JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

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Fermentation with *Aspergillus awamori* Enhanced Contents of Amino Nitrogen and Total Phenolics as Well as the Low-Density Lipoprotein Oxidation Inhibitory Activity of Black Soybeans

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ABSTRACT: A solid fermentation was performed on black soybeans with *Aspergillus awamori*. The effects of fermentation on the contents of total phenolics and amino nitrogen and on the inhibitory effect on low-density lipoprotein (LDL) oxidation of black soybeans were examined. Results revealed that fermentation significantly enhanced the LDL oxidation inhibitory activity and total phenolics and amino nitrogen contents of black soybeans. The increased content of amino nitrogen was closely related to the enhanced LDL oxidation inhibitory activity of fermented black soybeans and its water extract. Fermentation temperature and length affected the LDL oxidation inhibitory effect exerted by the prepared fermented black soybeans. The *A. awamori*-fermented black soybean prepared at 30 °C for 3 days exhibited the highest inhibitory effect on LDL oxidation. The bioactive principles related to the inhibitory effect on LDL oxidation in black soybeans, regardless of fermentation, could be most efficiently extracted with water rather than 80% methanol or 80% ethanol.

KEYWORDS: black soybean, fermentation, total phenolics, amino nitrogen, LDL inhibitory activity

INTRODUCTION

Fermentation is a bioprocess frequently employed to produce bioactive compounds or enhance the functional properties of foodstuffs that are further used to prepare healthy food. For example, Natto, a popular Japanese health food, is prepared with the fermentation of cooked soybeans. This fermented product contains nattokinase, a clot-dissolving agent and angiotensin converting enzyme (ACE) inhibitor, which is capable of lowering blood pressure and produced by *Bacillus subtilis* during fermentation.[1,2] Pyo and Lee[3] reported that soybean after fermentation with *Monascas* possessed enhanced potential antioxidant capacity and ACE inhibitory activity. They indicated that extracts from these fermented soybeans could be used as multifunctional food additives. Additionally, fermentation leading to an enhancement of vitamin K_2 content and the superoxide dismutase activity of black soybeans was reported.[4] Furthermore, it was suggested that a nutritious weaning food could be formulated by mixing rice with *Rhizopus azygosprous*-fermented black soybeans.[5]

Black soybeans are cultivated and consumed in many countries throughout the world. In China, black soybeans are used to produce traditional fermented condiments such as *In-yu* black sauce and *In-si* or *Ttou-si*, the dried byproduct of the mash of black bean sauce.[6] Unlike normal soybeans, black soybeans have a darker seed coat, which contains anthocyanin.[7] Although similar to common soybeans, black soybeans are considered to be a nutritionally rich food with a plentiful supply of protein and calories. In addition to anthocyanin, other constituents of black soybean such as isoflavone, vitamin E, carotenoid, and saponin were all reported to exert biological activity.[7,8] Furthermore, black soybeans were also found to inhibit low-density lipoprotein (LDL) oxidation,[9] to reduce the incidence of DNA damage by chlophosphamide,[10] and to suppress the mutagenesis induced by various mutagens.[11,12]

In an attempt to develop healthy food, we have conducted a series of studies to investigate the effect of fermentation on the functional properties of black soybeans in the past few years. We found that the functional properties of black soybeans, such as 2,2-diphenyl-1-picrylhydrozyl (DPPH) radical-scavenging activity and anmutagenicity, were enhanced significantly after fermentation with *Aspergillus awamori*.[11,13] Additionally, the *A. awamori*-fermented black soybean (koji) was noted to contain higher amounts of aglycone, the bioactive isoflavone, than did unfermented black soybeans.[14]

In the present study, we further investigated the effect of fermentation with *A. awamori* on the LDL oxidation inhibitory effect of black soybeans. Because amino acids, peptides, and phenolics might relate to the LDL oxidation inhibitory action,[15−20] changes of these contents in black soybeans were also examined. Furthermore, the efficiency of solvents (water, 80% methanol, and 80% ethanol) used to extract the bioactive principles related to LDL oxidation inhibitory effect of fermented and nonfermented black soybean were compared.

MATERIALS AND METHODS

Black Soybeans and *A. awamori*. In the present study, black soybeans, purchased from the local market, were used as the fermentation substrate. The filamentous fungus, *A. awamori*, provided by Professor Yu, Graduate Institute of Food Science and Technology, National Taiwan University, was used as the starter organism for the fermentation of black soybeans.

Received: January 12, 2011
Revised: March 2, 2011
Accepted: March 3, 2011
Published: March 03, 2011

dx.doi.org/10.1021/jf2001684 | *J. Agric. Food Chem.* 2011, 59, 3974–3979

Preparation of Fermented Black Soybeans. The solid fermentation procedure described by Lee and Chou[14] was followed to prepare the fermented black soybeans. Briefly, whole black beans were washed and soaked in distilled water that was 6 times the weight of the beans. They were left overnight at room temperature. After the water had been decanted, the black soybeans were steam-cooked in an autoclave at 121 °C for 15 min. After cooling, the steamed black bean substrate (50 g) was inoculated with 1.0 mL of spore suspension (approximately 10^6/mL) of *A. awamori*. The inoculated black bean substrate, after a thorough mixing, was placed on a round screen with a 60 mesh and then incubated for 3 days at 30 °C and 95% relative humidity. When effects of fermentation temperature and time were studied, the inoculated black soybeans were incubated at 25, 30, or 35 °C for 3 days or incubated at 30 °C for a period of 0−5 days. After fermentation, The prepared fermented black soybean was then freeze-dried in a freeze-dryer (Free Dry System/Freezone 4.5, Labconco, MO) and homogenized.

Preparation of Solvent Extracts. To prepare the extracts, the ground powder of the fermented black soybeans or nonfermented black soybeans was extracted twice by shaking at 25 °C for 24 h with solvents at a ratio of 1:10 (w/v). The solvents with various polarities used included distilled water, methanol (80%), and ethanol (80%). The solvent extracts obtained with the same solvent were then combined. They were then filtered through Whatman no. 1 filter paper (Whatman, Maidstone, U,K,), vacuum concentrated, and freeze-dried.

Measurement of the Inhibitory Effect against Cu^{2+}-Induced Oxidation of Human LDL. The procedure described by Puhl et al.[21] with minor modifications was followed to determine the inhibitory effect on LDL oxidation. Briefly, venous blood sample was first collected from a healthy and overnight fasted adult volunteer (male, 28 years old) and kept in vacuum tubes containing EDTA (1 mg/mL). Plasma was obtained from blood by centrifugation at 1770*g* for 10 min at 4 °C. LDL (d = 1.019−1.063 g/mL) was then isolated from the plasma by sequential ultracentrifugation, which was carried out using a Beckman LE-80K ultracentrifuge and 70 Ti rotor (Beckman, Palo Alto, CA). After centrifugation, the isolated LDL was dialyzed against 5 mM phosphate-buffered saline (PBS, pH 7.4) in the dark and purged with nitrogen gas before use. The cholesterol content of the dialyzed LDL was determined according to the method of Richmond[22] with the CHOK-PAP enzymatic kit (Merck, Darmstadt, Germany).

To measure the LDL oxidizability, the cholesterol concentration of LDL was first diluted with PBS (5 mM) to give a final concentration of 150 μg/mL. The diluted LDL (100 μL) was incubated with a 10 μL sample (20 μg/mL in 10% ethanol) or positive controls, 130 μL of PBS and 10 μL of 125 μM $CuSO_4$. After mixing, the kinetics of LDL oxidation were measured by monitoring the absorbance of conjugated diene formation at 232 nm with a multidetection microplate reader (Synergy HT, BIO-TEC, Atlanta, GA) every 15 min at 30 °C for 12 h. The positive controls tested included daidzein (LC Laboratories, Woburn, MA), Trolox (Sigma-Aldrich Co.), gallic acid (Sigma-Aldrich Co.), and equol (Extrasynthèse, Genay, France).

Determination of Total Phenolic Content. The method described by Singleton et al.,[23] with minor modifications, was followed to examine the total phenolic content of samples. An aliquot of 0.1 mL of extract dissolved in DMSO (Merck) was added to 1.9 mL of deionized water and 1.0 mL of Folin−Ciocalteu phenol reagent (Sigma-Aldrich Co.). After further mixing with 5.0 mL of 20% Na_2CO_3, the mixture was kept at room temperature in darkness for 20 min. Absorbance was then measured at 735 nm. The measurement was compared to a standard curve of prepared gallic acid solution, and the results were expressed as milligrams of gallic acid per milligram of extract.

Determination of Amino Nitrogen Content and Dried Weight. The content of amino nitrogen was analyzed according to the formal titration method as described by AOAC.[24] The AOAC[24] method was also used to determine the dried weight.

Table 1. Various Solvent Extraction Yields of Black Soybean and *A. awamori*-Fermented Black Soybean

	extraction yield[a] (%, w/w)	
extraction solvent	nonfermented black soybean	fermented black soybean
80% methanol	16.01 ± 0.31 aB	12.06 ± 0.02 bB
80% ethanol	14.55 ± 0.38 aB	12.23 ± 0.39 bB
water	32.88 ± 1.06 bA	38.67 ± 1.38 aA

[a] Each value represents the mean ± SD (n = 3), and means in the same row with different letters (a, b) were significantly different by t test (P < 0.05). Means in the same column with different letters (A, B) were significantly different by Duncan's multiple-range test (P < 0.05).

Statistical Analysis. Mean values and standard deviations were calculated from the data obtained from the three separate experiments. Means were analyzed with unpaired two-tailed Student's t test and compared using Duncan's multiple-range test method in SAS, version 8 (SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Extraction Yields of Black Soybeans with or without Fermentation. It is indicated that no single solvent can extract all of the bioactive principles from food because of variations in polarity and solubility.[25,26] For that reason, the extraction yields of various solvents were first investigated. It is expected that various amounts of phenolic compounds, proteins, sugars, organic acids, and pigments may be present in the prepared solvent extracts.[26] As shown in Table 1, extraction yields of the nonfermented or fermented black soybean varied with the extraction solvent. Generally, the extraction yield of the 80% methanol extract and the 80% ethanol extract of the nonfermented or fermented black soybeans showed no significant difference (P > 0.05). Yields from water extract are significantly higher (P < 0.05) than other solvent extracts. Furthermore, fermentation with *A. awamori* resulted in a reduced extraction yield by 80% methanol extract and 80% ethanol extract of black soybean. On the contrary, the water extraction yield noted with the fermented black soybean (38.67%) was significantly higher (P < 0.05) than that (32.88%) with the nonfermented black soybean.

Fermentation Affects the Inhibitory Effect of Black Soybeans on LDL Oxidation. Steinberg et al.[27] indicated that oxidized LDL plays a key role in the atherosclerotic process and suggested that diets containing antioxidants might be useful therapy to reduce LDL oxidation and the progression of atherosclerosis. Figure 1 shows the effect of various solvent extracts of black soybean and *A. awamori*-fermented black soybean on LDL oxidation. Generally, three phases including (a) lag phase, (b) propagation phase, and (c) decomposition phase were noted when extract samples were examined. The oxidation lag time (minutes) is defined as the interval between initiation of oxidation and the intercept of the tangent for the slope of the absorbance curve during the propagation phase.[21] The lag time of LDL oxidation was found to be approximately 123 min (control), whereas, generally, an increased lag time ranging between 193 and 366 min was noted in presence of solvent extract. Table 2 shows the relative prolongation rate of various solvent extracts of black soybean and fermented black soybean. The relative propagation rate of samples was obtained by dividing the lag time of the sample by that of the control, which was set at 1.00. In comparison to the control, the solvent extracts

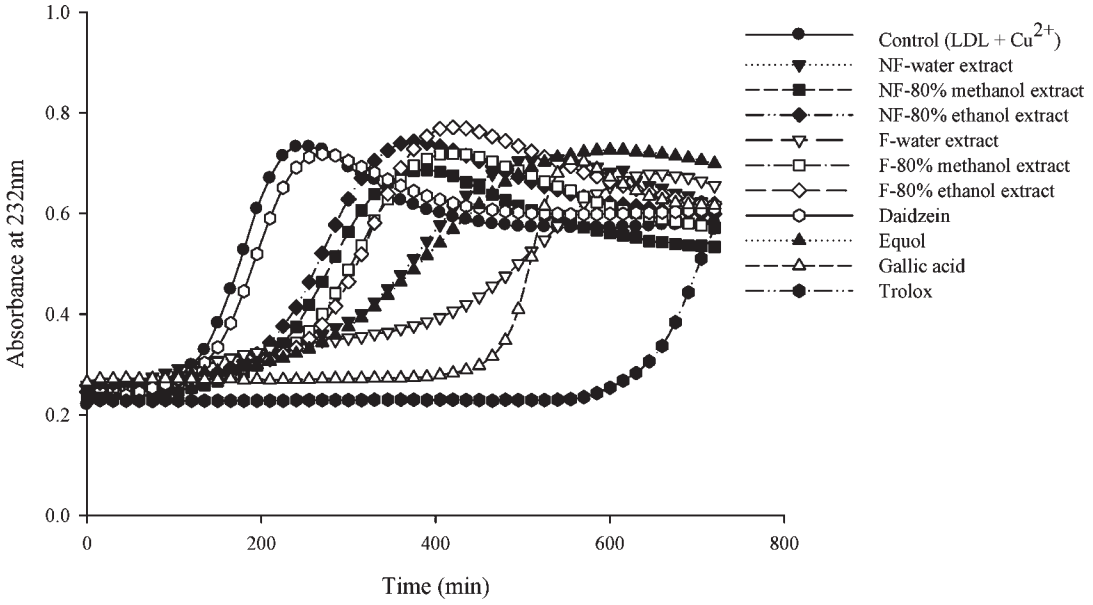

Figure 1. Effects of various extracts from black soybean and *A. awamori*-fermented black soybean on LDL oxidation. NF, nonfermented black soybean; F, fermented black soybean. The concentration of all the extracts was 20 μg/mL, whereas that of standards (positive controls) was 1 μg/mL.

Table 2. Inhibitory Effect of Various Extracts of Black Soybean and *A. awamori*-Fermented Black Soybean on LDL Oxidation

	inhibitory effect on LDL oxidation[a]	
sample (20 μg/mL)	relative prolongation rate[b] of nonfermented black soybean extract	relative prolongation rate of fermented black soybean extract
80% methanol extract	1.64 ± 0.07 bD	1.89 ± 0.04 aE
80% ethanol extract	1.57 ± 0.09 bD	2.06 ± 0.02 aD
water extract	2.16 ± 0.02 bC	2.98 ± 0.06 aC
control (LDL + Cu^{2+})	1	
daidzein (1 μg/mL)	1.33 ± 0.00 E	
equol (1 μg/mL)	2.07 ± 0.02 C	
gallic acid (1 μg/mL)	3.85 ± 0.03 B	
Trolox (1 μg/mL)	4.60 ± 0.06 A	

[a] Values are represented as the prolongation rate relative to control and the means ± SD (n = 3). Means in the same row with different letters (a, b) were significantly different by t test (P < 0.05) and in the same column with different letters (A–E) were significantly different by Duncan's multiple-range test (P < 0.05). [b] Relative prolongation rate was obtained by dividing the lag phase (min) of control. The value of control is regarded as 1.00, and the other samples are expressed as the relative values to the control.

of black soybean and fermented black soybean showed relative propagation rates of 1.57–2.16 and 1.89–2.98, respectively, depending on the solvent used for extraction. Regardless of extraction solvent, all of the extracts examined showed an inhibitory effect on LDL oxidation with a higher relative prolongation rate than that of control. Besides, the extract of fermented black soybean with a significantly higher relative prolongation rate (Table 2) exhibited a significantly higher (P < 0.05) inhibition on LDL oxidation than the respective extract of black soybean. Among the various extracts examined, the water extract of the fermented black soybean exhibited the highest inhibitory effect on LDL oxidation with the highest relative prolongation rate of 2.98. The LDL oxidation inhibitory effect noted with the nonfermented black soybeans is consistent with the results of Takahashi et al.,[9] who also indicated that LDL oxidation inhibitory effect exerted by the methanol extract of black soybeans was higher than that of yellow soybeans. Most importantly, the data shown in Table 2 demonstrate that the inhibitory effect of black soybeans on LDL oxidation can be further enhanced through fermentation with *A. awamori*.

Fermentation Temperature and Length Affect the LDL Oxidation Inhibitory Effect of Fermented Black Soybeans. Fermentation temperature and length have been reported to affect the antioxidative activity of the fermented products by various investigators.[17,28,29] Lee et al.[28] observed that the highest DPPH radical-scavenging effect of the *A. awamori*-fermented black soybeans could be obtained after fermentation at 30 °C for 3 days. Extending the fermentation time or conducting the fermentation at 25 or 35 °C resulted in reduced antioxidant activity. Zhu et al.[29] reported that the DPPH scavenging activity of Maitauza, a *B. subtilis* B_2-fermented soybean product, increased from the start of fermentation and reached its maximum after 24 and 12 h of fermentation, respectively. Further extending the fermentation period did not significantly increase the antioxidant activity. Therefore, the LDL oxidation inhibitory effect exerted by the water extract of fermented black soybeans, obtained with various fermentation temperatures and lengths, was further examined.

Table 3 shows the LDL oxidation inhibitory effect of the water extract and the relative LDL oxidation inhibitory effect of the fermented black soybean prepared with *A. awamori* at various temperatures for 3 days. It was found that the relative prolongation rate of the water extracts of fermented black soybeans varied with fermentation temperature. Regardless of fermentation temperature, they were all significantly higher (P < 0.05) than that of the unfermented black soybean extract. Besides, the black soybean extract fermented at 30 °C, showing the highest relative prolongation rate of 2.98, was found to exhibit the highest

Table 3. Effect of Fermentation Temperature on the Inhibitory Effect of *A. awamori*-Fermented Black Soybean on LDL Ixidation

	inhibition on LDL oxidation	
fermentation temperature (°C)	relative prolongation rate of extract[a] (20 μg/mL)	relative effect of fermented black soybean[b]
nonfermented	2.16 ± 0.02 D	1
25	2.53 ± 0.06 C	1.30
30	2.98 ± 0.06 A	1.62
35	2.65 ± 0.05 B	1.41
control (LDL + Cu^{2+})	1	

[a] Relative prolongation rate was obtained by dividing the lag phase (min) of control. The value of control is regarded as 1.00, and the other samples are expressed as relative values to the control. Each value represents the mean ± SD (n = 3), and means in the same column with different letters were significantly different by Duncan's multiple-range test (P < 0.05). [b] Relative antioxidative effects of black soybean and fermented black soybean were obtained by dividing the extract content added in experiment with extraction yield of the extract and then multiplying by relative prolongation rate. The value of minimum is regarded as 1.00, and the other extracts are expressed as relative values to the minimum.

Table 4. Effect of Fermentation Length on the Inhibitory Effect of *A. awamori*-Fermented Black Soybean on LDL Oxidation

	antioxidant activity on LDL oxidation	
fermentation period (day)	relative prolongation rate of extract[a] (20 μg/mL)	relative effect of fermented black soybean[b]
0	2.16 ± 0.02 F	1
1	2.27 ± 0.06 E	1.08
2	2.52 ± 0.06 C	1.22
3	2.98 ± 0.06 A	1.62
4	2.62 ± 0.03 B	1.42
5	2.40 ± 0.04 D	1.31
control (LDL + Cu^{2+})	1	

[a] Relative prolongation rate was obtained by dividing the lag phase (min) of control. The value of control is regarded as 1.00, and the other samples are expressed as relative values to the control. Each value represents the mean ± SD (n = 3), and means in the same column with different letters were significantly different by Duncan's multiple-range test (P < 0.05). [b] Relative antioxidative effects of black soybean and fermented black soybean were obtained by dividing the extract content added in experiment with extraction yield of the extract then multiplying by relative prolongation rate. The value of minimum is regarded as 1.00, and the other extracts are expressed as relative values to the minimum.

inhibitory effect on LDL oxidation among the various water extracts of the examined fermented black soybeans. Taking extraction yields into account (data not shown), the relative LDL oxidation inhibitory effect of fermented black soybean was calculated by multiplying the yield of water extract by the relative prolongation rate of the respective extract and compared with that of the nonfermented black soybean, which was designed 1.0. As shown in Table 3, the black soybeans fermented at 30 °C showed the highest relative inhibitory effect of 1.62 on LDL oxidation among the various fermented black soybeans prepared. This implies that the LDL oxidation inhibitory activity exerted by the same amount of black soybean fermented at 30 °C is 1.62-fold that of the nonfermented black soybean.

The effect of fermentation length on the LDL oxidation inhibitory effect exerted by the water extract of fermented black soybeans is shown in Table 4. The water extracts of black soybean fermented at 30 °C for various periods showed a relative prolongation rate ranging from 2.16 to 2.98 depending on fermentation length. With the highest relative prolongation rate, the water extract of fermented black soybeans prepared at 30 °C for 3 days showed the highest LDL oxidation inhibitory effect. Additionally, this 3-day-fermented black soybean also exhibited the highest relative inhibitory effect on LDL oxidation among the various fermented black soybeans prepared with different fermentation periods.

Fermentation Affects the Total Phenolic Content of Black Soybean. As shown in Table 5, the total phenolic content of black soybeans or fermented black soybeans varied with the extraction solvent. The observed phenomenon was consistent with the results of Zhao et al.[20] Total phenolic content was lowest in the water extract of black soybean, regardless of fermentation. Besides, the total phenolic content of the fermented black soybean extract is significantly higher (P < 0.05) than the respective extract of the nonfermented black soybeans. For example, the 80% methanol extract of fermented black soybean showed a total phenolic content of 42.61 mg gallic acid/g extract, which is significantly higher

Table 5. Total Phenolic Content of Various Black Soybean and *A. awamori*-Fermented Black Soybean Extracts

	mg gallic acid equivalent/g extract[a]	
extract	nonfermented black soybean	fermented black soybean
80% methanol	29.13 ± 0.81 bB	42.61 ± 1.03 aA
80% ethanol	35.06 ± 0.66 bA	38.59 ± 0.60 aB
water	23.80 ± 0.33 bC	28.29 ± 0.31 aC

[a] Each value represents the mean ± SD (n = 3), and means in the same row with different letters (a, b) were significantly different by t test (P < 0.05). Means in the same column with different letters (A–C) were significantly different by Duncan's multiple-range test (P < 0.05).

(P < 0.05) than that of 29.13 mg gallic acid/g extract noted with the respective extract of nonfermented black soybeans. Catalyzing the release of phenolics by β-glucosidase produced by *A. awamori* from black soybeans during fermentation might lead to the enhancement of total phenolic content.[13] In addition to the enhanced total phenolic content of the fermented black soybean noted in the present study, a higher content of aglycone and anthocyanin was found in fermented black soybeans than in nonfermented black soybeans.[13,14] These compounds possessed antioxidant activity.[15,17] Furthermore, aglycones (genistein and deidzein) showed stronger antioxidant activity than their glucosides (genistin and daidzin).[30] All may play a role in enhancing the antioxidant activity of the *A. awamori*-fermented black soybeans (Tables 2). It was reported that there was a close relationship between the content of total phenolic compounds and antioxidant activity.[26] Whereas we noted that the water extract of fermented and nonfermented black soybeans contained less total phenolics (Table 5), they showed higher antioxidant activity than the other solvent extracts (Table 2). It is therefore suggested that the phenolic compound is only a part of

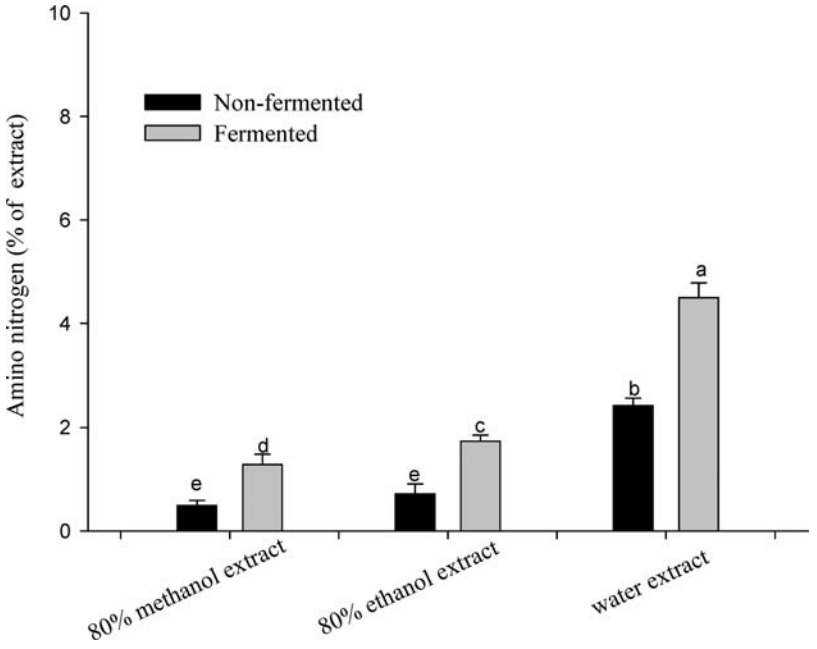

Figure 2. Amino nitrogen contents of various extracts of black soybean and *A. awamori*-fermented black soybean. Means (bars values) with different letters are significantly different by Duncan's multiple-range test ($P < 0.05$).

the total antioxidant ability in black soybeans and fermented black soybeans.

Fermentation Affects the Amino Nitrogen Content of Black Soybean. Peptides, especially histidine-containing peptides, and some amino acids such as lysine, glycine, valine, and histidine were reported to show antioxidant activity.[16,18,19] For that reason, the amino nitrogen content of the various solvent extracts of fermented black soybeans was examined with the results shown in Figure 2. A higher amino nitrogen content implied a higher degree of protein hydrolysis and higher contents of amino acid and peptides in the sample. It was found that fermentation resulted in the increased content of amino nitrogen in the extracts of black soybeans, regardless of extraction solvent. Apparently, this was due to the degradation of protein into peptides and amino acids through the action of protease produced by starter organisms during the fermentation. A similar phenomenon has also been observed for some oriental fermented products of soybeans.[31,32] As shown in Figure 2, it was noted that the amino nitrogen content of the extracts varied with the extraction solvent. The water extract contained a significantly ($P < 0.05$) higher content of amino nitrogen than other solvent extracts. Additionally, the water extract of fermented black soybeans contained the highest amino nitrogen content among the various solvent extracts examined. The high amount of peptides and amino acids detected in the water extract of black soybeans and nonfermented black soybeans was closely related to the high LDL oxidation inhibitory effect (Tables 2). Therefore, in accordance with the observation of Iwai et al.,[33] peptides and amino acids, in addition to phenolic compounds, are likely to play a major role in enhancing the LDL inhibitory effect of the water extract from fermented black soybeans.

On the basis of the data obtained, it is concluded that fermentation with *A. awamori* could further significantly ($P < 0.05$) increase the inhibitory LDL oxidation effect of black soybeans. The highest enhancement of the LDL oxidation inhibitory activities was obtained with the fermented black soybean prepared at 30 °C for 3 days. Water used as the solvent could most efficiently extract the bioactive principles related to the LDL oxidation inhibitory effect of black soybeans. In addition to phenolic compounds, increases of peptides and amino acids played an important role in the enhancement of antioxidant activity exerted by black soybeans, regardless of fermentation. In light of the enhanced aglycone content and antimutagenicity reported previously[11,14] and the results obtained in the present study, it is suggested that fermentation can be employed as a useful tool to enhance the functional properties of black soybeans. The fermented black soybeans may thus be used to develop food ingredients and diets with healthy and therapeutic values.

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Funding Sources

This research was financially supported by The National Science Council, ROC (Taiwan) (NSC 97-2313-B-002-015-MY3).

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