Research Article

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Effects of fermentation on antioxidant properties and phytochemical composition of soy germ

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Abstract

BACKGROUND: Traditional soy-fermented foods, such as miso, douche, natto, and tempeh have been widely used as a dietary supplement in Asian countries, and numerous reports on their phenolics and antioxidant activities have been published. Soy germ contains 10-fold higher phenolics than whole soybean, hence using soy germ as fermentation substrate will be more efficient than whole soybean.

RESULTS: Soy germ fermented with *Aspergillus niger* M46 resulted in a high-efficiency bio-transformation of phenolics and flavonoids to their metabolites, and a diverse secondary metabolic product was also found to response oxidation stress of fungal colonisation. Its antioxidant activity against hydroxyl radicals and superoxide radicals ($IC_{50} = 0.8$ and 6.15 μg mL^{-1}, respectively) was about 205-fold and 47-fold higher than those of unfermented soy germ ($IC_{50} = 164.0$ and 290.48 μg mL^{-1}), respectively. These results were similar to those observed for Trolox, and more active than those of BHT and hesperidin. The β-glucosidase and α-amylase produced during fermentation were mainly responsible for mobilisation of the phenolics.

CONCLUSION: Our results demonstrate that fermented soy germ has the potential to be a good dietary supplement for prevention of oxidative stress-related diseases, and the solid-state bioprocessing strategy could be an innovative approach to enhance the antioxidant activity of soy germ.
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Keywords: antioxidant; *Aspergillus*; fermented soy germ; phenolics

INTRODUCTION

Phenolic phytochemicals have been used as functional foods and dietary supplements in complementary therapies. Several reports showed that solid-state fermentation of plant substrates is an efficient biotechnological strategy that enriching plant substrate for phenolic phytochemicals. It has been reported that these phenolics of plants could be bio-transformed via different microbial fermentation, such as *Bacillus* spp.,[1,2] *Lentinus edodes*,[3] *Trichoderma hazianum*,[4] *Aspergillus* spp.[5−8] and *Rhizopus* spp.[9,10]

Traditional soy-based fermented foods, such as miso, douche, natto, tempeh, kinema and cheonggukjang, have been widely used as a dietary supplement in Asian countries; and there are numerous research on phenolics and antioxidant activities of these fermented soybeans.[2,5,7] Soy germ contains 10-fold higher phenolics than whole soybean, hence using soy germ as fermentation substrate is more efficient than whole soybean. Several publications have explored the use of soy germ plant as a substrate. Tipkanon *et al.*[11] used the β-glucosidase enzyme from Sigma Chemical for conversion of isoflavone glucosides to aglycones in soy germ, and in some studies bifidobacteria[12,13] and Aspergilli[14] liquid fermentation was used. However, no research used solid-state fermentation as a strategy for bioconversion of phenolic and flavonoid to elevated antioxidant activity of soy germ to date. Aspergilli are widely used in industry and biotechnology for production of enzymes and fermented foods[15] and our preliminary study demonstrated that solid-state fermented soy germ by *Aspergillus niger* M46 showed higher antioxidant activities compared to the unfermented soy germ.

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical is a stable radical source and is widely applied to evaluate the total antioxidant activity in samples. The hydroxide radicals, superoxide radicals and peroxyl radicals were more likely to be produced *in vivo*, hence, they were physiologically more relevant in free radicals scavenging tests *in vitro*. Among these, the hydroxyl radical is considered as

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the most reactive free radical in biological tissues. Polyphenols and flavonoids are widely distributed in plants and play a wide range of physiological functions. In this report, soy germ was fermented with *A. niger* M46 to produce biologically active compounds, and its antioxidative activity in different oxidation systems in comparison with those of synthetic antioxidant butylated hydroxytoluene (BHT) and the natural antioxidant Trolox was investigated. In addition, the relationships between the phenolic and flavonoid variation, the antioxidant activity and two carbohydrate cleaving enzyme activities of β-glucosidase and α-amylase were also investigated since these antioxidants are mobilised and modified during solid-state bioprocesses by fungi and are usually linked to the activities of carbohydrate cleaving enzyme.[16,17]

MATERIALS AND METHODS

Microbial fermentation

The *A. niger* M46, isolated from the Taiwan soil and identified by Food Industry Research and Development Institute (Hsinchu, Taiwan), was incubated to potato dextrose agar (PDA) to obtain spore suspensions in sterile distilled water. Soy germ was pulverised and sterilised at 121 °C for 15 min. Solid fermentation was performed by spraying spore suspension into sterilised soy germ (ca. 5×10^5 spores g^{-1} substrate), and the inoculated soy germ was cultured at 30 °C for 7 days.

Sample preparation

The fermented samples were pulverised and extracted exhaustively with 70% methanol at a mass:volume ratio of 1:10 at 30 °C, 120 rpm for 24 h. The extract was filtrated through Whatman No. 1 filter paper, concentrated under reduced pressure and successively lyophilised.

DPPH radical scavenging ability

DPPH radical scavenging activity of the fermented soy germ extracts were measured according to Huang and Mau.[18] A sample (4 mL) was mixed with 1 mL of methanol solution containing the 1 mmol L^{-1} $DPPH^{\bullet}$ radicals. The reaction mixture was vortex-mixed and incubated for 40 min in the dark, and absorbance was monitored at 517 nm. The decrease at 517 nm between the blank and a sample was used for calculating the scavenging activity. Trolox with different concentrations was used as standard for construction of the calibration curve. Each value is expressed as mean ∀ SD ($n = 3$).

Inhibition (%) of $DPPH^{\bullet}$ radical was calculated according to the following equation:

$$\text{inhibition} = \left[\left(\text{OD}_{\text{control}} - \text{OD}_{\text{standard}}\right) / \text{OD}_{\text{control}}\right] \times 100$$

where $\text{OD}_{\text{control}}$ is the optical density (OD) of the control at 0 µg mL^{-1}, and $\text{OD}_{\text{standard}}$ is the OD of standard or sample.

Hydroxyl radical scavenging ability

Hydroxyl radicals scavenging ability of the extracts was determined according to the assay described by Tsai *et al.*[19] The reaction mixture included 1.0 mL of 3 µmol L^{-1} IBG, 0.1 mL of 1.0 mmol L^{-1} $FeSO_4$, 1.6 mL of 3 % H_2O_2 and 0.05 mL of 10 mmol L^{-1} EDTA; and the ultra-weak photon was measured using BJL ultra-weak chemiluminescence (Jye Horn Co., Taipei, Taiwan). A 10 µL sample was

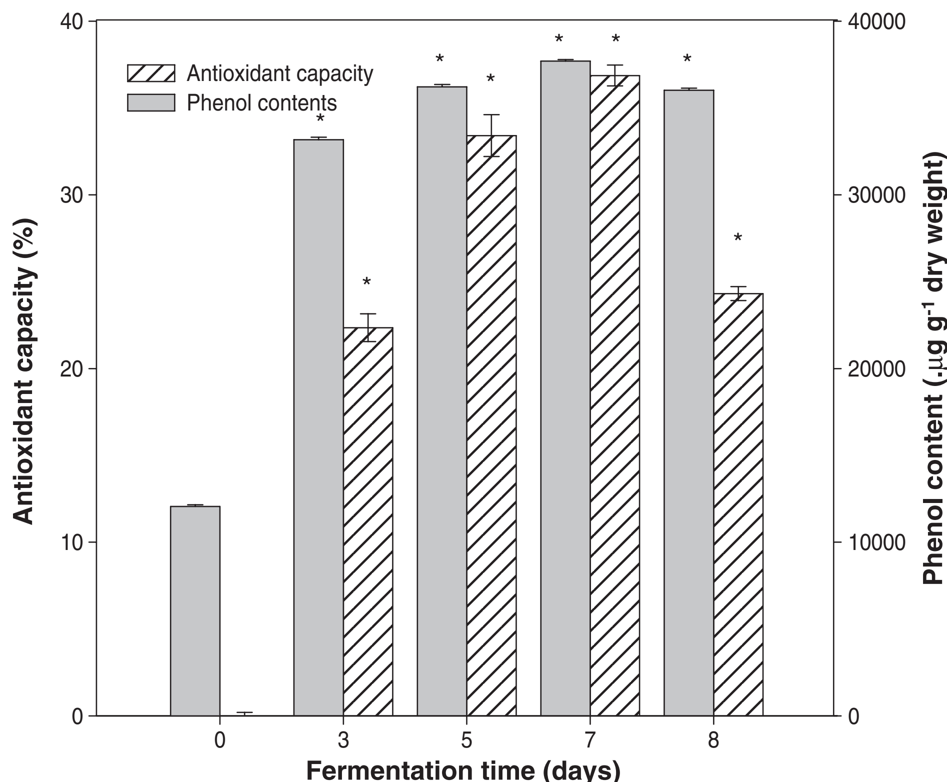

Figure 1. Total phenolic contents and antioxidant activity against DPPH radicals of *Aspergillus niger* M46-fermented soy germ during fermentation. Each value is expressed as mean ± SD ($n = 3$). The antioxidant activities of the samples were detected at 400 µg g^{-1} dry weight. *Significant difference from 0 day, $P < 0.05$.

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Table 1. Flavonoid content of soy germ fermented with *Aspergillus niger* M46 at 30 °C, and 50% water content during fermentation

Fermentation time (days)	Total flavonoid content
0	1406.3 ± 15.2
3	6128.4 ± 10.1*
5	8544.1 ± 8.5*
7	9684.4 ± 12.6*

Each value is expressed as mean ± SD ($n = 3$).
*Significant difference from 0 day, $P < 0.05$.
Flavonoid content was measured as mg catechin g^{-1} dry weight.

added to the reaction mixture, and the decrease in the degrees of the CL (chemiluminescence) counts represented the hydroxyl radicals scavenging abilities. Trolox and BHT were used as positive controls. Each value is expressed as mean ± SD ($n = 3$).

Superoxide radical scavenging ability

Superoxide anion radical scavenging activity was determined according to Tsai *et al.*[20] with slight modifications. The reaction mixture was included 1.0 mL of 2.0 mmol L^{-1} lucigenin, 0.05 mL of 1.0 mol L^{-1} arginine, 0.05 mL of 1.4 µmol L^{-1} methylglyoxal and 1.0 mL of PBS buffer (pH 7.4); and the ultra-weak photon was measured using BJL ultra-weak chemiluminescence (Jye Horn Co.). A 10 µL of sample was added to the reaction mixture, and decrease in the CL counts represented the superoxide radical scavenging ability. Trolox and BHT were used as positive controls. Each value is expressed as mean ± SD ($n = 3$).

Peroxyl radical scavenging ability

Peroxyl radicals scavenging ability of the sample was measured according to Alberto *et al.* [21] A 150 µL sample of 96 mmol L^{-1} fluorescein and a 20 µL of sample solution were pre-incubated for 5 min at 37 °C, and the oxygen radical absorbance capacity (ORAC) analyses was initiated by addition 30 µL of 320 mmol L^{-1} 2,2′-azobis(2-amidinopropane) dihydrochloride. Fluorescein decay curves between the blank and a sample were assayed.[19] Trolox was used as a positive control.

Total polyphenolic content

The total polyphenol content were determined according to the method described by Shetty *et al.* [22] The reaction mixture included 1 mL of the sample, 1 mL of 95% ethanol solution, 5 mL of distilled water and 0.5 mL Folin–Ciocalteu reagent. After 5 min, 1 mL of 5% Na_2CO_3 solution was added and allowed to incubate in the dark for 1 h. The absorbance was measured at 725 nm against a blank. Gallic acid with different concentrations was used as standard for construction of the calibration curve. Total polyphenol content was expressed as micrograms of gallic acid equivalent (GAE) per gram of sample. Each value was expressed as mean ± SD ($n = 3$).

Total flavonoid content

Total flavonoid content was determined according to the method of Jia *et al.* [23] In brief, 0.5 mL of sample supernatant was mixed with 3.0 mL of deionised water and subsequently with 0.15 mL of a 5% sodium nitrite solution. After 5 min, 0.3 mL of a 10% aluminium chloride solution was added and allowed to stand for 6 min, then 1.0 mL of 1 mol L^{-1} sodium hydroxide was added to the mixture. The mixture was thoroughly mixed and allowed to incubate for another 15 min. The intensity of the pink colour was measured at 510 nm in a U-2001spectrophotometer (Tokyo, Japan). Total flavonoid content was quantified by the standard curve of catechin (Sigma, St Louis, MO, USA) and expressed as mg L^{-1} of catechin equivalents (CE). Each value is expressed as mean ± SD ($n = 3$).

Flavonoid analysis

The lyophilised fermented samples were extracted with 70% methanol at 30 °C, 120 rpm for 4 h. Then they were filtrated through a 0.45 µm filter and analysed by HPLC method for the flavonoid content described by Hsu and Chiang.[24] The ODS C_{18} reverse-phase column (4.6 mm × 250 mm) was eluted with a linear gradient of acetonitrile (0–50% at 0–40 min and 50–90% at 40–60 min) containing 0.1% trifluoroacetic acid at 1 mL min^{-1}, and detected the pattern at 262 nm on a diode array detector. Daidzin, glycitin, genistin, malonyl daidzin, malonyl glycitin, malony genistin, acetyl daidzin, acetyl glycitin, acetyl genistin, daidzein genistein, glycitein were used as standards in the assay.

α-Amylase activity

The *α*-amylase activity of sample was determined using the procedure as developed by the Worthington Biochemical Co.[25] with little modification. A 0.5 mL of 1% starch in a 20 mmol L^{-1} phosphate buffer with 6 mmol L^{-1} sodium chloride (pH 6.9) was incubated with 0.1 mL sample for 30 min at 30 °C. The reaction was terminate by adding 1 mL of 3,5-dinitrosalicyclic acid solution in boiling water bath for 5 min. Afterward, the tube was cooled and 10 mL of dH_2O was added; the released maltose was measured at 420 nm and calculated from the standard curve of maltose. One unit of *α*-amylase was defined as the amount of enzyme that releases 1 µmol of maltose per minute under the assay conditions. Each value was expressed as mean ± SD ($n = 3$).

β-Glucosidase activity

The *β*-glucosidase activity of the sample was determined using the procedure developed by Matsuura *et al.*[26] *p*-Nitrophenol *β*-glucopyranoside (*p*-NPG) (2.0 mL, 1 mmol L^{-1}) in a 0.1 mol L^{-1} pH 5.0 phosphate–citrate buffer was pre-incubated for 5 min at 30 °C. The reaction was initiated by adding 0.5 mL sample and terminated by adding 2.5 mL of 0.5 mol L^{-1} sodium carbonate after 1 h of incubation. The *p*-nitrophenol released was measured at 420 nm and calculated from the standard curve of *p*-nitrophenol. One unit of *β*-glucosidase was defined as the amount of enzyme that releases 1 µmol of *p*-nitrophenol per minute under the assay conditions. Each value was expressed as mean ± SD ($n = 3$).

Statistical analysis

The significance of differences between two samples was analysed using the Student *t*-test. A *P* value of less than 0.05 was taken as significant.

RESULTS

Total antioxidative activity, polyphenol content and flavonoid content

As shown in Fig. 1 and Table 1, the soy germ fermented by *A. niger* M46 showed greatly increased amounts of phenolic and flavonoid constituents. This was accompanied by a significant increase in antioxidant activity compared to unfermented soy germ during the 7 day fermentation ($P < 0.05$). The scavenging activity of

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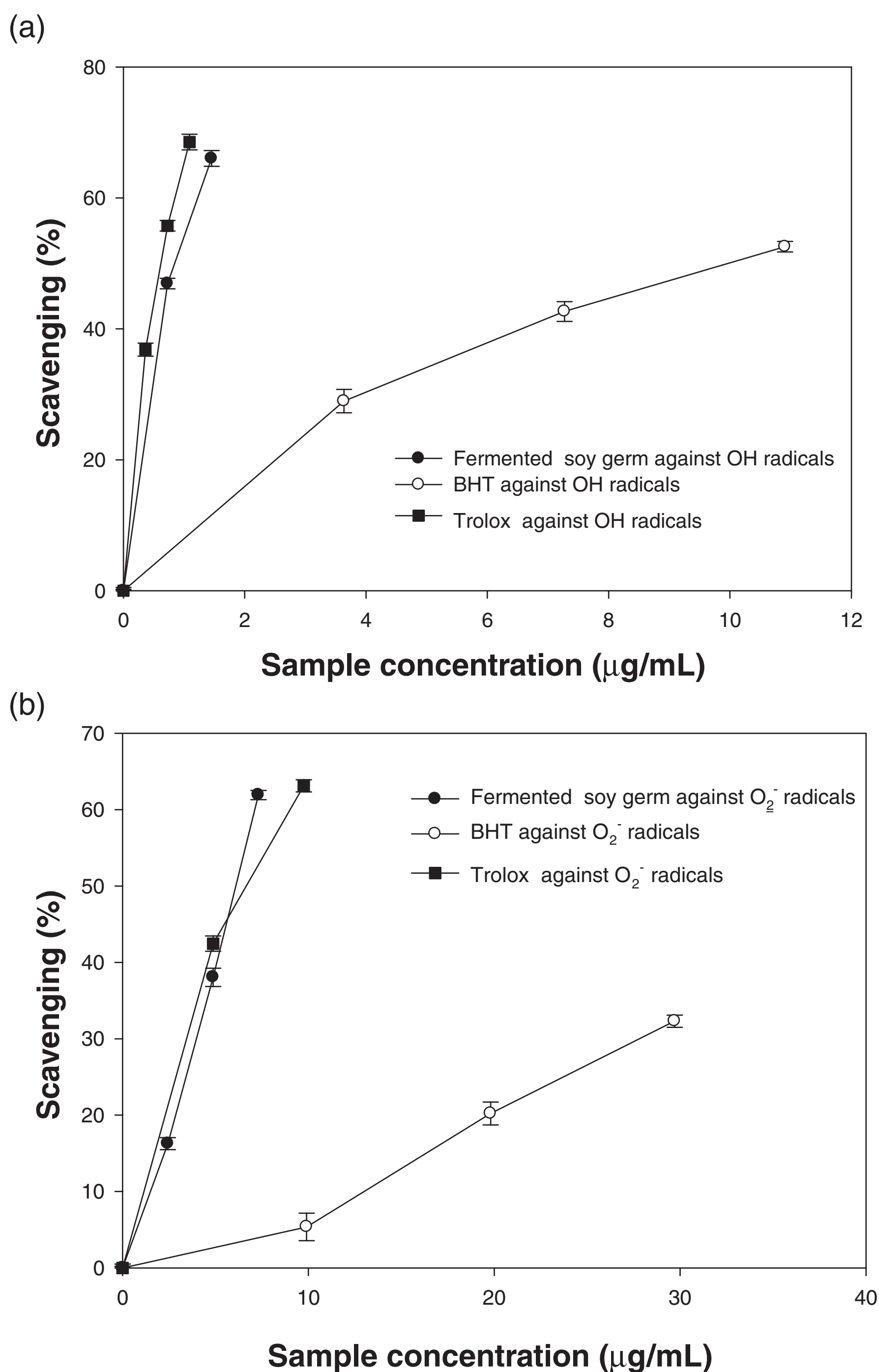

Figure 2. Radical scavenging activity of *Aspergillus niger* M46-fermented soy germ toward hydroxyl radicals (a) and superoxyl radicals (b). Each value is expressed as mean ± SD ($n = 3$).

soy germ fermented for 7 days ($IC_{50} = 0.41$ mg mL^{-1}) was about 6.5-fold greater than that of raw soy germ ($IC_{50} = 2.66$ mg mL^{-1}). The fermentation was necessary in order to release or bio-convert antioxidative constituents from a low active form of soy germ.

Antioxidative characterisation

The hydroxyl radical scavenging activity drastically increased with increasing sample concentration (Fig. 2a) and was far superior to the activity observed for unfermented soy germ ($IC_{50} = 164.0$ μg mL^{-1}) (data not shown). It was about 205-fold stronger than that of raw soy germ. The IC_{50} value of the fermented soy germ (0.8 μg mL^{-1}) was close in value to that observed for natural antioxidant Trolox (0.58 μg mL^{-1}), and synthetic antioxidant BHT was the lowest ($IC_{50} = 9.09$ μg mL^{-1}).

As shown in Fig. 2b, the antioxidant activity of fungal-grown soy germ also exhibited dose-dependent scavenging activities against superoxide radicals with varying capacity and was much more effective than that of observed for unfermented soy germ ($IC_{50} = 290.48$ μg mL^{-1}) (data not shown). It was about 47-fold stronger than that of raw soy germ. The IC_{50} value of the

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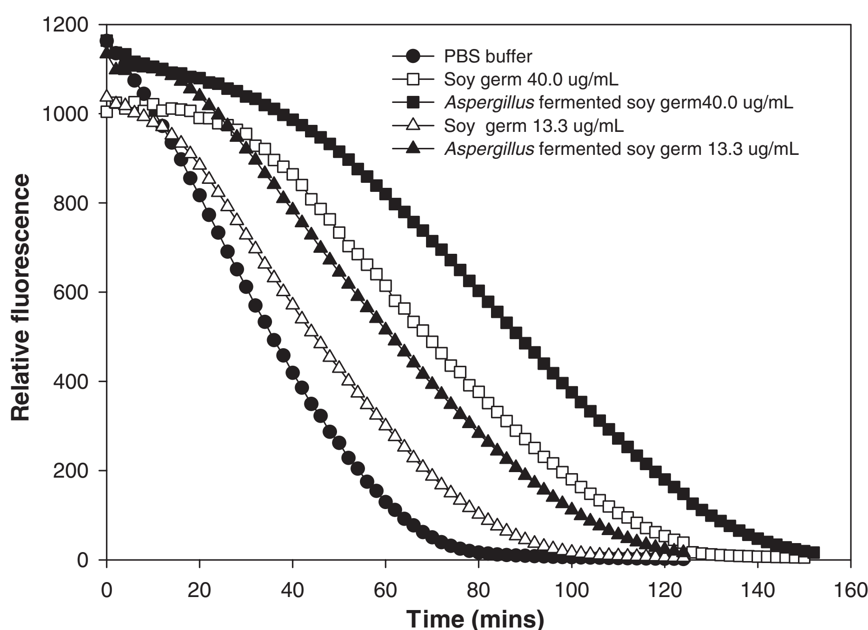

Figure 3. Radical scavenging activity of *Aspergillus niger* M46-fermented soy germ toward peroxyl radicals.

fungal-grown soy germ was 6.15 μg mL^{-1} compared with the IC_{50} values of Trolox and BHT at IC_{50} 6.42 μg mL^{-1} and 48.01 μg mL^{-1}, respectively. Fermented soy germ might act like superoxide dismutase, which neutralised superoxide anion and is one of the defence mechanisms in the living cell.

The ORAC assay was further used to test the antioxidative capacity to quench peroxyl radicals, and the ORAC value is usually reported as a Trolox equivalent.[19] All fungal-grown soy germ samples had a concentration-dependent increase in the inhibition of peroxyl radicals (Fig. 3), and the antioxidant activity of the fungal-grown soy germ against peroxyl radicals at a concentration of 13.3 μg mL^{-} (5.75 μg mL^{-1} Trolox equivalent) was nearly two-fold higher than that of raw soy germ (2.98 μg mL^{-1} Trolox equivalent) at the same concentration. The peroxyl radicals ($LOO^{\bullet}$) could be formed in the lipid peroxidation process, so the fermented soy germ could terminate the free radical chain reaction of lipid peroxidation.

To sum up the above antioxidant studies, the *Aspergillus*-fermented soy germ has antioxidant activity superior to unfermented soy germ against DPPH radicals, hydroxyl radicals, superoxide radicals and peroxyl radicals. As a result, fermented soy germ showed the potential to be a natural antioxidant for functional food.

α-Amylase and β-glucosidase activities during fermentation

The α-amylase reached the highest activity on the first day and remained generally high during fermentation as shown in Fig. 4a. On the other hand, almost no activity of β-glucosidase activity from *Aspergillus*-fermented soy germ was observed on the first day, and it had a significantly increase during the 7 day fermentation ($P < 0.05$). The β-glucosidase activity increased approximately 200-fold during the later stages of growth (Fig. 4b).

Flavonoid analysis

In this study, the soy germ fermented by selected *Aspergillus* sp. apparently increased flavonoid constituents (Fig. 5a), which were not present (or in smaller amounts, if present) in unfermented soy germ (Fig. 5b). The isoflavone glycosides were bio-converted by β-glucosidase into minor aglycones in the fermented soy germ, and a new fraction (labelled as P) was found in fermented soy germ (Fig. 5a). The antioxidant activity of fraction P exhibited appreciable antioxidant activity, and the IC_{50} value was 0.07 and 0.47 μg mL^{-1} for hydroxyl radicals and superoxide radicals scavenging, respectively (data not shown).

DISCUSSION

The antioxidant activity of fermented soy germ was similar to natural antioxidant Trolox, and was more active than those of the BHT synthetic antioxidants. Moreover, the antioxidant activity of fermented soy germ was 1325-fold and 377-fold stronger than natural phenolic antioxidant hesperidin, which is the most important flavonoid of *Citrus* sp., with IC_{50} values of 1.06 mg mL^{-1} for hydroxyl radicals and 2.32 mg mL^{-1} for superoxide radicals (data not shown). This study showed solid-state fermentation was a good strategy to increase the antioxidant activity of soy germ.

Some reports indicated that the phenolic antioxidants mobilized and modified during solid state bioprocess usually are linked to the activities of carbohydrate cleaving enzyme,[16,17] so the relationships between the phenolic variation, the antioxidant activity and the activities of these two carbohydrate cleaving enzymes (β-glucosidase and α-amylase) were also investigated. In this study, the amylase reached the highest activity on the first day; it possibly supported starch degradation of soy germ for energy needs of fungal growth during fermentation.[17] The β-glucosidase activity also apparently increased approximately 200-fold during the later stages of growth, suggesting it directly correlates for mobilization and recombination of conjugated phenolic and flavonoids.[10] It was postulated that the phenolics and flavonoids released from polymeric or conjugated forms and resulted in a corresponding increase in total antioxidant capacity. A higher antioxidant activity was produced during the early stage of growth because of oxidation stress of fungal colonisation.[10] The finding is also consistence with other beans after fermentation, such as black bean,[6] soybean[7] and fava bean.[10]

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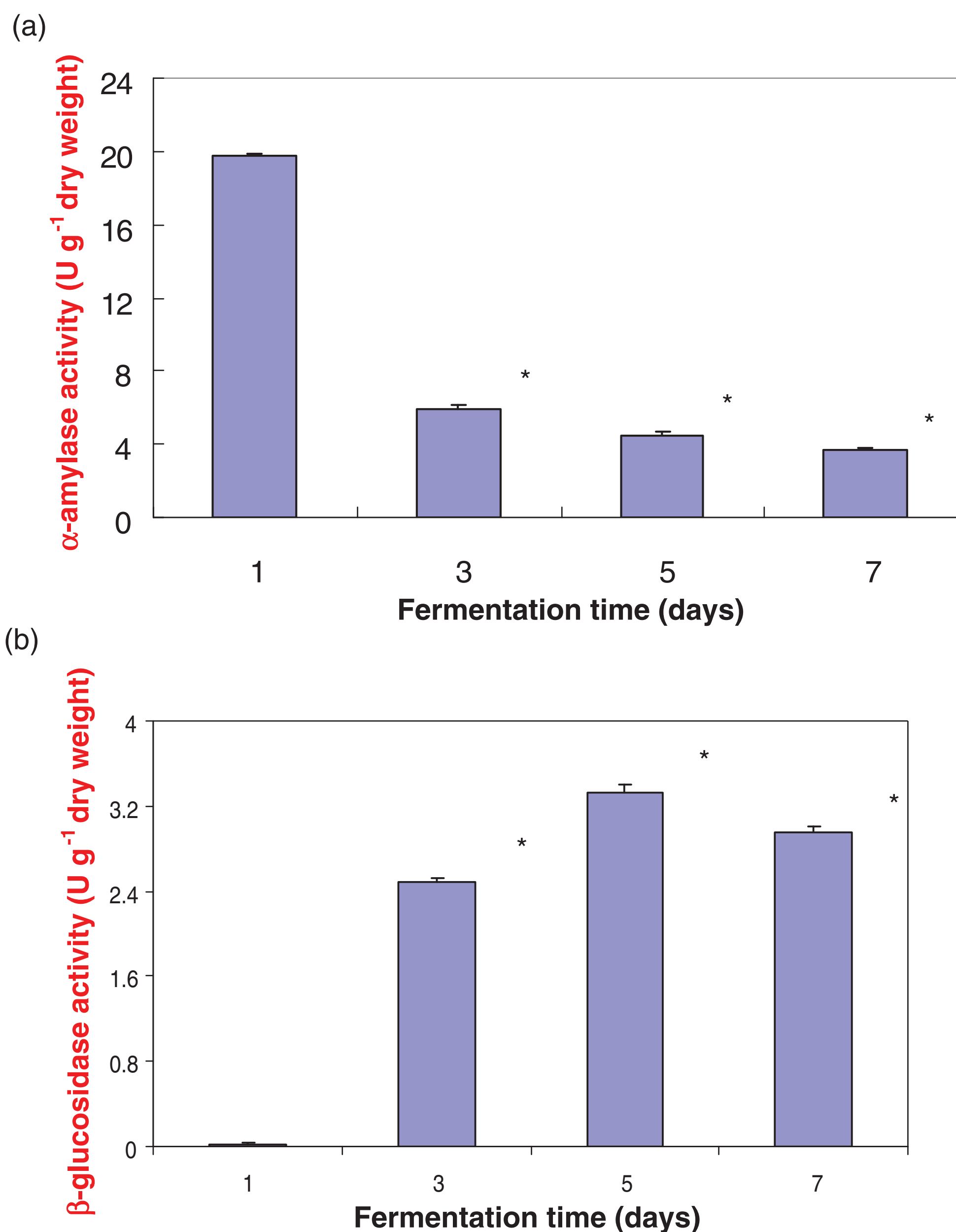

Figure 4. α-Amylase activity (a) and β-glucosidase activity (b) of *Aspergillus niger* M46-fermented soy germ during fermentation. Each value is expressed as mean $\pm$ SD ($n = 3$). *Significant difference from 1 day, $P < 0.05$.

To sum up the above studies, the novel antioxidants could be produced from the soy germ materials during *Aspergillus* fermentation. The soy germ fermented with *A. niger* M46 altered the composition of isoflavones, and the isoflavones were further converted into other microbial-induced metabolites. Some researchers reported that flavonoid aglycones and their metabolites are more effective antioxidants than flavonoid glycosides,[27–29] such as dihydrodaidzein, equol and *O*-desmethylangolensin, 8-hydroxydaidzein and 8-hydroxygenistein. A more diverse secondary metabolic product in fungi was found to response oxidation stress of fungal colonisation and growth. It is suggested that the increasing antioxidant capacity might be from symbiotic interaction of released flavonoid aglycones and their metabolites (fraction P) in this study. However, the components of fraction P are relative complex in further purification (data not shown), so the crude extract of fermented soy germ could be taken directly as health-promoting supplements in commercial forms concerning it needs much more time and cost of further purification. The results are different from other microbial fermented soy germ,[12–14] which the microbes were not be able to further metabolise the isoflavone aglycones into other metabolites.

CONCLUSION

The fermentation using fungus *Aspergillus* was necessary in order to release biological components for antioxidant activity from the low-activity soy germ material. The fungal-grown soy germ was far superior to raw soy germ in quenching DPPH radicals, hydroxyl radicals, superoxide radicals and peroxyl radicals. Its antioxidant activity is similar to that observed for Trolox, and more active than

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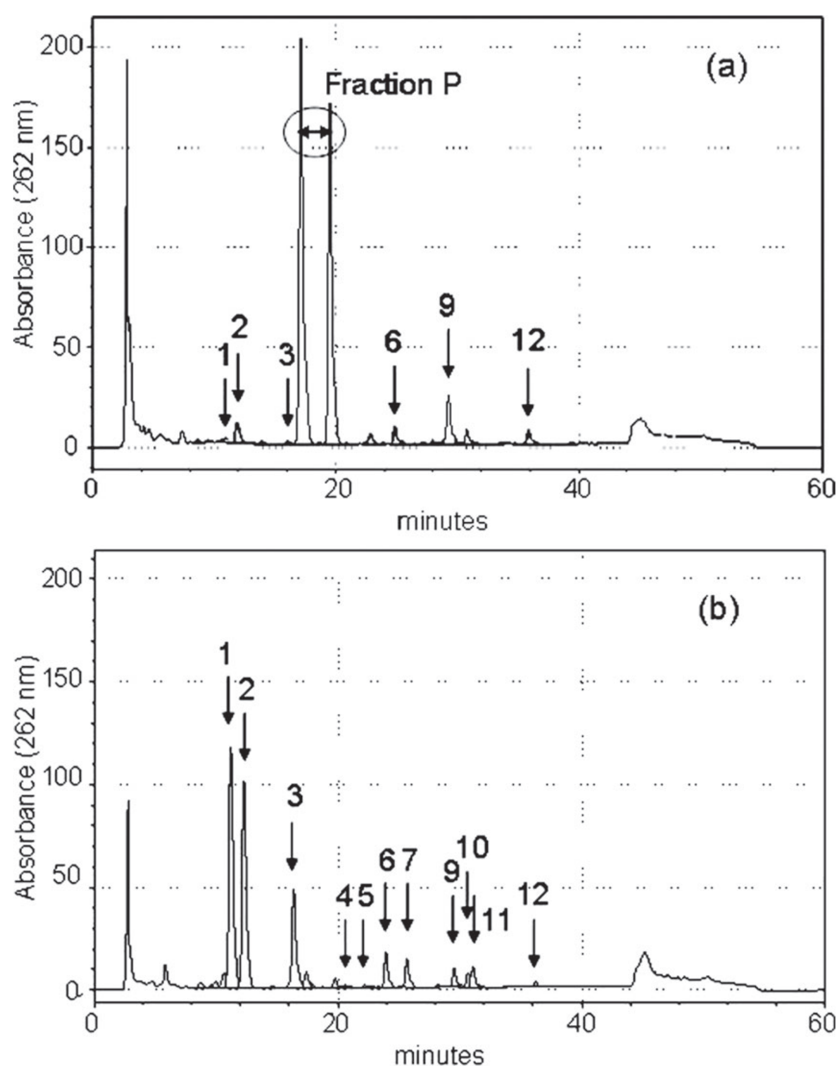

Figure 5. HPLC patterns of soy germ fermented with (a) and without (b) *Aspergillus niger* M46, on an ODS C18 reverse-phase column (4.6 mm × 250 mm) was eluted with a linear gradient of acetonitrile (0–50% at 0–40 min, and 50–90% at 40–60 min) containing 0.1% trifluoroacetic acid at 1 mL min^{-1} and detection at 262 nm. 1. daidzin, 2. glycitin, 3. genistin, 4. malonyl daidzin, 5. malonyl glycitin, 6. acetyl daidzin, 7. acetyl glycitin, 8. malony genistin, 9. daidzein, 10. glycitein, 11. acetyl genistin, 12. genistein.

those of BHT and hesperidin The β-glucosidase and α-amylase were mainly responsible for the mobilisation of phenolics during A. niger M46 fermentation. Our results demonstrate that fermented soy germ has the potential to be a good dietary supplement for prevention of oxidative stress-related diseases, and the solid-state bioprocessing strategy could be an innovative approach to enhance the antioxidant activity of soy germ.

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