Antioxidant and Neuroprotective Effects of *Doenjang* Prepared with *Rhizopus*, *Pichia*, and *Bacillus*

Su Jin Kang¹, Ji Yeon Seo¹, Kye Man Cho², Chang Kwon Lee³, Jeong Hwan Kim⁴, and Jong-Sang Kim¹,⁵

¹School of Food Science and Biotechnology (BK21 Plus Program) and ²Institute of Agricultural Science and Technology, Kyungpook National University, Daegu 41566, Korea
³Department of Food Science, Gyeongnam National University of Science and Technology, Gyeongnam 52725, Korea
⁴Department of Research and Development, Monggo Foods Co., Ltd., Gyeongnam 51395, Korea
⁵Division of Applied Life Science (BK21 Plus), Graduate School, and Institute of Agriculture and Life Science, Gyeongsang National University, Gyeongnam 52828, Korea

**ABSTRACT:** A new type of *doenjang* was manufactured by mixing soaked soybean, *koji* (*Rhizopus oryzae*), *cheonggukjang* (*Bacillus amyloliquefaciens* MJ1-4 and *B. amyloliquefaciens* EMD17), and *Pichia farinosa* SY80 as a yeast, salt, and water, followed by fermentation with *koji* that was made by fermenting whole wheat with *R. oryzae*. The mixed culture *doenjang* was designed to have a more palatable flavor and stronger biological activities than the conventional product. The extract of mixed culture *doenjang* showed higher antioxidant activity than the commercial *doenjang* as evaluated by the ferric reducing antioxidant power assay although it was not significantly different from the commercial product in 2,2-diphenyl-1-picrylhydrazyl and 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical scavenging activities. Further, the mixed culture *doenjang* reduced intracellular reactive oxygen species levels and protected cells from glutamate-induced cytotoxicity more efficiently in human hippocampal HT22 neuroblastoma cells than the commercial *doenjang*. In conclusion, a newly-developed mixed culture *doenjang* had a strong antioxidant activity *in vitro* and cultured cell model systems, exhibited a potential to prevent oxidative stress-associated disorders although animal and clinical studies are needed to confirm its in vivo efficacy.

**Keywords:** *Rhizopus oryzae*, *doenjang*, antioxidant, neuroprotective effect

**INTRODUCTION**

In Korea, soybean has been processed in various ways such as tofu, soymilk, soy sprout, and other fermented products. In particular, *doenjang*, *ganjang*, and *gochujang* are major traditional fermented soy foods, with annual production of 91,449, 116,458, and 140,832 metric ton, respectively, in 2012 (1). The main ingredient in *doenjang* is *meju*, which is normally made by fermenting cooked soybean with airborne fungi or artificial inoculation with *Aspergillus oryzae* or *Aspergillus sojae*.

It has been reported that *doenjang* has several health benefits including anticarcinogenic, antiobesity, anti-inflammatory, antioxidant, and anti-melanogenesis actions (2-5). The major components that contribute to the health benefits of *doenjang* are isoflavones and their derivatives, peptides, and undigested protein (6-8). A commercial *doenjang* could be further improved in terms of favor, quality, and bioactive function. Therefore, we developed a novel formula for mixed culture *doenjang* consisting of 2,000 g soaked soybean, 1,080 g *koji*, 300 g *cheonggukjang*, 120 g yeast, 500 g salt, and 1,000 g water. Yeast (*Pichia farinosa* SY80) and *cheonggukjang* containing *Bacillus amyloliquefaciens* MJ1-4 and *B. amyloliquefaciens* EMD17 were added to improve flavor and to confer antimicrobial and antioxidant activities. Our previous study showed that fermentation of soybean with *B. amyloliquefaciens* MJ1-4 and *B. amyloliquefaciens* EMD17 significantly increased fibrinolytic, anti-microbial, and antioxidant activity (9), and these *Bacillus* strains were utilized for manufacturing *cheonggukjang* that was used in preparing the mixed culture *doenjang*.

Oxidative stress is associated with numerous diseases including cancer, atherosclerosis, Parkinson’s disease, and Alzheimer’s disease (AD). The brain is particularly vulnerable to oxidative damage and has high oxygen con-
sumption. About 20% of all oxygen and 25% of all glucose are consumed by cerebral functions. The brain also contains a relatively high content of polyunsaturated fatty acids (PUFA) that are more sensitive to oxidation (10). On the other hand, levels of antioxidant defense in the brain are modest, which render neurons especially sensitive to a disturbance in the balance between antioxidants and production of ROS. Moreover, the brain also has a high content of redox active metals, which can promote the formation of reactive oxygen species (ROS) and has been linked to AD pathology.

Therefore, numerous attempts have been made to treat or prevent AD using antioxidants. Although most such trials failed, antioxidant compounds still remain as promising candidates for controlling AD.

While soybean contains a variety of bioactive components, fungal fermentation could lead to enhanced biological activity by converting glycoside forms of bioactive compounds into aglycones that are more bioavailable forms (11). Thus this study attempted to develop a new mixed culture doenjang with higher antioxidant and neuroprotective activities than the commercial product while keeping good sensory properties.

**MATERIALS AND METHODS**

**Materials**

All reagents used were of ACS grade, and were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). All cell culture reagents and fetal bovine serum (FBS) were obtained from Gibco BRL (Grand Island, NY, USA). 2',7'-dichlorofluorescein diacetate (DCFDA) used as a molecular dye was purchased from Welgene (Daegu, Korea) and Gibco BRL culture reagents and fetal bovine serum (FBS) were obtained from Invitrogen (Carlsbad, CA, USA).

**Preparation of doenjang extract**

Mixed culture doenjang was prepared by mixing soaked soybean (2,000 g), koji (1,080 g), cheonggukjang (300 g), yeast (120 g), salt (500 g), and water (1,000 g). An aliquot was stored in a deep freezer for the study while the rest of it was subjected to fermentation for 6 weeks at 25±3°C. For koji preparation, whole wheat was soaked in water for 6 h, autoclaved at 121°C for 30 min, cooled down to room temperature, followed by inoculation with Rhizopus oryzae (5%, w/w) and incubated for 5 days 25±1°C. Cheonggukjang containing B. amyloliquefaciens MJ1-4 and B. amyloliquefaciens EMD17 strains was used for its antioxidative, anti-bacterial, and fibrinolytic activities. Commercial doenjang was obtained from Monggo Foods Co., Ltd. (Changwon, Korea). Freeze-dried doenjang samples were extracted with 10 volumes of 80% (v/v) ethanol, filtered, and concentrated to a final concentration of 10 mg/mL, and filtered through 0.2 μm sterile syringe filter before assays.

**FRAP assay**

The ferric reducing antioxidant power (FRAP) of doenjang extract was determined as described previously (12). Briefly, 30 μL of H₂O, 30 μL of ferrous sulfate as standard, or samples were incubated at room temperature with 1 mL of FRAP reagent [300 mmol/L acetate buffer (pH 6.3), 10 mmol/L 2,4,6-tri(2-pyridyl)-1,3,5-triazine (TPTZ) solution, 20 mmol/L FeCl₃ solution, and H₂O₂], and the absorbance was recorded after 4 min. FRAP values of unknowns were calculated on the basis of standard curves established using standards at 0.1 ~ 1.0 mmol/L.

**DPPH radical scavenging assay**

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of doenjang extract was evaluated as previously described (13). Briefly, 50 μL of sample solution [or dimethyl sulfoxide (DMSO)] was added to 200 μL of 200 μM DPPH radical solution, which was freshly made. After 30 min of incubation at room temperature, the absorbance at 515 nm was measured.

A synthetic antioxidant reagent, L-ascorbic acid (AA), was used as a positive control, and all tests were carried out in triplicate.

**ABTS radical cation decolorization assay**

The 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) solution was prepared by reacting 5 mL of 7 mM ABTS and 80 μL of 2.45 mM potassium persulfate, and the mixture was shaken in the dark at room temperature for 12 h before use (14). It was diluted with ethanol so that its absorbance was adjusted to 0.7 at 734 nm. ABTS (1 mL) was added to glass test tubes containing 50 μL of samples and mixed by a vortex mixer for 30 s. The absorbance was measured at 734 nm after 5 min. The percentage of radical scavenging activity was calculated by comparing the absorbance values of the control without samples. All determinations were performed 3 times.

**Determination of total phenolic and flavonoid contents**

Total phenolics were determined using the Folin-Ciocalteu reagent (15). Briefly, 100 μL of extract was mixed with 50 μL of sodium bicarbonate solution (10%, w/v), followed by the addition of 15 μL of Folin-Ciocalteu reagent (previously diluted 5-fold with distilled water). After 5 min at room temperature, the sample mixture was transferred to a 96-well microplate, and the absorbance at 593 nm was measured using a microplate reader. Results are expressed as gallic acid equivalents.

Total flavonoid content was determined by aluminum chloride using a colorimetric method previously described with slight modifications (16). Briefly, 25 μL of the sample was mixed with 75 μL of 95% methanol in a 96-well
microplate. Then, 5 µL of 10% AlCl₃·6H₂O, 1 M potassium acetate, and 14 µL of distilled water were added, and the mixture was incubated for 40 min at room temperature. The absorbance readings were obtained at 415 nm with a microplate reader (Sunrise, Tecan Group Ltd., Männedorf, Switzerland). The total flavonoid content of the samples was extrapolated from standard curves established with quercetin at 0~50 µg/mL.

**Determination of intracellular ROS levels in HT22 cells**

Intracellular levels of ROS were quantified by the 2,7-dichlorofluorescein (DCF) assay according to Wang and Joseph (17), with slight modifications. A mouse hippocampal cell line (HT22 cells) was obtained from Professor Dong Seok Lee and Kyung Sik Song (Kyungpook National University, Daegu, Korea). For routine maintenance, cells were grown in α-medium essential media (Gibco BRL) supplemented with 10% heat-inactivated FBS in a humidified CO₂/95% air incubator (Thermo Fisher Scientific, Waltham, MA, USA). Most of the steps, including incubation of the samples, was extrapolated from standard curves established with quercetin at 0~50 µg/mL.

**Neuroprotective activity assay**

HT22 mouse hippocampal neuronal cells were routinely maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% FBS and 100 units/mL penicillin and 100 µg/mL streptomycin in a humidified CO₂ incubator (MCO-19-AIC, Sanyo, Osaka, Japan) at 37°C and 5% CO₂/95% air.

The cells were seeded in a 96-well culture dish in DMEM supplemented with 10% FBS at a density of 2×10³ cells per well for HT22. The next day, various doses of samples and 5 mM of glutamate were added to the cells (18). After culturing cells for 24 h, the cell survival rate was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay (18). Cell viability is presented as the percentage relative to the untreated control.

**Statistical analysis**

Statistical significance of the data was tested by analysis of variance, followed by Student’s t-test (for independent samples), using SPSS Statistics software version 22 (SPSS Inc., Chicago, IL, USA). Data are presented as mean±standard error (SE).

**RESULTS**

**Ferric reducing capacity of extracts from mixed culture and commercial doenjang**

When the Fe³⁺-TPTZ complex is reduced to the Fe²⁺-TPTZ form by an antioxidant under acidic conditions, an intense blue color with an absorption maximum develops at 593 nm. Therefore, the antioxidant effect (reducing ability) can be evaluated by monitoring the formation of a Fe²⁺-TPTZ complex using a spectrophotometer. The ferric reducing capacity of extracts from both newly-developed and commercial doenjang was dose-dependently increased. Furthermore, the mixed culture doenjang extract was found to have a higher FRAP value than the commercial doenjang (Fig. 1).

**Radical scavenging activity of extracts from mixed culture and commercial doenjang**

The aqueous ethanolic extracts of mixed culture and commercial doenjang showed scavenging activities from DPPH and ABTS⁺ radicals in dose-dependent manners.

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Fig. 1. Ferric reducing antioxidant power (FRAP) values of mixed culture and commercial doenjang extracts. Sample extracts were prepared by extracting freeze-dried doenjang samples with 10 volumes of 80% (v/v) ethanol, removing solvent by rotary evaporation and nitrogen flushing, and then redissolving the extract in the solvent at the concentration of 10 mg/mL. Significantly different from each other by Student’s t-test at **P<0.01.
(Fig. 2 and 3). However, there was no significant difference in radical scavenging activity of the extracts from the 2 different types of *doenjang*.

**Inhibition of glutamate-induced ROS production by *doenjang* extract**

In the absence of glutamate treatment, intracellular ROS levels were not significantly different in HT22 cells treated with either mixed-culture or commercial *doenjang* extract although it was lower in the cells treated with 200 µg/mL extract from commercial *doenjang* than the mixed culture *doenjang*. Treatment with 5 mM glutamate caused a significant increase of intracellular ROS levels that was suppressed by simultaneous treatment with the mixed culture *doenjang* extract but not by the commercial *doenjang* extract (Fig. 4).

**Protective effects of *doenjang* extract from glutamate-induced cytotoxicity in HT22 cells**

Mouse hippocampal HT22 cells treated with glutamate (5 mM), were killed by 60~70%. However, addition of mixed culture *doenjang* extract into the culture attenuated glutamate-induced cytotoxicity in a dose-dependent manner (Fig. 5). However, commercial *doenjang* extract did not recover glutamate-induced cytotoxicity and even further amplified growth inhibition caused by glutamate.
DISCUSSION

*Doenjang*, one of the most consumed traditional seasonings and condiments in Korea, is usually made from soybean fermented with fungi such as *Aspergillus oryzae* and salt. *Doenjang* is coarser in texture, like a chunky peanut butter, and it is an excellent source of free amino acids, organic acids, minerals, and vitamins (19). In particular, the taste of *doenjang* was found to be influenced by free amino acids, sugars, and organic acids (oxalic, succinic, fumaric, and citric acids), whereas the flavor was associated with acetic acid, ethyl alcohol, benzaldehyde, ethyl acetate, ethyl 2-methyl butanoate, 2,5-dimethylpyrazine, tetramethylpyrazine, isovaleric acid, 2-methylbenzaldehyde, tetramethylpyrazine, benzaldehyde, ethyl alcohol, ethyl caprylate, furfural, and butanoic acid (20).

As yeast was reported to improve the flavor of *doenjang* while *B. amyloliquefaciens* MJ1-4 and *B. amyloliquefaciens* EMD17 had anti-microbial, antioxidant, and fibrinolytic activities, we prepared the mixed culture *doenjang* using these *Bacillus* strains, yeast, and *R. oryzae*. In particular, *doenjang* manufactured with *R. oryzae* was reported to contain high levels of chitoooligosaccharides (171 μg/g) due to autolysis of fungal cell walls consisting of chitin during aging whereas *doenjang* made with koji of *A. oryzae* had low level of chitoooligosaccharides (0–37.2 μg/g) (21).

Overall sensory properties of the newly-prepared product were superior to commercial brands of *doenjang* (data not shown). Furthermore, it showed stronger antioxidant activity as measured by FRAP, compared to commercial *doenjang*, consistent with our previous data that the manufacturing of *cheonggukjang* using *B. amyloliquefaciens* MJ1-4 enhanced the levels of total phenolics and isoflavone aglycones as well as antioxidant activity (9). However, there was no significant difference in DPPH and ABTS radical scavenging activities between mixed culture and commercial *doenjang*. That is, the mixed culture *doenjang* had stronger ferric ion-reducing activity than commercial *doenjang*, but both kinds of *doenjang* showed similar potential to scavenge radicals from DPPH and ABTS. Although the FRAP assay was reported to be similar to the results obtained from the DPPH and ABTS radical scavenging assays, the antioxidant activities of the *doenjang* samples tested in the study showed discrepancies depending on the assays used, maybe due to mechanistic differences (22).

In addition, the mixed culture *doenjang* with *cheonggukjang* prepared by fermenting it with *B. amyloliquefaciens* MJ1-4 showed higher amounts of total phenolics than the commercial *doenjang* (Table 1), suggesting that isoflavone glycosides of soybean were extensively metabolized into aglycones during fermentation and/or the aging process of mixed culture *doenjang*. Our previous study showed that *B. amyloliquefaciens* MJ1-4 was capable of producing β-glycosidase in soybeans and was accompanied by rapid conversion of isoflavone glycosides into aglycones in the process of preparing *cheonggukjang* (9).

It is widely recognized that the aglycone forms of isoflavones had higher *in vivo* and *ex vivo* antioxidant capacity than their glycosides (23), consistent with our finding that the mixed culture *doenjang* with higher flavonoids inhibited intracellular ROS production and glutamate-induced cytotoxicity in mouse hippocampal HT22 cells more efficiently than the commercial counterpart. Several studies have shown that soy isoflavones have neuroprotective effects in various models including rat primary cortical neuron, hippocampus, and SH-SY5Y challenged with β-amyloid, glutamate, thapsigargin, and tert-butylhydroperoxide (24). In particular, genistein was reported to attenuate learning and memory deficits in amyloid β(1–40)-treated rat model of AD (25).

Meanwhile, soy isoflavone aglycones are found to be significantly more bioavailable than their glycosides in animal models, and thereby have increased potential to exert antioxidant action and health benefits including neuroprotection.

In conclusion, the mixed culture *doenjang* developed in the study had higher antioxidant and neuroprotective activities than the commercial product, and warrants further study to confirm its preventive effects against ROS-mediated chronic diseases including cancer, cardiovascular disease, cataract, age-related macular degeneration, and aging in general.

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**AUTHOR DISCLOSURE STATEMENT**

The authors declare no conflict of interest.

### Table 1. Total phenolics and flavonoids contents of mixed culture and commercial *doenjang*

| Samples          | Commercial doenjang | Mixed culture doenjang |
|------------------|---------------------|------------------------|
| Total phenolics (GAE mg/g) | 10.9±0.2          | 10.8±0.3               |
| Total flavonoids (μg/g)    | 273.2±23.2         | 525.9±79.4             |

Data represent mean±SD (n=3). *1Gallic acid equivalents.*
REFERENCES

1. MFDS. 2014. 2014 Production of food and food additives. Ministry of Food and Drug Safety, Osong, Chungbuk, Korea. p 86.

2. Lee DE, Lee KW, Byun S, Jung SK, Song N, Lim SH, Heo YS, Kim JE, Kang NJ, Kim BY, Bowden GT, Bode AM, Lee HJ, Dong Z. 2011. 7,3',4'-Trihydroxyisoflavone, a metabolite of the soy isoflavone daidzein, suppresses ultraviolet B-induced skin cancer by targeting Cot and MKK4. J Biol Chem 286:14246-14256.

3. Nam YR, Won SB, Chung YS, Kwak CS, Kwon DY. 2011. Inhibitory effects of Doenjang, Korean traditional fermented soybean paste, on oxidative stress and inflammation in adipose tissue of mice fed a high-fat diet. Nutr Res Pract 9: 235-241.

4. Jeong JK, Chang HK, Park KY. 2014. Doenjang prepared with mixed starter cultures attenuates azoxymethane and dextran sulfate sodium-induced colitis-associated colon carcinogenesis in mice. J Carcinog 13: 9.

5. Cha YS, Park Y, Lee M, Chae SW, Park K, Kim Y, Lee HS. 2014. Doenjang, a Korean fermented soy food, exerts antibesity and antioxidative activities in overweight subjects with the PPAR-γ2 C1431T polymorphism: 12-week, double-blind randomized clinical trial. J Med Food 17: 119-127.

6. Hwang KM, Jung KO, Song CH, Park KY. 2008. Increased antimutagenic and anticlastogenic effects of doenjang (Korean fermented soybean paste) prepared with bamboo salt. J Med Food 11: 717-722.

7. Park KY, Jung KO, Rhee SH, Choi YH. 2003. Antimutagenic effects of doenjang (Korean fermented soypaste) and its active compounds. Mutat Res Fundam Mol Mech Mutagen 523-524: 43-53.

8. Lovati MR, Manzoni C, Gianazza E, Arnoldi A, Kurowska E, Kim JE, Kang NJ, Kim BY, Bowden GT, Bode AM, Lee HJ, Dong Z. 2011. 7,3',4'-Trihydroxyisoflavone, a metabolite of the soy isoflavone daidzein, suppresses ultraviolet B-induced skin cancer by targeting Cot and MKK4. J Biol Chem 286:14246-14256.

9. Kim JH, Hwang CE, Lee CK, Lee JH, Kim GM, Jeong SH, Shin JH, Kim JS, Cho KM. 2014. Characteristics and antioxidant effect of garlic in the fermentation of cheonggukjang by Bacillus amyloliquefaciens MJ1-4. J Microbiol Biotechnol 24: 959-968.

10. Marszałek JR, Lodish HF. 2005. Docosahexaenoic acid, fatty acid-interacting proteins, and neuronal function: breastmilk and fish are good for you. Annu Rev Cell Dev Biol 21: 633-657.

11. Nam DH, Kim HJ, Lim JS, Kim KH, Park CS, Kim JH, Lim J, Kwon DY, Kim JH, Kim JS. 2011. Simultaneous enhancement of free isoflavone content and antioxidant potential of soybean by fermentation with Aspergillus oryzae. J Food Sci 76: H194-H200.

12. Benzie IFF. 1996. An automated, specific, spectrophotometric method for measuring ascorbic acid in plasma (EFTSA). Clin Biochem 29: 111-116.

13. Katsube T, Tabata H, Ohba Y, Yamasaki Y, Anuarad E, Shiwaku K, Yamane Y. 2004. Screening for antioxidant activity in edible plant products: comparison of low-density lipoprotein oxidation assay, DPPH radical scavenging assay, and Fol-in-Ciocalteu assay. J Agric Food Chem 52: 2391-2396.

14. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med 26: 1231-1237.

15. Kuhnen S, Moacyr JR, Mayer JK, Navarro BB, Trevisan R, Honorato LA, Maraschin M, Pinheiro Machado Filho LC. 2014. Phenolic content and ferric reducing-antioxidant power of cow's milk produced in different pasture-based production systems in southern Brazil. J Sci Food Agric 94: 3110-3117.

16. Chung HS, Chang LC, Lee SK, Shamon LA, van Breemen RB, Mehta RG, Farnsworth NR, Pezzuto JM, Kinghorn AD. 1999. Flavonoid constituents of Chorizanthe diffusa with potential cancer chemopreventive activity. J Agric Food Chem 47: 36-41.

17. Wang H, Joseph JA. 1999. Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. Free Radic Biol Med 27: 612-616.

18. Behl C, Widmann M, Trapp T, Holsboer F. 1995. 17β-Estradiol protects neurons from oxidative stress-induced cell death in vitro. Biochem Biophys Res Commun 216: 473-482.

19. Yang SH, Choi MR, Kim JK, Chung YG. 1992. Optimization of the taste components composition in traditional Korean soybean paste. J Korean Soc Food Nutr 21: 449-453.

20. Lee JE, Kang SH, Kim HR, Lim SI. 2015. Volatile compounds analysis of certified traditional Doenjang. J Korean Soc Food Sci Nutr 44: 944-950.

21. Eum BW, Kwak BY, Kim SY, Shon DH, Lee KH. 2003. Enhancement of chitooligosaccharides in doenjang (soybean paste) and kanjang (soy sauce) using Bacillus subtilis koji and Rhizopus oryzae koji. Korean J Food Sci Technol 35: 291-296.

22. Moon JK, Shibamoto T. 2009. Antioxidant assays for plant and food components. J Agric Food Chem 57: 1655-1666.

23. Lee CH, Yang L, Xu JZ, Yeung SYV, Huang Y, Chen ZY. 2005. Relative antioxidant activity of soybean isoflavones and their glycosides. Food Chem 90: 735-741.

24. Lee YB, Lee HJ, Soln HS. 2005. Soy isoflavones and cognitive function. J Nutr Biochem 16: 641-649.

25. Bagheri M, Joghataei MT, Mohseni S, Roghani M. 2011. Genistein ameliorates learning and memory deficits in amyloid β1-40 rat model of Alzheimer’s disease. Neurobiol Learn Mem 95: 270-276.