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Free Radical Scavenging Activity of Methanol Extracts of *Chungkukjang*

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Abstract

To further the goal of isolating *Bacillus sp.* from commercial chungkukjang (CKJ) for a development of a probiotic dietary adjunct using soymilk or milk, antioxidant activity of CKJ purchased from the Sunchang Traditional Village in Chunbook province was examined. Six CKJ samples were evaluated and 3 were selected based on the results of the physicochemical analysis and sensory evaluation for further antioxidant study. IC_{50} for DPPH scavenging activity of methanol extracts of CKJ ranged from 238.1 to 345.7 μg/mL. CKJ exhibited over 80% scavenging of •OH and ONOO- at concentrations of 100 μg/mL and 250 μg/mL, respectively. O_2^- and NO scavenging activities of three CKJ were increased in a dose dependent manner with the concentration tested from 100 to 1000 μg/mL. In this study, the methanol extract of CKJ exhibited a great reduction capability and powerful free radical scavenging activity, especially against OH^- and $ONOO^-$, which are the most toxic radicals responsible for oxidative damage in the body. However, radical scavenging effects of CKJ on DPPH, O_2^-, and nitrite radical were rather moderate. In conclusion, CKJ may reduce the oxidative stress in the body by scavenging the free radicals.

Key words: chungkukjang, free radical, soybeans, fermentation, hydroxyl radical, peroxynitrite

INTRODUCTION

Oxidative stress has been implicated as an important etiologic factor in human pathology, which can lead body to a degenerative state consequently causing diseases such as cardiovascular disease, cancer, diabetes, liver disease, arthritis and etc. Free radicals and other reactive oxygen species (ROS) are responsible for this oxidative damage. ROS are known to attack biomolecules in the body by oxidizing them, thereby leading to cell death and tissue damage (1). Foods having antioxidant properties or containing antioxidants are receiving much attention due to their ability to prevent oxidative damage to human body.

Soybeans are a rich source of the glycosylated isoflavons genistin and daidzin, which are converted to genistein and daidzein, respectively by microflora in the intestine. Due to their structural similarities to estrogen, there has been a rising interest in soybean's health benefits not only on sex hormone metabolism, but also on other biological activities including cholesterol-lowering properties (2). Despite its ubiquity in the Asian diet, soybean has some limitations in terms of bean flavor for some consumers and it contains non-digestible raffinose and stachyose. These limitations can be avoided by fermentation. Fermented soybean products such as doenjang, chungkukjang, miso, natto and tempeh are widely consumed in Asia and their health benefits are well documented.

Chungkukjang (CKJ), fermented soybean with *Bacillus subtilis*, is very similar to natto. Natto is usually consumed raw but CKJ is further fermented with garlic, red pepper powder, green onion and salt to prolong the storage time. The functional properties of CKJ are antioxidant, antimicrobial, blood pressure lowering and antidiabetic activities (3-8) which may come from isoflavons, peptides, phenols, and other flavonoids produced during the fermentation. The fermentation process increases these constituents (9,10). The quality of traditionally made CKJ varies depending on the activity of protease of *Bacillus subtilis* present in the rice straw. If the protease activity of *Bacillus subtilis* is high enough, then the taste and storage property of CKJ is good otherwise it gets rotten easily.

In this study, we first determined if CKJ has radical scavenging effects and what if these properties can be varied with by making different products. We collected 6 samples from different manufacturer located at the Sunchang Traditional Village where is famous for CKJ. Based on the results of the physicochemical analysis and

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sensory evaluation of the 6 samples, three CKJ were selected for the antioxidant study and evaluated for radical scavenging effects and lipid oxidation inhibition.

MATERIALS AND METHODS

Materials and chemicals

Six CKJ were purchased from different manufacturers located at the Sunchang Traditional Village in Jeonbuk province in Korea, which is famous for CKJ. CKJs manufactured no longer than 2 days were obtained. 1,1-Diphenyl-2-picrylhydrazyl (DPPH), nitroblue tetrazolium (NBT), malondialdehyde (MDA), 2-deoxyribose, and dihydrorhodamine (DHR) 123 were purchased from Sigma (Sigma-Aldrich, Korea). All other chemicals used were of analytical grade and were obtained from Merck and Sigma (Sigma-Aldrich, Korea).

Physicochemical analysis

CKJ was first diluted with 9 volumes of distilled water (w/v) before it was used for the physicochemical analysis. pH was measured with a pH meter (No. 735p-02002, Istek, Korea). Acidity of CKJ was determined by titrating it with 0.1 N NaOH until the pH of the solution reached 8.2. Amino-nitrogen (NH_2-N) content was measured by the Formol method (11). The content of ammonia-nitrogen (NH_3-N) and γ-glutamyltranspeptidase (γ-GTP) was measured with a commercial kit (AM 158-K and AM 505-K, respectively, Asan, Korea). For total bacteria counts, 1 mL of CKJ was serially diluted with 9 mL of sterilized saline and spread onto an agar plate (Oxoid Ltd., Hampshire, England) and then incubated for 24 hr for bacterial growth. The color of CKJ was determined by measuring the L* (black–white component, luminosity), a* (+ red to − green component) and b* (+ yellow to − blue component) values using a colourimeter (Minolta chroma meter, CT-310, Tokyo, Japan).

Sensory evaluation

Sensory evaluation was carried out with raw CKJ according to the replicated randomized complete block design with thirty untrained panel members. Descriptive characteristics (appearance, taste, aroma, flavor, texture, acceptability) were subjectively evaluated using grades diversified from 1 (dislike extremely) to 9 (like extremely)

Preparation of methanol extracts of CKJ

For the study of antioxidant activity, freeze-dried CKJ was extracted with 10 volumes of 100% methanol at room temperature for 24 hr for three times. After drying the extracts under vacuum, CKJ methanol extracts were obtained. The yields of three samples (S1, S2, and S3) were 19.3%, 23.9%, and 32.2% respectively.

Determination of radical scavenging activity of methanol extracts of CKJ

DPPH radical scavenging activity: DPPH radical scavenging activity was measured by the method of Hatano et al (12). The reaction mixture contained 100 μL of 60 μM DPPH and 100 μL of methanol extracts whose concentration was predetermined. The reaction mixture left to stand in a dark room for 30 min. Absorbance of the reaction mixture was determined at 540 nm. Ethanol (95%) was used as a control. The scavenging activity of DPPH radical was expressed as IC_{50}.

Superoxide radical scavenging activity: Superoxide radical (O_2^-) generated in the xanthine-xanthine oxidase system was determined spectrophotometrically via monitoring the product of NBT as an end product (13). The reaction mixture was prepared with 400 μL of each methanol extract (100～1000 μg/mL), 100 μM xanthine, 60 μM NBT, 0.05 U/mL xanthine oxidase and 0.1M phosphate buffer (pH 7.4) to make a final volume of 2.0 mL. After incubation at 37°C for 10 min, the absorbance was measured at 560 nm, compared with the control samples run without xanthine oxidsase. Percent inhibition was calculated from the optical density of the CKJ treated and control samples.

$$\text{Inhibitory rate (\%)} = \frac{(C-CB)-(S-SB)}{C-CB} \times 100$$

C: control, CB: control blank, S: sample, SB: sample blank

Hydroxyl radical scavenging activity: The oxidized 2-deoxyribose, oxidized by hydroxyl radical (•OH) produced by the Fenton reaction, is degraded to malondialdehyde (MDA) (14). Reaction mixture was prepared with 0.2 mL of 10 mM $FeSO_4$-$7H_2O$ with 10 mM EDTA, 10 mM 2-deoxyribose solution (0.2 mL) and methanol extracts (1.4 mL)/or 0.2 M phosphate buffer (1.4 mL, pH 7.4). The reaction was initiated adding 1 mM H_2O_2 (0.2 mL) followed incubation at 37°C for 4 hr. After incubation, 1 mL each of 2.8% trichloroacetic acid (TCA) and 1 mL of 1.0% thiobarbituric acid (TBA) were added to the incubation medium. It was boiled (95 ～100°C) for 10 min followed by immediate cooling in the ice water. MDA produced during the reaction was measured at 520 nm. Phosphate buffered saline (pH 7.0) was used as a control. The -OH scavenging activity was expressed as an inhibition rate as follow.

$$\text{OH-scavenging activity (\%)} = \frac{Abs_c - Abs_s}{Abs_c} \times 100$$

Abs_c: Absorbance of control
Abs_s: Absorbance of sample

Peroxynitrite radical scavenging activity: Peroxynitrite ($ONOO^-$) scavenging activity was measured by monitoring the oxidation of dihydrorhodamine (DHR) 123 (15). A stock solution of 5 mM DHR 123 in N,N-dimethylformamide was purged with nitrogen and stored at -20°C. Working solution (5 μM DHR 123) was prepared immediately prior to the each experiment in the dark room and placed on ice. The rhodamine buffer (90 mM sodium chloride, 50 mM sodium phosphate (pH 7.4), and 5 mM potassium chloride) including diethylenetriaminepenta acetic acid (DTPA, 5mM) was purged with nitrogen and placed on ice before use. The reaction medium was prepared with methanol extracts (100～1000 μg/mL), 180 μL of reaction solution [rhodamine buffer 175.8 μL＋4 μL DTPA (5 mM)＋0.2 μL DHR 123 (5 mM)] and 3-morpholinosydnomine (SIN-1, 200 μM) to be a total volume of 0.2 mL. This reaction medium was incubated at 37°C for 10 min. Changes in fluorescence of the reaction medium were monitored for 30 min at an excitation wavelength of 485 nm and emission wavelength of 535 nm.

Nitrite scavenging activity: Nitrite scavenging was measured by the method of Kato et al. (16). Methanol extracts of CKJ (100～1000 μg/mL) was added to 1 mL of $NaNO_2$, pH of the sample was adjusted to be 1.2 with 0.1 N HCl and then it was incubated at 37°C for 1 hr. Five milliliter of 2% acetic acid and 0.4 mL of Griess reagent were added to 1 mL of the incubated solution. It was left stand at room temperature in the dark for 15 min. The absorbance of the reaction mixture was determined at 520 nm on a microplate reader (ELx800, Bio-Tek Instruments, Inc., USA).

Lipid Peroxidation inhibition: Oxidation medium was prepared with 0.2 mL of methanol extract, 0.5 mL of 2.5% linoleic acid, 0.5 mL of phosphate buffer (pH 7.0), and 0.1 M glucose. The reaction mixture was incubated at 37°C for 3.5 hr. Ethanol (75%, 9.7 mL) and 30% ammonium thiocyanate (0.1 mL) were added to 100 μL of reaction mixture. One hundred microliter of 20 mM ferrous chloride (in 3.5% HCl) was added to the above solution and left to stand at room temperature for 3 min. The absorbance of each solution was determined at 500 nm (17).

RESULTS

Physicochemical results of CKJ

Physicochemical properties of 6 CKJ from different manufacture are shown in Table 1. As shown in the table, pH, acidity, γ-GTP, N content, total bacteria, and color of CKJ are significantly different among the samples. Sensory evaluation results were also dissimilar (Table 2). pH and acidity of CKJ were ranged between 6.1～7.0 and 1.0～2.8, respectively. Nitrogen contents of CKJ expressed as NH_2 and NH_3 were between 0.6～1.1 μg/dL and 59.4～168.3 mg%. γ-GTP was varied from 7.2～16.5 mU/mL. But the total bacterial count is different among samples. The color of the CKJ was very different according to the manufacturer. As shown in table 2, the appearance, flavor, take, texture of 6 CKJ were very dissimilar therefore the total acceptability, expressed as a preference, was also significantly different. In sensory evaluation, S1, S2, and S3 were scored over 5 out of 9 for preference. Based on the physicochemical analysis and sensory evaluation, we selected S1, S2 and S3 for the antioxidant study which fit into the criteria of high quality CKJ in terms of freshness, N content, smell and taste.

The effect of CKJ on scavenging DPPH, ROS and RNS

DPPH method is one of the widely used analytical tools for examining the antioxidant property of samples

Table 1. Physicochemical analysis of chungkukjang from different manufacturers[1)]

		S1	S2	S3	S4	S5	S6
pH		7.0 ± 0.0^{a}	6.8 ± 0.0^{c}	6.6 ± 0.0^{c}	6.8 ± 0.0^{c}	6.1 ± 0.0^{e}	6.9 ± 0.1^{b}
Acidity (%)		1.7 ± 0.5^{a}	1.0 ± 0.0^{ab}	1.3 ± 0.1^{b}	1.6 ± 0.1^{bc}	2.8 ± 0.2^{ca}	1.4 ± 0.4^{bc}
NH_2-N content (μg/dL)		1.1 ± 0.1^{a}	0.7 ± 0.1^{c}	0.6 ± 0.1^{b}	0.8 ± 0.1^{b}	0.6 ± 0.0^{e}	1.0 ± 0.0^{a}
NH_3-N content (mg%)		166.7 ± 2^{a}	76.7 ± 1.3^{d}	92.2 ± 1.0^{b}	146.0 ± 1.4^{b}	59.4 ± 2.7^{e}	168.3 ± 1.4^{a}
γ-GTP (mU/mL)		16.5 ± 0.1^{a}	7.5 ± 0.1^{d}	8.2 ± 0.1^{b}	12.5 ± 0.5^{b}	7.5 ± 0.5^{a}	7.2 ± 0.1^{d}
Total bacteria count (log CFU/mL)		9.5 ± 0.1^{a}	9.1 ± 0.1^{b}	9.2 ± 0.1^{b}	9.6 ± 0.0^{a}	8.8 ± 0.1^{c}	9.4 ± 0.3^{b}
	L	84.4 ± 1.4^{cd}	78.8 ± 2.8^{cd}	80.9 ± 0.1^{b}	87.0 ± 0.4^{b}	8.8 ± 0.1^{c}	85.6 ± 2.8^{bc}
Color[2)]	a	1.2 ± 0.4^{cd}	1.7 ± 0.7^{a}	0.9 ± 0.1^{a}	0.1 ± 0.1^{c}	0 ± 0.3^{d}	0.3 ± 0.9^{bc}
	b	14.4 ± 0.6^{cd}	36.2 ± 0.5^{c}	38.1 ± 0.1^{b}	31.1 ± 1.0^{d}	21.5 ± 0.5^{e}	41.4 ± 1.1^{a}

Values are mean±SD.
[a-d]Data are significantly different by one-way ANOVA followed Duncan's multiple range test at the 0.05 level of significance.
[1)]Chungkukjang samples (S1～6) were purchased from the Sunchang Traditional Village at Jeonbuk Province. The manufacturer are as follows; S1: Kim Young Sun, S2: Moon Ok Rae, S3: Hyang Guk Woon, S4: Moon Neung Ja, S5: Oh Bok, S6: Oh Sun E.
[2)]L: lightness, a: redness, b: yellowness.

Table 2. Sensory evaluation of chungkukjang from different manufacturers[1)]

	S1	S2	S3	S4	S5	S6
Appearance	5.55±1.53[b]	6.66±1.67[a]	4.90±1.76[bc]	3.41±1.84[d]	5.31±2.39[b]	4.03±1.72[cd]
Flavor	5.24±1.90[ab]	5.48±2.18[a]	5.17±1.81[ab]	4.31±1.87[b]	4.83±1.95[ab]	2.83±1.87[c]
Taste	5.59±1.62[a]	4.83±2.12[ab]	5.52±1.71[a]	4.03±1.70[bc]	4.69±2.22[ab]	3.62±1.84[c]
Texture	5.79±1.42[a]	5.66±1.54[ab]	4.97±1.82[bc]	4.52±1.15[c]	4.69±1.87[c]	4.21±1.52[c]
Preference	6.17±1.71[a]	5.31±2.17[ab]	5.31±2.16[ab]	4.14±1.62[c]	4.52±2.34[bc]	3.66±1.86[c]

Values are mean±SD.
[a-d]Data are significantly different by one-way ANOVA followed Duncan's multiple range test at the 0.05 level of significance.
[1)]See the legend of Table 1.

Table 3. IC_{50} for DPPH scavenging activity of methanol extracts of chungkukjang from different manufacturers[1)]

CKJ[2)]	IC_{50} (μg/mL)
S1	345.7±53.3[a]
S2	320.8±32.0[a]
S3	238.1±26.5[b]

Data are mean±SD. [a,b]Data are significantly different by one-way ANOVA followed Duncan's multiple range test at the 0.05 level of significance.
[1)]See the legend of Table 1.
[2)]Three samples were selected among 6 CKJ based on the physicochemical analysis and sensory evaluation.

(18). Table 3 shows IC_{50} values for DPPH radical scavenging activity of CKJ. IC_{50} for S3 was significantly lower than those of S1 and S2, exhibiting higher DPPH radical scavenging activity ($p<0.05$). To evaluate the health benefits of CKJ, ROS and reactive nitrogen species (RNS) scavenging activities of CKJ were examined. The concentration of methanol extracts of CKJ tested for the free radical scavenging activity ranged from 100 to 1000 μg/mL. Superoxide anion radical and NO scavenging activities of CKJ increased dose dependently. Among three samples, S3 showed the greatest effect on scavenging O_2^- (Fig. 1) while S2 revealed the greatest inhibition of NO generation (Fig. 2). Radical scavenging activity, expressed as an inhibition rate, of S3 CKJ against O_2^- was over 80% at 1000 μg/mL concentration, and this effect was significantly different with other samples ($p<0.05$). The inhibition rate of S2 CKJ against NO formation at 1000 μg/mL concentration was 77% ($p<0.05$). For •OH and $ONOO^-$ experiments, very powerful radical scavenging activity of CKJ was observed. At a concentration of 100 μg/mL of CKJ, over 80% of •OH was scavenged in all three samples although the inhibition rate was slightly increased as the sample concentration increased (Table 4). Among the three samples, S3 showed the greatest inhibition against hydroxyl radical. As shown in Table 5, $ONOO^-$ scavenging activity of S2 CKJ was 76% at 100 μg/mL concentration and it was reached over 90% inhibition at 250 μg/mL concentration tested. At a 250 μg/mL concentration, all three CKJs inhibited $ONOO^-$ by 80%. According to these results, CKJ is a very powerful antioxidant against hydroxyl and peroxynitrite radicals.

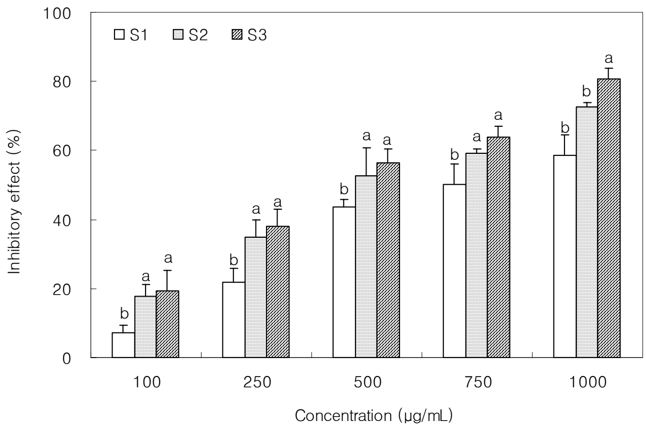

Fig. 1. Superoxide anion scavenging activities of methanol extracts of chungkukjang from different manufacturers. Values are mean±SD. See the legend of Table 3. [a,b]Data are significantly different by one-way ANOVA followed Duncan's multiple range test at the 0.05 level of significance.

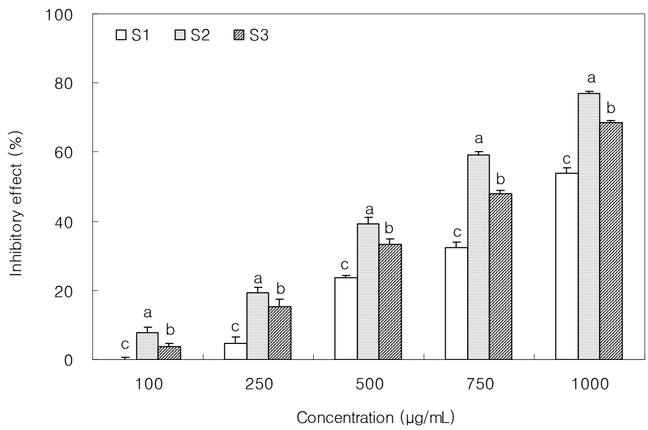

Fig. 2. Nitrite scavenging activities of methanol extracts of chungkukjang from different manufacturers. Values are mean ±SD. See the legend of Table 3. [a-c]Data were significantly different by one-way ANOVA followed Duncan's multiple range test at the 0.05 level of significance.

Table 4. Hydroxyl radical scavenging activities of methanol extracts of chungkukjang from different manufacturers

CKJ[1)]	Concentration (μg/mL)				
	100	250	500	750	1000
S1	80.2±0.6[b]	89.5±0.3[a]	92.4±0.1[b]	95.4±0.2[b]	93.7±0.3[b]
S2	83.4±0.8[a]	89.0±0.2[b]	92.7±0.4[b]	88.9±0.1[c]	94.1±0.3[b]
S3	84.3±0.4[a]	89.1±0.1[b]	96.3±0.3[a]	102.4±0.8[a]	105.0±0.5[a]

Values are mean±SD.
[a,b]Data were significantly different by one-way ANOVA followed Duncan's multiple range test at the 0.05 level of significance.
[1)]See the legend of Table 3.

Table 5. Peroxynitrite scavenging activities of methanol extracts of chungkukjang from different manufacturers

CKJ[1)]	Concentration (μg/mL)				
	100	250	500	750	1000
S1	56.4±0.9[c]	79.6±0.8[c]	89.0±0.3[c]	91.5±0.4[c]	93.3±0.2[c]
S2	75.9±2.4[a]	90.1±1.0[a]	95.7±0.5[a]	96.8±0.4[a]	97.6±0.2[a]
S3	63.4±2.5[b]	85.0±0.9[b]	93.1±0.8[b]	95.6±0.7[b]	97.0±0.4[b]

Values are mean±SD.
[a-c]Data were significantly different by one-way ANOVA followed Duncan's multiple range test at the 0.05 level of significance.
[1)]See the legend of Table 3.

Inhibition of unsaturated fatty acid peroxidation by CKJ

CKJ inhibited the Fe^{2+} induced linoleic acid oxidation in a dose dependent manner (Fig. 3). Among the three CKJs, S3 inhibited lipid oxidation the most. Unsaturated fatty acid peroxidation was retarded by over 50% in the presence of S3 at the 500 μg/mL concentration.

DISCUSSION

Oxidative damage is involved in the progression of degenerative disease. Many studies have focused on the prevention of oxidative damage by reducing oxidative stress in the body. Consequently foods having antioxidant properties or containing antioxidants are of great interest. In this study, methanol extracts of CKJ exhibited radical scavenging activity against DPPH, ROS, and RNS as well as unsaturated fatty acid peroxidation. One important finding observed in our study is that CKJ possesses a great potential for scavenging •OH and $ONOO^-$. Numerous researchers have cited that fermented soybean products such as natto (19), miso (20), dou-chi (21) doenjang (9), CKJ (9,22), and *Lactobacillus*-fermented soybean products (23) possess antioxidant activities. The antioxidant activity of fermented soybean product originally comes from the phenols and flavonoids present in the soybean (24), the concentrations of which are increased after fermentation (25). Besides this, metabolites such as amino acids, peptides, and aglycone isoflavons (genistein and daidzein) liberated during fermentation are also responsible for the elevated antioxidant properties of the fermented soybean products (26).

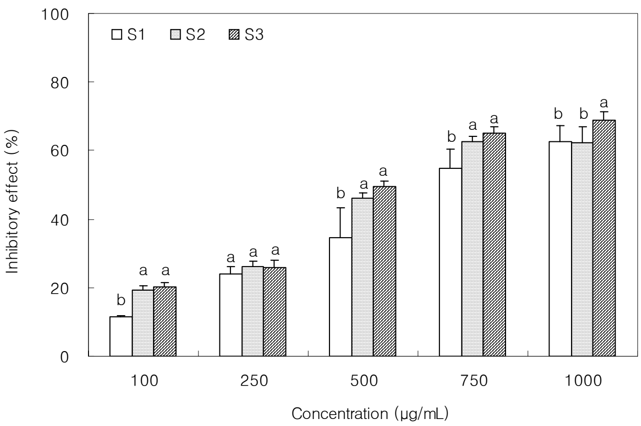

Fig. 3. The inhibition effect of methanol extracts of chungkukjang on lipid oxidation from different manufacturers. Values are mean±SD. See the legend of Table 3. [a,b]Data were significantly different by one-way ANOVA followed Duncan's multiple range test at the 0.05 level of significance.

Our results demonstrated that methanol extracts of all three CKJ from different manufactures had DPPH scavenging activity which is stronger (IC_{50}, 238.1～345.7 μg/mL) than ethanol extracts of CKJ (14.9～26.6% inhibition at concentration of 150～450 μg/mL) reported by Kim et al. (22). It seems that lipophilic compounds, such as phenolic compounds, isoflavons and lipid soluble nutrients are readily dissolved into the extracts which might exert stronger antioxidant activity.

Our results showed that methanol extracts of all three CKJ exhibited ROS (O_2^-, •OH) and RNS (NO, and $ONOO^-$) radical scavenging activity in a dose dependent manner, especially against •OH and $ONOO^-$. All three CKJ exhibited an 80% scavenging of •OH at the 100 μg/mL concentration and for $ONOO^-$ removal at the 250 μg/mL concentration, revealing a very powerful antioxidant activity. Low-molecular-weight viscous substance (<100,000) of natto showed a stronger •OH scavenging activity than that for O_2^-, determined by ESR at 10 mg/mL concentration (19). From these data we could conclude that fermented soybean products by *Bacillus species* have a great ROS scavenging activity, especially for •OH. The microorganisms responsible for making CKJ and natto are *Bacillus subtilis* and *Bacillus natto*, respectively. Therefore, researchers expect similar physiological activities from both products. Hydroxyl radical is formed from hydrogen peroxide by the Fenton reaction. This species is considered most toxic among all ROS. Due to this extremely short half-life, •OH interacts with bimolecular immediately after generation (27).

One notable finding in this study is that methanol extracts of CKJ possess RNS (NO and $ONOO^-$) scavenging activity. Peroxynitrite scavenging activity of CKJ is greater than that for NO. At least five differences in activity were observed in this study with CKJ from different manufactures. One of the more potent oxidants among nitrogen-derived free radicals is $ONOO^-$, which is formed by the reaction of two ubiquitous free radical species: O_2^- and NO (28). Peroxynitrite is generally considered to be more toxic than either of its precursors, NO or O_2^-, because of its powerful oxidative action, which can cause damage to proteins, lipids, and DNA via more subtle mechanisms referred to as nitration processes (29-31).

Unsaturated fatty acid peroxidation is another event that elevates radical reactions in the body, providing lipid radicals to either ROS or RNS or these radicals indirectly initiate the lipid oxidation, vice versa, as a result of superoxide and hydrogen peroxide serving as precursors of singlet oxygen and hydroxyl radical (32). Therefore prevention of lipid peroxidation is considered an important way to retard the oxidative damage.

In this study, the methanol extract of CKJ exhibited a great reduction capability and powerful free radical scavenging activity especially against •OH and $ONOO^-$, but radical scavenging effects of CKJ on DPPH, O_2^-, and nitrite radical were rather moderate. The degree of radical scavenging activity of the three CKJs was significantly different as we expected. Since traditional methods of making CKJ use naturally occurring bacteria in rice straw instead of inoculating the *Bacillus subtilis*, the quality of CKJ prepared the traditional method is varied depending on the activity of protease of *Bacillus subtitis* present in the rice straw. If the protease activity of *Bacillus subtitis* is high enough, then the taste and storage property of CKJ is good, otherwise it easily rots.

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