Metabolite Profiling and Bioactivity of Rice Koji Fermented by Aspergillus Strains

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In this study, the metabolite profiles of three Aspergillus strains during rice koji fermentation were compared. In the partial least squares discriminant analysis-based gas chromatography–mass spectrometry data sets, the metabolite patterns of A. oryzae (KCCM 60345) were clearly distinguished from A. kawachii (KCCM 60552) and only marginal differences were observed for A. oryzae (KCCM 60551) fermentation. In the 2 days fermentation samples, the overall metabolite levels of A. oryzae (KCCM 60345) were similar to the A. oryzae (KCCM 60551) levels and lower than the A. kawachii (KCCM 60552) levels. In addition, we identified discriminators that were mainly contributing tyrosinase inhibition (kojic acid) and antioxidant activities (pyranonigrin A) in A. oryzae (KCCM 60345) and A. kawachii (KCCM 60552) inoculated rice koji, respectively. In this study, we demonstrated that the optimal inoculant Aspergillus strains and fermentation time for functional rice koji could be determined through a metabolomics approach with bioactivity correlations.

Keywords: Rice koji, metabolite profiling, Aspergillus, gas chromatography–mass spectrometry, tyrosinase inhibition activity, antioxidant activity

The production of traditional Korean beverages involves two different steps: (i) glycolysis by filamentous fungi, and (ii) alcohol production by yeast fermentation. The first step plays an important role in the second alcohol fermentation step, since saccharification of cereals provides the yeast with a sufficient amount of sugar [28]. Koji is often used in the first step of the fermentation process during the production of various fermented foods such as shochu, and miso. In general, Aspergillus kawachii and Aspergillus oryzae are mainly used for koji preparation owing to their high antioxidant, tyrosinase inhibition, and glycolysis activities [2, 17, 18, 28]. In particular, A. oryzae was reported to be an ideal microorganism for koji fermentation because it is responsible for the synthesis of secretory hydrolytic enzymes, for amino acid metabolism, and also acts as a sugar uptake transporter [2, 17, 18]. In addition, its representative secondary metabolite is kojic acid, which has been used as a cosmetic and food additive for skin-lighting and preventing enzymatic browning [2, 6].

A. oryzae contains several different types of enzymes such as α-amylase, aminopeptidase, amyloglucosidase, lactase, and lipase [2, 28]. These enzymatic activities can be changed based on rice composition; therefore, in this study, we examined differences in metabolite profiles including fungal metabolites using gas chromatography–mass spectrometry (GC–MS). GC–MS is a comprehensive and non-biased technological system that can analyze various primary metabolites related to sugars, amino acids, and fatty acids, and it has been shown to reliably quantify primary metabolites from sample extracts [5, 7, 9, 15, 22, 29]. Principal component analysis (PCA) and supervised approaches such as partial least squares (PLS) are simple methods that have been widely used to analyze GC–MS data sets collected from various fungi-treated rice koji samples [10, 13, 14, 23, 27].

In this study, since A. oryzae (KCCM 60345) has been widely used to ferment rice koji, we compared the compositional changes between A. kawachii (KCCM 60552) and A. oryzae (KCCM 60551) during fermentation and found that these changes were dependent on the fermentation time and there was a correlation between metabolites and bioactivities.
**Materials and Methods**

**Chemicals and Reagents**
Trolox, kojic acid, mushroom tyrosinase, l-tyrosine, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), Folin-Ciocalteu’s phenol reagent, and 2,4,6-tripryidyl-s-triazine (TPTZ) were purchased from Sigma-Aldrich (St. Louis, MO). Pyranonigrin A was from Enzo Life Sciences International (Butler Pike, USA). Methanol was procured from Duksan Chemical (Ansan, Korea). For GC–MS analysis, pyridine, methoxyamine hydrochloride, and N,N-(trimethylsilyl) trifluoroacetamide (MSTFA) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

**Fermentation Conditions of Rice Koji Production**
A. kawachii (KCCM 60552) and A. oryzae (KCCM 60551) were purchased from the Fermented Food Company (Suwon, Korea). A. oryzae (KCCM 60345) was purchased from the Korean Culture Center of Microorganism (KCCM) (Seoul, Korea). Raw rice samples (Icheon, Korea) were washed and soaked for 12 h and then drained in water for 2 h. The samples were then steamed for 1 h and the temperature was reduced to 30°C. Each fungal spore (0.03%) was inoculated separately and fermented at 30°C and 85% humidity for 14 days. Fungi-treated rice samples were collected at 2-day intervals. The samples were freeze-dried for 48 h and stored at ~80°C until further use.

**Sample Preparation**
Freeze-dried rice koji samples were finely ground in a mortar, and 1 g of powder was extracted with 5 ml of 80% methanol. After 24 h, the sample mixture was centrifuged at 45,000 rpm for 5 min. Twenty µl of the supernatant was transferred to a 1.5 ml Eppendorf tube and the extract solution was concentrated using a speed vacuum machine (Incheon, Korea).

The extracted sample was derivatized before GC–MS analysis with 75 µl of methoxyamine hydrochloride in pyridine (20 mg/ml). The mixture was incubated at 65°C for 30 min. As a second derivatizing agent, 75 µl of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) was added to the mixture and incubated at 65°C for 60 min [11, 13, 14].

**Conditions of GC–MS Analysis**
Analysis was performed on a Varian 4000 GC–MS/MS system (Varian, Palo Alto, CA, USA) coupled with a CP-8400 injector. The system was equipped with a FactorFour capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness; Varian). The injector temperature was 250°C and the transfer line and ion source temperatures were 230°C and 200°C, respectively. The column was isothermally held at 100°C for 2 min, then raised to 300°C at 10°C/min, and the final temperature was maintained at 300°C for 10 min. One µl of the sample mixture was injected in the split mode [25:1 (v/v)] and the helium gas flow rate through the column was 1 ml/min. Ions were generated by a 70 kV electron impact (EI), and were recorded over the mass range of 50–1,000 m/z [11, 13, 14].

**Data Processing**
Data preprocessing was performed using Varian MS Workstation 6.9 software (Varian, Palo Alto, CA, USA). The GC–MS raw data files were converted to netCDF (*cdf) format with the Vx Capture (version 2.1; Adron Systems, Laporte, MN, USA) for further analysis. After conversion, automatic peak detection and alignment were performed using XCMS. The XCMS parameters were determined using R-program version 2.9.0 (R project for statistical computing, http://www.r-project.org, with http://massspec.scripps.edu/xcms/documentation.pup). The corresponding peaks were confirmed in the original chromatogram and were compared with the NIST mass spectral database (National Institute of Standards and Technology, FairCom, Gaithersburg, USA) [11, 13, 14].

**Statistical and Multivariate Analysis**
Statistical analyses were performed on all variables by SIMCA-P+ (version 12.0; Umetrics, Umeå, Sweden). Significance test and visualize date were described by STATISTICA (version 7.0; StatSoft Inc., Tulsa, OK, USA). Partial least squares discriminant analysis (PLS-DA) was done to determine the differences in the metabolite composition of the samples. The peak intensity of all variables was log10 transformed. All variables were scaled to unit variance for PLS-DA derived from the GC–MS data sets [9, 11, 13, 14].

**Tyrosinase Inhibitory Activity**
The mushroom tyrosinase inhibitory activity was determined as described previously with some modifications [12, 16]. The reaction mixture consisted of 153 µl of 0.1 M sodium phosphate buffer (pH 6.5), 5 µl of sample, 5 µl of mushroom tyrosinase (2,500 unit/ml), and 36 µl of 1.5 mM l-tyrosine, all of which were added to a 96-well plate. After the reaction mixture was incubated at 37°C for 20 min, the absorbance was measured at 490 nm using a microplate reader (Seoul, Korea). Kojic acid (IC50, half-maximal inhibitory concentration) was used as a positive control.

**DPPH Radical Scavenging Assay**
A 200 µM DPPH solution was made in ethanol. The DPPH ethanol solution was heated and dissolved for 30 min until the absorbance reached 1.0 at 515 nm. The samples (20 µl) and 180 µl of DPPH ethanol solution were incubated at room temperature for 20 min in 96-well microplates. The absorbance of the DPPH free radical was measured using a microplate reader. Results were expressed in millimoles of trolox equivalent concentration per gram of rice koji. The concentration of the standard solutions ranged from 0.156 to 2.5 mM. Experiments were carried out in triplicate [13, 14].

**Troxol Equivalent Antioxidant Capacity (TEAC)**
A 7 mM ABTS ammonium solution was prepared in water and treated with 2.45 mM potassium persulfate. The mixture was allowed to stand at room temperature for 12–16 h until it turned dark blue. The solution was then diluted until the absorbance reached 0.7 at 750 nm. Then 180 µl of the ABTS solution was mixed with 20 µl of the
sample solution in a 96-well microplate and incubated at room temperature for 6 min. Results were expressed in millimole of trolox equivalent concentration per gram of rice koji. The concentration of the standard solutions ranged from 0.25 to 2 mM. Experiments were carried out in triplicate [13, 16, 25].

Ferric Reducing/Antioxidant Power (FRAP) Assay
The FRAP reagent contained 2.5 ml of a 10 mmol/l TPTZ solution in 40 mmol/l HCl, 2.5 ml of 20 mmol/l FeCl₃·6H₂O, and 25 ml of 0.3 mol/l acetate buffer at pH 3.6. Three hundred µl of the FRAP reagent was mixed with 10 µl of sample and the absorbance was measured at 570 nm after 6 min using a microplate reader. Results were expressed in millimole of Trolox equivalent concentration per gram of rice koji. The concentration of the standard solutions ranged from 0.25 to 2 mM. Experiments were carried out in triplicate [3, 8, 14].

RESULTS
Multivariate Analysis of Rice Koji Fermentation by Different Aspergillus Strains
Rice koji fermented with different fungal species was collected at 2-day intervals and analyzed in triplicate by GC–MS. The data sets were analyzed using the PLS-DA model. The PLS-DA score plots were derived from the GC–MS data sets and the plots were separated into PLS1 (59.5%) and PLS2 (19.4%) (Fig. 1A). Fermentation patterns were clearly differentiated based on the fungal species used for fermentation. According to PLS2, the score plots of AK were largely separated from the A. oryzae species (Fig. 1A). Different metabolite profiles from the two different strains in the same species were also observed (Fig. 1B).

Metabolite Changes During Rice Koji Fermentation
The PLS-DA model explained the significant differences in the metabolite profile between 0 day and 2 days for all rice koji (Fig. 1). To identify the metabolite changes that occurred between 0 day and 2 days of fermentation, we compared the rice koji treated with all the fungi (Fig. 2). All of significantly different metabolites (p< 0.005) were increased during 2 days. Changes in the sugars and sugar alcohols (glycerol, arabitol, glucose, galactose, dulcitol, glucopyranose, fructose, and hexadecanoic acid), and organic acid (citric acid and oxaloacetic acid) were commonly observed in two or three of the rice koji samples. Among the specific metabolites of fungi, only pyranonigrin A was significantly changed in Aspergillus kawachii KCCM 60552 (AK552), whereas galactofuranoside, tyrosine, and phenylalanine were increased when Aspergillus oryzae
KCCM 60551 (AO551) was used. In the case of Aspergillus oryzae KCCM 60345 (AO345), turanose and kojic acid were increased during fermentation \( (p<0.005) \) (Table 1). To determine the optimal fermentation time, strain-specific metabolites were compared as a function of fermentation time. The metabolites sharply increased up to 2 days and no significant increases were observed at longer fermentation time (Fig. 3). Based on this result, 2 days was selected as the fermentation time to compare the different fungal characteristics (Fig. 4). As shown in Fig. 4B, a higher content of sugar alcohols (glycerol, dulcitol, and myo-inositol), and organic and fatty acids (pyruvic acid, citric acid, and hexadecanoic acid) was observed when AK552 was used. In addition, sugar levels (galactose, fructose, glucopyranose, and turanose) were also higher for AK552, except glucose. Kojic acid and pyranonigrin A were shown to be by-products of rice koji fermentation for AO345 and AK552, respectively.

**Antioxidant Activities and Tyrosinase Inhibition Activity of Rice Koji**

AK552 showed much higher antioxidant activities including DPPH, TEAE, and FRAP than AO551 and AO345. For AK552, the activity gradually increased with fermentation time up to 4 days. The three different antioxidant activities were correlated with one another. Pyranonigrin A, which is a well-known antioxidant, was most likely the source of antioxidant activity for AK552 (Table S2, Fig. 5A); in addition, the low antioxidant activity of AO345 and AO551 was most likely observed because these did not produce pyranonigrin A. AO345 had a higher tyrosinase inhibition activity than AK552 and AO551. In addition, the quantity of kojic acid was highly correlated with the tyrosinase inhibition activity, which increased in the AO345 sample with fermentation time (Fig. 5B). AK552 and AO551, which did not produce kojic acid, showed low levels of tyrosinase inhibition activity.

**DISCUSSION**

Fungi play an industrially important role in the fermentation process. Their specific metabolites, bioactivities, and enzymatic activities dictate food quality and add additional value \([4, 18, 20, 24, 28]\). In addition, rice koji is not only used in Korean traditional wine but also as a natural additive for cosmetics. Therefore, the types of fungal
inoculants and their bioactivity levels are the most important factors in preparing rice koji.

This study revealed that the main discriminators to characterize fungal inoculants were sugars and sugar alcohols. These compounds contribute to mouth-feel and sweet flavor; therefore, they are important to rice wine from a taste perspective [19].

In a previous study, we demonstrated that AO551 was an optimal inoculant for rice koji preparation in regard to glucose production because of its high glucoamylase level [11]. This study also confirmed that AO551 and AO345 could result in a higher production of glucose than AK552 (Fig. 4). In addition, we demonstrated that AO345 has a higher tyrosinase inhibition activity (Fig. 5). Through
During fermentation (Table S3), the antioxidant activity of AO345 and AO551 increased slightly enhanced antioxidant activity of AK552. In addition, the pyranonigrin A was the major factor contributing to the tyrosinase inhibition activity. After 2 days of fermentation, kojic acid, which acts as an antioxidant, was increased during fermentation. Several microbial metabolites in rice also have antioxidant levels of AO345 and AO551 increased slightly increased during fermentation. Polyglycals, pyruvic acid, turanose, and glycerols, except sugar compounds were more significantly changed than other compounds in all the rice koji samples. This indicates that the ultimate goal of koji fermentation and glycolysis for second fermentation as alcohol production were processed properly (Table 1). Therefore, we concluded that 2 days was an optimal time for rice koji fermentation. In addition, our results indicate that AO345 would be optimal for producing functional rice koji, since this fungus results in the production of non-aflatoxin and kojic acid, which has a skin-whitening effect [21, 26].

**Fig. 5.** Correlation with metabolites and bioactivities. (A) Correlation with FRAP activity and quantity of pyranonigrin A production as a function of fermentation time. Polygonal solid line graph: Quantitation of Pyranonigrin A in AK552; bar graph: FRAP activity of AO345 (white), AO551 (gray), and AK552 (black). The black bar graph corresponds to the solid line graph. (B) Correlation with tyrosinase inhibition activity and quantity of kojic acid production as a function of fermentation time. Polygonal dot line graph: Quantitation of kojic acid in AO345; bar graph: tyrosinase inhibition activity of AO345 (white), AO551 (gray), and AK552 (black). The white bar graph corresponds to the dot line graph.

Quantitative analysis, we concluded that kojic acid was the major factor contributing to the tyrosinase inhibition activity. Not all the strains produced kojic acid, even though they belong to the same species (Table 1). The production of kojic acid seemed to be dependent on microbial enzymatic activity related to dehydration and oxidation [1, 2]. AO551 and AO345 showed a similar fermentation pattern as AK552. The levels of most sugar-related metabolites such as glucose, pyruvic acid, turanose, and glycerols, except kojic acid, were increased during fermentation.

AK552, generally used in rice koji, produced pyranonigrin A, which acts as an antioxidant, after 2 days of fermentation. However, several microbial metabolites in rice also have antioxidant activity, so we could not conclude that pyranonigrin A was the major factor contributing to the enhanced antioxidant activity of AK552. In addition, the antioxidant levels of AO345 and AO551 increased slightly during fermentation (Table S3). The antioxidant activity of fermented food and their koji have already been reported, including rice koji with Aspergillus kawachii and Aspergillus oryzae. However, pyranonigrin A was detected only in Aspergillus kawachii fermentation.

As summarized in this research, the metabolomics approach is a useful tool to optimize fermentation time and fungal inoculants. As described in Fig. 1 and 4, the process of rice koji fermentation was nearly terminated after 2 days. Furthermore, the bioactivity levels including antioxidant and tyrosinase inhibition activity dramatically increased from 0 to 4 days and did not increase any further at longer fermentation times. During fermentation, we recognized that sugar compounds were more significantly changed than other compounds in all the rice koji samples. This indicates that the ultimate goal of koji fermentation and glycolysis for second fermentation as alcohol production were processed properly (Table 1). Therefore, we concluded that 2 days was an optimal time for rice koji fermentation. In addition, our results indicate that AO345 would be optimal for producing functional rice koji, since this fungus results in the production of non-aflatoxin and kojic acid, which has a skin-whitening effect [21, 26].

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**References**


