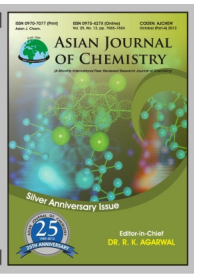
Asian Journal of Chemistry; Vol. 25, No. 18 (2013), 10565-10568

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2013.15988



Antioxidant Activities of Fermented Soybean Prepared with *Bacillus subtilis*

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(*Received*: 2 July 2013; *Accepted*: 27 November 2013) AJC-14448

The antioxidant activities of methanolic extract of fermented soybean prepared with *Bacillus subtilis* and cooked non-fermented soybean were investigated. The total phenolics increased markedly from 675 to 6629 mg/kg after 72 h of the fermentation time. At the same concentration level of lyophilized extract, soybean fermentation prepared with *Bacillus subtilis* showed a higher antioxidant activity than that cooked non-fermented soybean. Methanolic extracts of soybean fermentation prepared with *Bacillus subtilis* showed a higher reducing activity than that of cooked non-fermented soybean at the same concentration level of lyophilized extract. Ferrous ion chelating ability of methanol extracts from fermented soybean increased with the increase of the fermentation time. Soybean fermentation prepared with *Bacillus subtilis* showed a higher ferrous ion chelating ability than that of cooked non-fermented soybean at the same concentration level of lyophilized extract. Therefore, soybean preparation with *Bacillus subtilis* may be exploited as a functional food to alleviate oxidative stress.

Key Words: Antioxidant activity, Soybean fermentation, *Bacillus subtilis*.

INTRODUCTION

Microbial sources have been shown to be a potential means of producing natural antioxidants in various fermented products, such as Japanese miso, Korea cheonggukjang or koji, Indian kinema and Chinese furu or sufu. Most antioxidant nutrients in fermented soybean products are polyphenolic compounds, acting as reducing agents, metal chelators and radical scavenging activity[1]. Soybean and different soybean products are known to contain phenolic compounds. Concentrations of these compounds in soybean were reported to increase after fermentation. It was suggested that the increased antioxidative activity was due to the liberation of lipophilic aglycones of isoflavone glucosides such as daidzein and genestein by the catalytic action of β-glucosidase during fermentation and due to significant increase in the formation of methanol-soluble antioxidative fraction[2,3].

Soybean fermentation prepared with *Bacillus subtilis* is a traditional Chinese fermentation food, which is produced by solid-state fermentation. It has played important roles in Chinese diets and highly popular for centuries[4]. There were three different types of soybean fermentation products in China, based on the microorganisms used, which can be classified into *Aspergillus*-type soybean fermentation product, Mucor-type soybean fermentation product and *Bacillus*-type soybean fermentation product. *Aspergillus*-type and Mucor-type soybean fermentation product are the most common type in China[5,6]. There are a few information reported that these two types of soybean fermentation product have antioxidative activities. However, there is no report studies on the antioxidative activities of soybean fermentation product prepared with *Bacillus subtilis*. The objective of this study is to investigate the antioxidative properties of *Bacillus*-type soybean fermentation product by determining the 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging effect, the reduced activity and the chelating ability of ferrous ions.

EXPERIMENTAL

Bacillus subtilis was kindly provided by the Institute of Microbiology, Chinese Academy of Sciences (Beijing, China). 1,1-diphenyl-2-picrylhydrazyl (DPPH), 1,10-phenanthroline and gallic acid were purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade.

Preparation of soybean fermentation product: Soybean were washed, soaked in water (1:3, w/w) at 25 ± 2 °C for 12 h and steamed at 121 °C for 0.5 h in a steamer (Shanghai Shenan Medical Instrument Co., Beijing, China). After cooling to 30 °C, the cooked soybeans were inoculated with *Bacillus subtilis* (10^6 total cells per gram of cooked soybean) in sterile glass bottles (500 mL) plugged with cotton wool and incubated at 37 °C and 75 % relative humidity for 72 h. One hundred gram of soybean fermentation samples were obtained at 0, 24, 48 and 72 h after the start of the fermentation process. The samples of soybean fermentation as well as cooked non-fermented

soybean samples at different fermentation times were dried separately for 24 h at 60 °C in a hot air oven.

Extration of soybean fermentation samples: All samples were minced and extracted with 10 times methanol (w:v) at room temperature for 5 h (repeated three times) and was then filtered through Whatman No. 4 filter paper. The methanol extracts of each sample were concentrated at 40 °C under vacuum and freeze-dried. The lyophilized extracts at different fermentation time were diluted with methanol to 20 mg/mL. The lyophilized extracts of soybean fermentation product after fermentation for 72 h were diluted with methanol to 1, 2, 3, 4, 5, 10, 20, 30 mg/mL, all lyophilized extracts stored at 4 °C until use.

Total phenolics content analysis: The total phenolic content was determined using a modified Folin-Ciocalteu method[7]. Each test samples (50 µL) was added to a test tube (1 mL volume) that contained 400 µL of distilled water. After vortexing the tubes, 50 µL of Folin-Ciocalteu's phenol reagents was added to each tube. The tubes were vortexed and 6 min later, 500 µL of 7 % Na_2CO_3 was added to each tube. The tubes were vortexed again and then allowed to stand for 1.5 h at room temperature. Thereafter, the absorbance of each sample was measured against a blank at 750 nm using a spectrophotometer (Spectrum 754 PC, Shanghai spectrum instruments Co. LTD, China). A calibration curve was constructed using 125, 250, 500, 1000 mg/mL gallic acid as a standard. The total phenolic content is expressed as milligrams of gallic acid per gram of dry extract.

Measurement of DPPH radical-scavenging activity: The method of Shimada *et al.*[8] was used to determine the DPPH-radical-scavenging activity of douche extract. 0.5 mL methanol extract was added to methanolic solution of 100 µM DPPH (1 mL). The mixture was shaken and left to stand at room temperature for 0.5 h. The absorbance of the resulting solution was then measured spectrophotometrically at 517 nm. The inhibitory percentage of DPPH was calculated according to the following equation:

$$\text{Scavenging effect}(\%) = \left(1 - \frac{\text{Absorbance sample}}{\text{Absorbance control}}\right) \times 100$$

Measurement of reducing activity: The reducing activity of samples was determined essentially following the method of Oyaizu[9]. A sample (0.3 mL) was mixed with 1 % potassium ferricyanide (0.3 mL, Hanawa, Osaka, Japan) and sodium phosphate buffer (0.3 mL, 0.2 M, pH 6.6). The mixture was incubated at 50 °C for 20 min and then 10 % trichloroacetic acetic acid (0.3 mL, Ferak, Berlin, Germany) was added. The mixture was centrifuged (6000 rpm) at 4 °C for 10 min. The upper layer (0.6 mL) was mixed with 0.1 % ferric chloride (0.12 mL, Hanawa, Osaka, Japan) and deionized water (0.6 mL). After 10 min of vortexing the tubes, absorbance of this mixture was then measured at 700 nm. A higher absorbance of this mixture indicates a higher reducing activity.

Measurement of ferrous ion chelating ability: Ferrous ion chelating ability of the extract was determined according to the method of Decker and Welch[10]. The Fe^{2+} level was monitored by measuring the formation of the ferrous ion-ferrozine complex. The Douchi qu extract (1 mL) was mixed with methanol (3.7 mL), 2 mM $FeCl_2$ (0.1 mL) and 5mM 1,10-ferrozine (0.2 mL) and the mixture was shaken and left at room temperature for 10 min.

The absorbance of the resulting solution was measured at 562 nm. A lower absorbance indicates a stronger ferrous ion chelating ability. The ability to chelate the ferrous ion was calculated as follows:

$$\text{Chelating effect}(\%) = \left(1 - \frac{\text{Absorbance sample}}{\text{Absorbance control}}\right) \times 100$$

RESULTS AND DISCUSSION

Extract yield: The yield of lyophilized methanol extract from fermented soybean prepared with soybean fermentation prepared with *Bacillus subtilis* during the fermentation is shown in Fig. 1. The yield of lyophilized methanol extract of non-fermented soybean is 6.2 ± 0.4 g/100 g. The yield increased with increase of the fermentation time, the values in fermented soybean after 72 h of fermentation time is 15.7 ± 1.1 g/100 g. The result is similar to Lin *et al.*[11] who reported that the value in cooked no fermented soybean and soybean kojis (fermented by different molds) were 5.3 and 6.7-16.8 g/100 g, respectively. Recently, there have been reported that different polar and nonpolar solvent extracts from plant-derived foods had different antioxidant activity, the methanolic extract was reported to possess higher antioxidant activity than that of other extracts[12].

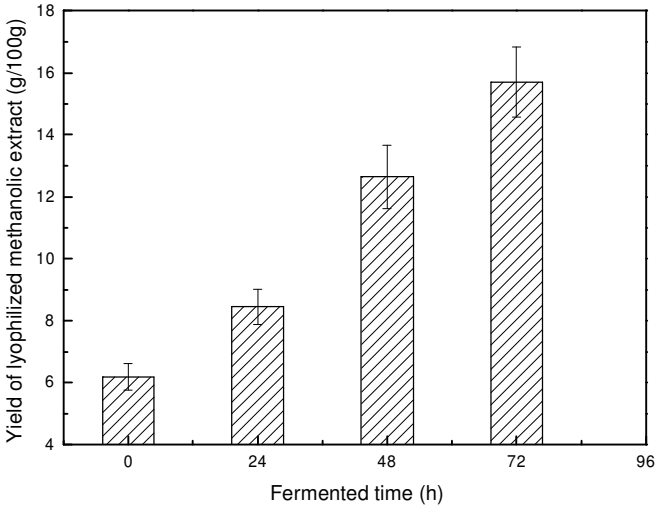

Fig. 1. Yield of lyophilized methanol extract at different fermentation time

Total phenolics and radical-scavenging activity: Changes in total phenolics and DPPH radical scavenging activity of fermented soybean with *Bacillus subtilis* during fermentation are shown in Fig. 2. The total phenolics increased markedly from the starting from 675 to 6629 mg/kg after 72 h of the fermentation time. DPPH free radical ability is frequently used in the determination of free radical scavenging ability[13]. The level of DPPH radical scavenging activity increased from 60.4 to 93.3 % after 72 h of the fermentation time. Shon *et al.*[14] reported that methanol extract of traditional Korea fermented soybean (cheonggukjang) showed DPPH radical scavenging activity of 69-87 % at total phenol concentration of 0.13-0.27 g/kg.

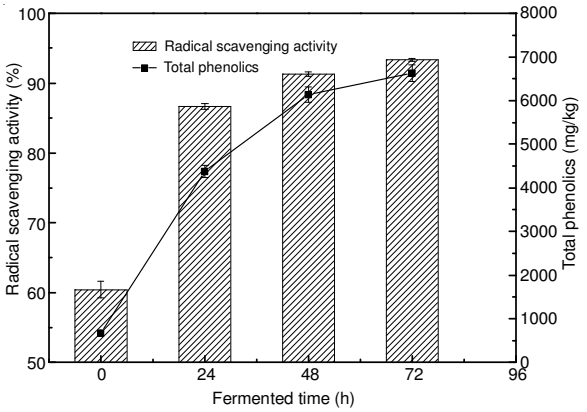

Fig. 2. Changes of total phenolic content and effect of radical scavenging activity of fermentation soybean during the fermentation

It has been reported that the antioxidant activity of plant materials strongly correlated with their content of the phenolic compounds, the increased antioxidative activity may be due to *B. subtilis* had the effect of increasing β-glucosidase and the aglycone contents during fermentation. Cho *et al.*[15] found that the starter *B. subtilis* CS90 can increase β-glucosidase activity of cheonggukjang. Even though specific key enzymes for enhanced phenolic compound were not elucidated. It was assumed that phenolic compounds produced by various enzymes during fermentation played an important role in the increased antioxidant activity.

The antioxidant effects of the lyophilized extract of cooked non-fermented soybean and fermented soybean, at different concentration on the DPPH radical scavenging activity were investigated (Fig. 3). At the concentration of 30 mg/mL, after 72 h, fermented soybean showed antioxidant activity with 95 %, while cooked non-fermented soybean showed 74 % antioxidant activity. At lower concentration, the lyophilized extracts of cooked non-fermented soybean and fermented soybean showed almost similar level of antioxidant activity. However, there were some reports that the methanol extracts of fermented soybean with *Aspergillus oryzane*, such as tempe, miso and koji, also had a better antioxidant than the non-fermented soybean[16-18].

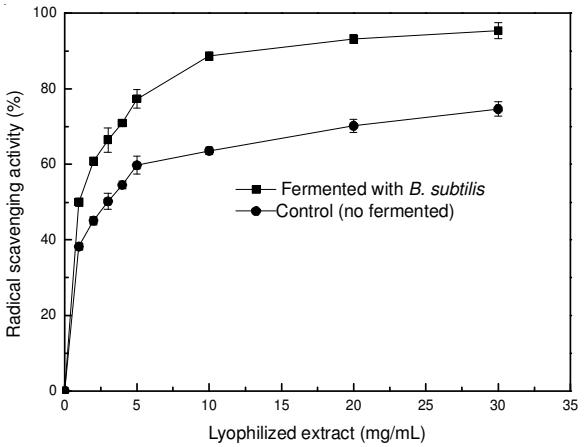

Fig. 3. Antioxidant activities of lyophilized extracts of cooked non-fermented and fermented soybeans in methanol

Reducing activity: The reducing activity indicates that these compounds are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes. In the present study, assay of reducing activity was based on the reduction of Fe^{3+}/ferricyanide complex to Fe^{2+} in the presence of reductant in the tested extract samples. The Fe^{2+} was then monitored by measure the formation of perl's Prussian blue at 700 nm[9].

The reducing activity of methanol extracts of fermented soybean during fermentation is shown in Fig. 4. Generally, it was found that the absorbance markedly increase from 0.97 to 2.81 after 72 h of the fermentation time. The increased reducing activity observed may be due to the formation of reductants that could react with free radicals to stabilize and terminate radical chain reaction during fermentation[19].

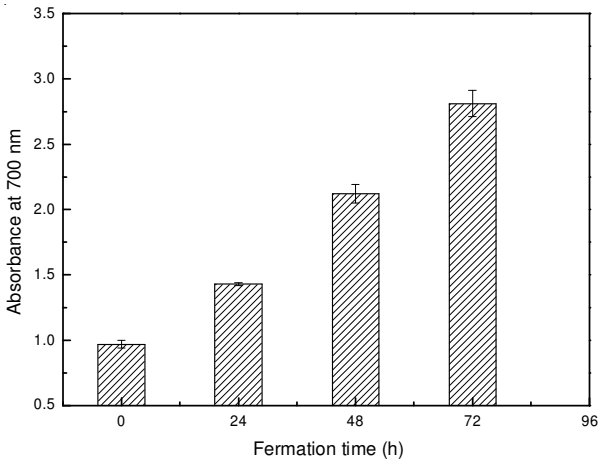

Fig. 4. Reducing activity of methanol extracts of fermented soybean at different fermentation time.

The dose-response curves for reducing activity of the lyophilized extract from cooked non-fermented soybean and fermented soybean is shown in Fig. 5. The reducing activity increased with the increasing concentration of the methanol lyophilized extract. At 30 mg/mL, after 72 h, methanol extracts of fermented soybean showed a higher absorbance at 700 nm with 2.7 than that of cooked non-fermented soybean with 2.2. Different amounts of reducing activity were observed with the soybean koji and kinema extracts, showed a higher absorbance than did the non-fermented soybean extracts at the same dosage level[11,20].

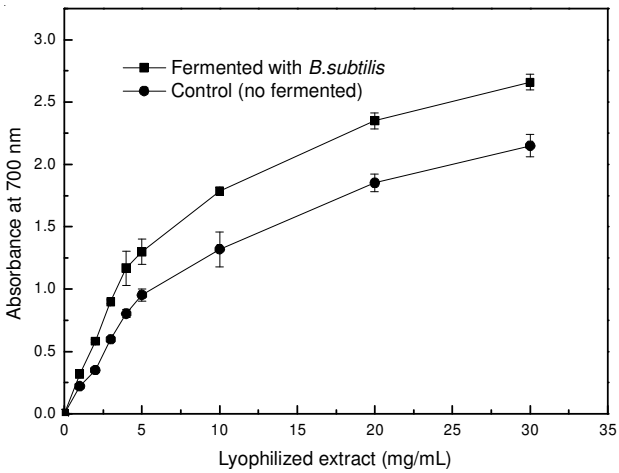

Fig. 5. Reducing power of the lyophilized extract from cooked non-fermented and fermented soybeans at different concentration

Ferrous ion chelating ability: Metal ions can initiate lipid peroxidation and start a chain reaction that leads to the deterioration of food. Ferrous ions, the most effective pro-oxidants, are commonly found in food systems[21]. In the present study, the chelating ability of methanol extracts from soybean fermentation product towards ferrous ions was investigated.

The ferrous ion chelating ability of methanol extracts from fermented soybean during fermentation is shown in Fig. 6. The ferrous ion chelating ability markedly increase with increase of fermentation time, at 30 mg/mL, after 72 h. The methanolic extracts of soybean fermentation product showed a higher ferrous ion chelating ability with 87.5 % than that of unfermented soybean with 31.2 %. Lin *et al.*[11] reported that the methanol extracts of fermented soybean prepared with *Asp. oryzae* exhibited a higher ferrous ion chelating ability than the extracts of cooked non-fermented soybean.

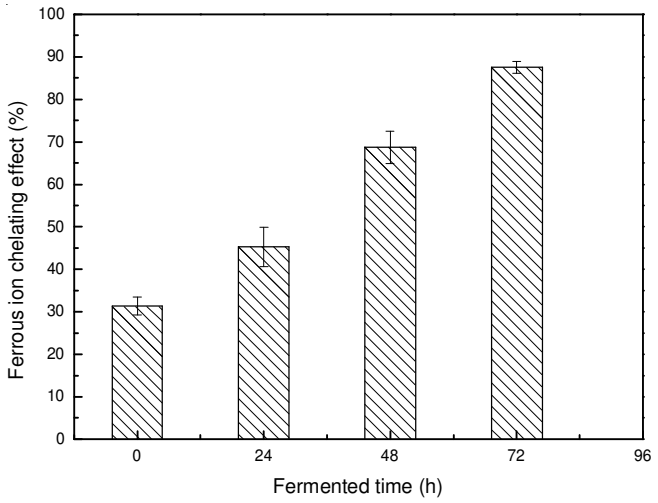

Fig. 6. Ferrous ion chelating effects of the lyophilized extract from fermented soybean at different fermentation time

The ferrous ion chelating ability of the lyophilized extract from cooked non-fermented soybean and fermented soybean is shown in Fig. 7. The ferrous ion chelating ability increased with the increase of the lyophilized extract of cooked non-fermented soybean and fermented soybean. At 30 mg/mL, after 72 h, methanol extracts of fermented soybean showed a higher ferrous ion chelating ability with 88.0 % than that of cooked non-fermented soybean with 66.2 %. The result showed that the ferrous ion chelating ability of cooked non-fermented soybean enhanced after fermentation, it is in consistence with the results obtained by Moktan *et al.*[21], where at the same dosage (30 mg/mL) the methanol extract of kinema prepared with *B. subtilis*, exhibited a higher ferrous ion chelating ability than the non-fermented soybean.

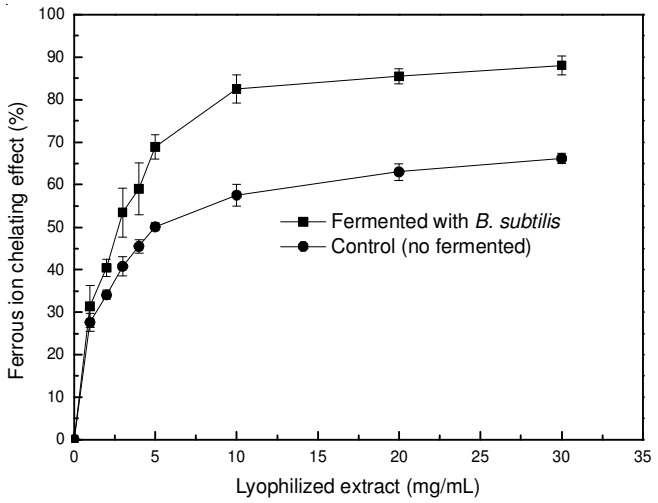

Fig. 7. Ferrous ion chelating effect of the lyophilized extract from cooked non-fermented soybean and fermented soybeans at different concentration.

Conclusion

The methanolic extracts of cooked non-fermented soybean and fermented soybean demonstrated that soybean fermentation prepared with *B. subtilis* enhanced free radical-scavening activity, reducing activity and ferrous ion chelating ability. All these antioxidant activity of fermented soybean increased with the increase of fermentation time and the concentration of methanolic extract, suggesting the fermentation with micro-organism play an important role in enhancing antioxidant activity. However, the characterization of the antioxidant compounds, their structure-activity relationship and possible mechanism remain to be investigated.

ACKNOWLEDGEMENTS

This work was supported by China Postdoctoral Science Foundation Grant (2012M520756) and Administration Office of Heilongjiang Postdoctoral Program (LBH-Z12111), from Chinese Postdoctoral Science Foundation Commission.

REFERENCES

1. S. Mathew and T.E. Abraham, *Food Chem*., **94**, 520 (2006).
2. H. Esaki, H. Onozaki and T. Osawa, in ed: M.T. Huang, Antioxidative Activity of Fermentation Soybean Products, Food Phyto-Chemicals for Cancer Prevention. Fruits and Vegetables, Washington DC; American Chemical Society, pp. 353-360 (1994).
3. H. Murakami, T. Asakawa, J.S. Terao and S. Matsushita, *Agric. Biol. Chem*., **48**, 2971 (1984).
4. J. Chen, Y.Q. Cheng, Y. Kohji and L.T. Li, *Food Chem*., **103**, 1091 (2007).
5. H.Y. Liang, J.J. Cheng and Y. Ma, *Chinese Food Sci*., **25**, 401 (2004).
6. T.J. Niu and Y. Ma, *China Brew*., **2**, 21 (2005).
7. V.L. Singleton and J.A. Rossi, *Am. J. Enol. Technol*., **32**, 936 (1965).
8. K. Shimada, K. Fujikawa, K. Yahara and T. Nakamura, *J. Agric. Food Chem*., **40**, 945 (1992).
9. M. Oyaizu, *Jpn. J. Nutr*., **44**, 307 (1986).
10. E. Decker and A.B. Welch, *J. Agric. Food Chem*., **38**, 674 (1990).
11. C.H. Lin, Y.T. Wei and C.C. Chou, *Food Microbiol*., **23**, 628 (2006).
12. C.C. Chyau, S.Y. Tsai, P.T. Ko and J.L. Mau, *Food Chem*., **50**, 2454 (2002).
13. G. Oboh and A.O. Ademosun, *J. Food Sci. Technol*., **49**, 729 (2012).
14. M.Y. Shon, K.I. Seo, S.W. Lee, S.H. Choi and N.J. Sung, *Korean J. Food Sci. Technol*., **32**, 936 (2000).
15. K.M.Cho, J.H.H. Lee, D. Yun, B.Y. Ahn, H. Kim and W.T. Seo, *J. Food Compos. Anal*., **24**, 402 (2011).
16. E. Berghofer, B. Grzeskowiad, N. Mundigler, W.B. Sentall and J. Walcak, *Int. J. Food Sci. Nutr*., **49**, 45 (1998).
17. H. Esaki, H. Onozaki and T. Osawa, *J. Agric. Food Chem*., **45**, 2020 (1997).
18. G.C. Yen, Y.C. Chang and S.W. Su, *Food Chem*., **83**, 49 (2003).
19. E.N. Herken and S. Guzel, *Int. J. Food Prop*., **13**, 1373 (2010).
20. J.H. Yang, J.L. Mau, P.T. Ko and L.C. Huang, *Food Chem*., **71**, 249 (2000).
21. B. Moktan, J. Saha and P.K. Sarkar, *Food Res. Int*., **41**, 586 (2008).