Antioxidant Activities of Soybean Fermented with *Aspergillus oryzae* BCC 3088

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ABSTRACT

The antioxidative activities of Thai native soybeans [Glycine max (L.) Merr. SJ2] fermented with Aspergillus oryzae BCC 3088 were investigated. Compared to naturally-fermented soybeans, the methanol extract of soybeans fermented with A. oryzae BCC 3088 was more effective in antioxidative activity by scavenging ability on 1,1-diphenyl-2-picrylhydrozyl (DPPH) radicals, inhibitory activities against linoleic acid peroxidation, Fenton reaction-induced breakage of DNA, and protein oxidation. The results suggest that the enhanced antioxidative activity of soybeans fermented with A. oryzae BCC 3088 observed in various antioxidative model systems could be related to the increased total phenolic and flavonoid contents, a significant bioconversion of the isoflavone glucosides (daidzin + genistin) into their corresponding bioactive aglycones (daidzein + genistein), and the formation of 8-hydroxygenistein (8-OHG). Fermentation of soybeans with A. oryzae BCC 3088 results in higher levels of isoflavone aglycones, which may enhance health benefits over naturally fermented soybeans.

Keywords: Fermented soybeans, *Aspergillus oryzae*, Isoflavones, Antioxidative activity

INTRODUCTION

Reactive oxygen species (ROS) formed in food systems and the human body not only induce oxidative stress that causes deterioration of foods but also causes oxidative damage to biomolecules, which are thought to be an important etiologic factor in carcinogenesis, formation of atherosclerotic plaques, aging, and development of chronic diseases (Steinberg, 1991; Jang et al., 1997; Moktan et al., 2008). Oxidative stress occurs when the formation of highly ROS increases, or when scavenging of ROS or repairing of oxidatively-modified molecules

decreases. Under normal physiological conditions, ROS can be scavenged by the cellular-defending systems. However, the dynamic balance between the generation and elimination of ROS may be disturbed under certain pathological conditions leading to cellular damage and death.

Dietary antioxidants have gained much interest due to their protective properties against free radicals that are known to be responsible for oxidative damage to living systems. Among various sources of natural antioxidants, soybean supplements have been developed because of several naturally occuring phenolic compounds and flavonoids, especially isoflavone (Hanasaki et al., 1994). Considerable evidence for a variety of health benefits associated with the consumption of cultured soy products has been reported (Lin and Yen, 1999; Marinova et al., 2005). Consequently, the intake of fermented soybean-derived antioxidants with free radical-neutralizing ability may be of importance in the prevention of some diseases, reducing oxidative damage, and providing benefits to human health (Cassidy, 1996).

Due to their broader nutrient profile and high bioavailability, traditional fermented soy foods are considered to have more health-promoting benefits. As a consequence of fermentation, soybeans are modified by microorganisms that not only grow and consume part of the substrate but also enrich them with the byproducts of their metabolism. In particular, fermentation with some GRAS microorganisms, especially filamentous fungi and bacilli, significantly increased the antioxidant concentration in fermented soybean products (Berghofer et al., 1998) such as miso, natto, tempeh and koji. Therefore, fermentation with an appropriate microorganism might be an important means in the development of high antioxidative soy products, contributing to overall disease prevention and enhancement of well-being.

A. oryzae is one of the most common fungi usually inoculated into the solid culture of steamed soybean, rice, or barley in koji preparation and then used to prepare traditional fermented food products (Chia et al., 2006). Besides producing an abundance of hydrolytic enzymes suitable for various industrial applications, *A. oryzae* has been employed extensively for the production of antioxidant compounds from soybeans (Wardhani et al., 2009, 2010). Nevertheless, discrepancies in the ability to increase antioxidative activity could be observed among various strains of *A. oryzae* used in previous studies. Therefore, the objective of this study was to investigate the antioxidant activity of soybean fermented with *A. oryzae* BCC 3088, previously identified as the strain with the highest ability to increase $ABTS^{\circ+}$ scavenging activity and ferric reducing ability (Punjaisee et al., 2011), and to identify the major components responsible for this increased antioxidative capacity after fermentation.

MATERIALS AND METHODS

Chemicals and reagents

2,2-Diphenyl-picrylhydrazyl (DPPH), linoleic acid, 2-thiobarbituric acid (TBA), ethylenediaminetetraacetic acid (EDTA), potassium phosphate, potas-

sium ferricyanide, linoleic acid, ferric chloride, gallic acid, catechin, and Folin Ciocalteu's phenol reagent were purchased from Sigma Chemical (St Louis, MO, USA). Hydrogen peroxide (H_2O_2, 30%, v/v) was obtained from Aldrich Chemical. (Milwaukee, WI, USA). 1,1,3,3-Tetraethoxypropane was purchased from Carlo Erba (Strada, Rodano, Italy). All other solvents and chemicals used were of analytical grade. Authentic standards of daidzin, genistin, daidzein, and genistein were purchased from Sigma Chemical (St Louis, MO, USA). 8-hydroxydaidzein (8-OHD) and 8-hydroxygenistein (8-OHG) were obtained from Plantech Chemical (Reading, Berkshire, England).

Preparation of microorganism

A. oryzae BCC 3088 was obtained from the BIOTEC-Culture Collection, National Center for Genetic Engineering and Biotechnology, Thailand. This microorganism was isolated from fermented soy sauce. The freeze-dried culture was rehydrated with 1 mL of sterile distilled water. A few drops of cell suspension were inoculated onto potato dextrose agar (PDA) from Difco (Franklin Lakes, MD, USA) and incubated at 37°C for 3 days. The growing colonies were transferred to the new PDA plate and incubated at 37°C for 5 days. Spores of the fungi were harvested by flooding the surface of the agar with sterile distilled water and aseptically filtered through three layers of sterile gauze. The turbidity of spore suspension was adjusted to 0.5 McFarland unit (Pfaller et al., 1995) and used as inoculum for the fermentation of soybeans.

Preparation of fermented soybeans

Soybeans [*Glycine max* (L) Merr SJ2] were obtained from Limsakdakun Co. Ltd. (Chiang Mai, Thailand). Whole soybeans were washed, soaked in water for 12 h and then autoclaved at 121°C for 30 min. After cooling, the cooked soybeans were inoculated with the spore suspension of *A. oryzae* BCC 3088 at the level of 1×10^6 spores per gram of cooked soybeans. The samples were then incubated at 30°C. Samples were collected at 24-h intervals for 4 days. The samples were immediately ground into powder in liquid nitrogen, using a blender (Model BBL550XL, Hawaii, USA). The samples were stored at -20°C until use. To prepare methanol extract, powderized sample (1 g) was extracted in 5 mL of methanol with shaking at 60 rpm in a water bath for 12 h at 37°C. The fermented soybean extracts were recovered by centrifugation, using a centrifuge model JE 25 (Beckman Coulter, Inc., CA, USA) at 12,000 Xg at 4°C for 15 min. The methanol extract was vacuum-concentrated at 40°C and dried by a freeze-dryer to dryness.

Measurement of DPPH radical-scavenging activity

The scavenging activity for the DPPH radical was determined, using the method of Velazquez et al. (2003). Varying amounts of fermented soybeans extract were dissolved in 0.1 mL of methanol and mixed with 2.9 mL of 0.1 mM DPPH in 80% methanol. The mixtures were left in the dark for 30 min at room temperature and the absorbance was then measured at 517 nm. The inhibitory

percentage of DPPH was calculated according to the following equation:

$$\text{Scavenging effect (\%)} = [1 - ((A_{sample} - A_{blank})/ A_{control})] \text{ X } 100\%$$

where, A_{sample} was the A_{517} of sample and $DPPH^{\bullet}$, A_{blank} was the A_{517} of sample without $DPPH^{\bullet}$, and $A_{control}$ was the A_{517} of $DPPH^{\bullet}$ without sample. The effective concentration at which 50% of the DPPH radicals were scavenged or EC_{50} value was obtained by interpolation from linear regression analysis from the plot of scavenging activity against the concentration of sample.

Lipid peroxidation inhibitory assay

Oxidation of linoleic acid was determined by modified method of Haraguchi et al. (1992). Different amounts of fermented soybean extract dissolved in 30 µL methanol were added to a reaction mixture, consisting of 0.57 mL of 2.51% linoleic acid in methanol and 2.25 mL of 40 mM phosphate (pH 7.0). After 5 days of incubation at 40°C, 0.2 mL of reaction mixture was taken and added with 1 mL of TBA solution containing 0.375% TBA, 15% TCA and 0.25 N HCl and boiled for 10 min. The mixture was centrifuged at 5,500 Xg for 25 min. The absorbance of the supernatant was measured at 532 nm, using a spectrophotometer UV-1601 (Shimadzu, Kyoto, Japan). The TBARS value was calculated from the standard curve of 1,1,3,3-tetraethoxypropane.

Plasmid relaxation assay

In vitro plasmid relaxation assay was determined by a modified version of the method of Ishikawa et al. (2004). DNA strand damages were measured by converting circular double-stranded supercoiled plasmid DNA into nicked circular and linear forms. Reactions were performed in 50 µL of solution containing 10 µL of supercoiled pUC18 plasmid DNA (750 ng), 10 µL of 10 mM phosphate (pH 7.8), 5 µL of 3.5% hydrogen peroxide, 5 µL of 100 mM ferric chloride and 20 µL of fermented soybean extracts at various concentrations (2.5, 5, 7.5 and 10 mg/mL). The mixtures were incubated at 37°C for 30 min and the reactions were stopped by adding 1 µL of 5 mM ethylenediaminetetraacetic acid (EDTA). Reaction mixtures (20 µL) were mixed with 5 µL of loading buffer containing 30% glycerol and 0.25% bromophenol blue. The mixture (15 µL) was loaded onto a 1% agarose gel. Electrophoresis was conducted using a constant voltage of 110V. The gel was stained using ethidium bromide solution for 10 min. The DNA bands were visualized under UV light and captured by a CCD camera (Genegenious, Singene, UK). DNA band intensity in digitized images was analyzed with the UTHSCSA Image Tool for Windows Version 3.0 software program (University of Texas Health Science Center, San Antonio, TX, USA).

Protein oxidation inhibition assay

Protein oxidation inhibition assay was determined by the method of Shacter (2000) with a slight modification. Reactions were performed in 85 µL of solution consisting of 25 µL of 2.5 mg/mL BSA, 10 µL of 1.5 mM $CuSO_4$, 10 µL of 37.5

mM H_2O_2 and 40 µL of fermented soybean extracts at various concentrations (0.25, 1.25, 2.5, 5.0 and 10.0 mg/mL). The mixtures were incubated at 37°C for 30 min, and the reactions were stopped by adding 85 µL of gel loading buffer, and then boiled for 3 min. Samples were subjected to electrophoresis (Laemmli, 1970). Proteins (5 µg) were loaded into the gel, producing 4% stacking and 12.5% separating gels, and then subjected to electrophoresis at a constant voltage of 75V, using a mini vertical Hoefer apparatus (Hoefer Scientific Instruments, San Francisco, CA, USA). After electrophoresis, the gels were fixed and stained with 0.125% Coomassie blue R-250 in 50% ethanol and 10% acetic acid and destained in 25% ethanol. Intensity of the protein bands in digitized images was analyzed with the UTHSCSA Image Tool for Windows Version 3.0 software program.

Total phenolic and flavonoid content assay

Total phenolic content was determined by the method of Marinova et al. (2005) using gallic acid as standard. One milliliter of fermented soybeans extract was added to 25-mL volumetric flask, containing 9 mL of deionized water. Folin-Ciocalteu's phenol reagent (1 mL) was added to the mixture and shaken. After 5 min, 10 mL of 7% Na_2CO_3 solution was added to the mixture. The solution was diluted to the final volume of 25 mL with distilled deionized water and mixed. After incubation for 90 min at room temperature, the absorbance against prepared reagent blank was measured at 750 nm. Total phenolic content of fermented soybeans was expressed as mg gallic acid equivalents (GAE)/g fermented soybeans.

Total flavonoid content was measured by the aluminum chloride colorimetric assay according to method described by Zhishen et al. (1999), using catechin as standard. One milliliter of fermented soybeans extract was added to 10-mL volumetric flask, containing 4 mL of distilled deionized water. To the flask was added 0.3 mL of 5% $NaNO_2$. After 5 min, 0.3 mL of 10% $AlCl_3$ was added. At the 6^{th} minute, 2 mL of 1 M NaOH was added and the total volume was increased to 10 mL with distilled deionized water. The solution was mixed well and the absorbance was measured against prepared reagent blank at 510 nm. Total flavonoid content of fermented soybeans was expressed as mg catechin equivalents (CE)/g fermented soybeans.

HPLC analysis for isoflavone compositions

In order to verify the presence of isoflavone composition, powderized sample was extracted with methanol as previously described and filtered through a 0.45 µm membrane (Millipore Co., Bedford, MA, USA) prior to analysis by HPLC (Griffith and Collison, 2001). Reversed phase HPLC analysis was carried out with Hewlett-Packard HP 1100 series equipped with an autosampler, DAD detector, and HP ChemStation Software (Scientific Equipment Source, Pickering, Canada), using a BSD Hypersil C-18 column (4.6 X 250 mm, 5 µm). For the analysis of isoflavones, the mobile phase was composed of solvent A (H_2O:methanol:acetic acid, 88:10:2, v/v) and solvent B (methanol:acetic acid, 98:2, v/v). Following the injection of 20 µL of sample, solvent A was increased from 90% to 100% over

20 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. The column temperature was controlled at 25°C. Quantitative data for daidzin, daidzein, genistin, genistein, 8-hydroxydaidzein (8-OHD), and 8-hydroxygenistein (8-OHG) were obtained from comparison with known standards.

Statistical analysis

All experiments were run in duplicate with triplicate determinations. Analysis of variance (ANOVA) and mean comparison were performed by Duncan's multiple range test (Steel and Torrie, 1980). Analysis was carried out using SPSS 11.0 for windows (SPSS Inc, Chicago, IL, USA).

RESULTS AND DISCUSSIONS

DPPH radical-scavenging activity

DPPH radical-scavenging activity of soybeans fermented with *A. oryzae* BCC 3088 was higher than that of soybeans naturally fermented (Figure 1) in which the EC50 values were 13.0 mg/mL and 22.5 mg/mL, respectively. Scavenging of DPPH free radical is the basis of a common antioxidant assay based on the reduction of 1,1-diphenyl-2-picrylhydrazyl (DPPH). As an odd electron becomes paired off in the presence of a hydrogen donor, the absorbance is decreased and the resulting decolorization is stoichiometric with respect to the number of electrons captured. The results show that soybeans fermented with *A. oryzae* BCC 3088 possibly contained substances that were more active as hydrogen donors and could react with free radicals to convert them to more stable products and terminate the radical chain reaction. Among naturally occurring antioxidative components in soybeans, antiradical activity was positively influenced by the phenolic compounds (Bors et al., 1990; Moktan et al., 2008). Along with previous reports on soybean koji fermented with other fungal strains, higher DPPH-scavenging effect would be a result of the ability of fungi to metabolize isoflavone precursors in soybeans to the more active isoflavone forms (Esaki et al., 1997; Chia et al., 2006). From a total of 21 fungal strains from 9 different genera tested, *Aspergillus* strains isolated from fermented soy foods, including five *A. oryzae* strains, one *A. sojae* strain, and one *A. tamarii* strain, were able to metabolize both daidzein and genistein to 8-hydroxydaidzein and 8-hydroxygenistein, respectively (Chang et al., 2007).

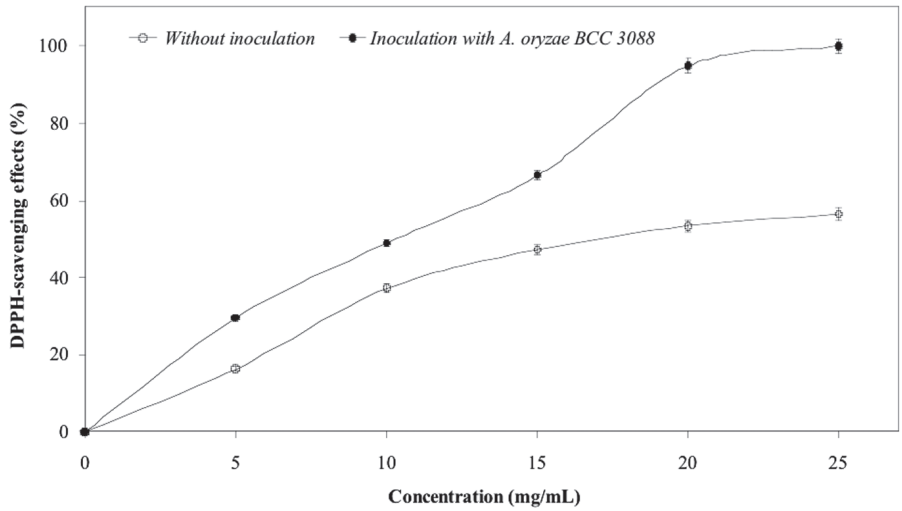

Figure 1. DPPH radical-scavenging activity of the methanol extract of soybeans fermented with and without inoculation of *A. oryzae* BCC 3088 at day 4 of fermentation.

Lipid peroxidation inhibitory assay

The soybeans fermented with *A. oryzae* BCC 3088 exhibited stronger inhibitory activity against linoleic acid peroxidation than soybeans naturally fermented (Figure 2), in which the EC50 values were 24.7 mg/mL and 45.5 mg/mL, respectively. The results conformed with Esaki et al. (1997) that fermented soybeans incubated with A. saitoi, which has been utilized for manufacturing, had the most antioxidative activity against lipid peroxidation. Lipid peroxidation leads to rapid development of rancid and stale flavors, and is considered as a primary mechanism of quality deterioration in lipid foods and oils (Güntensperger et al., 1998). Corresponding with DPPH-scavenging effect, the fermented soybeans with *A. oryzae* BCC 3088 was likely to contain substances that can function both as an antioxidant and as a free radical acceptor that can convert free radicals into harmless substances through an energy-decreasing procedure. This action is extensive and effective in eliminating free radicals ranging from the superoxide anion to H_2O_2 to lipid peroxide free radical, as well as having an antioxidant effect on unsaturated fatty acids and lipids. Previous research has shown that phenolic compounds formed, through different chemical mechanisms, including free radical quenching, electron transfer, and radical addition, are able to suppress lipid peroxidation (Mathew and Abraham, 2006). Rather than their mere presence, a synergism of various phenolic compounds and/or other components present in the extract may also contribute to the total antioxidant activity (Shahidi et al., 1994).

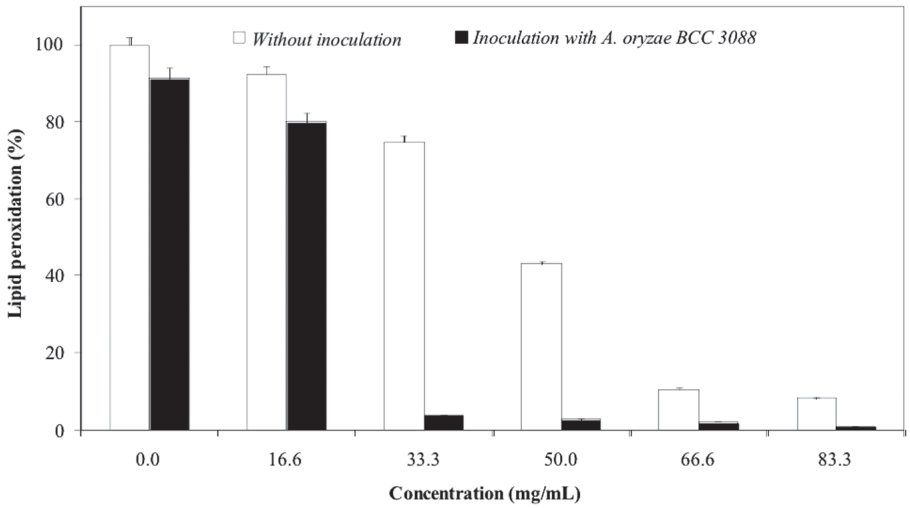

Figure 2. Comparison of the capacity of the methanol extracts of soybeans fermented with and without inoculation of *A. oryzae* BCC 3088 to inhibit the production of thiobarbituric acid reactive substances (TBARS).

Plasmid relaxation assay

Soybeans fermented with *A. oryzae* BCC 3088 showed higher antioxidative activity in inhibiting DNA relaxation than those naturally fermented without inoculation at any concentrations tested (Figures 3A and 3B). Based on the relative band intensity, treatment of DNA with fermented soybean extract of *A. oryzae* BCC 3088 significantly increased the concentration of supercoiled DNA retained after reaction in a dose-dependent manner ($P<0.05$). DNA strand breakage was induced in the presence of H_2O_2 and Fe^{2+} while DNA in the presence of H_2O_2 or Fe^{2+} alone did not show significant strand breakage. Induced oxidative damage in DNA through Fenton reaction is thought to arise via a site-specific mechanism, i.e., involving the interaction of a transition metal ion with DNA prior to its reaction with H_2O_2 to produce the damaged DNA species (Chevion, 1988). Apart from scavenging ability on hydroxyl radicals, this study implies that part of the antioxidant activity of the fermented soybeans extract might arise from their iron chelating ability.

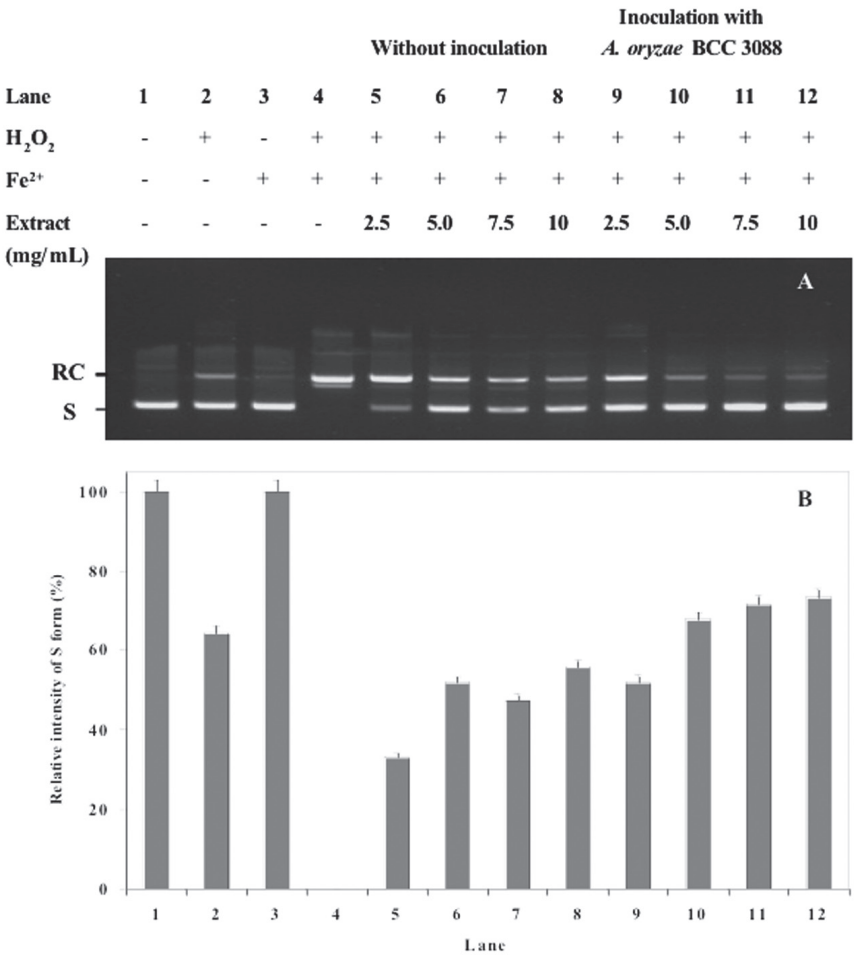

Figure 3. Inhibitory effect of the methanol extracts of soybeans fermented with and without inoculation of *A. oryzae* BCC 3088 on plasmid DNA relaxation in Fenton reaction system (A). RC and S represent relaxed circular and supercoiled forms of DNA, respectively. Relative band intensity of supercoiled DNA in comparison with total band intensity (RC+S) of DNA in the tested samples (B). The values, expressed as percentage, represent mean ± SD of triplicate measurements.

Protein oxidation inhibition assay

The dose-response inhibition of copper-induced protein oxidation of the methanol extracts from fermented soybeans incubated with *A. oryzae* BCC 3088 and the soybeans naturally fermented are shown in Figures 4A and 4B. Based on the percentage band intensity of BSA monomer (~67 kDA) retained after reaction, the extract of fermented soybeans inoculated with *A. oryzae* BCC 3088 showed higher antioxidative activity in inhibition than control group at any concentrations tested (Figure 4B). Oxidants such as H_2O_2 and various kinds of ROS

are implicated in mediating a wide array of damage to proteins (Shacter, 2000). Collectively, these ROS can lead to oxidation of amino acid residue side chains, formation of protein-protein cross-linkages, and oxidation of the protein backbone resulting in protein fragmentation (Berlett and Stadtman, 1997). Proteins highly influence the physical characteristics of foods and so oxidative changes of these biomolecules may have a significant effect on food integrity. According to Stagos et al. (2007), it is also speculated that phenolic compounds formed by the action of inoculated fungi may be a mechanism accounting for the protective activity of natural antioxidants against induced oxidation of proteins.

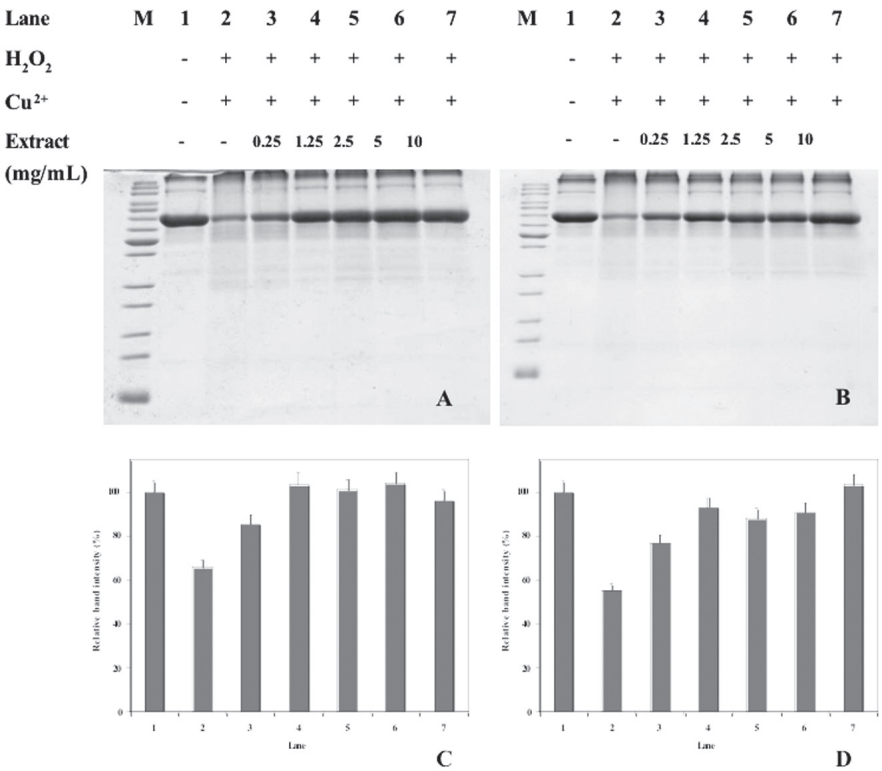

Figure 4. Protein oxidation inhibition assay of the methanol extracts of soybeans fermented with (A) and without (B) inoculation of *A. oryzae* BCC 3088. Relative band intensity of BSA monomer (~67 kDa) in the tested samples in comparison to that of control in the presence or absence of extracts of soybeans fermented with (C) and without (D) inoculation. The values represented as mean ± SD of triplicate measurements.

Total phenolic contents

The fermented soybeans incubated with *A. oryzae* BCC 3088 contained higher total phenolic contents than those fermented with no inoculation (Table 1). Total polyphenolic content increased dramatically during the 4-day fermentation. However, the larger increase was observed in the soybeans fermented with *A. oryzae* BCC 3088. Only correlation between total polyphenolic content and

DPPH-radicals scavenging activities was found to be statistically significant (P<0.01) among twenty soybean cultivars that were analyzed for their antioxidant activity and content of isoflavones, flavonoids, and total polyphenolics (Tepavcevic et al., 2009). Several studies revealed that phenolic compounds were responsible for a wide spectrum of biochemical activities such as antioxidant, antimutagenic, and anticarcinigenic as well as the ability to modify the gene expression (Bors and Saran, 1987; Lopes et al., 1999; Tapiero et al., 2002). The increased total phenolic content in soybean after fermentation is consistent with findings reported by Vettem and Shetty (2002), McCue and Shetty (2003) and Randhir et al. (2004). These studies suggested that β-glucosidase produced by fungi catalyzed the release of aglycones from the soybean substrate and thereby increased their phenolic content.

Total flavonoid contents

The total flavonoid content of soybeans fermented with *A. oryzae* BCC 3088 was higher than those naturally fermented without inoculation (P<0.05) (Table 1). The results corresponded with the increase in total phenolic contents after fermentation. Flavonoids represent the most common and widely-distributed group of water-soluble polyphenolic molecules which have the diphenylpropane (C_6-C_3-C_6) skeleton. The flavonoids exhibit a wide range of antioxidative effects as free radical scavengers, hydrogen-donating compounds, singlet oxygen quenchers, and metal ion chelators. Therefore, an increase in total flavonoid contents was positively correlated with the increased capacity in reducing power and DPPH radical-scavenging, the antioxidant activity in linoleic acid/water emulsion system and inhibition for lipid peroxidation by thiobarbituric acid reactive substances (Romero et al., 2004). The flavonoid family comprises 15 classes of compounds in which isoflavones are the predominant flavonoid in soybean seeds (Ho et al., 2002). Therefore, a larger increase in total flavonoid contents might be explained by the changes in isoflavone composition exerted by the activity of fungi during fermentation.

Table 1. Changes in total phenolic and flavonoid contents of soybeans fermented with and without inoculation of *A. oryzae* BCC 3088.

	Without inoculation		**Soybeans inoculated with *A. oryzae BCC* 3088**	
	Day 0	**Day 4**	**Day 0**	**Day 4**
Total phenolic (mg GAE/100g sample)	82.41 ± 0.001^{aA}	152.03 ± 0.002^{bB}	91.47 ± 0.001^{dC}	398.83 ± 0.002^{fD}
Total flavonoid (mg CE/100g sample)	15.72 ± 0.001^{bE}	30.20 ± 0.002^{cF}	19.36 ± 0.004^{eG}	90.36 ± 0.012^{dC}

Note: Means with different small letters in the same column and capital letters in the same row indicated significant differences (P<0.05) between treatments.

Isoflavone composition

The primary isoflavones in soybeans are daidzein, genistein, and their respective β-glycosides, daidzin and genistin, which are known to be the anti-oxidative components (Table 2). Most of the soy products have a total isoflavone concentration of 1-3 mg/g in which the isoflavones appear mostly as glycoside conjugates (Esaki et al., 1999a). After fermentation, total glucosides content of soybeans fermented with *A. oryzae* BCC 3088 decreased to about 2.9 fold, but the proportion of aglycone in total isoflavone fermented with and without *A. oryzae* BCC 3088 markedly increased about 10 and 3.4 fold, respectively (Table 2). Aglycone concentration was remarkably higher in soybeans fermented with *A. oryzae* BCC 3088 whereas glycoside concentration decreased significantly after 4 days of fermentation (Table 2). As for the increases of aglycone isoflavone during the fermentation, the proportion of aglycones in total isoflavones was markedly higher in soybeans fermented with *A. oryzae* BCC 3088 than those from uninoculated soybeans. The liberation of lipophilic aglycones of isoflavone glucosides such as daidzein and genistein by the catalytic action of β-glucosidase during fermentation resulted in the increased aglycone isoflavones and antioxidative activity of miso and tempeh (Esaki et al., 1994). The aglycones are present in the soybean seeds in small quantities, varying from 1 to 3% of the total isoflavones (Góes-Favoni et al., 2010). A higher content of aglycones might be a result of the action of β-glucosidase (β-D-glycoside glycohydrolase, EC 3.2.1.21), endogenous in soy (Matsura et al., 1995) and the associated β-glucosidase of the fermenting microbes (Kaya et al., 2008), which promote the hydrolysis of the β-glucoside conjugates, converting them to aglycones. In consideration of the possible radical scavenging activity of fermented soybean, isoflavone aglycones and the formation of *o*-dihydroxyisoflavones, especially 8-OHG, are considered responsible for the overall increased antioxidant properties. Being respectively liberated from genistin by β-glucosidase, 8-OHG was formed from genistein by microbial hydroxylation ((Esaki et al., 1999(a), (b); Chang et al., 2007). These isoflavones exhibited significantly stronger antioxidative activity than daidzein and genistein in both oil and lipid/aqueous systems.

Table 2. Changes in isoflavone content of soybeans fermented with and without inoculation of *A. oryzae* BCC 3088.

Amount (mg/100g sample)	Without inoculation		Soybeans inoculated with A. oryzae BCC 3088	
	Day 0	Day 4	Day 0	Day 4
Daidzin	149.9 ± 0.045^{dB}	85.3 ± 0.032^{bD}	119.0 ± 0.026^{cD}	37.0 ± 0.002^{aB}
Genistin	148.9 ± 0.041^{cB}	100.1 ± 0.044^{bE}	106.0 ± 0.011^{bC}	41.0 ± 0.004^{aC}
Daidzein	8.4 ± 0.005^{bA}	48.6 ± 0.001^{cB}	7.5 ± 0.00^{aA}	85.0 ± 0.002^{dF}
Genistein	12.8 ± 0.003^{bA}	24.2 ± 0.001^{cA}	7.4 ± 0.002^{aA}	66.0 ± 0.018^{dD}
8-hydroxygenistein	ND	ND	ND	0.048 ± 0.001^{A}
Total glucosides	298.8 ± 0.087^{dC}	185.4 ± 0.076^{bF}	225.2 ± 0.011^{cE}	78.0 ± 0.006^{aE}
Total aglycone	21.2 ± 0.001^{bA}	72.8 ± 0.003^{cC}	14.9 ± 0.001^{aB}	150.4 ± 0.016^{dG}
Total isoflavone	320.0 ± 0.088^{cD}	258.2 ± 0.079^{bG}	239.9 ± 0.010^{aF}	228.3 ± 0.010^{aH}

Note: Mean ± SD (standard deviation) from three determinations of two separate experiments. Different letters (a,b,c,d) in the same row and (A,B,C,...) in the same column denote significant differences ($P<0.05$) between treatments. ND: Not detected.

CONCLUSIONS

The present study suggests that *A. oryzae* BCC 3088 was a suitable fermenting strain to promote the antioxidant and free radical scavenging activities of soybeans. The results suggested that the increased total polyphenol content and aglycone isoflavone during fermentation mainly contributed to the enhanced antioxidant activity of soybean fermented with *A. oryzae* BCC 3088 inoculation. A significant bioconversion of the isoflavone glucosides (daidzin + genistin) into their corresponding bioactive aglycones (daidzein + genistein) in soybeans during fermentation was observed. However, it should be noted that the other antioxidants such as chlorogenic acid, caffeic acid, carotenoids, tri-terpenoids, saponin, melanoidin (a product from Maillard reaction during fermentation) and might also contribute to the increased antioxidant effects.

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