JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Antioxidant Activities of Black and Yellow Soybeans against Low Density Lipoprotein Oxidation

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Several studies have demonstrated that the daily intakes of soy foods were associated with a reduced cardiovascular risk. The aim of our study was to investigate the inhibitory effect of black soybeans on low density lipoprotein (LDL) oxidation in comparison to yellow soybeans. The extract from black soybean had a longer LDL oxidation lag time than that from yellow soybean (205 ± 16 and 65 ± 3 min, respectively). When both soybeans were divided into the seed coat and the mixture of the germ and cotyledon, the diluted extract solution from the black soybean seed coat prolonged the lag time significantly more than the original extract of the yellow soybean seed coat. On the other hand, antioxidant effects of the extract from the mixture of germs and cotyledons were similar in both soybeans. Regarding total polyphenol contents, the seed coat of black soybean had a higher polyphenol content than that of yellow soybean (29.0 ± 0.56 and 0.45 ± 0.02 mg/g, respectively). Interestingly, the mixture of the germ and cotyledon hydrolyzed by β-glucosidase in both soybeans showed a stronger inhibitory effect on LDL oxidation than that before being hydrolyzed by β-glucosidase. These results suggest that black soybeans may be more effective in inhibiting LDL oxidation than yellow soybeans because of total polyphenols contents in its seed coat. In addition, aglycones, which are rich in soybeans fermented or hydrolyzed by β-glucosidase, may play a crucial role in the prevention of oxidation-related diseases.

KEYWORDS: Soybean; LDL oxidation; free radical; aglycone; glucoside; polyphenol

INTRODUCTION

Soybeans and soy products, which are very rich in isoflavones and soy protein, are very popular foods in Japan. Previous studies have shown that daily intakes of soy foods were associated with a reduced cardiovascular risk, such as lower lipids and lipoproteins levels (*1*−*6*), lower blood pressures (*7*, *8*), lower homocysteine levels (*8*, *9*), and an improvement in both arterial compliance and the endothelial function (*10*−*13*).

Oxidized low density lipoprotein (LDL) is considered to play a key role in the atherosclerotic process (*14*). The intake of dietary antioxidants may be a useful therapy to prevent LDL oxidation and the progression of atherosclerosis. Soy isoflavones have been reported to decrease the LDL oxidizability both in vitro and ex vivo (*8*, *15*−*19*). Such studies in soybeans have been conducted with normal soybeans, which have a yellow seed coat, although antioxidant effects of soybeans with a darker seed coat on LDL oxidation have not yet been elucidated. Interestingly, the seed coat in black soybeans contains anthocyanins (*20*), which are a kind of polyphenol and is known to scavenge free radicals (*21*). Our study was thus carried out to evaluate the antioxidant effect of yellow and black soybeans on LDL oxidizability.

MATERIALS AND METHODS

Plant Materials. Black soybean seeds (Hyoukei-kuro-3goh) and yellow soybean seeds (Ryuhou) were obtained from the Hokubu Agricultural Technology Institute (Hyogo, Japan) and the Japan Speciality Agriculture Products Association (Tokyo, Japan), respectively. Both soybean seeds were harvested in 1999. The soybean seeds were freeze-dried and then divided into the seed coat and the mixture of the germ and cotyledon by hand. The separated samples were ground up and stored at −80 °C until analysis.

Extraction of Antioxidants from Whole Soybeans and Seed Coats. One hundred milligrams of powdered soybean seeds was sonicated for 20 min with 2 mL of 1% (v/v) trifluoroacetic acid (TFA; Wako Pure Chemical Industries, Ltd., Osaka, Japan) in methanol and extracted for 24 h at 4 °C. After centrifugation (1500*g*, 10 min, 4 °C), the supernatant was collected as crude extracts containing antioxidants. The residue was extracted again by the above protocol. The collected supernatant (1 mL) was evaporated and dissolved in 0.2 mL of 80% (v/v) acetone for analysis. This protocol was also used for extraction of the crude pigment from seed coat. This original extract from the

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10.1021/jf048062m CCC: $30.25
Published on Web 04/26/2005

seed coat of black soybeans was diluted by phosphate-buffered saline (PBS) from 1:10 to 1:30 in order to use in LDL oxidizability assay.

Extraction of Antioxidants from the Mixture of Germs and Cotyledons. The mixture of germs and cotyledons (100 mg) was sonicated for 20 min with 2 mL of 1% (v/v) TFA in methanol and centrifuged (1500*g*, 10 min, 4 °C). The supernatant was collected as crude extracts of the germ and cotyledon. The residue was re-extracted three times by the above protocol. The collected supernatant (1.5 mL) was evaporated and dissolved in 0.2 mL of 80% (v/v) acetone. After centrifugation, the clear supernatant was used for analysis.

Extraction of Aglycones from the Mixture of Germs and Cotyledons. The crude extract from the mixture of germs and cotyledons (1.5 mL) was dried under nitrogen and placed at room temperature for 10 min with 0.5 mL of 0.2 M acetate buffer (pH 4.0). This sample solution was hydrolyzed overnight at 37 °C with 0.3 mL of enzyme solution containing 150 mg of ascorbic acid, 10 mL of 0.2 M acetate buffer, and 0.5 mL of β-glucosidase (Sigma Chemical Co., St. Louis, MO). Five milliliters of cooled diethyl ether was added to the solution and centrifuged (1500*g*, 10 min, 4 °C) after being shaken by a hand for 1 min. The ether phase was collected as the solution containing aglycones. Diethyl ether added to the water phase again was extracted twice according to the above protocol. The evaporated ether solution (1.5 mL) was dissolved in 0.2 mL of 80% (v/v) acetone for analysis.

Free Radical Scavenging Activity. The free radical scavenging activity was determined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH, Wako Pure Chemical Industries, Ltd.) (*22*). An aliquot of the extracts from the germ and cotyledon was mixed with 2 mL of 0.1 mM DPPH in ethanol. Following incubation for 20 min at 37 °C, the absorbance was measured at 516 nm with a Beckman model DU 650 spectrophotometer.

Isolation and Preparation of LDL. Plasma samples were obtained from fasting normolipidemic volunteers with their informed consent. LDL was separated by single-spin density gradient ultracentrifugation (417000*g*, 40 min, 4 °C) using a TLA-100.4 fixed angle-rotor (Beckman Instruments Inc. CA). The LDL protein concentration was determined using a Micro BCA Protein Assay Kit (Pierce Laboratories Inc., Rockford, IL). Before the start of the oxidation experiments, LDL samples were diluted with PBS to give a final concentration of 70 μg/mL LDL protein.

Measurement of LDL Oxidizability. The LDL oxidizability was measured according to our previous report (*23*). The prepared LDL samples were oxidized with or without 5 μL/mL extracted solution from soybeans by 400 μM 2,2′-azobis(4-methozy-2,4-dimethylvaleronitrile) (AMVN−CH_3O; Wako Pure Chemical Industries, Ltd.), which is an oxidative inducer. The kinetics of LDL oxidation were obtained by monitoring the absorbance of conjugated dienes formation at 234 nm with a Beckman model DU 650 spectrophotometer at 4 min intervals at 37 °C. The lag time of lipid peroxidation is defined as the time interval between the initiation and the intercept of the two tangents drawn to the lag and propagation phase of the absorbance curve at 234 nm, and it was expressed in min.

Determination of Total Polyphenol Content. The total polyphenol content was determined by a Folin−Ciocalteu assay using (+)-catechin (Sigma Chemical Co.) as the standard. The mixture of sample solution (50 μL), distilled water (3 mL), 250 μL of Folin−Ciocalteu's reagent solution (Nakaraitesuku Co., Kyoto, Japan), and 10% (v/v) $NaCO_3$ (750 μL) was incubated for 8 min at room temperature, and then, 950 μL of distilled water was added. After the mixture stood for 2 h at 20 °C, the absorbance of 750 nm was measured. The total polyphenol content was expressed as a (+)-catechin equivalent.

Anthocyanin Analysis from Black Soybean Seed Coats. The cyanidin-3-O-glucoside of the seed coat in black soybean was carried out by HPLC using a Develosil ODS-HG-5 column (4.6 mm × 250 mm, Nomura Chemical Co. Ltd., Aichi, Japan) with a visible detector at 520 nm (Shimadzu Co., Kyoto, Japan) (*24*). The linear gradient was run from acetonitrile/0.01% TFA (1:9, v/v) to acetonitrile/0.01% TFA (5:5, v/v) for 40 min at a flow rate of 0.8 mL/min. The cyanidin-3-O-glucoside chloride was used as the standard.

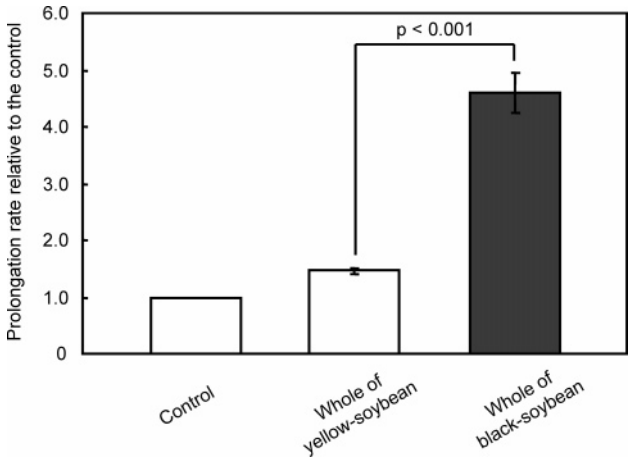

Figure 1. Antioxidant activities of crude extracts from the whole yellow and black soybeans on LDL oxidation. The values are represented as the prolongation rate relative to control and the means ± SD ($n = 5$).

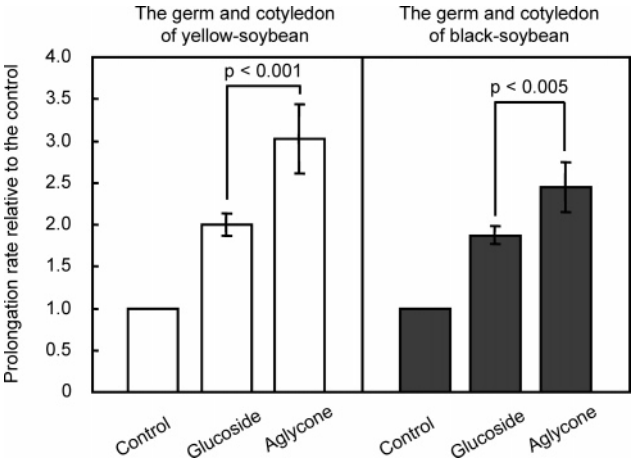

Figure 2. Antioxidant activities of the germ and cotyledon mixtures before and after β-glucosidase digestion in each soybean on LDL oxidation. The extract before and after β-glucosidase digestion is expressed as glucoside and aglycone, respectively. The values are represented as the prolongation rate relative to control and the means ± SD ($n = 5$).

Statistical Analysis. A statistical analysis was performed using Student's *t* test, and a *p* value of <0.05 was considered to be statistically significant.

RESULTS

Antioxidant Activity of the Extracts from Each Soybean. The antioxidant activities of the crude extracts from the whole yellow and black soybeans on LDL oxidation are shown in **Figure 1**. In comparison to control, the prolongation rate of whole yellow and black soybeans was 1.5 ± 0.1 and 4.6 ± 0.4, respectively. The extract of whole black soybean (205 ± 16 min) had a longer lag time than that of whole yellow soybean (65 ± 3 min).

The inhibitory effects of the germ and cotyledon mixtures from each soybean with or without enzymatic extraction on LDL oxidizability are shown in **Figure 2**. The prolongation rate relative to the control significantly increased in the aglycone for the germ and cotyledon of both soybeans. In comparison to control, the prolongation rate of glucoside and aglycone was 2.0 ± 0.1 and 3.0 ± 0.4 in the yellow soybeans and 1.9 ± 0.1 and 2.5 ± 0.3 in the black soybeans, respectively.

We performed further antioxidant analyses, to evaluate the radical scavenging capacity, in both glucosides of yellow and

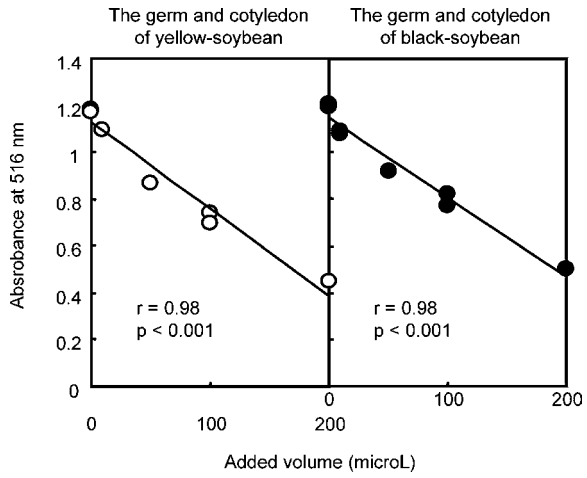

Figure 3. Radical scavenging activities of each germ and cotyledon mixture in yellow and black soybeans. The free radical scavenging capacity of each germ and cotyledon mixture was measured with DPPH.

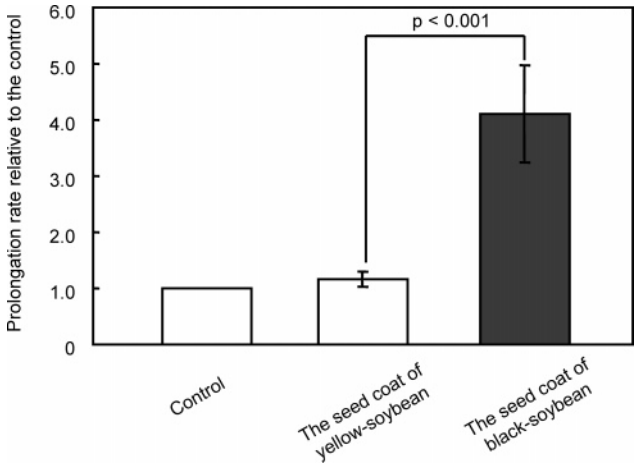

Figure 4. Antioxidant activities of seed coat extracts in each soybean on LDL oxidation. The extract from black soybeans seed coat was diluted by 1:30. The values represent the prolongation rate relative to control and the means ± SD ($n = 5$).

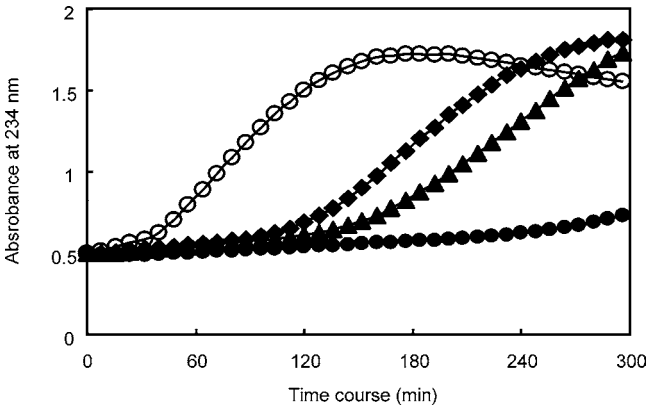

Figure 5. Dose-dependent effect of the extract from black soybeans seed coat on LDL oxidation. LDL (70 μg protein/mL) was incubated with 400 μM AMVN–CH_3O in the presence of the extract from black soybeans: ○, control; ◆, the extract solution diluted by 1:30; ▲, the extract solution diluted by 1:20; and ●, the extract solution diluted by 1:10.

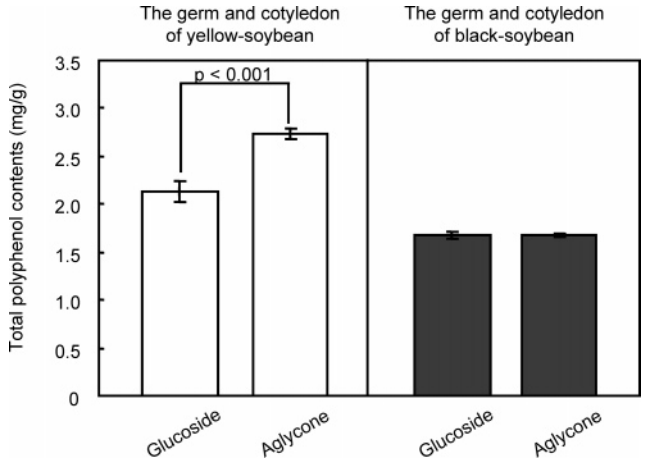

Figure 6. Total polyphenol contents in aglycone and glucoside from the mixture of the germ and cotyledon of each soybean. The values are the means ± SD ($n = 5$).

black soybean with the stable radical DPPH (**Figure 3**). The volume of crude glucoside extract required to cause a 50% decrease in the absorbance at 516 nm relative to control (100%) was calculated. The glucoside extract solution of yellow and black soybean required 146 and 161 μL, respectively, for scavenging 50% of the DPPH radicals. Because the extract solution of each whole soybean contained polyphenol [yellow soybean, 0.27 mg/mL; black soybean, 0.21 mg/mL (data not shown in figures)], 39 μg of the yellow soybean polyphenol (18 mg as the mixture of the germ and cotyledon) and 34 μg of the black soybean polyphenol (20 mg as the mixture of the germ and cotyledon) were needed in order to scavenge 50% of the DPPH radical.

As shown in **Figures 4** and **5**, the extract pigment from black soybean seed coat had a very strong antioxidant activity against LDL oxidation. The extract solution diluted by 1:30 from the black soybean seed coat significantly prolonged the lag time of LDL oxidation in comparison to the original extract from the yellow soybean seed coat (**Figure 4**). In comparison to control, the prolongation rate of seed coats in yellow and black soybeans was 1.2 ± 0.1 and 4.1 ± 0.9, respectively. The LDL oxidizability increased in a dose-dependent manner after the addition of the extract pigment from black soybean seed coat. As compared with control (36 min), the lag time was 119 min at the extract solution diluted by 1:30, 156 min at 1:20, and 246 min at 1:10 (**Figure 5**).

Total Polyphenol Content of the Extracts from Each Soybean. In yellow soybean, the aglycone of the germ and cotyledon (2.7 ± 0.1 mg/g) had a higher polyphenol content than the glucoside of the germ and cotyledon (2.1 ± 0.1 mg/g), although the total polyphenol content of glucoside and aglycone in black soybean was closely similar (**Figure 6**).

Regarding the total polyphenol content of the seed coat in **Figure 7**, black soybean had a higher polyphenol content than yellow soybean. The total polyphenol content was found to be 0.45 ± 0.02 mg/g in yellow soybean and 29.0 ± 0.56 mg/g in black soybean. The concentration of cyanidin-3-glucoside in the black soybean seed coat was 10.8 mg/g, which was measured with HPLC.

DISCUSSION

The present study demonstrated that black soybeans have a stronger inhibitory effect against LDL oxidation than yellow soybeans and that this effect was dependent on total polyphenol content in its seed coat. We could not find any significant difference between the mixture of germs and cotyledons in black and that in yellow soybeans regarding the antioxidant ability on DPPH radicals and LDL oxidizability.

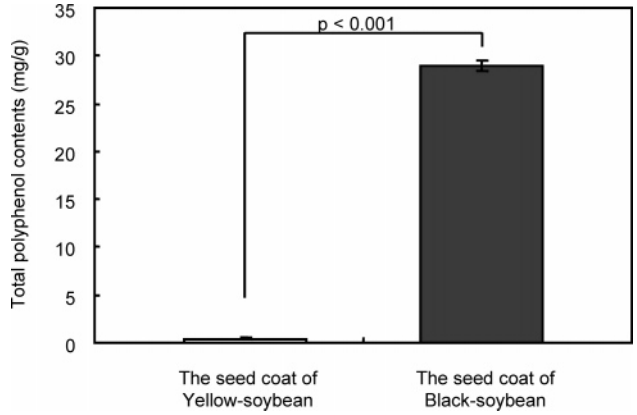

Figure 7. Total polyphenol contents in the seed coat of yellow and black soybeans. The values are the means ± SD ($n = 5$).

A few studies have reported the antioxidant effect of soybeans with darker seed coats such as those with brown and black colors. Tsuda et al. (*25*) evaluated the antioxidant activity of white, red, and black beans using a linoleic acid oxidation system and thiocyanate methods. The seed coat extract from white beans had no antioxidant activity, while those from red and black beans inhibited the oxidation of linoleic acid. The inhibitory effects were the same as that of 200 μg of tocopherol. Regarding germs, none of the extracts had any antioxidant activity. Takahata et al. (*26*) showed the DPPH radical scavenging activity to be the strongest in black soybeans among the three kinds of soybeans regarding the seed coat, which also included reddish-brown, brown, and black beans, and was closely dependent on the content of phenolic compounds. Regarding the antioxidant activity in the seed coat or the mixture of the germ and cotyledon, our results were compatible with the findings in their reports.

The antioxidant in the seed coat may thus play an important role in protection against oxidative damage for germination during storage (*27*). The major antioxidant in seed coats of the black soybean was polyphenolic compounds, anthocyanins, which ranged from 1.58 to 20.18 mg/g in volume (*20*). The cyanidin-3-O-glucoside content of the seed coat in black soybeans was 10.8 mg/g in our study. We analyzed only cyanidin-3-O-glucoside because Tsuda et al. (*25*) have shown that only cyanidin-3-O-glucoside exhibited a strong antioxidant activity in antioxidant assays among three anthocyanins isolated from red and black beans. Other soybean polyphenols such as isoflavones were not evaluated in the present study. The antioxidant ability of cyanidin-3-glucoside is a part of the whole antioxidant ability in soybeans. To elucidate the contribution of each soybean polyphenol regarding the antioxidant activities in soybeans, a further study on the varieties of soybean polyphenol will be needed.

The mixture of the germ and cotyledon before β-glucosidase treatment tended to prolong the LDL oxidation lag time as compared to the control. However, this change did not reach a statistically significant difference. Interestingly, the mixture of the germ and cotyledon after β-glucosidase treatment significantly prolonged the lag time in comparison to glucoside. In line with our results, isoflavone aglycones (genistein and daidzein) have been reported to have a stronger antioxidant activity than its glucosides (genistin and daidzin) according to the findings of $ABTS^{\bullet+}$ total antioxidant activity assay (*28*) or LDL oxidizability induced by the copper (*15*). These data may be the result of a lack of hydroxyl groups due to the existence of glucosidic linkage in the glucoside forms.

The major polyphenolic compounds of soybeans were isoflavones, which exist as glucosides (genistin, daidzin, and glycitin) in soybeans and in unfermented soy foods (*29*). The glucosides were converted to the corresponding aglycones by β-glucosidase of intestinal microflora and then are absorbed from the small intestine (*30*, *31*). The aglycones, which are in Japanese traditional fermented soy foods such as miso, natto, and soy sauce (*29*), are absorbed effectively from the small intestine without being affected by intestinal microflora (*31*). Regarding the bioavailability of anthocyanins, dietary anthocyanins are incorporated into plasma in structurally intact forms without enzymatic action of intestinal microflora in human (*32*, *33*), and the glucoside forms and its metabolites may contribute to antioxidant activities in plasma and tissue (*34*, *35*). To obtain a more effective antioxidant activity of soybeans, it might be a good idea to use aglycone and anthocyanin-rich soy foods, which are fermented dark-colored soy foods and those hydrolyzed by β-glucosidase, rather than using unfermented light-colored soy foods. Further studies will be needed to evaluate the antioxidant activity and bioavailability in aglycone-rich soy foods, especially to compare various physiological effects among fermented various colored soybeans both in vitro and in humans.

In conclusion, our results for two types of soybeans revealed the superior protective effect of aglycones in black soybeans on LDL oxidation. This effect of black soybeans was dependent on the total polyphenol content included in the seed coat of black soybeans. Therefore, the intake of dark-colored soybeans, such as black soybeans, among whole beans may be more useful for preventing such oxidation-related diseases as atherosclerotic diseases and various types of cancers.

ABBREVIATIONS USED

LDL, low density lipoprotein; TFA, trifluoroacetic acid; DPPH, 1,1-diphenyl-2-picrylhydrazyl; AMVN−CH_3O, 2,2′-azobis(4-methozy-2,4-dimethylvaleronitrile).

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Received for review November 19, 2004. Accepted April 5, 2005.

JF048062M