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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 βg (PubChem CID: 164453)

ABSTRACT

We performed mass spectrometry (MS)-based metabolite profiling of *Cheonggukjang* (CGJ) and three types of garlic (normal, sprouted, and fermented) added CGJ (G-CGJ). Metabolite profiling suggested that the major distinguishing factor between the four types of CGJ lay in whether garlic was added, although different metabolic states were observed in G-CGJ. Among the discriminant metabolites between CGJ and G-CGJ, the levels of four amino acids such as L-valine, L-isoleucine, L-leucine, and glycine were decreased in G-CGJ compared to CGJ because garlic inhibited the protease activity of *Bacillus subtilis* during fermentation. In addition, the relative contents of L-cysteine, S-allyl-cysteine, S-allyl-cysteine sulfoxide, soyasaponin βg, and soyasaponin γg, which showed positive correlation with antioxidant activities, were high in G-CGJ. These results suggest that MS-based metabolite profiling could be a useful tool for understanding the metabolic differences of fermented foods according to the additives, and their relationship with antioxidant activity.

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

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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 (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 metabolite 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). Trichloroacetic acid was purchased from Merck Millipore Co. (Darmstadt, Germany). Methoxyamine hydrochloride, N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA), potassium persulfate, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium 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 grinded garlic cultivated in South Korea, Namhae and Euisung 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*/*w*) *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* stocked 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, methoxymation and silylation, to volatilize the metabolites in CGJ. Methoxymation 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