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Antioxidant activity and active compounds of rice koji fermented with *Aspergillus candidus*

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Abstract

Antioxidant activity of ethyl acetate extracts from rice koji (EAERK) fermented with *Aspergillus candidus* CCRC 31543 was evaluated, and the antioxidant components in koji were isolated and identified. EAERK (200 μg/ml) showed a 90% inhibitory effect on peroxidation of linoleic as determined by the thiocyanate method. EAERK also showed a marked antioxidant activity on the oxidation of lard determined by using the Rancimat method. EAERK had a strong scavenging effect on the DPPH radical. Silica gel column chromatography was used to separate EAERK into eight fractions (A–H). Fractions C–F possessed significant antioxidant activity and showed more than 88% inhibition of peroxidation of linoleic acid. Fraction C was crystallized and purified to obtain compound **1**. Fractions D–F were combined and re-separated by means of column chromatography and the subfraction that exhibited strong antioxidant activity was crystallized to yield purified compound **2**. Compounds **1** and **2** were shown to be ergosterol and terphenyllin, respectively, on the basis of UV-vis spectral, MS and NMR analyses. Ergosterol showed no antioxidant activity on the peroxidation of linoleic acid. However, the antioxidant activity of terphenyllin was equal to that of BHA at 200 μg/ml.

Keywords: *Aspergillus candidus*; Rice koji; Fermentation; Antioxidant activity; Terphenyllin

1. Introduction

Antioxidants are usually applied to prevent lipid peroxidation in the food industries. Though synthetic antioxidants have been widely used, they have been reassessed for possible toxic and carcinogenic components that may form during degradation (Ito, Fukushima, & Tsuda, 1985). Due to these health concerns, natural antioxidants have been extensively employed instead of synthetic ones in recent years. Microbial sources have been shown to be a potential means of producing natural antioxidants (Ishikawa, 1992). Antioxidant substances (protocatechuic acid, gallic acid) can be derived from the solid or semi-solid culture of *Aspergillus terreus* S-4 (Kawakubo, Nishira, Aoki, & Shinke, 1993). *Aspergillus* spp. have been widely applied as a starter in the koji industries and are usually inoculated into the solid-culture of steamed rice, barley soybean and the mixture of wheat flour and soyflour (Esaki, Onazaki, Kawakishi, & Osawa, 1997). In traditional fermented foods (e.g. sake, soy sauce or miso), *Aspergillus* spp. possess the function of saccharification, and can be used to obtain special colours and flavours. Physiological substances including ferulic acid and its derivatives, associated with anti-tumour, antibacterial, and antioxidant activity, as well as hypotensive effects, were also produced by *Aspergillus* spp. (Ohshita, 1990; Yoshizawa, Komatsu, Takahashi, & Otsuka, 1979). In our previous studies, *Aspergillus candidus CCRC* 31543 was screened from 10 molds, and its broth filtrate extract (by ethyl acetate, EAEAC) was found to exhibit significant antioxidant activity (Yen & Lee, 1996). The antioxidant components of EAEAC were isolated and identified as 3,3″-dihydroxyterphenyllin, 3-hydroxyterphenyllin and candidusin B, and these three compounds were neither cyto/genotoxic toward human intestine 407 (Int 407) cells nor mutagenic toward *Salmonella typhimurium* TA98 and TA100 (Yen, Chang, Sheu, & Chiang, 2001). However, antioxidants from the solid-culture fermented with *A. candidus* have not been

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investigated. The objective of this study was to isolate and identify the antioxidant components in ethyl acetate extracts from the rice koji (EAERK) of *A. candidus*. The antioxidants of these *Aspergillus* metabolites were evaluated and compared with BHA and EAERK.

2. Materials and methods

2.1. Microorganism

Lyophilized culture of *A. candidus* CCRC 31543 was obtained from the Culture Collection and Research Center (CCRC, Food Industry Research and Development Institute, Hsinchu, Taiwan, ROC). The stock culture was grown on potato dextrose agar (PDA) and maintained at 25 °C. Spore suspension of *A. candidus* was prepared in sterile water and used for inoculation.

2.2. Medium and chemicals

Polished rice (Tsailai var. Taichung No. 10) was steamed and cooled for the solid-culture of *A. candidus*. Linoleic acid, butylated hydroxyanisole (BHA) and ferric chloride hexahydrate, were obtained from the Sigma Chemical Co. (St. Louis, MO). Ammonium thiocyanate, sodium dihydrogen phosphate, and disodium hydrogen phosphate were obtained from Wako Pure Chemicals (Osaka, Japan). Lard without added any antioxidant was obtained from the Chian-Kwuan Oil Co. (Kaohsiung, Taiwan). Silica gel 60 (70–230 mesh) was purchased from E. Merck (Darmstadt, Germany).

2.3. Culture conditions

Rice koji was produced according the method of Hoppe, Jha, and Egge (1997). Polished rice was steeped in distilled water for 1 h and steamed at 121 °C for 15 min for the purpose of sterilization. A suspension of *A. candidus* spores was sprayed onto the surface of the steamed (cooled) rice (inoculing of 10%, v/w) and incubated for 15 days.

2.4. Extraction and isolation of antioxidant substances from rice koji

The fermented rice (rice koji) was repeatedly blended and extracted with ethyl acetate. The mixture was shaken in a separatory funnel for 20 min. The organic layer was collected, dried over anhydrous sodium sulfate (1%, w/v), filtered, evaporated to dryness in vacuum, and weighed to determine the yields of soluble solids.

EtOAc extracts from the rice koji (20 g) were chromatographed on a silica gel column (600 × 66 mm i.d.). Among the eight fractions (A–H), fractions A–F were collected with the hexane/EtOAc gradient (80/20–0/100, v/v), and fractions G and H were collected with the EtOAc/MeOH (50/50–0/100, v/v) gradients. Fractions B and C were recrystallized with *n*-hexane/MeOH to obtain compound **1**. Fractions D, E and F were combined and further purified also using column chromatography, and compound **2** was derived through repeated column chromatography. Both compounds (**1** and **2**), were identified by means of spectrometry (Hayashi et al., 1995).

2.5. Spectrometry

The UV–vis absorption spectra of the active components in methanol were recorded on a Hitachi U-3000 spectrophotometer. The mass spectra of the components were obtained using the EI-MS mode on a JEOL-DMX 300 mass spectrometer. The nuclear magnetic resonance (NMR) spectra were measured in $CDCl_3$ and methanol-d_4 with a Brücker AC-250 NMR spectrometer operating at 299.95 MHz for ^1H NMR and 75.43 MHz for ^{13}C NMR with complete proton decoupling. The sweep width, pulse angle, repetition delay and acquisition time for ^1H NMR were 4500.0 Hz, 7.0 μs, 0 and 2.0 s, respectively, and for ^{13}C NMR were 25,000.0 Hz, 7.0 μs, 2.0 and 1.0 s, respectively. The chemical shifts are reported in parts per million (ppm) from tetramethylsilane.

2.6. Antioxidant activity determined using the thiocyanate method

The antioxidant activity of extract and purified compound on inhibition of linoleic acid peroxidation was assayed using the thiocyanate method (Mitsuda, Yasumodo, & Iwami, 1966). A 0.5 ml methanol solution of the isolated compounds was mixed with linoleic acid emulsion (2.5 ml, 0.02 M, pH 7.0) and phosphate buffer (2 ml, 0.2 M, pH 7.0). The linoleic acid emulsion was prepared by mixing 0.2804 g of linoleic acid, 0.2804 g of Tween 20 as emulsifier and 50 ml phosphate buffer, and then the mixture was homogenized. The reaction mixture was incubated at 37 °C to accelerate the oxidation process. The levels of oxidation were determined by measuring the absorbance at 500 nm on a Hitachi U-2000 spectrophotometer following reaction with ferrous chloride and ammonium thiocyanate. The antioxidant activity was expressed as a percentage of inhibition of peroxidation (IP%): IP% = [1−(absorbance of sample at 500 nm)/(absorbance of control at 500 nm)]×100. The antioxidant activities of BHA and α-tocopherol were also assayed at the same concentration for comparison purposes. All the tests were performed in triplicate and the results averaged.

2.7. Antioxidant activity determined using the Rancimat Method

Lard was used as the lipid substrate to evaluate the lipid oxidation inhibition activity of EAEAC, the isolated compounds and BHA. A Metrohm 679 Rancimat instrument was used in this experiment. A 0.5 ml methanol solution of EAERK was mixed with 2.5 g of lard in different glass cylinders. The oxidation experiments were carried out at 100 °C, and the flow rate of blowing air was 20 ml/min. For the control experiment, 0.5 ml of methanol was added to 2.5 g of lard, and the experiment was conducted under the same conditions as described earlier. The inhibitory activities of BHA and α-tocopherol dissolved in methanol (200 μg/ml) were also analyzed for comparison purposes. All the tests were performed in triplicate and the results averaged.

2.8. Scavenging effect on α,α-diphenyl-β-picrylhydrazyl (DPPH·) radicals

The scavenging effect of the isolated compounds on the DPPH radicals was estimated according to the method of Hatano, Kagawa, Yasuhara, and Okuta (1988). The samples of EAERK or BHA in 4 ml methanol were mixed with a 1.0 ml solution of DPPH radical in methanol. The mixture was shaken vigorously and left standing for 30 min, and the absorbance of the mixture at 517 nm was measured using a Hitachi U-2000 spectrophotometer. All the tests and analyses were performed in triplicate and the results averaged.

2.9. Statistical analysis

Statistical analyses were performed according to the SAS User's Guide. Analysis of variance was performed using the ANOVA procedure. Significant differences ($P < 0.05$) between means were determined using Duncan's multiple range tests.

3. Results and discussion

3.1. Antioxidant activity of EAERK on linoeic acid peroxidation

The antioxidant activity of EAERK, determined using the thiocyanate method, was compared with that of α-tocopherol, BHA and ethyl acetate extracts from steamed rice (EAESR), and the results are shown in Table 1. The antioxidant activity was evaluated in the methanol solvent system. At 200 μg/ml, EAERK exhibited 93% inhibition in the linoleic acid peroxidation system, which was significantly ($P < 0.05$) higher than that of α-tocopherol (82%) and EAESR (25%) but close to that of BHA (93%).

Ethyl acetate has been widely used as a solvent for the extraction of antioxidants from various sources, including 2,3-dihydroxy benzoic acid from *Penicillium roquefortii* (Hayashi et al., 1995), and indophenol-reducing phenol from *Moretierella* sp. (Hirota, Morimitsu, & Hijo, 1997). Both mycelial and broth filtrate extracts were found to exhibit high levels of antioxidant activity equal to that of BHA (99%) at a concentration of 200 μg/ml (Yen & Chang, 1999). It is suggested that the antioxidant activity of EAERK comes from the mycelia growing on the rice koji.

3.2. Prevention of lard oxidation

The Rancimat method is commonly used to evaluate the antioxidative potency of various antioxidants and is based on the increase of electrical conductivity due to the formation of volatile dicarboxylic acids as a result of lipid oxidation. The addition of EAERK to lard markedly extended the induction time of lipid oxidation (Table 2). The induction time of EAERK increased with increasing concentration and was significantly ($P < 0.05$) higher than that of control within the 50–200 μg/ml range. EAERK (100 μg/ml) exhibited strong antioxidant activity with a protection factor (PF) value of 16.4, which was substantially ($P < 0.05$) higher than that of BHA (5.3) at 200 μg/ml. Yen and Lee (1997)

Table 1
Antioxidant activity of ethyl acetate extracts from rice koji (EAERK) in the linoleic acid emulsion peroxidation system as measured by the thiocyanate method at 60 h

Sample[a]	Inhibition of lipid peroxidation (%)[b]
α-Tocopherol	81.5±2.0
BHA	93.3±0.8
EAESR	25.3±4.5
EAERK	93.2±0.1

[a] The concentration of all test samples was 200 μg/ml. EAESR is ethyl acetate extracts from steamed rice.
[b] Each value is mean±S.D. ($n = 3$).

Table 2
Prevention of lard oxidation by ethyl acetate extracts from rice koji (EAERK), as measured by the Rancimat method

Sample	Concentration (μg/ml)	Induction period (h)	Protection factor (PF)[a]
Control	–	2.3±0.1d[b]	1.0±0.0c
EAERK	50	14.5±0.6b	6.4±0.3b
	100	37.2±2.4a	16.4±1.8a
	200	>48.0	–
BHA	200	12.1±0.5c	5.3±0.4b

[a] PF (protection factor) = Induction period of sample (antioxidant)/Induction period of control (lard).
[b] Each value is the mean±S.D. ($n = 3$). Values in column with different superscripts are significantly different ($P < 0.05$).

reported that the extract has hydrogen donating ability and a synergistic effect with some known antioxidants. The higher antioxidant activity of the extract might result from the synergistic effect between the antioxidant components in the extract.

3.3. Scavenging DPPH radical

The DPPH radical is considered to be a model of a lipophilic radical. A chain reaction in lipophilic radicals was initiated by the lipid autoxidation. The scavenging effects of EAERK and BHA on DPPH radical are compared and shown in Table 3. According to the results shown in Table 3, EAERK had significant scavenging effects on the DPPH radical and the effects increased with increasing concentration in the range 200–800 μg/ml. There were no significant differences ($P > 0.05$) in the scavenging effects between 800 μg/ml and 1600 μg/ml (Table 3). Compared with that of BHA, the scavenging effect of EAERK was lower. Shimada, Fujikawa, Yahara, and Nukamura (1992) reported that the activity of antioxidants (compounds) corresponds to the number of hydrogens available for donation by hydroxyl groups. The scavenging effect of EAEAC is thought to be due to the antioxidative components, 3,3″-di-OH terphenyllin and 3-OH terphenyllin (Yen et al., 2001). The radical scavenging effects of EAERK might be due to the hydroxyl groups in the antioxidant extract.

3.4. Isolation and identification of antioxidant components from ethyl acetate extract of rice koji

Rice koji, cultured with *A. candidus*, was extracted with ethyl acetate and the extract yield (EAERK) was 0.84% (w/v). The major components in EAERK were isolated by means of silica gel column chromatography. Through column chromatographic separation on silica gel, the extract was separated into eight fractions (A–H). The antioxidant activities of fractions A–H are shown in Table 4. The IP% values of these fractions were C 89%, D 98%, E 99%, F 98%, G 91% and H 88% higher than that of α-tocopherol and close to that of BHA.

Fraction C was resolved in methanol and recrystallized from ethyl acetate to yield white noodle crystalline solid compound **1**. From UV–vis, MS, ^{1}H NMR and ^{13}C NMR data, compound **1** was identified as ergosterol, and the spectral characteristics of compound **1** were found to be identical to those reported by Arami, Hada, and Tada (1997).

Ergosterol is the main sterol component in the cell membrane of fungus. Though ergosterol and compound **1** exhibited no antioxidant activity in the linoleic acid emulsified peroxidation system (data not shown), they were reported to be capable of inhibiting lipid peroxidation in the cell membrane system (Wiseman, Laughton, Arnstein, Cannon, & Halliwell, 1992). The antioxidant activity of the sterol compound was thought to result from interaction between the hydrophobic aromatic ring of the sterols and the phospholipid residues in the cell membrane.

Fractions D–F were collected and combined for advanced column chromatography conducted using an integrated mobile phase *n*-hexane/ethyl acetate. The eluates were collected, combined and evaporated*in vacuum* to yield a pale yellow crystalline solid compound **2**. From UV–vis, MS, ^{1}H NMR and ^{13}C NMR data, compound **2** was identified as terphenyllin. The structure of compound **2** might be 2′,5′-dimethoxy-4,3′,4″-trihydroxy-*p*-terphenyl (terphenyllin), 3′,5′-dimethoxy-4,2′,4″-trihydroxy-*p*-terphenyl or 2′,3′-dimethoxy-4,5′,4″-trihydroxy-*p*-terphenyl. The spectral characteristics of compound **2** were found to be identical to those reported by Marchelli and Vining (Marchelli, Vining, & Terphenyllin, 1975) and identified as terphenyllin. The structure of compound **2** is shown in Fig. 1.

Table 3
Scavenging effect of ethyl acetate extracts from rice koji (EAERK) on α,α-diphenyl-β-picrylhydrazyl (DPPH•) radical

Sample	Concentration (μg/ml)	Absorbance at 517 nm	Scavenging effect (%)[a]
Control	–	0.4±0.0[b]	0.0±0.0
EAERK	200	0.27±0.00	25.5±0.8e
	400	0.18±0.05	58.5±2.6d
	800	0.08±0.00	78.0±0.3c
	1600	0.08±0.00	78.9±0.4c
BHA	200	0.04±0.00	88.4±0.4b
	400	0.03±0.00	90.9±0.4a
	800	0.03±0.00	91.3±0.1a
	1600	0.23±0.00	93.6±0.3a

[a] Scavenging effects (%) (capacity to scavenging DPPH radical) = [(absorbance of control at 517 nm) − (absorbance of sample at 517 nm)]/(absorbance of control at 517 nm) × 100.

[b] Each value is the mean ± S.D. ($n = 3$). Values in column with different superscripts are significantly different ($P < 0.05$).

Table 4
Antioxidant activity of fractions A–H of ethyl acetate extract from rice koji fractionated by silica gel column chromatography

Fractions[a]	Inhibition of lipid peroxidation (%)[b]
A	0±0
B	0±0
C	89.1±0.2
D	97.6±0.2
E	99.2±0.2
F	98.4±0.3
G	90.5±0.2
H	88.3±0.2

[a] The concentration of all test samples was 200 μg/ml.

[b] Each value is mean ± S.D. ($n = 3$).

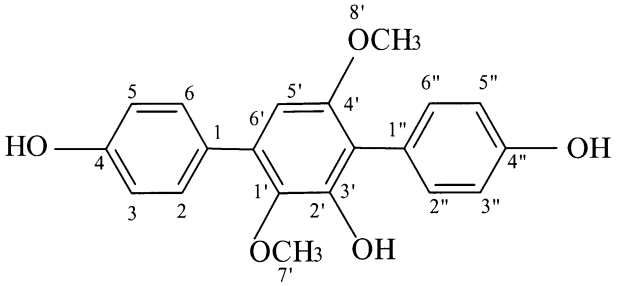

Fig. 1. Structure of antioxidant compound **2** isolated from the ethyl acetate extracts of rice koji fermented with *Aspergillus candidus* CCRC 31543.

3.5. Antioxidant activity of compound 2

The antioxidant activity of compound **2** is shown in Table 5. According to the results shown in Table 5, compound **2** exhibited an IP% of 99%, which was significantly ($P < 0.05$) higher than that of EAERK (97%) and close to that of BHA. The structure of terphenyllin includes three phenolic hydroxyl groups and an *ortho*-methoxy substitution relative to the hydroxyl group in the central aromatic ring. The strong antioxidant activities of these compounds are believed to be a result of these hydroxyl groups and the *ortho*-methoxy substituent in their chemical structures.

The activity of the compounds corresponds to the number of hydrogen atoms available for donation by hydroxyl groups (Shimada et al., 1992), and the radical scavenging effects of EAERK may also be due to the three hydroxyl groups of terphenyllin. Moreover, the *ortho*-methoxy group on the central aromatic rings also stabilizes the aryloxy radical by means of electron donation, thereby increasing both the antioxidant and antiradical efficiency (McMurry, 1984). In our previous study (Yen et al., 2001), *A. candidus* metabolites 3,3″-di-OH terphenyllin, 3-OH terphenyllin and candidusin B, were proved to have no cytotoxicity toward human intestine cells (Int 407) at 10–200 μg/ml and toward *typhimurium* TA98 or TA100 at 200 and 500 μg/ml per plate, either with or without S9 mix. Moreover, single cell gel electrophoresis assay (comet assay) also revealed that these three compounds caused no significant ($P >$ 0.05) DNA damage in Int 407 cells at 0–100 μg/ml. Though such a safety evaluation was not performed for compound **2** in this study, it can be expected that the results for compound **2** would be similar to those for the above three compounds, since the structure of compound **2** is similar to those of the three compounds.

Table 5
Antioxidant activity of isolated compound **2** from the ethyl acetate extracts of rice koji (EAERK) fermented with *Aspergillus candidus* CCRC 31543

Sample[a]	Inhibition of lipid peroxidation (%)[b]
BHA	99.6±0.1
Compound **2**	99.1±0.1
EAERK	96.6±0.3

[a] The concentration of all test samples was 200 μg/ml.
[b] Each value is mean±SD ($n = 3$).

Results obtained in this study clearly demonstrate that ethyl acetate extract of rice koji fermented with *A. candidus* exhibited a significant antioxidant activity. The compound terphenyllin, isolated from the ethyl acetate extract of *A. candidus* rice koji, had a high level of antioxidant activity and is believed to be the main antioxidant component of the extract. More safety evaluation of terphenyllin and EAERK should be conducted. Further investigations to understand their antioxidant mechanisms more thoroughly and to examine their biomedical effects are underway in our laboratories.

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