Changes in Physiochemical Properties during the Fermentation of 
Doenjang Prepared with Black Soybeans

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Abstract

A physicochemical assessment of Doenjang (traditional fermented soyfood) prepared with Korean black soybeans (Glycine max) was carried out. The T-N rate increased slowly during storage up to 120 days and the A-N rate increased up to 80 days of ripening and then decreased slightly. The caseinolytic activity increased slowly during storage up to 80 days and then decreased after 80 days. In addition, the fibrinolytic and β-glucosidase activities increased up to 80 and 30 days and then decreased. Genistein and daidzin concentrations gradually decreased with increased fermentation time. However, genistein and daidzin slowly increased with fermentation time. Genistein and daidzin reached maximum concentrations (316.8 and 305.2 μg/g, respectively) and plateaued thereafter. The anthocyanins increased greatly during fermentation up to 50 days and then remained constant between 50 and 90 days. Polyphenol contents showed a slight increase up to 80 days and then slowly decreased. The DPPH and ABTS radical scavenging activities increased linearly during storage up to 50 days, reached about 28.9% and 2.17 mg/g, respectively, and then slowly decreased. At 20 days of fermentation, macrophage-stimulating activity of the extract showed a maximum activity.

Key words: Doenjang, antioxidant, macrophage stimulating activity, fibrinolytic activity

INTRODUCTION

Great interest has recently been devoted to fermented foods as dietary contributors to longevity. In particular, special focus has been placed on fermented soybean foods made traditionally in Asian countries for their physiological activities. Traditionally, Koreans consumed high quantities of soybean and soy foods to overcome the protein deficiency risk resulting from a rice-based diet. Accordingly, the Korean diet should contain a significantly higher level of isoflavones than the normal Western diet. Consumption of soy products may be responsible for the low incidence of hormone-related diseases such as breast and prostate cancer in Korea (1).

Soybean-based fermented foods such as fermented whole cooked soybeans (Chungkookjang in Korea, Natto in Japan) and soybean paste (Doenjang in Korea, Miso in Japan) are very popular in Korea. It was reported that some of the components of soybean, especially trypsin inhibitor, isoflavones, saponin and phytic acids, showed various physiological activities, anticancer, fibrinolytic activity, ACE inhibition effects as well as other factors.

Thus, Doenjang may have different components from raw soybeans, which could be driven by chemical or biochemical reactions during fermentation process. Black soybeans (Glycine max (L.) Merr.) are a nutritionally rich foodstuff. The seed coats of black soybeans contain anthocyanin and so they are darker than the seed coats of other strains of other soybeans (2). They also contain isoflavone, vitamin E, saponin and anthocyanin which have been shown to exert biological activity (3-6). In China, black soybeans fermented by filamentous fungi are further processed to make traditional fermented condiments such as In-yu black sauce and In-si or Ttou-si, the dried by-product of black soybean sauce (7). The beneficial effects of the black soybean were described in Ben-Tsao Gong Mu, an ancient Chinese Botanical Encyclopedia, dating back to the early 16th century (8).

In an attempt to develop healthy food possessing functional properties, black soybeans were used to prepare the doenjang. To further evaluate physiochemical properties of Doenjang, changes in chemical components, antioxidant activities, and macrophage stimulating activities were studied.
MATERIALS AND METHODS

Doenjang preparation

The process of making Doenjang was as follows: black beans were purchased from Pajunonghuyup, sorted, washed, and soaked in water for 12 hr at 15°C and then cooked for 4 hr at 100°C. The cooked soybeans (500 g) were cooled to 30°C, inoculated with Aspergillus oryzae (0.2%), and fermented at 30°C for 36 hr. After drying at 40°C for 3 day, soybean meju was prepared to form globules (diameter 1.5~2 cm). The meju was then placed in a clay pot and brine was added, resulting in a 33:12:55 ratio of meju, salt, and water. The mixture was then fermented for 3 months. One hundred pots, each containing 10 kg of Doenjang, were prepared. Fresh Doenjang samples (100 g) were frozen, and extracted with water (20-fold, 50 g/L). Each extract was used to assay various physicochemical properties.

Enzyme assay and chemical analysis

Doenjang (10 g) was homogenized with 40 mL of water and the homogenate was centrifuged at 3,000 g at 5°C. The supernatant was used as a crude enzyme. Fibrinolytic activity was determined by the modified fibrin plate method (9) 10 mL 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 and was solidified at room temperature. Then, five holes were made on a fibrin plate by suction using a capillary glass tube (5 mm-diameter). 20 μL 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 the standard curve derived by using plasmin.

Caseinolytic protease was determined by the modified fibrin plate method (10). Caseinolytic activity was assayed by 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, mixed with 0.1 mL of 1.5 M trichloroacetic acid, allowed to stand for 20 min, and then centrifuged at room temperature. The A275 for 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 by a modified procedure of Peralta et al. (11). 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 extracts were incubated in a test tube for 30 min at 40°C. The reaction was stopped by addition of 2 mL of 0.25 M sodium carbonate and the amount of p-nitrophenol liberated was determined by the yellow color developed under alkaline conditions. The absorbance was measured at 420 nm. A unit of enzyme activity was defined as the amount of enzyme releasing 1.0 μmole of p-nitrophenol per min.

The contents of amino type nitrogen (A-N) and total nitrogen (T-N) were determined by the methods of TNBS (12) and semi-micro Kjeldahl (13), respectively.

Determination of isoflavones

Extraction of isoflavone glucosides and aglycones from fermented black soybean and quantification were performed similarly to previous reports with some modification (14). 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 then filtered with a syringe filter (0.45 μm, Millipore Co., Bedford, MA, USA) for HPLC analysis. Reversed phase HPLC analysis was carried out with JASCO (Tokyo, Japan), using 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 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. Quantitative data for daidzin, genistin and their aglycones were obtained by comparison to known standards.

Determination of total polyphenols (TP) and total anthocyanins

Total polyphenols (TP) content were determined using the Folin-Ciocalteu method (15), adapted to a micro-scale. In a 1.5-mL Eppendorf tube, 0.79 mL distilled water, 0.01 mL Doenjang ethanol extract appropriately diluted, and 0.05 mL Folin-Ciocalteu reagent was 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 for 120 min. The absorbance was read at 750 nm, and the total polyphenol concentration was calculated from a calibration curve (r²=0.999), using gallic acid as standard (50~800 mg/L).

Anthocyanins measurements were performed using well-established spectrophotometric methodology (16, 17). Analytically, Doenjang ethanol extract was placed in a 0.2-cm path length quartz cuvette, and the absorbance was measured at 520 nm (A520). Following this,
0.02 mL of a 20% sodium metabisulphite solution was added, the sample was mixed well, and after 1 min the absorbance was read at 520 nm. A 520 \( \text{SO}_2 \). A 12% ethanolic solution was used as a blank. All measurements were corrected to a 1.0-cm path length. Further, extract (0.02 mL) was mixed with 0.98 mL 1 N HCl solution in a 1.5-mL Eppendorf tube, mixed, and allowed to stand for 180 min at room temperature. The absorbance was read at 520 nm (A \( \text{HCl} \)), using a 1.0-cm path length cuvette. For the blank, 0.02 mL of a 12% ethanolic solution was used instead of extract. The concentration of total anthocyanins (TA) was calculated as follows:

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\text{TA (mg/L)} = 20 \times [\text{A}_{\text{HCl}} - (5/3) \times \text{A}_{\text{SO}_2}] \times \text{final extract volume (mL)/sample weight (g)}.
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**Macrophage-stimulating activity**

The 6- to 8-week-old mice (male ICR, Daihan-Biolink Co., Chungbuk, Korea) were intraperitoneally injected with 1 mL of 5% sterile thioglycollate medium. After 3 days, peritoneal exudates cells were harvested from the peritoneal cavity by injecting 5 mL of cold RPMI 1640 medium containing 5-mM HEPES, penicillin (100 U/mL) and streptomycin (100 \( \mu \text{g/mL})). The supernatant was discarded after centrifugation (250 \( \times \text{g} \), 10 min, 4°C), and then an aliquot (200 \( \mu \text{L} \)) of the cell suspension (1 \( \times \text{10}^6 \text{cells/mL} \) was allowed to adhere onto the surface of the wells of a flat-bottomed 96-well microplate. After incubation at 37°C for 2 hr in a humidified atmosphere of 5% CO\(_2\) and 95% air, nonadherent cells were removed by washing twice with RPMI 1640 medium containing 10% fetal bovine serum to prepare the adherent macrophage monolayer (18). After the macrophage cells (180 \( \mu \text{L} \)) were cultured in the presence of the test sample (20 \( \mu \text{L} \)) in a 96-well microplate for 24 hr, macrophage cells in a 96-well microplate were solubilized by the addition of 25 \( \mu \text{L} \) of 0.1% Triton X-100. One hundred fifty microliters of 10.0 mM p-nitrophenyl phosphate (Sigma) in 0.1-M citrate buffer (pH 5.0) and 50 \( \mu \text{L} \) of 0.2-M borate buffer (pH 9.8) were added to the reaction mixture, and the absorbance at 405 nm was photometrically measured using a microplate reader (Model 3550-UV, Bio-Rad, Hercules, CA) (19).

**RESULTS AND DISCUSSION**

**Changes of T-N and A-N**

Fig. 1 presents the change in the total and amino nitrogen during fermentation of *Doenjang* prepared with black soybean. The T-N rate increased slowly during fermentation up to 120 days and then decreased thereafter. In addition, the A-N rate increased during the first 80 days of fermentation and then decreased slightly thereafter. The soluble nitrogen rate increased linearly during fermentation up to 80 days.

From these results, it can be concluded that proteins in the *Doenjang* prepared with black soybean were hydrolyzed by koji enzymes during 2–3 months of fermentation and then terminated. The termination of hydrolysis may be due to the inactivation of enzymes over 3 months of fermentation, or due to the substrate specificity of enzymes. Further study is needed in order to elucidate this phenomenon. The changes in the A-N, which are important in fermentation, reached their maximum in the first 3 months and plateaued thereafter. However, the A-N reached a maximum in the first 2 months and plateaued thereafter in Korean traditional *Doenjang* prepared with soybean (20). The *Doenjang* prepared with black soybean might ripen more slowly than *Doenjang* prepared with conventional soybeans.

**Changes of fibrinolytic and caseinolytic protease and \( \beta \)-glucosidase**

Fig. 2 presents the change in the fibrinolytic and caseinolytic proteases and \( \beta \)-glucosidase during fermentation of *Doenjang* prepared with black soybean. The caseinolytic activity increased slowly during fermentation up to 80 days and then decreased in activity after 80 days. In addition, the fibrinolytic activity increased up to 80 days and then decreased thereafter. \( \beta \)-Glucosidase activity increased slowly up to 30 days and then slightly decreased thereafter.

Recent studies of proteolytic enzymes have focused on their regulatory roles in a variety of physiological processes. Among the most thoroughly studied regulatory proteases are those associated with coagulation, fibrinolysis, and the complement system (21). Fibrinolytic...
enzymes are the agents that dissolve fibrin clots. Three enzymes that are currently used for these purposes include urokinase, streptokinase, and genetically engineered tissue plasminogen activator. 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, occurrence of acute coronary reocclusion, and bleeding complications (22). Consequently, several lines of investigation are currently being pursued to enhance the efficacy and specificity of fibrinolytic therapy. Recently, fibrinolytic enzymes have been discovered from food sources.

The fungi used as the starter organisms in the present study were capable of producing \( \beta \)-glucosidase, which promotes cleavage of the \( \beta \)-glycosyl bond in the black soybean glucoside isoflavones to form aglycones (23, 24). It was found that the fermentation of black soybean with the starter organisms examined caused a marked increase in the content of aglycone and total anthocyanin (24,25). Therefore, these changes may all lead to the enhanced biological activity observed with the extracts of fermented black soybean.

Changes of isoflavone, anthocyanin and polyphenol contents during fermentation

Isoflavones, particularly genistein, have been demonstrated to exhibit anti-oxidative potential (26) and phytosteroidal activity (27). Compared to soybean products, fermented soybean contained larger amounts of genisteins than unfermented soy products. Daidzein and genistein were found in all sample extracts in the fermented soy products. In Fig. 3, genistin and daidzin were gradually decreased with increased fermentation time. Especially, daidzin decreased slowly during first 90 days and then was not present thereafter. However, genistein and daidzein were slowly increased with fermentation time. Genistein and daidzein showed maximum contents (316.8 and 305.2 \( \mu \)g/g, respectively) and plateaued out thereafter. Fermentation was also noted to increase the content of aglycone, the bioactive isoflavone (25).

Barnes et al. demonstrated that genistein could be formed from genistin during fermentation, while 6-\( O \)-malonyl genistin could be converted to 6-\( O \)-acetylgenistin or genistin during heating (28). It has also been reported that human saliva is capable of converting glucosides into aglycones (29), explaining the fact that aglycones can be absorbed to some extent in the stomach prior to the main absorption from the small intestine (30). The hydrolysis of the glucosides in the small intestine is necessary for their absorption (31). Only the aglycones are absorbed, and it is believed that the isoflavones, genistein and daidzein, are responsible for the observed benefits (32).

Fig. 4 presents the change in the anthocyanin and polyphenol contents during fermentation of Doenjang prepared with black soybean. The anthocynins increased greatly during fermentation up to 50 days and then showed similar contents between 50 and 90 days. However, the contents greatly decreased after 90 days. Polyphenol contents showed a slightly increase up to 80 days, and then decreased slowly after 80 days.

Previous workers have investigated the effect of fermentation length on the total phenolic content in some bean substrates (33,34). Randhir et al. (34) conducted a 20-day solid fermentation of fava bean with \( R. \) oligosporus and found that the total phenolic level was reduced during the first 8 days of fermentation, but in-
increased substantially thereafter. McCue and Shetty (33) reported that the total phenolic content in soybean extract did not increase until 4 days of cultivation. The trends in the variation of total phenolic content in black bean observed in the present study appear not to be consistent with those reported by McCue and Shetty (33) and Randhir et al. (34). These discrepancies may be attributed to differences in the starter organism and the bean substrate studied.

### Free radical scavenging activity

The stable free radicals DPPH and ABTS were used to measure the radical-scavenging activities of *Doenjang* extracts. DPPH and ABTS radical scavenging activities showed the same tendency in Fig. 5. The DPPH and ABTS radical scavenging activities increased linearly during storage up to 50 days, reached about 28.9% and 2.17 mg/g, respectively, and then slowly decreased thereafter. DPPH radical scavenging activities showed a similar tendency as those of polyphenols changes.

Isoflavones have direct free radical quenching ability, with genistein and daidzein being particularly effective (35-37). Soy isoflavones have been shown to exhibit antioxidant effects both *in vitro* and *in vivo*. The free radical-scavenging activity of all soybean isoflavones and their glycosides was also compared at a concentration of 12 μmol/L (38). Genistin had greater activity than other isoflavones and glycosides. Mitchell et al. (39) demonstrated that genistein and daidzein did not strongly scavenge DPPH or galvinoxyl free radicals.

Black soybean (*Glycine max*) also contains anthocyanin derived from soybean skin. It contains flavonoid and non-flavonoid molecules, including anthocyanins. Anthocyanins are polyphenolic compounds that have been shown to have anti-inflammatory (40) and anti-diabetic (41) effects, with a daily intake in humans estimated to be as much as 180~215 mg/per/day. The antioxidative activity of anthocyanin pigments data indicate that as the number of hydroxyl substituents on the B-ring is increased, higher activity is achieved with the glycosides, while in the case of the aglycones increased hydroxyl substituents produces weaker activity. It has been revealed that active oxygen agents such as OH· and O₂⁻ are thought to cause oxidative damage, and much attention has been focused on active oxygen scavenging agents such as natural phenolics like flavonoid and anthocyanin pigments in preventing cell damage. In general, the flavylium cation form of anthocyanin is stable in the acidic condition, but the structure changes in neutral and alkaline conditions and breaks down (42). When anthocyanins scavenge active oxygen or lipid hydroperoxide radicals, the structure would also be broken, and reaction products exhibiting antioxidative activity may scavenge the radicals.

### Macrophage-stimulating activity

The macrophage-stimulating activities of hot-water extracts obtained from *Doenjang* prepared with black soybean were investigated *in vitro* (Fig. 6). These results suggested that the hot-water extracts of *Doenjang* enhanced the stimulatory responses of macrophages. At 20 days of fermentation, macrophage-stimulating activity of the extract showed a maximum activity, and then decreased after 20 days of fermentation, which was coincident with the control.

Since macrophages act as a link between the innate and acquired immune system, fight infection, control inflammation and angiogenesis, and promote wound healing (43), they play critical roles in the immune response. Macrophages kill microorganisms, tumor cells, and damaged tissues during inflammation by two separate oxida-
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Anti-inflammatory cytokines belong to the T-cell-derived cytokines and are involved in the down-regulation of inflammatory reactions. Because of the potent and profound biological effects of cytokines, it is not surprising that their activities are tightly regulated, most notably at the levels of secretion and receptor expression. Additional regulatory mechanisms are provided by the concomitant action of different cytokines and the presence of biological fluids in specific inhibitory proteins, soluble-binding factors and specific autoantibodies. Although macrophage activation by Doenjang may also induce inflammation and atherosclerosis, these effects solely depend on the kinds of cytokines produced during stimulation and neighboring cells in the microenvironment where they have been released. Therefore, we will try to survey the relationship between macrophage stimulation and the produced cytokines in future study.

In an attempt to develop healthy food possessing functional properties, black soybean was used to prepare the doenjang. Fermentation was also noted to increase the content of aglycone, the bioactive isoflavone, and the DPPH and ABTS radical scavenging activities increased linearly during storage up to 50 days, reaching about 28.9% and 2.17 mg/g, respectively, and then slowly decreased. Therefore, Doenjang prepared from black soybean may be recommended as dietary supplement and functional food.

REFERENCES


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**Fig. 6.** Changes in macrophage stimulating activity during fermentation of Doenjang prepared with black soybean.


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