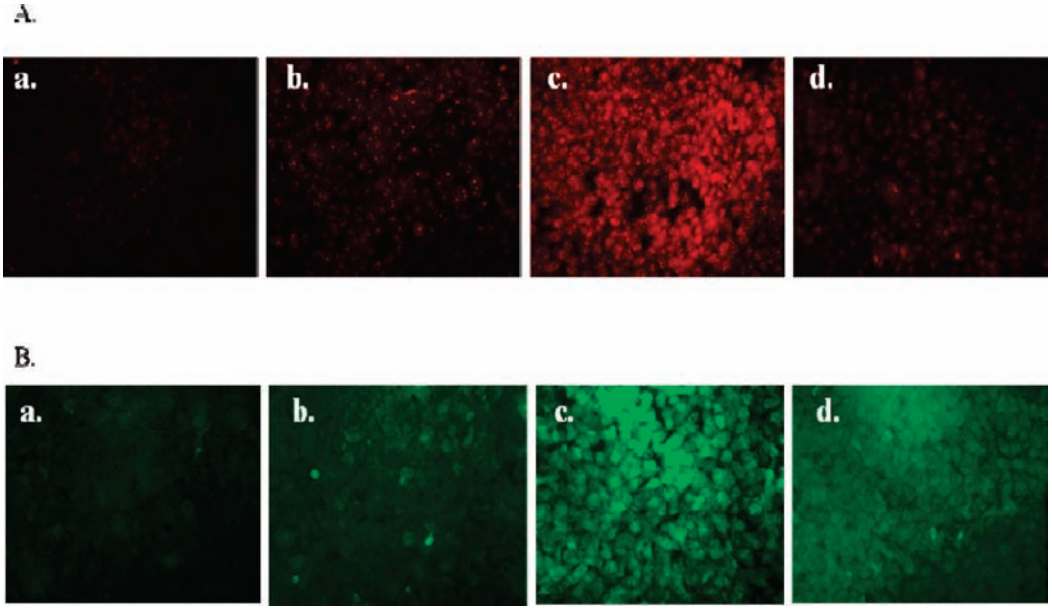

Figure 8. Inhibition of H_2O_2-induced ROS production in hepa1c1c7 cells by glyceollins. (**A**, **B**) Fluorescence micrographs of murine hepatoma cell line (hepa1c1c7 cells) stained with DHE (red) (**A**) and DCFDA (green) (**B**): a, control; b, H_2O_2 (500 μM); c, glyceollins (3 μg/mL); d, H_2O_2 (500 μM) plus glyceollins (3 μg/mL). Magnification 40×.

activity than isomers II and III (*26*, *27*). Furthermore, the antioxidant activity of glyceollins was found to be greater than its parent compound daidzein and even genistein at same concentrations (data not shown).

We have also compared the isoflavone profile in noninoculated soybean with inoculated with *A. sojae*. As expected, treatment of presoaked soybean with *A. sojae* increased the glyceollin precursor isoflavone content such as daidzein by 3.2-fold compared with noninoculated soybean, and total glyceollin isomers were also found to be highly elevated in the soybean sample inoculated with *A. sojae* (4 mg/g) (*6*). Additionally, to test the difference of the antioxidant properties between noninoculated and inoculated crude extracts, we have measured total phenol contents and conducted FRAP assay (data not shown). In accordance with the result of the total glyceollin contents, the phenol contents and ferric-reducing antioxidant power were higher in the extract of soybean treated with fungi than untreated soybean extract (data not shown). Therefore, the increased antioxidant capacity in biotic elicitor-treated soybean might be due to the increased levels of glyceollins and free isoflavones.

As it is recommended to use at least two different types of assays to improve reliability of the antioxidant effect of the sample, we evaluated the antioxidant potential of glyceollins using a combination of assays for scavenging electron or radical, such as DPPH, ABTS, and FRAP, and the assays associated with lipid peroxidations (*28*). It is not rare that different methods produce variable antioxidant values for even the same compound because chemically distinct methods are based on different reaction mechanisms (*29*). Thus glyceollins showed very strong singlet oxygen scavenging activity but had negligible inhibitory activity against hydroxy radical production.

The 1,2-diphenylpropane structures as well as phenolic hydroxyl group of glyceollins appear to be one of the most important factors that influence the chain breaking antioxidant activity (*30*) while the role of prenyl substitution is unclear in antioxidant activity.

Although glyceollins showed strong antioxidant activity using in vitro model systems, it is not clear whether it exerts the same antioxidant effect in vivo or not because polyphenols are usually extensively metabolized in the body and their physiological activities should be altered significantly. Even so, there are a number of reports that antioxidant phytochemicals contribute to benefits of human health. In conclusion, the glyceollin-rich fraction showed strong antioxidant potential from various assay systems and needs further study to examine in vivo activity.

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