한작지(Korean J. Crop Sci.), 56(4): 349~360(2011)

Evaluation of Antioxidant Activities of Peptides Isolated from Korean Fermented Soybean Paste, *Chungkukjang*

Sun-Lim Kim*†, Hee-Youn Chi****, Jung-Tae Kim*, On-Sook Hur**, Deog-Su Kim*, Sae-Jung Suh***, Hyun-Bok Kim**, and Ill-Min Cheong*****

*National Institute of Crop Science RDA Suwon 441-857, Korea
**National Academy of Agricultural Science, RDA, Suwon, 441-707, Korea
***International Technology Cooperation Center, RDA, Suwon, 441-707, Korea
****MWorks, Co., LTD. Geumcheon-Ku, Seoul, 153-769, Korea
*****Department of Applied Life Science, College of Life and Environment Science, Konkuk University, Seoul 143-701, Korea

ABSTRACT The objectives of present study were to characterize the peptides which were isolated from Korean fermented soybean paste, *chungkukjang*, and to determine their antioxidant activities. Four fractions were collected from the methanol extract of *chungkukjang* by using a recycling preparative HPLC. Among fractions, Fr-2 was identified to be highly potent free radical scavenging activity in the assay of 1,1-diphenyl-2-picryl-hydrazyl (DPPH) and nitroblue tetrazolium(NBT)-reduction inhibition. Base on antioxidant effects, fraction Fr-2 was employed for the refraction with a prep-column and separated into five fractions of which two fractions were identified to have higher antioxidant activity. To confirm the amino acid constituents of antioxidant fractions Fr-2-2 and Fr-2-3 were analyzed, and eight kinds of amino acids such as aspartic acid, threonine, serine, glutamic acid, glycine, lysine, histidine, and arginine were identified as the constituent amino acids. Antioxidant activities of the separated peptides were further assessed cell viability with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl terazolium bromide (MTT), and fluorescence-activated cell sorting (FACS) analysis of H4IIE cells treated with hydrogen peroxide (H_2O_2). *Chungkukjang* peptides have shown their ability to protect H4IIE rat hepatoma cells against H_2O_2-induced oxidative stress by concentration and time-dependent manner. Therefore, These results indicated that fermented soybean paste *chungkukjang* will be promoted the antioxidant and radical scavenging activities, and beneficial for health. The antioxidant peptide fractions Fr-2-2 and Fr-2-3 were denominated as *P-NICS-1* and *P-NICS-2*, respectively. However, further studies were required to clarify their amino acid sequences and molecular properties, and physiological significances.

Keywords : *chungkukjang*, antioxidant, peptide, amino acid, DPPH, NBT-reduction, cell viability, DCFH-DA oxidation

Soybean (*Glycine max* L.) contains approximately 40 percent protein, and this produces various peptide and amino acid during processes like fermentation.

Fermented soy foods such as *chungkukjang* and doenjang are important source of the Korean diet. Fermentation period of *chungkukjang* is relatively shorter than other traditional fermented soybean pastes.

Korean *chungkukjang* and Japanese natto are very similar products, but the differences between *chungkukjang* and natto are mainly found in the different soybean-seed size, fermentation process, and consumption method (Choe *et al.,* 1996).

Chungkukjang is produced by fermentation with a cooked soybean, but their quality are varies considerably with soybean varieties, fermenting bacterial strains, fermentation time, sub-ingredients, and so on (Lee *et al.,* 2005).

During the fermentation, *chungkukjang* produces considerable amounts of ferments by *Bacillus substilis*. The ferments decompose carbohydrates and protein, and simultaneously produce a sticky substance composed of fructan in the form of polyglutamate (Fujii *et al.,* 1975). Polyglutamates produced in sufficiently fermented *chungkukjang* are sticky mucilage like spun thread and have a soft texture and

†Corresponding author: (Phone) +82-31-290-6746 (E-mail) kimsl@korea.kr
<Received 19 September 2011; Revised 14 November 2011; Accepted 19 November 2011>

characteristic flavor and taste (Lee *et al.*, 1991; Thomas *et al.,* 2005).

The consumption of *chungkukjang* has been increasing rapidly in recent years due to its health beneficial properties. Several studies have demonstrated the biological activities of *chungkukjang* such as anticancer, antihypertensive activity, hypocholesterolemic and hypolipidemic effects (Chung *et al.,* 1997; Kim *et al.,* 1996; Lee *et al.,* 1996; Kang *et al.*, 2003).

Generally, peptide is known not only as a nutrient but for functions in numerous biological reactions (Mahmoud, 1994). Especially, various peptides derived from fermented soybean foods are known as the most potent physiological agents (Lee, *et al.,* 1998).

Oligopeptide is composed from bonding of 2 to 10 amino acids, and it means below 10,000 of normal molecular weight. Peptide existing inside human bodies shows many physiological activities, therefore interests in biological reactions and physiological activities in foods are increasing recently. Peptide in food becomes a supplier of amino acid which is vital for protein biosynthesis in nutrition and has a great affect on properties of foods. Especially, serine, glycine, alanine function in reducing blood cholesterol level and have antioxidative effect and functions related to immunity (Lee *et al.,* 1998).

Bioactive peptides can be released by the microbial activity of fermented food or through enzymes derived from microorganism (Korhonen & Pihlanto, 2003). Therefore, fermentation is considered to be an efficient way to produce bioactive peptides and may also synthesize new peptide sequences. Numerous peptides with various bioactive functions such as antioxidative, anticancer, immunomodulatory actions, opioid agonistic and antagonistic have been identified (Chung *et al.*, 1997; Kang *et al.,* 2003; Mahmoud, 1994; Lee, 1998; Lee *et al.*, 2002; Korhonen & Pihlanto, 2003; Wenyi & Elvira, 2005). Generally, fermentation is not enough to fully hydrolyze soybean proteins. Glycoproteins, phosphoproteins, and other modified constituents that contain a higher number of disulfide bridges are more difficult to hydrolyze, but the proteases in *Bacillus* and *Rhizopus* strains can successfully cleave soybean proteins into large peptides (Wenyi & Elvira, 2005).

Interest in antioxidant activity of bioactive peptides generated from the fermentation of various proteins has attracted much attention in recent years.

The peptides from soybean, rice bran, oil seeds, seafood, milk and other dairy products have been extensively studied as the promising precursors of the bioactive peptides (Wenyi & Elvira, 2005; Gibbs *et al.*, 2004; Yoshikawa *et al.*, 2003; Floris *et al.*, 2003). Also, several reports have indicated that fermented *chungkukjang* showed stronger antimutagenic effects than nonfermented cooked soybeans (Lee *et al.*, 2005; Chung *et al.*, 1997; Ko *et al.*, 1996; Kwon *et al.*, 2002; Park *et al.*, 2001). However, little research has been done on the antioxidant properties of *chungkukjang* derived peptides. Therefore, this study was to separate and characterize the antioxidant peptides from Korean fermented soybean pasta, *chungkukjang*.

MATERIALS AND METHODS

Preparation of *chungkukjang*

Soybean variety 'Taekwangkong' was grown and harvested at the field of National Institute of Crop Science, Suwon, Korea.

Chungkukjang was prepared following the traditional procedure. The 1,000 g of carefully selected soybeans were soaked in water overnight, and steamed for 5 hours at 120℃. After cooled to about 40℃, bacterial inoculation of steamed soybean was conducted with 35 g of rice straw and incubated at 38℃ for 48 hours in the commercial fermentation jar. Rice straw provided the fermenting microorganisms, *Bacillus substilis*. Fully fermented *chungkukjang* was freeze-dried and powdered, and then defatted with hexane by using automatic fat extraction system (Gerhardt Soxtherm 2000, German), Defatted *chungkukjang* powder was extracted with methanol for 24 hours, then the suspension was filtered with No 3 filter paper, and pass through by a 0.25 μm PTFE filter. The filtrate was evaporated in a rotary vacuum evaporator (EYELA Co, Tokyo, Japan) prior to use.

Separation of peptide fractions

Condensed methanol extract of *chungkukjang* was fractionated by a recycling preparative HPLC (Japan Analytical Industry Co., Ltd., Tokyo, Japan) equipped with a Jaigel-

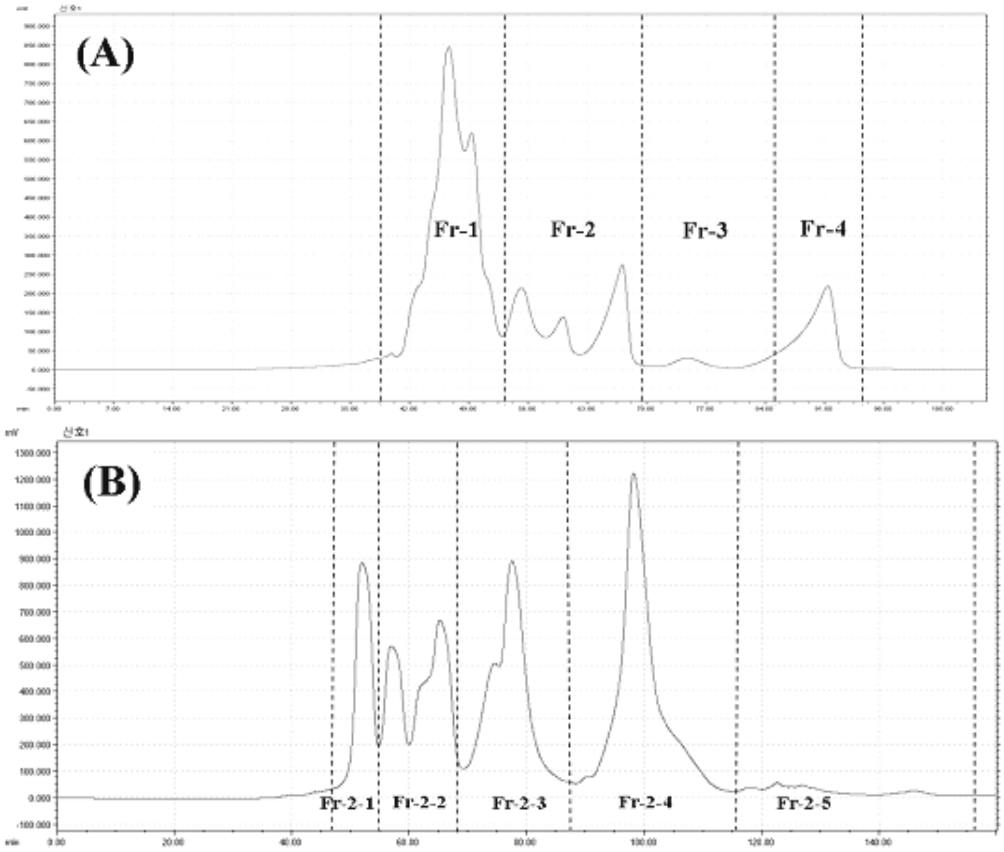


Fig. 1. Separation scheme of active fractions from methanol extract of *chungkukgang* by Jaigel-GS310 prep-column, and antioxidant fraction was selected by DPPH radical scavenging activity and NBT-reduction inhibition (A). Base on antioxidant effects, fraction Fr-2 was refractionated by Jaigel-W252 prep-column and five potent fractions were separated (B).

GS310 prep-column (20×500 mm, JAI, Japan).

As a mobile phase, methanol (100%, v/v) was applied at a flow rate of 5.0 ml/min in isocratic mode and the active fractions were detected at 280 nm (UV-3702, JAI, Japan). This procedure generated four fractions (Figure 1), and which were subjected to determine their antioxidant activities of DPPH free radical scavenging and NBT reduction inhibition. Considering antioxidative effects, the fractions which displayed the highest antioxidant activity were then further separated on a Jaigel-W252 prep-column (20×500 mm, JAI, Japan). To accomplish this, methanol (100%, v/v) was applied at a flow rate of 3.5 mL/min in isocratic mode and the active fraction was collected. Finally, five potent fractions were separated (Figure 1).

Assessment of radical scavenging activity

DPPH radical scavenging activities of separated fractions were measured by 1,1-diphenyl-2-picryl-hydrazyl (DPPH). Assessment of free radical scavenging activity was conducted by measuring the absorbance of DPPH as described by Yoshida *et al.* (2003). The reaction mixture, which comprised 2.5 mL of 0.35 mM DPPH (dissolved in 50% ethanol, v/v) and 0.2 mL of collected fractions, was incubated for 10 min at room temperature, and changes in DPPH absorbance were measured at 517 nm. Absorbance of ethanol (100%, v/v) was used as a blank. The antioxidant activity was calculated as per cent inhibition caused by the hydrogen donor activity of each sample: Inhibition (%)=(*1-absorbance of sample/absorbance of blank*)×100. The NBT-reduction inhibition was measured by a spectrophotometer at 560 nm (Asada *et al.,* 1974). Test tubes containing reaction solution were illuminated with 20-W Sylvania Groux fluorescent lamps for 7 min at 25℃. Antioxidant activity was represented as follows: Antioxidant activity (%)=(*1-A/B*) where A is

the absorbance of samples and B is the absorbance of the control, respectively.

Peptide characterization by reversed-phase HPLC

Fractions were separated with a reversed-phase C_{18} column (4.6×250 mm, 5u, Phenomenex, USA) and Millenium^{32} HPLC workstation system (Waters, USA). The mobile phase consisted of 0.1% trifluoroacetic acid (TFA) in water (eluent A) and of 0.1% TFA in acetonitrile (eluent B). The flow rate was 1 mL/min and a 90 min gradient of 20~45% eluent B was followed by elution with 45% eluent B for 20 min, and spectra were monitored at 210 nm. The injection volume for all fractions was 20 uL.

Amino acid analysis

The 3 mL of 6 N-HCl was added on the obtained fractions, and hydrolyzed for 24 hours at 110℃ in test tubes with nitrogen gas flushing. Afterwards, the hydrolyzed samples were diluted to the 100 mL of Milli-Q water and filtered with Millipore 0.45 um syringe filters (Waters, Milford, USA). Filtrated sample was put into an autosampler and injected into an amino acid autoanalyzer (Hitachi L8800, Japan). The amount of each amino acid present in the sample was calculated with reference to the standard amino acids (Ajnomoto-Takara Co., Japan).

Assessment of cell viability and DCFH-DA oxidation

Cell viability assay was performed using the modified 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) methods (Chung *et al.*, 1997). The rat hepatoma H4IIE cell line was cultured in the medium containing 10% fetal bovine serum and 1% penicillin/streptomycin in a humidified atmosphere with 5% CO_2 at 37℃. H4IIE cells were passaged every 3-4 days. After incubation, cells were stained with MTT (0.2 mg/mL) for 4 hours. After the medium was removed, formazan crystals formed in the wells were dissolved in 100 μL dimethylsulfoxide(DMSO). The absorbance was measured at 540 nm using an ELISA microplate reader (Multiskan EX, Thermo Labsystems). Cell viability was calculated using the following equation: Viability(% control)=*absorbance of sample/(absorbance of control)×100*.

For the assay of DCFH-DA oxidation, H4IIE cells (1×10^6 cells/mL) were washed twice with phosphate-buffered saline and 2',7'-dichlorofluorescein diacetate (DCFH-DA) was added to the cell suspension, than incubated in a 37℃ water bath for 10 min. After incubation, H4IIE cells were treated with peptide fractions of *chungkukjang* in the presence and absence of 250 μM H_2O_2-induced oxidative stress. The antioxidant activity was evaluated by measuring the intensity of dichlorofluorescein (DCF) fluorescence using fluorescence-activated cell sorting (FACS, Becton Dickinson, USA) analysis (Chi *et al.*, 2007).

RESULTS AND DISCUSSION

Separation of antioxidant peptides

In the present study, we tried to separate the peptide fractions from the methanol extract of *chungkukjang* by using a recycling preparative LC.

Recycling preparative LC consists of a switching-column which is accomplished by permitting the eluent to be directed from the column outlet back into the column inlet. Therefore, the separation process among peaks can be repeated to increase the effective separation power. For this, recycling preparative LC has been successfully applied to separate natural products and compounds (Martin *et al.*, 1976; Grill, 1998), but little has been done on the separation of peptides in the fermented soybean foods. Methanol extract of *chungkukjang* was loaded on Jaigel-GS310 prep-column and fractionated, and this work repeated until the peaks separated.

The columns applied to this study are a kind of gel filtration chromatography (GFC) column. They are based on the discrimination of individual components by the pores of the packing material. Therefore, large molecules can only partially penetrate the pores, whereas smaller molecules can access most of all pores. Thus, large molecules elute first, smaller molecules elute later.

As shown in Figure 1A, four fractions were collected with GS310 column, and they were subjected to determine the antioxidant activity according to the methods of DPPH free radical scavenging activity and NBT-reduction inhibition.

Among four fractions, the second fraction Fr-2 was identified to be highly potent in the assay of DPPH free radical scavenging activity and NBT-reduction inhibition.

Base on antioxidative effects, fraction Fr-2 was employed for the refraction with a Jaigel-W252 prep-column which has been frequently used for the removal of aggregates in a final polishing step. This procedure generated five fractions (Figure 1B), and they were also subjected to antioxidant activity assay according to the same methods. Considering the antioxidant activity assay results, two antioxidant peptide fractions were finally selected, then their amino acid constituents and molecular properties were determined by using amino acid autoanalyzer and reversed-phase HPLC, respectively.

Radical scavenging activity of separated fractions

The fractions separated from the methanol extract of *chungkukjang* by using GS310 and W252 prep-column were subjected to assay the antioxidant activities.

DPPH is a relatively stable organic radical that shows maximum absorbance at 517 nm, thus widely used as a substrate to evaluate the efficacy of antioxidants (Shimada *et al.*, 1992; Nanjo *et al.*, 1996). In our DPPH assay, separated fractions from the methanol extract of *chungkukjang* reduced the DPPH radical to a yellow-colored compound, apparently due to the DPPH radical accepting an electron or a hydrogen to become a stable diamagnetic molecule. The antioxidant activity of the second fraction Fr-2 was significantly higher as compared with other separated fractions in two methods. Fraction Fr-2 showed approximately 72.4% of radical scavenging activity in DPPH, and 81.4% in NBT reduction inhibition assay (Figure 2).

According to the antioxidant activity assay results, fraction Fr-2 was selected for the refractionation on a W252 prep-column, then finally separated into five fractions, and these partially purified peptide fractions were also subjected to antioxidant activity assay.

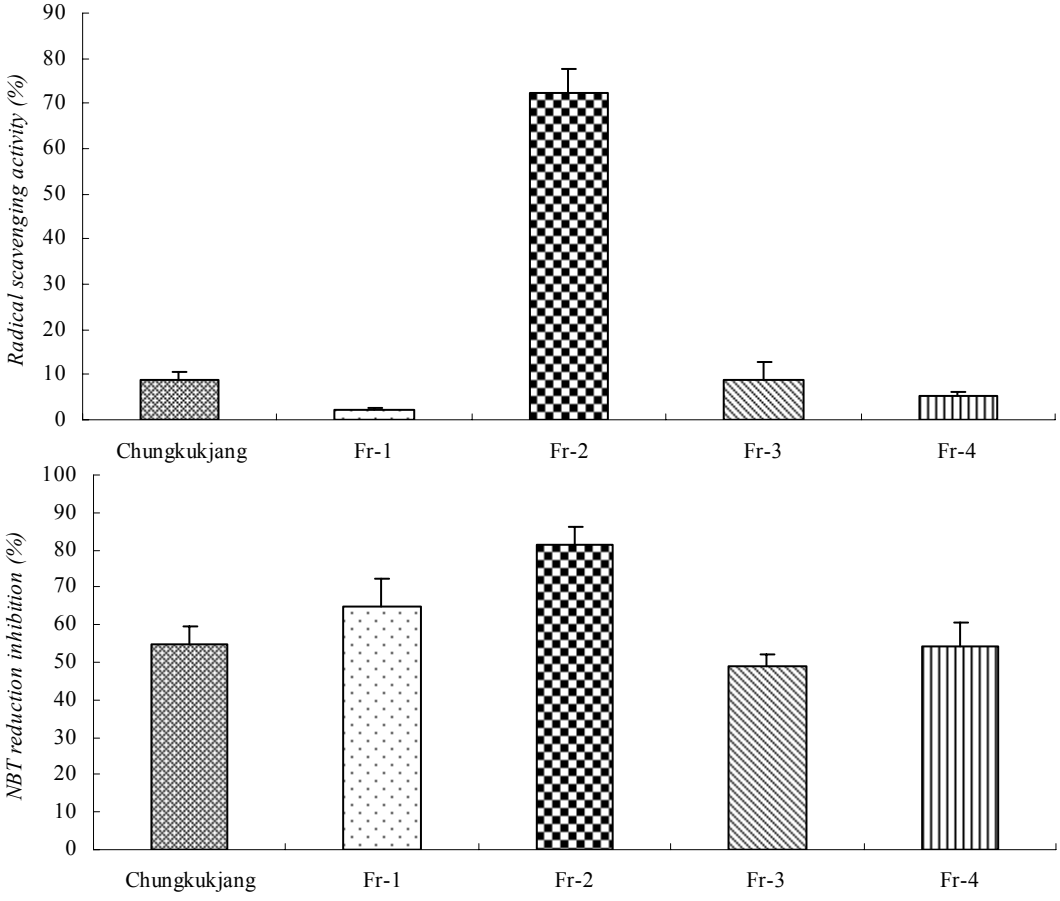


Fig. 2. DPPH radical scavenging activity (upper panel) and NBT-reduction inhibition (lower panel) of separated fractions from methanol extract of *chungkukjang*.

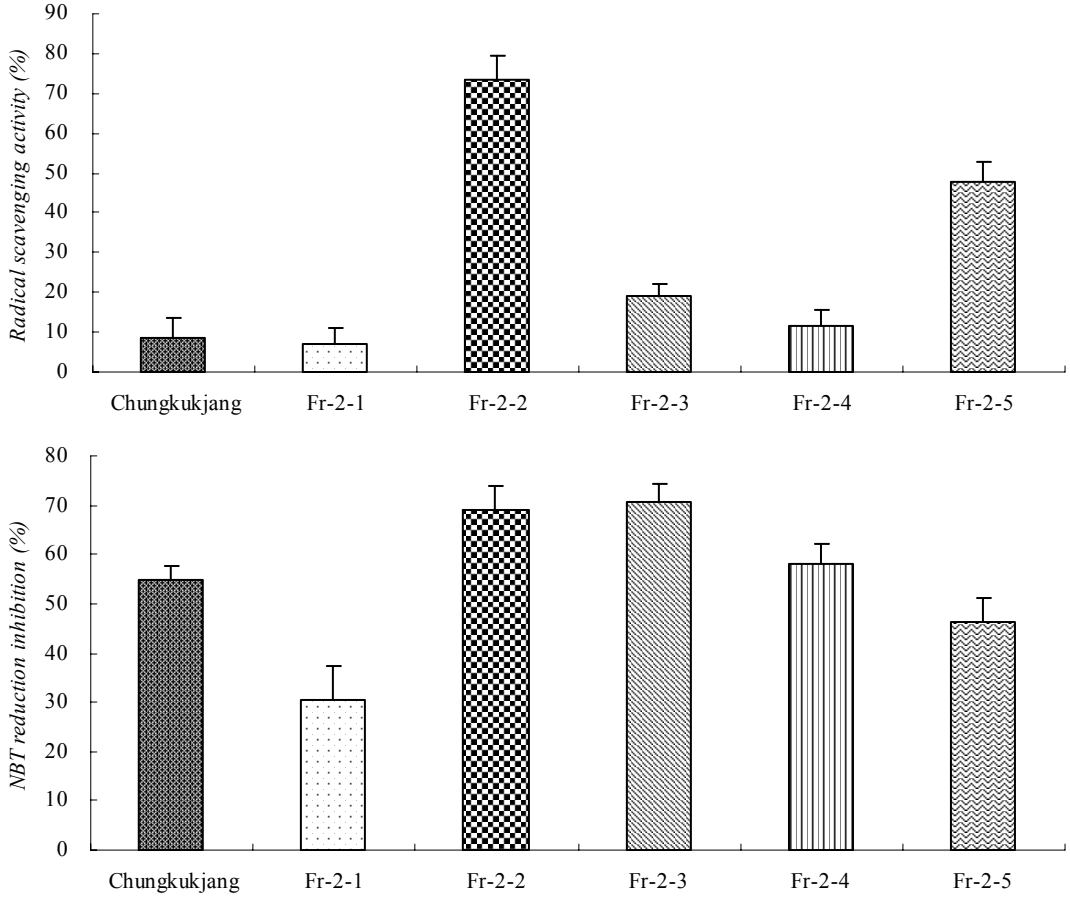


Fig. 3. DPPH radical scavenging activity (upper panel) and NBT-reduction inhibition (lower panel) of separated peptide fractions, *Fr-2-2* and *Fr-2-3*.

Among the five fractions, fraction Fr-2-2 was observed to be the most potent antioxidant activity of 73.3% in DPPH assay, furthermore fractions Fr-2-2 and Fr-2-3 showed their activities 69.1% and 70.5% in NBT reduction inhibition assay, respectively (Figure 3).

Several peptides are generally accepted to be antioxidative activity. Chen *et al.* (1998) reported soybean peptides have higher antioxidant activity than intact soybeans. Matoba (2002) also reported that after enzyme digestion of β-conglycinin and glycinin, the radical-scavenging activities were increased 3 to 5 times than intact soybean protein.

Fermentation is considered to be an efficient way to produce bioactive peptides. Bioactive peptides are released by the microbial activity of fermented food or through enzymes derived from microorganism (Korhonen & Pihlanto, 2003).

Therefore, this study results suggest that the peptide fractions separated from the methanol extract of *chungkukjang* have an antioxidant activity.

Peptide characterization by reversed-phase HPLC

We examined the peptide properties of selected antioxidant fractions by using analytical HPLC, and obtained results were presented in Figure 4.

The column utilized in the investigation was reversed-phase C_{18} (4.6×250 mm, 5u) column designed for separation of proteins and peptides (> 10,000 MW).

As shown in Figure 4, the fractions were composed of various peaks, but the HPLC elution profiles of two fractions Fr-2-2 and Fr-2-3 were apparently different between each other. Generally, peptide chromatographic profiles are determined by the characters of their side chains and substituent groups, which define their basic or acidic character, or the degree of hydrophobicity or hydrophilicity

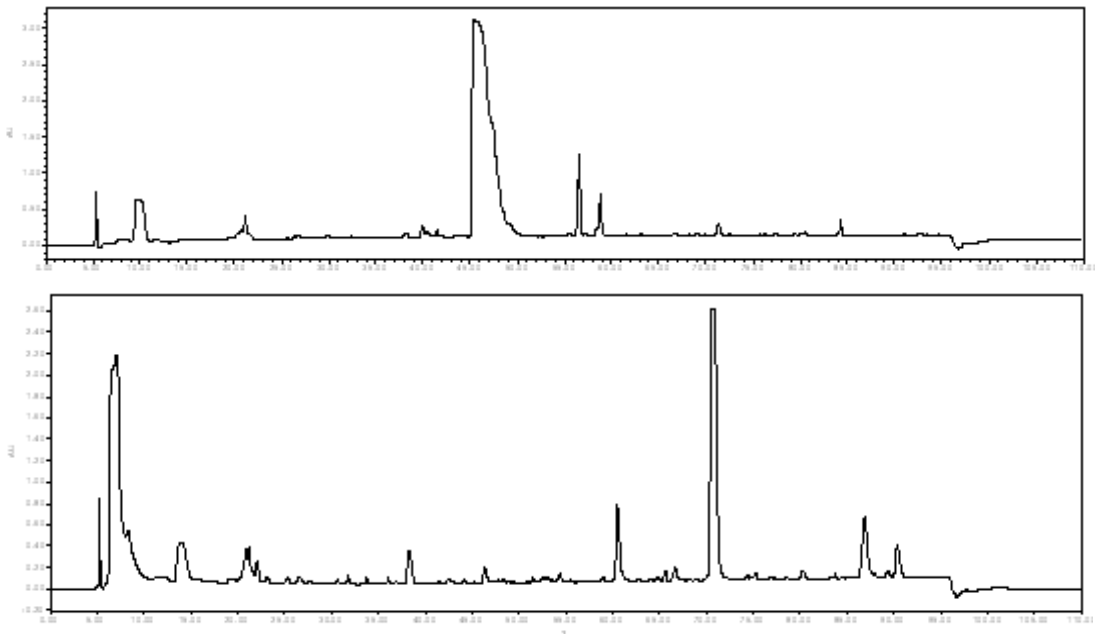


Fig. 4. Reversed-phase HPLC chromatogram profiles on a Jupiter C18 column (4.6×250 mm, 5 μ) of antioxidant fractions Fr-2-2 (upper chromatogram) and Fr-2-3 (lower chromatogram) which were fractionated by a recycling preparative LC equipped with a Jaigel-W252 prep-column (20×500 mm) as shown in Figure 1. Chromatogram was obtained by using a 0.1% TFA in water (eluent A) and 0.1% TFA in acetonitrile (eluent B). Flow rate was 1 mL/min and a 90 min gradient of 20~45% eluent B was followed by elution with 45% eluent B for 20 min, and spectra were monitored at 210 nm. The injection volume was 20 uL.

(Bogusław *et al.,* 2007). Moreover, the retention times of peptides are depended on specific interactions between stationary phase, mobile phase, and the investigated compounds. Therefore, as observed in the present study, different elution profiles of antioxidant fractions on retention can be explained the properties of peptide fractions. As presented in Figure 1, two antioxidant fractions Fr-2-2 and Fr-2-3 were separated with Jaigel-W252 prep-column, thus the large peptide molecules had eluted first, while the smaller peptide molecules had eluted later.

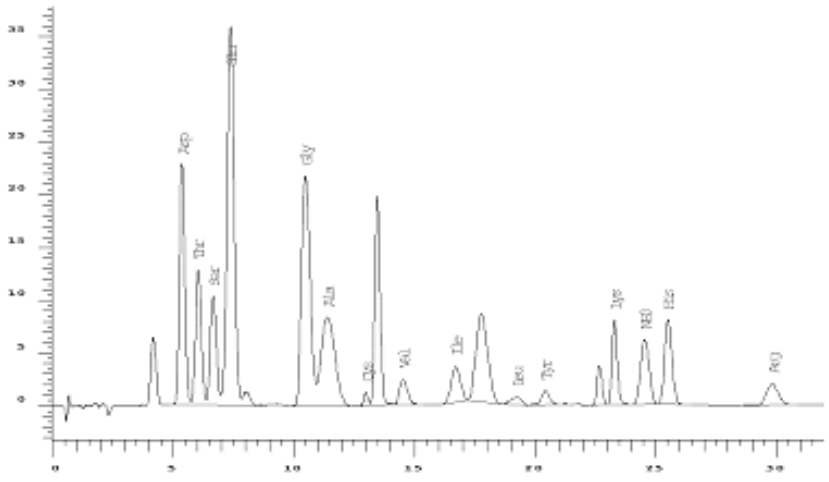
However, the reversed-phase HPLC elution profiles of two fractions were completely different from the profiles of a Jaigel-W252 prep-column. In general, the smaller and hydrophilic molecules were eluted earlier on the reversed-phase C_{18} column. Therefore, this study results implied that the antioxidant peptide fraction Fr-2-2 is composed of relatively large molecules and more potent hydrophobicity than those of fraction Fr-2-3. However, further investigations are required to clarify the nature of the peptide fractions.

Amino acid composition of *chungkukjang* and their antioxidant fractions

The amino acid compositions of *chungkukjang*, and their antioxidant fractions Fr-2-2 and Fr-2-3 were analyzed by amino acid autoanalyzer.

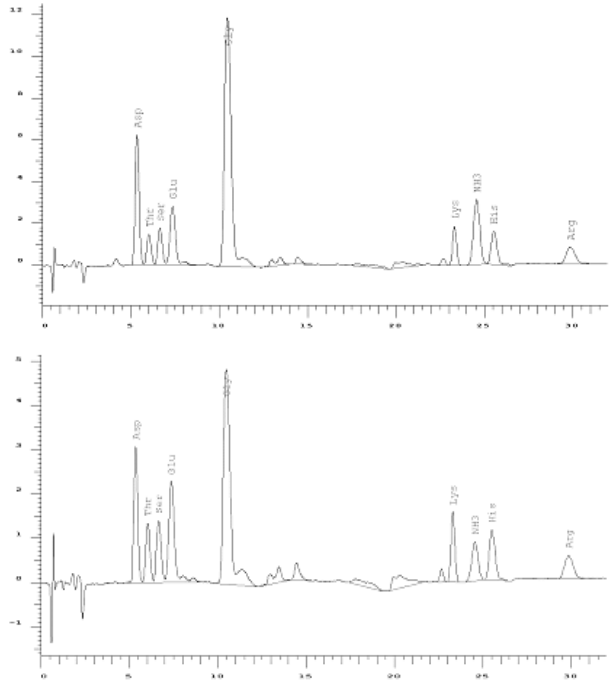
Eighteen amino acids were identified in *chungkukjang* (Figure 5). The obtained results showed that acidic amino acids such as glutamic and aspartic acid constitute approximately 32.6% of the total amino acids, and glutamic acid is the most abundant amino acid in *chungkukjang*. It is generally accepted that the specific savory taste of *chungkukjang* mainly comes from glycine, glutamic acid, arginine, and other free amino acids. Lee (1973) and An *et al.* (1987) also reported that fermented soybean products had more glutamic acid and aspartic acid in amino acid than soybeans, therefore our study results agreed with these findings.

To confirm the amino acid constituents of antioxidant fractions Fr-2-2 and Fr-2-3 were analyzed, and Figure 6 presented the results.



Amino acids	Composition (%)
Aspartic acid	12.68±0.71
Threonine	6.13±1.24
Serin	5.73±0.36
Glutamic acid	19.93±1.19
Glycine	10.14±0.80
Alanine	4.70±0.45
Cystein	0.70±0.22
Valine	9.01±1.26
Methionine	1.31±0.09
Isoleucine	1.87±0.24
Leucine	4.65±0.62
Tyrosine	0.38±0.06
Phenylalanine	2.79±1.63
Lysine	4.46±0.08
Histidine	4.47±0.55
Arginine	4.15±0.42
Proline	6.90±0.75

Fig. 5. Amino acid composition of Korean fermented soybean paste, *chungkukjang*.



Amino acids	Composition (%)	
	Fr-2-2	Fr-2-3
Aspartic acid	15.91±0.16	14.12±0.13
Threonine	4.11±0.40	6.61±0.46
Serine	5.13±0.09	7.43±0.05
Glutamic acid	9.70±0.52	15.04±0.39
Glycine	49.82±3.45	36.60±2.87
Lysine	4.68±0.11	7.20±0.55
Histidine	6.02±0.21	7.91±0.65
Arginine	4.62±0.31	5.09±0.25

Fig. 6. Amino acid compositions of antioxidant peptide fractions, Fr-2-2 (upper chromatogram) and Fr-2-3 (lower chromatogram) which were separated from Korean fermented soybean paste, *chungkukjang*.

Eight kinds of amino acids such as aspartic acid, threonine, serine, glutamic acid, glycine, lysine, histidine, and arginine were identified as the constituent amino acids of two peptide fractions. Among the eight amino acids, glycine was revealed as the most abundant constituent amino acid, and considerable amounts of aspartic acid, glutamic acid, histidine, serine, lysine, arginine, and threonine were found in Fr-2-2 and Fr-2-3, respectively.

Several peptides are generally accepted have an antioxidative activity, and these peptides usually composed of 2-20 amino acid residues per molecule, and the lower the molecular weight, the higher their chance to exert biological effects (Pihlanto-Leppala, 2001; Roberts *et al.,* 1999). The peptides derived from soybean protein hydrolysate

with 5-16 amino acid residues showed strong inhibition activities on the autoxidation of linoleic acid (Chen *et al.*, 1995). Kim *et al.* (1999) have obtained a glycopeptide from ethanol fractions of bromelain-defatted soybeans hydrolysate composed mainly of Asp, Glu, Pro, Gly and Leu with strong cytotoxic activity against P388D1 mouse lymphoma cells. Hydrophobic amino acids and one or more residues of His, Pro, Met, Cys, Tyr, Trp, Phe and Met are believed to enhance the activities of the antioxidant peptides (Chen *et al.*, 1998; Da'valos *et al.*, 2004; Herna'ndez-Ledesma *et al.*, 2005). Among the listed amino acids, histidine, methionine and cysteine are very important to the radical scavenging activity of peptides due to their special structure of characteristics. Histidine has the proton-donation ability, while methionine is prone to oxidation of the methionine sulfoxide, and cysteine donates the sulfur hydrogen (Herna'ndez-Ledesma *et al.*, 2005; Tsuge *et al.*, 1991). Some recent reports suggested that the peptide Gln-Gly-Ala-Arg, which exhibited the highest antioxidant activity from porcine skin collagen hydrolysates (Li *et al.*, 2007; Suetsuna *et al.,* 2000), and the Glu-Leu residue in peptides was recently reported to play an important role in radical scavenging (Jun *et al.*, 2004). Additionally, presence of aspartic acid seems to play a vital role irrespective of its position as observed in several antioxidative peptide sequences (Byuna *et al.*, 2009).

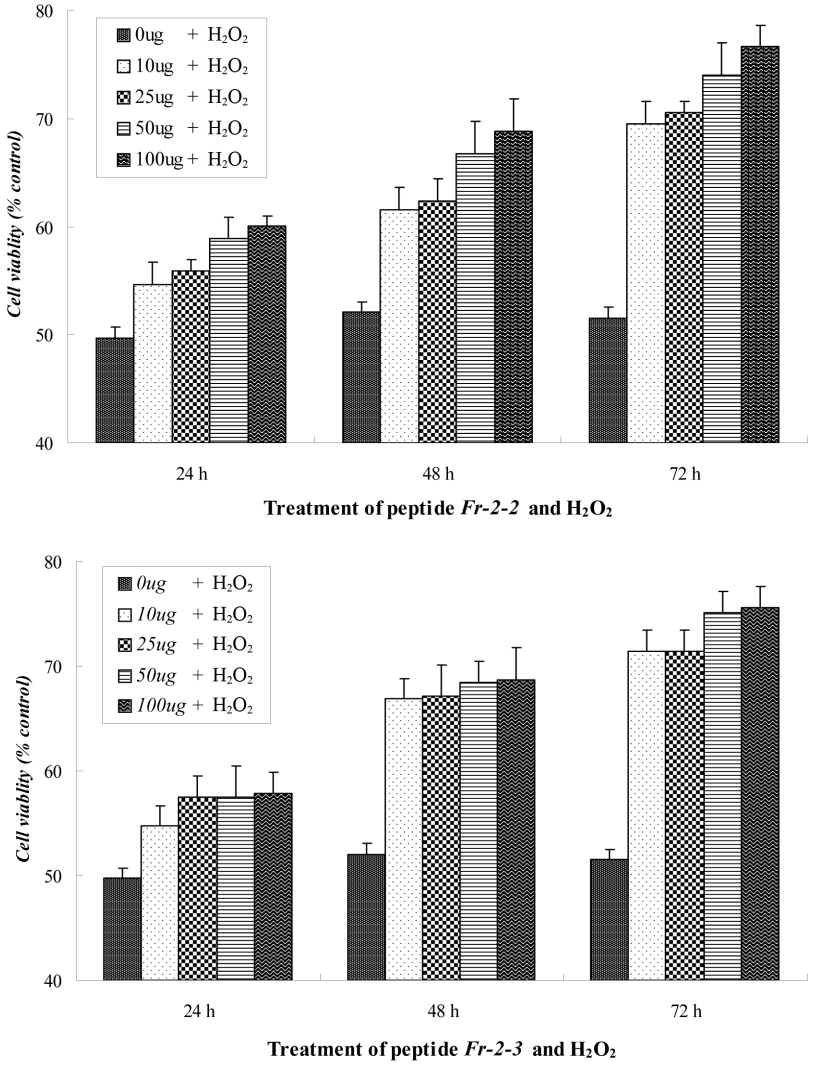


Fig. 7. Concentration and time-dependent antioxidant effects on H4IIE cells which were treated with H_2O_2-induced oxidative stress. Cell viability was expressed relative to the untreated control cell. Each value represents the mean±SD.

In the present study, we identified the eight amino acids (Gly, Asp, Glu, His, Ser, Lys, Arg, and Thr). Therefore, we speculate that these amino acids might play an important role on their antioxidant activity of the two peptide fractions Fr-2-2 and Fr-2-3.

The antioxidant activity of peptides is closely related to their amino acid constituents and their sequences (Chen *et al.,* 1998; Suetsuna *et al.,* 2000). However, this study didn't specifically investigate the amino acid sequence due to the separated fractions were overlayed with other peaks (Figure 1). Therefore, to obtain more information about antioxidant peptides derived from *chungkukjang*, further investigations of the amino acid sequences of the active peptide fractions Fr-2-2 and Fr-2-3 were required.

Effect of *chungkukjang* peptides on cell viability and DCFH-DA oxidation

In order to examine the effect of peptide fractions to protect from cell death, a MTT assay was performed using rat hepatoma H4IIE cell line.

Base on a preliminary MTT assay result (data not shown), we determined an optimal incubation condition that maintains approximately 50% cell viability upon exposure of the cells to 1 mM H_2O_2 stress.

In the absence of peptide fractions, cell viability was approximately 50% after the treatment of H_2O_2-induced oxidative stress. However, in the presence of peptide fractions, cell viability was remarkably higher as increasing the concentrations of peptides from 10 to 100 μg/mL, although the H4IIE cells were exposed to the H_2O_2-induced oxidative stress (Figure 7).

These results strongly suggested that the peptide fractions Fr-2-2 and Fr-2-3 have a significant effect on the cell viability in a concentration and time dependent manner. Therefore, the ability of *chungkukjang* peptides to protect from cell death was considered mainly due to their potent antioxidant activity that reduces the oxidative stress.

Conversion of non-fluorescent dichlorofluorescein diacetate

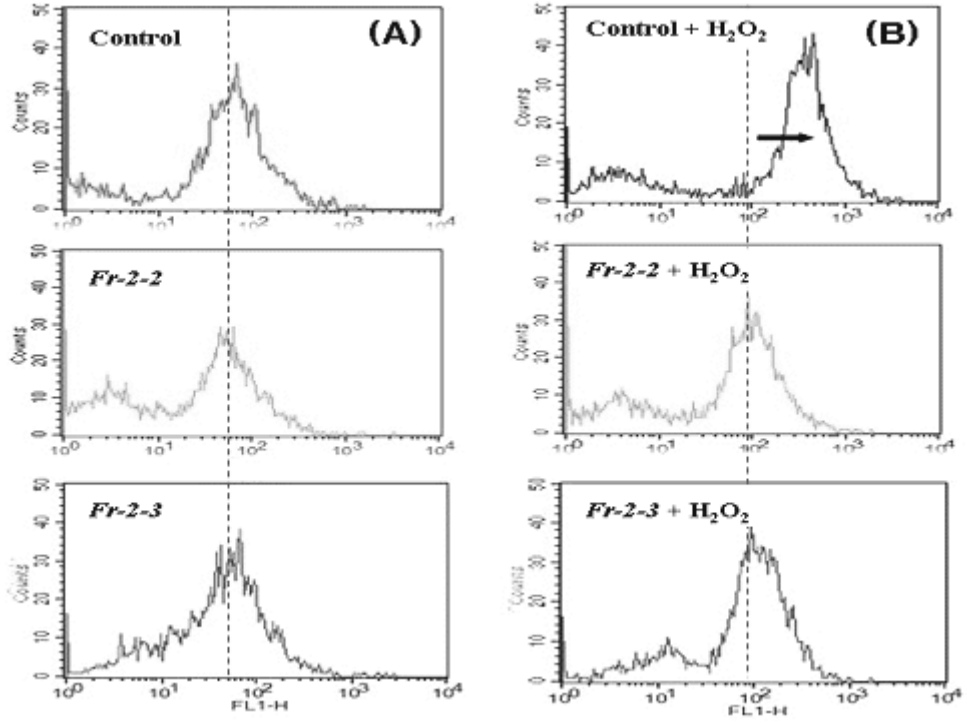


Fig. 8. Fluorescence activated cell sorter (FACS) analysis of *chungkukjang* peptide fractions. H4IIE cells were treated with peptide fractions in the presence and absence of 250 μM H_2O_2-induced oxidative stress. (A): H4IIE cells treated with peptide fractions, and control, *Fr-2-2 and Fr-2-3*. (B): H4IIE cells treated with peptide fractions and H_2O_2-induced oxidative stress.

(DCFH-DA) to fluorescent dichlorofluorescein (DCF) by hydrogen peroxide can be detected by a fluorescence activated cell sorter (FACS) assay. Once DCFH-DA is taken up by living cells, it is deacetylated to non-fluorescent dichlorofluorescein by the action of esterase. And, subsequent oxidation of DCFH by ROS produces DCF which can be easily visualized (Halliwell & Gutteridge, 1999). As shown in Figure 8A, H_2O_2-treated H4IIE cells increased DCF fluorescence significantly as compared with the control H4IIE cells.

However, in the addition of fractions Fr-2-2 and Fr-2-3, the DCF fluorescence of H4IIE cells was not increased, although the cells were exposed to H_2O_2-induced oxidative stress (Figure 8B). These results were well consistent with the results obtained by the observation of the antioxidant activity assays of DPPH scavenging activity, NBT-reduction inhibition, and MTT cell viability.

Therefore, the presented study results indicated that fermented soybean paste *chungkukjang* will promote the antioxidant and radical scavenging activities, and beneficial for health. The antioxidant peptide fractions Fr-2-2 and Fr-2-3 were denominated as *P-NICS-1* and *P-NICS-2*, respectively, and further studies are under way in our laboratory to clarify the amino acid sequences and molecular properties, and physiological significances of these peptides.

REFERENCES

An, H. S., J. S. Bae, and T. S. Lee. 1987. Comparison of free amino acids, sugars, and organic aicd in soybean paste prepared with various organisms. *J. Korean Agricultural Chemical.* 30 : 345-350.

Asada, K., M. Takahashi, and M. Nagate. 1974. Assay and inhibitors of spinach superoxide dismutase. *Agr. Biol. Chem.* 38 : 471-173.

Bogusław, B., K. Sylwia, K. Tomasz, R. Katarzyna, M. Monika, and J. Tobias. 2007. HPLC columns partition by chemometric methods based on peptides retention. *J. Chromatogr. B.* 845 : 253-260.

Byuna, H. G., L. K. Lee, H. G. Park, J. K. Jeon, and S.K. Kim. 2009. Antioxidant peptides isolated from the marine rotifer, *Brachionus rotundiformis*. *Process Biochemistry.* 44 : 842-846.

Chen, H. M., K. Muramoto, and F. Yamaguchi. 1995. Structural analysis of antioxidative peptides from soybean b-conglycinin. *J. Agric. Food Chem.* 43 : 574-578.

Chen, H. M., K. Muramoto, F. Yamaguchi, K. Fujimoto, and K. Nokihara. 1998. Antioxidative properties of histidine-containing peptides designed from peptide fragments found in the digests of a soybean protein. *J. Agric. Food Chem.* 46 : 49-53.

Chi, H. Y., C. H. Lee, K. H. Kim, S. L. Kim, and I. M. Chung. 2007. Analysis of phenolic compounds and antioxidant activity with H4IIE cells of three different rice grain varieties. *Eur. Food Res. Technol.* 225 : 887-893.

Choe, J. S., J. S. Kim, S. M. Yoo, H. J. Park, T. Y. Kim, C. M. Chang, and S. Y. Shin. 1996. Survey on preparation method and consumer response of Chung-Kuk-Jang. *Korea Soybean. Digest.* 13 : 29-43.

Chung, K. S., K. D. Yoon, D. J. Kwon, S. S. Hong, and S. Y. Choi. 1997. Cytotoxicity testing of fermented soybean products with various tumour cells using MTT assay. *Kor. J. Appl. Microbiol. Biotechnol.* 28 : 239-241.

Da'valos, A., M. Miguel, B. Bartolome', and R. Lo'pez-Fandino. 2004. Antioxidant activity of peptides derived from egg white proteins by enzymatic hydrolysis. *J. Food Protection.* 67 : 1939–944.

Floris, R.; I. Recio, B. Berkhout, and S. Visser. 2003. Antibacterial and antiviral effects of milk proteins and derivatives thereof. *Curr. Pharm. Des.* 9 : 1257-1275.

Fujii, H., A. Shiraishi, H. Kaba, M. Shibagaki, S. Takahashi, and A. Honda. 1975. In topics in secondary metabolism I *Bacillus subtillis*: *Molecular biology and industrial application. Hakkokogaku Kaishi.* 53 : 424.

Gibbs, B. F., A. Zougman, R. Masse, and C. Mulligan. 2004. Production and characterization of bioactive peptides from soy hydrolysate and soy-fermented foods. *Food Res. Int.* 37 : 123-131.

Grill, C. M. 1998. Closed-loop recycling with periodic intra-profile injection: a new binary preparative chromatographic technique. *J. Chromatogr.* 796 : 101-113.

Halliwell, B., J. and M. C. Gutteridge. 1999. Free radicals in biology and medicine, 3rd ed. *Oxford University Press, Oxford.* p. 381-385.

Herna'ndez-Ledesma, B., A. Da'valos, B. Bartolome', and L. Amigo. 2005. Preparation of antioxidant enzymatic hydrolysates from clactalbumin and a-lactoglobulin, identification of active peptides by HPLC–.MS/MS. *J. Agric. Food Chem.* 53 : 588-593.

Jun, S. Y., P. J. Park, W. K. Jung, and S. K. Kim. 2004. Purification and characterization of an antioxidative peptide from enzymatic hydrolysate of yellowfin sole (*Limanda aspera*) frame protein. *Eur. Food Res. Technol.* 219 : 20-26.

Kang, M. J., J. I. Kim, and T. W. Kwon. 2003. Effect of chongkukjang on blood glucose and lipid profile in neonatal streptozotocin-induced diabetic rats. *Food Sci. Biotechnol.* 12 : 544-547.

Kim, J. Y., H. J. Woo, C. W. Ahn, H. S. Nam, Z. I. Shin,

and H. J. Lee. 1999. Cytotoxic effects of piptides fractionated from bromelain hydrolyzates of soybean protein. *Foo Sci. Biotechnol.* 8 : 333-337.

Kim, W., K. Choi, Y. Kim, H. Park, J. Choi, Y. Lee, H. Oh, I. Kwon, and S. Lee. 1996. Purification and characerterization of fibrinolytic enzyme produced form Bacillus sp strain CK-11-4 screened from chungkukjang. *Appl. Environ. Microbiol.* 62 : 2482-2488.

Ko, H. S.; H. K. Ju, K. O. Jung, and K. Y. Park. 1996. Antimutagenic effect of chungkookjangs prepared with the different kinds of soybeans. *J. Korean Assoc. Cancer Preven.* 4 : 204-212.

Korhonen, H., and A. Pihlanto. 2003. Food-derived bioactive peptides-opportunities for designing future foods. *Curr. Pham. Des.* 9 : 1297-1308.

Kwon, E. Y., K. O. Jung, S. H. Moon, and K. Y. Park. 2002. Studies on enhancing chemopreventive effect of chungkookjangs-Antimutagenic activity of chungkookjangs prepared with the different fermentation periods and ingredient ratio. *J. Korean Assoc. Cancer Preven.* 7 : 200-209.

Lee, B. Y., D. M. Kim, and K. H. Kim. 1991. Physicochmical properties of viscous substances extracted from chungkookjang. *Korean J. Food Sci. Technol.* 23 : 599-604.

Lee, C. H. 1973. Studies on the amion acid composition of Korean fermented soybean Meju products and the evaluation of the protein qualiy. *Korean J. Food Sci. Technol.* 5 : 210-214.

Lee, H. J. 1998. Health fuctional peptides form soyben foods. *Korea Soybean Digest.* 15 : 16-22.

Lee, M. Y., S. Y. Park, K. O. Jung, K. Y. Park, and S. D. Kim. 2005. Quality and functional characteristics of chungkukjang prepared with various *Bacillus* sp. isolated from traditional chungkukjang. *J. of Food Sci.* 70 : 191-196.

Lee, S. H., B. N. Cho, C. K. Hyun, and S. S. Jew. 2002. Physiological, functional characterisetics of silk peptide: Antioxidant effect and immune fuction. *Food Science and Industry.* 35 : 57-62.

Lee, S. K.; S. Heo, D. H. Bae, and K. H. Choi. 1998. Medium optimization for fibrinolytic enzyme production by Bacillus subtilis KCK-7 isolated from Korean traditional chungkukjang. *J. Appl. Microbiol. Biotechnol.* 26 : 226-231.

Li, B., F. Chen, X. Wang, B. Ji, and Y. Wu. 2007. Isolation and identification of antioxidative peptides from porcine collagen hydrolysate by consecutive chromatography and electrospray ionization mass spectrometry. *Food Chem.* 102 : 1135-1143.

Mahmoud, M. I. 1994. Physicochemical and fucctional properties of protein hydrolysates in nutritional products. *Food Technology.* 48 : 89-95.

Martin, M., F. Verillon, C. Eon, and G. Guiochon. 1976. Theoretical and experimental study of recycling in high performance liquid chromatography. *J. Chromatogr.* 125 : 17-41.

Matoba, T. 2002. How does the radical-scavenging activity of soy protein food change during heating. *Daizu Tanpakushitsu Kenkyu.* 5 : 47-50.

Nanjo, F., K. Goto, R. Seto, M. Suzuki, M. Sakai, and Y. Hara. 1996. Scavenging effects of tea catechins and their derivatives on 1,1,-diphenyl-2-picrylydrazyl radical. *Free Radical Biology and Medicine*. 21 : 895-902.

Park, K. Y., E. Y. Kwon, and K. O. Jung. 2001. Studies on enhancing chemopreventive effect of chungkookjangs. Antimutagenic activity of chungkookjangs prepared with the different varieties of soybean and starter. *J. Korean Assoc Cancer Preven.* 6 : 36-43.

Pihlanto-Leppala A. 2001. Bioactive peptides derived from bovine whey proteins: opioid and ACE-inhibitory peptides. *Trends Food Sci. Tech.* 11 : 347-356.

Roberts, P. R., J. D. Burney, K. W. Black, and G. P. Zaloga. 1999. Effect of chain length on absorption of biologically active peptides from the gastrointestinal tract. *Digestion.* 60 : 332-337.

Shimada, K., K. Fujikawa, K. Yahara, and T. Nakamura. 1992. Antioxidative properties of xanthan on the antioxidation of soybean oil in cyclodextrin emulsion. *J. Agric. Food Chem.* 40 : 945-948.

Suetsuna, K., H. Ukeda, and H. Ochi. 2000. Isolation and characterization of free radical scavenging activity peptides derived from casein. *J. Nutrition and Biochemistry*. 11 : 128-131.

Thomas, C., M. Michèle, and F. Agnès. 2005 CapE, a 47-amino-acid peptide, is necessary for *Bacillus anthracis* polyglutamate capsule synthesis. *J. of Bacteriology.* 187 : 7765-7772.

Tsuge, N., Y. Eikawa, Y. Nomura, M. Yamamoto, and K. Sugisawa. 1991. Antioxidant activity of peptides prepared by enzymatic hydrolysis of egg-white albumin. *Nippon Nogeikagaku Kaishi.* 65 : 1635-1641.

Wenyi, W., and G. M. Elvira. 2005. A new frontier in soy bioactive peptides that may prevent age-related chronic diseases. *CRFSFS.* 4 : 63-78.

Yoshida, T., K. Mori, T. Hatano, T. Okumura, I. Uehara, K. Komagoe, Y. Fujita, and T. Okuda. 1989. Studies on inhibiton mechanism of autooxidation by tannins and related polyphenols on 1.1-diphenyl-2-picrylhydrazyl radical. *Chem. Pharm. Bull.* 37 : 1919-1923.

Yoshikawa, M., M. Takahashi, and S. Yang. 2003. Delta opioid peptides derived from plant proteins. *Curr. Pharm. Des.* 9 : 1325-1330.