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ARTICLE

Mass spectrometry-based metabolomic study of traditional *Doenjang* effects against hepatic fibrosis

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Abstract Korean traditional *Doenjang* are currently certified as traditional food. Certified *Doenjang* are valuable as a traditional food, however, only a few studies have evaluated and researched their quality characteristics and functionality. In the present study, we investigated the metabolite profile of *Doenjang* according to its α-SMA expression suppressing effect through mass spectrometry-based metabolomics. Also, the relationship between *Doenjang* metabolites and the suppression of α-SMA expression was identified. In the efforts to find traditional *Doenjang* metabolites related to liver functions, branched-chain amino acids, *p*-aminobenzoic acid, 7,4′-dihydroxyflavone, soyasaponin, and isoflavone aglycone were found to be higher in the *Doenjang* group and showed higher α-SMA expression suppression activity. The present study highlights the fact that comprehensive metabolite analysis of traditional *Doenjang* provides information on its ability to improve liver function as well as other useful information for better understanding of the factors related to the effects of *Doenjang* metabolites against hepatic fibrosis.

Keywords α-SMA expression inhibiting activity · *Doenjang* · MS-based metabolomics

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Introduction

Korean fermented soybean products are divided into soybean paste (*Doenjang*), ground fermented soybeans, soy sauce, and red pepper paste. The fermented soybean products have been used traditionally as a great source of nutrition, providing protein and essential amino acids that are insufficient in the grain-based Korean diet. Moreover, these fermented soybean products exhibit diverse physiological characteristics such as obesity-preventing effects (Kwon et al. 2006; Bae et al. 2013), anticancer effects (Park et al. 2003; Jung et al. 2006; Lim et al. 2007), anti-diabetic activities (Lim et al. 2004), enhanced immune function (Masilamani et al. 2012; Karasawa et al. 2013), and hepatoprotective effects (Kinjo et al. 1998; Soomro et al. 2008; Park et al. 2013). These characteristics of *Doenjang* are based on the fact that protein, fat, and the other contents in soybeans are changed into digestible forms during fermentation by various microorganisms. However, despite the existing research on various characteristics of *Doenjang* mentioned earlier, only a few studies on the functions related to liver diseases have been reported.

To increase the consumption and globalization of traditional *Doenjang*, a national certification system that assures the quality and safety of excellent traditional food should be established (Heo and Jin 2011). Therefore, 46 items and 472 food plants are currently certified as traditional food. Especially in the *Doenjang* category, 51 products (Standard No. T015) are currently produced and sold with the traditional food certification. Certified *Doenjang* is valuable as traditional food, and their consumption is expected to increase based on high consumer confidence. A recent study on the quality characteristics of commercially certified *Doenjang* (Kang et al. 2013)

evaluated the quality characteristics and isoflavone content of *Doenjang*.

This traditional fermented food has been reported to have a number of functions as a complex with various ingredients and substances derived from the raw materials to the metabolites by microorganisms. However, studies specifically focusing on changes occurring in certain metabolites in the food and related functions have not been reported. For such a study, it is necessary to use the metabolomics techniques. To date, however, metabolomics studies on traditional fermented foods have mostly been conducted only for fragmentary studies using certain ingredients for each fermented product, and global analysis has not yet been performed. In recent years, metabolomics has been applied to traditional foods to investigate the characteristics and patterns of metabolite changes according to the fermentation period and the methods used for Korean traditional fermented soy products (Namgung et al. 2010; Park et al. 2010; Kim et al. 2012), the antioxidative effects of cheonggukjang (fast-fermented bean paste) (Kim et al. 2011), and to investigate the relationship between biomarkers and health functions (Kwon et al. 2011).

In a previous study, we evaluated the α-SMA expression inhibiting effect of traditional *Doenjang* (fermented soybean paste), which has received a quality certification as a traditional food in Korea (Park et al. 2013). Based on the results of the preceding research, this study investigated the metabolite profiles of *Doenjang* according to its α-SMA expression suppressing effects through metabolomics for the purpose of discovering effective bioactive substances. These results will be used as basic material for investigating correlations between the improvement of liver function and *Doenjang*. Furthermore, this study will provide guidelines for the improvement in liver function induced by traditional *Doenjang*.

Materials and methods

Chemicals

All chemical reagents were of analytical grade. The standard reagents, all amino acids, and isoflavones were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Sample collection

The 24 certified traditional *Doenjang* samples used in this study were obtained from various areas, Gangwon (3), Gyeonggi (5), Gyeongnam (1), Gyeongbuk (3), Jeonbuk (6), Chungbuk (1), Chungnam (2), and Jeju (3) in Republic of Korea. Each *Doenjang* sample was fermented for a period of ~6 months to 2 years. Additionally, five types of *Doenjang* samples were produced from each single-strain inoculation to compare with the traditional *Doenjang*.

Doenjang extraction

Lyophilized *Doenjang* samples (300 mg) were extracted with 4 mL of 50 % methanol for 3 h at room temperature. The supernatants were diluted and filtered prior to UPLC analysis.

Doenjang metabolites analysis by UPLC-Q-TOF–MS

A UPLC system (Agilent 1290 Infinity, USA; Agilent, USA) coupled to a Q-TOF mass spectrometer (Agilent 6520 with Jet Stream Technology, Agilent) was used to analyze the metabolites of *Doenjang* extract using a C18 column ACE Excel 3 SuperC18 (3 μm, 4.6 × 150 mm). Chromatographic separation was performed at an injection volume and flow rate of 1 μL and 0.9 mL/min, respectively, using mobile phases A (0.1 % formic acid in DW) and B (0.1 % formic acid in acetonitrile) at a gradient mode within 30 min. The Q-TOF–MS was operated in the positive ESI mode within the mass scan range of 60–1000 m/z. The dry gas temperature was 350 °C with capillary, skimmer, and fragmentor voltages at 4, 65, and 170 V, respectively. The nebulizer and dry gas flow rates were 45 psi and 12 L/min, respectively.

Isoflavone analysis of *Doenjang*

Isoflavone contents of *Doenjang* were analyzed using Agilent technologies HPLC 1200 Series (Agilent Technologies, Palo Alto, CA, USA) equipped with a Phenomenex kinetex C18 100A (100 mm × 2.1 mm × 2.6 μm, Phenomenex, Torrance, CA, USA) column.

Data processing and multivariate analysis

All MS data were extracted using the MPP software package (Agilent). MS data were also aligned and normalized with the MassHunter Mass Profiler Professional software (v. B. 02. 01). The resulting datasets were then imported into SIMCA-P version 12.0 (Umetrics, Umeå, Sweden), and a mean-centered scaling method was applied for multivariate statistical analysis. At first, principal component analysis (PCA), an unsupervised pattern recognition method, was performed to investigate the intrinsic variation in the dataset. Furthermore, a supervised pattern recognition method and partial least squares-discriminant analysis (PLS-DA) were used to discriminate among each *Doenjang* group. PLS-DA provides a way to remove systematic variation from an input dataset *X* (-compounds or metabolites) not correlated with the

response set Y (discriminant classes). The quality of the models is described by R^2X and Q^2 values. R^2X is defined as the proportion of the variance in the data explained by the models and indicates a good fit, and Q^2 is defined as the proportion of variance in the data predictable by the model, indicating the predictability.

Results

The LC-TOF/MS data were used to investigate the differentiation in the metabolites among each *Doenjang* group. The values for 807 variables in the ESI positive mode mass spectra were used as the scale to unit variance for the multivariate statistical analysis. PLS-DA was performed to visualize the differences in the *Doenjang* samples over the certified traditional *Doenjang* and single-strain inoculation using the PLS-DA model shown in Fig. 1A. Additionally, the validation model is shown in Fig. 1B. PLS-DA score plots showed that the *Doenjang* samples were clearly separated into two parts: (1) dependence on natural fermentation and (2) single-strain inoculation before the fermentation of *Doenjang* with good statistical indication values of R^2X, R^2Y, and Q^2 of 0.25, 0.50, and 0.23, respectively. The movement of the score plots from the left to the right indicates a metabolic change in the single-strain inoculation to the natural fermentation effect. To validate the PLS-DA model, we generated a permutation test with 200 random permutations, and found that most R^2Y and Q^2Y values in the permuted models were lower than the corresponding values in the original model.

At the beginning of comparative interpretations on the variation in *Doenjang* metabolites, PCA analysis was applied to visualize the metabolic discrimination between higher and lower α-SMA expression inhibitions of *Doenjang* (Fig. 2). The PCA model indicated that higher α-SMA expression inhibition for *Doenjang* samples other than no. 1 and 8 was clustered on the left side, with 0.43 and 0.25 predictability for R^2X and Q^2, respectively.

The *Doenjang* samples with higher α-SMA expression inhibiting activity were characterized by higher levels of

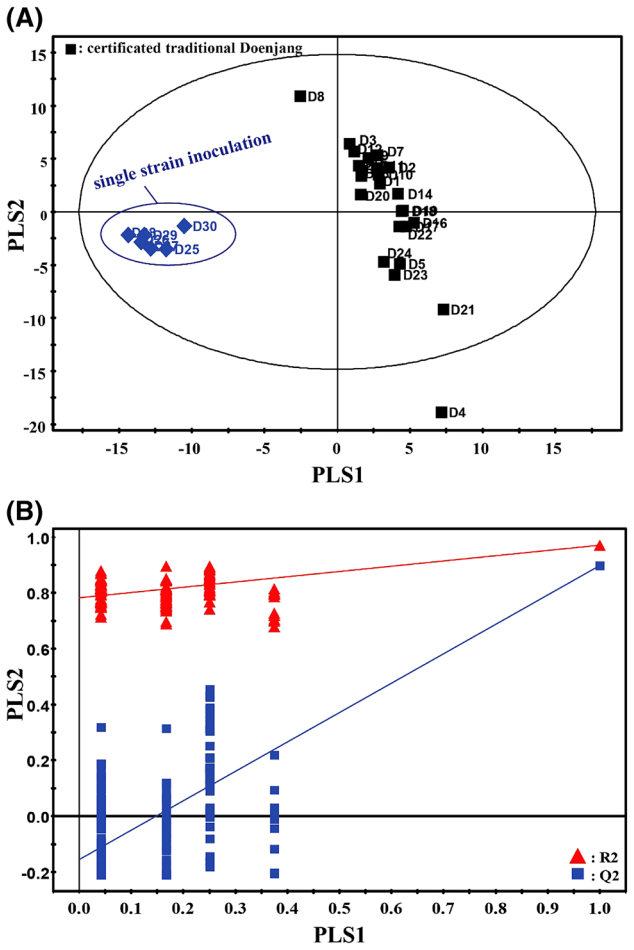

Fig. 1 PLS-DA score plot (**A**) and its validation model (**B**) derived from 807 metabolites on UPLC-Q-TOF/MS of *Doenjang* extracts (R^2X 0.328, R^2Y 0.989, Q^2 0.92). *Symbols* in **A**; *black square*, natural fermentation certificated traditional *Doenjang* and *blue diamond*, *Doenjang* produced with single-strain inoculation

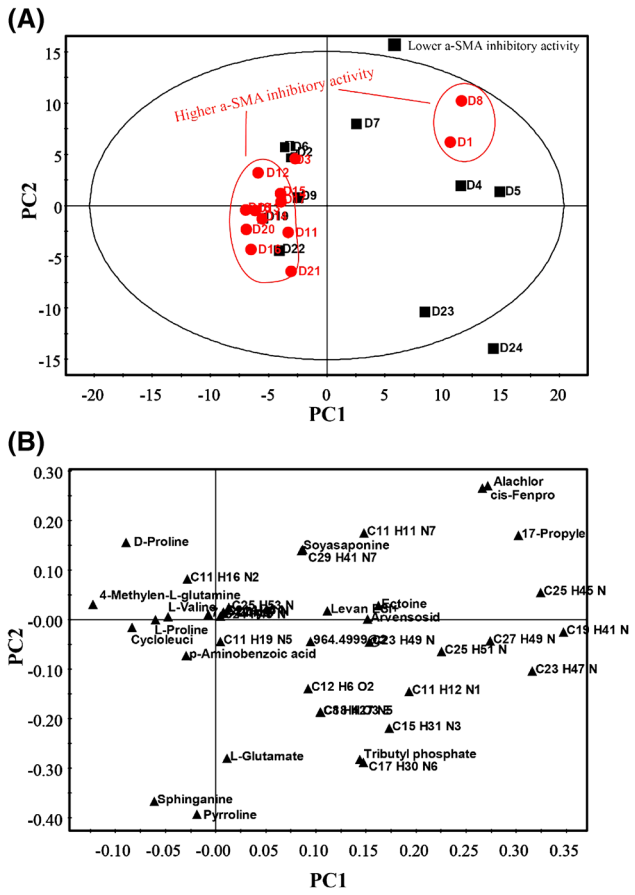

Fig. 2 PCA score plot (**A**) and scatter loading (**B**) plots derived from 43 metabolites on UPLC-Q-TOF–MS of *Doenjang* extracts (R^2X 0.429, Q^2 0.248)

proline, 4-methylene-L-glutamine, valine, cycloleucine, *p*-aminobenzoic acid, and soyasaponin III compared to those with lower inhibition activities.

As shown in Fig. 2A, the PCA model had a weak predictability value ($Q^2 = 0.25$); however, it showed a general tendency, indicating several effective metabolites with α-SMA expression inhibiting activity. Moreover, some quantitative metabolites with significance were also analyzed and shown. The correlations between the major metabolites and the α-SMA inhibition activities of *Doenjang* are listed in Table 1.

To investigate the amino acids and isoflavone compound levels related to α-SMA inhibition activities in the traditional *Doenjang*, PLS-DA analysis was performed as shown in Fig. 3. The PLS-DA score plot showed a clear separation into three clusters with good statistical indication. The R^2X, R^2Y, and Q^2 values were 0.55, 0.69, and 0.55, respectively. Each cluster in the PLS-DA score plot presented the two traditional *Doenjang* groups with lower and higher α-SMA inhibition activities as well as the single-strain inoculation *Doenjang* group in short fermentation periods. Most of the amino acids and isoflavone levels were highly correlated with the fermentation time and α-SMA expression inhibiting activities of the *Doenjang* samples.

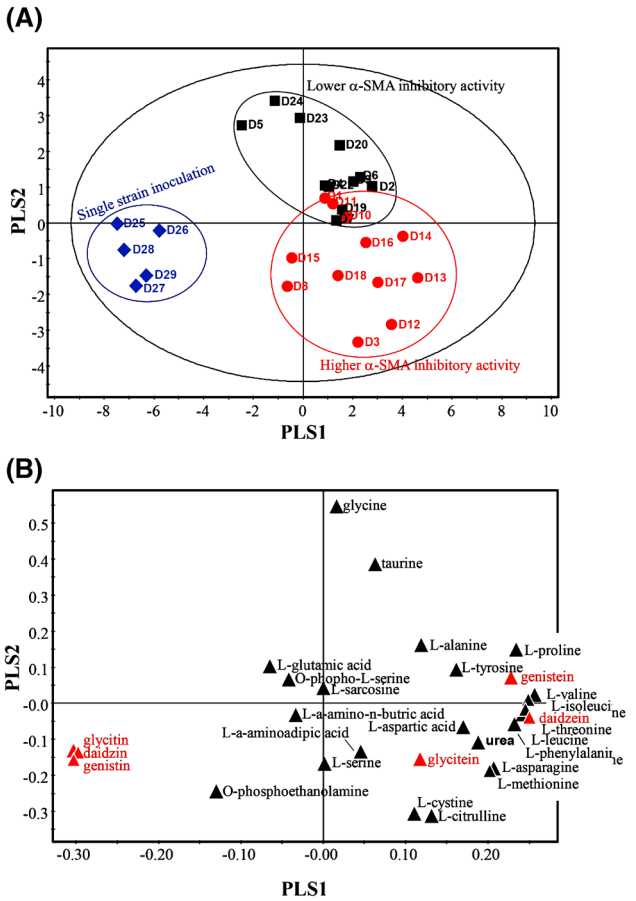

Fig. 3 PLS-DA score (**A**) and scatter loading (**B**) plots derived from amino acids and isoflavone on UPLC-Q-TOF–MS of *Doenjang* extracts (R^2X 0.55, R^2Y 0.688, Q^2 0.552)

Discussion

Effects of α-SMA inhibition activity and doenjang metabolite changes

In our previous report, we evaluated the α-SMA inhibition effect of the certified traditional *Doenjang* (Park et al. 2013). Based on the previous results, the present study provides the metabolite profiles of *Doenjang* based on its effect on α-SMA expression inhibiting activity to find the effective bioactive substances. The *Doenjang* samples with higher α-SMA expression inhibiting activity were characterized by higher levels of proline, 4-methylene-L-glutamine, valine, cycloleucine, *p*-aminobenzoic acid, and soyasaponin III compared to those with lower inhibition activities. Also some quantitative metabolites with significance were also analyzed and shown. The correlations

Table 1 Summary of the correlations between the major metabolites identified and the α-SMA inhibition activities of *Doenjang*

Compounds	Formula	Mass	RT	Higher group	Characteristics
p-Aminobenzoic acid	$C_7H_7NO_2$	137.0478	1.78	Higher α-SMA Inhibitory activity	Synthesis from folate by bacteria Vitamin B complex
L-Valine	$C_5H_{11}NO_2$	117.0790	1.74	Higher α-SMA Inhibitory activity	Essential amino acid
L-Proline	$C_5H_9NO_2$	115.1364	1.82	Higher α-SMA Inhibitory activity	essential amino acid
7,4-Dihydroxyflavone	$C_{15}H_{10}O_4$	254.0518	19.73	Higher α-SMA Inhibitory activity	Flanone
Pyrroline hydroxycarboxylic acid	$C_5H_7NO_3$	129.1140	18.72	Higher α-SMA Inhibitory activity	Metabolites of arginine and proline
Soyasaponin III	$C_{42}H_{68}O_{14}$	796.9809	22.25	Sample no 1, 8	Physicochemical and pharmacological properties

between the major metabolites and the α-SMA inhibition activities of *Doenjang* are listed in Table 1. *p*-aminobenzoic acid is an intermediate in the bacterial synthesis of folate and is also a B vitamin. It is found in foods, mainly in grain, yeast, and meat products. In the human body, it supports folic acid production by intestinal bacteria; therefore, it is likely that the amount of *p*-aminobenzoic acid increases according to the number of microorganisms in *Doenjang*, which contains higher levels of amino acids, indicating that strong enzyme activity was initiated during the fermentation of *Doenjang*. Most amino acids, especially L-valine and L-proline, were abundant in the *Doenjang* samples, which had high α-SMA expression inhibiting activity. It has been reported that the increased free proline levels observed in cirrhotic livers did not induce increased collagen accumulation, and a moderate correlation between free proline and the amount of collagen in cirrhotic human livers has been reported (Forsander et al. 1983). 7,4-dihydroxyflavone was also associated with higher α-SMA inhibition activity in *Doenjang*, consistent with the results on the hepatic protective effects of the phenolic compound 7,8-dihydroxyflavone regarding bromobenzene-induced toxicity (Payá et al. 1993). Pyrroline hydroxycarboxylic acid is an intermediate in arginine and proline metabolism. It is converted into *trans*-4-hydroxy-L-proline via pyrroline-5-carboxylate reductase and to L-erythro-4-hydroxyglutamate via δ-1-pyrroline-5-carboxylate dehydrogenase. Higher levels of pyrroline hydroxycarboxylic acid were detected in the *Doenjang* samples with high proline contents, indicating that proline metabolism was activate during the fermentation. Abundant proline levels in the *Doenjang* samples were correlated with high α-SMA expression inhibition activity. Soyasaponins are triterpenoid glycosides found in soybeans and other legumes. A lot of studies indicate that soyasaponins are bioactive (Gurfinkel and Rao 2003). Although some of the hepatoprotective effects of soyasaponins have been studied (Ikeda et al. 1998; Kinjo et al. 1998), few studies on hepatic fibrosis have been reported. In this study, soyasaponin III was abundant in *Doenjang* samples 1 and 8, which had high α-SMA expression inhibition activity. This indicates that soyasaponin may be a bioactive compound for the treatment of hepatic fibrosis. Further studies are required to understand soyasaponin's hepatoprotective effects.

Amino acids and isoflavone metabolites

To investigate the amino acids and isoflavone compound levels related to α-SMA inhibition activities in the traditional *Doenjang*, PLS-DA analysis was performed in Fig. 3. Most of the amino acids and isoflavone levels were highly correlated with the fermentation time and α-SMA expression inhibiting activities of the *Doenjang* samples.

Genistin, daidzin, and glycitein are the major isoflavones in soybean. Both genistin and daidzin are conjugated to sugar as glycosides in soybeans, and isoflavone glycosides are hard to absorb unless hydrolyzed and converted to their bioactive forms, genistein and daidzein. Both are converted to aglycones by intestinal microflora or in vitro fermentation (Xiao 2008). Most traditional Asian soybean fermentation products contain high levels of isoflavone aglycones, which are more bioavailable and active than the corresponding glycoside form, because of the long-term fermentation. The levels of isoflavone aglycones have been known to increase during fermentation into soybean products. The fermentation time seems to be positively related to a significant increase in the levels of isoflavone aglycone forms together with a decrease in the isoflavone glycoside forms. Therefore, Chungkukjang, a short-term fermented soy product, has significantly higher levels of large molecules than long-term fermented products such as *Doenjang* (Yamabe et al. 2007; Shin et al. 2014).

In this study, the levels of most of the amino acids and isoflavone aglycones were much higher in the traditional *Doenjang*, which had higher α-SMA inhibitory activity, whereas higher levels of isoflavone glycosides, glycitin, daidzin, and genistin, were found in the *Doenjang* samples subjected to short-term fermentation using a single strain. The hydrolysis of soy proteins and the deglycosylation of isoflavone glycosides convert them to amino acids and aglycones through microbial activities during the fermentation process.

Soy protein is reported to be primarily responsible for physiological activity related to the reduction of hepatic lipogenesis rather than soy isoflavone. However, isoflavones regulate hepatic fatty acid oxidation and adipose tissue gene expression (Takahashi and Ide 2008). Moreover, soy isoflavone has been reported to reduce hepatic lipid deposition and increase antioxidant capacity. The mechanism may be related to the inhibition of SREBP-1c and the activation of PPARα expression in the liver (Leng et al. 2011). In this study, higher levels of amino acids and isoflavone aglycone in the traditional *Doenjang* with higher α-SMA expression inhibiting activity indicated that these compounds are strongly related to the physiological activity. In the efforts to investigate *Doenjang* amino acids related to liver function, valine, isoleucine, and leucine, the branched-chain amino acids (BCAAs) were found to possess significantly higher activity in the *Doenjang* group, which showed higher α-SMA inhibitory activity. It is interesting to note that other studies have reported the benefits of BCAAs on overall prognosis in patients with liver cirrhosis (Kawaguchi et al. 2013). These studies indicated that BCAA administration stimulates hepatic protein synthesis in patients with chronic liver disease and

could contribute significantly to improving their nutritional status, resulting in an improvement in liver function (Khanna and Gopalan 2007; Soomro et al. 2008). Therefore, in this study, higher levels of isoflavone aglycones and BCAAs were found to be beneficial for the treatment of hepatic fibrosis.

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