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Effect of Foliar Application of Selenium on the Antioxidant Activity of Aqueous and Ethanolic Extracts of Selenium-Enriched Rice

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Selenium fertilizer was foliar applied to determine the effects of antioxidant activity of selenium-enriched rice assessed by α,α-diphenyl-β-picylhydrazyl (DPPH) radical scavenging and the ferric thiocyanate (FTC) method. Results showed that selenium concentration in rice was significantly enhanced dose dependently. Aqueous or ethanolic extracts of rice displayed significantly higher antioxidant activity against lipid peroxidation. The activities of aqueous extracts were significantly higher than those of ethanolic extracts and increased with the increasing selenium concentration in rice. The DPPH assay showed that the kinetic behaviors of aqueous extracts were complex and slow, while ethanolic extracts reacted quickly with DPPH radical. Aqueous extracts of rice exhibited higher antiradical efficiencies than ethanolic extracts, and rice (1.275 mg Se kg^{-1}) presented the lowest EC_{50} values of 533.46 ± 0.58 μg mL^{-1}. As compared to rice extracts, all of the reference antioxidants showed more than 4-fold antiradical efficiencies than rice extracts. This radical scavenging activity was significantly correlated with selenium concentrations in rice ($R = 0.862$, $p < 0.05$), while ethanolic extracts were inversely correlated with selenium concentration in rice.

KEYWORDS: Selenium; extracts of Se-enriched rice; antioxidant activity

INTRODUCTION

Selenium has received considerable attention as an essential micronutrient for animals and the human body. It functions in the active site of a large number of selenium-dependent enzymes such as GSH-Px (*1*) and in anticancer and other physiological functions (*2*, *3*). A lower selenium level in body is reported to be responsible for high incidences of cancer and disease. However, selenium concentration of a particular food may be variable and dependent on the geographic origin of the soil where the agricultural crops are grown (*4*). Selenium deficiency is still a very serious nutritional and health problem in China (*5*). Therefore, the supply of selenium to livestock through forage and to human beings through food has been a practice adopted to prevent selenium deficiencies in several areas (*6*).

Rice is one of the leading food crops of the world and is the staple food of over half of the world's population (*7*). Rice is an excellent source of complex carbohydrates, fiber (brown rice), and vitamins, and after it is hulled, the polished rice has fewer nutrients than brown rice (*8*). Many studies have shown the antioxidant and low cholesterol levels of oil extracts from rice bran (*9*−*11*). However, little is reported on the physiological properties of polished rice. In addition, the selenium content of rice was lower, and the mainly rice-based diet contributed an inadequate amount of selenium for Chinese inhabitants (*12*). In our previous study, we have shown that selenium application could increase selenium content from 0.025 ± 0.011 μg g^{-1} in regular polished rice to 0.471−0.640 μg g^{-1} in Se-enriched polished rice (*13*). The in vivo antimutagenic assay of Se-enriched rice also exhibited a significantly higher activity than that of regular rice.

The objective of this study is to explore the effect of foliar application of selenium fertilizer on selenium content in rice and determine whether Se-enriched rice presented higher antioxidant activity than regular rice at various selenium concentrations using α,α-diphenyl-β-picylhydrazyl (DPPH) radical scavenging and the ferric thiocyanate (FTC) method.

MATERIAL AND METHODS

Chemicals. Chemicals were used as follows: linoleic acid (ca. 99%) (Wako Chemical Pure Chemical Industries Ltd., Osaka, Japan); DPPH (Aldrich Chemical Co., Milwaukee, WI); 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (Aldrich Chemical Co. Milwaukee, WI); α-tocopherol (Sigma Chemicals Co., St. Louis, MO); and butylated hydroxyanisol (BHA), ammonium thiocyanate, and ferrous chloride (Nanjing Chemical Industry, Nanjing, China). Ethanol and other reagents were of analytic grade produced in Nanjing.

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Preparation of Selenium Fertilizer. Lobster waste (20%), chicken excreta (30%), silkworm excreta (15%), pig excreta (34%), and EM (effective microorganism) bacterium (1%) were mixed and allowed to ferment for about 2 weeks in a methane-generating pit. Se, as sodium selenite and water, was added to the mixed fertilizer and well-distributed, and then, fermentation was allowed to continue for 4 weeks. The fermented solution was filtered. The final solution contained 50 g Se L^{-1}. The Se-enriched fertilizer was bottled in a plastic container as a volume of 100 mL (*12*, *13*).

Preparation of Regular Rice and Selenium-Enriched Rice Samples. The plot experiment was conducted in May, 2001, in Kunshan Country, Jiangsu Province. The cultivar of rice was R 109. The soil pH in this region was 5.7, and the total selenium content was 0.326 μg Se g^{-1} of soil. Application of selenium fertilizer to rice was detailed by Chen (*13*) on September 9, 2001. Rice samples with three different selenium concentrations were obtained by varying the concentrations of selenium fertilizer. When being thoroughly tasseled, the grain was hand harvested on October 30, 2001. After it was harvested, the grain was dried at 50 °C, then hulled, and milled into powder for further use. Selenium concentration of rice was analyzed using atomic fluorescence spectroscopy (*12*).

Preparation of Rice Aqueous and Ethanolic Extracts. Five grams of ground rice was extracted with 100 mL of distilled water or 100 mL of 75% ethanol, respectively. Extractions were conducted in a water bath with a constant temperature of 60 °C for 3 h. Each sample was extracted twice with the same volume of solvents. The mixture was filtered and combined. The filtrate was evaporated to dryness in vacuo and kept frozen for antioxidative assays.

Standard Aqueous and Ethanolic Rice Extracts Solution. Twenty micrograms of rice extract was solublized in 75% ethanol in an ultrasonic water bath to a final concentration of 1000 μg mL^{-1}.

Determination of Antioxidant Activity with the FTC Method. Two milliliters of 1000 μg mL^{-1} rice extract or 2 mL of 200 μg mL^{-1} antioxidants, 2 mL of 2.51% (w/v) linoleic acid in ethanol, 4 mL of 0.05 mol L^{-1} of phosphate buffer (pH 7.0), and 2 mL of distilled water were mixed in a vial of 10 mL with a screw cap and then kept in a 40 °C water bath in the dark. A 0.1 mL amount of the above mixture was added to 9.7 mL of 75% (v/v) ethanol and 0.1 mL of 30% (w/v) ammonium thiocyanate. After 5 min, 0.1 mL of 0.02 mol L^{-1} ferrous chloride in 3.5% (v/v) hydrochloric acid was added to above mixture and then kept in a 40 °C water bath in the dark. The absorbance of mixture was measured every 24 h at 500 nm until an unchangeable absorbance value arrived. The FTC method was described in detail by Kikuzaki (*14*). All of the tests were performed in triplicate, and the results were averaged.

Assay of DPPH Radical Scavenging Activity. The antioxidant activities of rice extracts, BHA, α-tocopherol, and Trolox were determined using the stable radical, DPPH (*15*, *16*). Briefly, 0.1 mL of rice extracts, BHA, Trolox, and α-tocopherol was added to 3.9 mL of 2×10^{-4} mol L^{-1} ethanol solution of DPPH in cuvette. Absorbance measurements commenced immediately. The decrease in absorbance was determined at 517 nm and continuously at every 5 min interval with a spectrophotometer until the reaction reached steady state. The percentage of DPPH remaining at the steady state was calculated as a function of the molar ration of antioxidant to DPPH. The EC_{50} value, which is defined as the amount of antioxidant necessary to decrease the initial DPPH concentration by 50%, was calculated from the results. All of the tests were performed in triplicate, and the results were averaged.

Statistics Analysis. One way analysis of variance was performed on the data using SPSS (Release 11.0). Student's *t*-LSD (least significant difference) ($P < 0.05$) was calculated to compare the means of the different samples.

RESULTS AND DISCUSSION

Effect of Selenium Application on Rice Selenium Concentration. Table 1 shows that selenium foliar application to rice can significantly increase its total selenium concentration ($P < 0.05$). The total selenium concentration in regular rice was increased from 0.027 to 0.435 ~ 1.275 μg g^{-1}. Statistics analyses showed that selenium concentrations in rice were significantly increased with the increasing selenium dose of applied fertilizer ($R = 0.993$, $P < 0.007$). This suggested that application of selenium fertilizer can effectively enhance the selenium content in rice.

Table 1. Effect of Selenium Application on Total Selenium in Polished Rice[a]

applied Se concn (mg Se L^{-1})	applied Se dose (g Se ha^{-1})	total Se concn (mg kg^{-1})
0	0	0.027 ± 0.001^a
20	15	0.435 ± 0.220^b
60	45	0.890 ± 0.017^b
80	60	1.275 ± 0.099^c

[a] Within the same column, means followed by different letters are significantly different at $P < 0.05$.

Antioxidant Activities of Rice Aqueous or Ethanolic Extracts Assayed by FTC Method. The antioxidant activity of rice extracts, determined using the FTC method, was compared with that of BHA, α-tocopherol, and Trolox, and the results are shown in **Figure 1**. The individual activity of samples showed low absorbance values, which indicated a high level of antioxidant activity. All of the rice extracts delayed oxidation of linoleic acid and, on the basis of low absorbance values, exhibited higher activity than control. Also, lipid inhibitive activities of rice aqueous extracts were higher than those of ethanolic extracts except of rice aqueous extract (0.027 mg Se kg^{-1}). However, aqueous extracts and ethanolic extracts showed the different pattern of activity (**Figure 1a**). Antioxidant activities of rice aqueous extracts decreased in the following order: rice (1.275 mg Se kg^{-1}) > rice (0.890 mg Se kg^{-1}) > rice (0.435 mg Se kg^{-1}) > rice (0.027 mg Se kg^{-1}).

All ethanolic extracts displayed significantly higher activity than control without antioxidants. However, there is no significant difference between all ethanolic exgtracts (**Figure 1b**).

Comparisons of antioxidant activities were also made between rice extracts and reference antioxidants including BHA, α-tocopherol, and Trolox (an aqueous analogue of α-tocopherol) (**Figure 1c**). The activity decreased in the following order: aqueous extract of rice (1.275 mg Se kg^{-1}) > BHA > Trolox > ethanolic extract of rice (0.435 mg Se kg^{-1}) > α-tocopherol. α-Tocopherol appeared to exert a lower antioxidant potential than rice extracts, BHA, and Trolox. During the first 3 days, the aqueous extract of rice (1.275 mg Se kg^{-1}), BHA, and Trolox showed no difference in delaying oxidation of lipid. From the fourth day, however, the antioxidant effect of aqueous extract on lipid oxidation was significantly higher than that of BHA and Trolox.

Antioxidant Activity of Rice Extracts Assessed by Scavenging DPPH Radical Method. The DPPH radical is considered to be a model of a stable lipophilic radical. Antioxidants react with DPPH, reducing a number of DPPH molecules equal to their number of available hydroxyl groups. Therefore, the absorption at 517 nm was proportional to the amount of residual $DPPH^{\bullet}$. The kinetic classification according to the time at the steady state has been reported as follows: rapid, <5 min; intermediate, 5−30 min; and slow, >30 min (*16*).

Figure 2 illustrates the kinetic behavior of rice extracts and antioxidants as radical scavengers toward $DPPH^{\bullet}$. Different kinetics was observed. In our study, all of the rice samples exhibited significant antiradical activity. During the first 15 min,

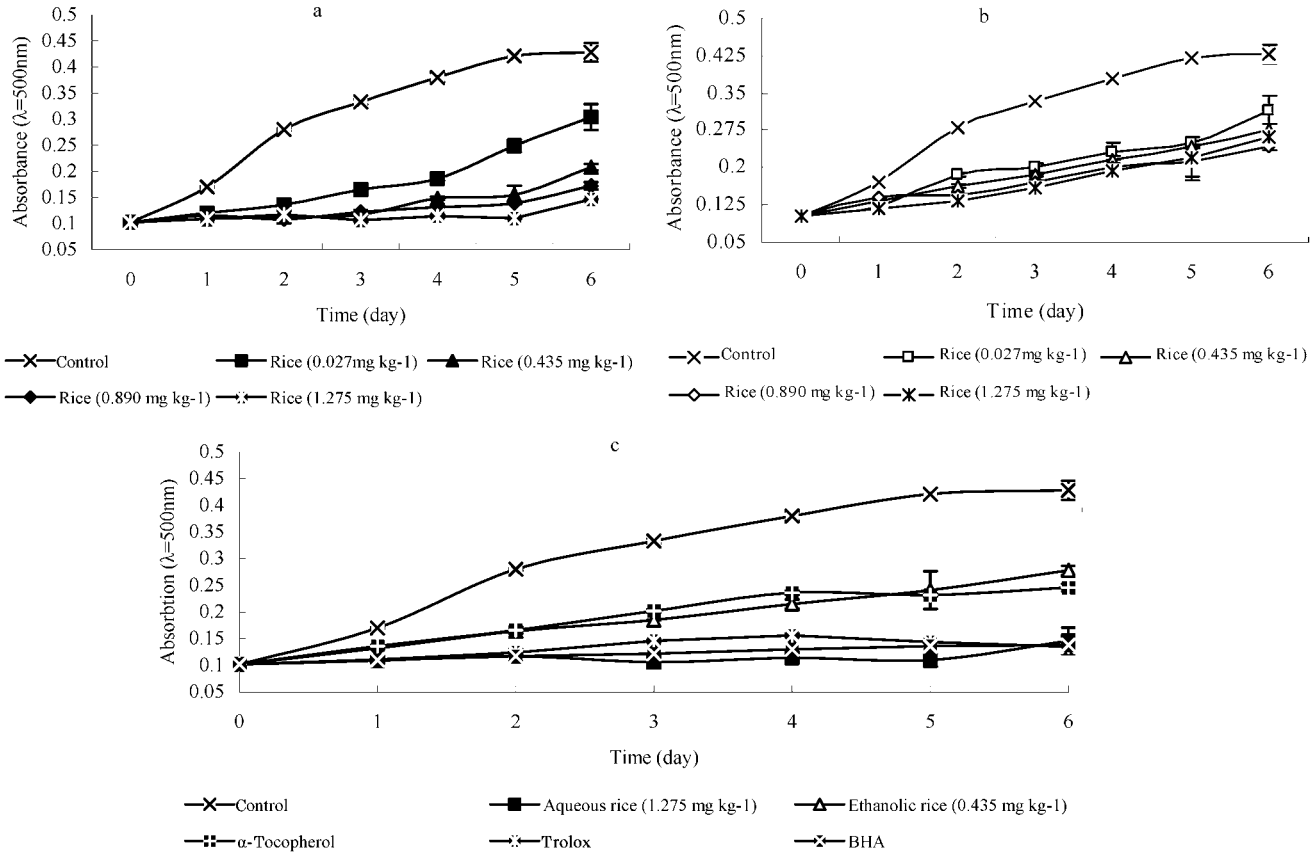

Figure 1. Antioxidant activity of rice aqueous extracts and ethanolic extracts, BHA, Trolox, and α-tocopherol, as measured by the thiocyanate method. (**a**) Aqueous extracts of rice with various selenium concentrations; (**b**) ethanolic extracts of rice with various selenium concentrations; and (**c**) comparison of antioxidant activities of aqueous extract of rice (1.275 mg Se kg^{-1}), ethanolic extract of rice (0.435 mg Se kg^{-1}), and reference antioxidants.

the reaction between DPPH radical and aqueous extracts was rapid and rice (0.027 mg Se kg^{-1}) reached to the steady state in 15 min (**Figure 2a**). Afterward, the absorbance decrease for all extracts was rather weak. However, for ethanolic extracts, the reaction reached a plateau in 5 min (**Figure 2b**). Therefore, the rice aqueous extracts and ethanolic extract (0.027 mg Se kg^{-1}) were slow and the rest of the ethanolic extracts were rapid. Three antioxidants reacted rapidly with DPPH radical and BHA, and Trolox reached the steady state in 5 min while the absorbance of α-tocopherol was stable till 15 min (**Figure 2c**). This result was a little different from data reported by Sánchez-Moreno (*16*) in that the kinetic classification of BHA was slow and α-tocopherol was intermediate.

EC_{50}, meaning the concentration of antioxidant needed to decrease by 50% the initial substrate concentration, is a parameter widely used to measure the antiradical efficiency (*17*−*19*). The lower the EC_{50} is, the higher the antioxidant power is. The values of rice extracts, BHA, α-tocopherol, and Trolox are compared and shown in **Table 2**. Except for rice aqueous extract (0.027 mg Se kg^{-1}), all of the aqueous extracts possessed the lower EC_{50} than their ethanolic extract. Rice aqueous extract (1.275 mg Se kg^{-1}) was the most efficient by the lowest EC_{50} values of 553.46 mg kg among all of the extracts, and the activity decreased in the order: rice (1.275 mg Se kg^{-1}) > rice (0.890 mg Se kg^{-1}) > rice (0.435 mg kg^{-1}) > rice (0.027 mg Se kg^{-1}). For ethanolic extracts, the activity followed the increasing order: rice (1.275 mg Se kg^{-1}) < rice (0.890 mg Se kg^{-1}) < rice (0.027 mg Se kg^{-1}) < rice (0.435 mg Se kg^{-1}). This behavior was similar to the above results determined by the FTC method. However, reference antioxidants provided rather lower EC_{50} values than rice extracts, in other words, meaning more than 4-folds antiradical efficiencies than rice extracts. BHA has the lowest EC_{50} of 114.45 ± 0.50 μg mL^{-1} among the three antioxidants and Trolox followed as the second. This result was in agreement with the previous repot that BHA and α-tocopherol had EC_{50} values of 93 g antioxidant kg^{-1} $DPPH^{\bullet}$ and 201 g antioxidant kg^{-1} DPPH (*16*). Shimada reported that the activity of antioxidants (compounds) corresponds to the number of hydrogens available for donation by hydroxyl groups (*20*). Also, it is well-known that monophenols are less efficient than the polyphenols, but in BHA, the methoxy substitution increases substantially the antioxidant power of monophenols.

Because of the complex nature of plants, extracts cannot be evaluated by only a single method. In this study, we adopted the FTC method to determine the ability to inhibit lipid oxidation and DPPH radical scavenging assay. On the basis of **Figure 1c**, there was little gap between the rice extracts and the reference antioxidants assayed by the FTC method. However, large gaps were found in **Table 2**. This difference may be due to the unequal concentrations of antioxidants. The antioxidant activity of rice extracts was assayed at the concentrations of 1 mg mL^{-1}, and antioxidants were assayed at 200 μg mL^{-1}. Therefore, it is noteworthy that the FTC method and DPPH radical scavenging kinetics gave false-negative results. EC_{50} values present the real antioxidative potential of antioxidants, regardless of their concentrations tested.

Effect of Selenium Concentration in Rice on Its Antioxidant Potential. The associations between rice selenium concentration and its antioxidant activity were also investigated in this study. **Table 2** shows that the EC_{50} values of rice aqueous

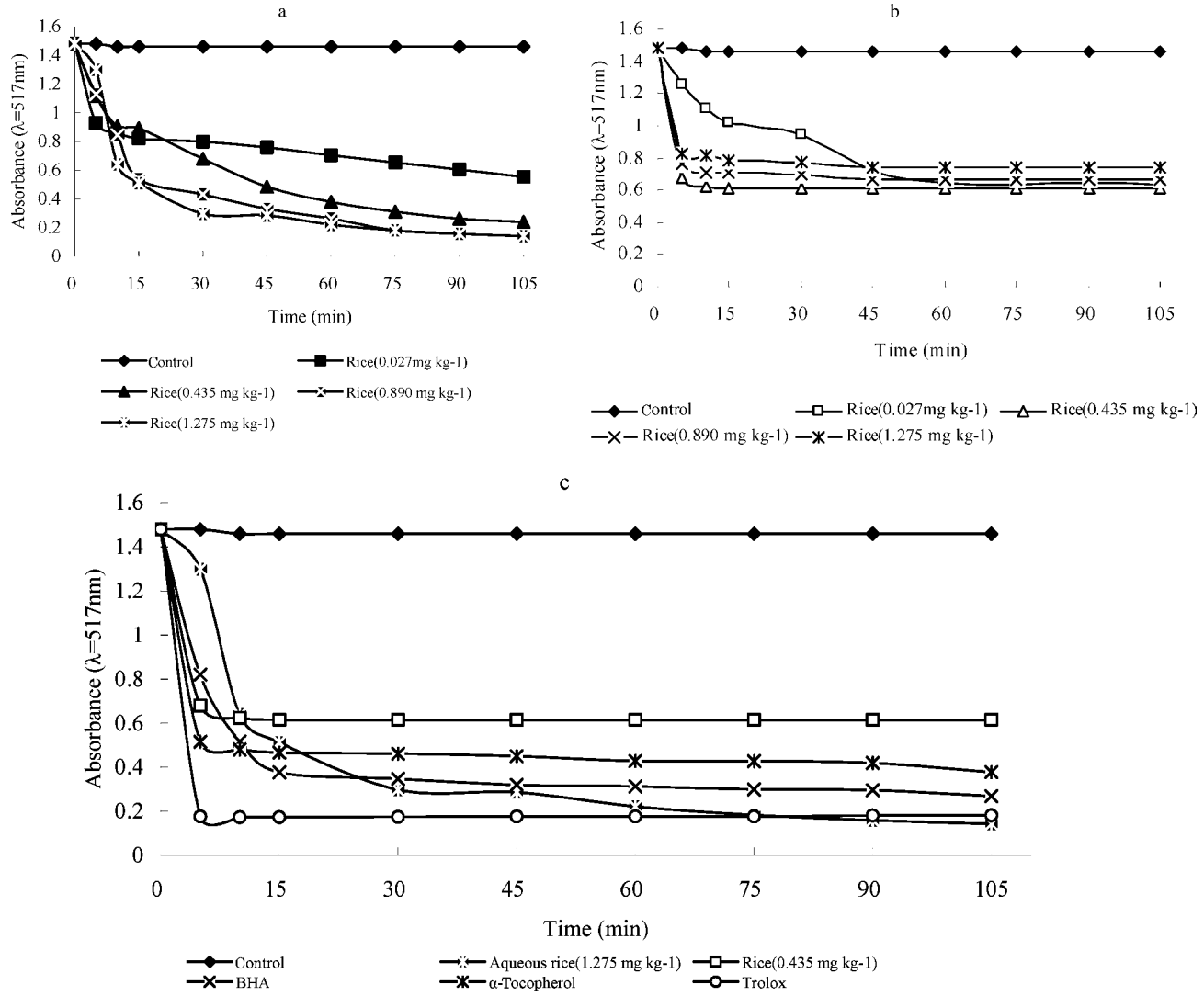

Figure 2. Kinetic behaviors of radical scavenging activity of rice extracts and three antioxidants. (**a**) Rice aqueous extracts; (**b**) rice ethanolic extracts; and (**c**) aqueous extract of rice (1.275 mg Se kg^{-1}), ethanolic extract of rice (0.435 mg Se kg^{-1}), and antioxidants.

Table 2. Radical Scavenging Activities of Rice Extracts and Antioxidants Expressed by EC_{50}[a]

	EC_{50} ($\mu g\ mL^{-1}$)	
sample	aqueous extract	ethanolic extract
rice (0.027 mg kg^{-1})	802.91 ± 3.69[a]	868.33 ± 9.35[a]
rice (0.435 mg kg^{-1})	597.65 ± 0.68 [b]	860.23 ± 4.94[a]
rice (0.890 mg kg^{-1})	557.23 ± 4.74[c]	918.90 ± 7.25[b]
rice (1.275 mg kg^{-1})	533.46 ± 0.58[c]	996.77 ± 8.53[c]
Trolox	122.39 ± 0.14	
α-tocopherol	133.65 ± 1.02	
BHA	114.45 ± 0.50	

[a] Within the same column, means followed by different letters are significantly different at $P < 0.05$.

extracts decreased with the increasing selenium concentration, and it is opposite in ethanolic extracts that EC_{50} increases with selenium concentrations in rice. EC_{50} values of aqueous extracts are lower than ethanolic extracts. After statistical analyses, we found that the selenium concentration in rice was significantly inversely correlated with its EC_{50} values, which indicated that selenium has a dose-dependent effect on antioxidant activities of rice aqueous extracts ($R = 0.862$, $P < 0.006$). However, in ethanolic extracts, it is interestingly found that with increasing selenium concentration in rice, the antioxidant activity of ethanolic extracts significantly decreased ($R = 0.906$, $P < 0.006$).

Selenium has been reported to enhance the antioxidative system in ryegrass at low concentrations dose responsively (*21*). Oxidative stress in Se-enriched garlic was significantly lower than that in regular garlic, and the antioxidant activity increased with selenium concentration in a hydroponic soulition (*22*). Selenium can also enhance the total antioxidant capacity property in "Seoul" lettuce in hydroponics without any nutritional loss (*23*). Our previous studies have reported that selenium enhanced the antioxidant activity of green tea extracts and that the effect is dose-dependent (*24*). In polished rice, starch accounted for 76.60% and crude protein and lipid accounted for 7.80 and 1.30%, respectively. Therefore, selenium might be responsible for the higher antioxidant activity of Se-enriched rice. Further studies are needed to clarify the components in Se-enriched rice responsible for its antioxidant activity.

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