Screening of Potential *Aspergillus* spp. for Production of Fermented Soybean with High Antioxidative Activity

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ABSTRACT

Thirty two strains of Aspergillus spp. were screened by their ability to increase antioxidative capacity of fermented soybean broth. Among all strains tested, soybean inoculated with A. oryzae BCC 3088 exhibited the highest ABTS$^{\cdot+}$ scavenging activity, followed by those inoculated with A. terricola BCC 3026, A. ornatus BCC 3101 and A. oryzae BCC 3083, respectively. The similar results were observed when these strains were inoculated in solid-state fermentation of soybeans as shown by both ABTS$^{\cdot+}$ scavenging activity and ferric reducing ability power (FRAP). Analysis of aglycone isoflavones in fermented soybeans after fermentation suggested that the proportion of aglycones in total isoflavones was highest in soybeans inoculated with A. oryzae BCC 3088, followed by A. terricola BCC 3026, A. ornatus BCC 3101, A. oryzae BCC 3083 and non-inoculated fermented soybeans, respectively. Assay of ß-glucosidase activity indicated that the high ß-glucosidase activity was related to the high antioxidative activity which was a culture- dependent characteristic of starter organisms. The results indicated the potential of A. oryzae BCC 3088 for production of fermented soybean with high antioxidative activity.

Key words: Fermented soybeans, *Aspergillus*, β-Glucosidase, Antioxidative activity, ABTS radical- scavenging activity, Ferric reducing ability power, Isoflavone substances

INTRODUCTION

Dietary antioxidants have gained much interest due to the preventive effects from free radicals that are known to be responsible for an oxidative damage to the living systems. Amongst various sources of natural antioxidants, the supplements from soybeans have been developed as a result of several naturally-occuring phenolic compounds and flavonoids, especially isoflavone (Hanasaki et al., 1994). Considerable evidences for a variety of health benefits associated with the consumption of cultured soy products have 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 for the prevention of oxidative damages and has a corresponding beneficial effect on human health (Steinberg, 1991; Jang et al., 1997). Therefore, the development of high antioxidative soybean products might play an important role in overall disease prevention and enhancement of well-being (Cassidy, 1996).

A filamentous fungus, *Aspergillus oryzae*, is the key organism in the production of several traditional foods. Its solid-state cultivation (SSC) has been confirmed to be the secret for the high productivity of secretory hydrolases, vital for the fermentation process and for effective degradation of raw materials. In oriental countries, *Aspergillus* spp. are usually inoculated into the solid culture of steamed soybean, rice or barley in koji preparation of various traditional fermented food products. In addition, fermentation with some strains of *Aspergillus oryzae* has been known to produce antioxidative substances. For example, the antioxidative activity of fermented soybean products such as miso inoculated with *Aspergillus oryzae* was significantly higher than in non-fermented steamed soybeans (Esaki et al., 1999a). Therefore, the objective of this study was to select *Aspergillus* strain displaying the highest ability to increase $ABTS^{\cdot+}$ scavenging activity and ferric reducing ability power. Besides, the amounts of potential antioxidative substances, isoflavones aglycones (daidzein and genistein), were also investigated.

MATERIALS AND METHODS

Chemicals and reagents

2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), *p*-nitrophenyl-β–D-glucopyranoside (*p*NPG), and *p*-nitrophenol were purchased from Wako Pure Chemical Industries, Osaka, Japan. Ferrous sulphate ($FeSO_4$) was purchased from Carlo Erba, Italy. Authentic standards of daidzin, genistin, daidzein and genistein were purchased from Sigma Chemical (St. Louis, MO, USA). HPLC-grade methanol was purchased from Fisher Scientific (UK). All other chemicals were of analytical grade.

Preparation of microorganisms

Thirty-two pure isolates of *Aspergillus* spp. were obtained from the BIOTEC Culture Collection, National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand. All these microorganisms were isolated

from fermented food products such as soy sauce, miso, koji and Japanese sake (Table 1). The freeze-dried culture was rehydrated with 1 mL of sterile distilled water. 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 the 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.

Table 1. Aspergillus strains used in the screening test

***Aspergillus* spp.**	**Strains**	**Sources**
A. oryzae	BCC 3103	Soy sauce
A. oryzae	BCC 3083	Koji
A. oryzae	BCC 3373	Soy sauce, Miso
A. oryzae	BCC 3088	Koji
A. oryzae	BCC 3087	Koji
A. oryzae	BCC 3048	Fermented soybean
A. oryzae	BCC 3102	Soy sauce
A. oryzae	BCC 13295	Koji
A. oryzae	BCC 17102	Unknown
A. oryzae	BCC 17103	Unknown
A. oryzae	BCC 17104	Unknown
A. oryzae	BCC 14613	Unknown
A. oryzae	BCC 14615	Sake koji
A. oryzae	BCC 14616	Koji
A. oryzae	BCC 6128	Koji
A. oryzae	BCC 7238	Unknown
A. oryzae	BCC 7051	Unknown
A. sojae	BCC 3037	Unknown
A. niger	BCC 3344	Soy sauce
A. niger	BCC 3025	Koji
A. terricola	BCC 3026	Fermented soybean
A. flavas	BCC 3041	Koji
A. ornatus	BCC 3101	Koji
A. awamori	BCC 13292	Koji
A. kawachii	BCC 13291	Unknown
A. japonicus	BCC 18313	Unknown
Aspergillus sp.	BCC 17548	Koji
Aspergillus sp.	BCC 17549	Koji
Aspergillus sp.	BCC 17550	Koji
Aspergillus sp.	BCC 17551	Koji
Aspergillus sp.	BCC 17552	Koji
Aspergillus sp.	BCC 17553	Koji

Preparation of soybean broth

Soybeans [*Glycine max* (L) Merr SJ2] were obtained from Limsakdakun Co. Ltd. (Chiang Mai, Thailand). Whole soybeans were ground into powder by a blender (Tomex model A328, Thailand), mixed with 1 L of distilled water and then autoclaved at 121˚C for 30 min (Hirayama model HVA-85/110, Japan). After cooling at the room temperature, the supernatant of autoclaved soybean mixture was recovered by centrifugation, using a centrifuge model JE 25 (Beckman Coulter, Inc., CA, USA) at 12,000 xg at 4˚C for 15 min and referred to as soybean broth. Soybean broth (5 mL) was inoculated with 1 mL of spore suspension with concentration of $1x10^6$ spores/mL and incubated at 30˚C for 4 days in an incubator shaker (Innova model 4100, Germany). The culture filtrates were centrifuged at 12,000 xg at 4˚C for 15 min. The supernatant was recovered and used for analysis.

Solid state fermentation of soybeans

Whole soybeans were washed, soaked in water for 12 h and then autoclaved at 121˚C for 30 min. After cooling, the autoclaved soybeans were inoculated with the spore suspension of selected strains of fungi at the level of $1x10^6$ spores/g of cooked soybeans. The inoculated soybeans were then incubated at 30˚C and sampled at 24 h interval up to 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, powdered 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 at 12,000 xg at 4˚C for 15 min. The methanol extract was vacuum-concentrated at 40˚C and dried to dryness by a freeze-dryer (Labconco Corporation, USA).

$ABTS^{\cdot+}$ scavenging activity assay

The ABTS radical cation ($ABTS^{\cdot+}$) scavenging activity was determined by the method of Roberta et al., (1998). $ABTS^{\cdot+}$ was produced by reacting ABTS stock solution (7 mM) in distilled water with 2.45 mM potassium persulfate. The mixture was allowed to stand in the dark at the room temperature for 12-16 h before use. The $ABTS^{\cdot+}$ solution was diluted with distilled water to the absorbance of 0.70-0.90 at 734 nm. In the tested reaction, fermented soybean broth, fermented soybean extract (20 µL) or standard (Trolox) were mixed with distilled water (80 µL) and 2 mL of $ABTS^{\cdot+}$ working solution. After 3 min incubation at the room temperature, absorbance was then measured at 734 nm. Scavenging effect on ABTS radical ability of fermented soybean broths and fermented soybean extracts were expressed as mg Trolox/ mL sample and mg Trolox/ g fermented soybeans, respectively.

Ferric reducing ability power (FRAP) assay

Ferric reducing ability power (FRAP) was determined by the method of Benzie and Strain (1996). FRAP reagent was prepared by mixing 300 mM acetate buffer pH 3.6 with 10 mM TPTZ and 20 mM ferric chloride. The mixture was mixed for 15 seconds and its absorbance was recorded at 593 nm. Fermented soybean extract or standard ($FeSO_4$) solution was added in freshly prepared FRAP reagent. After 4 min of mixing, absorbance was then measured at 593 nm. The change in absorbance ($\Delta A_{593\ nm} = A_{593\ nm\ after} - A_{593\ nm\ before}$) was calculated for each sample and related to $\Delta A_{593\ nm}$ of Fe^{2+} standard solution. The FRAP of fermented soybeans was expressed as mg $FeSO_4$/g fermented soybeans.

β-glucosidase activity assay

β-glucosidase activity was determined by the method of Esaki et al., (1999b) by using *p*-nitrophenyl-β–D-glucopyranoside (*p*-NPG) as a substrate. Fermented soybean powder was extracted with phosphate-citrate buffer pH 6.0 (1:5, w/v). Extraction was carried out by sonicating the mixture for 20 min at 4˚C. The fermented soybean extract was recovered by centrifugation at 12,000 xg at 4˚C for 15 min. The fermented soybean broth or fermented soybeans extract 0.5 mL was mixed with 2.0 mL of 1 mM *p*-NPG in a 0.1 M phosphate-citrate buffer (pH 6.0). The reaction mixture was incubated at 30˚C for 20 min in a water bath, then stopped reaction by adding 2.5 mL of 0.5 M sodium carbonate. The resulting *p*-nitrophenol was immediately monitored at 420 nm. One unit of β-glucosidase was defined as the amount of enzyme which liberated 1 μmol of *p*-nitrophenol per min with specified condition.

The protein content of fermented soybean broths and fermented soybean extracts was determined by the method of Lowry et al., (1951), using bovine serum albumin (BSA) as a standard.

HPLC analysis for isoflavone compositions

In order to verify the presence of isoflavone composition, powdered 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, 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). Scatter plot was performed to determine the correlation between $ABTS^{\cdot+}$ scavenging activity and β-glucosidase activity or total aglycones. Correlation coefficients of linear regression (R^2) were computed by using the Statistical Package for Social Science (SPSS for windows version 17.0: SPSS Inc.).

RESULTS AND DISCUSSION

Screening of *Aspergillus* strains in fermented soybean broth

Almost soybean broths inoculated with fungal inoculation exhibited stronger $ABTS^{\cdot+}$ scavenging activity than non-inoculated soybean broth (control) (Figure 1). However, the enhanced effect on antioxidative activity of fermented soybean broths varied depending on the strains of *Aspergillus* strains inoculated. Amongst 32 strains tested, the fermented soybean broth inoculated with A. *oryzae* BCC 3088 exhibited the highest antioxidative activity (0.48 TEAC/mL sample), followed by *A. terricola* BCC 3026 (0.46 TEAC/mL sample), *A. ornatus* BCC 3101 (0.45 TEAC/mL sample) and *A. oryzae* BCC 3083 (0.44 TEAC/mL sample), respectively. The ABTS radical scavenging assay is one of the popular indirect methods of determining the antioxidative capacity of compounds (Roberta et al., 1998). In the absence of antioxidants, the ABTS radical is rather stable, but it reacts energetically with an H atom donor and is converted into a non-colored form of ABTS. Therefore, these 4 *Aspergillus* strains were selected for further study.

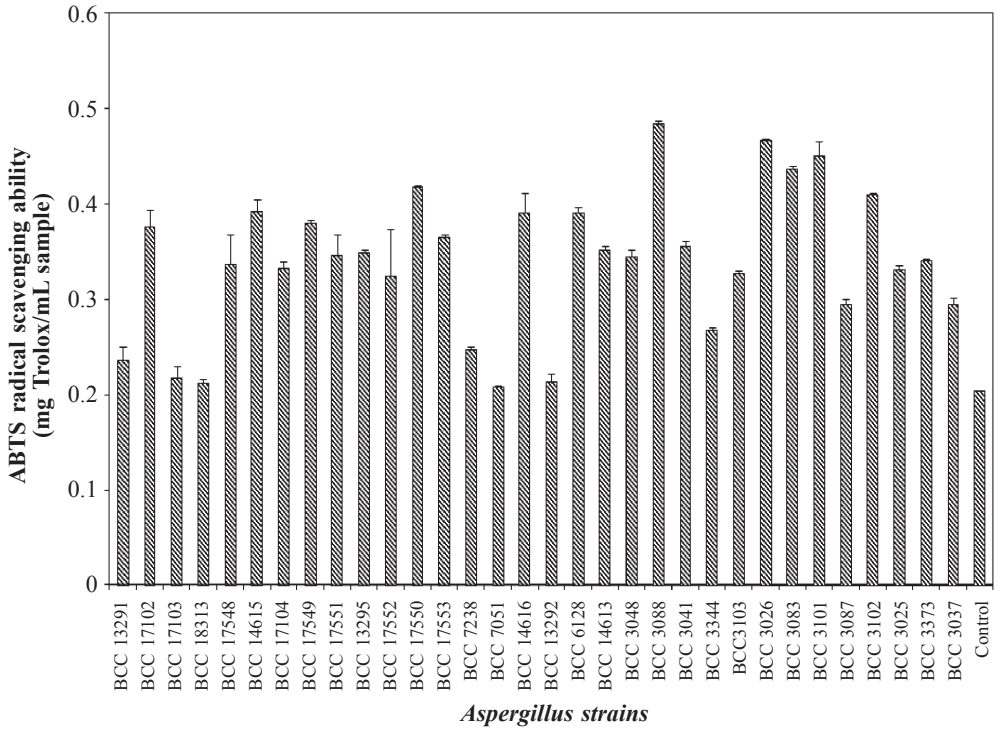

Figure 1. Antioxidative activity by scavenging effect on ABTS radical ability assay of fermented soybean broths with 32 strains of *Aspergillus*. Each fungal strain code represented in Table 1. A control contained only soybean broths without inoculation. The antioxidative activity of fermented soybean broths was measured at the fourth day of fermentation. Each value was the averages of 4 replicates.

Antioxidative activities of fermented cooked soybeans

The scavenging effects on $ABTS^{\cdot+}$ and FRAP of cooked soybeans fermented with 4 selected *Aspergillus* strains are shown in Figures 2a and 2b, respectively. Soybeans fermented with 4 selected *Aspergillus* strains exhibited stronger antioxidative activity than soybeans without inoculation (control). The results obtained were in agreement with those previously observed in fermented soybean broth. Furthermore, the fermented soybeans incubated with *A. oryzae* BCC 3088 at the fourth day possessed the highest antioxidative activity (1.59 TEAC/g fermented soybeans) among all strains selected from the fermented soybean broth.

FRAP of fermented soybeans inoculated with 4 selected *Aspergillus* strains at different times ranged from 0.097 and 0.650 mg $FeSO_4$/g fermented soybeans (Figure 2b). The fermented soybeans incubated with *A. oryzae* BCC 3088 at the fourth day possessed the highest FRAP (0.65 mg $FeSO_4$/g fermented soybeans) among all strains selected, followed by those inoculated with *A. terricola* BCC 3026, *A. ornatus* BCC 3101 and *A. oryzae* BCC 3083, respectively. The results were correlated with $ABTS^{\cdot+}$ scavenging activity of fermented soybeans. The FRAP assay is a method for assessing antioxidative activity based on reducing ferric to ferrous ion (Benzie and Strain, 1996). The samples reduced Fe^{3+}/tripyridyltriazine complex, present in stoichiometric excess, to the blue-colored ferrous complex form with an increase in absorbance at 593 nm. The results were in accordance with Santiago et al., (1992), Berghofer et al., (1998) and Chung et al., (2002) who reported that antioxidative activity of fermented soybean products could be enhanced through fermentation with certain microorganisms, especially the filamentous fungi such as *Aspergillus* and *Rhizopus*.

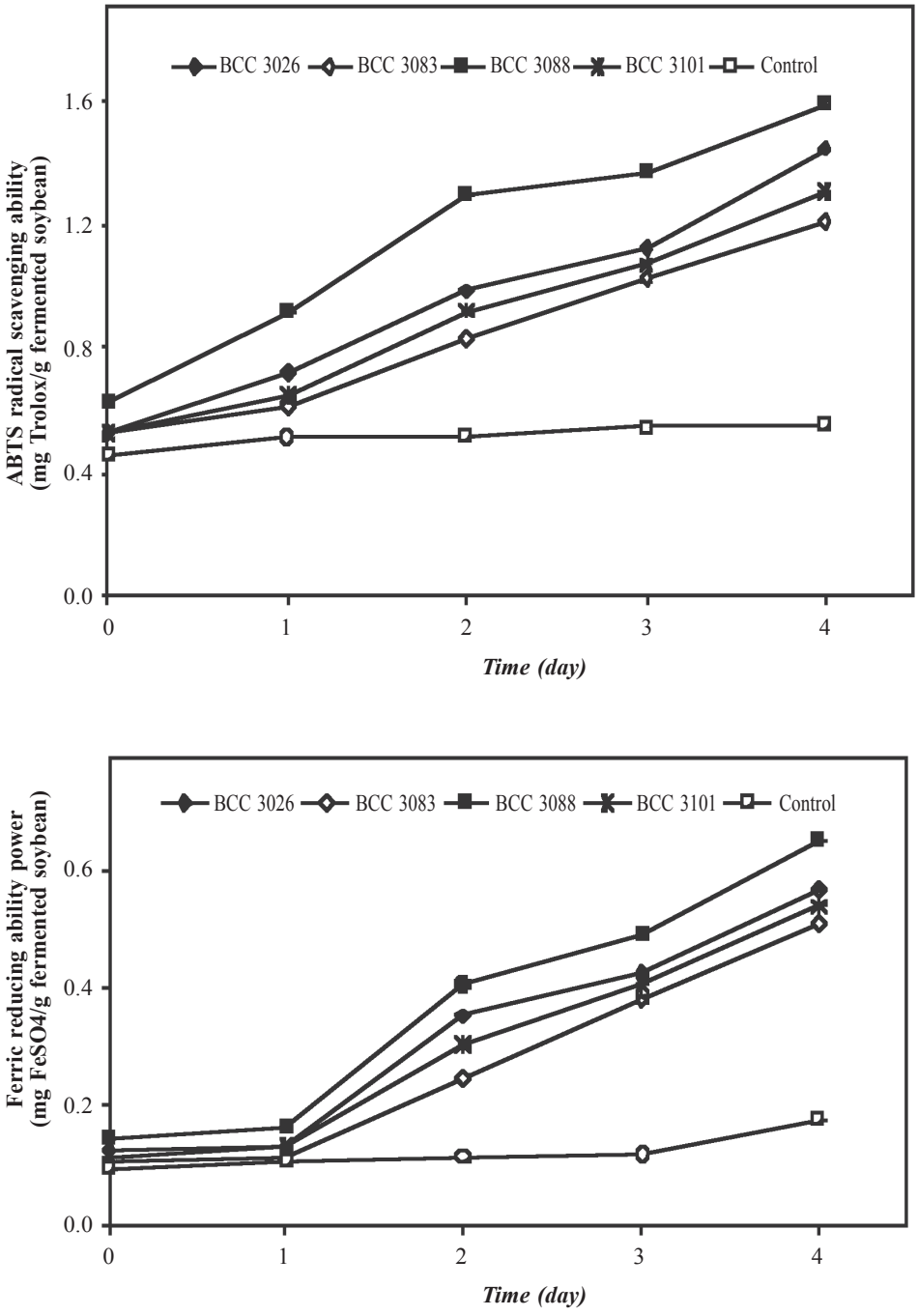

Figure 2. Antioxidative activity by scavenging effect on ABTS radical cation ($ABTS^{\cdot+}$) scavenging activity assay (A) and ferric reducing ability power assay (FRAP) (B) of fermented soybeans with 4 strains of *Aspergillus* during fermentation. A control contained only steamed soybeans without inoculation. Each value was the averages of 2 separate experiments.

Isoflavones content of fermented soybeans

The changes in isoflavones content of fermented soybeans inoculated with all selected fungal strains at day 0 and day 4 of fermentation are shown in Table 2. The primary isoflavones in soybeans are daidzein, genistein and their respective β-glycosides, daidzin and genistin. Almost soy products have a total isoflavone concentration of 1-3 mg/g in which the isoflavones appeared mostly as the glycoside conjugates (Góes-Favoni et al., 2010). After fermentation, aglycone concentration of soybeans fermented with *A. oryzae* BCC 3088 became remarkably high whereas glycoside concentration decreased significantly after 4 days of fermentation (Table 2). As for the increases of isoflavone aglycone during the fermentation, the proportion of aglycones in total isoflavones was highest in soybeans fermented with *A. oryzae* BCC 3088, followed by those inoculated with *A. terricola* BCC 3026, *A. ornatus* BCC 3101, *A. oryzae* BCC 3083 and non-inoculated fermented soybeans, respectively. In consideration of the possible free radical scavenging activity of fermented soybean, isoflavone aglycones are considered to be responsible for the overall increased antioxidant properties in both oil and lipid/aqueous systems. A higher content of aglycones might be a result from the action of β-glucosidase (β-D-glycoside glycohydrolase, EC 3.2.1.21), endogenous in soybeans (Matsura et al., 1995), and the associated β-glucosidase of the fermenting microbes (Kaya et al., 2008) which promoted the hydrolysis of the β-glucoside conjugates, converting them to aglycones.

Table 2. The changes in isoflavone glucosides (daidzin and genistin) and isoflavone aglycones (daidzein and genistein) content of soybeans at day 0 and day 4 of fermentation.

Strains	Time	mg/g fermented soybeans					
		Daidzin	genistin	Total glucosides	daidzein	genistein	Total aglycones
BCC 3026	Day 0	1.439 ± 0.012^{dB}	1.397 ± 0.001^{cC}	2.836^{dA}	0.081 ± 0.023^{jF}	0.122 ± 0.002^{iE}	0.203^{iD}
	Day 4	0.154 ± 0.027^{ghiD}	0.264 ± 0.066^{fgD}	0.418^{ghC}	0.727 ± 0.002^{cB}	0.778 ± 0.008^{bB}	1.505^{bA}
BCC 3088	Day 0	1.466 ± 0.052^{dB}	1.374 ± 0.060^{cdB}	2.84^{dA}	0.114 ± 0.00^{hiD}	0.145 ± 0.002^{hCD}	0.259^{ghC}
	Day 4	0.168 ± 0.004^{ghF}	0.295 ± 0.006^{fE}	0.463^{ghD}	1.167 ± 0.007^{aB}	0.918 ± 0.00^{aC}	2.085^{aA}
BCC 3083	Day 0	1.290 ± 0.027^{eB}	1.305 ± 0.018^{deB}	2.595^{eA}	0.069 ± 0.006^{jD}	0.088 ± 0.002^{kD}	0.157^{jC}
	Day 4	0.103 ± 0.004^{hiF}	0.169 ± 0.004^{hE}	0.272^{iD}	0.629 ± 0.003^{eC}	0.708 ± 0.00^{dB}	1.337^{dA}
BCC 3101	Day 0	1.343 ± 0.005^{eC}	1.359 ± 0.001^{cdB}	2.702^{deA}	0.082 ± 0.002^{jF}	0.105 ± 0.002^{jE}	0.187^{iD}
	Day 4	0.124 ± 0.006^{hiE}	0.234 ± 0.053^{fghD}	0.358^{hiC}	0.675 ± 0.005^{dB}	0.741 ± 0.002^{cB}	1.416^{cA}
Control	Day 0	1.876 ± 0.057^{aB}	1.863 ± 0.052^{aB}	3.739^{aA}	0.107 ± 0.007^{iD}	0.163 ± 0.005^{gCD}	0.27^{gC}
	Day 4	1.069 ± 0.040^{fC}	1.254 ± 0.055^{eB}	2.323^{fA}	0.609 ± 0.001^{fE}	0.306 ± 0.001^{fF}	0.915^{fD}

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

Correlation between antioxidative activity and β-glucosidase activity

β-glucosidase activity of the fermented soybean broths and cooked soybeans incubated with *Aspergillus* are shown in Figures 3a and 3b. The β-glucosidase activities of fermented soybean broths inoculated with all fungal strains were higher than that of the non-inoculated soybean broth (control). The fermented soybean broths incubated with *A. oryzae* BCC 3088 showed the highest β-glucosidase activity, followed by *A. terricola* BCC 3026, *A. ornatus* BCC 3101 and *A. oryzae* BCC 3083, respectively. β-Glucosidase catalyzes the hydrolytic cleavage of β-glycosidic linkages of low molecular mass glycosides and is also to be a key enzyme in the enzymatic release of aromatic compounds from glucosidic precursors found in fruits and fermented products (Gueguen et al., 1996; Christine et al., 1998). *Aspergillus* strains are known for their ability to produce β-glucosidase with significantly higher yields than the other species. Esaki et al., (1999a) reported that β-glucosidase produced from *A. saitoi* in the fermented soybean extract gradually hydrolyzed the glucoside isoflavones into aglycone isoflavones. It was suggested that the catalytic action of β-glucosidase during fermentation liberated aglycones of isoflavone glucosides, resulting in the increased antioxidative activities (Esaki et al., 1994). Hence, the higher antioxidative activities observed could thus be related to their high β-glucosidase activity.

For solid-state fermentation, the specific activity of β-glucosidase activity of fermented soybeans ranged between 0.34 and 6.87 unit/g fermented soybeans (Figure 3). The β-glucosidase activity of all fermented soybeans was higher than that of the soybeans without inoculation (control). Additionally, the fermented soybeans incubated with *A. oryzae* BCC 3088 at the fourth day showed the highest β-glucosidase activity. β-Glucosidase activity gradually increased with fermentation time, especially after 2 days which was the stage of sporulation. Murakami et al., (1984) reported that the β-glucosidase from filamentous fungi during the fermentation liberated the isoflavones in soybeans, resulting in the increased antioxidative activity in miso and tempeh. β-glucosidase produced from the *A. saitoi* fermentation gradually converted glucosides into aglycones, potential antioxidative substances (Esaki et al., 1994). Therefore, the high β-glucosidase enzyme activity in fermented soybeans caused by filamentous fungi was related to antioxidative activity as well.

In the present study, antioxidative capacity of fermented soybeans was positively correlated with total aglycone concentration (R^2= 0.71, P<0.05) and β-glucosidase activity (R^2= 0.84, P<0.05). These results confirmed that increased total aglycone isoflavone during fermentation mainly contributed to the enhanced antioxidant activity of soybean fermented with starter culture. Summarily, the results indicated that the fermented soybeans incubated with *A. oryzae* BCC 3088 gave the highest amount of isoflavone aglycones content, resulting in the highest antioxidative activities since β-glucosidase hydrolyzed daidzin and genistin and then released daidzein and genistein, the potential antioxidative substances, during fermentation. The finding in this study corresponded with Chia-Hung et al., (2006) who reported that soybean fermented with filamentous fungi containing abundance of β-glucosidase enzyme possessed enhanced antioxidative activities in various model systems.

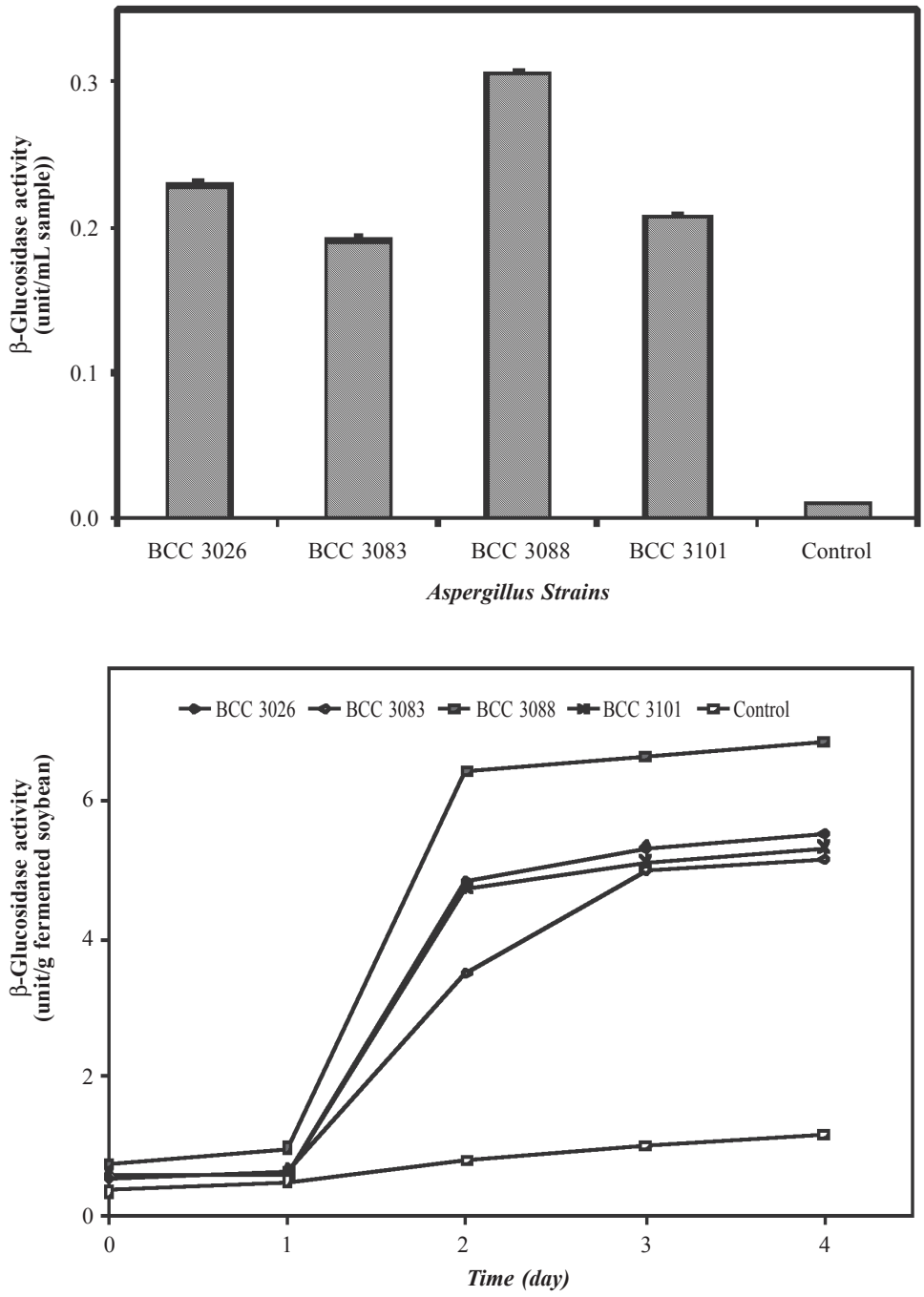

Figure 3. β-Glucosidase activity of fermented soybean broths measured at the fourth day of fermentation (A) and in fermented soybean during fermentation (B) with 4 selected strains of *Aspergillus*. A control contained only soybean broths without inoculation. β-glucosidase activity of fermented soybean broths was measured at the fourth day of fermentation. Each value was the averages of 4 replicates.

CONCLUSION

A. oryzae BCC 3088 had the most potential antioxidative activities among the 32 strains of *Aspergillus* tested and possessed enchanced $ABTS^{\cdot+}$-scavenging effect, FRAP and higher amount of potential antioxidative substances, isoflavone aglycones (daidzein and genistein). Our results also suggested the possibility to enhance antioxidative activity of fermented soybeans by using *Aspergillus* strain that was capable of producing β-glucosidase. The fermented soybeans inoculated with *Aspergillus* showed higher β-glucosidase enzyme and isoflavones content in comparison with those of control, indicating the higher antioxidative activities. However, the mechanism and the essential biofactors contributing to the antioxidative activity remain to be further clarified.

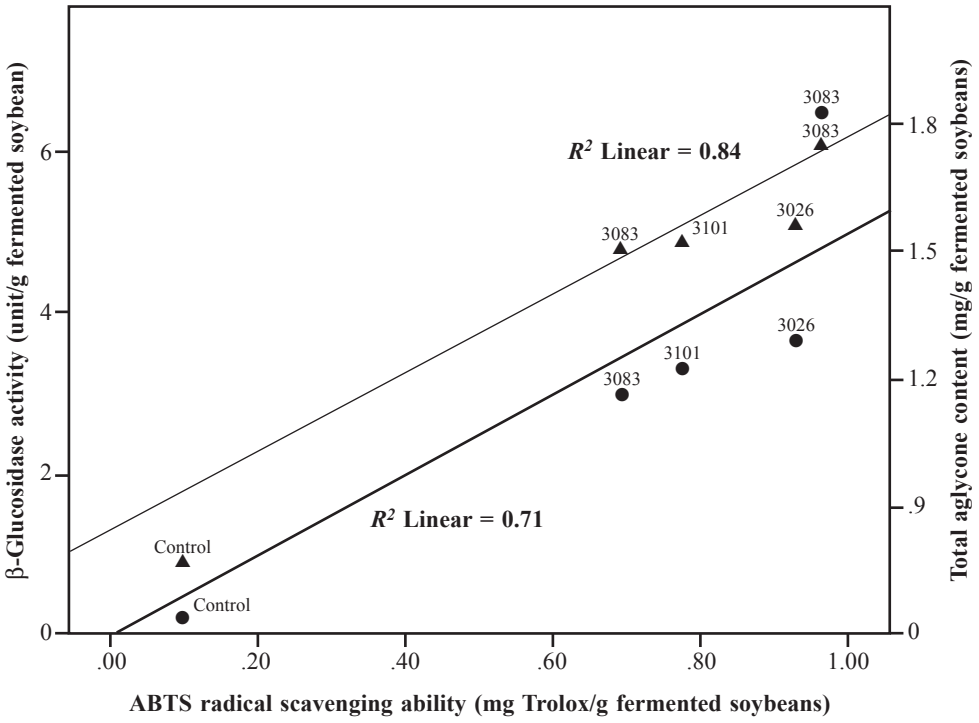

Figure 4. Scatter plot depicting correlation between scavenging effects on $ABTS^{\cdot+}$ with β-Glucosidase activity (▲)and total aglycone concentration (●).

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