EPR and HPLC Investigation of Pigments in Thai Purple Rice

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Abstract: We investigated the pigments in Thai purple rice using electron paramagnetic resonance (EPR), X-band (9 GHz) EPR imaging (EPRI), and HPLC. The location and spatial distribution of the paramagnetic species in purple and white rice were determined by EPR and EPRI. EPR primarily detected three paramagnetic species in purple rice, which were identified as stable radicals, Mn2+, and Fe3+ based on the g-values and hyperfine components of the EPR signals. Subsequent noninvasive two-dimensional (2D) EPRI revealed that these stable radicals are primarily located in the pigmented region of purple rice, while very few radicals were observed in the interior of the rice. HPLC revealed that the major compounds were cyanidin-3-O-glucoside and peonidin-3-O-glucoside. Scavenging activities, EPR, and EPRI imaging results indicate that the stable radicals contain the radical state of anthocyanins and are mostly found within the pigmented embryo region of purple rice. They could be either associated with scavenging activities or could be one of the products of their oxidative decomposition.

Key words: purple rice, anthocyanin, pigment, HPLC, EPR, imaging

1 INTRODUCTION

Thai purple rice is attracting attention for its antioxidant effects and health benefits1–3. The purple color of this rice is due to the deposition of large amounts of the anthocyanin pigment. Certain compounds in the rice have been recognized as health-enhancing substances because of their antioxidant, anti-inflammatory, and anticancer effects1–3. Although the total contents of useful chemicals in such foodstuffs have been determined via analyses of powdered food crops, we have very limited knowledge about the distribution and concentration of useful chemicals within the crops.

Free radicals are generated in plants as a result of antioxidant scavenging activities and biochemical processes4–7. In most cases, stable paramagnetic species are found in the pigmented (colored) regions of plant seed coats5–7. These pigmented regions usually contain various organic compounds such as antioxidants. Electron paramagnetic resonance (EPR) can be used for the nondestructive detection of free radicals. The EPR spectrum appears either as an asymmetric line shape or as a series of multiple overlapping lines, depending on the sample being assessed4–7.

The X-band (9 GHz) EPR imaging (EPRI) technique exhibits good spatial resolution and sensitivity. Several reports have described its application to investigate free radicals in naturally occurring high-value crops4–7. Noninvasive EPRI and EPR spectroscopy have provided detailed information regarding the location and concentration of paramagnetic species (e.g., transition metal ions, transition metal complexes, and stable organic radicals) in naturally occurring biological samples. Application of these techniques has revealed that the stable radicals are primarily located in the seed coat, while very few radicals were observed in the seed cotyledon. More specifically, these results indicate that stable radical species are only found within the seed coat, and few radical species are found in other seed parts5–7. These stable radicals could be the products of antioxidant reaction processes.

In addition to Thai purple rice, Japanese black rice (Shi-kokumai) also contains pigments, particularly anthocyanins8. Many papers have reported the radical scavenging and other beneficial functions of anthocyanins8. EPR detected paramagnetic species and the aforementioned functions of anthocyanin in black rice9. EPRI revealed that stable radicals are distributed in the exterior of rice. However, little is known about endogenous paramagnetic species (e.g., Mn2+) and organic radicals present in rice. EPRI could be a useful tool for obtaining such information.

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In this study, paramagnetic species in physically and chemically untreated rice were investigated using X-band EPR, noninvasive two-dimensional (2D) EPR, scavenging effect, and HPLC. EPR was carried out to detect paramagnetic species in whole rice, whereas 2D EPR was used to demonstrate the spatial distribution of stable (unreactive) organic radicals within the rice grains. Possible antioxidants present in the extracted purple rice pigment fraction were also characterized using HPLC. The localization and concentration of the endogenous stable radicals within the rice are also discussed.

2 EXPERIMENTAL

2.1 Samples

Khao Gam Pah E-Kaw (purple rice) was collected from the Mae Hong Son Rice Research Center, Mae Hong Son Province, and Niaw San-Pah-Taung (white rice) was harvested from the Chiang Mai Rice Research Center, Chiang Mai, Thailand, in November 2016. These samples were used for EPR without any chemical or physical treatment. Black rice (Murasaki no kimi) was harvested from a rice paddy located in the far north (Hirosaki, Aomori Prefecture) of the main island in Japan, in the autumn of 2015, and was milled after harvesting. For EPR measurements, the rice grains (0.0230–0.0385 g/rice) were sequentially inserted into an EPR tube (outer diameter, 5.0 mm; inner diameter, 4.0 mm; Wilmad LabGlass, Buena, NJ, USA) or an EPR rod (outer diameter, 5.0 mm).

Chemicals for EPR analyses were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Cyanidin-3-O-glucoside chloride and peonidin-3-O-glucoside chloride were purchased from Extrasynthese Co., Ltd. (Genay, France) and used as received.

2.2 EPR and EPRI measurements

A JEOL RE-3X 9 GHz EPR spectrometer (JEOL Co. Ltd., Tokyo, Japan) was used for continuous wave (CW) measurements. The system was operated at 9.43 GHz using a 100-kHz modulation frequency. All CW EPR spectra were obtained in a single scan. Typical CW EPR settings were as follows: microwave power, 5 mW; time constant, 0.1 s; sweep time, 4 min; magnetic field modulation, 0.32 mT; and magnetic field sweep width, 5 – 300 mT.

A modified JEOL RE-3X 9 GHz EPR spectrometer was used for EPR imaging. A detailed description is available elsewhere. All measurements were performed at ambient temperature.

2.3 Anthocyanin quantification by HPLC

The extracted samples of the pigmented part of purple rice were analyzed using HPLC (Agilent 1100), in accordance with a modified version of the method reported by Pengkumsri et al. and Prior et al. Briefly, purple rice (5.0 g) was extracted with 2% HCl in methanol (100 mL) using a shaking incubator at 150 rpm and 50°C for 30 min. Subsequently, the supernatant was filtered through a 0.45-μm filter for HPLC analysis. The wavelength for UV detector analysis was set at 520 nm. Symmetry Shield RP18 column (diameter = 250 x 4.6 mm) obtained from Waters Co., Ltd. was used for this purpose. The mobile phase consisted of acetonitrile and 4% phosphoric acid. The linear gradient elution was operated from 0 to 40 min, with acetonitrile of 10–20% (flow rate of 1.0 mL/min, injection volume of 10 μL). The anthocyanin standards including delphinidin-3-glucoside, cyanidin-3-O-glucoside, delphinidin, peonidin-3-O-glucoside, and malvidin-3-O-glucoside were purchased from Extrasynthese Co., Ltd. (Genay, France).

2.4 Determination of antioxidant activity

2.4.1 ABTS assay

2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) free radical cation decolorization assay was carried out using an improved version of the method reported by Saenjum et al. Briefly, ABTS” was generated by the oxidation of 7.0 mM ABTS with 2.5 mM potassium persulfate for 16 h in the dark at room temperature (stock solution). Then, the ABTS” stock solution was diluted with absolute ethanol to give an absorbance of 0.7 ± 0.2 at 734 nm before being used (ABTS” working solution). Different concentrations of the tested samples, along with the standard L-ascorbic acid, Trolox, and quercetin, were mixed with the ABTS” working solution. The decrease in the absorbance was measured after incubation in the dark for 5 min at room temperature. All measurements were carried out in triplicate. The results are expressed as vitamin C equivalent antioxidant capacity (VCEAC), Trolox equivalent antioxidant capacity (TEAC), and quercetin equivalent antioxidant capacity (QEAC).

2.4.2 Scavenging effects on superoxide anion

The scavenging effects of Thai purple rice and white rice extract on superoxide anions were assayed following the method of Saenjum et al.2. Superoxide anion radicals were generated in a phenate methosulfate (PMS)–β-nicotinamide adenine dinucleotide (NADH) system by the oxidation of NADH and analyzed by the reduction of nitroblue tetrazolium (NBT). The reaction was performed in 200 μL of PBS buffer (pH 7.4, containing NADH, NBT, and EDTA) in a 96-well plate, with different concentrations of the tested sample. PMS was added to initiate the reaction. After 5 min of incubation in the dark at room temperature, the absorbance was measured at 560 nm using a Beckman Coulter microplate reader. L-ascorbic acid and cyanidin-3-O-glucoside were used as positive controls. All samples were tested in triplicate. The results are expressed as the 50% inhibition concentration value (IC50, μg/mL).
3 RESULTS AND DISCUSSION

3.1 EPR of rice

Figure 1 shows the EPR spectra of (A) purple and (B) whole white rice, which were obtained with a 300 mT sweep width. The EPR spectrum of purple rice exhibited three distinct signals, which were stable for at least a few months, and corresponded to Fe$^{3+}$, Mn$^{2+}$, and organic radicals. The first signal was characteristic of the Mn$^{2+}$ paramagnetic center ($M_i = 5/2$)-related sextet. The apparent increases in the hyperfine couplings while moving from low to high fields were attributed to the Mn$^{2+}$ moiety and the overlap of other paramagnetic centers.

The second signal was strong and reproducible. The relatively broad single peak observed at $g = 2.00_0$ was indicative of stable organic radicals, suggesting the possibility of the radical being generated during scavenging activities and the presence of antioxidant-related organic compounds in the rice. The featureless EPR signal for the organic radicals can be due to the delocalization of unpaired electrons throughout the aromatic ring and relatively weak interactions with the neighboring nuclei. Figure 2 shows the EPR spectrum of the central region ($g = 2.00_0$). The distorted baselines of the spectra occur owing to the overlap with the Mn$^{2+}$ signal and other paramagnetic species. The peak-to-peak line width ($\Delta H_{pp}$) of the signal was $\sim 0.63$ mT. In contrast, the EPR spectrum of white rice showed a very small signal. The radicals can be organic radicals and/or carbon centered radicals based on the $g$-value obtained. The concentration was estimated by comparison with a TEMPO solution (known concentration) in a capillary tube (outer diameter, 1.0 mm; inner diameter, 0.9 mm). The number of spins per sample for the purple rice sample was $\sim 3.2 \times 10^{17}$.

The third signal was characteristic of Fe$^{3+}$ (the $g$-values of $\sim 4.34_4$ and $\sim 3.21_4$ at a lower magnetic field with filled triangles, as shown in Fig. 1). The signal at $g = 4.34_4$ shows the characteristic peak for high-spin iron, while that at $g = 3.21_4$ could be low-spin iron; however, this could not be confirmed without knowing the other components of the signals ($g_x$ and $g_y$). In order to further consider the relationship between Fe$^{3+}$ and organic radicals, we analyzed black rice. Figure 1 (C) shows the EPR spectrum of milled black rice. The Fe$^{3+}$ signal was not observed in black rice at a low magnetic field. The EPR spectrum of black rice revealed two distinct signals, which corresponded to Mn$^{2+}$ and organic radicals. In addition, we identified other paramagnetic species in the central region with very broad and intense signals. We also found similar signals for the husk.

In the case of white rice, we observed all three distinct signals, but the intensity of the organic radicals was lower than that seen in purple rice (Figs. 1 and 2). The signal intensity of the organic radicals was also very low in the endosperm region. Additionally, in white rice, the signal of the organic radicals in the husk was more intense than that

![Fig. 1](image1.png)  
**Fig. 1** EPR spectra of (A) whole purple, (B) white, and (C) black rice. The sweep width was 300 mT. The filled triangles indicate the Fe$^{3+}$ signal at $g = 4.34_4$. Each spectrum was taken at a single scan.

![Fig. 2](image2.png)  
**Fig. 2** EPR spectra of (A) purple and (B) white rice at the central region ($g = 2.00_0$). The sweep width is 10 mT.
K. Nakagawa, W. Yooin, and C. Saenjum

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The EPR spectra of the husks of purple and white rice. The spectra show the presence of Fe\(^{3+}\), Mn\(^{2+}\), and the organic radicals, as well as strong paramagnetic species. The EPR spectra are similar to those in Fig. 1.

The signal of the organic radicals for white rice is much less intense than that seen in the purple rice spectra. In addition, when the sample weight is taken into account, the intensity of the Fe\(^{3+}\) signal for white rice is stronger than that for purple rice. In some cases, the iron ion is involved in iron-mediated reactions such as the Fenton reaction.

The reactions produce reactive oxygen species (ROS) as follows:

\[
\text{Fe}^{2+}\text{-complex} + \text{M} \rightarrow \text{Fe}^{3+}\text{-complex} + \text{ROS} \quad (1)
\]

\[
\text{ROS} + \left(\text{Antioxidants and/or others}\right) \rightarrow \text{Stable organic radicals} \quad (2)
\]

If the iron-mediated reaction is the main source of ROS, the Fe\(^{3+}\) concentration increases with increasing stable antioxidant radicals (reaction 2). However, we observed a less intense Fe signal for the purple rice than the white rice. In the case of black rice, we observed relatively strong signals from organic radicals, although the signal from iron was absent (Fig. 1(C)). We concluded that the iron-mediated reactions may not be the main reaction in this case. Moreover, Mn\(^{2+}\) signals are weaker than those of the endosperm, in both types of rice. Hence, for further investigation, we focused on the organic radicals in purple rice.

3.2 2D EPRI of purple rice

To study the organic radicals present in purple rice in further details, we performed EPRI studies because the signal intensity of white rice was very weak. Figure 4 shows a sample image and an EPR image of the purple rice obtained using a scan width of 5 mT at the central region (\(g = 2.00\)) of the spectrum in Fig. 2. The dashed line shows the approximate size of the rice. Based on the \(\Delta H_{pp}\) value (\(\sim 0.63\) mT), the spatial resolution of the rice EPRI was estimated to be \(\sim 0.19\) cm. It is noted that not all pigments are EPRI-detectable radicals (at low levels), especially for EPRI.

The signal overlap around the central region has been mentioned. The EPR baseline of the purple rice is not straight. Although we adjusted the baseline before data processing (e.g., convolution and deconvolution), the background of the overlapped signals tends to create artifacts.

Notably, the EPRI results showed that the organic radicals are mostly distributed near the embryo region of the purple rice. EPRI studies of dry embryos of rice seeds (\textit{Oryza sativa} L.) stored in a natural (warm and humid) environment showed free radical accumulation\(^{14}\). We speculate that the embryo region may have higher scavenging and/or oxidant activities than other regions. While the results are very different from those in black rice\(^5\), the EPRI observation is similar to that reported for sesame seeds\(^5\). We observed organic radicals, which were located on the outer surface region of the rice (Fig. 3(B)).
3.3 HPLC analyses of purple rice

Figure 5 shows an HPLC chromatogram of purple rice extract. The largest peak in the chromatogram was assigned to cyanidin-3-O-glucoside, as determined by comparison with the specific retention time and absorption spectra of the authentic standard, which usually corresponds to anthocyanin. The anthocyanins were quantified by determining the peak areas in HPLC chromatograms; the concentrations of cyanidin-3-O-glucoside and peonidin-3-O-glucoside were found to be 87.5 and 32.3 mg/100 g dry weight, respectively. The anthocyanin content of Khao’Gam Pah E-Kaw (purple rice) extract. HPLC conditions were the same for both.

Fig. 5  (1) HPLC elution profiles of authentic anthocyanin standards and those of the mixed ones. A: delphinidin-3-O-glucoside; B: cyanidin-3-O-glucoside; C: delphinidin; D: peonidin-3-O-glucoside; and E: malvidin-3-O-glucoside. (2) HPLC chromatogram of Khao’Gam Pah E-Kaw (purple rice) extract. HPLC conditions were the same for both.

Scavenging activities may be responsible for the origin of stable radicals in the rice sample. The scavenging reaction scheme presents a possible explanation for stable radical production. We propose that intermediate stability (i.e., an unreactive state) plays a key role in antioxidant reactions or scavenging activity\textsuperscript{16}. Physiological processes of plants produce reactive oxygen species (ROS)\textsuperscript{16, 17}, which together with nitric oxide are involved in regulating various processes\textsuperscript{18, 19}. ROS react with antioxidants such as anthocyanins (e.g., cyanidin-3-O-glucoside and peonidin-3-O-glucoside) to produce stable radicals, which may not easily propagate further as shown in the scavenging reaction scheme\textsuperscript{3}.

Fig. 6  EPR spectra of the purple rice without husk and reagent powder in Para-film (cyanidin-3-O-glucoside, ~0.0004 g). The EPR conditions were the same.

The EPR spectra are very similar to each other with respect to line shape and $\Delta H_p$. The featureless spectra could be attributed to the delocalization of unpaired electrons throughout the aromatic ring. The EPR signal intensity of the reagent is much higher than that of purple rice. The signal contribution of anthocyanin and other paramagnetic species is roughly 60% (per gram) of the purple rice, based on comparison with white rice (Fig. 2). Only a small amount of the reagent is in radical form. The result indicates the scavenging potential of the reagent. In addition, the EPR spectrum of peonidin-3-O-glucoside is the same (data not shown).

This suggests that the unpaired spin of the reagent may delocalize the anthocyanin frame. Thus, the compounds are likely to contribute significantly to stable radicals in purple rice.
Anthocyanins are polyphenol compounds that form stable radical intermediates. EPR detects such resultant radical intermediates. EPR and EPRI provided further insight into the intermediate species of pigmented seeds. Our speculations about the possible compounds and the reaction scheme are based on the results obtained from EPR, EPRI, HPLC, and previous reports. Our consideration regarding the radicals, in relation to the antioxidant reaction scheme, is valid. However, we have modified the scheme in order to account for the possibilities of oxidants.

Previous studies on purple rice containing anthocyanin pigment in the bran showed that this pigment mainly comprises cyanidin-3-O-glucoside and peonidin-3-O-glucoside. These studies also measured ABTS and superoxide radical scavenging activities of purple rice. The scavenging activities of purple rice are approximately 3.5 times higher than those of white rice under the experimental conditions. Thus, the present results reveal that stable radicals can be produced during the scavenging activities of antioxidant compounds in purple rice. The EPR results suggest that cyanidin-3-O-glucoside of purple rice is detectable.

Our HPLC results show that the major compound in purple rice is cyanidin-3-O-glucoside. This agrees with previous reports on black rice. The EPR signal intensity of purple rice in our study is approximately 3.7 times stronger (per gram of the sample) than that of white rice. In addition, the scavenging activities of purple rice are approximately 3–4 times higher than those of white rice. Our results are consistent with those of previous studies. However, we have not excluded other contributions (e.g., paramagnetic species) to the signal.

In summary, when plant seeds have pigments, strong and stable radical signals were obtained, suggesting that the radicals are related to the pigment. X-band EPR detected at least three different paramagnetic species (Mn²⁺, Fe³⁺, and stable radicals) in both the rice. The spatial distribution of endogenous organic radicals was imaged using noninvasive 2D EPRI, which revealed that the stable radicals are located in the pigmented embryo region of the rice and not in the rice interior. The possible stable organic radicals were inferred from EPR, EPRI, scavenging effect, and HPLC results.

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REFERENCES

1. Shao, Y.; Xu, F.; Sun, X.; Bao, J.; Beta, T. Identification and quantification of phenolic acids and anthocyanins as antioxidants in bran, embryo and endosperm of white, red and black rice kernels (Oryza sativa L.). J. Cereal Sci. 59, 211-218 (2014).
2. Naenjum, C.; Chatthongpisut, R.; Schwartz, S.J.; Bao, J.; Beta, T. Location of radical species in a black pepper investigated by CW EPR and 9 GHz EPR imaging. Spectrochim. Acta Part A 131, 342-346 (2014).
3. Chatthongpisut, R.; Schwartz, S.J.; Yongsawatdigul, J. Antioxidant activities and anti-inflammatory activities of gamma-oryzanol rich extracts from Thai purple rice bran. J. Med. Plant Res. 6, 1070-1077 (2012).
4. Nakagawa, K.; Hara, H. Investigation of radical locations in various sesame seeds by CW EPR and 9 GHz
EPR imaging. Free Radi. Res. 49, 1-6(2015).
6) Nakagawa, K.; Epel, B. Investigating the distribution of stable paramagnetic species in an apple seed using X-band EPR and EPR imaging. J. Oleo Sci. 66, 1375-1379 (2017).
7) Nakagawa, K.; Promjareet, A.; Priprem, A.; Netweera, V.; Hara, H. Investigation of scavenging activities and distribution of paramagnetic species in Zanthoxylum limonella seeds. Free Radic. Res. 50, 1432-1440 (2016).
8) Watanabe, S.; Imawaka, N.; Katsube, T.; Yamasaki, Y. Radical scavenging activity and identification of anthocyanin pigments in Shikokumai (Oryza sativa). Nippon Shokuhin Kagaku Kogaku Kaishi 56, 419-429 (2009).
9) Nakagawa, K.; Maeda, H. Investigating pigment radicals in black rice using HPLC and multi-EPR. J. Oleo Sci. 66, 543-547 (2017).
10) http://epr-it.specman4epr.com/
11) Pengkumsri, N.; Chaiyasut, C.; Saenjum, C.; Sirilun, S.; Peerajan, S.; Suwannalert, P.; Sirisattha, S.; Sivamartthi, B.S. Phytochemical and antioxidative properties of black, brown, and red rice varieties of northern Thailand. Food Sci. Technol. 35, 331-338(2015).
12) Prior, R.L.; Wu, X.; Schaich, K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. J. Agric. Food Chem. 53, 4290-4302 (2005).
13) Nakagawa, K. Effects of low dose X-ray irradiation of eggshells on radical production. Free Radi. Res. 48, 679-683 (2014).
14) Nandi, S.; Sen-Mandi, S.; Sinha, T.P. Active oxygen and their scavengers in rice seeds (Oryza sativa cv. IET 4094) aged under tropical environmental conditions. Seed Sci. Res. 7, 253-260 (1997).
15) Yamuangmorn, S.; Thebault, P.C. Variation of anthocyanin content and antioxidant capacity among local Thai purple glutinous rice genotypes. J. Agric. Sci. 32, 191-199 (2016).
16) Nakagawa, K. Photochemical reactions of antioxidant sesamol in aqueous solution. J. Am. Oil Chem. Soc. 77, 1205-1208 (2000).
17) Mittler, R.R. Oxidative stress, antioxidants and stress tolerance. Plant Sci. 7, 405-415 (2002).
18) Gill, S.; Tuteja, N. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol. Biochem. 48, 909-930 (2010).
19) Nakagawa, K.; Matsumoto, K.; Chaiserm, N.; Priprem, A. X-band electron paramagnetic resonance investigation of stable organic radicals present under cold stratification in ‘Fuji’ apple seeds. J. Oleo Sci. 66, 1375-1379 (2017).
20) Nakagawa, K.; Tero-Kubota, S.; Ikegami, Y.; Tsuchihashi, N. EPR and TREPR Spectroscopic studies of antioxidant sesamolyl and related phenoxyl radicals. Photochem. Photobiol. 60, 199-204 (1994).