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Characteristics of Korean Soybean Paste (*Doenjang*) Prepared by the Fermentation of Black Soybeans

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Abstract

The changes in components and biological activities of *doenjang* samples prepared with black soybeans and fermented with *Bacillus subtilis* SCB were investigated. The amino nitrogen (A-N) contents of samples increased with increasing black soybean content. A *doenjang* product made using a 1:1 ratio of soybeans-black soybeans showed a maximum level of genistein and daidzein isoflavones (1111.6 μg/g) at 110 days of fermentation, along with decreasing contents of genistin and daidzin due to the conversion to aglycones. The black soybean-only *doenjang* sample showed higher protease activity, including caseinolytic and fibrinolytic enzyme activities, than the other samples, and had relatively higher polyphenol content and DPPH radical scavenging activity. Therefore, *doenjang* made with additions of black soybeans and fermented by *B. subtilis* SCB may have improved physiological properties, suggesting this to be a valuable method of preparation.

Key words: *doenjang*, *B. subtilis* SCB, black soybeans, isoflavones, DPPH radical scavenging activity

INTRODUCTION

Fermented foods are important components of traditional diets around the world. *Doenjang* (Korean fermented soybean paste) is an important fermented food in Korea. *Doenjang* has been traditionally manufactured from *meju*, which is a fermented rectangular block of crushed cooked soybeans. The primary microorganisms involved in *meju* fermentation are *Bacillus subtilis* and molds such as *Rhizopus*, *Mucor*, and *Aspergillus* species (1).

Recently, *doenjang* has received attention from both the public and industry, as many studies have reported its healthful physiological effects. *Doenjang* contains protease inhibitors, phytic acid, and isoflavones, which have antioxidation, antimutation, and anticancer activities (2). In particular, soy isoflavones have attracted much attention due to their potential ability to prevent and treat chronic diseases, postmenopausal complications, and sex-hormone related cancers (3,4). In unprocessed soybeans, isoflavones are mostly present in their glycoside forms, and are metabolized into aglycones during fermentation processes, such as in the preparation of *cheonggukjang*, *doenjang*, *miso*, *tempeh*, and other fermented soy foods. Isoflavonoids from legumes are reported to be hydrolyzed by microorganisms in the large intestine prior to absorption. It has been suggested that the aglycone forms of isoflavones are more effectively absorbed than the glycosides, although recent studies have demonstrated that the glycosides have equal bioavailability to the aglycones (5). Therefore, soybean fermentation can enhance the health promotion functions of isoflavones through the conversion of glycosides into aglycones.

Black soybeans [*Glycine max* (L.) Merr.] are a nutritionally rich foodstuff. The seed coats of black soybeans contain anthocyanins, so they are darker than the seed coats of other strains of soybeans (6). Black soybeans also contain isoflavones, vitamin E, saponins, and anthocyanins, which have been shown to exert biological activity (7-10). In China, black soybeans fermented by filamentous fungi are further processed to make traditional fermented condiments such as In-yu black sauce as well as In-si or Ttou-si, the dried by-products of black soybean sauce (11). The beneficial effects of black soybeans were described in Ben-Tsao Gong Mu, an ancient Chinese Botanical Encyclopedia, dating back to the early 16th century (12).

In order to investigate and determine a possible *doenjang* product with healthful functional properties, we prepared *doenjang* samples made with different additions of black soybeans and examined changes in chemical components, antioxidant activities, and isoflavone contents during fermentation.

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MATERIALS AND METHODS

Doenjang preparation

The following four *doenjang* products were prepared using different ratios of soybeans and black soybeans: soybean-only (1.5 kg), soybean-black soybean (1 kg:0.5 kg), soybean-black soybean (0.75 kg:0.75 kg), and black soybean-only (1.5 kg). The *doenjang* preparation process consisted of the following: the soybeans and black soybeans were purchased from Paju-nonghyup, sorted, washed, and soaked in water for 12 hr at 15°C; they were then cooked for 4 hr at 100°C. The cooked soybeans (1.5 kg) were cooled to 30°C and inoculated with *Bacillus subtilis* SCB (9×10^8 cell/mL, 20 mL) (13). Next, salt (150 g) was added to the soybeans and they were fermented at 30°C for 3 months. One hundred pots, each containing 10 kg of *doenjang*, were prepared. The fresh *doenjang* samples (100 g) were frozen and extracted with water (20-fold, 50 g/L). Each extract was used to assay various physiochemical properties.

Enzyme assay and chemical analysis

The *Doenjang* (10 g) samples were each homogenized with 40 mL of water and the homogenate was centrifuged at $3,000\times g$ and 5°C. The supernatants were used as the crude enzyme. Fibrinolytic activity was determined by the modified fibrin plate method (14). Ten milliliters of plasminogen-free fibrinogen (Sigma, St. Louis, USA) in 0.1 M borate buffer (pH 7.5) was mixed with 0.1 mL of thrombin solution (200 NIH U/mL, Sigma) in a Petri dish (100×15 mm) and solidified at room temperature. Then, five holes were made on a fibrin plate by suction using a capillary glass tube (5 mm-diameter). Twenty microliters of sample solution was dropped into each hole and incubated at 37°C for 8 hr. After measuring the dimension of the clear zone, the number of units was determined according to a standard curve derived using plasmin.

The caseinolytic protease activities of samples were determined by the modified fibrin plate method (15). The caseinolytic activity was assayed using the following procedure: a mixture (1 mL) containing 0.7 mL of 0.1 M sodium phosphate buffer (pH 7.5), 0.1 mL of 2% casein, and 0.1 mL of enzyme solution was incubated for 5 min at 37°C. It was then mixed with 0.1 mL of 1.5 M trichloroacetic acid, allowed to stand for 20 min, and then centrifuged at room temperature. The A_{275} of the supernatant was measured and converted to the amount of tyrosine equivalent. One unit of caseinolytic activity was defined as the amount of enzyme releasing 1 μmol of tyrosine equivalent per min.

β-Glucosidase activity was determined using a modified procedure of Peralta et al. (16). For the enzymatic reaction, 200 μL of the substrate [1 mM *p*-nitrophenyl-β-D-glucopyranoside in 0.1 M sodium phosphate buffer (pH 6.7)] and 200 μL of the respective extracts were incubated in a test tube for 30 min at 40°C. The reaction was stopped by the addition of 2 mL of 0.25 M sodium carbonate and the amount of *p*-nitrophenol that was liberated was determined by the yellow color developed under alkaline conditions. The absorbance was measured at 420 nm. One unit of enzyme activity was defined as the amount of enzyme releasing 1.0 μmole of *p*-nitrophenol per min.

The content of amino nitrogen (A-N) was determined by the TNBS method (17).

Determination of isoflavones

The extraction of isoflavone glucosides and aglycones from the *doenjang* samples and their quantification were performed similarly to a previous report with some modification (18). Each culture was extracted with 80% aqueous methanol for 24 hr with shaking at room temperature. The insoluble residue was separated by centrifugation and the supernatant was filtered with a syringe filter (0.45 μm, Millipore Co., Bedford, MA, USA) for HPLC analysis. Reversed phase HPLC analysis was carried out with a JASCO system (Tokyo, Japan), using a YMC AM 303 ODS-A column (4.6×250 mm, Kyoto, Japan). The mobile phase was composed of 0.1% acetic acid in acetonitrile (solvent A) and 0.1% acetic acid in water (solvent B). Following the injection of 20 μL of sample, solvent A was increased from 15% to 35% over 50 min, and then held at 35% for 10 min. The solvent flow rate was 1 mL/min and the eluted isoflavones were detected at 254 nm. The quantitative data for daidzin, genistin, and their aglycones were obtained by comparison to known standards.

Determination of total polyphenols (TP) and DPPH radical scavenging activity

Total polyphenol (TP) content was determined using the Folin-Ciocalteu method (19), adapted to a microscale. In a 1.5-mL Eppendorf tube, 0.79 mL of distilled water, 0.01 mL of *doenjang* ethanol extract appropriately diluted, and 0.05 mL of Folin-Ciocalteu reagent were added and mixed. After exactly 1 min, 0.15 mL of sodium carbonate (20 g/100 mL) was added, and the mixture was mixed and allowed to stand at room temperature in darkness, for 120 min. The absorbance was read at 750 nm, and the total polyphenol concentration was calculated from a calibration curve (r^2=0.999), using gallic acid as the standard (50～800 mg/L).

The DPPH radical scavenging activity of the free and bound extracts was measured according to the method

of Cheung et al. (20) with some modifications. A 0.8 mL aliquot of 0.2 mM DPPH ethanolic solution was mixed with 0.2 mL of the respective *doenjang* ethanol extracts. The mixture was then vigorously shaken and left to stand for 10 min under subdued light. The absorbance was measured at 520 nm.

Radical scavenging activity (%) $= (1 - A_{sample}/ A_{control}) \times 100$, where A_{sample} is the absorbance in the presence of sample and $A_{control}$ is the absorbance in the absence of sample.

RESULTS AND DISCUSSION

Changes in amino nitrogen (A-N)

Fig. 1 shows the changes in amino nitrogen content of the prepared *doenjang* samples during fermentation according to different ratios of soybean and black soybean. With the exception of the 2:1 soybean-black soybean and soybean-only samples, the A-N rate slowly increased during fermentation until 80 days and slightly decreased thereafter. The 2:1 soybean-black soybean *doenjang* showed a maximum A-N value (5.6 mg/g) at 110 days and then its content slightly decreased (Fig. 1). The soybean-only *doenjang* had a dramatic increase in content up to 30 days and then did not show differences thereafter. And the black soybean-only sample generally showed higher A-N values than the other samples over the entire fermentation time, and the soybean-only sample showed lower values. Overall, A-N content increased with increasing black soybean content in the *doenjang*.

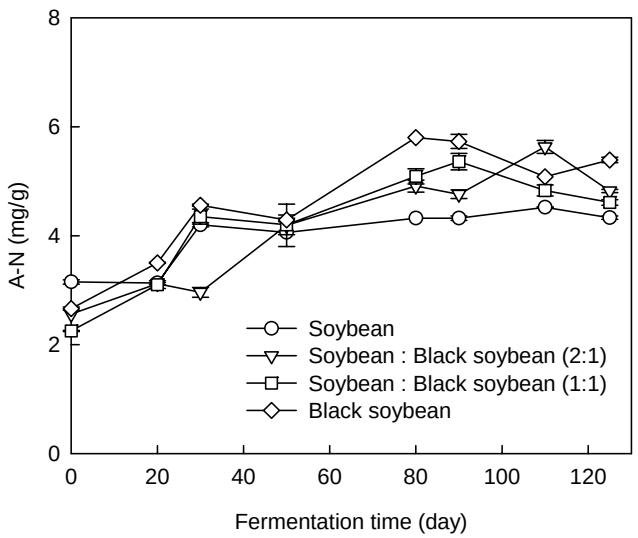

Fig. 1. Changes in amino nitrogen (A-N) during fermentation of *doenjang* samples prepared with soybeans and black soybeans. The *doenjang* were prepared with different ratios of soybean and black soybean: soybean (1.5 kg), soybean-black soybean (1 kg:0.5 kg), soybean-black soybean (0.75 kg:0.75 kg), and black soybean (1.5 kg).

The inositol phosphate (phytate) content of soybeans is typically higher than that of black soybeans. The nutritional importance of phytate lies in its ability to chelate several minerals, especially divalent metals such as Ca, Fe, Zn, and Mo, thereby reducing their availability in the intestinal tract. Phytates may also interact with proteins to form insoluble complexes that inhibit the peptic digestion of ovoalbumin and elastin (21). Thus, black soybean proteins may be more rapidly hydrolyzed by proteolytic enzymes than soybean proteins.

Changes in isoflavones

Soy isoflavones act as weak estrogens or anti-estrogens depending on their concentration in the medium (22). The physiological functions of isoflavones appear to be mediated by a variety of mechanisms, including estrogenic activity, the inhibition of topoisomerase and tyrosine kinase, cell cycle arrest, and so forth (23). There are 12 chemical forms of isoflavones in soybeans and soy foods, which include genistin, daidzin, glycitin, and their aglycones. Fermented soybeans contain larger amounts of genistein than unfermented soy products. Genistein and genistin are major components in fermented soy foods, constituting more than 50% of the total isoflavones (24). Generally, the isoflavone aglycones have greater bioavailability than their glucoside counterparts (25). Among the isoflavone aglycones, genistein has shown better health functional effects on cancer, osteoporosis, and climacterium than other isoflavones (26).

Fig. 2 shows the changes in isoflavone contents during the fermentation of *doenjang* samples prepared with different ratios of soybeans and black soybeans. Levels of daidzin and genistin decreased with increasing fermentation time regardless of the soybean to black soybean ratio used in preparation. On the other hand, the amounts of daidzein and genistein increased with increasing fermentation time. The 2:1 soybean-black soybean *doenjang* sample showed relatively high amounts of daidzein and genistein until 80 days of fermentation time, with decreases thereafter. And the 1:1 soybean-black soybean sample had increases in content up to 110 days of fermentation followed by decreases thereafter; in addition, it had the highest total value of daidzein and genistein (1111.6 μg/g) among all the *doenjang* samples over the entire fermentation time. Whereas the black soybean- only sample presented the lowest amounts of daidzein and genistein throughout fermentation.

Barnes et al. (27) demonstrated that genistein could be formed from genistin during fermentation, while 6-*O*-malonyl genistin could be converted to 6-*O*-ace-

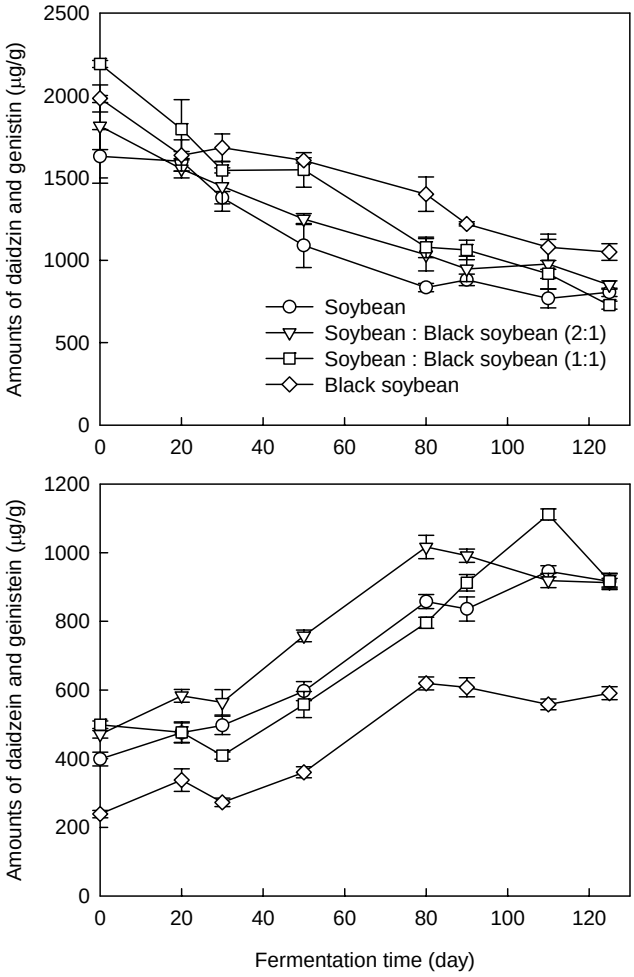

Fig. 2. Changes in isoflavones during fermentation of *doenjang* samples prepared with soybeans and black soybeans. The *doenjang* were prepared with different ratios of soybean and black soybean: soybean (1.5 kg), soybean-black soybean (1 kg:0.5 kg), soybean-black soybean (0.75 kg:0.75 kg), and black soybean (1.5 kg).

tylgenistin or genistin during heating. This report agrees with our results in that amounts of daidzein and genistein increased with increasing fermentation time, while amounts of daidzin and genistin decreased (Fig. 2). A genistein-enriched *doenjang* would have greater health effects than a typical *doenjang* product. Glucoside isoflavones can be converted to isoflavone aglycones by intestinal microorganisms (28). However, intestinal bacteria also metabolize and degrade isoflavones (29).

Therefore, it is important to consume isoflavones in their aglycone forms, which are easily absorbed in the intestine. The transformed aglycones in our results (Fig. 2), namely genistein and daidzein, are absorbed and have potential antimutagenic and anticancer properties (30).

Changes in fibrinolytic and caseinolytic protease and β-glucosidase activities

Fig. 3 presents the changes in fibrinolytic and caseinolytic protease and β-glucosidase activities in the prepared *doenjang* samples during fermentation according to different ratios of soybean and black soybean.

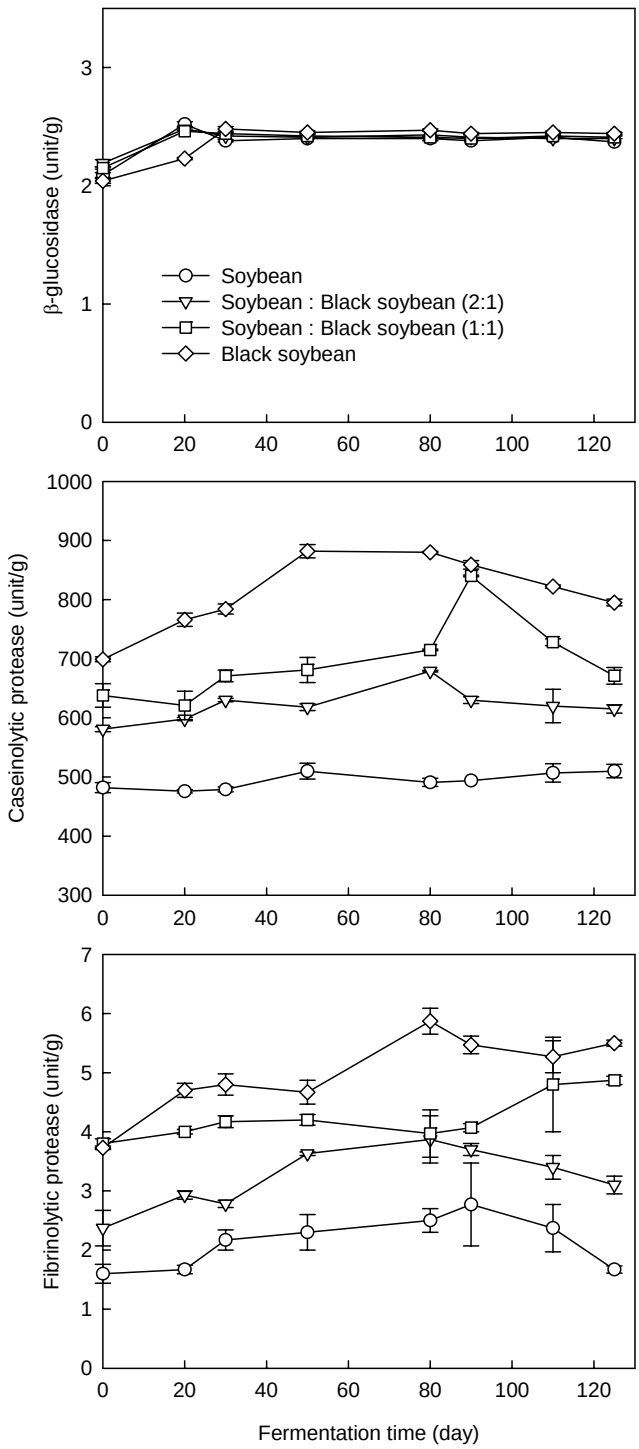

Fig. 3. Changes in β-glucosidase, caseinolytic, and fibrinolytic activities during fermentation of *doenjang* samples prepared with soybeans and black soybeans. The *doenjang* were prepared with different ratios of soybean and black soybean: soybean (1.5 kg), soybean-black soybean (1 kg:0.5 kg), soybean-black soybean (0.75 kg:0.75 kg), and black soybean (1.5 kg).

Genistin is an isoflavone containing glucose by a β-glucosidic linkage, and can be converted to genistein by releasing glucose. Therefore, genistin can be converted to genistein by treatment with β-glucosidase. However, in this study, β-glucosidase activity increased slightly up to 20 days of fermentation and then plateaued, and significant differences were not shown among the samples.

The caseinolytic protease activity of the black soybean-only sample increased until 50 days of fermentation and then decreased. The 1:1 soybean-black soybean *doenjang* showed a maximum activity (840 unit/g) at 90 days of fermentation. The soybean-only sample did not show differences in caseinolytic protease activity during fermentation, and activity was generally higher with increasing black soybean content. The fibrinolytic protease activities of the black soybean-only and 2:1 soybean-black soybean samples increased until 80 days of fermentation and then decreased, whereas the activity of the 1:1 soybean-black soybean sample was not different up to 80 days and then slightly increased thereafter. The mechanism for this increase in its fibrinolytic activity will be elucidated in further studies.

The fibrinolytic activity of the soybean-only sample increased slightly until 90 days of fermentation and then decreased thereafter. All *doenjang* samples showed similar tendencies in terms of their caseinolytic and fibrinolytic protease activities. Namely, the sample made with a relatively high black soybean content presented high caseinolytic and fibrinolytic protease activities.

Recent studies on proteolytic enzymes have focused on their regulatory roles within a variety of physiological processes (31). Fibrinolytic enzymes are the agents that dissolve fibrin clots. Yet, fibrinolytic enzyme therapy, such as the intravenous administration of urokinase, is expensive and patients may suffer from undesirable side effects such as resistance to reperfusion, the occurrence of acute coronary reocclusion, and bleeding complications (32). Consequently, several lines of investigation are currently being pursued to enhance the efficacy and specificity of fibrinolytic therapy. And recently, fibrinolytic enzymes have been discovered in food sources.

The *doenjang* prepared with a relatively high content of black soybeans showed higher caseinolytic and fibrinolytic protease activities (Fig. 3). Thus, these results suggest that black soybean products fermented with *B. subtilis* SCB may have enhanced biological activities and could have potential therapeutic uses for patients suffering from fibrin clots.

Changes in total polyphenols and DPPH radical scavenging activity

The changes in polyphenol content and DPPH radical scavenging activity during the fermentation of *doenjang* samples prepared with different ratios of soybean and black soybean are presented in Fig. 4 and 5, respectively. The polyphenol contents of the samples slightly increased until 80 days of fermentation, with the exception of the soybean-only sample, and then no differences were shown thereafter (Fig. 4). The soybean-only sample had increases in polyphenol content until 50 days of fermentation and then content slightly decreased thereafter. Pearl millet showed increases in polyphenol content sim-

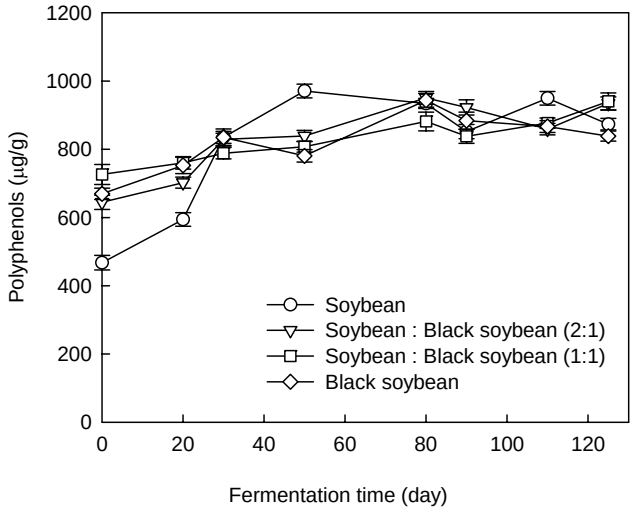

Fig. 4. Changes in polyphenol content during fermentation of *doenjang* samples prepared with soybeans and black soybeans. The *doenjang* were prepared with different ratios of soybean and black soybean: soybean (1.5 kg), soybean-black soybean (1 kg:0.5 kg), soybean-black soybean (0.75 kg:0.75 kg), and black soybean (1.5 kg).

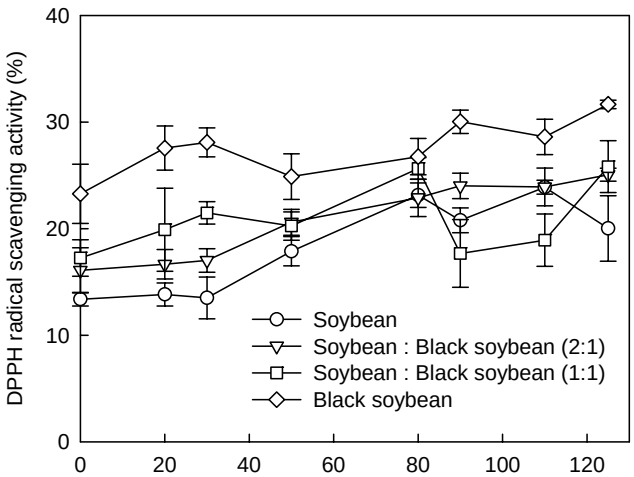

Fig. 5. Changes in DPPH radical scavenging activity during fermentation of *doenjang* samples prepared with soybeans and black soybeans. The *doenjang* were prepared with different ratios of soybean and black soybean: soybean (1.5 kg), soybean-black soybean (1 kg:0.5 kg), soybean-black soybean (0.75 kg:0.75 kg), and black soybean (1.5 kg).

ilar to our study with fermentation (33), and increases as well as decreases have been reported after fermentation (33-35). In black soybeans, increases in amounts of polyphenols are attributed to protease activity. When protein networks are hydrolyzed with protease, polyphenols within the protein networks are easily liberated. Thus, polyphenol levels may increase during fermentation. A decrease in polyphenol content might be caused by browning reactions or oxidation, or by microbial conversion to polyphenol metabolites.

DPPH radical scavenging activity was relatively higher in the black soybean-only sample as compared to the other samples during fermentation (Fig. 5). The 1:1 soybean-black soybean and soybean-only samples had increases in activity until 80 days and then activity decreased thereafter, whereas the scavenging activity of the 2:1 soybean-black soybean sample increased slightly over the entire fermentation time.

The observed effect of fermentation length on the antioxidative activity of fermented black beans was reported previously (36). McCue and Shetty (36) observed that the DPPH-scavenging effects of a soybean ethanol extract fluctuated over a 10-day fermentation period. While Randhir et al. (37) reported that the DPPH-scavenging effect of fava beans fermented with *R. oligosporus* decreased from the start of fermentation to a low level on the 8th day, and then increased until the 20th day of fermentation. Polyphenols are frequently reported to covary with antioxidative activity (36,37). However, in the present study, total polyphenol content and the extent of antioxidative activity were not strongly correlated in the samples

Isoflavones are flavonoid components of soybean seeds with three or more phenol hydroxyl residues, and are therefore called soybean polyphenols (38). Black soybeans (*Glycine max*) also contain anthocyanins that are derived from the soybean skins, which contain flavonoid and non-flavonoid molecules, including anthocyanins. Among three anthocyanins isolated from black beans, only cyanidin-3-*O*-glucoside exhibited strong antioxidant activity in antioxidant assays (39). Isoflavones contain three aglycones: genistein, daidzein, and glycitein. In particular, genistein and daidzein exert various effects, including estrogen-like activity, anti-oxidant effects, and anti-cancer effects. These data may be attributable to the lack of hydroxyl groups due to the existence of glucosidic linkages in the glucoside forms (40). The glucosides are converted to the corresponding aglycones by β-glucosidase that is produced by intestinal microflora and are then absorbed from the small intestine (41). The aglycones are effectively absorbed from the small intestine without being affected by the intestinal microflora (42). Regarding the bioavailability of anthocyanins, in humans, dietary anthocyanins are incorporated into the plasma in structurally intact forms without the enzymatic action of intestinal microflora (43), and the glucoside forms and their metabolites may contribute to antioxidant activity in the plasma and tissue (44). To obtain more effective antioxidant activity from soybeans, one may recommend the use of aglycone- and anthocyanin-rich soy foods, which are fermented dark-colored soy foods hydrolyzed by β-glucosidase, rather than using unfermented light-colored soy foods.

To conclude, in this study we prepared *doenjang* samples using fermentation with *B. subtils* SCB, in which the samples were made with different ratios of soybeans and black soybeans. The results demonstrated that isoflavone metabolism occurred during the fermentation process. In particular, the 1:1 soybean-black soybean sample showed a maximum content (1111.6 μg/g) of genistein and daidzein as well as physiological activities at 110 days of fermentation. Significant decreases in levels of genistin and daidzin were also presented along with comprehensive conversions of glycosides into aglycones. Furthermore, the black soybean-only *doenjang* showed higher protease activity, including caseinolytic and fibrinolytic enzyme activities, than the other samples, as well as relatively high polyphenol content and DPPH radical scavenging activity. Therefore, the addition of black soybeans for *doenjang* preparation using fermentation with *B. subtilis* SCB may improve the physiological properties of products, suggesting this to be a valuable preparation method.

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