Nutritive and bioactive components in rice fermented with the edible mushroom *Pleurotus eryngii*

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The present research was the first to compare nutritive and bioactive components of *Pleurotus eryngii*-fermented rice with untreated rice. After fermentation, the total content of crude protein, crude fat, polysaccharides, reducing sugar, and polyphenols was significantly increased in fermented rice. The total content of amino acid in fermented rice was enhanced to 557.71 mg/g in comparison with 258.83 mg/g in non-fermented rice. The content of essential amino acid increased from 79.32 mg/g to 140.36 mg/g. The content of adenosine in fermented rice (175.64 µg/g) is much higher than that of non-fermented rice (14.38 µg/g). In antioxidant assay, the ethanol extract of the *P. eryngii*-fermented rice showed a strong reducing ability with EC\(_{50}\) value less than 0.25 mg/ml. These results indicated that rice fermented by *P. eryngii* could be useful as a new functional food.

**Keywords:** *Pleurotus eryngii*; nutritive components; adenosine; antioxidation

1. Introduction

Mushrooms have been used as food and food-flavoring material for centuries due to their nutritional and medicinal value and the diversity of their bioactive components (Ng 1998). Recently, they have attracted much more attention as functional foods or physiologically beneficial medicine (Mau et al. 2004). The technique of solid state fermentation (SSF) involves microbial growth on moist solid medium without free water; the traditional Japanese food “natto” (*Bacillus*-fermented soybean) and the Chinese food “anka” (*Monascus*-fermented rice) were produced by this method. Many studies have shown that mushrooms fermented with this technique could enhance the production of bioactive secondary metabolites. The SSF of *Hericium erinaceus* improved its nutritive value by increasing the amount of proteins (Han 2003), and the SSF of Shiitake increased the content of phenolic compounds (McCue & Shetty 2005).

*P. eryngii* is a saprophytic mushroom that is taxonomically related to Basidomycotina, Agricales, Pleurotaceae, and Pleurotus. The proximate composition, flavor compounds, and taste components in *P. eryngii* have been studied (Mau et al. 1998). The extract of *P. eryngii* has been reported to exhibit anti-aging effect, anti-tumor activity (Guillen et al. 2000; Hawang et al. 2003), blood glucose-lowering property (Kang et al. 2001), inhibitory effects on angiogenesis-related enzymes (Kang et al. 2003), antioxidant activity (Hui et al. 2002), and immune regulatory activity (Kang et al. 2004). In our current study, the edible mushroom *P. eryngii* was inoculated into cooked rice and fermented by the SSF technique to produce a fermented rice product. The ethanol extract of *P. eryngii*-fermented rice (PER), when subjected to HPLC analysis, is found to be rich in secondary metabolites in comparison with that of the non-fermented rice (R). Previously, we reported the isolation and identification of a new cytotoxic macrocyclic diterpenoid eryngiolide A and two known lactones from the ethyl acetate extract of PER (Wang, Li, et al. 2012). In this work, we compared the variation of nutritive and bioactive components, and the antioxidant property between the PER and R.

2. Materials and methods

2.1. Fungal material and cultivation

The strain of *P. eryngii* used in this work is kept in the culture collection at the Institute of Microbiology, Chinese Academy of Sciences, Beijing (access number: CGMCC 5.775). The fungal strain was cultured on slants of potato dextrose agar at 25°C for 10 days. Agar plugs were inoculated into 500 ml Erlenmeyer flask which contained 120 ml medium before sterilization. The medium consisted of 0.4% glucose, 1% malt extract, and 0.4% yeast extract, and the final pH of the medium was adjusted to 6.5. Large-scale fermentation was carried out in twenty 500 ml flasks each...
containing 80 g of rice and 120 ml of distilled water. Each flask was inoculated with 5.0 ml of the culture medium and incubated at 25°C for 40 days. As a blank control, cooked rice without fungal inoculation was treated in the same way.

2.2. General methods
UV data were recorded on a Shimadzu Biospec-1601 spectrophotometer (Shimadzu, Indonesia). NMR data were acquired with Bruker Avance-500 spectrometer using solvent signals (dimethyl sulfoxide-d₆: δIH 2.49/δC 39.7; Bruker, Karlsruhe, Germany) as references. ESIMS and HRESIMS data were obtained using a Bruker APEX III 7.0T spectrometer. Thin layer chromatography (TLC) was carried out on Silica gel 60F₂₅₄ and the spots were visualized by spraying with 10% H₂SO₄ and heating. Octadeclisylsil (ODS; Lobar, 40–63 μm, Merck) were used for column chromatography. Preparative HPLC was performed on an Agilent 1200 HPLC system using an ODS column (RP-18, 250 × 10 mm, YMC Pak, 5 μm; detector: UV; Agilent Technologies Ltd., Santa Clara, CA, USA) with a flow rate of 2.5 ml/min.

2.3. Preparation of sample for component analysis and bioassay
The lyophilized powders of PER and R were used for the analysis of nutritive components. For bioassay, the lyophilized powder (5 g) was refluxed with 50 ml of water or ethanol for two times to afford the water extract or ethanol extract, respectively. For analysis of the polysaccharides, a UV–V is spectrophotometer was used at 625 nm using a standard curve prepared with 10–70 μg/ml (final concentrations) of glucose (Sigma Chemical Co., Louis, MO, USA) (Morris 1948). The total amount of phenolic compounds was quantitatively determined according to a procedure described by Singleton and Rossi with gallic acid as the reference standard at 760 nm (Singleton & Rossi 1965).

2.4. Determination of amino acid content
A reversed-phase high performance liquid chromatographic (RP-HPLC) method was used for the determination of amino acids. The sample was mixed with norleucine as the internal standard, then derivatized with phenylisothiocyanate (PITC) and analyzed on a Venusil-AA column at 40°C with detection at 254 nm. The mobile phase, composed of (A) 0.10 M NaAc buffer (pH 6.5)/acetonitrile (93:7, v/v) and (B) 80% acetonitrile (v/v), was at a flow rate of 1.0 ml/min using a gradient program: 0–2 min, 0% B; 2–15 min, 0–10% B; 15–25 min, 10–30% B; 25–33 min, 30–45% B; 33–33.1 min, 45–100% B; 33.1–38 min, 100% B.

2.5. Determination and preparation of adenosine
2.5.1. Detection and determination of adenosine by HPLC
The detection and determination of adenosine in PER and R was carried out using reversed-phase Agilent 1200 HPLC system equipped with an ODS column (Agilent Technologies, 5 μm, 4.6*150 mm). Ten per cent methanol-distilled water was used to elute with a flow rate of 1.0 ml/min; the column temperature was maintained at 30°C and the detection was set at 260 nm.

To determine the adenosine in PER, the lyophilized powders of PER and R (2.0 g) were ultrasonically extracted with 50 ml of 15% methanol-distilled water for 30 min at room temperature. The supernatant was centrifuged at 3000 rpm for 5 min and filtered through a 0.45 μm syringe filter prior to HPLC analysis. The HPLC analysis was calibrated against a standard curve obtained from adenosine solutions of 1–20 μg/ml.

2.5.2. Extraction and preparation of adenosine
The PER (100 g) was extracted with 15% methanol in water by exhaustive maceration (3 × 300 ml), and the extract solution was evaporated to dryness under vacuum to afford the crude extract (487 mg). The crude extract was subjected to ODS column chromatography using a gradient of increasing methanol concentration (0%, 20%, 60%) in water to afford 3 subfractions (Fr. 1–3). Fr. 2 (132 mg) was purified by preparative HPLC using 8% methanol in water to afford adenosine (16.2 mg, tR 6.95 min).

2.6. Antioxidative activity
2.6.1. Reducing power
The reducing power was determined according to the method of Oyaizu (Oyaizu 1986). Each sample
(1–7 mg/ml, 2.5 ml) in deionized water was mixed with 2.5 ml of 1% potassium ferriacyanide (Sigma), and the mixture was incubated at 50°C for 20 min. After adding 2.5 ml of 10% trichloroacetic acid (w/v, Wako), the mixture was centrifuged at 200 g for 10 min. The upper layer (5 ml) was mixed with 5 ml of deionized water and 1 ml of 0.1% ferric chloride (Wako), and the absorbance was measured at 700 nm against a blank. A higher absorbance indicated a higher reducing power. Ascorbic acid was used as the reference standard, and its EC$_{50}$ value was 3.96 ± 0.25 µg/ml.

2.6.2. Chelating ability on ferrous ions

The chelating ability was determined according to the methods of Dinis, Madeira, and Almeida (Dinis et al. 1994). Each sample (1–5 mg/ml, 1.0 ml) in deionized water was mixed with 3.7 ml of methanol and 0.1 ml of 2 mM ferrous chloride (Merck). The reaction was initiated by the addition of 5 mM ferrozine (0.2 ml, Sigma). At room temperature for 10 min, the absorbance of the mixture was determined at 562 nm against a blank. A lower absorbance indicates a higher chelating power. EDTA was used for comparison; EC$_{50}$ value of EDTA was 0.13 ± 0.00 mg/ml.

2.6.3. Scavenging ability on 1,1-diphenyl-2-picrylhydrazyl radical

Each sample (0.5–2 mg/ml, 1.0 ml) in deionized water was mixed with 1.0 ml of ethanol solution containing 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Sigma) radicals (to a final concentration of 0.2 mM DPPH). The mixture was shaken vigorously and left to stand for 30 min in the dark; the absorbance was then measured at 517 nm against a blank (Shimada et al. 1992). The scavenging ability was calculated as follows: scavenging ability (%) = [(A$_{517}$ of control – A$_{517}$ of sample)/A$_{517}$ of control]*100. Ascorbic acid was used for reference comparison; the EC$_{50}$ value is 19.83 ± 0.04 µg/ml.

Table 1. Main nutritive components of fermented product of P. eryngii (PER) and non-fermented rice (R).

|                  | R        | PER      |
|------------------|----------|----------|
| Crude Protein (mg/g) | 28.17 ± 1.41 | 133.42 ± 1.27** |
| Crude fat (mg/g)   | 2.16 ± 0.21  | 13.92 ± 0.32** |
| Polysaccharide (mg/g) | 11.46 ± 0.02  | 22.46 ± 0.03** |
| Reducing sugar (mg/g) | 15.67 ± 1.33  | 40.67 ± 1.25** |
| Polyphenol (mg/g)  | 0.55 ± 0.00  | 4.84 ± 0.01** |

Notes: PER: P. eryngii-fermented rice, R: non-fermented rice. The results represent the mean ± SD from three repeats, and significant of difference compared with Rice control at **p < 0.001 (Student’s t-test).

In this work, the total amino acid in the PER and R was analyzed by HPLC method. Seventeen amino acids were found in both fermented as well as R (Figure 1). The total amino acid content in the fermented rice was increased to 557.71 mg/g; compared to 258.83 mg/g in the R (Table 2). Glutamic acid was the major amino acid with a concentration of 51.57 mg/g in the R. After fermentation, tyrosine became the major amino acid with a concentration of 213.17 mg/g (Figure 2); it has been reported tyrosine can improve stress, cognitive and physical performance of a human being (Hao et al. 2001; Mahoney et al. 2007). We also found that the total content of essential amino acids was enhanced from 79.32 mg/g to 140.36 mg/g after fermentation. As we know, the essential amino acids cannot be synthesized in vivo; therefore, they must be acquired from food. High content of essential amino acids in rice fermented by P. eryngii provides beneficial effect to human health, and further supports it to be a new functional food.

3.2. Determination of adenosine

Adenosine is known as an important bioactive ingredient present in edible and medicinal fungi, such as Ganoderma lucidum (Yoswathana et al. 2010) and Cordyceps militaris (Lim et al. 2012); it is a naturally occurring purine nucleoside and formed by the breakdown of adenosine triphosphate (ATP), serving as a building block for nucleic acid and energy storage molecules (Alam et al. 1999). Adenosine was detected and prepared by HPLC from the PER (Figure 3). The content of adenosine between fermented rice and R was analyzed by HPLC method. The linearity equation of adenosine is $y = 180.67 \times -7.256$, the high correlation coefficient value ($R^2 = 1.0000$) indicated good correlations between adenosine concentrations and their peak areas within the test ranges ($x = 1–20$ µg/ml). The content of adenosine in the R is 14.38 µg/g, while that of PER reached 175.64 µg/g. After fermentation, the content of adenosine was increased to 12-fold.

Adenosine, a colorless oil, was assigned as C$_{10}$H$_{13}$N$_2$O$_4$ on the basis of positive HR-ESI-MS at $m/z$ [M + Na]$^+$ 268.1074 (calcd. for C$_{10}$H$_{13}$N$_2$O$_4$Na, 268.1040) and $^1$H-NMR(500 MHz, in DMSO-$d_6$) of
Figure 1. Chromatograms of total amino acids in PER and R, (A) R: non-fermented rice, (B) PER: *P. eryngii*-fermented rice, (C) reference standard.
Table 2. Content of total amino acid in fermented product of *P. eryngii* (PER) and non-fermented rice (R).

| Essential amino acid (mg/g) | Nonessential amino acid (mg/g) |
|-----------------------------|-------------------------------|
|                             | R                             | PER                        | R                                 | PER                        |
| Thr                         | 7.66 ± 0.27                   | 13.53 ± 0.25**             | Asp                               | 14.26 ± 0.13               |
| Val                         | 2.88 ± 0.06                   | 5.66 ± 0.28**              | Glu                               | 51.57 ± 0.87               |
| Met                         | 16.52 ± 0.22                  | 21.44 ± 0.31*              | Ser                                | 13.87 ± 1.36               |
| Ile                         | 19.86 ± 0.24                  | 35.53 ± 0.32**             | Gly                                | 16.14 ± 0.69               |
| Leu                         | 6.57 ± 0.29                   | 13.43 ± 0.36**             | His                                | 0.66 ± 0.01                |
| Phe                         | 10.75 ± 0.23                  | 9.25 ± 0.09                | Arg                                | 13.46 ± 0.28               |
| Lys                         | 15.36 ± 0.02                  | 40.53 ± 0.51**             | Ala                                | 15.07 ± 0.14               |
| Trp                         | —                             | —                          | Pro                                | 13.59 ± 0.27               |
|                             |                                |                             | Tyr                                | 33.53 ± 0.30               |
|                             |                                |                             | Cys                                | 7.34 ± 0.08                |

Notes: PER: *P. eryngii*-fermented rice, R: non-fermented rice. The results represent the mean ± SD from three repeats, and significant of difference compared with Rice control at *p* < 0.05 and **p** < 0.001 (Student’s t-test).

3.3. **Antioxidative activity evaluation**

Various mushrooms have been reported to possess strong antioxidant activity (Yang et al. 2002). In the current study, the reducing ability, chelating ability, and DPPH scavenging ability of the PER and R was compared (Table 3). The PER and R were extracted with ethanol and water to afford the ethanol extract and the water extract, respectively. The reducing powder determined with the potassium ferricyanide reduction method is a significant indicator of the antioxidant activity. After fermentation, the ethanol extract of the PER showed strong reducing ability with 

\[ EC_{50} < 0.25 \text{ mg/ml} \] (the ethanol extract of the R has \[ EC_{50} = 0.55 \text{ mg/ml} \]). The difference of the chelating ability and the DPPH scavenging activity between the fermented rice and R was small. The enhancement of the reducing effect in the PER further evidenced the potential of the PER to be a new functional food.

4. **Conclusions**

Our current findings strongly support the application of the PER as a novel functional food to prevent the occurrence of stress-related diseases and carcinogenesis. The main nutritional and bioactive components including crude protein, crude fats, polysaccharides, reducing sugars, polyphenols, total amino acids, and adenosine were significantly increased in fermented rice of *P. eryngii*. Further studies on appropriate animal models and the structure–activity relationship for bioactive metabolites are needed, which would extend our understanding of this fermented product to human health. In combination with our earlier study on the mushroom *Flammulina velutipes* (Wang, Bao, et al. 2012), it can be concluded that SSF of the edible mushrooms can greatly explore their potential of producing bioactive secondary metabolites.
Figure 3. Chromatograms of adenosine in PER and R, (A) Adenosine reference, (B) R: non-fermented rice, (C) PER: P. eryngii-fermented rice.

Table 3. Antioxidant effect of fermented product of P. eryngii (PER) and non-fermented rice (R).

|                  | EC<sub>50</sub> value (final concentration: mg/ml) |
|------------------|--------------------------------------------------|
|                  | R       | PER     |
| Ethanolic extract| Reducing power   | 0.55 ± 0.11 | <0.25   |
|                  | Scavenging ability | 0.95 ± 0.13 | >1      |
|                  | Chelating ability  | <0.25   | 0.31 ± 0.03 |
| Water extract    | Reducing power   | <0.25   | 0.27 ± 0.08 |
|                  | Scavenging ability | >1      | >1      |
|                  | Chelating ability  | 0.56 ± 0.03 | 0.47 ± 0.07 |

Note: EC<sub>50</sub>: Potassium ferricyanide was reduced by 50%, DPPH radicals were scavenged by 50%, and ferrous ions were chelated by 50%.

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