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Metabolomics Reveal Optimal Grain Preprocessing (Milling) toward Rice *Koji* Fermentation

Sunmin Lee, Da Eun Lee, Digar Singh, and Choong Hwan Lee*

Department of Bioscience and Biotechnology, Konkuk University, Seoul 05029, Republic of Korea

Supporting Information

ABSTRACT: A time-correlated mass spectrometry (MS)-based metabolic profiling was performed for rice *koji* made using the substrates with varying degrees of milling (DOM). Overall, 67 primary and secondary metabolites were observed as significantly discriminant among different samples. Notably, a higher abundance of carbohydrate (sugars, sugar alcohols, organic acids, and phenolic acids) and lipid (fatty acids and lysophospholipids) derived metabolites with enhanced hydrolytic enzyme activities were observed for *koji* made with DOM of 5−7 substrates at 36 h. The antioxidant secondary metabolites (flavonoids and phenolic acid) were relatively higher in *koji* with DOM of 0 substrates, followed by DOM of 5 > DOM of 7 > DOM of 9 and 11 at 96 h. Hence, we conjecture that the rice substrate preprocessing between DOM of 5 and 7 was potentially optimal toward *koji* fermentation, with the end product being rich in distinctive organoleptic, nutritional, and functional metabolites. The study rationalizes the substrate preprocessing steps vital for commercial *koji* making.

KEYWORDS: *rice koji, degree of milling, fermentation, mass spectrometry, enzyme activity*

1. INTRODUCTION

Fermentation, a customary food processing and preserving method, can be traced back to thousands of years in prehistoric era.[1] Considering the modern perspectives, the fermented food products are globally relished as functional foods with the capacity to function as health aids.[2] Typically, a food fermentative bioprocess is carried out using diverse microflora, including fungi, yeast, and bacteria, to improve the nutritional value and food safety with enhanced organoleptic properties.[3,4]

Koji, being an inextricable component or starter for numerous fermented foods, condiments, and beverages typical to East Asia, was employed largely in food fermentative bioprocesses.[5] *Koji* is made through solid-state fermentation by inoculating steamed rice grains with mold (*Aspergillus oryzae*), which germinates to secrete hydrolytic enzymes triggering fermentation.[6] Since nearly two millennia, *Aspergillus* strains have been used for the *koji* fermentation, employed typically for rice wines (*makgeoli* and *sake*), soy sauce (*ganjang* and *shoyu*), fermented soybean (*doenjang* and *miso*), soy−pepper paste (*gochujang*), and distilled spirits (*shochu*).[7] Particularly, rice *koji* fermentation with *A. oryzae* primarily occurs through carbohydrate metabolism by secreted hydrolytic enzymes.[8] In particular, the hydrolytic enzymes secreted by *A. oryzae* (*koji* mold) modulates substrate textures through affecting the release of metabolic gamut, such as monosugars, amino acids, and fatty acids.[9]

In the fermentation process of any product, the starting substrate and inoculum are critical to the quality of fermentation end products. Previously, we correlated structural contours and metabolic compositions of different rice varieties with culture growth, secreted enzymes, and metabolites in *koji*.[10] Yoshizaki et al. have also reported the effects of three types of rice *koji* (yellow, white, and black) on the volatile aroma compounds using gas chromatography−mass spectrometry (GC−MS)-based profiling.[11] Functionally, the rice is a valuable and rich source of antioxidants, including phenolic compounds, and has a hard husk (hull) that protects the endosperm. Most of these compounds are distributed in different portions of rice grain, particularly accessible through the milling process. The commercial rice-milling process generates products, such as husk and bran (pericarp), with low-value fractions. After the husk is removed, the remainder is known as brown rice, which includes the bran, embryo, and endosperm.[12] Brown rice retains its bran layer and embryo bud, while the bran and embryo of white rice are removed during complete milling. Liu et al. have highlighted the effects of varying degrees of milling (DOMs) on total flavonoid and phenolic compositions as well as the associated antioxidant activities for brown rice.[13] Determining an appropriate DOM is vital for improving nutrient utilization and controlling potential hazards associated with antinutritional factors to avoid nutrient loss as a result of overmilling.[14]

Metabolomics have increasingly been used to study the nutritional, functional, and organoleptic aspects of fermented foods in a high-throughput manner, owing to the technological and computational advancements in mass spectrometry (MS) and related databases, respectively. In recent years, the metabolomic perspectives of numerous fermented foods, viz., *koji*, *doenjang*, and *gochujang*, have been comprehensively studied to gain insights of the corresponding nutritional, functional, and quality biomarkers.[10,15,16] Herein, we examined the subtle effects of rice substrate milling on metabolomic profiles and associated biochemical phenotypes, viz., enzyme activities and antioxidant phenotypes.

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2. MATERIALS AND METHODS

2.1. Chemicals and Reagents. High-performance liquid chromatography (HPLC)-grade water, methanol, and acetonitrile were obtained from Thermo Fisher Scientific (Waltham, MA, U.S.A.). Analytical-grade chemicals, viz., acetic acid, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), casein from bovine milk powder, Folin−Ciocalteu's phenol, formaldehyde solution, formic acid, methoxyamine hydrochloride, *p*-nitrophenol, *p*-nitrophenol *β*-D-glucopyranoside (*p*-NPG), potassium persulfate, pyridine, sodium hydroxide, sodium acetate, starch, *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and tyrosine were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Sodium carbonate, sodium dihydrogen phosphate, and disodium hydrogen phosphate were purchased from Junsei Chemical Co., Ltd. (Tokyo, Japan). Trichloroacetic acid was obtained from Merck Millipore Co. (Darmstadt, Germany).

2.2. Rice *Koji* Fermentation with Substrates Having Varying DOMs and Sample Harvest. Brown rice samples of a Korean cultivar, 'Jinsang', were used in this study toward the fermentative production of rice *koji* samples and differentially milled using a polishing machine (model MP-220, Yamamoto Co., Tokyo, Japan) to obtain rice samples with different DOM. Each DOM was represented in terms of the proportion of embryo bud and bran layer that remained in rice (Table 1). The five milled rice categories, viz., DOMs of 0, 5, 7, 9, and 11, were collected and stored at −20 °C. The *koji* mold *A. oryzae* (KCCM 12698), procured from the Korean Culture Center of Microorganisms (KCCM, Seoul, Korea), was used as the culture inoculum. *A. oryzae* was maintained on malt extract agar (malt extract, 20 g; glucose, 20 g; peptone, 1 g; and agar, 20 g/L) at 28 °C. The fermentative bioprocess steps of *koji* production were adapted from Lee et al.[10] The fermented rice *koji* samples were harvested at every 12 h (up to 96 h) and stored at deep freezing conditions (−80 °C) until analyses.

Table 1. Information for DOM of Rice

DOM	proportion of the embryo bud and bran layer in rice (%)
0	100
5	50
7	30
9	15
11	5

2.3. Gas Chromatography Time-of-Flight Mass Spectrometry (GC−TOF−MS) Analysis. All sample extraction and derivatization steps were performed as described by Lee at al.[10] GC−TOF−MS analysis was performed on an Agilent 7890A GC system (Santa Clara, CA, U.S.A.) with a Pegasus HT TOF-MS (Leco Corporation, St. Joseph, MI, U.S.A.). A RTx-5MS (30 m length × 0.25 mm inner diameter, J&W Scientific, Folsom, CA, U.S.A.) was used as a carrier gas (helium) at a constant flow rate of 1.5 mL/min. The injector and ion source temperatures were maintained at 250 and 230 °C, respectively. The oven temperature was maintained at 75 °C for 2 min and then increased to 300 °C at 15 °C/min, which was sustained for 3 min. Next, 1 μL of sample was injected with a mass scan range of *m*/*z* 50−800. Overall, three samples as well as analytical replicates were maintained for each variant.

2.4. Liquid Chromatography Electrospray Ionization Mass Spectrometry (LC−ESI−MS) Analysis. The samples were extracted and analyzed for secondary metabolites using ultrahigh-performance liquid chromatography linear trap quadrupole ion trap tandem mass spectrometry (UHPLC−LTQ−IT−MS/MS) and ultraperformance liquid chromatography quadruple time-of-flight mass spectrometry (UPLC−Q−TOF−MS), using the protocols described by Lee at al.[10] Samples were separated on a Syncronis C18 column with 100 × 2.1 mm, 1.7 μm particle size (Thermo Scientific). The mass spectra and photodiode array range in both positive- and negative-ion modes were tuned for *m*/*z* 100−1000 and 200−600 nm, respectively.

2.5. Data Processing and Multivariate Statistical Analysis. The raw data sets from GC−TOF−MS and UHPLC−LTQ−IT−MS/MS analyses were transformed to netCDF (*.cdf) format using Leco ChromaTOF and Thermo Xcalibur software, respectively. The respective netCDF (*.cdf) files were subjected to MetAlign software (http://www.metalign.nl) mediated data processing as described previously by Lee et al.[10,15] The resulting data matrix, which contained the suitable peak mass (*m*/*z*), retention times (min), and peak area information as variables, was evaluated using SIMCA-P+ 12.0 software (Umetrics, Umea, Sweden) for multivariate statistical analysis. The data sets were log-transformed, and unit variance was scaled prior to principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) to compare the five rice *koji*. Significant differences (*p* value of <0.05) were tested by one-way analysis of variance using PASW Statistics 18 (SPSS, Inc., Chicago, IL, U.S.A.). The putative metabolite identification was performed through matching respective molecular weights and formula, retention time, mass fragmentation patterns, and ultraviolet (UV) absorbance data available in the literature and our in-house library (Tables S1 and S2 of the Supporting Information).

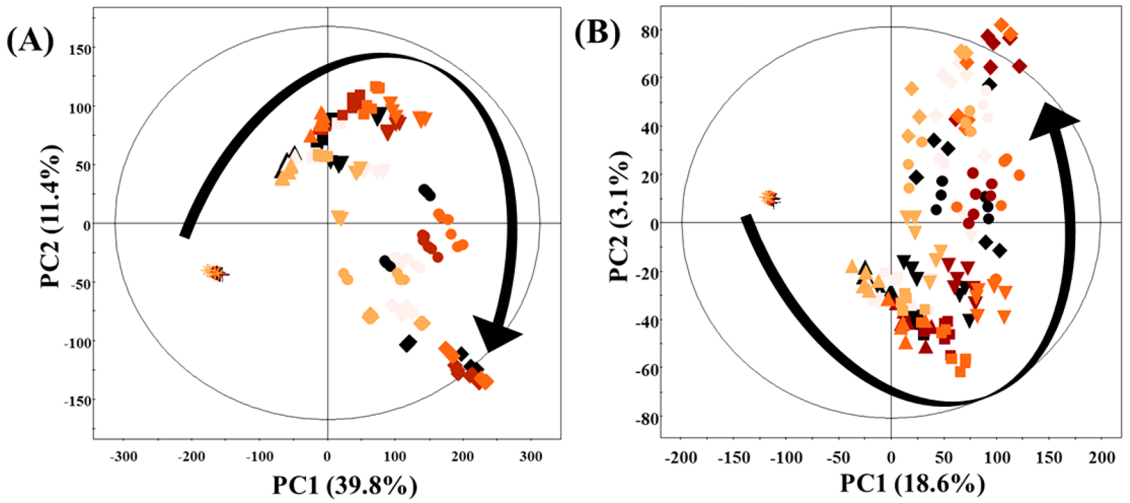

Figure 1. PCA score plot from the (A) GC−TOF−MS and (B) UHPLC−LTQ−IT−MS/MS data sets of rice *koji* cultured with *A. oryzae* according to the DOM (black, 0; brown, 5; dark orange, 7; light orange, 9; pink, 11; +, 0 h; ∗, 12 h; ▲, 24 h; ■, 36 h; ▼, 48 h; ●, 72 h; and ◆, 96 h).

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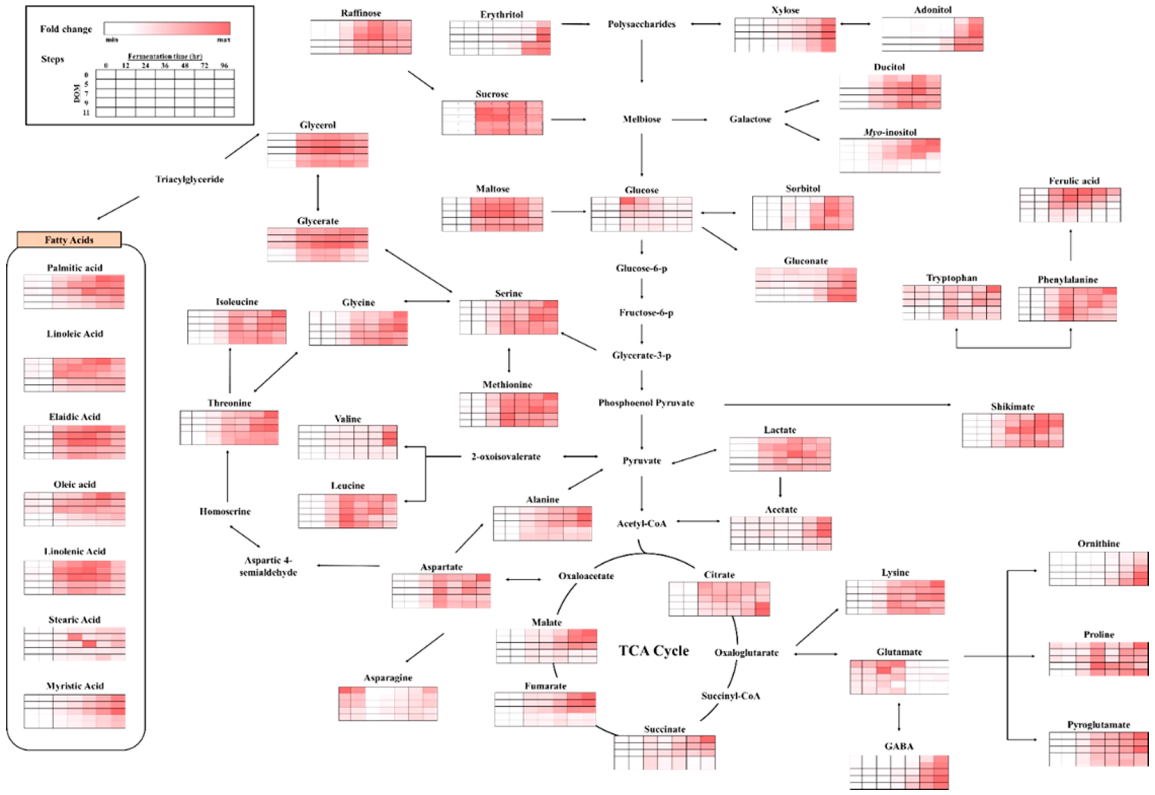

Figure 2. Pathway for the relative levels of discriminant metabolites at different times of rice *koji* with DOM as determined using the PLS-DA data sets (VIP of >1.0 and p value of <0.1) for GC−TOF−MS analyses. The discriminant metabolites were further correlated with corresponding steps in the biosynthetic pathways adapted from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The values represent the fold change with respect to unfermented rice.

2.6. Enzyme Activities. The enzymatic activity assays, including amylase, protease, and β-glucosidase, were conducted according to the protocols described by Lee et al.[10] Each rice *koji* sample (10 g) in 90 mL of water was extracted by shaking in an incubator at 120 rpm and 30 °C for 1 h. To evaluate enzymatic activities, filtered supernatants were used.

2.7. Determination of the Antioxidant Activity. To determine the antioxidant activity of rice *koji* samples, ABTS and ferric reducing antioxidant power (FRAP) assays were performed in triplicate. The ABTS antioxidant assay was performed using the method partially adapted from Re et al. and Lee et al.[10,17] For analysis, the ABTS stock solution was diluted using distilled water, achieving a solution with an absorbance of 0.7 ± 0.02 at 750 nm. Each sample extract (20 μL) was incubated with ABTS solution (180 μL) in a 96-well plate; the reaction was incubated at room temperature for 6 min under dark; and the absorbance was measured at 750 nm. The FRAP assay was performed using a mixture of 300 mM acetate buffer (pH 3.6), 20 mM iron(III) chloride, and 10 mM 2,4,6-tripyridyl-*S*-triazine (TPTZ) solution in 40 mM HCl (10:1:1, v/v/v). For analysis, 10 μL of sample was added to 300 μL of FRAP reagent and incubated at room temperature for 6 min. Absorbance was measured at 570 nm. The results are represented as the Trolox equivalent antioxidant capacity concentration (mM) per milligram of *koji*. The standard curves of Trolox ranged from 0.0156 to 0.5 mM.

2.8. Physicochemical Characteristics. To evaluate physicochemical characteristics, 3 g of *koji* samples were extracted with 30 mL of distilled water at 30 °C for 1 h and 120 rpm. The content of total sugar contents at 25 °C expressed as °Brix was determined using a digital refractometer (HI 96811, Hannah Instruments, Woonsocket, RI, U.S.A.). pH was measured using a pH meter (Thermo). Titratable acidity was measured by titrating the samples with 0.1 N NaOH solution to a pH value of 8.4. The amino-type nitrogen contents were estimated by adding 36% formaldehyde solution (20 mL) to the titrated samples, followed by sample retitration to pH 8.4 using 0.1 N NaOH solution. Amino-type nitrogen contents were quantitatively estimated on the basis of the titrated volume of 0.1 N NaOH and transformed to the total amino acid content.

3. RESULTS

3.1. Metabolic Profiling and Multivariate Analyses for Rice *Koji* Fermented Using the Substrates with Varying DOMs. Differential metabolomes among the rice *koji* samples made using the substrates with varying DOMs were examined using multivariate analyses, i.e., PCA and PLS-DA, based on GC−MS and LC−MS data sets. The PCA and PLS-DA models based on rice *koji* samples according to the DOM are shown in Figure 1 and Figure S1 of the Supporting Information, respectively. The PCA is an unsupervised, multivariate statistical method for transforming an original set of correlated variables to a new set of uncorrelated variables, called principal components (PCs). On the other hand, PLS-DA is commonly applied to evaluate the clear distinctions between groups (variables) within the observed data sets.

The PCA score plot derived from GC−TOF−MS (Figure 1A) and UHPLC−LTQ−IT−MS/MS (Figure 1B) data sets showed 51.2% (PC1, 39.8%; PC2, 11.4%) and 21.7% (PC1, 18.6%; PC2, 3.1%) variances, respectively. The PLS-DA model showed a similar pattern of metabolic profiles compared to the PCA model, indicating a steady alteration in rice *koji* metabolomes during the course of fermentation. Intriguingly, each rice *koji* with different DOMs of substrates exhibited different metabolite changes. However, similar patterns were observed between rice *koji* with substrates having DOM of 5 and 7 as well as DOM of 9 and 11 samples.

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Table 2. Column Representations for the Highest Levels of Discriminant Metabolites in Five Rice *Koji* According to the DOM[a]

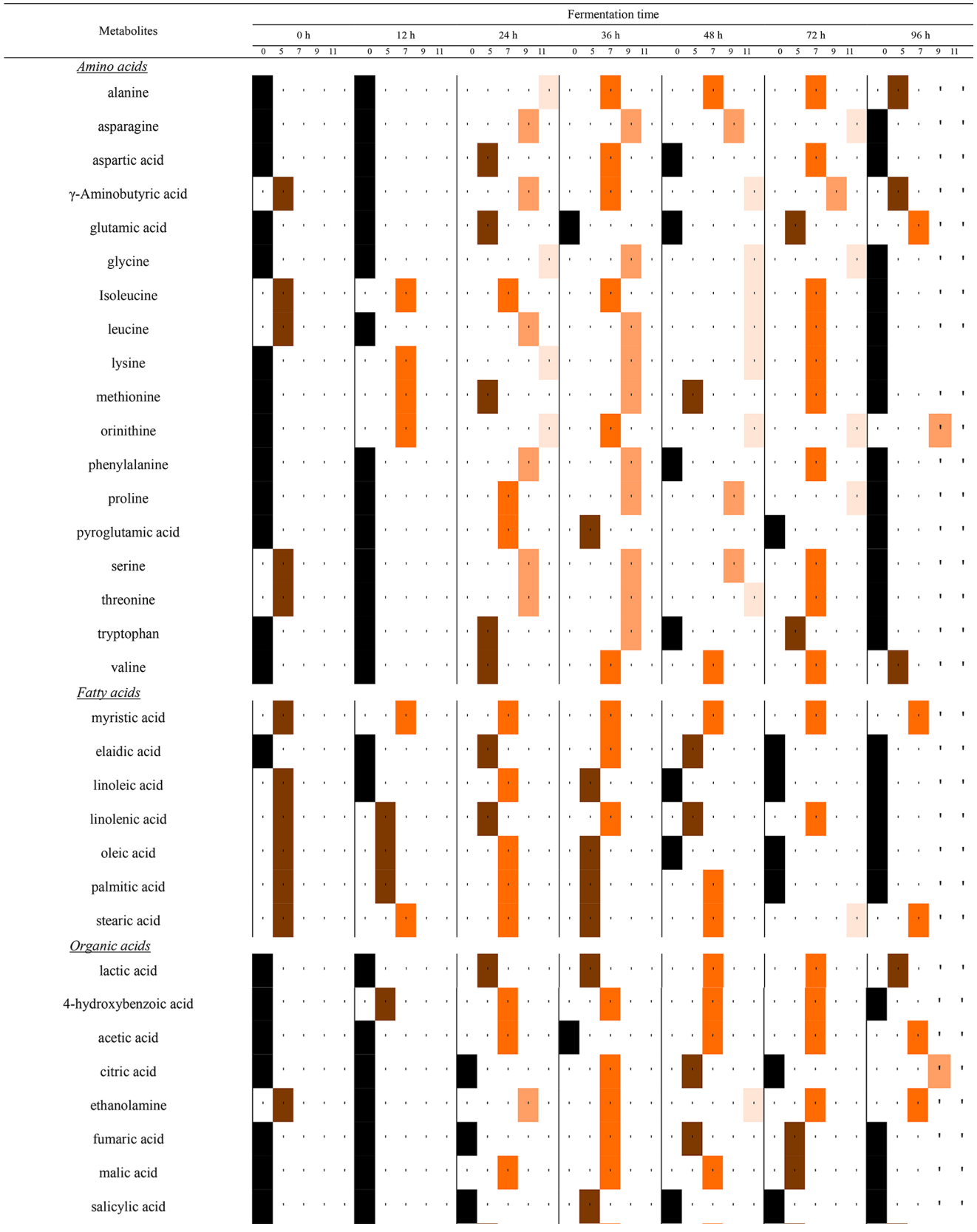

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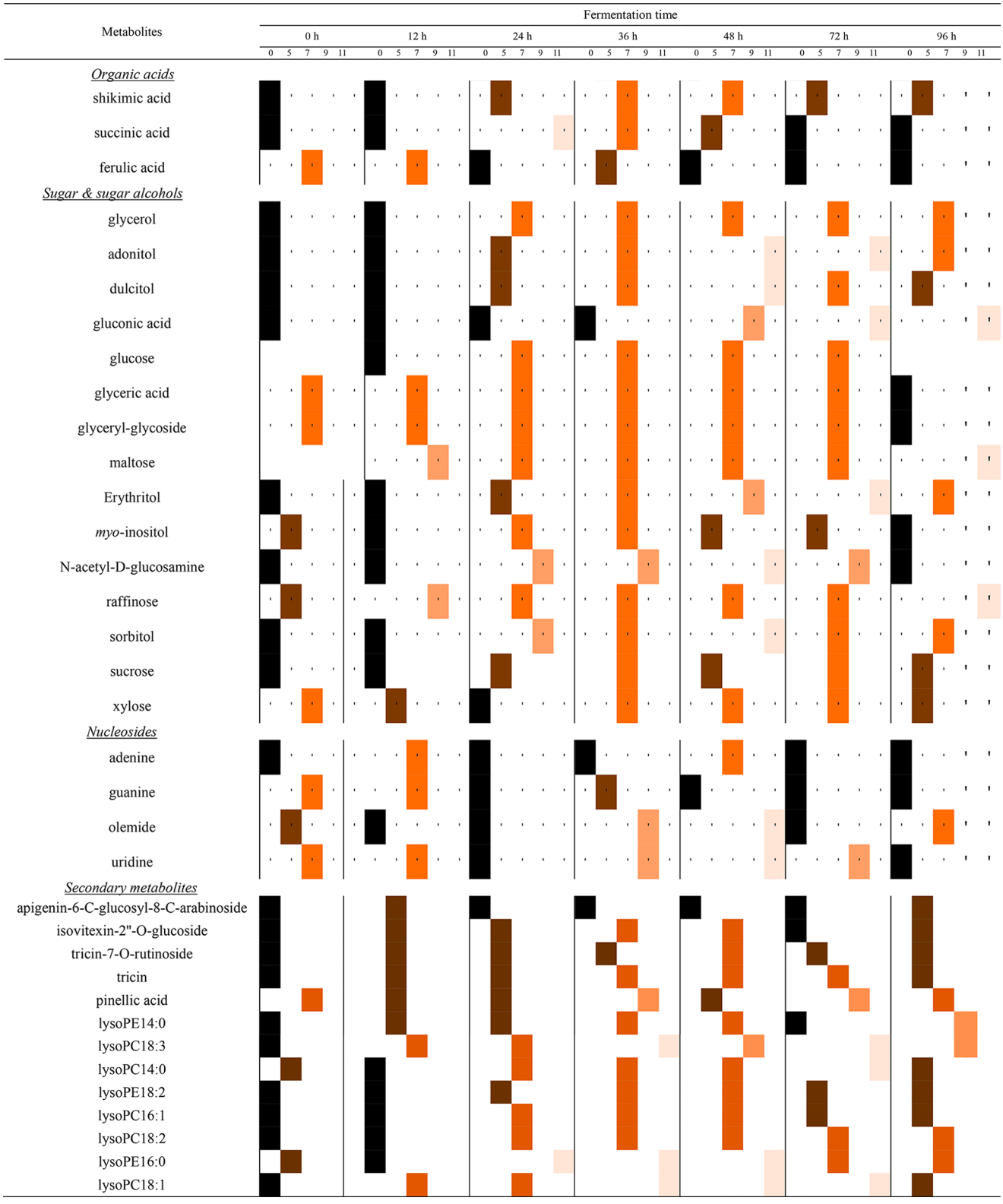
Table 2. continued



[a]Each column represents the DOM color having a metabolite of the highest content at each time (black, 0; brown, 5; dark orange, 7; light orange, 9; and pink, 11).

Differential variables were selected on the basis of variable importance in projection (VIP > 1.0) values and p values ($p < 0.05$) obtained by the PLS-DA model. The variable importance in projection (VIP) values reflect the statistical significance of each variable in the model. A total of 54 primary metabolites by GC−TOF−MS data (18 amino acids, 7 fatty acids, 11 organic acids, 15 sugars and sugar alcohols, and 4 others) were identified using standard compounds, mass fragment patterns, of the National Institute of Standards and Technology (NIST) and an in-house library (Table S1 of the Supporting

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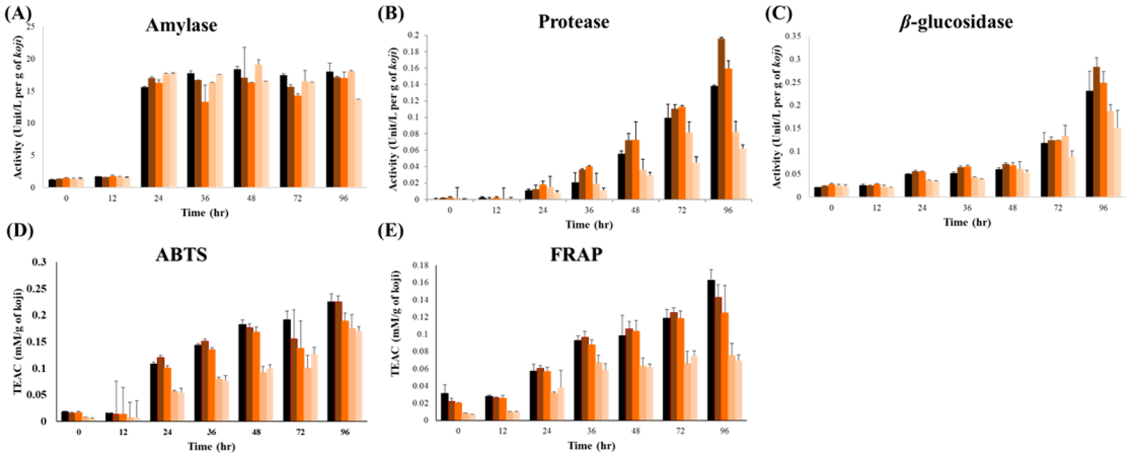

Figure 3. Changes in (A) amylase, (B) protease, (C) β-glucosidase, (D) ABTS, and (E) FRAP of rice *koji* cultured with *A. oryzae* according to the DOM during fermentation (black, 0; brown, 5; dark orange, 7; light orange, 9; and pink, 11).

Information). Further, 13 secondary metabolites by UHPLC−LTQ−ESI−IT−MS/MS data were selected as major compounds related to the discrimination between rice *koji* with different DOMs (Table S2 of the Supporting Information). In particular, apigenin-6-*C*-glucosyl-8-*C*-arabinoside, isovitexin-2″-*O*-glucoside, tricin-*O*-rutinoside, tricin, pinellic acid, and 8 lysophospholipids, including lysophosphatidylcholine (lysoPC) and lysophosphatidylethanolamine (lysoPE), were identified by comparing the data to an in-house library and published literature.

3.1.1. Temporal Primary Metabolomes for Rice Koji Fermented Using Substrates with Varying DOMs. To visualize discriminative primary metabolites according to fermentation time, a heatmap was made based on the GC−TOF−MS data. The trends of variations in metabolite levels according to fermentation time are shown in Figure 2, with their relative levels represented as fold changes normalized to the respective value of unfermented rice with different DOMs. The trends of sugar and sugar alcohol derivatives, viz., adonitol, dulcitol, gluconate, glycerate, maltose, *myo*-inositol, raffinose, sucrose, sorbitol, and xylose, were gradually increased. The relative abundance of glucose was increased until 24 h and then decreased until 96 h. In amino acid metabolism, the relative levels of serine, glycine, isoleucine, threonine, valine, methionine, aspartate, alanine, phenylalanine, tryptophan, lysine, γ-aminobutyric acid (GABA), ornithine, and pyroglutamate, were increased, whereas those of asparagine and glutamate were gradually decreased. Among the fatty acids, particularly palmitic, linoleic, elaidic, oleic, linolenic, stearic, and myristic acids were increased. Metabolites related to the tricarboxylic acid (TCA) cycle, such as citrate, malate, fumarate, and succinate, increased with the fermentation time.

3.1.2. Relative Disparity in the Levels of Discriminant Metabolites in Rice Koji Made Using the Substrates with Varying DOMs. To illuminate the relative levels of discriminant metabolites among rice *koji* types made using the substrate with varying DOMs representing each fermentation time point, a heatmap was used for indicating the highest levels of respective metabolites (Table 2). The comparison with substrates (unfermented rice) revealed that most metabolites, such as amino acids, organic acids, sugars and sugar alcohols, and secondary metabolites were relatively higher in DOM of 0 *koji*, whereas fatty acids were highest in DOM of 5 *koji*. Dependent upon the fermentation time point, the levels of metabolites in various rice *koji* types changed from DOM of 0 to 5−7 at 24 h and subsequently from DOMs of 5−7 to 7−9 at 36 h. Between 48 and 72 h, these metabolic trends changed in the rice *koji* made with substrates having DOM of 7−11. In contrast, the highest levels of amino acids, sugars and sugar alcohols, nucleotides, fatty acids, and organic acids, except secondary metabolites, were observed in DOM of 0 rice *koji* samples at 96 h.

3.2. Variations in Enzymatic Production, Bioactivities, and Physiological Characteristics for Rice *Koji* Types Made Using the Substrates with Varying DOMs. The fermentation-time-correlated enzymatic activities for different rice *koji* types made using the substrates with varying DOMs were determined to estimate the differential secretion of hydrolytic enzymes by *A. oryzae* (panels A−C of Figure 3). In general, the amylase, protease, and β-glucosidase activities were increased linearly with the fermentation time in different rice *koji* types. The protease and β-glucosidase activities were highest in the *koji* samples with DOM of 7 substrates, whereas amylase activity showed higher values in *koji* samples with DOM of 0−9 substrates, except for samples with DOM of 11. Interestingly, amylase activity for all *koji* types, regardless of DOM, was highest at 24 h, while protease and β-glucosidase activities increased linearly until 96 h. Measurement of the functional phenotypes, i.e., ABTS and FRAP, to determine antioxidant activities showed that the rice *koji* samples with DOM of 0 substrates had the highest antioxidant levels, followed by DOM of 5 > DOM of 7 > DOM of 9 and 11 substrates made rice *koji* (panels D and E of Figure 3). Hence, we assume that the antioxidant activities of *koji* increased linearly until 96 h, regardless of DOM of the substrates.

4. DISCUSSION

We employed the metabolomic approaches to evaluate the comprehensive metabolic and biochemical events underlying fermentative *koji* preparation with rice substrates having varying DOMs. We observed that the differential metabolomes of rice *koji* types were the direct biochemical functions of (1) time-correlated metabolism and (2) enzyme activities, by *koji* mold subjected to fermentation on rice substrates with varying DOMs. During the course of fermentative growth, the spore count for *A. oryzae* was steadily increased, while the moisture content was decreased. There were no significant differences observed for mold growth and sporulation among the *koji*

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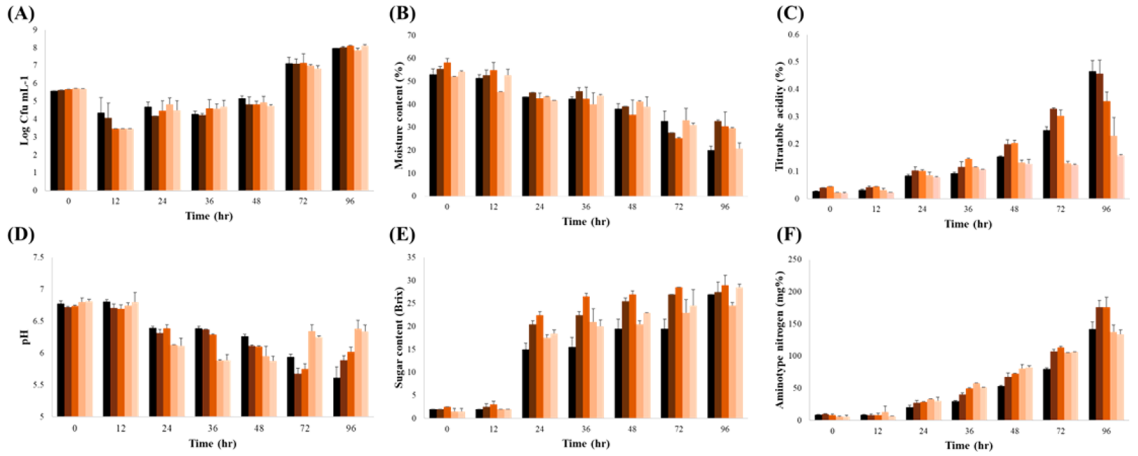

Figure 4. (A) Total mold count, (B) moisture content, (C) pH, (D) titratable acidity, (E) total sugar content, and (F) total amino acid content of *koji* cultured with *A. oryzae* according to the DOM during fermentation (black, 0; brown, 5; dark orange, 7; light orange, 9; and pink, 11).

samples with five different DOMs. We assumed that the decrease of the moisture content in *koji* was probably due to consumption of water by *Aspergillus* growth as well as through evaporation.[18]

The multivariate analysis for various rice *koji* extracts based on GC−TOF−MS data sets indicated a fermentation time-correlated metabolic profile for primary metabolites, regardless of the DOM (Figure 1A). On the other hand, the multivariate analysis based on UHPLC−LTQ−IT−MS/MS data sets showed a secondary metabolite profile depending upon various DOMs of rice substrates (Figure 1B). As shown in Figure 2, the primary metabolites were altered briskly with enzyme activities over time, i.e., increased abundance of metabolites related to sugar metabolism with an increase in amylase and β-glucosidase activities at 24 and 72 h, respectively. Similarly, the gradual changes in amino acid levels were recorded with enhanced protease activity. The fatty acid levels were also increased at 24 h.

4.1. Time-Correlated Metabolic Alterations for Rice *Koji* Independent of DOM of Substrates. The organic acids produced in the TCA cycle, viz., citrate, fumarate, malate, and succinate, acts as intermediates to various metabolic pathways (Figure 2). With an increase in organic acid contents, the pH decreased with an accompanying increase in titratable acidity of the rice *koji* samples (panels C and D of Figure 4). The production of citric acid used in the dairy, food, beverage, pharmaceutical, and biochemical industries is extensively carried out using *Aspergillus* species.[19] Fumaric acid is used as a starting metabolite for the synthesis of polymers, while malate and succinate have wide applications in the food, beverage, and pharmaceutical industries.[20]

Among the sugars, the glucose levels showed the highest levels at 24 h, unlike other sugar metabolites, followed by its steep decline of levels during the later stages of *koji* fermentation. In particular, glucose is a major carbon source hydrolyzed from the substrate starch by fungal enzymes, including amylase and β-glucosidase. The sugars are further metabolized to sugar alcohols through alcoholic fermentation.[21] The sugar alcohols are produced during microbial fermentation through multiple fermentation pathways.[22] For example, erythritol, a four-carbon sugar alcohol that is widely distributed in nature, such as in foods, is produced industrially via the pentose phosphate pathway, beginning with enzymatic hydrolysis of the starch from rice to generate glucose. Similarly, sorbitol can be obtained by simultaneous hydrolysis and reduction of glucose to change the aldehyde group to a hydroxyl group.

The proteolytic enzymes produced by *Aspergillus* species release the organic nitrogen contents from complex proteins to be used as a nitrogen source essential for growth and metabolism.[23,24] The rice *koji* fermentation with *Aspergillus* spp. effectively increased the contents of free amino acids (alanine, glycine, and serine) related to sweetness and nutritional properties.[25] The total amino acid contents, including those of aromatic amino acids (phenylalanine and tryptophan) and branched-chain amino acids (leucine, isoleucine, and valine), were increased with enhanced protease activities over time (Figures 2, 3B, and 4F). Functionally, the biosynthesis of aromatic amino acids in *Aspergillus fumigatus* has been linked with its antifungal properties, while the branched-chain amino acids in *Aspergillus nidulans* are reportedly vital for building proteins.[26,27] In our study, we observed a decrease in glutamate contents coupled with an increase in other amino acid contents, especially those of GABA over time. It has been reported earlier that *A. oryzae* contains a gene for glutamate decarboxylase (GAD, EC 4.1.1.15), which produces GABA via decarboxylation of glutamic acid during cultivation of the spore suspension.[28] GABA was employed in functional foods for reducing blood pressure and promoting better sleep and diuretic effects.[29−31] The relative levels of unsaturated fatty acids, saturated fatty acids, and lysophospholipids were increased with the biosynthesis as well as bioconversion of fatty acids (Figure 2). Whole rice bran lipids induce the lipid metabolism. This means that fungi generate excess lipids by fermenting the substrate materials as well as synthesize their own lipids for fungal biomass production.[32] Earlier, Abu et al. have reported that solid-state fermentation with *A. oryzae* typically increases the concentrations of lipids, primarily including C16:0, C18:0, and C18:1.[33]

4.2. Metabolic Alterations for Rice *Koji* Depending upon DOM of Substrates. A rice grain has different biomolecular compositions for its different parts, arranged from surface to inside, including outer husk or hull, bran, embryo, and innermost endosperm. A variety of metabolites in rice, viz., phenolic acids, cinnamic acids, anthocyanins, flavonoids, steroidal compounds, polymeric carbohydrates, and proteins, are nutritionally vital as health-promoting functional foods.[34] Following the milling process, rice contains

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less of the embryo bud and bran layer (Table 1). We detected higher proportions of amino acids, organic acids, sugars and sugar alcohols, and lysophospholipids, together contributing to the high antioxidant activities for DOM of 0 rice *koji* compared to those for DOM of 5−11 *koji* samples (Table 2 and panels D and E of Figure 3), maintaining similar pH and titratable acidity (panels C and D of Figure 4). Although the relative abundance for most metabolites in rice *koji*, made using the substrates with intermediate DOM, were increased during the course of fermentation, the metabolite abundance for unfermented DOM of 0 substrate was comparatively higher than substrates with increased DOM (Table 2).

Given the proportion of the bran layer in rice after different DOMs, rice *koji* was relatively affected by various microbial enzymes and associated bioactivities. The protease and *β*-glucosidase activities were observed varying in the order of DOM of 5 > DOM of 7 > DOM of 0 > DOM of 9 > DOM of 11 substrates, while the amylase activity was observed unaffected by the DOM. Accordingly, amino acids, sugars, and sugar metabolism were highest in DOM of 5−7 samples during the middle of fermentation at 24−72 h. Intriguingly, most of the primary metabolites were highest in the *koji* samples with DOM of 0 at the end 96 h of fermentation. Mechanistically, *β*-glucosidase cleaves glucosidic linkages in polysaccharides and flavonoid glycosides and hydrolyzes them into oligo- or monosaccharides and corresponding flavonoid aglycones to improve enzymatic saccharification.[35] In the *koji* samples with DOM of 5 substrate, enhanced *β*-glucosidase activities in rice *koji* were mainly correlated with a decrease in the flavonoid glycosides and tricin rutinoside with a corresponding increase in the relative levels of sugars and flavonoid aglycone, tricin. The changes observed in the metabolite levels for DOM of 0 *koji* samples might have been impeded by the rice bran wall, impervious to enzymatic hydrolysis. Hence, following the rice bran hydrolysis, the relative levels of most metabolites was altered in DOM of 0 *koji* samples. The delay in metabolite release in the case of brown rice *koji* fermentation was reportedly linked to the impenetrable brine layer, which limits enzymatic penetration and subsequent hydrolysis.[10] Earlier, Yu et al. have reported that the protease and glucoamylase activities for rice *koji* made using a brown rice substrate with 50−70% DOM reach a maximum following 5 days of fermentation with *A. oryzae*.[36]

The ABTS and FRAP assays that determined antioxidant activities were considerably higher in rice *koji* using substrates with DOMs of 0, 5, and 7, in comparison to those for DOM of 9 and 11 *koji* samples, during the course of fermentation. Hence, increasing the DOM of substrates allows for the enzymatic penetration through impervious rice bran and endosperm, hence releasing the polyphenols, flavonoids, and free amino acids affecting the antioxidant activities.[13,14] Antioxidant activity prevents or limits oxidation, which is considered beneficial for improving food quality and health. Evaluating antioxidant compounds from different DOMs of rice substrates provides useful information for commercial *koji* making, owing to the nutritional and functional properties of antioxidant metabolites.

Previously, it has been shown that flavonoid and phenol compounds derived from plants possess high antioxidant activities.[37] Pérez et al. have reported that the amino acid composition of honey affects its free radical scavenging capacity.[38] A known antioxidant phytochemical, i.e., ferulic acid, is commonly found in rice bran, which reportedly alleviates oxidative stress in various organic systems.[39] We observed that the 96 h rice *koji* with DOM of 0 substrate contained the highest levels of antioxidant compounds. We conjecture that antioxidant potentials of rice *koji* are mainly influenced by its flavonoids and phenolic acid components, which synergistically constitute its various functional properties.

In conclusion, we observed that *A. oryzae* exhibited a unique overall metabolism, maneuvering the secretion of hydrolytic enzymes (amylase, *β*-glucosidase, and protease) ergo the metabolomes in rice *koji* fermented using the substrates with varying DOMs. Typically, at the initial and final stages of *koji* fermentation, a variety of functional metabolites were relatively higher in *koji* made with substrates having DOM of 0, in comparison to *koji* types made with substrates subjected to varying DOMs. However, in the middle stages of *koji* fermentation, the substrates with DOMs of 5−7 showed relatively higher contents of metabolite intermediates of carbohydrate metabolism, viz., sugars and sugar alcohols, organic acids, phenolic acids, and lipid metabolism intermediates, such as fatty acids and lysophospholipids. The enhanced release of free metabolites in the *koji* samples was influenced by the relatively higher amylase, *β*-glucosidase, and protease levels in rice substrates with DOMs of 5−7 compared to DOM of 0. The present findings provide useful insights for large-scale commercial production of rice *koji* that relies heavily upon the DOM to rationalize its raw substrate processing.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.7b05131.

List of significantly distinct metabolites from rice *koji* with different DOMs during fermentation identified by GC−TOF−MS (Table S1), list of significantly distinct metabolites from rice *koji* with different DOMs during fermentation identified by UHPLC−LTQ−ESI−IT−MS/MS (Table S2), and PLS-DA score plot derived from the GC−TOF−MS and UHPLC−LTQ−ESI−IT−MS/MS data sets of rice *koji* cultured with *A. oryzae* according to the DOM (Figure S1) (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Telephone: +82-2-2049-6177. Fax: +82-2-455-4291. E-mail: chlee123@konkuk.ac.kr.

ORCID

Choong Hwan Lee: 0000-0002-2311-185X

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Notes

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