



The Comparative Evaluation of Fermented and Non-fermented Soybean Extract on Antioxidation and Whitening

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The present study was performed to compare the antioxidative and whitening activities of fermented soybean extract (FSB) and non-fermented soybean extract (SB). Antioxidative and whitening activities of FSB and SB were evaluated by the determination of DPPH, superoxide radical and hydroxyl radical scavenging activities, linoleic acid inhibition activity, and tyrosinase inhibition activity. FSB showed the higher effect than SB in the antioxidative activities. Also FSB showed the better effect than SB in whitening activity. These results demonstrated that the fermentation played a more excellent role than the non-fermentation in antioxidation and whitening. Therefore, this study suggested that FSB could be a useful cosmetic ingredient for antioxidation and skin whitening.

Key words: Soybean, Fermentation, Antioxidation, Whitening, Cosmetic ingredient

INTRODUCTION

Nowadays, the skin aging is attracting growing interest because the preservation of the youthful appearance of skin is the prerequisite for beauty (Rittié and Fisher, 2002; Zouboulis and Makrantonaki, 2011).

Skin aging was caused by various environmental, physical, chemical and mechanical insults (Makrantonaki and Zouboulis, 2008). It was divided into intrinsic aging and photoaging. Intrinsic aging of the skin is a naturally occurring process relating to chronological age, and photo aging results from cumulative sun exposure. It is commonly associated with increased dryness, roughness, laxity, dyschromia, telangiectasia, wrinkles, and irregular pigmentation (Trookman *et al.*, 2009; Zhong *et al.*, 2011).

The causes and mechanisms of intrinsic aging are far less understood than photoaging (Oender *et al.*, 2008). Photoaging of the skin is mediated by the sensitization of reactive oxygen species (ROS) and lipid peroxides (Miyachi, 1995; Peres *et al.*, 2011). Among several reactive oxygen species, superoxide and hydroxyl radicals play important roles in photoaging (Kim *et al.*, 2011).

Recently, many studies have focused on the development of the ROS- eliminating natural antioxidants which are not

harmful to human body and have strong antioxidative effects (Kim *et al.*, 2010). Soybean (*Glycinemax* (L.) Merrill) is one of the major crops containing antioxidant components such as phenolic acids, tocopherols, phytic acids, trypsin inhibitor, amino acid and isoflavones containing daidsein, genistein, daidzin and genistin (Hayes *et al.*, 1977; Lee *et al.*, 2008). It has been reported that these nutritional components in soybean were associated with human health benefits such as decreased risks of various cancers, heart disease, cardiovascular disease, and increased antioxidative effects (Choi *et al.*, 2010; Wang *et al.*, 2008).

Fermentation has been reported to cause a general improvement in the natural nutritive values of soybean products, increasing total soluble solids, vitamins, free fatty acids, soluble nitrogen, free amino acids and vitamins E and C (Frias *et al.*, 2005; Wang *et al.*, 2011). For example, the antioxidative activities of fermented soybean products such as miso, tempeh and natto, inoculated with *Aspergillus oryzae*, *Rhizopus oligosporum* and *Bacillus natto* were significantly higher than those of non-fermented steamed soybean (Lin *et al.*, 2006).

The present study was performed to make the comparative evaluation on the antioxidation and whitening activities of fermented soybean extract (FSB) and non-fermented soybean extract (SB). Antioxidation and whitening activities of FSB and SB were investigated by the determination of DPPH, superoxide radical and hydroxyl radical scavenging activities, linoleic acid inhibition activity and tyrosinase inhibition activity.

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MATERIALS AND METHODS

Strains and growth conditions. *Bacillus subtilis* 168 (ATCC 33234, KCTC 2217) was obtained from Korean Culture Center of Microorganisms (KCCM). *B. subtilis* 168 were maintained in Luria-Bertani broth (LB Difco Laboratories, Detroit, MI) at 35°C.

Fermentation and extraction. 20 g of soybean (SB) was sterilized in the autoclave for 15 min and *Bacillus subtilis* 168 was proliferated for 3 hr. The sterilized SB was inoculated with 1% of *Bacillus subtilis* 168 at absorbance of 1.0 at 600 nm, and the inoculated SB was fermented at 40°C for 36 hr. The fermented SB was extracted by reflux-condensing in 80% ethanol for 4 hr. The extract of SB was concentrated with an evaporator and freezing-dried. The non-fermented SB was extracted by reflux-condensing in 80% ethanol for 4 hr. Then it was concentrated with an evaporator and freezing-dried.

Measurement of free radical scavenging activity. The free radical scavenging activity was evaluated by method of Blois (Zhu *et al.*, 2001). One ml of sample and 0.5 ml of 1,1-diphenyl-2-picrylhydrazyl (DPPH) alcoholic solution (0.2 mM) were mixed and incubated in a test plate at 37°C for 10 min. The absorbance at 517 nm was measured on automated microplate reader Synergy HT (Bio-Tek instruments Inc, Vermonts, USA).

Measurement of O₂⁻ scavenging activity. This test was measured by method of superoxide radical scavenging (Kang *et al.*, 1959). 10 µl of FSB and SB, 130 µl of 50 mM potassium phosphate buffer, and 10 µl of 20 mM menadion (Sigma-Aldrich, St. Louis, MO), were mixed and to which was added 50 µl of DCFH (Sigma-Aldrich, St. Louis, MO) solution. Fluorescence of DCF was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm for 30 min on microplate fluorescence spectrophotometer Synergy HT (Bio-Tek instruments Inc, Vermonts, USA).

Measurement of OH scavenging activity. This test was measured by method of hydroxyl radical scavenging (Kang *et al.*, 1959). 10 µl of FSB and SB, and 190 µl of reagent A (540 µl of 10 mM FeSO₄ and 20 ml of 1.35 mM H₂O₂ were mixed) were mixed and to which was added 50 µl of DCFH solution. Fluorescence of DCF was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm for 40 min on microplate fluorescence spectrophotometer Synergy HT (Bio-Tek instruments Inc, Vermonts, USA).

Autoxidative effect using linoleic acid. This test was measured by method of autoxidation (Cho *et al.*, 2006). The

sample were settled in 1:250 volumes of D.D.W. 0.75 ml of pre-treated sample was added 3 ml of 10 mM linoleic acid (Fluka, Buchs, Switzerland) and the solution was left at 4°C for 24 hours. 0.1 ml of its were added to 4.7 ml of 75% ethanol, 0.1 ml of 30% ammonium thiocyanate (Sigma-Aldrich, St. Louis, MO), and 0.1 ml of 20 mM iron chloride (I) (Sigma-Aldrich, St. Louis, MO). And then, it was kept at room temperature for 3 min. The absorbance at 500 nm was measured on automated microplate reader Synergy HT (Bio-Tek instruments Inc, Vermonts, USA).

Inhibition of tyrosinase activity. 15 µg of sample was add to 150 µl of 0.1 M PBS (pH 6.86), 2.5 µl of 1.5 mM L-tyrosinase (Sigma-Aldrich, St. Louis, MO) and 7 µl of mushroom tyrosinase (2,380 unit/ml), (Sigma-Aldrich, St. Louis, MO) at Elisa' well. The absorbance at 490 nm was measured on automated microplate reader Synergy HT (Bio-Tek instruments Inc, Vermonts, USA). And then, it was kept at 30°C for 3 min and absorbance at 490 nm was measured on automated microplate reader Synergy HT (Bio-Tek instruments Inc, Vermonts, USA).

$$\text{Inhibition activity (\%)} = \frac{(\text{A control} - \text{A sample})}{\text{A control}} \times 100$$

Statistics. All data were expressed as mean ± SD. Statistical analysis was performed using one-way ANOVA followed by Duncan's post hoc test (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Optimal condition of fermentation from *Bacillus subtilis* 168 in soybean extract. The optimal condition of fermentation was established by DPPH radical scavenging

Table 1. DPPH radical scavenging effect of fermented soybean extract

Time	DPPH radical scavenging effect (%)	
	FSB at 40°C	FSB at 30°C
6	52.17 ± 1.27 ^a	35.58 ± 1.41 ^b
12	59.90 ± 0.84 ^b	35.12 ± 1.41 ^b
24	64.50 ± 0.00 ^c	58.75 ± 0.98 ^f
30	81.76 ± 1.55 ^g	69.25 ± 0.42 ^g
34	80.07 ± 0.84 ^f	74.08 ± 1.27 ^h
36	83.50 ± 0.56 ^h	60.12 ± 0.56 ^f
48	79.46 ± 1.27 ^f	43.16 ± 0.70 ^e
54	72.50 ± 1.12 ^e	31.62 ± 0.65 ^a
60	74.00 ± 0.28 ^e	38.25 ± 0.28 ^{cd}
66	69.50 ± 1.41 ^d	39.16 ± 0.14 ^d
72	60.00 ± 0.00 ^b	37.15 ± 0.02 ^c

a, b, c, d, e, f, g, h are different group by one-way ANOVA followed by Duncan's post hoc test.

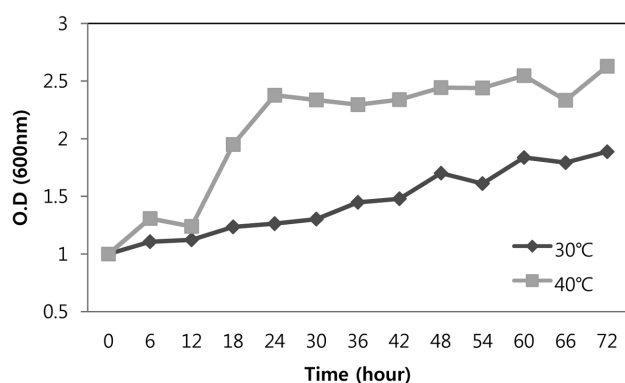


Fig. 1. *Bacillus subtilis*168 growths of fermented soybean extract. O.D : optical density, variation of O.D at 40°C (■), variation of O.D at 30°C (◆).

activity. DPPH free radical scavenging activities of fermented SB (FSB) at temperature and time are shown in Table 1. FSB at 40°C showed much higher antioxidative activity than FSB at 30°C. The highest free radical scavenging effect was 83.5% at 36 hr. So the optimal condition of fermentation was 40°C and 36 hr. Cell growth rates at different fermentation temperature in SB fermented by *Bacillus subtilis*168 are shown in Fig. 1. The rates of cell growth at 40°C are higher than those of cell growth at 35°C.

The comparative evaluation of fermented soybean (FSB) and non-fermented soybean (SB) on antioxidation.

Free radicals are known to be a major factor in biological damages and DPPH has been used to evaluate the free radical scavenging activity of natural antioxidants (Yokozaawa *et al.*, 1998). DPPH radical scavenging activities of FSB, SB, and vitamin C were presented in Table 2. They showed the increasing scavenging activity in a dose-dependent manner from 1 to 100 µl/ml of FSB, SB, and vitamin C. DPPH scavenging activity of FSB was 20% higher than SB. In comparison with vitamin C (1 µl/ml), FSB (1 µl/ml) was much higher. It was reported that fermented *Codonopsis lanceolata* increased DPPH radical scavenging activity, compared to non-fermented *Codonopsis lanceolata* (Park, 2009). Antioxidative effect was showed in proportion to

Table 2. DPPH scavenging activity of fermented soybean extract, non-fermented soybean extract and vitamin C

Concentration (µl/ml)	DPPH scavenging activity (%)		
	FSB	SB	Vitamin C
100	78.60 ± 0.38 ^d	57.16 ± 0.35 ^a	83.39 ± 1.02 ^c
10	74.33 ± 3.06 ^c	55.82 ± 0.79 ^a	83.01 ± 1.39 ^c
1	72.60 ± 3.71 ^c	55.44 ± 0.26 ^a	66.79 ± 1.02 ^b

FSB: fermented soybean extract, SB: non-fermented soybean extract, Vitamin C : positive control, a, b, c, d, e are different group by one-way ANOVA followed by Duncan's post hoc test.

Table 3. O₂⁻ scavenging activity of fermented soybean extract, non-fermented soybean extract and vitamin C

Concentration (µl/ml)	O ₂ ⁻ scavenging activity (%)		
	FSB	SB	Vitamin C
100	89.73 ± 1.13 ^g	45.72 ± 0.00 ^e	76.41 ± 0.36 ^f
10	80.14 ± 0.00 ^f	30.26 ± 4.93 ^b	69.39 ± 1.32 ^e
1	48.79 ± 5.70 ^{cd}	18.58 ± 1.13 ^a	53.36 ± 2.97 ^d

FSB: fermented soybean extract, SB: non-fermented soybean extract, Vitamin C: positive control, a, b, c, d, e, f, g are different group by one-way ANOVA followed by Duncan's post hoc test.

cell growth rate (Choi *et al.*, 2008) This study demonstrated the similar pattern of the result.

Superoxide radical generates metabolic processes of many biological cells (Masaki *et al.*, 1995). Superoxide anion radical is a precursor to active free radicals that have potential of reacting with biological macromolecules and thereby inducing tissue damage (Gülçin, 2006). Xanthine oxidase response with xanthine conversing uric acid, resulting superoxide radical (Kuppusamy and Zweier, 1989). The superoxide radical scavenging activities of FSB, SB, and vitamin C are shown in Table 3. FSB showed the higher scavenging activity of superoxide radical than SB and vitamin C.

Hydroxyl radical was one of the highest reactive free radical, which was known to play important role in oxidation of various tissue and cell membrane *in vivo* (Heo and Wang, 2008). Hydroxyl radical scavenging activities are shown in Table 4. Scavenging activities of FSB in all kinds of concentrations were higher than SB and vitamin C. It was reported that the increase of radical scavenging activity resulted from physiological active substance such as peptide, maillard browning material and mucilages during the fermentation with multiple mechanisms (Shon *et al.*, 2007).

Autoxidative inhibition effect on linoleic acid. Lipid peroxidation can be catalyzed by enzymes (lipoxygenases, cyclooxygenases) or initiated by sources of reactive oxygen species (Goupy *et al.*, 2007). Linoleic acid also influences skin pigmentation by stimulating epidermal turnover and increased desquamation of melanin pigment from the

Table 4. OH⁻ scavenging activity of fermented soybean extract, non-fermented soybean extract and vitamin C

Concentration (µl/ml)	OH ⁻ scavenging activity (%)		
	FSB	SB	Vitamin C
100	79.19 ± 0.39 ^f	44.76 ± 1.52 ^c	65.66 ± 1.26 ^c
10	63.85 ± 4.17 ^f	36.54 ± 3.60 ^b	32.35 ± 0.23 ^b
1	51.93 ± 2.89 ^d	30.22 ± 5.13 ^{ab}	24.17 ± 1.57 ^a

FSB: fermented soybean extract, SB: non-fermented soybean extract, Vitamin C: positive control, a, b, c, d, e, f are different group by one-way ANOVA followed by Duncan's post hoc test.

Table 5. Inhibition of autoxidation by using linoleic acid of fermented soybean extract, non-fermented soybean extract and vitamin C

Concentration ($\mu\text{l}/\text{ml}$)	Inhibition of autoxidation by using linoleic acid (%)		
	FSB	SB	Vitamin C
100	79.60 \pm 0.38 ^d	57.16 \pm 0.35 ^a	83.78 \pm 1.09 ^e
10	74.33 \pm 3.06 ^c	55.85 \pm 0.79 ^a	82.43 \pm 1.36 ^e
1	72.60 \pm 3.71 ^c	55.44 \pm 0.26 ^a	66.79 \pm 1.02 ^b

FSB: fermented soybean extract, SB: non-fermented soybean extract, Vitamin C: positive control, a, b, c, d, e are different group by one-way ANOVA followed by Duncan's post hoc test.

epidermis (Ebanks *et al.*, 2009). Table 5 shows the inhibition effect of linoleic acid of FSB, SB, and vitamin C. FSB showed the higher inhibition activity of linoleic acid than SB, but the lower than vitamin C.

Inhibitory effect on tyrosinase. Tyrosinase (monophenol,3,4-dihydroxyphenylalanine: oxidants, E.C.1.14.18.1), which catalyses the synthesis of the melanin precursor dihydroxyphenylalanine (DOPA), has been reported to be specific for melanocytes (Brichard *et al.*, 1993). The inhibitory effects of FSB, SB, and vitamin C are shown in Table 6. Tyrosinase catalyzed biosynthesis of melanin which the hydroxylation of monophenols to o-phenols (monophenolase activity), and the oxidation of the o-phenols to o-quinones (diphenolase activity), both using molecular oxygen (Nerya *et al.*, 2004). Antioxidants was considered preventing skin damage by theses oxidation progress. The inhibitory effects of FSB were higher than those of SB in the dose-dependent manner, which suggested that FSB could play a role in the skin whitening by the inhibition of melanin production and supported previous reports that antioxidants are good inhibitors of tyrosinase.

Collectively, on the basis that the fermented soybean extract had the better effects than the non-fermented soybean extract in DPPH scavenging activity, superoxide radical, hydroxyl radical, inhibition of autoxidation and inhibitory tyrosinase, it could be concluded that the fermentation played the important role in the improvement of antioxidant

Table 6. Tyrosinase inhibition of fermented soybean extract, non-fermented soybean extract and vitamin C

Concentration ($\mu\text{l}/\text{ml}$)	Tyrosinase inhibition (%)		
	FSB	SB	Vitamin C
100	75.55 \pm 0.38 ^d	53.21 \pm 4.36 ^b	79.09 \pm 0.58 ^d
10	72.22 \pm 3.06 ^d	52.14 \pm 4.56 ^b	73.24 \pm 0.44 ^d
1	32.22 \pm 3.71 ^a	50.71 \pm 6.06 ^b	60.86 \pm 2.52 ^c

FSB: fermented soybean extract, SB: non-fermented soybean extract, Vitamin C: positive control, a, b, c, d are different group by one-way ANOVA followed by Duncan's post hoc test.

tion and skin whitening.

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