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Antioxidative and Lipofuscin-Formation Inhibitory Effects of Soybean and *Chungkukjang*

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Abstract

To compare antioxidative and antiaging effects between yellow soybean (YS) and *Chungkukjang* (CK) *in vivo* system, male Sprague-Dawley rats (n=24) were fed the diets containing YS and CK for 8 weeks, respectively. The YS and CK groups showed the preventive effects on lipid and protein oxidations in liver and plasma. Hepatic SOD and GSH-peroxidase activities were significantly inhibited in CK group. Superoxide anion radicals in cytosol significantly lowered in YS and CK groups compared with control group. In addition, dietary YS and CK effectively inhibited formation of the lipofuscin, the indicator of aging in heart and eye, especially the CK group had a stronger preventive activity in eye. The results of this study showed that YS and CK diet effectively suppressed the superoxide anion radical formation and tissue oxidation.

Key words: *Chungkukjang*, soybean, antioxidative activity, lipofuscin

INTRODUCTION

Soybean is an important edible plant as a source of protein and oil as well as phytochemicals such as genistein, phytic acid, tocopherol, and saponin (1). In epidemiological studies, the consumption of soybean-containing diets has been associated with low incidence of certain human cancers, osteoporosis, hormone-related diseases, cholesterolemia, atherosclerosis and many other diseases (2).

Of the many physiological effects of soybean, antioxidative activity has been pointed out as one of the potential role of soybean. Soybean flour and its derivatives stabilized lipid oxidation by interacting primary antioxidants and synergists (3). Pratt (4) reported that flavonoids were primary antioxidants in soybean, and the mechanism of antioxidative action of flavonoids derived from free radical scavenging capability. In normal condition, there is a balance between the generation of free radicals and the antioxidant defense mechanisms *in vivo* (5). Antioxidants are essential in preventing cellular damage caused by free radicals and free radical-mediated lipid peroxidation (6), thus antioxidative compounds contained soybean are available to improve the body health due to the action of suppressing oxidative stress.

Chungkukjang (CK), the fermented soybean paste with *bacillus subtilis*, is similar to *natto* in Japan. The research about the physiological properties of CK is rare, while Japanese researchers performed several studies about *natto*. Iwai et al. (7,8) reported that the main components showing antioxidative functions of *natto* were presented in water-soluble fraction, which was effective on inhibition of LDL oxidation *in vitro* and *in vivo*. The antioxidant activity of *natto* was associated with concentrations of water-soluble compounds produced during fermentation, rather than the amount of isoflavone aglycones (9). It suggests that peptides or amino acids produced from fermentation is major components in *natto*. Therefore, we expected CK has similar antioxidative activity to *natto*.

With a strong suggestion, we compared the antioxidative effects of CK and soybean *in vivo* system. Diets containing YS and CK were fed to the Sprague-Dawley (SD) rats for 8 weeks and the values of lipid and protein oxidation were measured in plasma and liver. Hepatic antioxidative enzyme activities and levels of superoxide anion and hydroxyl radicals were also measured. In addition, the contents of lipofuscin in heart and eye were determined to evaluate the anti- aging activity of YS and CK.

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MATERIALS AND METHODS

Materials

Yellow soybean (Danwon variety) was provided by Yeongnam Agricultural Research Institute. It was powdered and steamed at 121°C for 15 min to inhibit the activity of trypsin inhibitor. In order to make CK, same variety of yellow soybeans were soaked in water for 12 hr, steamed at 121°C for 15 min, cooled till 65°C, and then inoculated with 2% suspension of spores of *Bacillus circulans* K-1, which was isolated and identified by Hong et al. (10). The incubation was carried out at 40°C for 3 days using *Chungkukjang* fermenter® (Cookoo, Seungkwang Elec., Korea). After the fermentation, CK was lyophilized and powdered. Then, its proximate compositions were analyzed by AOAC methods (11).

Animal experiment

Sprague-Dawley rats, 10～12 weeks of age (200～300 g), were obtained from the Experimental Animal Center (Daejeon, Korea) and were randomly divided into three groups (n=10 per group): control, yellow soybean (YS), and *Chungkukjang* (CK) groups. We analyzed the composition of soy flour (Table 1), then added to the flour the other AIN-93 diet components to keep the same proportion of the macronutrients above and the same amount of calories. Composition of experimental diet was shown in Table 2, and dietary compositions were designed with same protein, oil, and dietary fiber by controlling casein, soybean oil, and cellulose based on the proximate composition of samples as shown in Table 1. Animals fed with experimental diet for 8 weeks, and subsequently, body weight gain, the amount of feed intakes, and feed efficiency ratio (FER) were examined. At the end of experiment, all of the rats were sacrificed under CO_2 anesthesia. Blood and tissues including liver, heart and eye were removed immediately from the animal models with dry ice and stored at -70°C until the analysis. Blood samples were centrifuged for 10 min at 3,000 rpm after collection and used for lipid peroxidation and protein oxidation analysis. The degree of lipid peroxidation and protein oxidation, antioxidative enzyme activities, and the concentrations of reactive oxygen species (ROS) were measured in the liver and the accumulation of lipofuscin in heart and eye tissues was determined.

Table 1. Proximate compositions of yellow soybean and *Chungkukjang* (%)

	Yellow soybean	*Chungkukjang*
Moisture	5.84	4.39
Crude protein	30.91	37.31
Crude lipid	18.55	19.12
Ash	4.81	4.88
Total dietary fiber	17.30	18.00
Carbohydrate	22.59	16.31

Table 2. Compositions of experimental diets for the animal study

Ingredients	Groups		
	Control	YS[1)]	CK[2)]
Casein	20.0	−	−
Yellow soybean	−	54.0	−
Chungkukjang	−	−	44.7
D·L-Methionine	0.3	0.5	0.5
Sucrose	30.0	30.0	30.0
Corn oil	10.0	−	1.5
Corn starch	25.7	12.5	18.8
Cellulose	9.3	−	1.3
AIN-76 mineral mixture	3.5	0.9	1.3
AIN-76 vitamin mixture	1.0	1.0	1.0
Choline bitartrate	0.2	0.2	0.2
$CaCO_3$	−	0.8	0.6
NaCl	−	0.1	0.1
Total	100.0	100.0	100.0

[1)]Group fed yellow soybean. [2)]Group fed *Chungkukjang*

Measurement of lipid peroxidation and protein oxidation

The contents of hepatic lipid peroxide were measured by thiobarbituric acid reactive substances (TBARS) method described by Ohkawa et al. (12) using 1,1,3,3-tetramethoxypropane (TMP) as a standard, and expressed contents of malondialdehyde (MDA). The homogenate was mixed with 8.1% sodium dodecyl sulfate, 20% acetic acid, and 0.8% TBA and the mixture was boiled for 1 hr. After that, it was cooled and extracted with n-butanol-pyridine. The absorbance of the upper layer was measured at 532 nm. Lipid peroxidation in plasma was also measured using method described by Buege and Aust (13) and values were expressed nmole MDA per mg protein. Protein oxidations in plasma and liver were measured by the method of Oliver et al. (14). The protein was precipitated from aliquots of plasma with trichloroacetic acid (TCA) and reacted for 60 min at room temperature with 2,4-dinitrophenylhydrazine (DNPH) in 2 N HCl. The derivative proteins were isolated with TCA, washed with ethyl acetate and ethanol, then dissolved in 6 M-guanidine hydrochloride. The absorbance at 370 nm was measured and converted to molar quantities using the absorption coefficient, 22,000 $M^{-1}cm^{-1}$. Values were expressed nmole carbonyls per mg protein and standardized with bovine serum albumin with modified Lowry method (15).

Measurement of antioxidative enzymes activities

To investigate the effect of soybean and CK on anti-

oxidative system *in vivo*, the activities of the hepatic antioxidative enzymes, superoxide dismutase (SOD), catalase, and glutathione peroxidase (GSH-px) were measured. SOD activity was determined according to the method of Marklund and Marklund (16) and one unit of total SOD activity was defined as the activity of enzyme that inhibited the oxidation of pyrogallol by 50%. Mn-SOD activity was measured in the addition with 1 mM KCN to inhibit Cu·Zn-SOD. Catalase activity was determined by measuring a decrease in absorption of hydrogen peroxide at 240 nm in reaction medium consisting of 50 mM phosphate buffer (pH 7.4) and 30 mM hydrogen peroxide (H_2O_2) (17). One unit of catalase activity was defined as the micromoles of degraded H_2O_2 per mg protein for 1 min. GSH-px was determined by the method of Lawrence and Burk (18). One unit of activity was equal to the micromole of oxidized NADPH per mg protein for 1 min. Protein concentration in liver homogenates was measured by the modified Lowry method (15) using bovine serum albumin as the standard.

Determination of superoxide anion and hydroxyl radical

To measure the amount of ROS accumulations in liver tissue, mitochondria and cytosol fractions were separated as follow; Livers were homogenized in 1:10 tissue weight-to-buffer volume ratio in 1.15% KCl-10 mM phosphate buffer (pH 7.4) and homogenates were centrifuged at $600\times g$ for 10 min, and then at $3{,}000\times g$ for 15 min. The pellets were re-suspended with homogenation buffer and this fraction was used as mitochondrial fraction for measurement of hydroxyl radical content. Supernatant was centrifuged at $10{,}500\times g$ for 1 hr and the resulting supernatant was used as cytosolic fraction for measurement of superoxide anion.

Generation of superoxide anion was achieved using the hypoxanthine-xanthine oxidase system (19). Reaction mixtures contained 0.1 mL of 30 mM hypoxanthine, 0.1 mL of 0.3 mM EDTA, and 0.1 mL of 3 mM cytochrome C and massed up to final volume of 3 mL with 88 mM KH_2PO_4-KOH buffer (pH 7.4). The reaction was started by addition of 0.3 mL of xanthine oxidase and the rate of cytochrome C reduction was measured at 550 nm.

Formation of hydroxyl radical was measured using the method with degradation of deoxyribose (19). The reaction mixture, in a total volume of 1.2 mL, contained the following reagents at the final concentrations stated; 10 mM potassium phosphate buffer (pH 7.4), 63 mM NaCl, and 0.8 mM deoxyribose. The reaction was initiated by adding 21 mM ferrous ammonium sulfate and the mixture incubated at 37°C for 15 min. 1 mL of 1% TBA and 1 mL 2.8% TCA were added, and then the mixture was heated at 100°C for 10 min, and cooled. The absorbance was measured at 532 nm.

Measurements of lipofuscin in heart and eye tissues

The content of lipofuscin was determined by Fletcher's method (20). After blotting the surface of tissues to remove excess moisture, chloroform-methanol, 2:1 (v:v), was added in a volume-to-weight ratio of 20:1. The eye and heart tissues were homogenized for 1 min, an equal volume of water was added, mixed thoroughly on a vortex mixer, and centrifuged for 2 min at 3,000 rpm. 1 mL aliquot of the chloroform layer was mixed with 0.1 mL of methanol and the fluorescence spectra was measured with excitation at 365 nm and emission at 450 nm (F-4500, Fluorescence Spectrophotometer, Hitachi, Japan). Relative fluorescence of lipofuscin was calculated as compared with value of control.

Statistical analysis

Statistical comparison of differences among the different groups was carried out one-way ANOVA test followed by Turkey's test ($p<0.05$) using SPSS (Statistical Analysis Program, version 12.0).

RESULTS AND DISCUSSION

Changes of food intake, weight gain, and organ weight in experimental rats

Food intakes, weight gains, food efficiency ratios and liver weight in rats fed experimental diets were shown in Table 3. Body weight gain and food intake of YS

Table 3. Body weight gain, food intake, and food efficiency ratio in rats fed experimental diet for 8 weeks

	Groups[1)]		
	Control	YS	CK
Weight gain (g/day)	4.43 ± 0.36^{a}	3.84 ± 0.39^{b}	4.39 ± 0.29^{a}
Food intake (g/day)	18.81 ± 1.16^{a}	16.93 ± 0.98^{b}	17.76 ± 1.24^{ab}
Food efficiency ratio	23.54 ± 1.18^{ab}	22.70 ± 2.08^{b}	24.74 ± 0.85^{a}
Liver (g)	10.66 ± 0.83^{NS}	9.96 ± 1.06	11.00 ± 0.71

[1)]Groups refer to Table 2.
Values are mean±SD (n=8). [a,b]Value with different superscripts within a column are significantly different by Turkey's test at the level of 0.05. [NS]Data are not significantly different.

group were significantly lowered than those of control and CK group ($p<0.05$). Food efficiency ratio of CK group was higher than that of YS group. Some studies showed that weight gain was lower in the animals fed soybean protein in those fed casein (21) while dietary soybean protein increased weight gain in rat as compared with casein (22). Madani et al. (23) reported weight gain and body and liver weights were lower in the 20% soybean protein-fed group than in the 20% casein-fed group. They discussed the impaired amino-acid contents (especially lysine and methionine) in soybean protein may be responsible for this low growth. In our study, soybean diet may affect the eating preference of rats because of high content in diet, although soybean was heated to exclude the effect of trypsin inhibitor and bean flavor with the addition of 0.5% methionine was added in the YS and CK diet to compensate the insufficient sulfur amino acids.

Bacillus fermentation of legumes reportedly resulted in improved digestibility (24). Kiers et al. (25) also suggested that considerable pre-digestion during *bacillus* fermentation could lead to higher bioavailability because the need for degradation of nutrients by gastro-intestinal enzymes is minimal. Thus, fermentation process to make CK could be helped the bioavailability of proteins and/or other nutrients in soybean.

Liver weights were not different among the experimental groups, although liver weights of rats fed soybean diets were slightly lowered than those of control and CK groups, which may be related to lowed body weight gains and food intakes.

Lipid oxidation in plasma and liver of rats

Lipid peroxidation plasma and liver of rats fed dietary YS or CK for 8 weeks are shown in Fig. 1. MDA contents in plasma were not statistically different among groups, but dietary soybean versus casein tended to suppress the MDA formation in plasma. Hepatic MDA contents in YS (0.77 ± 0.22 nmole MDA/mg protein) and CK group (0.60 ± 0.01 nmole MDA/mg protein) were significantly lower than that of control group (0.92 ± 0.23). CK diet suppressed the formation of lipid peroxides in the liver compared to soybean diet. It suggested that dietary CK exerts strong antioxidative activity *in vivo* system due to the antioxidative compounds existed in soybean and the byproducts produced by fermentation.

Protein oxidation in plasma and liver of rats

The contents of protein carbonyl were measured by the reaction with a classical carbonyl reagent 2,4-dinitrophenylhydrazine (DNPH). As shown in Fig. 2, the protein oxidation in plasma of group fed YS and CK

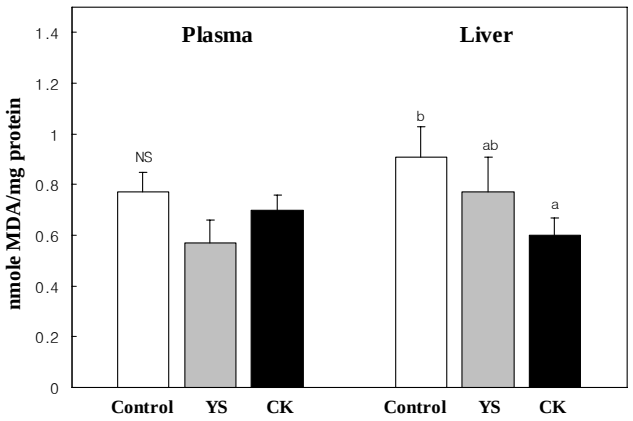

Fig. 1. TBARS values of plasma and liver in rats fed experimental diets for 8 weeks. YS: Yellow soybean group, CK: *Chungkukjang* group. [NS]Data are not significantly different. [a,b]Data are significantly different analyzed by one-way ANOVA followed Turkey's test at the level of 0.05.

diet was significantly inhibited compared with control group ($p<0.05$). There was no significance in liver, but carbonyl contents of YS and CK groups were tended to decrease compared with control group.

Active oxygen species generated in a variety of biological systems have been implicated in the mechanisms of several diseases (26), and have been able to induce damage to cells in tissue. Introduction of carbonyl groups into amino acid residues of proteins is a hallmark for oxidative modification (27). Oxidative modifications of amino acid residues include derivatization of those such as proline, arginine, and lysine to carbonyl derivatives (14). From the results, dietary YS and CK suppressed the lipid and protein oxidation, which suggested that antioxidative components such as isoflavones, phenolic acids, peptides and amino acids in soybean exerted antioxidative effect in rats. Especially, CK diet sig-

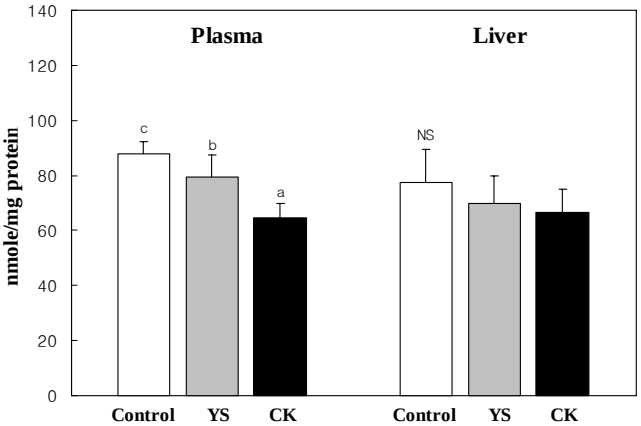

Fig. 2. Protein carbonyl values of plasma and liver in rats fed experimental diets for 8 weeks. Groups refer to Table 2. [NS]Data are not significantly different. [a-c]Data are significantly different analyzed by Turkey's test at 0.05 level.

Table 4. Antioxidative enzyme activities of liver in rat fed experimental diets for 8 weeks (unit/mg protein)

Groups[1)]	Antioxidative enzymes				
	Total SOD	Mn-SOD	Cu·Zn-SOD[2)]	Catalase	GSH-px
Control	15.05±2.65[b]	10.19±1.54[NS]	4.86±1.97[NS]	4.20±0.64[NS]	333.4±41.1[c]
YS	14.81±2.14[ab]	10.02±1.21	4.78±1.69	4.27±0.41	146.3±34.9[a]
CK	12.29±1.43[a]	9.53±0.97	2.75±1.35	4.49±0.35	204.2±33.4[b]

[1)]See the legend of Table 2.
[2)]Activities of Cu·Zn-SOD were subtracted Mn-SOD from total SOD.
Values are means±SD (n=8). [a-c]Values with different superscripts within a column are significantly different by Turkey's test at the level of 0.05. [NS]Data are not significantly different.

nificantly inhibited the lipid peroxidation in liver and protein oxidation in plasma ($p<0.05$).

Antioxidative enzymes activity in liver

The effects of YS and CK diet on the activities of the hepatic antioxidative enzymes such as SOD, catalase, and GSH-px are shown in Table 4. There are antioxidative enzymes systems for detoxifying on oxidative stress from free radical in tissues, especially a lot of enzymes existed in liver. Total SOD activity was significantly decreased in CK group (12.29±1.43 unit/mg protein) compare with control group (15.05±2.65). Mn-SOD, Cu·Zn-SOD, and catalase activities were not significantly different between control group and experimental groups. However, GSH-px activity was significantly decreased in YS and CK groups compare with control group. Antioxidative enzymes prevent cells from oxidative stress by scavenging ROS and lipid peroxides. In this experiment, lipid peroxidation and protein oxidation were decreased in liver of experimental groups (YS and CK groups), although antioxidative enzymes were not induced. These results suggested that YS and CK supplementation attenuated hepatic oxidative stress through the direct antioxidative effects of related compounds and those activities were enhanced with the fermentation process to make CK.

Scavenging effect of free radical

To confirm the antioxidative effects of YS and CK, contents of reactive oxygen species (ROS), superoxide anion and hydroxyl radicals were measured in liver (Table 5). Contents of superoxide anion radical of YS (1.37±0.40 nmole/mg protein) and CK (1.14±0.29) groups were significantly reduced relative to control group (2.13±0.38), whereas hydroxyl radical of YS group (2.59±1.04) was slightly lower than those of control (3.56±1.74) and CK groups (3.51±0.75).

Oxygen is essential for the survival of aerobic cells, but it has long been known to be toxic when supplied at concentrations greater than those in normal air. The biochemical mechanisms responsible for O_2 toxicity include lipid peroxidation and the generation of H_2O_2 plus

Table 5. Superoxide anion and hydroxyl radical contents of liver in rat fed experimental diet (nmole/mg protein)

Groups[1)]	Superoxide anion radical	Hydroxyl radical
Control	2.13±0.38[b]	3.56±1.74[NS]
YS	1.37±0.40[a]	2.59±1.04
CK	1.14±0.29[a]	3.51±0.75

[1)]See the legend of Table 2.
Values are means±SD (n=8). [a,b]Values with different superscripts within a column are significantly different by Turkey's test at the level of 0.05. [NS]Data are not significantly different.

the superoxide radical (26). In biochemical systems, superoxide anion and H_2O_2 react together to form the hydroxyl radical, which can attack and destroy almost all known biomolecules and transform the DNA structure (28). Thus, the suppression of ROS formation can ultimately prevent or delay the diseases caused by oxidative stress in the body. In this point of view, YS and CK suppressed the formation of superoxide anion, so it could be used for preventing disease related with oxidative stress and enhancing the health condition.

Lipofuscin level in eye and heart

Lipofuscin is an end-product of lipid peroxidation reaction induced by reactive oxygen radicals in cells (29), which is increased with aging. Along with antioxidative activities of soybean and *Chungkukjang*, suppressing effect of lipofuscin accumulation was also studied in this experiment. Relative concentrations of lipofuscin in the heart tissue of the YS and CK groups were 58.0% and 64.3% of the concentration of control group, respectively (Fig. 3). In the eye, compared with control group, dietary YS and CK group versus control group effectively inhibited the relative contents of lipofuscin by 75.3% and 50.8%, and especially CK was more effective for suppressing the accumulation of lipofuscin.

Many studies have supported that aging was induced by oxidative stress such as ROS and reactive nitrite species, thus antioxidative activity is considerably related to the anti-aging (6). Even though the anti-aging mechanism of soybean and CK is not clear, those diets sig-

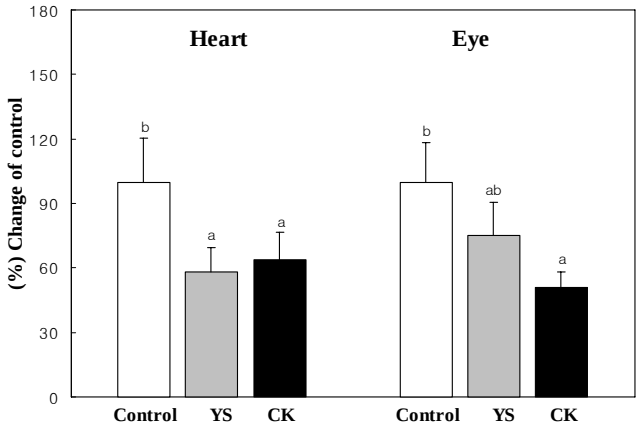

Fig. 3. Relative fluorescence of lipofuscin of heart and eye in rat fed experimental diets for 8 weeks. Groups refer to Table 2. [a,b]Data are significantly different analyzed by Turkey's test at 0.05 level.

nificantly suppressed the formation of lipofuscin. In our study using streptozotocin-induced diabetic rats, lipofuscin level was increased by the condition of oxidative stress such as diabetes, but the supplementation of soybean and CK effectively suppressed the accumulation of lipofuscin in eyes (data not shown). These results support that supplementation of soybean and CK can modulate the oxidative stress related diseases, promote the body health, and finally retard the aging.

Over the past decades, scientists have conducted the considerable research on the physiological properties of soybeans, especially isoflavones, the characteristic components in soybeans (30). Soybeans and soy foods are the main natural dietary sources that can provide nutritionally relevant amounts of isoflavones (31). Furthermore, soybean contains many antioxidative compounds including phenolic acids, α-tocopherol, saponin, phytic acid, and amino acids.

Fukutake et al. (32) also reported the contents of genistein in fermented soybean products, natto and misso, were higher than in soybean. It is due to microbes used in the fermentation, which could hydrolyze isoflavones or high molecules into the aglycones or low molecular compounds. Moreover, some researches suggested that isoflavones exist in aglycone form, produced during fermentation process, are absorbed faster and efficiently compared to isoflavone glycosides (33). Hutchins et al. (34) also suggested that supplementation of tempeh, fermented soybean product, would increase urinary isoflavonoid excretion compared with that of an equivalent amount of unfermented soybean because of structural alteration in the soybean cotyledon. It indicates that the hydrolysis of the isoflavones glycosides and the increased digestibility as a result of fermentation could make the isoflavones more available in a fermented product than in a non-fermented product.

In this study, we found dietary YS and CK suppressed lipid and protein oxidation, and prevented the accumulation of ROS and lipofuscin. It is not clear which compounds is the most dominant antioxidant in YS and CK this experiment. We suggest that the superior antioxidative activity of CK is due to the hydrolyzed compounds and the new components formed during fermentation with synergistic effects.

Isoflavones undergo acidic and enzymatic hydrolysis and demethylation to yield the aglycones genistein and daidzein and these aglycones may then be further metabolized by got flora (35). It has also been reported that daidzein was readily metabolized to monohydroxylated compounds such as a equol (36). Equol does not naturally occur in plants but is a specific intestinal bacterial metabolite of ingested isoflavones and is a major contributor to the known protective benefits including protection against hormone-dependent (breast and prostate cancer) and age-related disease (cardiovascular disease and osteoporosis) (37). Soy protein, the soy-derived isoflavones genistein and daidzein, and the metabolite equol are hypothesized to impart antioxidant protection contributing to reduction in oxidative stress.

Moreover, dietary protein level and origin play an important role in lipoprotein metabolism and the antioxidative defense statue. Madani et al. (23) reported that feeding 20% soybean protein versus 20% casein involved lower plasma TBARS concentration. Thus, origin of protein may responsible for suppressing effect on lipid oxidation. In addition, content of free amino acids in CK was higher than that of steamed soybean flour, which might be bioavailability of CK protein is better to soybean (38).

In this study, dietary YS and CK suppressed the oxidative stress, such as lipid and protein oxidation, the formation of superoxide anion, and the accumulation of lipofuscin. Especially, dietary CK showed a stronger antioxidative effect than soybean, which speculates that the useful components of soybean can be easily absorbed by degradation during fermentation to make CK. In the future, more detailed studies are needed to clarify the major antioxidative compounds of CK and the changes during metabolism because it is possible that this activity may be associated with metabolites processed in fermentation.

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