In Vitro Antidiabetic and Antiobesity Activities of Traditional Kochujang and Doenjang and Their Components

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ABSTRACT: In this study we investigated the antidiabetic and antiobesity effects of aqueous ethanol extracts of traditional kochujang and doenjang. The average α-glucosidase inhibitory activity and adipogenesis inhibitory activity for the kochujang samples were 29.6% and 20.8%, respectively, while those of the doenjang samples were 46.3% and 11.6%, respectively. Therefore, antidiabetic activity is high in doenjang and antiobesity activity is high in kochujang. Kochujang and doenjang components responsible for suppressing the functional effects were investigated by metabolomic analysis. For kochujang, *p*-coumaric acid, N6,N6,N6-trimethyllysine, threonine, and methionine positively correlated with inhibition of adipogenesis activity, whereas for doenjang, betaine and betaine aldehyde were thought to be responsible for the antidiabetic effects. As *p*-coumaric acid and betaine were the most probable candidates with functional effects, these two compounds were selected for further analysis. Inhibition of adipogenesis was shown to be $14.0\pm1.85\%$ for betaine chloride and $38.3\pm3.27\%$ for *p*-coumaric acid, suggesting that *p*-coumaric acid is more effective than betaine against obesity. However, betaine exhibited higher α-glucosidase inhibitory activity than *p*-coumaric acid. Our results suggest that both kochujang and doenjang can be used against diabetes and obesity. However, clinical trials are necessary to support these results.

Keywords: fermented foods, antidiabetic, antiobesity, betaine, *p*-coumaric acid

INTRODUCTION

For over two thousand years, traditional Korean cuisine has consisted of boiled rice (bap) served with side dishes, collectively named as "banchan", that are made from various vegetables and wild edible greens (Kim et al., 2016). To enhance savory taste of vegetable-based dishes, Koreans often use "jang" condiments as their seasoning of choice (Lee, 2016). Jang is the term used for condiments or soup bases, which contain fermented soybean (maju) as their basic ingredient called meju; these include doenjang (soybean paste), kochujang (chili paste), and ganjang (soy sauce) (Jeon et al., 2002; Joo et al., 1992; Kim et al., 1998). Meju is made by kneading, pounding, and shaping steamed soybean into bricks, and then allowing them to air-dry while fermentation takes place by the action of numerous microorganisms, including *Aspergillus oryzae* and *Bacillus subtilis* (Choi et al., 2009; Kim et al., 2011). Meju is then washed and sun-dried before being formulated as the basic ingredient of various jangs. Meju, and thus jang, contains microbial hydrolysates of soybean, such as amino acids, peptides, free fatty acids, and phytochemicals, which are responsible for the umami taste of banchans (Kim, 2004; Lee, 1976). Although the study was conducted using tempeh, a mold-fermented soybean, Sparringa and Owens (1999) demonstrated that only 9 to 17% of crude protein remains at the end of the fermentation process, whereas the majority of soy protein gets degraded into amino acids, peptides, and other nitrogenous compounds. In addition, meju and jang are rich in functional compounds such as isoflavones, phytic acid, trypsin inhibitor, phenolic compounds, and saponin (Choi et al., 2007).

During the past two decades, numerous scientists have explored the beneficial effects of doenjang and kochujang, especially against life-style-related diseases such as cancer (Jang et al., 2014; Jung et al., 2006), obesity (Bae et al., 2013; Kwak et al., 2012), and type 2 diabetes mellitus (Bhathena and Velasquez, 2002; Kwon et al., 2010; Kwon et al., 2011). Obesity and type 2 diabetes mellitus

are two of the most wide-spread diseases in both under-developed and industrialized nations, and seriously threaten quality of life. Obesity and type 2 diabetes mellitus are associated with metabolic abnormalities and could be managed or alleviated to some extent by a careful diet (Perseghin, 2001; Walker et al., 2007). Several studies indicate that the main etiology of both these ailments can be attributed to nutrition. Type 2 diabetes mellitus refers to a group of diseases that are characterized by both peripheral insulin resistance and insulin deficiency, and which results in high levels of blood glucose (American Diabetes Association, 2009; Koh, 1998). Obesity is a medical condition where excessive fat is stored in adipose tissues, either by increased cell volume or number (Karageorgi et al., 2013).

Kim et al. (2008) demonstrated that feeding rats with jang alleviates diabetic symptoms and that this effect may be attributed to increases in the concentrations of aglycones, which are microbially produced during the meju making process. Kwon et al. (2007a; 2007b; 2011) reported that long-term feeding of jang enhances production of insulin and attenuates insulin resistance in diabetic rats. According to animal studies by Soh et al. (2008), feeding fermented soybean paste to high-fat diet-fed mice induces increased mRNA expression of hepatic enzymes involved in oxidation of fatty acids, which reduces body fat accumulation and improves serum lipid profiles. Roh et al. (2015) recently stated possible uses for 7,3',4'-trihydroxyisoflavone, which is isolated from soybean paste, as a therapeutic agent for overcoming obesity. *In vivo* rat studies have shown that doenjang effectively induces adiponectin production and suppresses expression of nuclear factor-κB, a transcription factor associated with the pathophysiology of obesity (Carlsen et al., 2009).

The growing pandemic of diabetes and obesity has resulted in increased demand for functional foods that can help counteract both disorders. Soybean-derived jangs are one such readily available "medicinal foods" and are therefore gaining renewed public attention. However, studies examining jang constituents responsible for its antiobesity and antidiabetic activities are limited. Thus, in this study we investigated the chemical constituents of doenjang and kochujang responsible for alleviating obesity and type 2 diabetes mellitus. We further determined possible mechanisms of action of these compounds.

MATERIALS AND METHODS

Extraction, lyophilization, and preparation of test samples
Kochujang (K1~K22) and doenjang (D1~D22) samples were purchased from traditional markets and refrigerated. For extraction, a 10-fold volume of 70% ethanol was added to samples then tubes were continuous shaken for

24 h at 25°C. The precipitates were then separated by centrifugation at 8,000 g for 30 min, and the supernatants were lyophilized. All lyophilized samples were then stored in a deep freezer. Prior to experimentation, these extracts were dissolved in distilled water and then filtered through a 0.45 μm syringe. Different dilutions of sample were prepared using distilled water (or 100% EtOH for *p*-coumaric acid) for antidiabetic screening. For antiobesity screening, samples were diluted in cell culture media. All diluted samples were stored in the refrigerator for up to 7 days, at which point they were replaced with fresh stock.

α-Glucosidase inhibition assays
α-Glucosidase inhibition assays were carried out following a method proposed by Tibbot and Skadsen (1996). Yeast α-glucosidase and *p*-nitrophenyl-α-D-glucopyranoside (Sigma, St. Louis, MO, USA) were used as the enzyme and substrate, respectively. To prepare the enzyme solution, α-glucosidase was dissolved in 100 mM phosphate buffer (pH 7.0) containing 0.2% bovine serum albumin and 0.02% NaN₃. To prepare 10 mM substrate solutions, *p*-nitrophenyl-α-D-glucopyranoside was dissolved in 100 mM phosphate buffer (pH 7.0). 50 μL of each sample and 100 μL α-glucosidase were then added into wells of a microplate, and the plate was incubated at room temperature (25°C) for 5 min. The absorbance was then measured at 405 nm using a multi-detection reader (Infinite 200, Tecan Group AG, Männedorf, Switzerland). Substrate solution (50 μL) was then added to the reaction mixture in each well, incubated for 2 min, and the absorbance was measured again at 405 nm. The rate of enzyme inhibition was calculated as follows:

$$\text{Inhibitory activity (\%)} = \frac{\text{(absorbance of control group} - \text{absorbance of experimental group)}}{\text{absorbance of control group}} \times 100$$

OP9 adipocyte differentiation assays
OP9 pre-adipocyte cells (fat cell) were procured from American Type Culture Collection (ATCC, Manassas, VA, USA), and cell culture reagents were purchased from Gibco (Carlsbad, CA, USA). Cells were grown in minimum essential medium (MEM)-α supplemented with 20% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin, and incubated at 37°C in a humidified incubator with 5% CO₂.

Cytotoxicity analysis
Viability of cells treated with different concentrations of extracts was measured using water-soluble tetrazolium salt (WST)-1 assays. Cells were seeded at a density of 1×10^4 cells/well in 96-well plates. After 24 h of incuba-

tion, cells were treated with different concentrations of extracts and incubated for 48 h. For each 100 μL of cell culture media, 10 μL WST-1 (Daeil Lab, Seoul, Korea) was then added. The absorbance was measured at 540 nm using a microplate reader. Cell viability was calculated using the following equation:

$$\text{Cell viability (\%)} = \frac{\text{absorbance of experimental group}}{\text{absorbance of control group}} \times 100$$

Adipogenesis induction

OP9 cells were cultured in MEM-α containing 20% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. When confluent, cells were induced to differentiate. At day 0, cells were cultured in MEM-α containing 10% FBS, 175 nM insulin, 0.25 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. At day 2, media was replaced with fresh MEM-α containing 10% FBS, 175 nM insulin, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin and cells were cultured for a further 3 days. Undifferentiated cells were maintained in MEM-α containing 20% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. During differentiation, cells were treated with differentiation induction media to identify inhibition. The untreated samples that differentiated were used as the control group.

Oil red O staining

Cell culture media was removed and cells were washed with phosphate-buffered saline (Gibco) and fixed with 4% formaldehyde (Sigma) at room temperature (25°C) for 20 min. Cells were then washed twice with distilled water following treatment with 60% isopropanol for 1 min, and then stained using Oil red O solution (Sigma) for 10 min. After staining, differentiated fat cells were identified using a phase-contrast microscope. To quantify the intracellular lipid content, leaching was performed using isopropanol, and the absorbance was measured at 520 nm using microplate reader.

Target metabolite extraction

Kochujang and doenjang samples were homogenized in 500 μL methanol containing internal standards (50 μM) by a homogenizer (1,500 rpm, 120 s×1 times) (Beckman Coulter, Inc., Brea, CA, USA). Homogenates were then added and mixed thoroughly with chloroform (500 μL) and Milli-Q water (200 μL), and centrifuged (2,300 g, 4°C, 5 min). The separated water layer (200 μL×2) was filtered through a 5-kDa-cutoff filter [ULTRAFREE-MC-PLHCC, Human Metabolome Technologies (HMT), Tokyo, Japan] to remove macromolecules. The filtrate was

centrifugally concentrated and resuspended in 50 μL ultrapure water and measurements were taken.

Capillary electrophoresis-time-of-flight mass spectrometry (CE-TOFMS) system

Kochujang and doenjang metabolites were measured in the cationic and anionic modes of a CE-TOFMS system (Agilent Technologies Inc., Santa Clara, CA, USA) based on the metabolome analysis protocol by HMT. Cationic and anionic metabolites were analyzed using a fused-silica capillary (50 μm i.d.×80 cm total length), with commercial cation and anion electrophoresis buffers (solution ID: H3301-1001 and H3302-1021, respectively, HMT), as the electrolytes. The sample was injected at 50 mbar pressure for 10 and 25 s to detect cationic and anionic metabolites, with applied voltage of 27 and 30 kV, respectively. Electrospray ionization-mass spectrometry was conducted in the positive and negative ion modes with the capillary voltage set at 4,000 and 3,500 V for the cationic and anionic mode analyses, respectively. The spectrometer scanned from m/z 50 to 1,000.

CE-TOFMS data analysis

Target metabolites were assigned from HMT standard library and Known-Unknown peak library on the basis of m/z and migration time. Metabolite concentrations were calculated by normalizing the peak area of each metabolite to the area of the internal standard and standard curves, which were obtained by single-point (100 μM) calibrations.

Statistical analysis

To determine statistical significance between different groups, one-way analysis of variance (ANOVA) was carried out, followed by Duncan post-hoc test (SPSS version 12, SPSS Inc., Chicago, IL, USA). P-values <0.05 were considered statistically significant.

RESULTS

Antidiabetic effects of traditional kochujang and doenjang extracts

Antidiabetic effects of kochujang and doenjang extracts were investigated by α-glucosidase inhibition assays. Among kochujang samples, K2 at 10 mg/mL was the most effective in suppressing α-glucosidase activity, inducing inhibition by 48.8% (Fig. 1). K7, K3, and K21 samples showed 40% inhibition, which was comparable with the positive control, acarbose. The average inhibitory activity of all the kochujang samples was 29.6%. Compared to kochujang extracts, doenjang extracts showed stronger α-glucosidase inhibitory activity, with D1 exhibiting 70.9% inhibition. At 10 mg/mL, doenjang inhibited
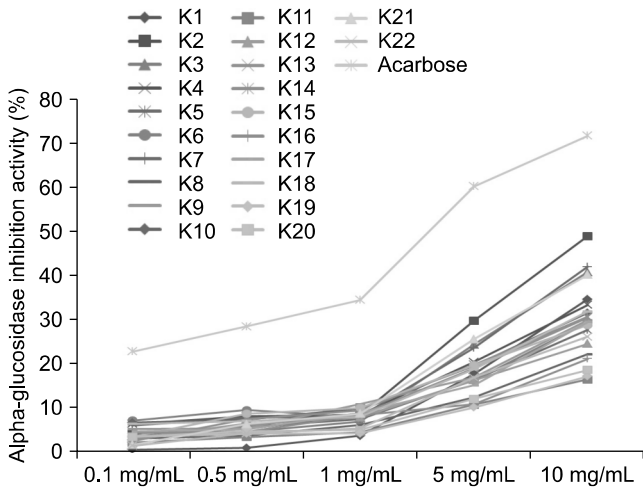
Fig. 1. α-Glucosidase inhibitory activities of traditional kochu-jang (K1~K22) products in Korea.
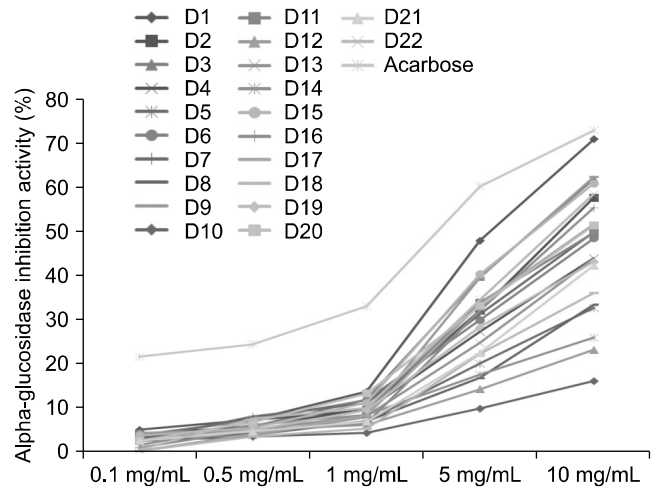

Fig. 2. α-Glucosidase inhibitory activities of traditional doenjang (D1~D22) products in Korea.

α-glucosidase activity by an average of 46.3% (Fig. 2).

Antiobesity effect of traditional kochujang and doenjang extracts

To examine cytotoxicity caused by doenjang and kochu-

jang extracts, cell viability assays were performed by incubating pre-adipocytes OP9 with different extract concentrations at 37°C for 24 h and 48 h; results were evaluated using the equations described previously. For both jangs, cell viability remained unchanged when incubated
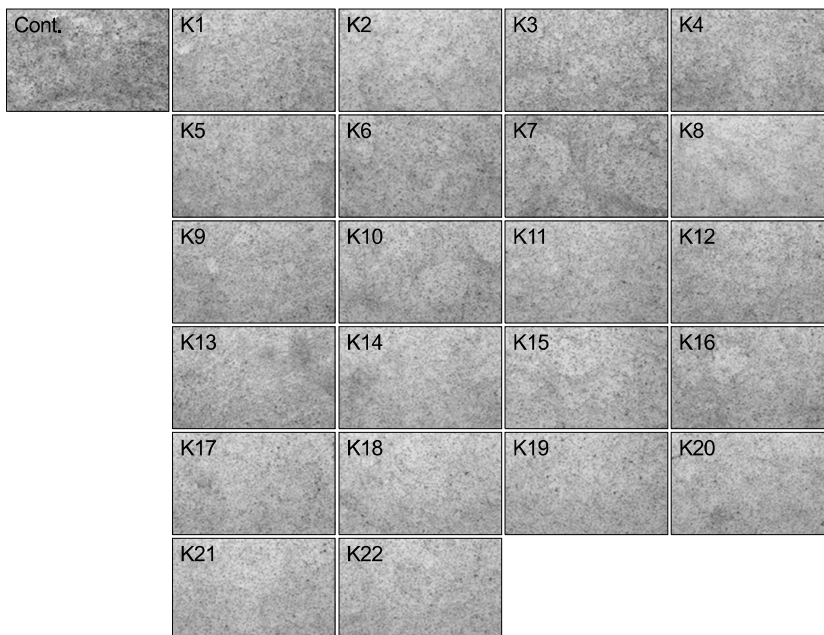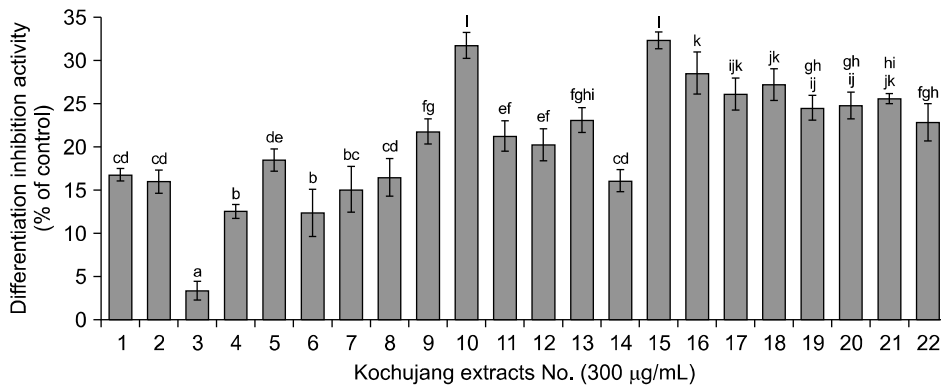

Fig. 3. Differentiation inhibitory activities of traditional kochujang. The data are shown as mean±SE. Mean values not sharing a common letter (a–l) are significantly different among the groups (*P*<0.05).

with extracts at concentrations ranging from 10 to 300 mg/mL. However, cell viability decreased significantly to 85∼90% at concentrations above 500 mg/mL, indicating the toxicity of high concentrations. Thus, 300 mg/mL of both kochujang and doenjang extracts were used in further experiments.

Oil red O was used to stain lipids that accumulated in OP9 cells treated with kochujang and doenjang extracts to explore their inhibitory effects on cellular differentiation (Fig. 3 and 4). Kochujang samples K15, K10, and K16 inhibited differentiation by 32.3±0.93%, 31.7±1.46%, and 28.5±2.46, respectively. In addition, the inhibitory activity of doenjang extracts on adipogenesis was determined in OP9 cells. At 300 µg/mL concentrations, D9 suppressed pre-adipocyte adipogenesis by 24.8±1.52%; in cells treated with D15 and D22, inhibition was 23.0± 1.36% and 18.8±1.4%, respectively. The average inhibitory activity for all the kochujang samples on adipogenesis was 20.8%, whereas the doenjang samples inhibited adipogenesis by 11.6%. Compared to the doenjang extracts, kochujang extracts showed stronger inhibition of adipogenesis.

Potential antidiabetic compounds

The components of kochujang and doenjang responsible for suppressing the functional effects were investigated by metabolomic analysis: p-coumaric acid, N6,N6,N6-trimethyllysine, threonine, and methionine present in kochujang positively correlated with inhibition of adipogenesis activity; for doenjang, the compounds possible attributing to its antidiabetic effects were betaine and betaine aldehyde (Table 1 and 2). As p-coumaric acid and betaine were the most probable candidates with a functional effect, these two compounds were selected for further analysis. Acarbose and betaine showed dose dependent inhibition of α-glucosidase activity, and acarbose suppressed carbohydrase activity by 72% by 10 mg/mL (Fig. 5). Betaine exhibited lower inhibitory activity at low concentrations (0.1∼1.0 mg/mL) when compared to acarbose; however, at higher concentrations (5∼10 mg/mL), trimethylglycine showed greater carbohydrase inhibitory activity. However, the inhibitory activity of p-coumaric acid was marginal (only 11∼15%) when compared to that of acarbose at all concentrations. We further studied the inhibitory activity of betaine chloride and p-coumaric
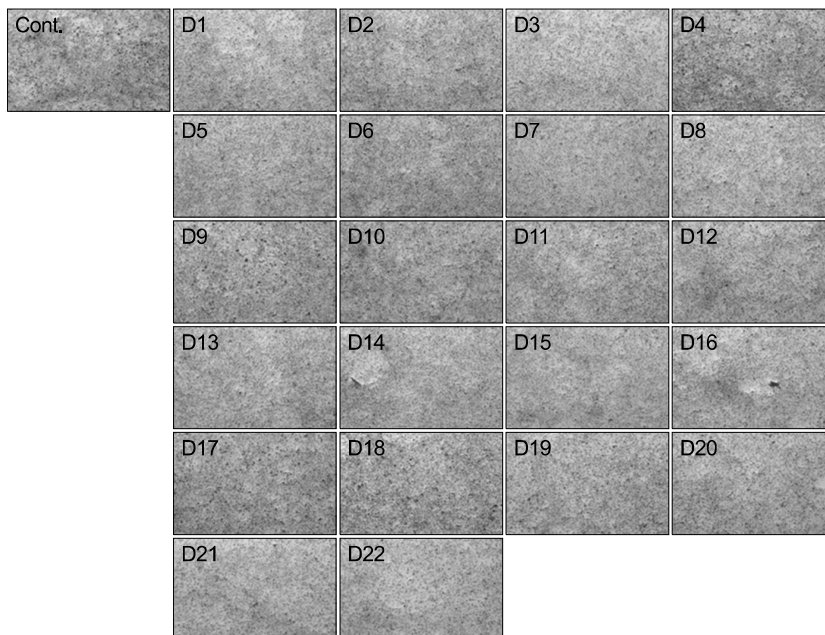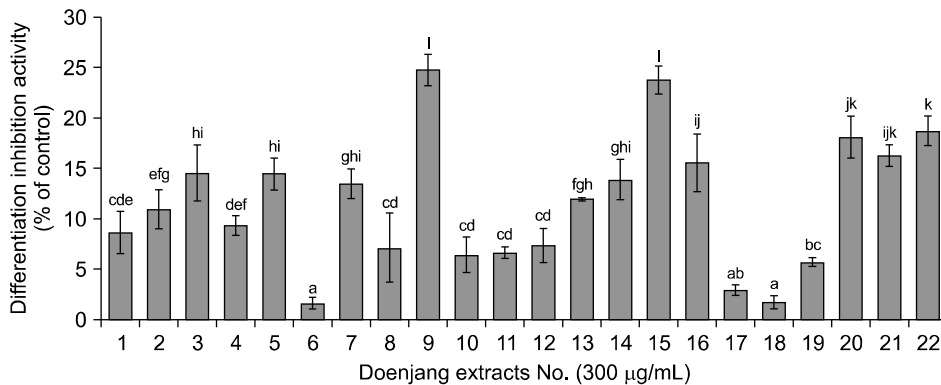

Fig. 4. Differentiation inhibitory activities of traditional doenjang. The data are shown as mean±SE. Mean values not sharing a common letter (a–l) are significantly different among the groups (P<0.05).

Table 1. Responsible candidates for functional effects of traditional Kochujang

Compound name	High group		Low group		t Value	Pr>|t|
	Mean	SD	Mean	SD		
p-Coumaric acid	1.8E-03	8.0E-04	3.0E-04	5.9E-04	4.32[#]	0.0003
N6,N6,N6-Trimethyllysine	1.3E-02	8.7E-04	1.0E-02	3.4E-03	2.79[*]	0.0116
Threonine	1.3E-01	4.4E-02	8.3E-02	2.8E-02	2.96[#]	0.0077
Methionine	3.6E-02	9.1E-03	2.1E-02	9.5E-03	3.03[#]	0.0067

Significant differences between means by t-test at [*]$P<0.05$ and [#]$P<0.01$.
SD, standard deviation.

Table 2. Responsible candidates for functional effects of traditional Doenjang

| Compound name	High group		Low group		t Value	Pr>|t|
	Mean	SD	Mean	SD		
Betaine	2.0E-01	4.3E-02	1.4E-01	3.7E-02	3.49[#]	0.0023
Betaine aldehyde+H_2O	3.0E-05	8.9E-05	1.6E-04	1.9E-04	−2.15[*]	0.0455

Significant differences between means by t-test at [*]$P<0.05$ and [#]$P<0.01$.
SD, standard deviation.
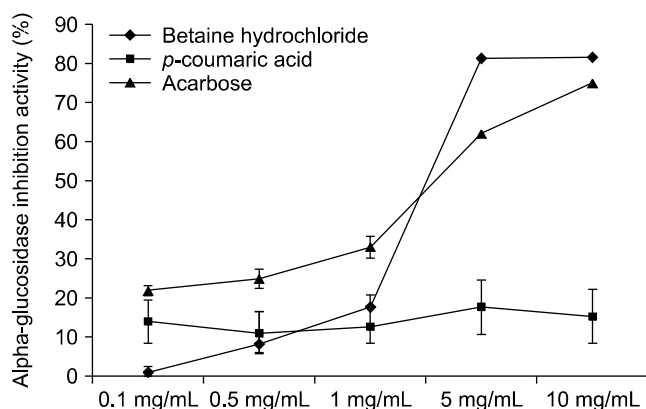
Fig. 5. α-Glucosidase inhibitory activities of potential functional compounds. The data are shown as mean±SE.

Fig. 6. Differentiation inhibitory activities of potential functional compounds. The data are shown as mean±SE.

acid on pre-adipocyte differentiation. Betaine chloride (10 μM) inhibited pre-adipocyte differentiation by 14.0 ±1.85%, whereas p-coumaric acid inhibited differentiation by 38.3±3.27%. This therefore suggests that p-coumaric acid may be more effective in preventing obesity (Fig. 6).

DISCUSSION

In this present study we investigated the antidiabetic effects of aqueous ethanol extracts of traditional kochujang and doenjang. The average α-glucosidase inhibitory activity of all kochujang samples was 29.6%, whereas doenjang extracts (10 mg/mL) showed higher inhibition with average value of 46.3%. The types of soybean paste and red pepper paste samples used in these experiment and their respective characteristics are mentioned in detail in previous publications (Park et al., 2016; Park et al., 2017). However, it is difficult to describe the correlation between
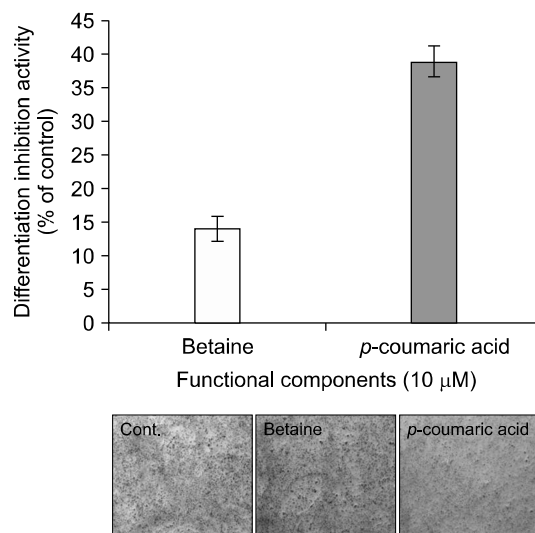
these and the positive effect we observed in the current study on α-glucosidase inhibitory activity. In addition, the reasons for the differences in α-glucosidase inhibitory activity of kochujang and doenjang is uncertain (Shukla et al., 2016; Hwang et al., 2017). Thus, in this paper, we aimed to identify the potential active ingredients through metabolic comparative analysis of active and inactive kochujang and doenjang samples; p-coumaric acid and betaine were found to the most probable candidates, and were selected for further studies. Although the reasons for the differences in α-glucosidase inhibitory activity is uncertain, the discrepancy is due to differences in soybean content of these jangs, and their preparation; doenjang contains more than tenfold higher content of legumes than kockujang (Park et al., 2016; Park et al., 2017). Doenjang is made solely from meju and sea salts,

while kochujang is prepared from meju, sodium chloride, glutinous rice, and red pepper. α-Glucosidase inhibitors function by preventing or slowing down the breakdown of polysaccharides to monosaccharides by binding reversibly or competitively to carbohydrase at the brush border of the small intestine, thereby slowing down rises in blood glucose levels (Önal et al., 2005). Kwon et al. (2011) reported that β-cells treated with doenjang extracts show higher viability and improved insulin secretory capacity than those treated with soybeans alone. This enhancement was attributed to the insulin/insulin-like growth factor-1 signaling pathway, which is activated by phosphorylation of both insulin receptor substrate-2 and Akt, resulting in a series of reactions and, eventually, increased pancreatic and duodenal expression. In contrast to our results, Lee et al. (2012) reported that α-glucosidase inhibitory activity of doenjang methanolic extracts in C2C12 myoblasts was not significant but, in comparison with the control, glucose uptake was increased by 1.23~1.25 fold (Lee et al., 2012). However, additional research, especially clinical trials, are required to confirm the previously reported hypoglycemic effects of kochujang and doenjang. The advantages of jangs are that, unlike acarbose, they do not induce any gastrointestinal disturbances such as flatulence or diarrhea, which are known side effects of acarbose (Hoffmann and Spengler, 1997). Both these jangs may therefore be used as a safe and efficacious alternative antidiabetic diet.

We assumed that *p*-coumaric acid and betaine are the most probable candidates to have antidiabetic effects, and thus the compounds were selected for further analysis. Interestingly, betaine inhibited α-glucosidase activity in a dose-dependent manner. Recently, several studies have explored the association between betaine and metabolic syndromes, including diabetes; however, none were related to α-glucosidase inhibition. According to Konstantinova et al. (2008), patients with diabetes excrete excess amount of betaine via urine due to choline dehydrogenase malfunctioning in mitochodria. Jeong et al. (2005) suggested that betaine helps damaged pancreatic β-cells recovery in streptozotocin-diabetic rats, therefore functioning as a hypoglycemic agent. Moreover, Jallal's patent revealed that combining betaine and its derivatives with other antidiabetic drugs reinforces their efficacy while decreasing side effects (Jallal, 2007). We propose that doenjang with higher betaine contents is a better diet for diabetic patients than kochujang. Reasons include the considerably higher content of betaine in doenjang as soybean is classified as a low accumulator of this compound. Since soil salinity stress is known to restrict growth of soybean plants, exogenous betaine may be added as an osmoprotectant to improve crop yield during cultivation (Rezaei et al., 2012). Moreover, considerable amounts of choline are present in soybean, which acts as a precursor of betaine.

Although some speculative reports have been published, the antiobesity mechanisms of betaine and *p*-coumaric acid remain unclear. Osmoregulation and methylation of homocysteine to methionine are two well-known functions of betaine (Finkelstein and Martin, 1984; Lang, 2007). However, animal studies have suggested that betaine facilitates reduction of lipid accumulation in tissues by increasing production of apolipoportein B and plasma triglycerides (Hayes et al., 2003; Sparks et al., 2006). Hsu et al. (2009) stated that intake of coumaric acid is beneficial for relieving high-fat diet-induced dyslipidemia, and reduces body, liver, and adipose tissue weights of experimental rats. In addition, Seo et al. (2015) reported that coumaric acid shows antiadipogenic effects *in vitro* and *in vivo*, effectively preventing weight gain and fat mass in male mice. The results of this study strongly suggest that both betaine chloride and *p*-coumaric acid suppress accumulation of lipids in adipose tissue. However, further studies are needed to elucidate the mechanism of their antiobesity effect.

In this study, we report direct but limiting evidence that fermented soyfoods, such as kochujang and doenjang, are effective in decreasing the risk of diabetes and obesity. We demonstrated that betaine and *p*-coumaric acids, the constituents of doenjang and kochujang, are responsible for the antidiabetic and antiobesity effects of these jangs. However, to confirm these findings, further research including human studies are needed, which consider the alarming increase of diabetes and obesity worldwide.

ACKNOWLEDGEMENTS

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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