Korean J. Crop Sci. 46(5): 411~415(2001)

Antioxidative Activity of Rice Grain using FI-CL and ESR Methods

Ill Min Chung*†, Kwang Ho Kim*, Joung Kuk Ahn*, Si Kyung Lee and Jin Ohk Lee***

**Dept. of Crop Science, Konkuk Univ., Seoul 143-701, Korea*
***Dept. of Appl. Biol. & Chem., Konkuk Univ., Seoul 143-701, Korea*

ABSTRACT: Fifty-four Korean native and 28 foreign rice varieties harvested in 1998 and 1999 were examined for antioxidative activity that is measured to a chemiluminescence and superoxide radical intensity, by the flow injection chemiluminescence (FI-CL) system and an electron spin resonance (ESR) spectrophotometer, respectively. In the chemiluminescence measurement by FI-CL, radical scavenger activity did not differ significantly among rice varieties between origin types of rice varieties, and between storage periods. Ginsun and Hongchoengdo, colored rice exhibited high electron scavenging effect by ESR. Therefore, these results indicate that the pigments of rice varieties may play important antioxidative roles and that it may be possible to breed rice varieties with higher antioxidative potentials.

Keywords : rice, variety, antioxidative activity, chemiluminescence, ESR

A free radical is defined as any atom or molecule that possesses an unpaired electron (Punchard and Kelly, 1996). In biology and in related fields, the major free radical species of interest are those of oxygen, referred to as oxygen free radicals (OFRs). OFRs are part of a greater group of molecules often called reactive oxygen species (ROS). They all oxidize more strongly than molecular oxygen itself and include superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxy radical (HO^-) (Sies, 1993). The ROS generated by environmental stresses such as low and high temperatures and pollutants are associated with a number of physiological disorders in plants. The plants have evolved to a wide range of enzymatic and non-enzymatic antioxidants to escape the oxidative stress by ROS, since antioxidants have common properties to be produced for a self-defense mechanism.

Superoxide dismutase (SOD, EC 1.15.1.1), discovered by McCord and Fridovich (1969), reacts with superoxide radicals at almost diffusion-limited rates to produce hydrogen peroxide ($2O_2^- + 2H^+ = H_2O_2 + O_2$). This enzyme is unique in that its activity determines the concentrations of O_2^- and H_2O_2. SOD is present in practically all organisms, from bacteria to human beings, and there are three types, classified by their metal cofactor, which depend on the metal found at the active site. The types are copper/zinc (Cu/Zn-SOD), manganese (Mn-SOD) and iron (Fe-SOD). In higher plants, the most prominent SODs are Cu/Zn isozymes found in the cytosol and plastids (Bannister *et al.*, 1987; Sakamoto *et al.*, 1992). The activity of plant SOD is known to increase in response to a variety of environmental and chemical stimuli (Fridovich, 1986; Peri-Treves & Galun, 1988). This enzyme is indispensable for protecting organisms from damage caused by active oxygen molecules (O_2^-, OH^-). To solve these shortcomings, more specific and reliable analyses of malonaldehyde were attempted using chemiluminescence (FI-CL) and electron spin resonance (ESR). These methods have been applied to the determination of radical scavenging activity. Chemilumine scence has advantages over other techniques as it is rapid, simple and highly sensitive (Choi *et al.*, 2000). Electron spin resonance or trapping has been the most successful method for the detection of highly reactive free radicals *in vivo* (DeGray & Mason, 1994). This technique involves the addition of a primary free radical across the double bond of a diamagnetic compound (the spin trap) to form a radical adduct more stable than the primary free radical. This technique involves the indirect detection of primary free radicals that cannot be directly observed by conventional ESR due to low steady-state concentrations or to very short relaxation times, which lead to very broad lines.

Rice is one of the most important food crops in Korea. The hope for improved nourishment of the world's population depends on the development of better rice varieties and improved methods for rice production and utilization. The quality of rice seeds selected for breeding and cultivation is of great agricultural importance, especially in the context of a decrease in nutritional quality and the oxidation of harvested grain during storage. The conditions for post-harvest storage of paddy rice vary from place to place depending on the technological properties of rice seeds, biochemical properties of rice constituents, and the quality of cooked rice kernels. Rice seeds contain phenolic compounds such as flavonoids, isovitexin, cyanidin, oryzanol, α-tocopherol, and phytic acid, which exhibit strong natural antioxidant properties (Ramarathnam *et al.*, 1986, 1989; Wu *et al.*, 1994; Choi & Oh, 1996;

†Corresponding author: (Phone) +82-2-450-3730 (E-mail) imcim@konkuk.ac.kr
<Received October 31, 2001>

Osawa *et al.*, 1985). Amongst these antioxidant compounds, isovitexin and phytic acid isolated from rice hulls were found to be very strong antioxidants for inhibiting lipid peroxidation (Ramarathnam *et al.*, 1989). For this reason, rice hulls have received much attention as an economically attractive source of natural antioxidants. It is common practice to store rice after harvest to age or mature it sufficiently before it is consumed. The rate of aging depends partly on the moisture content, but mainly on the temperature (Beyer & Fridovich, 1987). Storage conditions influence the loss of dormancy in freshly harvested rice seeds after short-term storage (Navasero *et al.*, 1975). It is therefore thought that the higher the antioxidative ability, the longer the rice will store. In order to breed rice varieties containing high antioxidative substances, screening methods need to be evaluated.

FI-CL and ESR methods were examined for the measurement of radical scavenging ability and radical intensity, respectively. Therefore, the objective of this study was to investigate antioxidative activity for screening rice varieties with high antioxidative activities by FI-CL and ESR in Korean native and foreign brown rice varieties.

MATERIALS AND METHODS

Fifty-four Korean native varieties including 8 colored rice 'Jangsamdo', 46 common rice 'Arongbyeoand' 28 foreign rice varieties including 13 colored rice 'Hweiju' 15 common rice 'GPNO 12856' were cultivated at the experimental farm, College of Agriculture and Life Science, Konkuk University, in 1998 and 1999. The seeds harvested in 1998 were stored for one year at room temperature. The rice was ground in a milling machine through a 40-mesh screen, and then stored at -35°C.

The test samples were prepared by the method of Chung *et al.* (2000a, b). Brown rice powder samples (10 g) of the respective rice varieties were extracted with 200 ml of 80% MeOH for 24 hours shaking in water bath at 20°C, followed filtration (filter paper No. 4), and then evaporated the filtrate to dryness *in vacuo* at 40°C. The separated fractions were weighed to determine the respective yields of soluble component. The crude samples thus obtained were redissolved with 80% HPLC grade MeOH to make 1% (g 100 ml^{-1}; w/v) extract solution.

Activity Measurement by the FI-CL System and ESR Spectrum Chemical reagents

Luminol, hypoxanthin, xathin oxidase, and superoxide dismutase (SOD) were purchased from the Sigma Chemical Company, USA. Hydrogen peroxide was obtained from the Hayashi Pure Chemical Industry, Osaka, Japan and 5,5-Dimethyl-1-pyrroline-N-oxide (DMPO) from Aldrich Chemicals, USA.

Measurement of chemiluminescence

Analysis of CL was performed with some modifications of the method of Choi *et al.* (2000). The CL was measured with a filter-equipped photon counting type spectrophotometer (Autolumat LB953; EG & G Dertholdl, Germany). Dispersed light at the grating was simultaneously detected on the photocathode with the image sensor in the range of 300-650 nm. The scheme of the FI-CL system is in Fig. 1. The mobile phase was 50 mM phosphate buffer (pH 7.4), containing 50% MeOH (for solvent-soluble samples), cytochrome *c* (10 mg l^{-1}), and luminol (2 mg l^{-1}). The pump was used to maintain the flow rate at 1.0 min^{-1}. In order to measure the ability of radical scavengers, the mixture of 0.006% H_2O_2 (5 μl) and scavenger solution (5 μl) was injected. The reduced CL intensity of the mixture compared to the CL intensity of 0.006% H_2O_2 (5 μl) enabled the quantitative analysis of radical scavenging activity. The percentage of radical scavenging activity was calculated from the following equation:

$$\text{Radical scavenging activity (\%)} = [1-(B/A)] \times 100$$

where A is the CL intensity generated from the injection of H_2O_2 only and B is the CL intensity generated from the injection of the H_2O_2 + scavenger.

SOD activity test by ESR spectrometer

The JES-TE200 ESR spectrometer (Japan Electron Optics

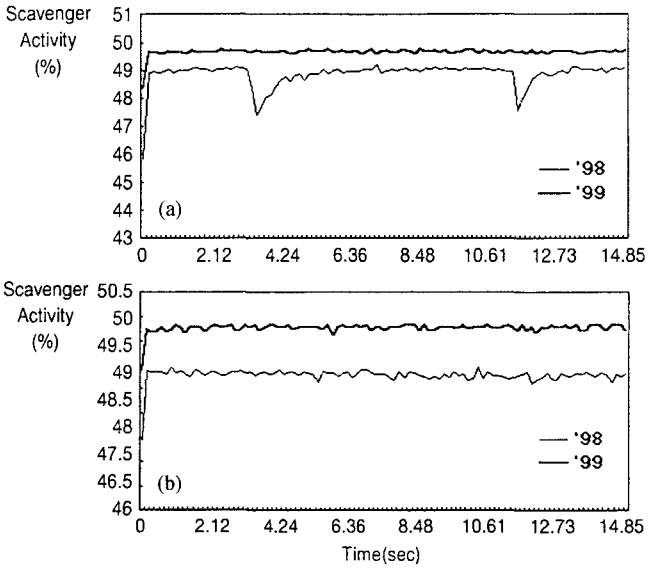

Fig. 1. FI-CL chromatograms of Oegukbyeo (a) and Red khosha cerma (b) seed extracts.

Laboratory Co. Ltd., Tokyo, Japan) was used to measure SOD activity on Ginsun, Hongcheongdo and Dabaegjo which was showed high and low SOD activity in nitro blue tetrazolium (NBT) reduction method. O_2^- radicals generated from the hypoxanthine-xanthine oxidase system were trapped by DMPO and the effect of the scavenger was compared with that of standard SOD by the method of Mitsuta *et al.* (1990) and Hiramatsu & Kohno (1987). For actual measurements, 50 μl of hypoxanthine (2 mM), 20 μl of DMPO (9.2 M) and 50 μl of scavenger or SOD were mixed. The reaction was started with the addition of 0.4 units ml^{-1} xanthine oxidase (50 μl) and an aliquot of the mixture then transferred into the cell and the ESR signal measured after 45 seconds. The intensity of the ESR signal was corrected as a ratio to the intensity of Mn^{2+}, which was used as an internal standard. Increases in ESR signal intensities were expressed in units mg of rice grain.

Statistical analysis

Analysis of variance was accomplished for all data using the general linear model procedure of the Statistical Analysis System program (SAS, 1986). All experiments were repeated three times. The pooled mean values were separated on the basis of least significant difference (LSD) at the 0.05 probability level.

RESULTS AND DISCUSSION

Antioxidative activity tests on seed extracts by Flow Injection-Chemiluminescence (FI-CL)

In the chemiluminescence measurement by FI-CL, radical scavenger activity did not differ significantly among either Korean native or foreign rice varieties (Table 1). In comparison to the storage period and variety type within each storage period, radical scavenger activity was similar between Korean native (49.3%) and foreign (49.2%) rice varieties. The chemiluminescence chromatogram of Oegukbyeo (Korean native rice) and Red khosha cerma (Foreign rice) is given in Fig. 1. CL intensity was decreased in the presence of rice

Table 1. Comparison of chemiluminescence on Korean native and foreign rice varieties.

Storage term	Type	No. of var		FI-CL
				Activity(%)
One year	Korean native rice varieties	8	Colored rice	49.4
		46	Common rice	49.1
			Mean	49.3
	Foreign rice varieties	13	Colored rice	49.1
		15	Common rice	49.4
			Mean	49.2
	Total	82		
	CV (%)			0.9
	LSD (0.05)			0.72
Three months	Korean native rice varieties	8	Colored rice	49.3
		46	Common rice	49.3
			Mean	49.3
	Foreign rice varieties	13	Colored rice	49.2
		15	Common rice	49.2
			Mean	49.2
	Total	82		
	CV (%)			0.4
	LSD (0.05)			0.35
	CV (%)†			0.7
	LSD (0.05)†			0.38

†between storage term

crude extracts (80% MeOH), indicating that extracts scavenger radicals that may be generated in the presence of luminol, hydrogen peroxide (H_2O_2), and cytochrome *c*, although the mechanism of CL generation is uncertain. In this study, the radical scavenger activity of rice grain extracts was measured, and it was thought that many compounds existing in the brown rice extracts used would play an important role in the contribution of antioxidative activity.

Measurement of SOD activity by Electron Spin Resonance (ESR)

To evaluate the NBT reduction method, high and low SOD activity extracts were tested with the ESR spin trapping method. The activity of SOD, as measured by ESR, is given in Fig. 2. Among the Korean native rice varieties studied in 1999, Hongcheongdo (17.1%) and Ginshun (16.5%), which had high SOD activity, and Dabaegjo, which had low SOD activity (4.8%), were tested by ESR for their superoxide radical intensity. Fig. 2 shows the electron spin resonance spectra of DMPO-O_2^- formed from hypoxanthin-xanthin oxidase system in DMPO and rice samples, with (a) representing the ESR signal intensities of DMPO. Hongcheongdo, Ginshun and Dabaegjo are represented by (b), (c) and (d), respectively. Dabaegjo formed the 1 : 2 : 2 : 1 spectrum type, which was similar to DMPO, known to have low SOD activity. However, Hongcheongdo and Ginshun did not form this spectrum type, indicating that they had high SOD activity. These figures show the same trend as those obtained by the SOD activity test with NBT reduction method, and underline the reliability of our measurement methods.

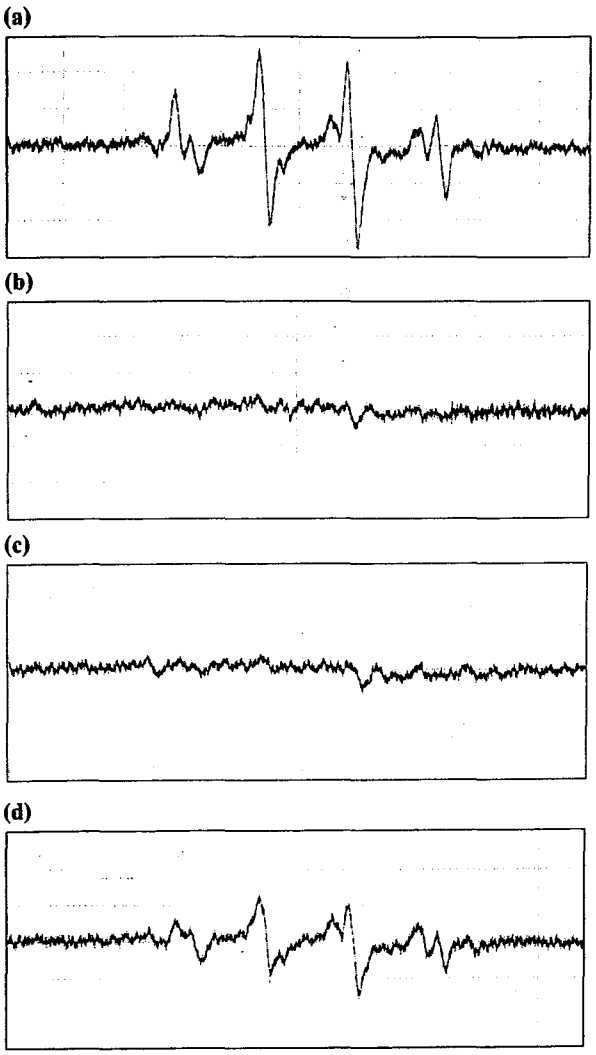

Fig. 2. ESR signal of superoxide anion radicals generated from the hypoxanthin-xanthin oxidase system in the DMPO. (a), DMPO; (b), high SOD activity variety, Ginshun; (c), high SOD activity, Hongcheongdo; (d), low SOD activity variety, Dabaegjo.

The superoxide radical is generated from molecular oxygen or hydrogen peroxide by a one-electron transfer reaction. The ESR method is known to be one of the most common and specific techniques for measuring radical scavenging activity. Mitsuta *et al.* (1990) reported that the generated O_2^- molecule was trapped stoichiometrically as the spin adduct of DMPO-O_2^- by using a spin trap DMPO. Rice seed extracts contain phytochemicals, plant phenolics and flavonoids and react with active oxygen radicals, such as superoxide anion radicals and hydroxy radicals to inhibit lipid oxidation. The result is similar to Torel *et al.* (1986), who concluded flavonoids, one of the most important antioxidant compounds inhibited lipid oxidation.

Our results point to the possibility that antioxidative activity of rice may be due in part to the presence of antioxidative compounds, including phenolic compounds would be involved in the inhibition of lipid oxidation. This compound was essentially identical to that obtained for authentic standard. In the present study, however, it was not possible to determine specifically which antioxidative compounds were present in the seed extracts. Further purification and using mass spectrometer and nuclear magnetic resonance would help identify antioxidative compounds.

ACKNOWLEDGMENTS

This study was conducted with support of the 2000-2001 ARPC research fund.

REFERENCES

Bannister, J.V., W. Bannister and H.G. Rotilio. 1987. Aspects of

the structure, function and applications of superoxide dismutase. *CRC Crit. Rev. Biochem.* 22 : 111-180.

Beyer, W.F. Jr. and I. Fridovich. 1987. Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. *Anal. Biochem.* 161 : 559-566.

Choi, H.C. and S.K. Oh. 1996. Diversity and function of pigments in colored rice. *Korean J. Crop Sci.* 41 : 1-9.

Choi, H.Y., E.J. Jhun, B.O. Lim, I.M. Chung, S.H. Kyung and D.K. Park. 2000. Application of flow injection-chemiluminescence to the study of radical scavenging activity in plant. *Phytotherapy Research.* 14 : 250-253.

Chung, I.M., K.H. Kim, J.K. Ahn and J.O. Lee. 2000a. Varietal Variation in Antioxidative Activity of Rice Grain by DPPH and TBA Methods. *Korean J. Crop Science.* 45 : 261-266.

Chung, I.M., K.H. Kim, J.K. Ahn and J.O. Lee. 2000b. Comparison of SOD and POD Activities in Rice Varieties. *Korean J. Crop Science.* 45 : 277-281.

DeGray, J.A. and R.P. Mason. 1994. (ed. N.M. Atherton, M.J. Davies, and B.C. Gilbert). *In* Specialist periodical report-electron spin resonance. Royal Society of Chemistry, Cambridge. 14 : 246-301.

Fridovichi, I. 1986. Superoxide dismutases. *Adv. Enzymol.* 58 : 61-97.

Hiramatsu, M. and M. Kohno. 1987. Determination of superoxide dismutase activity by electron spin resonance spectrometry using the spin trap method. *JEOL News.* 23 : 7-9.

MaCord, J.M. and I. Fridovich. 1969. Superoxide dismutase. An enzyme function for erythrocuprein (hemocuprein). *J. Biol. Chem.* 244 : 6049-6055.

Mitsuta, K., Y. Mizuta, M. Kohno, M. Hiramatsu and A. Mori. 1990. Application of ESR spin trapping technique to the evaluation of SOD like activity of biological substances. *Bull Chem. Soc. Jpn.* 63 : 187-191.

Navasero, E.P., L.C. Baun and B.O. Juliano. 1975. Grain dormancy, peroxidase activity and oxygen uptake in *Oryza sativa*. *Phytochem.* 14 : 1899-1902.

Osawa, T., N. Ramarathnam, K. Shunro, N. Mitsuo and T. Toru. 1985. Antioxidative defense system in rice hull against damage caused by oxygen radicals. *Agric. Biol. Chem.* 49 : 3085-3087.

Peri-Treves, R. and E. Galun. 1988. The Cu/Zn-superoxide dismutase genes are developmentally regulated and respond to light and stress. *Plant Mol. Biol.* 17 : 745-760.

Punchard, N.A. and F.J. Kelly. 1996. Free Radicals: A practical approach. IRL Press. pp.1-6.

Ramarathnam, N., T. Osawa, M. Namiki and T. Tashiro. 1986. Studies on the relationship between antioxidative activity of rice hull and germination ability of rice seeds. *J. Sci. Food Agric.* 37 : 719-726.

Ramarathnam, N., T. Osawa, M. Namiki and T. Tashiro. 1989. Chemical studies on novel rice hull antioxidants. 2. Identification of isovitexin, a *c*-glycosyl flavonoid. *J. Agric. Food Chem.* 37 : 316-319.

Sakamoto, A., H. Ohsuga and K. Tanaka. 1992. Nucleotide sequences of two cDNA clones encoding different Cu/Zn-superoxide dismutases expressed in developing rice seed. *Plant Mol. Biol.* 19 : 323-327.

SAS Institute. 1986. SAS User's Guide; Basic. 5th ed. SAS Institute, Cary, NC.

Sies, H. 1993. Strategies of antioxidant defense. *Eur. J. Biochem.* 2 : 213-215.

Torel, J., J. Cillard and P. Cillard. 1986. Antioxidant activity of flavonoids and reactivity with peroxyradicals. *Phytochem.* 25 : 383-385.

Wu, K., W. Zhang, P.B. Addis, R.J. Epley, A.M. Salih and J. Lehrfeld. 1994. Antioxidant properties of wild rice. *J. Agric. Food Chem.* 42 : 34-37.