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

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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 ozyzae*, *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 anti-microbial 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-

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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 Welgene (Daegu, Korea) and Gibco BRL (Grand Island, NY, USA). 2',7'-dichlorofluorescein diacetate (DCFDA) used as a molecular dye was purchased 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 *koij* 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 H_2O, 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 $FeCl_3$ solution, and H_2O], 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-sulphonic 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% $AlCl_3 \cdot 6H_2O$, 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-minimum essential media (Gibco BRL) supplemented with 10% heat-inactivated FBS at 37°C in an atmosphere of 5% CO_2/95% air under saturating humidity and passaged every other day (1:4 split ratio) by trypsinization with 0.25% trypsin/0.02% ethylenediaminetetraacetic acid sodium salt solution (Thermo Fisher Scientific, Waltham, MA, USA).

The cells (2,000 cells per well) were seeded into a black-bottom 96-well plate and cultivated for 24 h. After cell attachment, the plates were washed with phosphate buffered saline (PBS) and treated with increasing concentrations of sample extracts suspended in 10% FBS containing media for 12 h in the absence or presence of 5 mM glutamate. The cells treated with samples were washed with PBS and incubated for 30 min with DCFDA dissolved in DMSO (final concentration 50 μM). Fluorescence was measured at 0 and 40 min using an excitation wavelength of 485 nm and emission of 535 nm in a fluorescence microplate reader (Infinite 200, Tecan Group Ltd.). Most of the steps, including incubation of reaction mixture containing dye and oxidant, washing, and fluorimetric determination, were performed in the dark. The intensity of fluorescence was calculated as $[(F_{40\,min}-F_{0\,min})/F_{0\,min}]\times 100$ as described elsewhere (17). Results are expressed as the relative intensity of fluorescence (in % of negative control).

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 CO_2 incubator (MCO-19-AIC, Sanyo, Osaka, Japan) at 37°C and 5% CO_2/95% air.

The cells were seeded in a 96-well culture dish in DMEM supplemented with 10% FBS at a density of 2×10^3 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 Fe^{3+}-TPTZ complex is reduced to the Fe^{2+} 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 Fe^{2+}-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

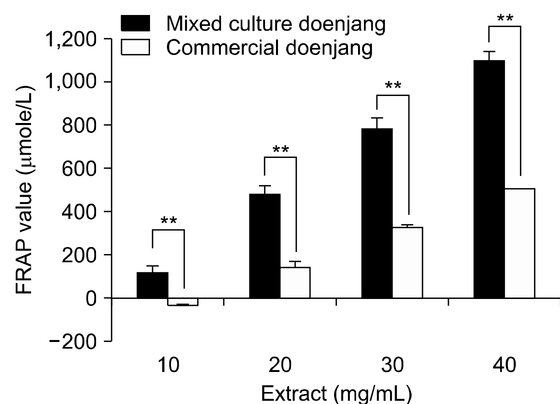

Fig. 1. Ferric reducing antioxidant power (FRAP) values of mixed culture and commercial *doenjang* extracts. Sample extracts were prepared by extracting freeze-dried *doenjang* samples with 10 volumes of 80% (v/v) ethanol, removing solvent by rotary evaporation and nitrogen flushing, and then redissolving 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.

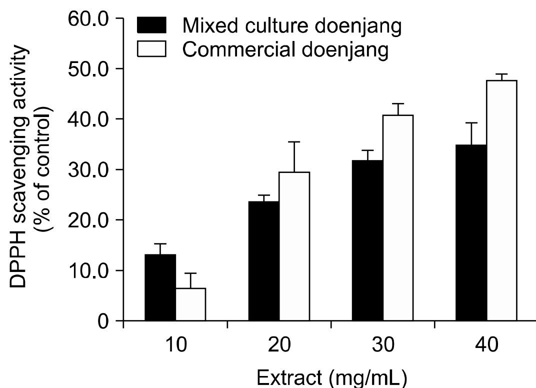

Fig. 2. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activities of mixed culture and commercial *doenjang* extracts. Refer to the legend under Fig. 1 for sample preparation for the assay.

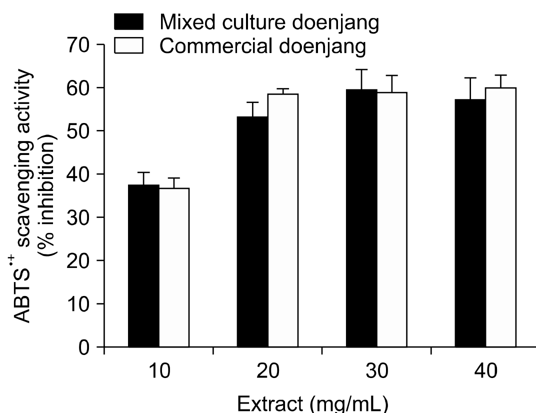

Fig. 3. 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activities of mixed culture and commercial *doenjang* extracts. Refer to the legend under Fig. 1 for sample preparation for the assay.

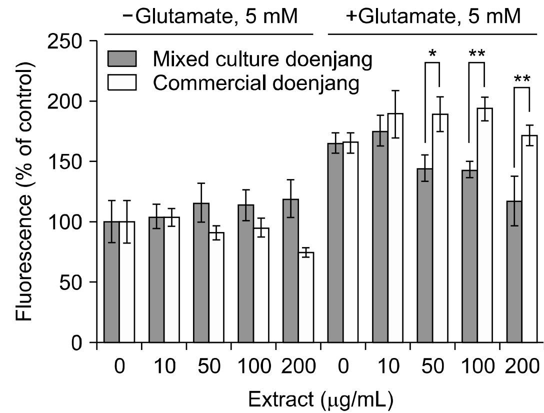

Fig. 4. Effect of mixed culture and commercial *doenjang* extracts on glutamate-induced reactive oxygen species (ROS) production in HT22 cells. HT22 cells were incubated with *doenjang* extracts (0, 30, 50, 100, and 200 μg/mL) in the absence or presence of 5 mM glutamate for 12 h. ROS production was monitored by incubating with 2′,7′-dichlorofluorescein diacetate for 30 min and measuring fluorescence at an excitation of 485 nm and emission of 535 nm. Fluorescence values are presented as the percentage relative to the untreated control (neither treated with glutamate nor *doenjang* extract). Significantly different from each other by Student's *t*-test at $^{*}P<0.05$ and $^{**}P<0.01$.

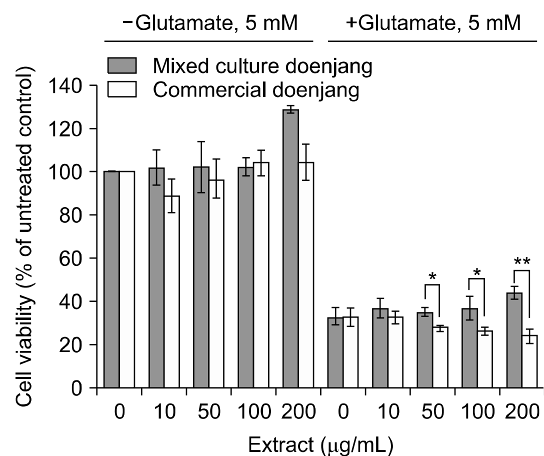

Fig. 5. Protective effect of mixed culture and commercial *doenjang* extracts against glutamate-induced cytotoxicity in HT22 cells. HT22 cells were seeded in a 96-well culture dish in DMEM supplemented with 10% fetal bovine serum at a density of 2,000 cells per well. The next day, various doses of samples (0, 10, 50, 100, and 200 μg/mL) and 5 mM of glutamate were added to the cells. After culturing cells for 24 h, the cell survival rate was determined by using MTT assay. Cell viability is presented as the percentage relative to the untreated control (neither treated with glutamate nor *doenjang* extract). Significantly different from each other by Student's *t*-test at $^{*}P<0.05$ and $^{**}P<0.01$.

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 sources 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 chitooligosaccharides (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 chitooligosacchardes (0∼37.2 μg/g) (21).

Overall sensory properties of the newly-developed 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).

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

	Samples	
	Commercial *doenjang*	Mixed culture *doenjang*
Total phenolics (GAE mg/g)[1]	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).
[1]Gallic acid equivalents.

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 $\beta_{(1\text{-}40)}$-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.

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