Investigation of Pigments in Thai Purple Rice Using Electron Paramagnetic Resonance Imaging and HPLC

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Summary Paramagnetic species (radicals) related pigments in Thai purple rice were investigated by electron paramagnetic resonance (EPR), X-band (9 GHz) EPR imaging (EPRI), and HPLC. The location and 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 organic radicals, Mn²⁺, and Fe³⁺ based on the g-values and hyperfine components of the EPR signals. 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. EPR, EPRI imaging, and HPLC 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 antioxidant activities or could be one of the products of their oxidative decomposition.

Key Words purple rice, pigment, anthocyanin, HPLC, EPR imaging

Free radicals are generated in plants as a result of antioxidant (scavenging) activities and biochemical processes (1–4). In most cases, stable paramagnetic species are found in the pigmented (colored) regions of plant seed coats (husk) (2–4). 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 in plants. The EPR spectrum appears either as an asymmetric line shape or as a series of multiple overlapping lines, depending on the sample being assessed (1–4).

The X-band (9 GHz) EPR imaging (EPR) technique exhibits good spatial resolution and sensitivity. Several reports have described its application to investigate free radicals in naturally occurring high-value crops (1–4). 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. EPR and EPRI techniques have 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 pigmented seed coat, and few radical species are found in other seed parts (2–4). These stable radicals could be the products of antioxidant reaction processes.

Thai purple rice is attracting attention for its antioxidant effects and health benefits (5–7). 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 effects (5–7). Although the total content of useful chemicals in such foodstuffs has been determined via analyses of powdered food crops, we have very limited knowledge about the distribution and concentration of useful chemicals within the crops.

In addition to Thai purple rice, Japanese black rice (Shikokumai) also contains pigments, particularly anthocyanins (8). Many papers have reported the radical scavenging and other beneficial functions of anthocyanins (8). EPR detected paramagnetic species and the aforementioned functions of anthocyanin in black rice (9). 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.

In this investigation, paramagnetic species (free radicals) in physically and chemically untreated rice were investigated using X-band EPR, noninvasive two-dimensional (2D) EPRI, scavenging effect, and HPLC. EPR was performed to detect paramagnetic species (radicals) in whole rice, whereas 2D EPRI was used to demonstrate the spatial distribution of stable (unreactive) organic radicals within the rice grains. Possible antioxidants

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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.

Materials and Methods

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-Tawng (white rice) was harvested from the Chiang Mai Rice Research Center, Chiang Mai, Thailand, in November 2016 (Fig. 1). These samples were used for EPR without any chemical or physical treatment. 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.

EPR measurements

A JEOL RE-3X 9 GHz EPR spectrometer (JEOL Resonance Inc., 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.

EPR imaging

A modified JEOL RE-3X 9 GHz EPR spectrometer was used for EPR imaging. We used 16 equal-angle spaced projections obtained with a maximum gradient of ~3.4 mT/cm (10, 11). First-derivative EPR spectra were numerically integrated to obtain the corresponding absorption spectra. The 2D images were reconstructed from a complete set of projections, which were collected as a function of the magnetic field gradient. Before reconstruction, each projection was deconvolved using fast Fourier transformation with the measured zero-gradient spectrum to improve the image resolution. The 2D image reconstruction was performed using the back-projection algorithm in the EPR-IT software package from the Center for EPR Imaging in Vivo Physiology at the University of Chicago (12). A detailed description is available elsewhere (1, 3, 10). All measurements were performed at ambient temperature.

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. (11) and Prior et al. (12). 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=250×4.6 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-glucoside were purchased from Extrasynthese Co., Ltd. (Genay, France).

Results

EPR of purple rice

Figure 2 shows the EPR spectra of (A) purple and (B) white rice. The sweep width was 300 mT. The filled triangles indicate the Fe3+ signal at g=4.345. The asterisk indicates free radical. Each spectrum was taken at a single scan.

![Fig. 1. Khao’ Gam Pah E-Kaw (purple rice) and Niaw San-Pah-Tawng (white rice) (husk and rice) were investigated.](image)

![Fig. 2. EPR spectra of (A) whole purple, and (B) white, rice. The sweep width was 300 mT. The filled triangles indicate the Fe3+ signal at g=4.345. The asterisk indicates free radical. Each spectrum was taken at a single scan.](image)
Investigating the possibility of the radical being generated during scavenging activities and the presence of antioxidant-related organic compounds in the rice (9). 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. 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 ~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 third signal was characteristic of Fe$^{3+}$ (the $g$-values of ~3.21_4 and ~3.34_5 at a lower magnetic field with filled triangles, as shown in Fig. 2). The signal at $g=4.34_5$ 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 (9). Stable radical was primarily located in the pigmented region of black rice, while very few radicals were observed in the rice interior.

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 (Fig. 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 in the endosperm.

We studied the husk of purple rice because the husk contains pigment. Figure 3 shows 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. 2. We also found similar signals for the husk (Fig. 3).

**EPR imaging 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 ($g\approx2.00$) of the spectrum in Fig. 2. The dashed line shows the approximate size of the purple rice. Based on the $\Delta H_{pp}$ value (~0.63 mT), the spatial resolution of the rice EPRI was estimated to be ~0.19 cm. The color scale indicates that the high radical concentration is red color. The background and not detectable level are blue color. It is noted that not all pigments are EPR-detectable radicals (at low levels), especially for EPRI. In the case of the husk, the original EPR signal is weak, the partial spatial distribution was observed by EPRI. An artifact at the center of the image can be due to the weak signal
during the data manipulations.

Notably, the EPR results showed that the organic radicals are mostly distributed near the embryo region of the purple rice. EPR studies of dry embryos of rice seeds (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.

**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 arrow corresponds to the cyanidin-3-O-glucoside in the chromatogram. The similar result was obtained for the husk although the signal-to-noise ratio was poor. 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 was higher than that reported by Yamaoangmorn and Thebault; they observed that local Thai purple glutinous rice genotypes, which were measured at different pH values and wavelengths, were associated with the total anthocyanin (15). It is also noted that there is a small peak approximately 25 min. At his point, we do not know it for sure.

**Discussion**

In order to establish the link between organic radicals obtained by EPR and cyanidin-3-O-glucoside determined by HPLC, we carried out additional experiments. The EPR spectra of the reagent powder (cyanidin-3-O-glucoside, ~0.0002 g) (Supplemental Online Material, Fig. S1). Autodxidation of the radical was observed for the compound. The reagent was purchased on November 16, 2016. Both spectra are very similar to each other with respect to line shape and $Delta H_{pp}$. 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 lower 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 antioxidant potential of the reagent. 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.

Antioxidant activities may be responsible for the origin of stable radicals in the rice sample. The antioxidant 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 (16). Physiological processes of plants produce reactive oxygen species (ROS) (16, 17), which together with nitric oxide are involved in regulating various processes (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 antioxidant reaction scheme (1).

Anthocyanins are polyphenol compounds that form stable radical intermediates. EPR detects such resultant radical intermediates (16, 20). EPR and EPRI provided further insight into the intermediate species of pigmented seeds. We were not able to identify the exact chemical compounds corresponding to the radicals detected. We speculated about the possible compounds and the reaction scheme based on the current EPR, EPRI, HPLC, and the previous results. Our consideration about the radicals, in relation to the antioxidant reaction scheme, is valid.

In conclusion, 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 (Mn$^{2+}$, Fe$^{3+}$, 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, and HPLC results.

**Disclosure of State of COI**

There is no conflict of interest to be declared.

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**Supporting information**

Supplemental Online Material is available on JSTAGE.

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