Investigating Pigment Radicals in Black Rice Using HPLC and Multi-EPR

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Abstract: We investigated the location and distribution of paramagnetic species in black and white rice using electron paramagnetic resonance (EPR), X-band (9 GHz) EPR imaging (EPRI), and HPLC. EPR primarily detected two paramagnetic species in black rice, which were identified as a stable radical and Mn⁴⁺ species, based on the g values and hyperfine components of the EPR signals. The signal from the stable radical appeared at g ≈ 2.00 and was relatively strong and stable. Subsequent noninvasive two-dimensional (2D) EPRI revealed that this stable radical was primarily located in the pigmented region of black rice, while very few radicals were observed in the rice interior. Pigments extracted from black rice were analyzed using HPLC; the major compound was found to be cyanidin-3-glucoside. EPR and HPLC results indicate that the stable radical was only found within the pigmented region of the rice, and that it could either be cyanidin-3-glucoside, or one of its oxidative decomposition products.

Key words: EPR, black rice, EPR imaging, pigment radical, anthocyanin, HPLC

1 INTRODUCTION

Free radicals are generated in plants as a result of antioxidant activities and biochemical processes¹–⁴. In most cases, stable paramagnetic species are found in the pigmented regions of plant seed coats¹–⁴. These pigmented regions usually contain various organic compounds like antioxidants. Electron paramagnetic resonance (EPR) can be used to detect such 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 assessed¹–⁴.

X-band (9 GHz) EPR imaging (EPRI) has good spatial resolution and sensitivity. Several reports describe the use of X-band EPR to investigate free radicals in naturally occurring samples¹–⁵. Noninvasive EPR and EPR spectroscopy can provide detailed information regarding the location and concentration of paramagnetic species (e.g., transition metal ions, transition metal complexes, stable organic radicals, etc.) in naturally occurring biological samples. The subsequent noninvasive EPR of the radicals present in each seed revealed that the stable radicals were primarily located in the seed coat, while very few radicals were observed in the seed cotyledon. These results indicate that stable radical species were only found within the seed coat, and few radical species were found in other seed parts³.

These stable radicals could be the products of antioxidant reaction processes.

Black rice is a type of rice that contains pigments, particularly anthocyanins. Various radical scavenging and other beneficial functions of anthocyanins have been suggested⁵. 1,1-diphenyl-2-picrylhydrazyl (DPPH) and superoxide radical scavenging activities in an organic solvent extract of Shikoku-mai black rice were measured using colorimetry and EPR spectroscopy to examine these anthocyanin functions in Shikoku-mai. However, little is known regarding the endogenous paramagnetic species (e.g., Mn²⁺) and organic radicals present in rice. EPR could be a useful tool for obtaining such information.

In this study, paramagnetic species in chemically untreated rice were investigated using X-band EPR, noninvasive two-dimensional (2D) EPR, and HPLC. EPR was carried out to detect paramagnetic species in rice, whereas 2D EPR demonstrated the distribution of the stable radical within rice. Possible antioxidants present in the extracted black rice pigment fraction were characterized using HPLC. The localization and concentration of the stable radical species within the rice were also discussed.
2 EXPERIMENTAL

2.1 Samples
Black rice (Murasaki no kimi) and white rice (Tsugaru Roman) were harvested from a rice paddy located in the far north (Hirosaki, Aomori Prefecture) of the main island of Japan in the fall of 2015, and were used without any chemical treatment. Both rice types were milled after harvesting. For measurements, the rice (approximately 0.0150 g/rice) was sequentially inserted into an EPR tube (outer diameter, 5.0 mm; inner diameter, 4.0 mm; Wilmad LabGlass, Buena, NJ, USA).

Chemicals for HPLC analysis were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan), while cyanidin-3-glucoside was obtained from Nacalai Tesque, Inc. (Kyoto, Japan). A spin probe reagent, 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPOL), and DPPH was purchased from Nacalai Tesque, Inc. (Kyoto, Japan), and were used as received; DPPH was used as a reference sample.

2.2 EPR measurements
A JEOL RE-3X 9 GHz EPR spectrometer (JEOL 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 – 10 mT.

2.3 EPR imaging measurements and data processing
A commercially available JEOL RE-3X 9 GHz EPR spectrometer was modified for use as an EPR imager. A detailed description is available elsewhere. All measurements were performed at ambient temperature.

We used 16 equal-angle-spaced projections obtained with a maximum gradient of ~3.3 mT/cm. The first-derivative EPR spectra were numerically integrated to obtain the corresponding absorption spectra. The two-dimensional (2D) images were reconstructed using the back-projection algorithm of the EPR-IT software package from the Center for EPR Imaging In Vivo Physiology at the University of Chicago.

2.4 Anthocyanin quantification by HPLC
Extracted samples of the pigmented part of black rice were analyzed using HPLC, according to a modification of the method reported by Martinelli et al. In brief, black rice (2.0 g) was extracted with 2% HCl in ethanol (100 mL) and mixed using a vortex mixer for 10 min. The mixture was then sonicated using an ultrasonicator (SND Co., Ltd., Nagano, Japan) for 10 min. Subsequently, the supernatant was filtered through a 0.45-μm filter for the HPLC analysis. The anthocyanin content was determined using HPLC. The HPLC system comprised a Hitachi 7000 HPLC system (Hitachi High-Tech Science Corporation, Tokyo, Japan) composed of an L-7000 pump, an L-7300 column oven (at 30 °C), and an L-7455 diode array detector set at 535 nm. The reverse-phase column used was a Mightysil RP-18GP (250 × 4.6 mm, 5 μm; Kanto Chemical Co., Inc., Tokyo, Japan). The HPLC mobile phases were water:formic acid [9:1 (v/v); solvent A] and water:methanol:acetonitrile:formic acid[400:225:225:100 (v/v/v/v); solvent B]. The linear gradient elution was performed as follows: time t (min): (t, A%): (0 min, 93%), (35 min, 75%), (45 min, 35%), (46 min, 0%), and (50 min, 0%), with a flow rate of 1.0 mL/min and an injection volume of 10 μL.
EPR imaging and HPLC of black rice

3 RESULTS AND DISCUSSION

3.1 EPR of rice

Figure 1 shows the EPR spectra of (A) black and (B) white whole rice, which were obtained with a 100 mT sweep width. The EPR spectrum of black rice comprised two distinct signals, which were stable for at least a few months, and corresponded to Mn$^{2+}$ and an organic radical. The first signal was characteristic of the Mn$^{2+}$ paramagnetic center (M$_t$=5/2-related sextet$^{9}$, 100% natural abundance of $^{55}$Mn isotope). The hyperfine coupling of the sextet was also consistent with the previously reported value$^{9}$. The apparent increases in hyperfine couplings while moving from low to high fields were because of the Mn$^{2+}$ moiety and the overlap of other paramagnetic centers. Similar EPR spectra were previously reported for black pepper seeds$^{3}$ and apple seeds$^{3}$.

The second signal was strong and reproducible. The relatively broad single peak observed at $g \approx 2.00$ was indicative of a stable organic carbon-centered radical$^{1,10}$, suggesting the possibility of the radical being generated during scavenging activities and the presence of antioxidant-related organic compounds in the seed$^{4}$. In contrast, the EPR spectrum of white rice showed no recognizable signal.

3.2 2D EPRI of black rice

To study the paramagnetic species present in black rice in more detail, we performed EPRI of the rice. Figure 2

Fig. 3 The left-hand panel shows the EPR measurement set-up. The right-hand panel shows a 2D EPR image of the black rice.

Fig. 4 EPR spectrum of the rice interior around $g \approx 2.00$, acquired with (A) 10.0 mT and (B) 5.0 mT sweep widths. The spectrum was obtained with a single scan.

Fig. 5 Upper panel is an HPLC chromatogram detected at 535 nm. Lower panel is a photodiode array chromatogram.
shows the EPR spectrum of the central region \((g = 2.00)\). The peak-to-peak line width \((\Delta H_p)\) of the signal was \(\sim 0.50\) mT. Figure 3 shows the EPR image of black rice obtained using a 5 mT scan width and the central region \((g = 2.00)\) of the spectrum shown in Fig. 2. Based on the \(\Delta H_p\) value, the spatial resolution of the rice EPR was estimated to be 0.15 cm. The concentration was estimated by comparing with a TEMPOL solution \((\text{known concentration})\) in a capillary tube \((\text{outer diameter}, 1.0 \text{ mm}; \text{inner diameter}, 0.9 \text{ mm})\). The number of spins per gram for milled black rice was \(\sim 2 \times 10^6\); this number may depend on the milling of the rice and the overlap of the Mn\(^{2+}\) species.

Figure 3 shows the 2D EPR image of a whole rice seed, in which the stable radical is located in the pigmented region of the rice. Very few radicals were observed in the interior of the rice. It is noted that the obtained EPR image was slightly unclear because of the slight fluctuations in the static magnetic field, which were stabilized by a magnetic current and not by using a Hall probe. More specifically, for magnetic field monitoring, the NMR probe was removed to enable the application of the magnetic field gradients for obtaining EPR images.

To confirm the 2D EPRI results, we removed the pigmented region of black rice to leave an interior similar to white rice, and subjected this rice interior to analysis. Figure 4 shows the EPR spectra of the rice interior with 100 mT and 5 mT sweeps. The EPR spectra of the rice interior showed a very weak signal at \(g = 2.00\). In addition, the Mn\(^{2+}\) signals were reduced to nearly zero. These results prove that the organic radical at \(g = 2.00\) and Mn\(^{2+}\) signals were concentrated in the pigmented part of the rice.

3.3 HPLC analyses of black rice

Figure 5 shows an HPLC chromatogram of black rice extract. The strongest peak in the chromatogram was assigned to cyaniding-3-glucoside by comparing with the specific retention time and absorption spectra of the authentic standard. Maximum absorption wavelength of the sample is 530 nm, which usually corresponds to anthocyanins. Anthocyanins were quantified by determining the peak areas in HPLC chromatograms; the concentration of cyaniding-3-glucoside was found to be 215.7 mg/100 g dry weight. These results were the same as those previously reported for black rice \((\text{Shikokumai 105})\).

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\text{Reactive radical (e.g. ROS)} \quad + \quad \text{Antioxidants} \quad \rightarrow \quad \text{Stable radical} \quad + \quad \text{H}_2\text{O}
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The scavenging reaction scheme shows a possible explanation for the stable radical production. We propose that an intermediate stability (or reactivity) is a key role for antioxidant reactions or scavenging activity. Plant physiological processes produce reactive oxygen species (ROS), which together with nitric oxide is involved in regulating various processes in plants. ROS react with antioxidants like phenolic compounds (e.g. cyaniding-3-glucoside) to produce stable radicals, which may not easily propagate further. Previous studies of black rice containing anthocyanin pigment in its rice bran showed that this pigment mainly comprises cyanidin-3-glucoside and peonidin-3-glucoside. These studies also measured DPPH and superoxide radical scavenging activities of black rice, and revealed that the stable radical can be produced during the scavenging activities of antioxidant compounds in the rice.

In summary, X-band EPR detected two different paramagnetic species in rice. The distribution of the organic radicals was imaged using noninvasive 2D EPRI, which showed that the stable radical is located in the pigmented region of the rice, and not in the rice interior. The stable organic radical was identified as cyaniding-3-glucoside using HPLC. Moreover, we showed that EPR, EPRI, and HPLC are very useful techniques for evaluating the distribution of stable paramagnetic species in biological samples.

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REFERENCES


6) Watanabe, S.; Imawaka, N.; Katsube, T.; Yamasaki, Y. Radical scavenging activity and identification of antho-

7) http://epri.uchicago.edu or http://epr-it.specman4epr.com/


