Evaluation of Free Radical Scavenging Activities of Antioxidants with an H_2O_2/NaOH/DMSO System by Electron Spin Resonance

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An H_2O_2/NaOH/DMSO system has been developed for the formation of three free radicals, and the application of the system was examined with the antioxidants ascorbic acid and tocopherol. Superoxide anion, hydroxyl radical,and methyl radical are simultaneously generated in this system. The scavenging activity of ascorbic acid and tocopherol for these radicals was estimated by 5,5′-dimethyl-1-pyrroline-*N*-oxide spin trapping electron spin resonance. Both water-soluble and oil-soluble antioxidants could be evaluated by using this system. Ascorbic acid specifically inhibited the superoxide anion and hydroxyl radical, whereas tocopherol suppressed the methyl radical.

Keywords: *Electron spin resonance; superoxide anion; hydroxyl radical; methyl radical; antioxidant; free radicals*

INTRODUCTION

Reactive oxygen species (ROS) are believed to play a critical role in many diseases such as cancer (Muramatsu et al., 1995; Leanderson et al., 1997), arteriosclerosis (Steinberg, 1989), gastric ulcers (Sussman and Bulkley, 1990; Debashis et al., 1997), and other conditions (Brown et al., 1996; Oliver et al., 1987; Busciglio and Yankner, 1996; Smith et al., 1996; Babizhayev and Costa, 1994). The intake of antioxidants such as ascorbic acid (AsA), tocopherol (Toc), carotene (Car), and polyphenols from food has been seen as a very attractive method of preventing these diseases (Vinson et al., 1995; Roginsky et al., 1996; Teissedre et al., 1996; Wiseman et al., 1997; Lotito and Fraga, 1998; Cohly et al., 1998).

Several methods to evaluate antioxidants have been reported and adopted (Fujita et al., 1988; McCord and Fridovich, 1969; Beauchamp and Fridovich, 1971; Dechatelet et al., 1974; Smith et al., 1990; Jorge et al., 1991; Puppo, 1992). These include 1,1,-diphenyl-2-picrylhydrazyl (DPPH) (Kirigaya et al., 1971), peroxide value (POV), thiobarbituric acid value (TBA) (Buege and Aust, 1978; Fraga et al., 1988), and others (Kumazawa and Oyama, 1965; Olcott and Einsett, 1958; Yagi, 1970; Yamamoto and Niki, 1989; Nishikimi et al., 1972; Babbs and Gale, 1987). The targets of the measurement were not necessarily specified in these methods, and in some cases there is no strict correlation between the values from different methods.

In electron spin resonance (ESR) studies on the radical scavenging activity of antioxidants, the xanthine/xanthine oxidase system and the Fenton reaction system are frequently adopted as sources of free radical generation (Mitsuya et al., 1990; Kohno et al., 1994; Ogawa et al., 1994). Because the xanthine/xanthine oxidase system is based on an enzymatic reaction, polyphenolic antioxidants might prevent superoxide anion generation by nonspecific interaction with the protein. In the Fenton reaction, which is a metal ion catalyzed system, polyphenolic antioxidants might also prevent the hydroxyl radical formation by chelation with the catalytic metal ion.

From the background mentioned above, we intended to establish a nonenzymatic and non-Fenton type ROS generating system and to apply it to the evaluation of antioxidants.

MATERIALS AND METHODS

Materials. DMPO was obtained from LABOTEC Co. (Tokyo, Japan). TEMPO was obtained from Aldrich Chemical Co. (Milwaukee, WI). Other chemicals were obtained from Wako Pure Chem. Ind., Ltd. (Osaka, Japan) unless otherwise mentioned.

Instruments. ESR spectra were recorded on a JEOL JES-RE1X spectrometer using a flat quartz cell designed for aqueous solution.

ESR Conditions. Conditions of ESR spectrometry were as follows: magnetic field, 336.3 ± 5 mT; power, 8.0 mW; modulation frequency, 100 kHz; frequency, 9.425GHz; modulation amplitude, 0.063 mT; gain, 500; time scan, 1 min; time constant, 0.03 s.

Procedure for Free Radical Generation. Fifty microliters of DMSO and the same volume of 25 mM NaOH and sample solution (aqueous) were mixed in a disposable plastic tube, followed by the addition of 5 μL of DMPO and 50 μL of 30% hydrogen peroxide. In the case of an oil-soluble antioxidant, the sample was dissolved in DMSO and 50 μL of it and an equal volume of water prepared by a Milli-Q reagent water system (Millipore) were mixed, followed by the addition of 5 μL of DMPO and 50 μL of 30% hydrogen peroxide.

The reaction mixture was put into the flat quartz cell and set in the ESR apparatus, then the scanning was begun after an appropriate period.

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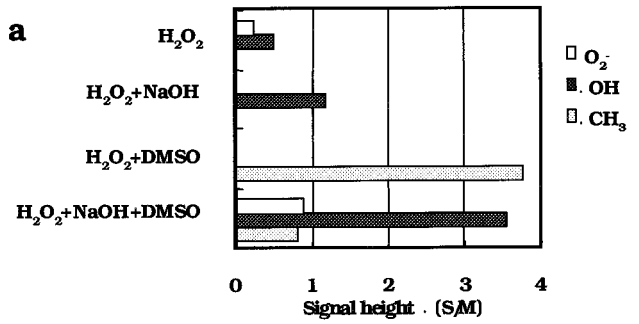

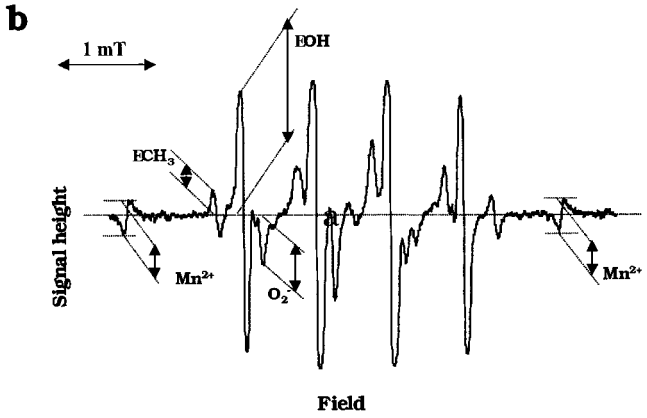

Hyperfine coupling constants of spin adducts

adducts	hfcc, mT		
	a^N	a^H_β	a^H_γ
DMPO-OH	0.143	0.117	0.013
DMPO-OOH	0.149	0.149	
DMPO-CH_3	0.164	0.224	

Figure 1. Free radical formation in the H_2O_2/NaOH/DMSO system: (a) Each component of the H_2O_2/NaOH/DMSO system added to the reaction is indicated in the vertical axis. Signal height indicates the ratio of signal intensity to Mn^{2+}, represented as S/M in text. (b) is a typical ESR spectrum in the H_2O_2/NaOH/DMSO system. ESR conditions are described in the text. Each free radical derived signal was assigned from the hyperfine structure constant (hfsc), which is shown under the spectrum, and the signal height is indicated at the double-sided arrows.

The signal height was calculated using a radical analyzer program (ESR data analyzer, LABOTEC Co.) attached to the instrument. Calculation was done for the positive signal height of methyl radical−DMPO adducts and hydroxyl radical−DMPO adducts and for the negative signal height of superoxide anion−DMPO adducts in the lowest magnetic field. The ratio of signal intensity against Mn^{2+} was shown by S/M.

RESULTS AND DISCUSSION

Mechanism of the Reaction of the H_2O_2/NaOH/DMSO System. To study the mechanism of radical generation, the signal height of three free radical adducts generated from separate and combined components of the H_2O_2/NaOH/DMSO system are shown in Figure 1a. In the case of only hydrogen peroxide alone, small signals of superoxide anion and hydroxyl radical were observed. When hydrogen peroxide and NaOH were mixed, only hydroxyl radical was observed. When hydrogen peroxide and DMSO were mixed, only methyl radical was observed. When all of the components were mixed, a typical spectrum of superoxide anion, hydroxyl radical, and methyl radical were visible as shown in Figure 1b. From these observations, the mechanisms of these radical-generating reactions were speculated to be as follows. Superoxide anion and hydroxyl radical are generated from the degradation of hydrogen peroxide. This reaction may be facilitated by alkaline. Superoxide anion changes into the hydroxyl radical by catalytic action of contaminated trace iron so that the amount of hydroxyl radical is relatively larger than that of superoxide anion (Baker and Gebicki, 1984). In this system, a one-electron reductant facilitates the generation of superoxide anion under alkaline conditions, and some samples may increase the amount of superoxide anion.

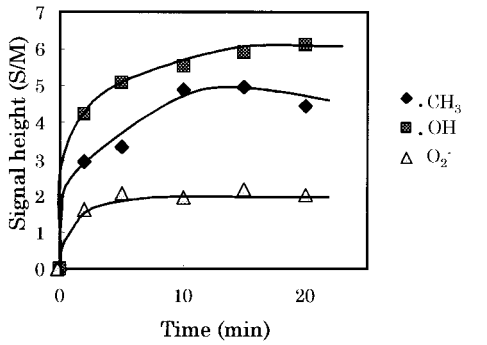

Figure 2. Time course of free radical generation in the H_2O_2/NaOH/DMSO system. Scanning was started at each point, i.e., 2, 5, 10, 15, and 20 min.

The hydroxyl radical can generate the methyl radical from DMSO. A sample such as L-ascorbic acid (AsA), which can be changed to a relatively stable radical itself, may also attack DMSO directly and generate the methyl radical. A free radical scavenger, which can scavenge free radicals directly, has no relationship with these reactions mentioned above.

There were no significant changes of the signals under conditions in which the concentration of NaOH was changed from 10 to 50 mM (data not shown). Then we fixed the concentration of NaOH at 25 mM. In many cases, the pH value was between 8 and 9 when the antioxidants were added to the system. Therefore, we believe that the effect of pH change on radical formation is minimal in this system.

Thus, the H_2O_2/NaOH/DMSO system is considered to be useful for the evaluation of radical scavengers.

Time Course of the Reaction in the H_2O_2/NaOH/DMSO System. After all of the reaction compounds were mixed, scanning was done at an appropriate time and signal height (ratio against internal Mn marker) of each radical−DMPO adduct recorded (Figure 2). Each S/M intensity was increased and reached a plateau in 10 or 15 min. The reaction time was then fixed for 10 min in the following experiments.

Calibration Curve of Radical Concentration and Signal−Height Using TEMPO. A series of different concentrations of 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) solution were prepared, and their ESR spectra were recorded. The concentration of the radical and the ESR signal height were linearly correlated between 0.1 and 0.4 μM, and the square of the correlation coefficient was 0.995. Under this condition, the linear correlation between free radical concentration and signal height (S/M) was confirmed.

Coefficient of Variation in the Measurement. In five measurements, the average and standard deviation of each signal height were calculated. The coefficient of

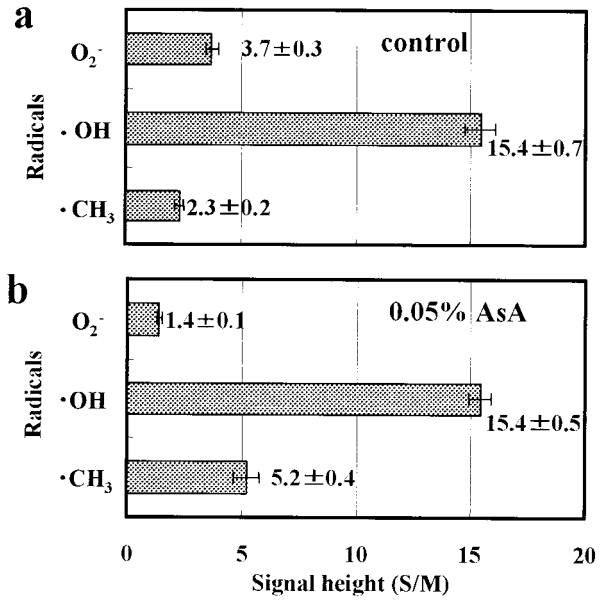

Figure 3. Coefficient of variation in the H_2O_2/NaOH/DMSO system. Radical species are indicated in the vertical axis. The values of average and standard deviation are indicated to the right of each bar.

variation (CV; percent of standard deviation and average) was below 10 (Figure 3a). When 0.05% AsA was added to the system, the signals of superoxide anion and hydroxyl radical were suppressed, and the signal of the methyl radical was increased. The CV of each signal was below 10 (Figure 3b).

Free Radical Scavenging Activity of Ascorbic Acid and Tocopherol. A series of different concentrations of ascorbic acid was prepared, and each of them was added to the H_2O_2/NaOH/DMSO system. Ascorbic acid reduced the signal height of the superoxide anion, and the concentration of added ascorbic acid and log(S/M) was linearly correlated (Figure 4a). Ascorbic acid also reduced the signal height of the hydroxyl radical, and a linear dose dependency was observed between ascorbic acid concentration and log(S/M) (Figure 4b). In contrast, ascorbic acid increased the signal height of the methyl radical. When a high concentration of ascorbic acid was used, the signal of the ascorbyl radical was observed (data not shown).

Therefore, it could be speculated that the reactions of ascorbic acid and free radicals generated ascorbyl radical, which attacked DMSO and generated methyl radical, or that the ascorbyl radical degraded into the methyl radical directly. In another possibility, ascorbic acid may react with a trace of contaminated metal ion and play a role as pro-oxidant (Uchida and Kawakishi, 1986; Sakagami et al., 1997; Podmore et al., 1998).

Unlike ascorbic acid, DL-α-tocopherol (Toc) reduced the signal height of the methyl radical. A linear dose dependency was observed between the concentration of Toc and log(S/M) (Figure 4c).

In this system, actually the ascorbic acid radical was observed when ascorbic acid was added at relatively high concentration (~>0.5%). Ascorbic acid showed its radical scavenging activity at a concentration below 0.05% as shown in Figure 4. Generally radical scavengers show their radical scavenging activity at much lower concentrations than that of their own radical formation in this system. Therefore, this system could be available under a condition in which the antioxidant's radical is not formed.

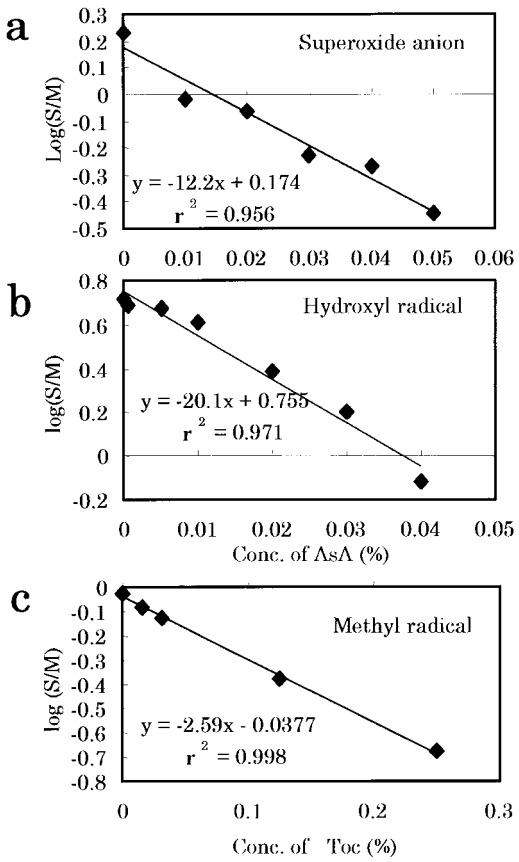

Figure 4. Radical scavenging activity of AsA and Toc. Concentration of AsA refers to that in the prepared solution before mixing with other components of the reaction system.

From the above observations, it is concluded that the H_2O_2/NaOH/DMSO system may be very useful for quantification of the radical scavenging activity of both water-soluble and oil-soluble antioxidants. Actually, the pH value of this system (almost 9) was not a physiological one, so the utility of this method is limited.

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