Metabolomics-Based Optimal Koji Fermentation for Tyrosinase Inhibition Supplemented with Astragalus Radix

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The present study was focused on improving the quality of rice koji by fermentation with a selected Aspergillus oryzae strain and a plant Astragalus radix. A. oryzae KCCM 60345 was used as main inoculant and the Astragalus radix was added as supplement in rice koji preparation. LC-MS based metabolite analysis and tyrosinase inhibitory activities were studied for different time periods. A. oryzae KCCM 60345 fermented rice koji supplemented with Astragalus showed higher tyrosinase inhibition activity at 4 d of fermentation and metabolite analysis with PCA and PLS-DA indicated differences in inhibition activity at 4 d of fermentation and metabolite analysis with PCA and PLS-DA indicated differences in

Key words: koji; fermentation; Astragalus radix; metabolomics

The traditional method of food fermentation is good for health, and repeated use of the same culture adapts it to the fermentation process without any harmful effects, but value-added products are attracting more attention from the manufacturing and consumers. Koji is an early stage in the fermentation in the process of traditional wine preparation. Molds have been used from olden days. Recently, medicinal plants or mushrooms are used to improve the functionality of traditional rice wine. Therefore, in this study, we have selected A. oryzae strain and Astragalus radix for rice fermentation to improve tyrosinase inhibition activity. The fermentation conditions were optimized, and the metabolites were analyzed by liquid chromatography-mass spectrometry (LC-MS) by the metabolomics approach. In addition, a correlation between tyrosinase inhibitory activity and the metabolites of rice koji was determined. This approach should help in monitoring the compositional changes in rice koji with Astragalus radix and A. oryzae in different fermentation processes as well as different fermentation periods.

Materials and Methods

Chemicals and reagents. Kojic acid, mushroom tyrosinase, L-tyrosine, and formic acid were purchased from Sigma-Aldrich (St. Louis, MO). Methanol as extraction solvent was from Duksan Chemical (Ansan, Korea). Acetonitrile and water as LC-MS solvent were from Fisher Scientific (Pittsburgh, PA).

Fermentation conditions for rice koji supplemented with Astragalus radix. A. oryzae KCCM 60345, a kojiic acid-producing strain, was purchased from the Korean Culture Center of Microorganisms, (KCCM) (Seoul, Korea). A. oryzae KCCM 60551, a kojiic acid less-producing strain, was purchased from the Fermented Food Company (Suwon, Korea) and Astragalus radix (100 g) (Jecheon, Korea) was freeze-dried and homogenized, and then mixed with 1 kg of raw rice (Jecheon, Korea). It was washed and soaked in water for 12h and then drained for 2h. The samples were steamed for 1h, and the temperature was reduced to 30°C. These processes were repeated 3 times. The rice koji fermented with A. oryzae KCCM 60345 and supplemented with Astragalus radix was labeled RK_345 and RKA_345, respectively.

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Similarly, the rice koji with A. oryzae KCCM 60551 and Astragalus radix supplement was labeled RK_551 and RKA_551, respectively. Fungal spores (0.03%) were inoculated separately and fermented at 30°C at 85% humidity for 14 d. Fungi-treated rice samples were collected at 2-d intervals. The samples were freeze-dried for 48 h and stored at −80°C until used.

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 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 modulspin speed-vacuum machine (Hanil, Incheon, Korea). The extracted sample was dissolved in 80% methanol then filtered through a 0.2-μm PTFE filter for LC-MS analysis.

Tyrosinase inhibitory activity. Mushroom tyrosinase inhibitory activity was described previously, with some modifications.6,11 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 placed in a 96-well plate. The reaction mixture was incubated at 37°C for 20 min, and then the absorbance was measured at 490 nm using a microplate reader (Biotek, Seoul, Korea). Kojic acid (IC50, half-maximal inhibitory concentration) was used as positive control.

HPLC analysis. In rice koji fermentation with A. oryzae KCCM 60345 and Astragalus radix, bioactive compounds, with tyrosinase inhibitory activity were purified by Preparative High Performance Liquid Chromatography (Prep HPLC) (Hitachi, Tokyo) with diode array detector L-2455, and pump L-2130. Separation was performed on YMC-Pack pro C18 (250×4.6 mm i.d., 5 μm). The mobile phase consisted of acetonitrile (A) and 5% acetonitrile in water (B) by gradient elution of 5% B at 0 min, 0–95% B at 0–50 min, 95–100% B at 50.1–55 min, 100–5% B at 55.1–55.1 min, and 5% B at 55.1–60 min. The flow rate was 1 mL/min and the injection concentration was 20 μg/mL.

UPLC/Q-TOF MS analysis. To identify the differences in secondary metabolites of the rice koji extracts, the UPLC/Q-TOF system, Ultra Performance Liquid Chromatography (UPLC) connected to a Micromass Q-TOF ultima API Time-of-Flight mass spectrometer (Waters, Milford, MA) was used with an autosampler at 4°C. The ESI source was operated in positive ionization mode with the capillary voltage at 1.2 kV. All data were processed with Masslynx V4.1 software (Waters, Milford, MA). Separation was carried out on an acuity UPLC BEH C18 column (100×2.1 mm i.d., 1.7 μm) at a column oven temperature of 25°C. The mobile phase consisted of acetonitrile (A) and water containing 0.1% formic acid (B) by gradient elution of 10% B at 0–1 min, 10–40% B at 1–5 min, 40–70% B at 5–11 min, 70–90% B at 11–12 min, 90–100% B at 12–14 min, and 10% B at 14–16 min. The flow rate was 0.3 mL/min, and the injection volume was 5 μL. Leucine-enkephalin in 50% acetonitrile (0.2 ng/mL) was used as the lock mass (m/z 556.2273), and sodium formate was used for calibration.

LC/ion trap ESI MS analysis. To obtain more information on variables from multivariate analysis, we used a 212-LC Binary Solvent Delivery System, a Meta Therm HPLC Column Heater, a Prostar 410 Autosampler, a Prostar 335 photodiode array detector, and a 500-ion trap mass spectrometer from Varian Technologies (Palo Alto, CA). Separation was performed on a PurSuit XR3 C18 (100×2.1 mm i.d., 3 μm) at a column oven temperature of 55°C. The mobile phase consisted of acetonitrile (A) and water containing 0.1% formic acid (B) by gradient elution of 5% B at 0 min, 5% B at 0–5 min, 5–100% B at 5–40 min, 100% B at 40–40.5 min, 100–5% B at 42.5–42.51 min, and 5% B at 42.51–45 min. The flow rate was 0.2 mL/min and the injection volume was 5 μL. A photo diode array was used to record the absorbance from 200 to 600 nm, and the UV spectrum was set at 220 nm. Mass spectra were simultaneously acquired using electrospray ionization in positive ionization modes at 70 V over a range of m/z 50–1,000. A drying gas pressure and a temperature of 20 psi and 350°C, a nebulizer pressure of 40 psi, and a capillary voltage of 70 V were imposed. MSn analysis was done with Turbo DDS, data-dependent scanning for a 500-MS system.12

Data processing. Data preprocessing was done using Masslynx V4.1 software (Waters, Milford, MA). The LC-MS raw data files were converted to netCDF (’.cd2’) format with the Databridge (Waters Corp., MA) for further analysis. After conversion, automatic peak detection and alignment were done using XCMS. The XCMS parameters were determined using R-program version 2.9.0 (The R project for statistical computing, www.r-project.org, with http://massspec.scripps.edu/xcms/documentation.ppl). The corresponding peaks were identified using the Accurate Mass Report Generator, which indicated ppm and i-fit value.12,13

Statistical and multivariate analysis. Statistical analyses were done on all variables by SIMCA-P+ (version 12.0, Umetrics, Umeå, Sweden). A significance test and multiple regression models, and visualized data were described by STATISTICA (version 7.0, StatSoft, Tulsa, OK). Principal component analysis (PCA) was done to obtain a general overview of the metabolites’ variance, and partial least squares discriminant analysis (PLS-DA) was done to determine the differences in the metabolite compositions of the samples. The peak intensities of all variables were log10 transformed. All variables were scaled to unit variance for PCA and PLS-DA derived from the LC-MS data sets.12,14 A heat map was constructed by means of R-program version 2.9.0. The variables included significantly changed metabolites and bioactivities according to fermentation time. Each square indicated r (Pearson’s correlation coefficient of a pair of metabolite, and tyrosinase inhibitory activity).14,15

Results
Effect of Astragalus radix on the tyrosinase inhibition activity of rice koji
According to the tyrosinase inhibition test, rice koji fermented with A. oryzae KCCM 60345 had a higher level of tyrosinase inhibitory activity than A. oryzae KCCM 60551. Moreover, A. oryzae KCCM 60345 showed the highest level of tyrosinase inhibition activity with added Astragalus radix (Fig. 1).

PLS-DA analysis of the metabolites of rice koji supplemented with Astragalus radix and treated with two different A. oryzae strains showed different fermentation patterns, except for 0 d (Fig. 2A). Loading plots and p-values revealed that kojic acid, calycosin-7-O-β-D-glucoside, ononin, formononetin, and 3-hydroxy-9,10-dimethoxypterocaran were responsible for recognizing RKA_345, whereas 2′,3′-7,3′,7,3′,7-trihydroxy-4′-methoxyisoflavan, calycosin-7-O-β-p-glucoside-6′O-malate, kojisatin A, 9,12,13-trihydroxy-10-octadecanoic acid, and 1-palmitoylglycerophosphocholine were more recognizing metabolites for RKA_551 (Fig. 2B).

To determine the effects of Astragalus radix in rice koji, the metabolites of RK_345 and RKA_345 were compared. On PCA analysis, RK_345 and RKA_345 were clearly separated by PC 2 (18.5%) (Fig. 3A). The production of major loading plots and p-values revealed that calycosin-7-O-β-p-glucoside, calycosin-7-O-β-D-glucoside-6′O-malate, ononin, formononetin-7-O-β-D-glucoside-6′O-malate, formononetin, 3-hydroxy-9,10-dimethoxypterocaran, and 2′,7-dihydroxy-3′,4′-dime-thoxyisoflavan were responsible for discriminating RKA_345. On the other hand, 1-linoleoylglycerophosphocholine, 1-palmitoylglycerophosphocholine, and 1-oleoylglycerophosphocholine were observed in RK_551 (Fig. 3B).
Time-dependent metabolite variation

The production of major tyrosinase inhibitory compounds kojic acid and calycosin was studied with respect to fermentation time. The relative quantity of kojic acid gradually increased up to 4 d and held steady until 14 d of fermentation (Fig. 4). Similarly, the amount of calycosin increased initially, but the concentration was lower after 4 d of fermentation.
Fig. 3. Metabolite Differences in Rice Koji Fermented with *Aspergillus oryzae* KCCM 60345 and Supplemented with Astragalus Radix. A. Score plot; and B, loading plot of PCA obtained from UPLC-Q-TOF data. (△) RK_345 (rice koji with *A. oryzae* KCCM 60345); (○) RKA_345 (rice koji fermented with *A. oryzae* KCCM 60345 + Astragalus radix). C. Total ion chromatogram for 2 d of rice koji fermentation. RK_345 (upper chromatogram), RKA_345 (bottom chromatogram). *p*-values: calycosin-7-O-β-D-glucoside (0.000), calycosin-7-O-β-D-glucoside-6'-O-malate (0.000), ononin (0.000), formononetin-7-O-β-D-glucoside-6'-O-malate (0.000), formononetin (0.000), 3-hydroxy-9,10-dimethoxypterocarpan (0.000), 7,2'-dihydroxy-3',4'-dimethoxyisoflavan (0.000), 1-inooleylglycerophosphocholine (0.000), 1-palmitoylglycerophosphocholine (0.001), 1-oleoylglycerophosphocholine (0.000).

Fig. 4. Box and Whisker Plots Showing Relative Metabolite Changes in Kojic Acid (A) and Calycosin (B) during RKA_345 Fermentation. The X axis indicates fermentation time and the Y axis indicates the peak area (log10 scale) (line, mean; box, standard error; whisker, standard deviation). (C) Chemical structures of kojic acid and calycosin.
min. Retention time; Meas. mass, measured mass; Cal. mass, calculated for the mass; Δ max, absorbance maxima in the UV range; Mol Form, molecular formula; error (ppm), deviation between averages of observed accurate mass and true accurate mass, in ppm; i-FIT, isotope fitting pattern; Ref., references; Putative ID, putative identification of metabolite; Metabolite ID, internal metabolite ID; discriminator R, metabolites from rice koji fermented with Aspergillus oryzae; A, metabolites from rice koji fermented with Astragalus radix.

### Table 1.
Metabolites That Have Previously Been Reported in Literature, Identified by UPLC-Q-TOF-MS and LC-ESI-Ion Trap-MS (Positive Ionization Mode) in RKA345

<table>
<thead>
<tr>
<th>Metabolite ID</th>
<th>Putative ID</th>
<th>UPLC-Q-TOF-MS</th>
<th>LC-ESI-Ion trap MS</th>
</tr>
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<tr>
<td>R1</td>
<td>kojic acid</td>
<td>C6H4O2</td>
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<tr>
<td>R2</td>
<td>kojistatin A</td>
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<td>7,2,3-trihydroxy-4′-methoxyisoflavan</td>
<td>C9H14O5</td>
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<td>calycosin-7-O-β-d-glucoside</td>
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</tr>
<tr>
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<td>calycosin-7-O-β-d-glucoside-6′-O-malate</td>
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<td>4.3</td>
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<tr>
<td>A4</td>
<td></td>
<td>C10H12O6</td>
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</tr>
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<td>A6</td>
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<td>C23H26O12</td>
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</tr>
<tr>
<td>R3</td>
<td>9,12,13-trihydroxy-10-octadecanoic acid</td>
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<td>A7</td>
<td>Formononetin</td>
<td>C14H10O4</td>
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<tr>
<td>A8</td>
<td>3-hydroxy-9,10-dimethoxythoxyarcarpan</td>
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<td>R7</td>
<td>1-oleoylglycerophosphocholine</td>
<td>C24H31NO5</td>
<td>11.9</td>
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### Correlation of metabolites and tyrosinase inhibition activity
In RKA345, 16 secondary metabolites were identified by UPLC-Q-TOF-MS and LC-ESI-ion trap-MS (Table 1). To identify the major metabolites contributing to tyrosinase inhibition activity, we used a heat map and a multiple regression model (MLR) (Fig. 5, Tables 2 and 3). Statistically, these compounds contributed to the increase in tyrosinase inhibition activity (Table 3). In addition, we confirmed that kojic acid and calycosin were the major metabolites and that they possessed tyrosinase inhibitory activity. The IC₅₀ values were 50.1 μM and 30.8 μM, respectively.

### Discussion
To improve the quality of the rice koji used in a variety of products such as traditional Korean wine, biofuel, and natural additives for cosmetics, two strains of A. oryzae with Astragalus powders were compared to those without the additive as to their production of secondary metabolites and biological activities. Due to high kojic acid production, A. oryzae KCCM 60345 has a higher level of tyrosinase inhibitory activity than A. oryzae KCCM 60551, which is used widely in koji fermentation. In addition, Astragalus radix, which has been reported to contain calycosin (3′,7-dihydroxy-4′-methoxyflavone), another tyrosinase inhibitor, had a synergistic effect with A. oryzae KCCM 60345 in improving the productivity of kojic acid.

In the case of kojic acid, A. oryzae KCCM 60345 produced the highest amount (0.8 mg/mL of MEA media) by comparison with the other 22 Aspergillus species we had. In addition, 10–15 g/g of Astragalus radix produced the highest amount (0.8 mg/mL of MEA media) by comparison with the other 22 Aspergillus species we had. In addition, Astragalus radix, which has been reported to contain calycosin (3′,7-dihydroxy-4′-methoxyflavone), another tyrosinase inhibitor, had a synergistic effect with A. oryzae KCCM 60345 in improving the productivity of kojic acid. In a previous study, calycosin was isolated from Astragalus radix (3.6 mg kg⁻¹), which was the most active compound. However, in the present work, calycosin was not detected in the rice koji. In a previous study, calycosin was isolated from Astragalus radix (3.6 mg kg⁻¹), which was the most active compound. However, in the present work, calycosin was not detected in the rice koji.

In addition, tyrosinase inhibitory activity was more improved in RKA345 than in RKA551, and the reason appears to be strong enzymatic activity related the conversion of calycosin derivatives. However, the present work failed to confirm the conversion of calycosin-7-O-β-d-glucoside and calycosin-glucoside-
6'-O-malate to calycosin by *A. oryzae* during fermentation. A decreased level of calycosin after 4 d of fermentation might be due to microbial enzyme activity or resistance to heat, oxygen, and moderate degrees of acidity. Even though the amount of calycosin was lower after 4 d of fermentation, the tyrosinase inhibitory activity in the rice koji (RKA_{345}) was maintained due to increased levels of kojic acid.

*A. oryzae* KCCM 60345 produced more of kojic acid with added Astragalus radix due to the growth of *A. oryzae* KCCM 60345. Some studies have reported that solid-state fermentation such as that of koji can lead to increased levels of kojic acid. In this study, kojic acid of *A. oryzae* KCCM 60345 made major contribution to bioactivity in rice koji. We conclude that the Astragalus radix supplement and inoculants *A. oryzae* KCCM 60345 improved the tyrosinase inhibition property of rice koji, due to their synergistic effects with kojic acid and calycosin. These applications can be used in the industrial production of rice koji.

### Acknowledgments

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