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Antioxidant activity of pigments derived from *Monascus purpureus*-fermented rice, corn, and sorghum

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Abstract

A mixture of pigments derived from *Monascus* has been shown to possess antioxidant activity, but specific pigments responsible for the activity have not been identified yet. This is the first investigation on the contribution of *Monascus* pigment compounds to the antioxidant activity. This research focused on the antioxidant activity of *Monascus* pigments (MP) produced by a solid state fermentation with rice, corn, whole sorghum grain (WSG), dehulled sorghum grain (DSG) and sorghum bran (SB) substrates. The antioxidant activity of the pigments was evaluated in vitro by 2,2-diphenyl-1-picrylhydrazyl (DPPH)-scavenging activity method. The profile and compounds of pigments were analyzed by using thin layer chromatography and liquid chromatography coupled with tandem mass spectrometry, respectively. These results showed that DPPH-scavenging activities of MP were in a range of 588 and 2,950 µmol Trolox Equivalent (TE)/100g. Rice-MP showed the highest DPPH-scavenging activity, followed by DSG-MP, WSG-MP, SB-MP and Corn-MP, while no DPPH-scavenging activity was detected in a commercial Beni Koji pigments. The higher amount of pigments was found to have the higher antioxidant activity. Pearson correlation analysis of the scavenging activity of DPPH and the amount of individual pigment showed that 2 compounds those are Monapilol B and rubropunctamine have a high correlation with the r value of 0.973 and 0.968, respectively. It was estimated that Monapilol B possesses higher contribution (64%) to the DPPH scavenging activity than that of Rubropunctamine (33%).

Introduction

Monascus pigments that consist of yellow, orange, and red pigments have been produced by the solid state fermentation with rice substrate and utilized by people in Asian countries since centuries ago (Pattanagul *et al.*, 2007). The pigments have been used in the development of natural food colorants and health products since the pigments bear various bioactivities. Historically, Asian people had utilized *Monascus*-fermented rice as a traditional medicine for its potential health benefits such as healthy heart and circulatory system, and for the treatment of blood stasis, disorders related to dyslipidemia, and atherosclerosis (Wang and Lin, 2007). Consequently, scientific investigation on health benefit of the pigments increases gradually.

Aniya *et al.* (1999) have initiated the investigation on the antioxidant activity of *Monascus* fermented product through the screening of 40 fungi species and showed 13 species with a high DPPH-scavenging activities. Antioxidant activity of the pigments extracted from *Monascus*-fermented rice (Aniya *et al.*, 2000; Yang *et al.*, 2006; Dhale *et al.*, 2007; Chairote *et al.*, 2009) and *Monascus*-fermented corn extract (Konbangkerd *et al.*, 2014; Kraboun *et al.*, 2013) has been reported. Sorghum (in the form of whole sorghum grain, dehulled sorghum grain and sorghum bran) (Srianta and Harijono, 2015) was also utilized for the *Monascus* fermentation, but the antioxidant acivity of the pigments derived from the fermented sorghum has not been studied. In studies on the antioxidant activity of the ethanolic extract from *Monascus*-fermented rice, Chairote *et al.* (2009) have

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shown that the darker red pigment exhibits the higher antioxidant activity. Kraboun *et al*. (2013) reported that the antioxidant activity of ethanolic extract from *Monascus*-fermented corn is consistent with the intensity of pigment. Our previous finding showed that pigments derived from *Monascus purpureus*-fermented rice, corn and sorghum contains a mixture of yellow, orange and red pigments. Twelve pigment compounds were detected in pigments extracted from those fermented cereals (Srianta *et al*., 2016). However, there is no report on the identification of pigments contributing to the antioxidant activity.

The objectives of this research were to compare antioxidant activities in pigments from *Monascus purpureus*-fermented rice, corn, and sorghum substrates, and to identify pigment compound(s) that are responsible for the antioxidant activity.

Materials and Methods

Microorganism

M. purpureus was isolated from commercial *Monascus*-fermented rice (MFR) and identified as *M. purpureus* M9 (NCBI Accession Number: HM188425.1). *M. purpureus* culture was maintained on potato dextrose agar (PDA) slant and sub-cultured monthly. *M. purpureus* starter was prepared by inoculating *M. purpureus* culture stock onto a PDA slant, incubated at 30˚C for 7 days, and then used for solid state fermentation.

Solid state fermentation

Substrates of rice, corn, and sorghum were individually prepared. In the case of sorghum, three samples of whole sorghum grain (WSG), dehulled sorghum grain (DSG) and sorghum bran (SB) were prepared. About 20 g of each substrate were added into a jar containing 15 mL of distilled water and sterilized at 121˚C for 20 min. Solid state fermentation was carried out by the inoculation of 1.5 mL of *M. purpureus* starter culture containing 5 x 10^5 spores/mL into the sterilized substrate, followed by incubation at 30˚C for 14 days. A sample of fermented material was taken daily, dried at 45˚C for 24 h and subjected to analysis of the biomass amount and pigment extraction.

Preparation of Monascus-*pigment extracts and pigments separation*

Preparation of *Monascus*-pigment extracts were carried out according to Chairote *et al.* (2009). One hundred milligram of each *Monascus*-fermented sample was transferred into a tube and mixed with 2 mL of 75% ethanol. The mixture was treated in an ultrasonic bath for 60 min, followed by centrifugation at 3,000 rpm for 15 min. The solid was re-extracted twice by the same procedure. The collected supernatants were mixed with 75% ethanol and mixed with solvent up to 10 mL in a volumetric flask. Pigment extracts from the solid fermented materials of *M. purpureus* on rice, corn and sorghum were called as MPE-rice, -corn and –sorghum, respectively, and on WSG, DSG and SB of sorghum as MPE-WSG, -DSG and -SB, respectively. A commercial *Monascus* pigment (purchased from Kanto Chemical Co., Tokyo, Japan) was used as a control.

Analysis of pigments in MPE and Beni Koji Pigment (Kanto Chemicals, Japan) was carried out by using two methods, thin layer chromatography (TLC) for the comparison of pigment profiles and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) for the determination of molecular weight of pigments. The TLC method was performed according to Nimnoi and Lumyong (2011). Three microlitter of ethanol extracts prepared as described above was applied onto a Silica Gel 60 F254 plate (Merck, Germany) and pigments were separated with a mobile phase consisting of chloroform:methanol:water = 90:25:4. Two bands of separated pigments that showed a high correlation to antioxidant activity were scrapped and collected. The collected sample was dissolved in 75% ethanol and centrifuged at 3,000 rpm for 15 min. The supernatant was used for antioxidant activity assay and pigment composition by using LC-MS/MS. Peak area was estimated from data recorded by using Analyst 1.5.1 software version.

Antioxidant activity assay and pigment composition analysis of MPE

In vitro antioxidant activity of *Monascus*-pigment extracts (MPEs) was measured by the 2,2-diphenyl-1-picrylhydrazyl (DPPH)-scavenging method (Brand-Williams *et al*., 1995). In this assay, the purple chromogen radical is reduced by antioxidant/reducing compounds to the corresponding pale yellow hydrazine. The reducing ability of antioxidants towards DPPH was evaluated by monitoring the decrease of absorbance at 517 nm (Karadag *et al*., 2009). Fifty microliter of sample solution of MPE or standard Trolox solution put into microplate wells and measured the absorbance at 517 nm as sample blank. After that, 100 µL of DPPH solution was added into each well and incubated at room temperature for 30 min. The absorbance of the incubated mixture was measured at 517 nm by using a multi-detection microplate reader power scan HT

(BioTek Instrument DS Pharma Biomedical, USA) and Gen 5TM microplate data collection with analysis software. The antioxidant activity was expressed as µmol Trolox Equivalent (TE)/100 g of sample and IC_{50}. IC_{50} was determined through antioxidant analysis of a series of concentration of MPE in a range of 1-12 mg/mL. Pigment composition of MPE was analyzed according to Miyake *et al*. (2008) with some modifications as described by Srianta *et al.* (2016).

Analysis of pigment contribution to the antioxidant activity

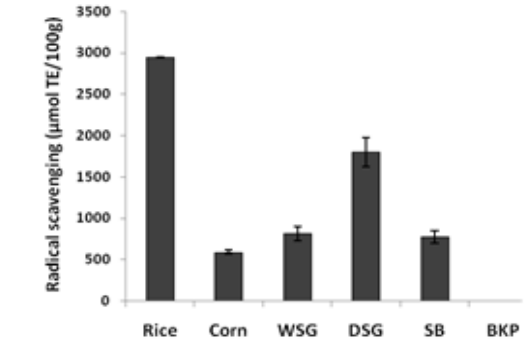
Contribution of pigments in MPEs to the antioxidant activity was estimated from correlation value between total of all pigment detected in MPE and the antioxidant activity in MPEs. The correlation was calculated by using Pearson correlation analysis (Kraboun *et al.*, 2013). If the pigment in MPEs possess high correlation value, contribution of each detected pigment to the antioxidant activity was then estimated. Contribution of each detected pigment compound to the antioxidant activity was estimated from correlation value between each pigment quantity and the antioxidant activity in various MPE. Higher correlation value means higher contribution of the pigment to the antioxidant activity.

Pigment compounds with significant correlation were then further analyzed to confirm the contribution. Separated pigment of MPE-rice on TLC sheets was used for the confirmation analysis. Two suspected bands were collected from a number of TLC sheets to get the equivalent amount of pigment in MPE. The collected band was solubilized in 75% ethanol, and then was analyzed for the antioxidant activity and pigment compound analysis according to Miyake *et al.* (2008) with some modifications as described by Srianta *et al.* (2016).

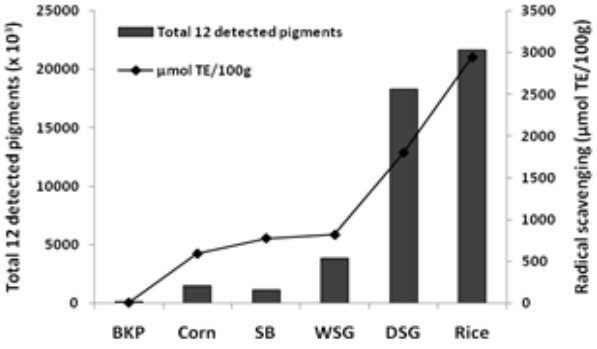
Results and Discussion

Antioxidant activity of MPE-rice, -corn, and -sorghum

Antioxidant activity of natural resources generate great interests due its potential health effects in preventing the negative effect of free radicals in human body and deterioration of foodstuffs (Molyneux, 2004). In this research, the DPPH-scavenging activity as an antioxidant activity of MPE-rice, -corn, -WSG, -DSG and -SB and of a commercial beni koji pigment (BKP) as a control (Figure 1(a)) were analyzed. The results showed that their DPPH-scavenging activities were in a range of 588 and 2,950 µmol TE/100g with IC_{50} value in the range of 1.79 and 10.6 mg/mL.The antioxidant activity levels of the MPEs used in this study are comparable to those of extracts of *Monascus*-fermented rice (IC_{50} of 4.38 mg/mL) and *Monascus*-fermented adlay (IC_{50} of 12.45 mg/mL) (Tseng *et al*., 2006; Yang *et al*., 2006) and of some foods such as red onion (IC_{50} of 1.89 mg/mL), guava (2,520 µmol TE/100g), red grapes (1,350 µmol TE/100g) and garlic (IC_{50} of 6.40 mg/mL) (Prakash *et al*., 2001; Thaipong *et al*., 2006; Qusti *et al*., 2010).



(a)



(b)

Figure 1. (a) DPPH-scavenging activities of MPE-rice, -corn, -WSG, -DSG and -SB. Sample preparation and analysis of DPPH-scavenging activity were performed as described in Materials and Methods. Standard deviation was calculated from the obtained data in triplicate experiments. (b) Correlation between DPPH-scavenging activity and amount of total 12 detected pigments in various MPEs. DPPH-scavenging activity (dotts and lines) and amount (columns) of total 12 detected pigments in various MPEs were plotted.

Among those MPEs, MPE-rice showed the highest DPPH-scavenging activity, followed by MPE-DSG, MPE-WSG, MPE-SB and MPE-corn, whereas BKP showed no DPPH-scavenging activity. Pearson correlation analysis between the antioxidant activity and the total of 12 pigment compounds detected in MPEs showed a significant possitive correlation ($r=0.948$, $p\leq0.05$) describe in Figure 1(b). This result is consistent with previous reports that the antioxidant activities of MPE-rice and -corn are correlated to their color and pigment contents

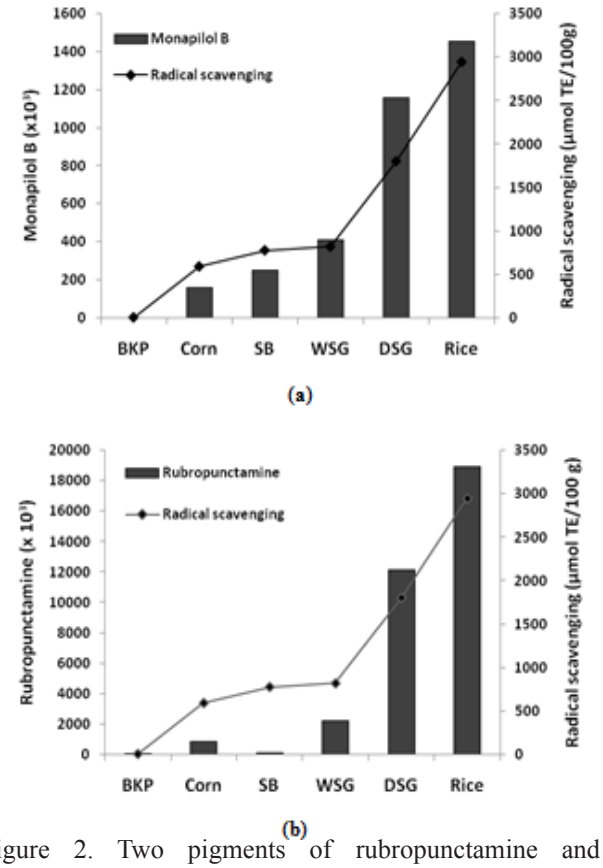


Figure 2. Two pigments of rubropunctamine and monapilol B showing a highest correlation between its DPPH-scavenging activity and amount in various MPEs. DPPH-scavenging activity (dotts and lines) and amount (columns) of monapilol B (a) and rubropunctamine (b) in various MPEs were plotted.

(Chairote *et al.*, 2009; Kraboun *et al.*, 2013). MPEs *Monascus* pigment is a mixture of many polyketide pigment compounds. Since there was no report on compounds of antioxidant pigments in *Monascus*-fermented materials, pigment compounds in MPEs was analyzed by using LC-MS/MS.

Antioxidant pigments contribution to the antioxidant activity of MPEs

The data of pigment composition of MPEs (data not shown) were used to analyze the contribution of each pigment compound to the antioxidant activity of MPEs. The 12 detected pigments were rubropunctatin, monascorubrin, rubropunctamine, monascorubramine, monascin, ankaflavin, xanthomonascin A, xanthomonascin B, monascopyridine A, monascopyridine B, yellow II and monapilol B. Among the 12 detected pigments, only 2 pigment compounds showed significant correlation i.e. rubropunctamine ($r=0.968$, $p\leq0.01$) and monapilol B ($r=0.973$, $p\leq0.01$), whereas r values of other pigments were in a range of -0.145 and 0.898, suggesting that the 2 pigment compounds are main antioxidants in the MPEs. Figure 2 showed the

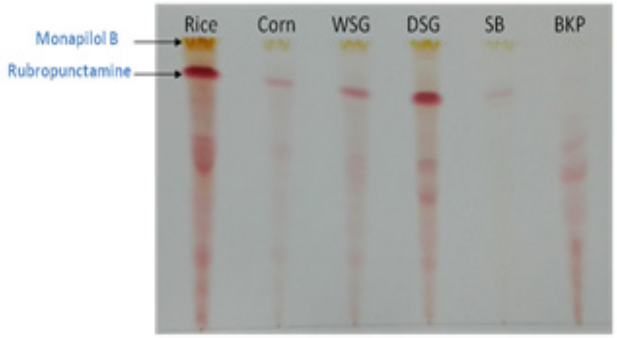


Figure 3. Pigments profile of MPE-rice, -corn, -WSG, -DSG and -SB on TLC. Sample preparation and TLC analysis were performed as described in Materials and Methods.

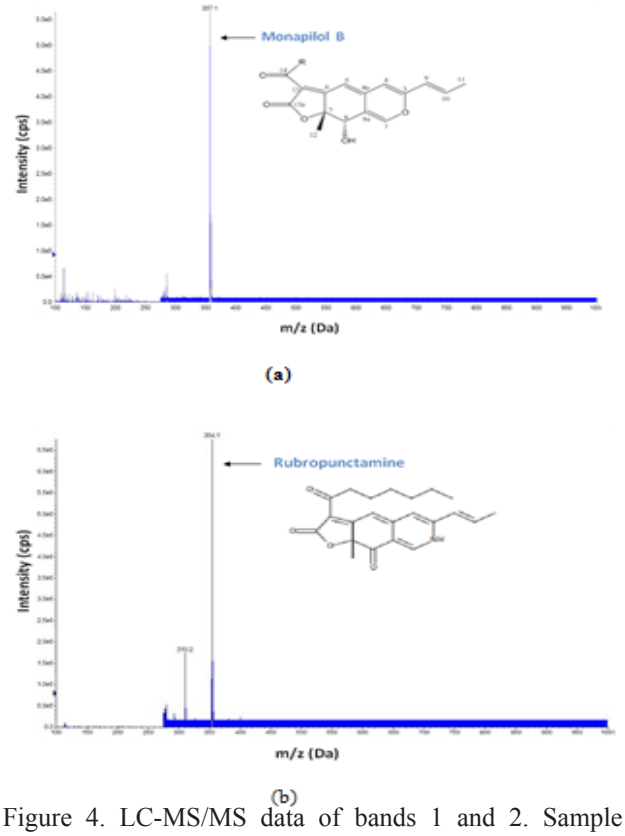


Figure 4. LC-MS/MS data of bands 1 and 2. Sample preparation and LC-MS/MS analysis were performed as described in Materials and Methods. (a) Band 1 was found to be rich in Monapilol B with +EMS of 357.1 *m/z* and (b) band 2 rich in Rubropunctamine with +EMS of 354.1 *m/z*. Chemical structures of monapilol B ($R=C_5H_{11}$) in figure 4(a) and rubropunctamine in figure 4 (b) were reproduced from references of Hsu *et al.* (2011) and Patakova (2013), respectively.

correlation of antioxidant activity and the pigment compounds. Pigment pattern on TLC-silica gel as shown in Figure 3 and pigment composition data allowed us to consider that top two bands were pigments responsible for antioxidant activity of MPEs since BKP as a control that had no antioxidant activity (Figure 1) showed no corresponding bands.

Thus, to confirm their antioxidant activity, the two bands in yellow-orange and red were subjected to analyses of antioxidant activity and LC-MS/MS.

As shown in Table 1, both bands showed DPPH-scavenging activity and the yellow-orange band (band 1) exhibited higher antioxidant activity than that of the red band (band 2). The chromatogram of LC-MS/MS (Figure 4) revealed that there were one major peak from each band and the molecular weights of the peaks from band 1 and band 2 were identical to those of monapilol B and rubropunctamine, respectively, indicating that bands 1 and 2 were rich in monapilol B pigment and rubropunctamine pigment, respectively. Based on these findings, it is suggested that monapilol B and rubropunctamine are antioxidant pigments, with relative specific activity of 1.28 and 0.05, respectively (Table 1). Monapilol B was the highest contributor to the antioxidant activity in MPEs tested in this study. Notably, it has been reported that monapilol B and rubropunctamine possess an anticancer activity (Akihisa *et al*., 2005; Hsu *et al*., 2011). From the chemical structures (Figure 4), the hidrogen donating groups, -NH and -OH of rubropunctamine and monapilol B, respectively, might play an important role in the DPPH radical scavenging. Those antioxidant pigments are structurally categorized in antioxidants having weak O-H or N-H bonds (Flora, 2009). Further study on the production and application of both antioxidant pigments as well as their basic research are required.

Conclusions

MPEs possess DPPH-scavenging activities in the range of 588 and 2,950 µmol TE/100g, being equivalent to some foods such as red onion, guava, red grapes and garlic, and MPE-rice showed the highest DPPH-scavenging activitiy, followed by MPE-DSG, MPE-WSG, MPE-SB and MPE-corn. High possitive correlation between antioxidant activity and pigment content was found in MPEs tested and monapilol B and rubropunctamine are suggested to be antioxidant pigments. Monapilol B was a main contributor to the antioxidant activity in MPEs.

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