Metabolomics reveals the effect of garlic on antioxidant- and protease-activities during Cheonggukjang (fermented soybean paste) fermentation

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Chemical compounds studied in this article:
L-Valine (PubChem CID: 6287)
L-Isoleucine (PubChem CID: 6306)
L-Leucine (PubChem CID: 6106)
Glycine (PubChem CID: 750)
L-Cysteine (PubChem CID: 5862)
s-Allyl-cysteine (PubChem CID: 9793905)
s-Allyl-cysteine sulfoxide (PubChem CID: 9576089)
Soyasaponin β (PubChem CID: 164453)

1. Introduction

Cheonggukjang (CGJ) is a representative fermented soybean paste used in Korea. CGJ has been consumed in Korea for a long time owing to its health benefits, such as antioxidant capacity (Choi et al., 2012), anticancer effect (Zhao et al., 2013), anti-obesity effect (Bae, Byun, Yu, Park, & Cha, 2013), and anti-inflammatory activity (Choi, Lim, Heo, Kwon, & Kim, 2008). Several steps are involved in the preparation of CGJ. The washed soybeans are soaked in water for one day, and then steamed. The cooled soybeans are fermented with Bacillus subtilis at 37 °C for 3–4 days. Smell, taste, and bioactivities of CGJ are attributed to the degraded compounds including amino acids, soyasaponins, and isoflavone aglycones during the soybean fermentation, which was mediated by the enzymes of B. subtilis (Kim et al., 2012; Cho et al., 2009). Lately, various additives such as berries (Kim et al., 2008), ginsengs (Shin, Lee, & Kim, 2008), and garlic (Kim, Hwang, et al., 2014; Kim, Jung, et al., 2014) were added to enhance the bioactivity of CGJ. Above all, bioactivities of garlic such as antioxidant activity (Banerjee, Mukherjee, & Maulik, 2003), anti-tumor effect (Tsubura, Lai, Kuwata, Uehara, & Yoshizawa, 2011), and antimicrobial activity (Harris, Cottrell, Plummer, & Lloyd, 2001) have been reported and garlic added food fermentation has been tried to develop functional food (Cho, Park, Jung, & Jo, 2001).

Based on chromatographic separation combined with mass spectrometry (MS), metabolomics provides qualitative and quantitative metabolic information of analyzed samples (Rochat, 2012). Due to...
these advantages, metabolomics is valuable for analysis in various fields such as plant sciences (Kim, Hwang, et al., 2014; Kim, Jung, et al., 2014), human diseases (Madsen, Lundstedt, & Trygg, 2010), drug discovery (Wishart, 2008a), and microbial research (Han, Antunes, Finlay, & Borchers, 2010). In particular, metabolomics has been applied to food sciences including nutritional analysis, food quality control, and health related effects of food (Crupi, Genghi, & Antonacci, 2014; Wishart, 2008b). Recently, metabolite changes in fermented food like soybean pastes (Park et al., 2010), cheeses (Izco & Torre, 2000), and vinegar (Cerezo, Cuevas, Winterhalter, Garcia-Parrilla, & Troncoso, 2010) have been analyzed by using GC-MS or LC-MS. Generally, GC-MS is favorable for analysis of primary metabolites (Osorio, Do, & Fernie, 2012) and LC-MS is profitable for analysis of compounds over a wide range of polarity and molecular weight (Lee & Kerns, 1999). Previous research studied the health related effect of CGJ by using GC-MS (Bae et al., 2013; Choi et al., 2008, 2012; Zhao et al., 2013). Also changed metabolic states of fermented soybean pastes based on processing steps have been reported (Lee et al., 2014). However, few studies have attempted to reveal the effect of garlic adding on CGJ fermentation as well as the relationship between changed metabolic state and bioactivity.

In this study, we performed the metabolism profiling of four types of CGJ by using GC-MS and LC-MS combined with multivariate statistical analysis and revealed their different metabolic states. Further, we correlated the results with antioxidant activities. This approach could be applied to the chemical analysis of fermented foods in accordance with different additives and relating the metabolic results with bioactivity.

2. Materials and methods

2.1. Chemicals and reagents

Ethanol, acetonitrile, and water were purchased from Fisher Scientific (Pittsburgh, PA, USA). Dipotassium hydrogen phosphate, potassium dihydrogen phosphate, and sodium carbonate were purchased from Junsei Chemical Co., Ltd. (Tokyo, Japan). Methoxyamine hydrogen chloride, N-methyl-N-(trimethylsilyl) trimethylsilyl trifluoroacetamide (MSTFA), potassium persulfate, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) dianionium salt (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,4,6-tripyridyl-s-triazine (TPTZ), Folin–Ciocalteu’s reagent, formic acid, pyridine, hydrochloric acid (HCl), iron (III) chloride, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), acetic acid, casein from bovine milk, l-tyrosine (non-animal source), and standard compounds were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Samples

2.2.1. Four types of CGJ samples

Fully mature soybeans harvested in autumn 2011 in South Korea were used in preparing CGJ. Soybeans were soaked in distilled water for 24 h at room temperature, and autoclaved at 121 °C for 20 min. Then, the steamed soybeans were cooled down to 50 °C. Three types of ground garlic cultivated in South Korea, Nambae and Euisin variety, were mixed to soybeans (5% w/w): peeled raw garlic, garlic sprouted for 5 days, and garlic fermented with Lactobacillus Plantarum S65 at 20 °C for 10 days in media containing 3% salt and 3% sucrose. The garlic added soybean paste was inoculated with 3% (v/v) B. subtilis which had been stored at −80 °C in 50% glycerol, and fermented at 37 °C for 72 h. The control group was equally processed except for garlic addition. After finishing fermentation, the four types of CGJ were freeze-dried and powdered. Before experiment, each sample was stored at below −70 °C. All the CGJ samples were provided from Kyungpook National University (702–701, Daegu, Korea) and the processing methods are briefly visualized in Fig. S1.

2.2.2. Soybean fermentation for measuring protease activity and analyzing primary metabolites

Soybeans purchased from local market were washed and soaked in water for one day, then autoclaved at 121 °C for 60 min. B. subtilis stock in −80 °C in 50% glycerol were cultivated in Nutrient Broth medium at 37 °C for one day until the OD_{600} reached 0.5. Then B. subtilis cultured in Nutrient Broth medium were inoculated to the crushed soybean (5% v/w). Followed by B. subtilis inoculation, crushed garlic (1.5 g) and two standard compounds including s-allyl-cysteine (0.375 mg and 0.75 mg) and s-allyl-cysteine sulfoxide (2.5 mg and 5.0 mg) were added. The weight of standard compounds was semi-quantified by comparing data from garlic and standard compounds analyzed by ultra-high performance liquid chromatography–mass spectrometry/mass spectrometry (UHPLC–MS/MS). Fermentation was conducted at 37 °C for three days in an incubator. The overall processing methods are indicated in Fig. S2.

2.3. Metabolite extraction of four types of CGJ and soybean fermentation samples

One gram of each freeze-dried CGJ powder was extracted with 10 mL of solvent mixture (ethanol/water, 8:2) at room temperature for 24 h by using a shaker. The mixture was centrifuged at 5000 rpm, 4 °C for 5 min. After centrifugation, the supernatants were filtered through a 0.22 μm filter and 1 mL of filtrates was dried by using a speed vacuum concentrator. Before gas chromatography-time of flight-mass spectrometry (GC-TOF-MS), 20 mg of the dried filtrates was dissolved in 1 mL of the solvent mixture. Then, 100 μL of each dissolved filtrates was re-dried by using a speed vacuum concentrator for derivatization. Derivatization was performed in two steps, methoxyamination and silylation, to volatilize the metabolites in CGJ. Methoxyamination was carried out by dissolving the re-dried filtrates in 50 μL of methoxyamine hydrochloride (20 mg/mL in pyridine) and incubating at 30 °C for 90 min. Then, silylation was performed by adding 50 μL of MSTFA to the methoximated samples and incubating at 37 °C for 30 min. Before UHPLC–MS/MS, 20 mg of the dried supernatants was dissolved in 1 mL of the solvent mixture. Then, 100 μL of each dissolved filtrates was filtered through a 0.2 μm PTFE filter before analysis. The fermented soybean samples were freeze-dried and powdered, and then similarly prepared according to CGJ samples before GC-TOF-MS analysis.

2.4. GC-TOF-MS analysis

An Agilent 7890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) fitted with an Agilent 7693 Autosampler and a Pegasus® High-Throughput (HT)-TOF-MS (LECO, St. Joseph, MI, USA) system was used for GC-TOF-MS analysis. GC separation was performed on an Rtx-5MS column (30 m length × 0.25 mm i.d., 0.25 μm particle size; Restek Corp., Bellefonte, PA, USA), with helium as a carrier gas at a constant flow rate of 1.5 mL/min. A 1 μL aliquot of the sample was injected into the GC. The front inlet, transfer line, and ion source temperature were 250, 240, and 230 °C, respectively. The oven temperature was maintained at 75 °C for 2 min, increased to 300 °C at a rate of 15 °C/min, and then held at 300 °C for 3 min. Electron ionization was performed at 70 eV, and full scanning over a mass-to-charge ratio (m/z) range of 45–1000 was used for mass data collection. Five analytical replications of each sample were obtained.

2.5. UHPLC–MS/MS analysis

Thermo Fisher Scientific LTQ XL linear ion trap mass spectrometer consisting of an electrospray interface (Thermo Fisher Scientific, San José, CA) coupled with DIONEX UltiMate 3000 RS Pump, RS Autosampler, RS Column Compartment, and RS Diode Array Detector (Dionex Corporation, Sunnyvale, USA) was used. Samples were
separated on a Thermo Scientific Syncrion C18 UHPLC column (100 mm length × 2.1 mm i.d., 1.7 μm particle size). The mobile phase consists of A (0.1% [v/v] formic acid in water) and B (0.1% [v/v] formic acid in acetonitrile) and the gradient conditions were increased from 10% to 100% of solvent B over 18 min, and re-equilibrated to the initial condition for 4 min. The flow rate was 0.3 mL/min and the injection volume was 10 μL. The photodiode array was set at 200–600 nm for detection and managed by 3D field. Ion trap was performed in positive and negative full-scan ion modes within a range of 150–2000 m/z. The operating parameters were as follows: source voltage, ± 5 kV; capillary voltage, 39 V; capillary temperature, 275 °C. Tandem MS analysis was performed by scan-type turbo data-dependent scanning under the same conditions used for MS scanning. Five analytical replications of each sample were obtained.

2.6. Data processing and multivariate statistical analysis

Raw data files of GC-TOF-MS were converted to NetCDF (*.cdf) format using Leco ChromaTOF software. The UHPLC–MS/MS data were acquired with Xcalibur software (version 2.0.0, Thermo Fisher Scientific, San José, CA), and raw data files were converted to NetCDF (*cdf) format using Xcalibur software. After conversion, the NetCDF files were subjected to preprocessing, correction of retention time and baseline, and peak extraction by using the MetAlign software package (http://www.metalign.nl). The resulting data were exported to a Microsoft Excel (Microsoft, Redmond, WA, USA). Multivariate statistical analysis was performed using SIMCA-P+ 12.0 software (Umetrics, Umeå, Sweden). Principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), orthogonal partial least squares discriminant analysis (OPLS-DA), loading S-plots, and loading scatter plot were performed to analyze metabolite differences between CGJ samples. The variables were selected based on variable importance in the projection (VIP) value and significant differences were tested by analysis of variance (ANOVA). After multivariate statistical analysis, variables were identified using authentic standard compounds by comparing both mass spectra and retention time. When standard compounds were not available, a tentative identification was performed based on the MS spectra using the national institute of standards and technology (NIST, 2005), combined chemical dictionary version 7.2 (Chapman & Hall/CRC), references, and an in-house library. Box and whisker plot analysis were performed using STATISTICA7 (version 7.0, StatSoft Inc., Tulsa, OK, USA). SPSS for Windows (version 12.0; SPSS Inc., Chicago, IL, USA) was used to calculate Pearson’s correlation coefficient between metabolites and antioxidant activity. A heatmap was applied to visualize the relative contents of discriminant metabolites by using Multiexperiment Viewer (version 4.9.1).

2.7. Antioxidant activity using ABTS, DPPH radical scavenging, and FRAP assay

ABTS, DPPH radical scavenging activity and FRAP assays were carried out with some modifications (Kim, Hwang, et al., 2014; Kim, Jung, et al., 2014). Dried CGJ samples (100 mg) were dissolved in 1 mL of solvent mixture (ethanol/water, 8:2). ABTS ammonium (7 mM) was dissolved in 2.45 mM potassium persulfate solution. The mixture was maintained at room temperature for one day in order to allow it to turn into a dark blue solution. The mixture was diluted with distilled water until the absorbance reached 0.7 ± 0.02 at 734 nm. Reaction mixtures containing 10 μL of each dissolved CGJ sample and 190 μL of ABTS solution were incubated at room temperature for 6 min in 96-well plates. After 6 min, the absorbance at 734 nm was recorded using a microplate reader. The concentration of Trolox standard solutions ranged from 0.0625 to 0.5 mM. Experiments were carried out in triplicate.

Reaction mixtures containing 20 μL of each dissolved CGJ sample and 180 μL of DPPH ethanol solution (0.2 mM) were incubated at room temperature for 20 min in 96-well plates. The absorbance of the DPPH free radical was measured at 515 nm using a microplate reader. Experiments were carried out in triplicate. A FRAP reagent was prepared by mixing 2.5 mL of 10 mM TPTZ solution in 40 mM HCl, 2.5 mL of 20 mM FeCl₃, and 25 mL of 0.3 M acetic buffer (pH 3.6). Reaction mixtures containing 10 μL of each dissolved CGJ sample and 300 μL of FRAP reagent were incubated at room temperature for 6 min in 96-well plates. The absorbance was measured at 593 nm using a microplate reader. Experiments were carried out in triplicate. The antioxidant activity of the four types of CGJ was shown as Trolox equivalent antioxidant activity (TEAC, mM).

2.8. Protease activity assay of fermented soybean paste

Protease activity assay of fermented soybean paste was carried out with some modifications (Sevinc & Demirkiran, 2011). One gram of fermented soybean pastes was extracted with 10 mL of solvent mixture (ethanol/water, 8:2) at room temperature for one day by using a shaker. Then, the mixture was centrifuged at 5000 rpm, 4 °C for 5 min, and the supernatants were used for protease activity assay. Equivalent volume (1 mL) of the culture supernatants and 1 M phosphate buffer (pH 7) containing 0.6% casein were mixed and incubated at 37 °C for 10 min. Then, 5 mL of 0.4 M trichloroacetic acid was added and the mixtures were incubated at 37 °C for 30 min to stop the reaction. After 30 min, the mixtures were filtered through a 0.2 μm PTFE filter, and 2 mL of filtrate was mixed with 5 mL of 0.4 M sodium carbonate and 1 mL of Folin solution (3-fold dilution of 2 N Folin stock solution with water). After mixing, the solution mixtures were incubated at 37 °C for 30 min, and then the optical density was measured at 660 nm by using a UV spectrometer. The protease activity was defined as nanomoles of L-tyrosine produced per unit time (nanokatals).

3. Results and discussion

3.1. Metabolite profiling of four types of CGJ by using GC-TOF-MS and UHPLC–MS/MS

During the soybean fermentation, macromolecules of soybean such as proteins, isoflavone glycosides, and soyaasaponins are hydrolyzed to small molecules and these processes are mediated by various enzymes of B. subtilis (Lee et al., 2014). However, due to its complexity, fermentation is affected by many conditions such as different processing steps (Dakwa, Sakyi-Dawson, Diako, Annan, & Amoa-Awuwa, 2005), kind of inoculated Bacillus species (Terlabie, Sakyi-Dawson, & Amoa-Awuwa, 2006), and various additives (Kim, Hwang, et al., 2014; Kim, Jung et al., 2014). In this research, a metabolite profiling of Cheonggukjang (CGJ) and three types of garlic added CGJ (G-CGJ) linked with antioxidant activity and protease activity was conducted to reveal the influence of garlic on CGJ fermentation. Metabolite profiling of the four types of CGJ were obtained by using GC-TOF-MS and UHPLC–MS/MS combined with multivariate statistical analysis and revealed the different metabolic states of each CGJ sample. In PLS-DA score plots derived from GC-TOF-MS as well as UHPLC–MS/MS data set (Fig. 1), clear separation was shown between CGJ and three types of garlic added CGJ (G-CGJ) by PLS1 (38.50% and 22.75%, respectively). Also, PCA score plots showed similar separation pattern (Fig. S3). Thirty-nine compounds were selected by VIP > 0.7 and p-value < 0.05. Among them, twenty-eight metabolites including eighteen amino acids, five sugars and sugar alcohols, three organic acids, one fatty acid, and urea were tentatively identified from GC-TOF-MS data by comparing mass fragment patterns and retention time with standard compounds, in house library, and NIST (Table 1). The relative contents of twenty-eight compounds were visualized as box and whisker plots (Fig. S4). As shown in Fig. S4, the levels of several amino acids such as l-valine (2), l-leucine (3), l-isoleucine (4), glycine (6), l-serine (7), l-methionine (9), GABA (10), l-phenylalanine (13), dl-ornithine (14), l-lysine (15), l-histidine (16),
L-tyrosine (17), and L-tryptophan (18) were high in CGJ but dramatically decreased in G-CGJ, while the levels of L-alanine (1) and L-cysteine (11) were high in G-CGJ. Eleven metabolites including two organosulfur compounds, three isoflavones, and six saponins were tentatively identified from UHPLC–MS/MS data by comparing mass to charge ratio, mass/mass fragment patterns, and UV absorbance according to Fig. 1.

Fig. 1. PLS-DA score plots derived from non-targeted metabolite profiling of four types of Cheonggukjang analyzed by (A) GC-TOF-MS and (B) UHPLC–MS/MS; RX is all the Xs explained by the component, RY is all the Ys explained by the component, and Q2 is total variation of the Xs and Ys that can be predicted by the component. ( • Cheonggukjang (CGJ), • Garlic added CGJ (G-C), ◆ Sprouted garlic added CGJ (SG-C), and ◼ Fermented garlic added CGJ (FG-C)).

Table 1
List of primary metabolites analyzed by GC-TOF-MS.

<table>
<thead>
<tr>
<th>No.</th>
<th>RT* (min)</th>
<th>Compounda</th>
<th>Unique mass</th>
<th>Mass fragment pattern</th>
<th>TMSb</th>
<th>IDc</th>
<th>VIPd</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.36</td>
<td>L-Alanine</td>
<td>116</td>
<td>147, 116, 103, 100, 75, 73, 59</td>
<td>2</td>
<td>STD</td>
<td>0.93</td>
<td>0.00E+00</td>
</tr>
<tr>
<td>2</td>
<td>6.60</td>
<td>L-Valine</td>
<td>218</td>
<td>218, 147, 144, 100, 73, 59</td>
<td>2</td>
<td>STD</td>
<td>1.62</td>
<td>0.00E+00</td>
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<tr>
<td>3</td>
<td>7.16</td>
<td>L-Leucine</td>
<td>158</td>
<td>158, 147, 102, 73, 59</td>
<td>2</td>
<td>STD</td>
<td>1.61</td>
<td>0.00E+00</td>
</tr>
<tr>
<td>4</td>
<td>7.34</td>
<td>L-Isoleucine</td>
<td>158</td>
<td>158, 147, 100, 73, 59</td>
<td>2</td>
<td>STD</td>
<td>1.63</td>
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<td>5</td>
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<td>L-Proline</td>
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<td>147, 142, 133, 75, 73, 59</td>
<td>2</td>
<td>STD</td>
<td>1.12</td>
<td>1.00E−05</td>
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<td>6</td>
<td>7.50</td>
<td>Glycine</td>
<td>174</td>
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<td>STD</td>
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<td>STD</td>
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<td>189</td>
<td>189, 147, 99, 75, 73, 66, 59</td>
<td>2</td>
<td>STD</td>
<td>1.62</td>
<td>0.00E+00</td>
</tr>
</tbody>
</table>

a Retention time.

b Compounds were selected by VIP > 0.7 and p-value < 0.05.

c Number of hydrogen atoms derivatized.

d Identification: STD, standard compound; mass pattern, comparing mass fragment pattern within house library and NIST; Variable importance in the projection.
references (Dalluge, Eliason, & Frazer, 2003; Fang, Yu, & Badger, 2004; Jin, Yang, Su, & Ren, 2006; Lee et al., 2014) or standard compounds (Table 2). The relative amounts of eleven metabolites are shown as box and whisker plots (Fig. S4). As shown in Fig. S5, except for soyasaponin I (34) and soyasaponin IV (35), the levels of other nine secondary metabolites were high in G-CGJ compared to CGJ. The overall results suggested that there were metabolic differences between CGJ and G-CGJ. Among G-CGJ, sprouted garlic added CGJ (SG-C) and fermented garlic added CGJ (FG-C) were distinguished from garlic added CGJ (G-C) by PLS2. A loading scatter plot derived from secondary metabolites were high in G-CGJ compared to CGJ. The overall results suggested that there were metabolic differences between CGJ and G-CGJ. Among G-CGJ, sprouted garlic added CGJ (SG-C) and fermented garlic added CGJ (FG-C) were distinguished from garlic added CGJ (G-C) by PLS2. A loading scatter plot derived from

Table 2
List of secondary metabolites analyzed by UHPLC–MS/MS.

<table>
<thead>
<tr>
<th>No.</th>
<th>RT&lt;sup&gt;a&lt;/sup&gt; (min)</th>
<th>UHPLC–MS/MS</th>
<th>[M + H]&lt;sup&gt;b&lt;/sup&gt;</th>
<th>[M – H]&lt;sup&gt;b&lt;/sup&gt;</th>
<th>MW&lt;sup&gt;c&lt;/sup&gt;</th>
<th>MS&lt;sup&gt;d&lt;/sup&gt; fragment patterns</th>
<th>UV (nm)</th>
<th>p-Value</th>
<th>VIP&lt;sup&gt;e&lt;/sup&gt;</th>
<th>ID&lt;sup&gt;f&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>29</td>
<td>1.02</td>
<td>S-allyl-cysteine sulfoxide</td>
<td>178</td>
<td>176</td>
<td>177</td>
<td>–</td>
<td>267</td>
<td>0.00E – 06</td>
<td>2.25</td>
<td>STD</td>
</tr>
<tr>
<td>30</td>
<td>1.29</td>
<td>S-allyl-cysteine</td>
<td>162</td>
<td>160</td>
<td>161</td>
<td>145, 73</td>
<td>221</td>
<td>0.00E + 00</td>
<td>7.02</td>
<td>STD</td>
</tr>
<tr>
<td>31</td>
<td>7.81</td>
<td>Malonyl daidzin</td>
<td>503</td>
<td>501</td>
<td>502</td>
<td>417, 255</td>
<td>259</td>
<td>4.00E – 07</td>
<td>1.90</td>
<td>Lee et al. (2014)</td>
</tr>
<tr>
<td>32</td>
<td>8.58</td>
<td>Malonyl genistin</td>
<td>519</td>
<td>517</td>
<td>518</td>
<td>433, 271</td>
<td>258</td>
<td>1.40E + 03</td>
<td>1.60</td>
<td>Lee et al. (2014)</td>
</tr>
<tr>
<td>33</td>
<td>10.56</td>
<td>Genistein</td>
<td>271</td>
<td>269</td>
<td>270</td>
<td>253, 215, 169</td>
<td>217</td>
<td>3.40E + 03</td>
<td>0.88</td>
<td>STD</td>
</tr>
<tr>
<td>34</td>
<td>11.50</td>
<td>Soyasaponin I</td>
<td>944</td>
<td>942</td>
<td>943</td>
<td>–</td>
<td>218</td>
<td>5.04E + 02</td>
<td>2.05</td>
<td>Fang et al. (2004)</td>
</tr>
<tr>
<td>35</td>
<td>12.10</td>
<td>Soyasaponin IV</td>
<td>767</td>
<td>765</td>
<td>766</td>
<td>543</td>
<td>219</td>
<td>2.00E + 04</td>
<td>1.43</td>
<td>Fang et al. (2004)</td>
</tr>
<tr>
<td>36</td>
<td>12.37</td>
<td>Soyasaponin VI</td>
<td>1070</td>
<td>1068</td>
<td>1069</td>
<td>575</td>
<td>216, 277</td>
<td>5.00E – 07</td>
<td>2.02</td>
<td>Dalluge et al. (2003)</td>
</tr>
<tr>
<td>37</td>
<td>12.65</td>
<td>Soyasaponin VIa</td>
<td>1040</td>
<td>1038</td>
<td>1039</td>
<td>550</td>
<td>220</td>
<td>2.00E + 05</td>
<td>1.87</td>
<td>Dalluge et al. (2003)</td>
</tr>
<tr>
<td>38</td>
<td>12.94</td>
<td>Soyasaponin Vg</td>
<td>923</td>
<td>921</td>
<td>922</td>
<td>–</td>
<td>220</td>
<td>2.00E + 08</td>
<td>2.67</td>
<td>Jin et al. (2006)</td>
</tr>
<tr>
<td>39</td>
<td>13.14</td>
<td>Soyasaponin Vga</td>
<td>893</td>
<td>891</td>
<td>892</td>
<td>557</td>
<td>221</td>
<td>1.00E + 06</td>
<td>1.48</td>
<td>Jin et al. (2006)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Retention time.

<sup>b</sup> Tentative metabolites based on variable importance projection (VIP) > 0.7 and p-value < 0.05.

<sup>c</sup> Molecular weight.

<sup>d</sup> Variable importance in the projection.

<sup>e</sup> Identification: STD, standard compounds; references.

![Fig. 2](image-url)
UHPLC–MS/MS data set of G-CGJ (Fig. S6) showed that the main metabolites responsible for this separation were S-allyl-cysteine sulfoxide (29) and S-allyl-cysteine (30).

3.2 Differences in the metabolite composition between CGJ and G-CGJ as well as antioxidant activity

Antioxidant activity of the four types of CGJ was measured using three different assays (ABTS, DPPH, and FRAP) (Fig. 2). The results indicated that G-CGJ showed higher antioxidant activity than CGJ in ABTS (Fig. 2A), DPPH (Fig. 2B), and FRAP (Fig. 2C) assays. To visualize the correlation between metabolites and antioxidant activities, a correlation map using Pearson’s correlation coefficient was produced (Fig. 2D). In total, 21 metabolites showed positive correlation \((0 < r < 1)\) with antioxidant activity:

- Five sugars and sugar alcohols (D-fructose (23), xylitol (24), D-rhamnose (25), D-mannose (26), and myo-inositol (27)),
- Two amino acids (L-alanine (1) and L-cysteine (11)),
- Two organic acids (lactic acid (19) and D-gluconic acid (21)),
- One fatty acid (linoleic acid (22)),
- Two organosulfur compounds (S-allyl-cysteine sulfoxide (29) and S-allyl-cysteine (30)),
- Three isoflavones (malonyl daidzin (31), malonyl genistein (32), and genistein (33)), and six soyasaponins (soyasaponin I (34), soyasaponin IV (35), soyasaponin ßg (36), soyasaponin ßa (37), soyasaponin ñg (38), and soyasaponin ña (39)).

To determine the significantly different metabolites between CGJ and G-CGJ, an orthogonal partial least squares discriminant analysis (OPLS-DA) model and a loading S-plot were applied (Fig. 3). Nine metabolites were selected as significantly different metabolites between CGJ and G-CGJ (variable importance in the projection (VIP) \(> 0.7\) and \(p\)-value \(< 0.05\) in OPLS-DA model) and indicated on the loading S-plots. The metabolites were divided into three categories: amino acids, organosulfur compounds, and DDMP-soyasaponins. Relative composition of the metabolites was proposed as a heat map using the value of fold changes normalized by an average peak area (Fig. 3E). Among them, two organosulfur compounds (S-allyl-cysteine sulfoxide (29) and S-allyl-cysteine (30)) and two DDMP-soyasaponins (soyasaponin ßg (36) and soyasaponin ñg (38)) are known to possess antioxidant activity (Chung, 2006; Okubo & Yoshikì, 1996), and the levels of them were relatively high in G-CGJ. In addition, those metabolites showed positive correlation \((0 < r < 1)\) with antioxidant activity, which might be related to the enhanced antioxidant activity of G-CGJ. The antioxidant activity of sulfur containing amino acids is reported (Atmaca, 2004) and L-cysteine is the precursor of organosulfur compounds (Jones et al., 2004). In case of L-cysteine (11), it showed positive correlation with antioxidant activity and relatively high contents in G-CGJ, which might account for promoted...
antioxidant activity in G-CGJ. The other metabolites (L-valine (2), L-leucine (3), L-isoleucine (4), and glycine (6)) showed relatively low levels in G-CGJ compared to CGJ. Previous research reported that thiol group at the active site of protease, especially cysteine protease, plays a major role in the hydrolytic cleavage of peptide bonds (McKerrow, 1999). However, organosulfur compounds such as allicin, S-allyl-cysteine, and S-allyl-cysteine sulfoxide can bind to thiol groups and inhibit the enzymatic activity of protease (Waag et al., 2010). Concerning this previous research, lower levels of four metabolites in G-CGJ might be related to the inhibited protease activity by garlic because the soy proteins are converted to amino acids by proteolytic activity of B. subtilis during soy fermentation (Chantawannakul, Oncharoen, Klanbut, Chukeatirote, & Lumyong, 2002).

3.3. Measurement of protease activity and metabolite profiling of fermented soybean pastes by using GC-TOF-MS

To analyze whether garlic affects the protease activity of B. subtilis during fermentation, we conducted soybean fermentation with garlic and standard compounds (S-allyl-cysteine and S-allyl-cysteine sulfoxide) by applying the manufacturing methods of existing CGJ samples (Fig. S2). Protease activity of soybean pastes fermented with B. subtilis was measured and the activity was shown as nanokatals (Fig. 4). As indicated in the results, when garlic was added during soybean fermentation, the protease activity was decreased (C, 0.142 nanokatals) compared to the positive control (B, 0.187 nanokatals), as well as after the adding of S-allyl-cysteine (D and E) and S-allyl-cysteine sulfoxide.

Fig. 5. Box and whisker plots of ten amino acids analyzed by GC-TOF-MS. A, Glycine max; B, Glycine max + B. subtilis; C, Glycine max + garlic + B. subtilis; The Y-axis of box and whisker plots indicates the peak area of each metabolite. (Line, mean; box, standard error; whisker, standard deviation).
In addition, the inhibition was concentration dependent as the protease activities were more inhibited when the weight of added compounds was doubled (Fig. 5D vs E and 5F vs G). In addition, the inhibition was concentration dependent as box and whisker plots (Fig. 5). By the fermentation, the relative contents of amino acids were drastically increased (B respect A), while the relative contents of amino acids were decreased (C compared to B) when garlic was added during fermentation. The results suggested that garlic inhibited the hydrolytic processes of soy proteins to amino acids.

4. Conclusion

Hyphenated techniques (GC-TOF-MS and UHPLC-MS/MS), which are different but complementary each other, were used to assess the metabolic profile of Cheonggukjang (CGJ). Metabolomics approach aiming at characterization of metabolites and their correlation with antioxidant activity is valuable and widely used for food research. In this study, metabolomics approach combined with protease activity assay was performed and revealed the different metabolic states of CGJ by adding garlic. These analytical methods would bring new way to analyze fermented foods.

**References**


