Free Radical Scavenging Activity of Grape Seed Extract and Antioxidants by Electron Spin Resonance Spectrometry in an H_2O_2/NaOH/DMSO System

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The scavenging effects of grape seed extract (GSE) on free radicals formed in an H_2O_2/NaOH/DMSO system were examined using a spin-trapping electron spin resonance (ESR) method and compared with other natural antioxidants, ascorbic acid, *dl*-α-tocopherol, and *β*-carotene. GSE reduced greatly the ESR signal intensity of superoxide radical−5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) adducts. GSE also exhibited weak scavenging activity on hydroxyl radical and a little scavenging activity on methyl radical. Ascorbic acid exhibited strong superoxide and hydroxyl radical scavenging activities, but it increased the amount of methyl radical at high concentration. *dl*-α-Tocopherol reduced the amount of superoxide anion, especially the amount of methyl radical. However, it slightly reduced the amount of hydroxyl radical. *β*-Carotene reduced the amount of hydroxyl radical and methyl radical, but it also slightly reduced superoxide anion. In the case of combination use of *β*-carotene and *dl*-α-tocopherol, all radical species were suppressed. Combination of GSE and *dl*-α-tocopherol also could reduce all radical species. *β*-Carotene and *dl*-α-tocopherol could reduce the methyl radical formation induced by ascorbic acid.

Keywords: *Electron spin resonance; reactive oxygen species; superoxide anion; hydroxyl radical; methyl radical; grape seed extract*

INTRODUCTION

In recent years, it has been regarded that reactive oxygen species (ROS) play a critical role in many diseases such as cancer (Muramatsu et al., 1995), arteriosclerosis (Steinberg et al., 1989), gastric ulcer (Das et al., 1997), and other diseases (Babizhayev and Costa, 1994; Sussman and Bulkley, 1990; Busciglio and Yankner, 1996; Brown et al., 1996; Smith et al., 1996; Oliver et al., 1987). To prevent these diseases, the intake of antioxidants such as polyphenols in tea and red wine has been greatly emphasized (Vinson et al., 1995; Teissedre et al., 1996; Leandrson et al., 1997; Wiseman et al., 1997; Lotito and Fraga, 1998; Cohly et al., 1998; Cao et al., 1997). Especially, it is suggested that the intake of polyphenols from red wine reduces the risk of cardiovascular disease and arteriosclerosis from epidemiological studies (Renaud and De Lorgeril, 1992).

Grape seed extract (GSE) is manufactured with aqueous ethanol extraction from grape seed, and it is rich in polyphenols (proanthocyanidin). Antioxidative and radical scavenging activity of proanthocyanidins had been reported (Fujita et al., 1988; Ricardo da Silva et al., 1991; Ariga, 1990; Ariga and Hamano, 1990; Ariga et al., 1988). We have been studying the biochemical and pharmacological properties of GSE. We have already reported its anticataract effect (Yamakoshi et al., 1998a,b), antiulcer effect (Saito et al., 1998), anticancer effect (Arii et al., 1998), and antiarteriosclerosis effect (Yamakoshi et al., 1998a,b) in vivo. We intend to clarify the free radical scavenging property of GSE in vitro and to analyze the mechanisms of its pharmacological effects.

In several reports (Mitsuya et al., 1990; Ogawa et al., 1994), the reduction of 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) spin adducts in electron spin resonance (ESR) was applied to the determination of the radical scavenging activities, but it was difficult to apply this method to water-soluble substances. The H_2O_2/NaOH/DMSO system has been developed for the evaluation of antioxidative ability of both water-soluble and oil-soluble antioxidants (Yoshimura et al., unpublished results). The mechanisms of free radical formation in the H_2O_2/NaOH/DMSO system assumed that superoxide anion and hydroxyl radical were generated from degradation of hydrogen peroxide and that methyl radical was generated from the degradation of DMSO by hydroxyl radical. This reaction may be facilitated by alkaline. Superoxide anion changes into hydroxyl radical by catalytic action of contaminated trace iron, so that the amount of hydroxyl radical is relatively larger than that of superoxide anion consequently. Using this system, radical scavenging activities for superoxide anion, hydroxyl radical, and methyl radical can be evaluated at the same time. Therefore, this system can be used for the characterization of the radical scavenging property of antioxidants. We show here the radical scavenging property of GSE in this system.

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Table 1. Analytical Data of GSE

components	%
proanthocyanidins	38.7
monomeric flavanols	2.40
moisture (by Karl Fischer's method)	2.10
crude protein (total N × 6.25)	3.70
fat (method with Soxhlet extractor)	0
fiber	0
ash	5.00
glucose	7.79
fructose	8.85
other sugars	2.66
organic acid	11.60
other acids	5.05
other components from grape seeds	11.99

MATERIALS AND METHODS

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

Preparation of GSE. GSEs from grape seed (*Vitis vinifera* were obtained from Kikkoman Corp. (Tokyo). GSE was extracted by 20% ethanol solution from the seeds. Typical chemical analysis data of GSE are shown in Table 1.

Preparation of Synthetic Procyanidin Oligomers. Synthetic procyanidin oligomers (dimers, trimers, tetramers, and pentamers) were prepared according to methods described in our previous paper (Saito et al., 1998). In brief, (+)-catechin and (+)-taxifolin were condensed in the presence of $NaBH_4$ in ethanol. The reactant was separated using Sephadex LH-20 column chromatogrhaphy.

Instruments. ESR spectra were recorded on a JEOL JES-RE1X spectrometer using a quartz flat 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, 9.425 GHz; modulation amplitude, 0.063 mT; gain, 500; time scan, 1 min; time constant, 0.03 s.

Experimental Procedure. Fifty microliters of dimethyl sulfoxide (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 oil-soluble antioxidant, the sample was dissolved in DMSO and 50 μL of it and the same volume of pure water (prepared by Milli-Q reagent water system) and the same volume of 25 mM NaOH were mixed, followed by the addition of 5 μL of DMPO and 50 μL of 30% hydrogen peroxide.

The reaction mixture was sucked into the quartz flat cell and set in the ESR apparatus; scanning was started at 10 min after the addition of hydrogen peroxide. The signal intensities of three radical species−DMPO adducts reached plateaus between 10 and 20 min after the start of the reaction.

Generation of ROS. The signal height was calculated using a radical analyzer program attached to the instrument. The calculation was done for the positive signal height of the methyl radical−DMPO adduct and the hydroxyl radical−DMPO adduct and for the negative signal height of the superoxide−DMPO adduct in the lowest magnetic field. The ratio of signal intensity against Mn^{2+} as reference represented S/M (Figure 1). Identification of three ESR spectra was made by hyperfine coupling constant of DMPO adducts as shown in Table 2.

RESULTS AND DISCUSSION

Ascorbic acid (AsA) suppressed superoxide anion at the concentration of 0.01% (Figure 2a). AsA also suppressed hydroxyl radical in almost the same manner, but it enhanced methyl radical formation at high concentration (1%). In such case, the ESR signal of the ascorbyl radical was also observed (Figure 3). Therefore, it is speculated that the ascorbyl radical attacked DMSO and generated the methyl radical. This observation may be in vitro evidence that suggests the harmfulness of excess intake of AsA (Uchida and Kawakishi, 1986; Sakagami et al., 1997; Podmore et al., 1998).

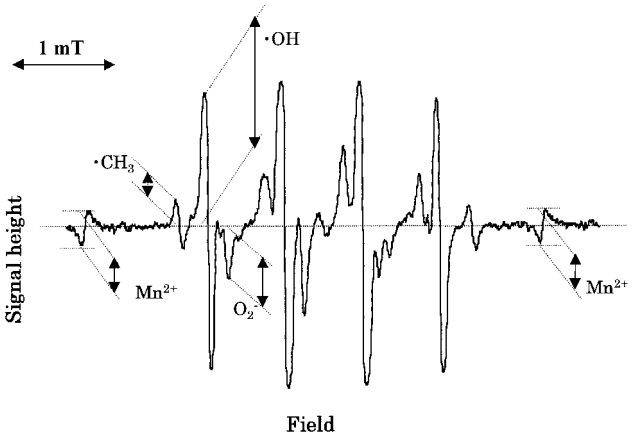

Figure 1. Typical ESR spectrum in the H_2O_2/NaOH/DMSO system. ESR conditions were described in the text. The scannning was started at 10 min after the mixing of all reagents. Each free radical derived signal was assigned, and signal heights were calculated at double-sided arrows in the figure. The intensity of the hydroxyl radical was so strong that the second and third peaks of the quartet signal of the hydroxyl radical−DMPO adduct were out of the upper limit of the display of the ESR apparatus. If the amplification rate of the apparatus was adjusted to obtain the proper spectrum of the hydroxyl radical−DMPO adduct (1:2:2:1), the signal height of the superoxide anion−DMPO adduct might be so small that it would be difficult to show the signal in the figure.

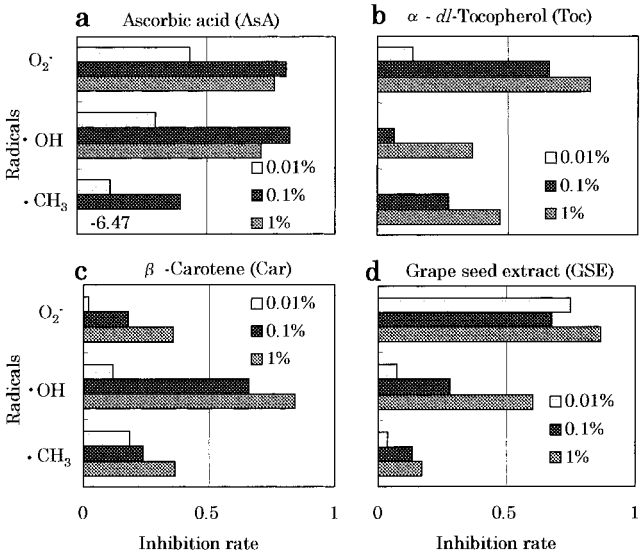

Figure 2. Radical scavenging property of AsA, Toc, Car, and GSE. Each antioxidant was added in the reaction system at three concentrations described in the figure. Inhibition rate was defined as the following equation: inhibition rate = 1 − signal height (sample)/signal height (control). The signal heights were normalized to the Mn^{2+} resonance.

Table 2. Hyperfine Coupling Constants of Spin Adducts

	hfcc, mT		
DMPO adduct	a_N	$a\square_H$	$a\square_H$
DMPO−OH	0.143	0.117	0.013
DMPO−OOH	0.149	0.149	
DMPO−CH_3	0.164	0.224	

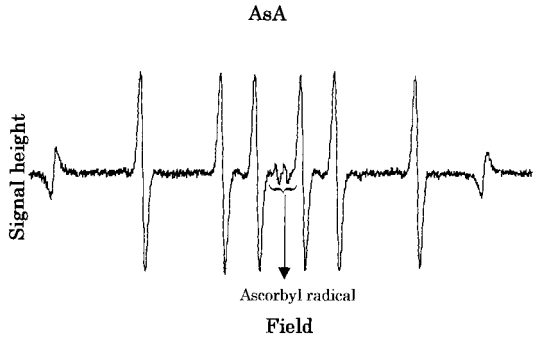

Figure 3. ESR spectra of AsA-induced methyl radical enhancement in the H_2O_2/NaOH/DMSO system. AsA (1.5%) was added to the reaction mixture. The signals of six-split methyl radical−DMPO adduct and the signals of doublet ascorbyl radical were observed. Each signal was assigned by its *g* value.

dl-α-Tocopherol (Toc) suppressed superoxide anion almost in the same manner as AsA, but the suppression rate against hydroxyl radical was smaller (Figure 2b). On the other hand, Toc exhibited potent specificity on methyl radical. This result may explain the evidence that Toc is used frequently as a powerful antioxidant for food preservation.

β-Carotene (Car) exhibited specificity on hydroxyl radical, although its suppression rate for superoxide anion was smaller than that of other antioxidants (Figure 2c).

Indeed, the possibility that the reduction of the hydroxyl radical−DMPO adduct with AsA resulted in the reduction of the methyl radical−DMPO adduct remained. However, in an appropriate condition, although Toc suppressed the superoxide anion−DMPO adduct and the methyl radical−DMPO adduct, it slightly affected the hydroxyl radical−DMPO adduct. With Car, the suppression rate of the methyl radical−DMPO adduct was relatively small compared with the suppression rate of the hydroxyl radical−DMPO adduct. Therefore, it was suggested that the effects of those antioxidants on methyl radical−DMPO adducts were independent from or preceded the reduction of hydroxyl radical−DMPO adducts.

Actually, methyl radical production depends on the reaction of hydroxyl radical and DMSO or other secondary reactions such as the reaction of ascorbyl radical with DMSO; the change of the methyl radical−DMPO adduct may not reflect the methyl radical scavenge immediately. Therefore, some hydroxyl radical scavenger may reduce the methyl−DMPO adduct and the formation of ascorbyl radical may enhance methyl radical−DMPO adduct. However, in the case that some antioxidants such as TOC can suppress the methyl−DMPO adduct and can slightly suppress the hydroxyl−DMPO adduct, it can be supposed that the antioxidant can preferably scavenge the methyl radical. Therefore, comparison of the changes of each signal, methyl−DMPO, hydroxyl−DMPO, and superoxide−DMPO, is useful for studying the radical scavenging property of antioxidants in the H_2O_2/NaOH/DMSO system.

GSE exhibited potent superoxide anion suppression, and it slightly suppressed hydroxyl radical. GSE exhibited a little inhibitory effect on methyl radical (Figure 2d). At alkaline pH, polyphenols in GSE may autoxidize, producing an additional source of superoxide anion, but enhancement of the superoxide anion−DMPO adduct with GSE was not observed. Therefore, some component

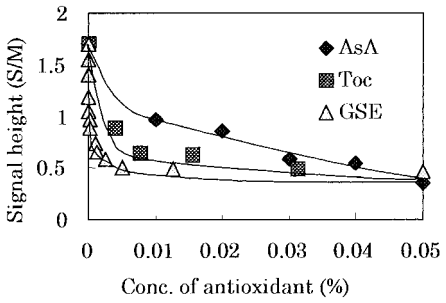

Figure 4. Dose dependency of superoxide anion suppression by AsA, Toc, and GSE in the H_2O_2/NaOH/DMSO system. The dilution series of each antioxidant was prepared. Each was added to the reaction system. The values indicated on the *x*-axis are the concentration in the prepared antioxidant solution (not the final concentration in the reaction system).

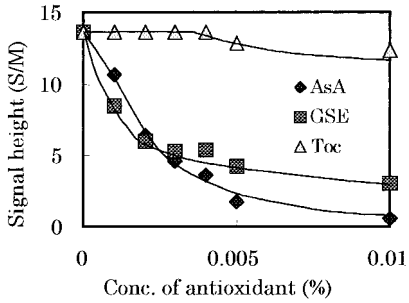

Figure 5. Dose dependency of superoxide anion suppression by AsA, Toc, and GSE in the hypoxanthin/xanthin oxidase system. The values indicated on the *x*-axis are the concentration in the prepared antioxidant solution (not the final concentration in the reaction system) as in Figure 4. Superoxide anion generation was done as described as follows. Fifty microliters of 100 mM HEPES buffer (pH 7.4), an equal volume of antioxidant solution, an equal volume of 2 mM hypoxanthin (Sigma Chemical Co.), and 5 μL of DMPO were mixed in a test tube. Scanning was started at 1 min after the addition of 50 μL of 0.4 unit/mL xanthin oxidase (Sigma) in 100 mM HEPES buffer. The signal height of the superoxide anion−DMPO adduct observed in the lowest magnetic field was calculated as the ratio against Mn^{2+}.

coexisting in GSE may prevent the autoxidation of polyphenol at alkaline pH.

Dose response curves of AsA, Toc, and GSE against superoxide anion in the H_2O_2/NaOH/DMSO system are shown in Figure 4. GSE was the most effective in those three antioxidants at the lowest concentration. The IC_{50} of GSE was estimated at almost 0.0005%, while that of Toc was almost 0.004% and that of AsA was 0.015%. It was concluded that GSE was a potent superoxide anion inhibitor in the H_2O_2/NaOH/DMSO system. The IC_{50} of GSE against superoxide anion was estimated to be almost 30 times smaller than that of AsA and 10 times smaller than that of Toc from the figure.

Indeed, it is difficult to extrapolate those results at alkaline pH to that of physiological pH. Therefore, our results should be understood as limited information. For comparison, measurement of the superoxide anion scavenging activity of antioxidants in a hypoxanthine/xanthine oxidase system at physiological pH was done. In this system, a physiological superoxide generating system, GSE was also the most effective at the lowest concentration (Figure 5). Toc was emulsified with sodium dodecyl sulfate and added to the system, and then it was less effective. Therefore, the lesser effectiveness of Toc may be caused by its solubility in an aqueous solution. Thus, an aqueous system such as the hypoxanthine/xanthine oxidase system may be disadvanta-

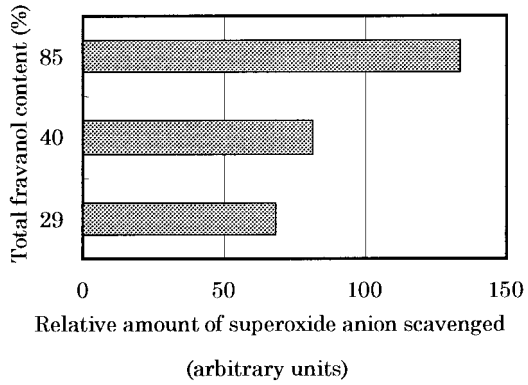

Figure 6. Superoxide anion scavenging activity of different preparations of GSE in the H_2O_2/NaOH/DMSO system. The definition of scavenged radical molecules in Figures 6 and 7 was a tentative one for convenience. The intensity (normalized to Mn^{2+}) of the signal in the lowest magnetic field of the Tempol triplet for a particular concentration was simply applied as radical concentration. Superoxide anion scavenging activity was determined as the rate of the dose response curve that indicated the linear relationship between sample concentration and signal height.

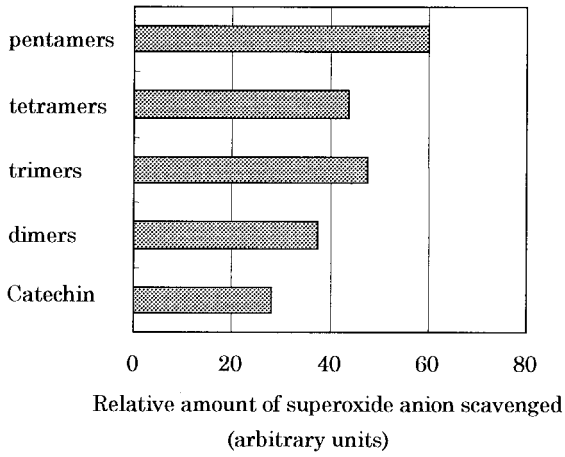

Figure 7. Superoxide anion scavenging activity of synthetic procyanidin (PC) oligomers in the H_2O_2/NaOH/DMSO system. The calculation was done in the same way as in the legend of Figure 6. Catechin was used as a flavanol monomer.

geous to oil-soluble antioxidants like tocopherol as we mentioned in the Introduction.

In the H_2O_2/NaOH/DMSO system, the relationship between superoxide scavenging activity and flavanol contents in GSE was examined by using different preparations of GSE. The flavanol contents in different preparations of GSE were determined according to the method of Broadhurst and Jones (1978). Dose dependence between superoxide scavenging activity and flavanol contents was observed (Figure 6). Thus, it was speculated that superoxide anion scavenging activity mainly depended on flavanols (proanthocyanidins) in GSE.

Flavanols in GSE are a mixture of several kinds of procyanidin oligomers that contains different compositions in the degree of polymerization. The relationship between superoxide scavenging activity and degree of polymerization of procyanidins was examined using synthetic oligomers (Ariga, 1990; Saito et al., 1998) in the H_2O_2/NaOH/DMSO system. The degree of polymerization became higher, and the superoxide anion scavenging activity became stronger (Figure 7). Therefore, it can be expected that the flavanol content and its degree of polymerization become higher and the superoxide anion scavenging activity of GSE becomes stronger.

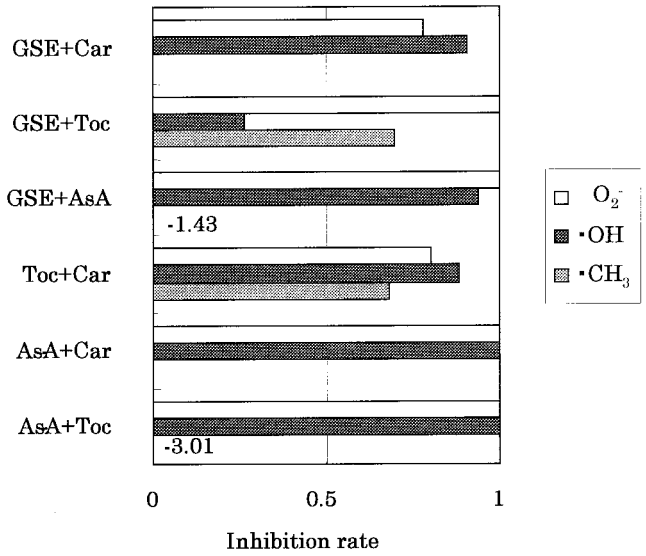

Figure 8. Effect of combination use of antioxidants. AsA solution was prepared at the concentration of 0.05%. Other antioxidant solutions were prepared at the concentration of 1.0%. Fifty microliters of each solution was added in 200 μL of reaction mixture. Inhibition rate was defined as in the legend of Figure 2.

Because antioxidant shows its own specificity against each radical species, combination use of different kinds of antioxidants may be effective to inhibit complex radical formation such as cellular membrane peroxidation triggered by superoxide anion in biological systems. Therefore, we tried to apply the H_2O_2/NaOH/DMSO system to the evaluation of combination use of antioxidants (Figure 8). Actually, AsA and Toc almost inhibited both superoxide anion and hydroxyl radical and reversed AsA-induced methyl radical enhancement. In the case of Toc and Car, all kinds of free radicals were well suppressed. Toc and GSE also showed suppression of all radical species.

From these observations, the relationship between the molecular structure of an antioxidant and its specificity against free radicals could be speculated. The ene-diol structure in AsA is effective for the superoxide anion and hydroxyl radical. The phenolic hydroxyl group in Toc and GSE (procyanidins) is effective for superoxide anion and methyl radical. The conjugated double bonds in Car are effective for hydroxyl radical and methyl radical.

Besides these partial structures of the molecules, the polarity also may affect the specificity against free radicals. Relatively low polarity molecules such as Toc and Car may specifically scavenge methyl radical.

In conclusion, there is no antioxidant that can scavenge all kinds of free radicals. Therefore, radical scavenging properties of antioxidants should be estimated using a suitable method as shown here. Moreover, combination use of antioxidants should be appropriately designed for preventing free radical induced diseases. We are convinced that the spin-trap ESR method in an H_2O_2/NaOH/DMSO system is very useful for such designing. GSE may act not only as a potent superoxide anion scavenger but also as a synergist for AsA, Toc, and Car.

ABBREVIATIONS USED

ESR, electron spin resonance; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; AsA, L-ascorbic acid; Toc, *dl*-α-tocopherol; Car, *β*-carotene; ROS, reactive oxygen species.

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