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Primary and secondary metabolite profiling of *doenjang*, a fermented soybean paste during industrial processing

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ABSTRACT

In this study, a comprehensive metabolite profile analysis of *doenjang*, a fermented soybean paste, at various steps of its industrial 5-step production process was conducted, by combining gas and liquid chromatography–mass spectrometry techniques with multivariate analysis. From the partial least squares discriminant analysis of primary and secondary metabolites, the patterns were clearly distinguishable between the various processing steps (step 1: steaming, step 2: drying, step 3: *meju* fermentation, step 4: brining, step 5: *doenjang* aging). Of the primary metabolites, most of the monosaccharides, amino acids, and fatty acids increased in steps 3–5. Isoflavone and soyasaponin derivatives were major secondary metabolites identified during the processing of *doenjang*. Isoflavone glycosides gradually decreased after step 1, while isoflavone aglycones distinctly increased in steps 4–5. Soyasaponins generally decreased during processing after step 2. Increased isoflavone aglycones, such as daidzein, glycitein, and genistein, were observed in steps 4–5 showed the strongest positive correlation with *doenjang*'s antioxidant potential and total phenolic content.

1. Introduction

Doenjang is a fermented soybean processed food produced from *meju*, a fermented soybean starter found in a typical Korean meal. In general, *doenjang* is produced in a 2-part process; first, for making *meju*, soybeans are pretreated by steaming, shaping, and drying, followed by fermentation using microorganisms (Kwon et al., 2011). In the second step, *meju* is fermented again after adding salt water, and the mixture is then separated into the solid portion, referred to as *doenjang* (soy paste), and the liquid portion *kanjang* (soy sauce). During processing, the qualitative characteristics of the soybean products change in accordance with the metabolites present, which vary due to the physicochemical or enzymatic activities occurring during each step (Prinyawiwatkul, Beuchat, McWatters, & Phillips, 1996; Villares, Rostagno, García-Lafuente, Guillamón, & Martínez, 2011).

Metabolite profiling, which aims to monitor all metabolites within a biological sample, has been used in previous studies to assess changes in metabolites of several types of foods and biological systems (Lee, Do, et al., 2012; Lee, Kim, et al., 2012). Mass spectrometry (MS) coupled with separation techniques, such as gas chromatography (GC) and liquid chromatography (LC), is a potential analytical tool for metabolomics (Lee, Do, et al., 2012). While GC–MS is mainly suited for primary metabolism products, such as amino acids, fatty acids, carbohydrates, and organic acids, LC–MS covers the large group of secondary metabolites, including alkaloids, flavonoids, saponins, phenolic acids, phenylpropanoids, glucosinolates, and polyamines (t'Kindt, Morreel, Deforce, Boerjan, & Bocxlaer, 2009). Therefore, a combination of different spectroscopic platforms, such as GC–MS and LC–MS, could detect broader classes of metabolites (Arbona, Iglesias, Talon, & Cadenas, 2009).

In fermented soybean products, primary metabolites, such as sugars, amino acids, organic acids, and salt, are thought to add to the sensory qualities of the food, yielding tastes that are sweet, savory, flavorful, and salty; in contrast, secondary metabolites, such as isoflavones, saponins, and tocopherols, contribute to the functional and antioxidant properties of soybean-based foods (Namgung et al., 2010; Wardhani, Vázquez, & Pandiella, 2010). Soybean-based products are the best examples of potential antioxidant activity. Antioxidant compounds found in soybeans and soybean products, including isoflavones, saponins, phytic acid,

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and phytosterols, have been reported to possess potential health benefits, such as reduction of oxidative damage (Wardhani et al., 2010).

In this study, we carried out a comprehensive metabolic profiling of *doenjang* during industrial processing. GC–TOF-MS and UPLC–Q-TOF-MS were used to detect broader classes of metabolites, and changes in metabolites were analyzed by multivariate analysis. Even though several studies pertaining to soy-based food products have examined metabolite changes and antioxidant potential, no studies have thoroughly assessed primary and secondary metabolite changes during the industrial stepwise processing of soybeans and their effects on the antioxidant capacity of the food product. In our study, changes in primary metabolites at different processing steps were examined in terms of the metabolic pathway, and correlations between secondary metabolites and antioxidant activities were investigated.

2. Materials and methods

2.1. Chemicals and reagents

Ethanol, methanol, isopropanol, acetonitrile, and water were purchased from Fisher Scientific (Pittsburgh, PA, USA). Methoxyamine hydrochloride, N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA), gallic acid, potassium persulfate, 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,4,6-tripyridyl-s-triazine (TPTZ), Folin–Ciocalteu's phenol, formic acid, pyridine, dimethyl sulfoxide, hydrochloric acid (Hcl), iron (III) chloride, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), acetic acid, and standard compounds were obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Sample preparation

Samples were obtained from CJ Cheiljedang Corporation (Seoul, South Korea). The processing method and basic information (pH and salinity) used to produce *doenjang* are shown in Fig. 1a. In brief, for making *meju*, the soybeans were sorted, washed, and soaked overnight in water. They were steamed for 1 h at 110 °C, and then crushed (step 1, steaming). This paste was pressed into a block (17 × 10 × 8.5 cm) and dried at 40–50 °C for one day (step 2, drying). These soybean blocks are called "*meju*". These *meju* blocks were fermented with naturally occurring microorganisms for 15 days (step 3, *meju* fermentation). Fermented blocks were brined in salt water (21%–24%) for 34 days (step 4, brining), and the liquid was then separated from the mixture. The residue was further subjected to an aging process for an additional 90 days (step 5, *doenjang* aging). The samples were collected at different steps: soybean, steaming (1 day) and drying (2 days), *meju* fermentation (3, 4 and 17 days), brining (22, 40, and 51 days), and *doenjang* aging (81 and 141 days). Each sample was collected at 6 different batches during the stepwise processing. These samples were lyophilized and stored in a −80 °C freezer until analysis.

2.3. GC–TOF-MS analysis

Lyophilized samples (100 mg) were extracted with 1 mL of mixture solvent (isopropanol:acetonitrile:water = 3:2:2, v/v/v) in a 2-mL eppendorf tube using a Retsch MM400 Mixer Mill (Retsch GmbH & Co, Germany) at 30 Hz/s for 2.5 min and then centrifuged at 8000×*g* for 8 min at 4 °C. After centrifugation, the supernatant was separated, and the same procedure was repeated twice. Subsequently, 200 μL of the supernatant was dried using a Speedvac (Biotron, Korea). The dried residue was methoximated with 200 μL of methoxyamine hydrochloride in pyridine (20 mg/mL) at 30 °C for 90 min. Derivatization for silylation was performed by adding 100 μL of MSTFA containing 1% trimethylchlorosilane, and the samples were incubated at 37 °C for 30 min. Analysis was performed using an Agilent 7890A gas chromatograph coupled with a Pegasus HT TOF-MS (Leco Corporation, St. Joseph, MI, USA). Metabolites were separated on RTx-5MS (30 m × 0.25 mm; film thickness, 0.25 μm), and helium was used as the carrier gas at a constant flow rate of 1.0 mL/min. The injector temperature was 250 °C, and the transfer line and ion source temperatures were 250 and 200 °C, respectively. The column temperature was held at 80 °C for 2 min, raised to 300 °C at a rate of 12 °C/min, and then held isothermally at the final temperature for 6 min. The injection volume was 1 μL in split mode (25:1, v/v), and mass spectra were recorded over the mass range of 50–600 *m*/*z*. Six biological replications from each of the samples from different processing steps were used.

2.4. UPLC–Q-TOF-MS analysis

Lyophilized samples (100 mg) were extracted with 1 mL of ethanol:dimethyl sulfoxide:water (14:1:5, v/v/v) in a Mixer Ball Mill (Retsch) at 30 Hz/s for 2.5 min, followed by centrifugation at 8000×*g* for 8 min at 4 °C. This procedure was repeated twice. Then, 400 μL of the extracted supernatant was dried using a Speedvac. The residues were dissolved in 800 μL of 80% methanol and filtered through a 0.22-μm filter (Woongki, Korea). Analysis was performed using a Waters ACQUITY UPLC system with Waters Q-TOF micro MS (Micromass MS Technologies, Manchester, UK). Five microliters of each sample was injected into an ACQUITY BEH C18 column (100 mm × 2.1 mm i.d., 1.7 μm particle size; Waters, Milford, MA, USA) with a gradient system at a flow rate of 0.3 mL/min. The mobile phases consisted of 0.1% v/v formic acid in water (solvent A) and 0.1% v/v formic acid in acetonitrile (solvent B). Gradient elution was as follows: 5% B for 1 min then increased to 100% B up to 10 min, held for 1 min, followed by decrease to 5% B, and maintained at 5% B for 1 min. Total run time was 12 min, including re-equilibration of the column to the initial conditions. The capillary voltage was set at −2300 V in negative ionization mode and 2500 V in positive ionization mode. The scan range was from 100 to 1000 *m*/*z*. The nebulization gas was set to 350 L/h (−) and 650 L/h (+) at a temperature of 300 °C, and the source temperature was 80 °C. Cone voltage was set at 50 V and the detector voltages were −1850 and 1800 V in negative and positive ion modes, respectively. The Q-TOF micro MS acquisition rate was set to 0.2 s with a 0.02-s interscan delay. Tune page was used to regulate the sample cone voltage. Leucine enkephalin was used as the lock mass (*m*/*z* 554.2615(−) and *m*/*z* 556.2771(+)) at a flow rate of 10 μL/min. Data was collected in centroid mode during acquisition. Six biological replications from each of the sample from different processing steps were used.

2.5. Data processing and multivariate analysis

GC–TOF-MS raw data was converted to netCDF (*.cdf) format using Leco ChromaTOF software (version 4.44). UPLC–Q-TOF-MS raw data sets were converted to netCDF using the Masslynx conversion tool, DataBridge (version 2.1). The netCDF data were aligned using metAlign, and the resulting file (*.CSV) was exported to Microsoft Excel (Microsoft, Redmond, WA, USA). Metalign software was used to provide deconvolution functions such as peak selection, baseline correction, and peak alignment for large metabolite data sets. Baseline correction and noise calculations were performed from scan number 80 to 1348 and the maximum amplitude was set to 10^5 for UPLC–Q-TOF data analysis. In GC–TOF-MS analysis, scan ranges were 300–13,200 and maximum amplitude was set to 10^8. Peak slope factor was set to 1, peak