Improvement of the Functional and Organoleptic Properties of the Fermented Soymilk with *Bacillus subtilis* 2829PNU015 in Addition of *Corni fructus* Extracts

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Abstract

To improve the functional and organoleptic properties of the fermented soymilk with *Bacillus subtilis* 2829PNU015 which has chungkukjang like flavor, functional materials from the edible plant extracts with well-known health promoting effects were examined by fibrinolytic activity and sensory evaluation. Among five samples such as *Corni fructus*, garlic, purple sweet potato, aged garlic, and pumpanini were tested, *Corni fructus* showed the highest fibrinolytic activity. The appearance and taste of the fermented soymilk (FS) was varied to the amount of *Corni fructus* added, and the highest acceptability of FS was observed from FS containing 1% *Corni fructus* extracts (10°Brix) in it. Best way of applying *Corni fructus* to the fermented soymilk (FFS) was found to be incubating the mixture of soymilk and *Corni fructus* with *Bacillus subtilis*. FFS demonstrated dose dependent radical scavenging activities and these activities were found to be higher than those of FS at designated concentration.

Key words: *Bacillus subtilis*, fermented soymilk, *Corni fructus*, sensory evaluation, free radical

INTRODUCTION

To develop probiotic dietary adjuncts of soymilk, many researchers have demonstrated the health promoting benefits of the fermented soymilk with lactic acid bacteria and/or bifidobacteria. However, fermentation of soymilk with *Bacillus subtilis* which is a major microorganism in chungkukjang (CKJ) has been tried yet. Since soybean is a suitable medium for *Bacillus subtilis*, soymilk should be a good nutrient for growing with *Bacillus* spp. as well as lactic acid bacteria (1). Although fermented soymilk has demonstrated antioxidative activities (2), cholesterol lowering effects (3), anti-mutagenic/anti-carcinogenic actions (4), and immunogenic activities (5), poor organoleptic properties of the fermented soymilk drawback to the use of it. Therefore, great efforts have been made to improve the existing starter cultures or to select new strains in specific fermentation process in order to develop new products with present flavor. The beany flavor and the indigestible oligosaccharides such as raffinose or stachyose responsible for causing flatulence can be reduced by certain bacterial strain or by additives once they used in the fermentation (6).

Chungkukjang (CKJ) is a fermented soybean product having antioxidant activity (7), antimicrobial, anti-hypertensive (8), hypolipidemic effect (9,10), and anti-diabetic activity (11). The *Bacillus* species responsible for CKJ fermentation secrete protease which degrades the soy proteins into peptides and amino acids. Certain microorganism secretes fibrinolytic enzyme strongly (12,13). Nattokinase from natto, chonggokkinase from CKJ, and subtilisin DFE from Chinese dou-chi are well known enzymes with strong fibrinolytic activity.

*Corni fructus* (*Corni officinalis Sieb et Zucc*) is a widely grown *Corni* spp. which has been used in Chinese herbal medicine and known for its tonic, analgesic and diuretic activities (14). Moreover, *Corni fructus* revealed beneficial effects on renal damage induced by advanced diabetes (15), hypoglycemic (16) and hypolipidemic effect (17). The physiological activity of *Corni fructus* might be due to the anthocyanin in this plant which had antioxidant effects (18). In this study, in order to improve the anti-atherogenic activity and organoleptic properties of the fermented soymilk with *Bacillus subtilis* 2829PNU015, fibrinolytic activities of several edible plant extracts were examined and application of the functional material to the fermented soymilk was studied. The radical scavenging activities of the fermented soymilk with or without the functional material were compared.
MATERIALS AND METHODS

Edible plant extracts
The concentrated extracts of *Corni fructus*, garlic, purple sweetpotato, aged garlic, and pumpkins were kindly provided from CH Food Company located in Busan. These edible plant extracts are on the market as a supplement for improving the general health. The concentrations of the plant extracts were adjusted to be 10° Brix with deionized water before use.

Fermentation of soymilk
Cultures were activated through 2 successive transfers in Nutrient broth (Becton, Dickinson & Co., Sparks, MD, USA) at 37°C for 24 hr using a 2% inoculums. Soymilk (Donghwa Food Co., Ltd., Yangsan, Korea) containing 4.0% soyprotein was dispersed into 500-mL bottles and 1% glucose (w/v) were added to it and heat treated at 121°C for 15 min. Each bottle was then inoculated with *Bacillus subtilis* 2829PNU015 of total to be 7~8 logCFU/mL before incubation at 40°C about 8 hr. Fermentation was terminated when the pH of the soymilk reached 5.5±0.2. Temperature and time duration for the fermentation were decided from pre-test (data not shown). For FFS preparation, *Corni fructus* which demonstrated the highest fibrinolytic activity was added to the soymilk (1%, v/v) before inoculating the microorganism. *Bacillus subtilis* 2829PNU015 isolated from Chongkukjang (KYS Co., Soonchang, Korea) was kindly provided from microbiology lab at P University (Busan, Korea)

Preparation of methanol extracts
For freeze-drying, the sample was first poured in to a fast-freeze flask (500 mL, Samduk Co., Seoul, Korea) and frozen at -20°C. The frozen samples were then lyophilized with a freeze drier (Samwon Freeze Dry System, Model SFDSM06, Seoul, Korea) at a condenser temperature of -80°C. Vacuum was automatically established once the container was attached to the freeze-dryer. It usually takes about one week to be completely lyophilized. Freeze-dried sample were extracted with 10 volumes of methanol (w/v) at room temperature for 24 hr. Methanol extracts were obtained under vacuum evaporator up to dryness. Methanol extracts were stored at 5°C for further analysis.

Fibrinolytic activity
Fibrinolytic activity of edible plant extracts was determined by fibrin plate method using 0.5U of plasmin (Sigma, St. Louise, MO, USA) per mL as a standard fibrinolytic protease (19). The fibrinogen solution [10 mL of 0.3% of human fibrinogen (type 1, Sigma)] in 10 mM phosphate buffer (pH 7.8) was mixed with 50 μL thrombin (100 NIH/mL, Sigma) followed by immediate pouring it on petri dish with slow stirring. After dish was allowed to stand at room temperature for 40 min to form fibrin clots, five holes were made on a fibrin plate using capillary glass tube. Fifty micro-liter of plant extracts and plasmin (0.5 U/mL, Sigma) were dropped on the hole and incubated for 18 hr at 37°C. The fibrinolytic activity of plant extract was expressed as plamin activity (PU/mL) which was calculated based on the ratio of dimensions of the clear zone by sample to that by plasmin.

1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity
DPPH scavenging activity was measured by the method of Hatano et al (20). The reaction mixture containing 100 μL of 60 μM DPPH and 100 μL of methanol extracts of the fermented soymilk in 96 well plate was left stand in the dark at room temperature for 30 min. Absorbance of the reaction mixture was determined with ELISA Microplate Reader (Bio-Rad, model 680, California, USA) at 540 nm.

Superoxide anion radical
Superoxide radical (O₂⁻) generated in the xanthine-xanthine oxidase system was determined spectrophotometrically via monitoring the product of nitroblue tetrazolium (NBT) as an end product (21). The reaction mixture was prepared with 400 μL of each methanol extract (100~1000 μg/mL), 100 μM xanthine, 60 μM NBT, 0.05 U/mL xanthine oxidase and 0.1 M phosphate buffer (pH 7.4) to make a final volume of 2.0 mL. After incubation at 37°C for 10 min, the absorbance was measured at 560 nm, compared with the control samples run without xanthine oxidase. Percent inhibition was calculated from the optical density of the fermented soymilk treated and control samples.

\[
\text{Scavenging activity (\%)} = \frac{(C - CB) - (S - SB)}{C - CB} \times 100
\]

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

Nitrite scavenging activity
Nitrite scavenging was measured by the method of Kato et al. (22). Methanol extracts of CKJ (100~1000 μg/mL) was added to 1 mL of NaNO₂, pH of the sample was adjusted to be 1.2 with 0.1 N HCl and then it was incubated at 37°C for 1 hr. Five milliliter of 2% acetic acid and 0.4 mL of Griess reagent were added to 1 mL of the incubated solution. It was left it stand at room temperature in the dark for 15 min. The absorbance of the reaction mixture was determined at 520 nm on a
microplate reader (ELx800, Bio-Tek Instruments, Inc., USA)

Sensory evaluation
Sensory evaluation of the fermented soymilk was carried out according to the replicated randomized complete block design with thirty trained panel members. Descriptive characteristics (appearance, taste, flavor, texture, acceptability) were subjectively evaluated using grades diversified from 1 (the least preferred) to 9 (the most preferred).

Statistical analysis
One-way analysis of variance (Anova) was followed by Duncan’s multiple range test in order to determine the statistical significance of measurements between groups, using the SAS software (SAS Institute, Cary, NC, USA; p<0.05). The radical scavenging activity between the fermented soymilk (FS) and functional fermented soy-milk (FFS) was analysis with student t-test.

RESULTS
Fibrinolytic activity of plant extracts
Fibrinolytic activities of plant extracts used in this study were significantly different (Table 1, p<0.05). Corni fructus showed the highest fibrinolytic activity (1.12 PU/mL) followed by pumpanini (0.78 PU/mL). Rest of the plants extracts demonstrated relatively weak fibrinolytic activity.

Table 1. Fibrinolytic activities of edible plant extracts

<table>
<thead>
<tr>
<th>Plant extracts</th>
<th>Fibrinolytic activity (PU/mL)</th>
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</thead>
<tbody>
<tr>
<td>Corni fructus</td>
<td>1.12 ± 0.20*</td>
</tr>
<tr>
<td>Garlic</td>
<td>0.28 ± 0.10b</td>
</tr>
<tr>
<td>Aged garlic</td>
<td>0.30 ± 0.05b</td>
</tr>
<tr>
<td>Purple sweetpotato</td>
<td>0.28 ± 0.05b</td>
</tr>
<tr>
<td>Pumpanini</td>
<td>0.78 ± 0.57ab</td>
</tr>
</tbody>
</table>

PU: Plasmin unit. Plasmin of 0.5 U per milliliter was used for the standard.
*The edible plant extracts (10°Brix) were kindly provided from CH Food Company (Busan, Korea) which are used to produce a commercial product.

Practical applications of Corni fructus to the fermented soymilk
In order to determine the proper concentration of Corni fructus, sensory evaluation was carried out with Corni fructus extracts added fermented soymilk (FS). Table 2 shows the sensory evaluation results of FS containing various amounts of Corni fructus. Appearance, flavor, taste and texture of FS were compared. The highest score for appearance and taste of FS was observed when 1% of Corni fructus added. The color of FS gets darkened as the amount of Corni fructus increased. On the other hand the flavor and texture of the FS were not affected by the amount of Corni fructus added. Thus, the score for the overall acceptability of 1% Corni fructus added FS was the highest among concentrations tested.

For further study on manufacturing FFS, the way of applying Corni fructus to FFS was examined. Corni fructus was added to the FS before (Fermented soymilk containing Corni fructus, FSCC) or after fermentation (Corni fructus added fermented soymilk, CAFS). The organoleptic properties of FSCC and CAFS were compared with FS and Chungkukjang powder added soymilk (CPAS). Table 3 demonstrates the sensory evaluation results of various FFS. The score for the appearance was found to be the highest in FS which is in line with the results of Table 2. The color of FS gets darkened as the amount of Corni fructus in the FS increased. But scores for the flavor, taste and overall acceptability of FSCC were comparable to those of FS. The scores for of taste and overall acceptability of FSCC were higher than FS, although they are not significantly different. The scores for flavor, taste and overall acceptability of CAFS were lower than those of FSCC informing that the way of adding Corni fructus in the process of manufacturing FS is very important. When organoleptic properties of FSCC and CAFS were compared with those of CPAS significant differences were observed in flavor and taste. CPAS is an easy way of consuming Chungkukjang in daily life by people who cannot cook Chungkukjang every day.

Table 2. Sensory evaluation of the fermented soymilk varied with the amounts of Corni fructus added

<table>
<thead>
<tr>
<th>Corni fructus extracts</th>
<th>Appearance</th>
<th>Flavor</th>
<th>Taste</th>
<th>Texture</th>
<th>Overall acceptance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1%</td>
<td>7.2 ± 0.9*</td>
<td>4.3 ± 2.0*</td>
<td>5.6 ± 1.4*</td>
<td>4.0 ± 1.8*</td>
<td>6.1 ± 1.3*</td>
</tr>
<tr>
<td>2%</td>
<td>5.6 ± 1.3b</td>
<td>4.3 ± 1.9</td>
<td>5.4 ± 2.0*</td>
<td>4.5 ± 1.7</td>
<td>5.0 ± 2.1ab</td>
</tr>
<tr>
<td>3%</td>
<td>3.9 ± 1.5c</td>
<td>4.1 ± 1.8</td>
<td>4.2 ± 1.9*</td>
<td>4.1 ± 1.7</td>
<td>3.7 ± 1.9abc</td>
</tr>
<tr>
<td>4%</td>
<td>3.5 ± 1.7c</td>
<td>4.0 ± 2.1</td>
<td>4.7 ± 2.0*</td>
<td>5.0 ± 1.8</td>
<td>4.4 ± 2.0abc</td>
</tr>
<tr>
<td>5%</td>
<td>3.2 ± 2.3c</td>
<td>3.3 ± 1.2</td>
<td>3.3 ± 1.8*</td>
<td>4.0 ± 1.5</td>
<td>3.1 ± 1.8*</td>
</tr>
</tbody>
</table>

Values are mean ± SD (n=30). *Data were significantly different with one-way ANOVA followed by Duncan’s multiple range test at the 0.05 level of significance. NS: not significant.
Table 3. Sensory evaluation of the fermented soymilk prepared with 1% *Corni fructus* with different methods of application

<table>
<thead>
<tr>
<th>Fermented soymilk</th>
<th>Appearance</th>
<th>Flavor</th>
<th>Taste</th>
<th>Texture</th>
<th>Overall acceptance</th>
</tr>
</thead>
<tbody>
<tr>
<td>FS</td>
<td>7.4 ± 1.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.7 ± 1.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.3 ± 1.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.1 ± 2.7&lt;sup&gt;NS&lt;/sup&gt;</td>
<td>5.5 ± 1.8&lt;sup&gt;NS&lt;/sup&gt;</td>
</tr>
<tr>
<td>FS&lt;sub&gt;CC&lt;/sub&gt;</td>
<td>4.2 ± 1.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.7 ± 1.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.8 ± 1.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.0 ± 1.6</td>
<td>5.8 ± 1.5</td>
</tr>
<tr>
<td>CAFS</td>
<td>4.1 ± 1.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.6 ± 1.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.8 ± 1.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.2 ± 1.4</td>
<td>4.4 ± 1.2</td>
</tr>
<tr>
<td>CPAS</td>
<td>4.9 ± 2.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.1 ± 2.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.0 ± 2.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.8 ± 2.3</td>
<td>4.4 ± 2.2</td>
</tr>
</tbody>
</table>

Values were mean ± SD (n=30). <sup>a,b</sup>Data were significantly different with one-way ANOVA followed by Duncan's multiple range test at the 0.05 level of significance. NS: not significant.

FS: Fermented soymilk with *Bacillus subtilis* 2829PNU015, FSCC: Fermented soymilk containing *Corni fructus*. The mixture solution of *Corni fructus* extracts (1%, 10°Brix) and soymilk were fermented with *Bacillus subtilis* 2829PNU015. CAFS: *Corni fructus* extracts added fermented soymilk. 1% *Corni fructus* extracts was added to the fermented soymilk with *Bacillus subtilis* 2829PNU015. CPAS: Chungkukjang powder (5%) added soymilk.

The effect of fermented soymilk with *Corni fructus* on radical scavenging activity

Radical scavenging activities of FSCC were examined and compared with those of FS. The concentration ranged from 100 to 1000 μg/mL of methanol extracts of FSCC and FS were tested. DPPH scavenging activity of FSCC was increased dose dependently and their activities were greater than those of FS at designated concentration (Fig. 1, p<0.05). FSCC showed approximately 25% elevated DPPH scavenging activity than FS at 1000 μg/mL concentration tested. FSCC and FS showed a great effect on scavenging superoxide anion radical than DPPH or NO, demonstrating over 60% inhibition at 100 μg/mL concentration tested. When the superoxide scavenging activity of FSCC was compared with that of FS, FSCC did not demonstrate greater effect on scavenging superoxide anion radical except at 1000 μg/mL concentration tested (98.8 % inhibition), which was 22.5% elevated activity than FS (p<0.05, Fig. 2). Dose dependent NO scavenging activities were observed from FS and FSCC. But NO scavenging activities of FS and FSCC were found to be rather weak than DPPH and superoxide anion radical scavenging activity, especially at low concentration tested. The NO scavenging activity of FSCC at 1000 μg/mL concentration was significantly higher by 33.8% than that of FS (p<0.05), but the radical scavenging activity of FSCC and FS below this concentration were not different (Fig. 3).

DISCUSSION

The fermented soymilk containing *Corni fructus* with *Bacillus subtilis* found to have higher antioxidant activity than the fermented soymilk with *Bacillus subtilis* in terms of DPPH, superoxide and NO scavenging activity. For the production of FSCC, a mixture of *Corni fructus* and soymilk was fermented together by *Bacillus subtilis*. *Corni fructus* (*Corni officinalis* Sieb et Zucc) has been used in Chinese herbal medicine due to its tonic, analgesic and diuretic activities (14) as well as beneficial

![Fig. 1. DPPH scavenging activity of the fermented soymilk containing *Corni fructus* with *Bacillus subtilis*.](image1)

![Fig. 2. Superoxide radical scavenging activity of the fermented soymilk containing *Corni fructus* with *Bacillus subtilis*.](image2)
effects on renal damage (15), diabetes (16), and hyperlipidemia (17) are well known. The physiological activity of Corni fructus might be due to the anthocyanin in this plant which had antioxidant effects (18). The fibrinolytic activity of Corni fructus (1.12 PU/mL) tested in this study was the highest followed by Pumpanini (0.78 PU/mL). The fibrinolytic activity of garlic, aged garlic, or purple sweetpotato extracts used in this study was relatively weak (approximately 0.3 PU/mL). Low fibrinolytic activity observed in garlic, aged garlic, or purple sweetpotato might be due to the heat application during sample extraction. The fibrinolytic activity of raw onion or garlic was reduced once it was cooked (23). Total phenolic contents of puree and steamed purple sweetpotato were significantly lower than raw sample or steamed sliced sample (24).

The health promoting functional properties of soymilk will be ameliorated by fermentation with probiotic bacteria (25). Therefore, extensive researches have been performed to produce the fermented soymilk with various species of lactic acid bacteria or bifidobacteria individually or together to promote the functional property (1-5), but not many researchers have tried with Bacillus spp. to produce the fermented soymilk. Chungkukjang (CKJ) is a Korean traditional fermented soybean with Bacillus subtilis which is usually consumed in winter time as a stew type. Recently people are interested in consuming CKJ more often since the health benefits of CKJ have been reported for antioxidant, antimicrobial, blood pressure lowering, lipid lowering, and anti-diabetic activities (11,26-29). Subsequently, alternative way of consuming CKJ was introduced on a market as a powdered or pill type. However, consumers are demanding even higher quality products with an easy application top of the elevated probiotic activity. Thus we tried first to produce the fermented soymilk with Bacillus subtilis, inoculating with 7~8 logCFU/mL and succeeded. And also, we tried to improve the sensory quality of the FS which revealed distinct taste and flavor like CKJ. One of the biggest concerns for the fermented soymilk by lactic acid bacteria is poor organoleptic properties especially unpleasant flavor produced during fermentation (6). In this study, organoleptic quality for FS was improved, when Corni fructus was fermented with soymilk, especially with taste characteristics. The fermented soymilk with Bacillus subtilis demonstrated CKJ like taste and flavor. A mixture of Corni fructus and soymilk fermented together with Bacillus subtilis (FSCC) produces better taste than FS (soymilk alone) or CAFS (Corni fructus added to FS after fermentation) or CPAS (CKJ powder added soymilk). Score for taste of FSCC were increased by 10% than FS and by 53% than CAFS. Therefore, overall acceptability referring a personal ‘preference’ for the FSCC was the highest among various products of FSs. According to these results, a use of additives to improve the quality of FS is found to be one of a good trial. There are needs for new starter culture to improve the probiotic benefits as well as sensory quality of FS with lactic acid bacterial or bifidobacteria. In this study we also found that practical method of applying additives to FS is very important. When we add Corni fructus extracts to the fermented soymilk after fermentation, the sensory quality of CAFS was not improved at all. The CKJ like taste of FS was not diminished by simple adding of Corni fructus extracts and flavor of CAFS worsened, just like CPAS. It seems that the metabolites of Corni fructus produced during fermentation compensate the distinct taste of FS with Bacillus subtilis. Further study for the mechanisms of improving taste of FSCC than CAFS is required.

ACKNOWLEDGMENTS

This research was financially supported by the Ministry of Education, Science Technology (MEST) and Korea Industrial Technology Foundation (KOTEF) through the Human Resource Training Project for Regional Innovation (M-020-20070131134352).

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Fermented Soymilk with *Bacillus subtilis*


(Received March 9, 2009; Accepted March 13, 2009)