Determination of Peroxyl Radical-Scavenging Activity in Food by Using Bactericidal Action of Alkyl Peroxyl Radical

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A novel bioassay for antioxidant activity based on bactericidal actions of alkyl peroxyl radicals (ROO•) was developed. ROO• was generated by the reaction of heme irons and various alkyl hydroperoxides. A Gram-positive bacterium, *Staphylococcus aureus*, was subjected to cytotoxic treatment with ROO• with or without various antioxidants. This bacterial suspension was serially diluted, on a 96-well multiplate, in mannitol broth containing phenol red as a pH indicator. After overnight incubation, the growth of ROO•-treated bacteria produces acid, resulting in a change in color of the broth, which permits easy quantitation of viable bacteria protected by antioxidants. Accordingly, ROO• scavenging by various antioxidants could be reproducibly quantitated. Among 22 antioxidants tested, cytotoxicity was substantially inhibited by α-tocopherol, glutathione, L-cysteine, probucol, butylated hydroxyanisole, and butylated hydroxytoluene. Furthermore, the antibactericidal activity of various teas and nonalcoholic drinks showed very high correlation with their antioxidant activities determined by luminol-dependent chemiluminescence assay ($r = 0.91$). By this simple bioassay, a large number of samples can be analyzed simultaneously to determine ROO•-scavenging activity.

Keywords: *Alkyl peroxyl radical; antioxidant; peroxyl radical scavenger; bactericidal action; bioassay*

INTRODUCTION

Lipid peroxidation has been reported to have many deleterious effects on membrane structure and cellular function (Tappel, 1973; Weiss, 1986; Niki *et al.*, 1988), which may have implications in the pathogenesis of various diseases and perhaps in carcinogenesis and aging (Pryor, 1982; Vuillaume, 1987). Various transition metals catalyze the generation of alkoxyl (RO•) and alkyl peroxyl (ROO•) radicals from lipid hydroperoxides (Davies, 1988; Taffe *et al.*, 1987; Akaike *et al.*, 1992). These organic radicals derived from lipid hydroperoxides abstract hydrogen atoms from polyunsaturated fatty acids, resulting in the generation of carbon-centered radicals (R•). Furthermore, R• readily reacts with molecular oxygen to again produce ROO• (Tappel, 1973; Weiss, 1986). Therefore, transition metals as catalysts, together with the products RO• and ROO•, promote the chain reaction of lipid peroxidation as shown below (Tappel, 1973; Weiss, 1986; Halliwell and Gutteridge, 1984; Braughler *et al.*, 1986; Ursini *et al.*, 1989):

$$\mathrm{ROOH} \xrightarrow{\text{transition metals}} \mathrm{ROO^{\bullet}\ (RO^{\bullet})} \quad (1)$$

$$\mathrm{RH + ROO^{\bullet}\ (RO^{\bullet}) \rightarrow R^{\bullet} + ROOH\ (ROH)} \quad (2)$$

$$\mathrm{R^{\bullet} + O_2 \rightarrow ROO^{\bullet}} \quad (3)$$

The importance of oxygen radical scavengers has been known for some time (Fridovich, 1978; Halliwell, 1990). Electron spin resonance (ESR) spectroscopy or chemiluminescence analysis may be applicable to quantitation of radical-scavenging activities (Akaike *et al.*, 1991, 1992; Mitsuta *et al.*, 1990; Cadenas and Sies, 1984). However, few convenient or simple methods of quantifying ROO• are available, in particular, for the screening of antioxidant activity in many samples of foods and other biological materials.

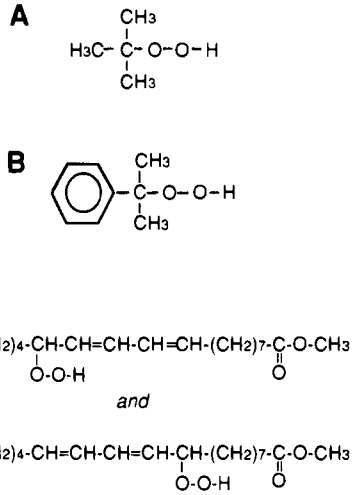

Figure 1. Structures of three organic hydroperoxides used in this experiment: (A) *t*-BuOOH; (B) CumOOH; (C) MeLOOH.

We recently reported the potent bactericidal action of ROO• generated by heme-iron-catalyzed decomposition of organic alkyl hydroperoxide (Akaike *et al.*, 1992). In this paper, we report a simple method for measurement of the scavenging capacity of various substances against ROO• based on the cytocidal action of this radical.

MATERIALS AND METHODS

Reagents. *tert*-Butyl hydroperoxide (*t*-BuOOH) and cumene hydroperoxide (CumOOH) were gifts from NOF Co., Ltd., Tokyo, Japan. Methyl linoleate hydroperoxide (MeLOOH) from autoxidized methyl linoleate was purified by silica gel chromatography as described previously (Gamage *et al.*, 1971). Structures of these three organic hydroperoxides are shown in Figure 1. α-Tocopherol, probucol, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), *n*-propyl gallate,

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N-tert-butyl-α-phenylnitrone (PBN), hemin, and Cu,Zn-superoxide dismutase (SOD) from bovine erythrocytes were purchased from Sigma Chemical Co., St. Louis, MO. *N,N*-Diphenyl-1,4-phenylenediamine (DPPD), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), and luminol were obtained from Aldrich Chemical Co., Inc., Milwaukee, WI. Uric acid, diphenylamine, β-carotene, and hyaluronic acid were obtained from Wako Pure Chemical Industry, Ltd., Osaka, Japan. L-Cysteine, glutathione, hydroquinone, and dimethyl sulfoxide (DMSO) were products of Nacalai Tesque Inc., Kyoto, Japan. L-Ascorbate was from Katayama Chemical Industries, Co., Ltd., Osaka, Japan. Ebselen was a kind gift from Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan. Egg yolk lecithin (phospholipids) was provided by NOF. To quantitate radical-scavenging activity of α-tocopherol, one part of α-tocopherol was mixed with 1.3-parts of 20% egg yolk lecithin in ethanol, followed by removal of ethanol *in vacuo*; the residual ethanol in lecithin and lecithin itself were tested as a control for the antioxidant or antibactericidal effect, and no appreciable antioxidant activity against ROO• was found at the concentration used in this experiment. This mixture was then diluted with phosphate-buffered saline (PBS; pH 7.3) to give homogeneous suspensions even after a prolonged standing. 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) of the highest purity and diethylenetriaminepentaacetic acid (DTPA) were obtained from Dojindo Laboratories, Kumamoto, Japan. Hemoglobin (Hb) was purified from healthy human red blood cells by using ion-exchange column chromatography (DE-52, Whatman, Maidstone, England) and molecular sieve column chromatography (Sephacryl S-200, Pharmacia Fine Chemicals, Uppsala, Sweden), and metHb was prepared according to a method recently reported (Akaike *et al.*, 1992). The growth medium (mannitol broth) used for determination of bacterial viability was prepared by adding D-mannitol (10 g) and phenol red (35 mg) to nutrient broth (18 g powder) (Eiken Chemical Co., Ltd., Tokyo, Japan); this mixture was dissolved in 1.0 L of pure water, and the pH was adjusted to 7.4, followed by sterilization in an autoclave (121 °C for 20 min).

Various kinds of tea, coffee, and their related nonalcoholic drinks were prepared as described previously (Maeda *et al.*, 1992). Briefly, 1 g of various dried leaves of tea or ground coffee, all for household use, were added to 100 mL of distilled water and boiled for 5 min followed by filtration through Millipore membrane (0.45 μm).

Assay for Antioxidant Activity Based on Bactericidal Action of ROO•. A Gram-positive bacterium, *Staphylococcus aureus* strain 209P, was used in the experiment. The bacteria were cultured overnight in a brain–heart infusion broth and were washed three times with PBS (pH 7.3) before use in the cytotoxicity assay. Alkyl peroxyl radicals were generated via heme-iron-catalyzed decomposition of various alkyl hydroperoxides as reported recently (Akaike *et al.*, 1992). To test for antioxidant activity of various substances, *S. aureus* (final concentration 1×10^6 CFU/mL) was treated in the reaction mixture of heme iron plus alkyl hydroperoxides, e.g., *t*-BuOOH (20 mM), CumOOH (5.0 mM), or MeLOOH (1.0 mM), in PBS (pH 7.3), in the presence or absence of the antioxidants. MetHb (100 μg/mL, 1.5 μM) was used as a catalyst for *t*-BuOOH and CumOOH, and hemin (5.0 μM) was used as a catalyst for MeLOOH. The antioxidant potential of various substances after serial dilution was assessed by determining their ability to prevent the killing of *S. aureus* in the presence of ROO• generated by the reaction of metHb (hemin) plus organic peroxides. The detailed procedural sequence of addition of a series of antioxidants and hydroperoxides is as follows: (1) Aliquots (100 μL) of the bacterial suspension (1×10^7 CFU/mL in PBS) were placed in the glass test tubes containing 500 μL of PBS. (2) To the bacterial suspension were added serially diluted antioxidants (100 μL). Lipid-soluble components such as probucol, DPPD, BHA, *n*-propyl gallate, trolox, diphenylamine, ebselen, and β-carotene were dissolved in DMSO, which had no effect on bactericidal action at less than 20%. (3) metHb (100 μL) (final concentration, 100 μg/mL) or hemin solution (100 μL) (final concentration, 5.0 μM) was then added. (4) After mixing, 100 μL of the appropriate peroxide was added and the mixture incubated for 30 min at 37 °C. After the bacteria were exposed to the ROO•-generating system, the bacterial suspension was serially diluted on a 96-well plastic multiplate in mannitol broth containing phenol red as a pH indicator followed by overnight incubation at 37 °C as shown in Figure 2. Bacterial growth after cultivation generally produces some organic acids, resulting in a change in the color of the culture medium from red to yellow. This allows rapid quantitation of viable bacteria. Accordingly, the antioxidant capacity of various substances against ROO• could be determined quantitatively (Figure 3). More specifically, the minimal inhibitory concentration (MIC) of the serially diluted antioxidant against ROO•-induced cytotoxicity was clearly observed on a multiplate, where the end point of bacterial growth indicates the amount of bacterial cells protected from the bactericidal action. The bacterial growth was quantified by colorimetric measurement of the bacterial medium on the multiplates by using an automatic multiplate reader (Model 450, Bio-Rad Laboratories, Richmond, CA) with dual wavelengths of 570 and 655 nm. Effective growth of the bacteria results in decreased A_{570}. The difference between A_{570} and A_{655} decreases in parallel with the bacterial growth, reaching 0.1 at maximum; A_{570} and A_{655} represent absorbance of red color and turbidity, respectively.

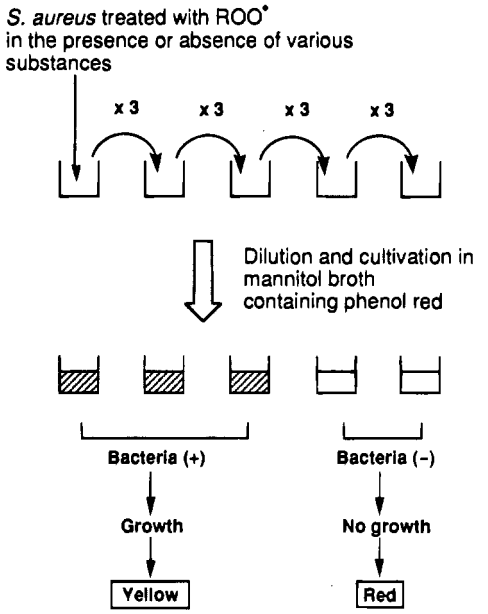

Figure 2. Scheme for quantitation of antioxidant activity against ROO• based on bactericidal effects of ROO•. The viability of the bacteria, which had been treated with the ROO•-generating system, was quantified by end point dilution of the bacteria in mannitol broth containing phenol red as a pH indicator, followed by cultivation on 96-well multiplates. Bacterial growth was easily identified by macroscopic observation as well as by instrumental measurement of the change in color of the medium. See text for details.

Detection of Peroxide-Degrading Activity of Various Substances Based on ROOH-Induced Cytotoxicty against *Pseudomonas aeruginosa*. To identify the peroxide-degrading potential of antioxidants, i.e., peroxidase-like activity, a system was developed that included an ROOH-sensitive bacterium *P. aeruginosa*, and *t*-BuOOH. *P. aeruginosa* (final concentration 1×10^6 CFU/mL) was treated directly with 20 mM *t*-BuOOH without metHb in the presence or absence of various antioxidants in a manner similar to that for the ROOH/metHb/*S. aureus* system just described. After treatment of *P. aeruginosa* with or without antioxidants, the bacterial cells were subjected to end point dilution on a 96-well plate, followed by overnight incubation at 37 °C. The viable cells were quantitated similarly to the ROOH/metHb/*S. aureus* system. We verified that no appreciable generation of ROO• occurred during the incubation of *P. aeruginosa* with *t*-BuOOH by using ESR spin trapping (data not shown).

Identification of ROO• by Using ESR Spin Trapping. ROO• generated in the reaction with the alkyl hydroperoxide

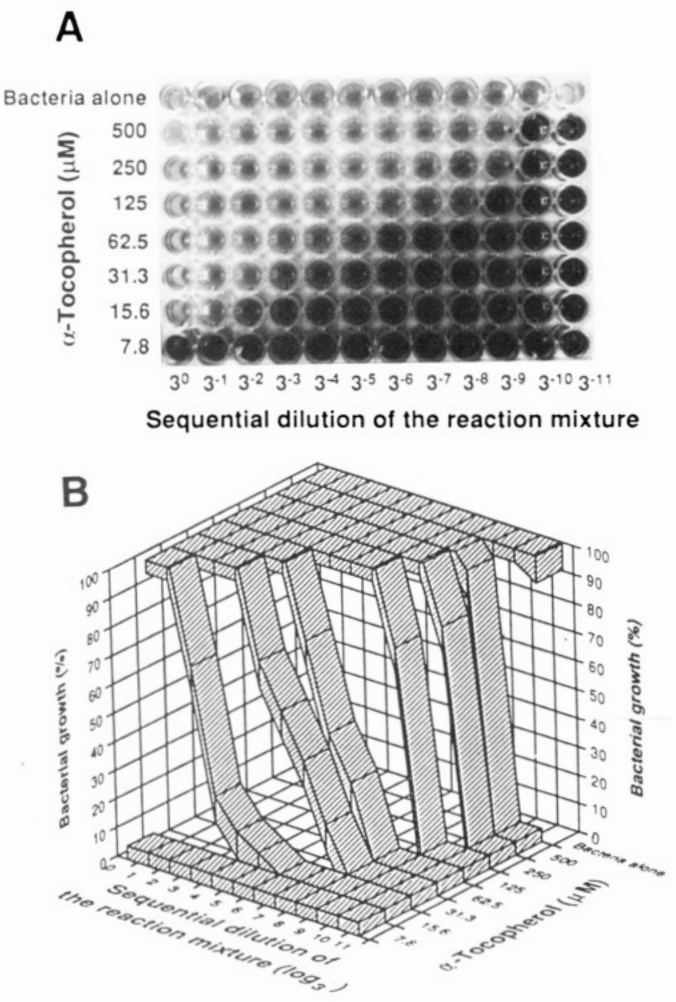

Figure 3. (A) Photograph showing antibactericidal activity of the antioxidant α-tocopherol determined by our method as described in Figure 2 and the text. In this monochromatic photograph, the red color, which was found in wells without cell growth, is seen as a darker tone (lower right area), and the yellow color, which resulted from the growth of bacteria, is seen as more transparent (upper left area). (B) Three-dimensional expression of photograph A. Bacterial growth was assessed with a multiplate reader by measuring the absorbance intensity of the wavelength pair 570 minus 655 nm of each well shown in part A. The percentage of bacterial growth in each well, which was calculated by comparing the decrease in the absorbance with that in the well showing the maximal decrease (maximal bacterial growth), was plotted at the vertical axis in each horizontal coordinate. The serial changes in bacterial growth efficacy are demonstrated by the ribbons along the abscissa. See text for colorimetric measurement.

plus metHb were identified by ESR spin trapping using the spin trap DMPO (4.5 mM) in 10 mM sodium phosphate buffer (pH 7.3) as described recently (Akaike *et al.*, 1992). ESR spectra were recorded with a JES-RE1X spectrometer (JEOL, Tokyo, Japan) using quartz flat cells (inner size 60 × 10 × 0.31 mm), with an effective sample volume of 160 μL, at room temperature under the following conditions: modulation frequently, 100 kHz; modulation amplitude, 0.079 mT; scanning field, 336.1 ± 5 mT; receiver gain, 1000; response time, 0.3 s; sweep time, 2 min; microwave power, 40 mW; and microwave frequency, 9.421 GHz. A quantitative analysis of the spin adducts of alkyl peroxyl radicals was performed according to the method described by us recently (Akaike *et al.*, 1992).

Inhibition Assay of Various Natural Substances against Luminol-Dependent Chemiluminescence Induced by *t*-BuOO•. Anti-alkyl peroxyl radical activities of a series of antioxidants and natural substances were assessed by measuring their inhibitory potentials against luminol-dependent chemiluminescence response generated by the reaction of *t*-BuOOH and metHb using a six-channel luminometer equipped with a data-analyzing computer system (Model LB 9505C, Laboratorium Berthold AG, Wildbed 1, Germany) as reported previously (Maeda *et al.*, 1992). In this reaction system, the light emission generated by luminol is induced mostly by *t*-BOO• because DMPO, which is a potent scavenger of *t*-BOO• (Akaike *et al.*, 1992), shows almost 100% inhibition of the chemiluminescence evoked in the reaction of luminol/*t*-BuOOH/metHb (data not shown). The reaction was initiated by the addition of 100 μg/mL of metHb to the reaction mixture which contained 20 mM *t*-BuOOH, 11.3 μM luminol, and 1.0 mM DTPA in PBS (pH 7.3) in the presence or absence of various natural test substances, and the chemiluminescence response was continuously recorded at 37 °C for a 30-min incubation period.

The dilution factor of each substance which gave a 50% reduction in chemiluminescence intensity (peak counting) was defined as 1 unit of 50%-inhibitory activity of peroxyl radical ($IPOX_{50}$).

RESULTS AND DISCUSSION

Generation and Inhibition of ROO•-Induced Bactericidal Action. Twenty-two substances were tested for antioxidant activity against the bactericidal action of ROO•. ROO• were generated via heme-iron-catalyzed decomposition of ROOH represented by the following equation (Davies, 1988; Akaike *et al.*, 1992):

$$\text{Fe(III)(porphyrin)} + \text{ROOH} \rightarrow [\text{Fe(IV)=O}]^{2+}(\text{porphyrin})^{\bullet+} + \text{ROH} \quad (4)$$

$$[\text{Fe(IV)=O}]^{2+}(\text{porphyrin})^{\bullet+} + \text{ROOH} \rightarrow [\text{Fe(IV)=O}]^{2+}(\text{porphyrin}) + \text{ROO}^{\bullet} + \text{H}^{+} \quad (5)$$

In this method, the viability of bacteria protected by antioxidants can be quantitated by colorimetric measurement of the culture on the multiplates by using a multiplate reader, as demonstrated in Figure 3. This method thus allows simple and rapid determination of antioxidant activity of many samples.

The radical scavenging effect of α-tocopherol determined by ESR spin trapping (Figure 4) correlated well with that determined in this bioassay system (Figure 4C). The inhibition of ROO•-induced cytotoxicity becomes apparent at a concentration of 15.6 μM of α-tocopherol, which was almost compatible to minimal concentration of α-tocopherol to inhibit the DMPO-OO*t*Bu formation based on ESR signal. Similar results were observed with other antioxidants showing inhibition of cytotoxicity by ROO• (*t*-BuOO• and CumOO•) (data not shown). These data suggest that the effect of antioxidants on ROO•-induced cytotoxicity can be attributed to direct scavenging of ROO•.

Effect of Various Antioxidants on ROO•-Induced Bactericidal Action and Molecular Species Involved in Cytotoxicity. MICs (or radical-neutralizing concentrations) of various antioxidants against ROO• are shown in Table 1. Determination of MIC values was highly reproducible in each hydroperoxide system. For the 22 substances tested, strong inhibition of bactericidal action (i.e., alkyl peroxyl or lipid peroxyl radical scavenging effects) was observed with α-tocopherol, probucol, glutathione, L-cysteine, BHA, and BHT. A second group including DMPO (a spin trap), DPPD, L-ascorbate, *n*-propyl gallate, luminol, trolox, and uric acid showed moderate inhibition. All of these compounds are well-known potent antioxidants, especially against lipid peroxidation involving ROO• (Halliwell,

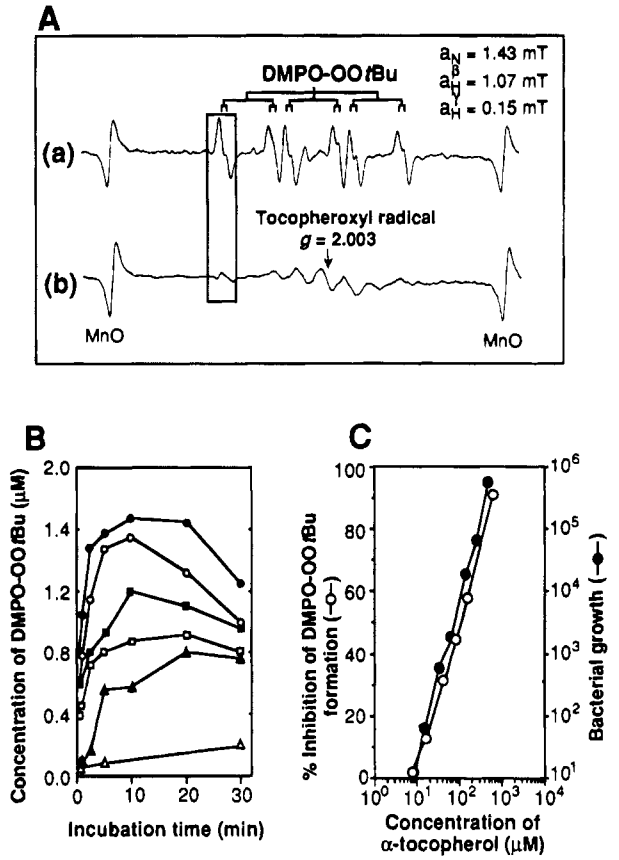

Figure 4. (A) Experimental spectra obtained in the reaction systems generating DMPO-OO*t*Bu in the presence (b) or absence (a) of α-tocopherol (660 μM). Radical-scavenging effects of α-tocopherol were tested by using ESR spin trapping with DMPO as the spin trap. The reaction was initiated by addition of *t*-BuOOH (final concentration 20 mM) to mixtures containing metHb (100 μg/mL) and DMPO (4.5 mM) in 25 mM sodium phosphate buffer (pH 7.3) in the presence or absence of α-tocopherol. ESR spectra were obtained 5 min after incubation at room temperature by using an ESR spectrometer (JES1X, JEOL, Japan). The hyperfine splitting of DMPO-OO*t*Bu is shown. Note that the ESR signal of tocopheroxyl radical is readily apparent in (b). (B) Time course of the concentration of DMPO-OO*t*Bu in the reaction of *t*-BuOOH/metHb in the presence or absence of α-tocopherol. The signal intensity of DMPO-OO*t*Bu, which appears in the magnetic field indicated by the box in (a), was normalized as a relative height against the standard signal intensity of the manganese oxide (MnO) marker, and the concentration of DMPO-OO*t*Bu was determined by double integration of the ESR spectra obtained in the same reaction system as (A) with 16.5 (○), 41.3 (■), 82.3 (□), 165 (▲), or 660 μM (△) of α-tocopherol, respectively; without α-tocopherol (●). After various incubation periods, ESR measurement was performed 30 s after the addition of DMPO to the individual reaction mixture. (C) Correlation between the radical scavenging effect of α-tocopherol as determined by ESR spin trapping as shown in (A) and (B) and that determined by the bacterial growth/bioassay as shown in Figure 3. The amount of DMPO-OO*t*Bu generated with or without α-tocopherol during 30-min incubation periods was quantitated by integrating the area under the curve shown in (B), and the inhibition profile of DMPO-OO*t*Bu formation by various concentrations of α-tocopherol was compared with the protective effect of α-tocopherol against *t*-BuOO•-induced cytotoxicity.

1990; Leibovitz *et al.*, 1990; Ames *et al.*, 1981). In contrast, β-carotene, ebselen, hydroquinone, L-methionine, hyaluronic acid, diphenylamine, PBN (a spin trap), DMSO, and Cu,Zn-SOD all provided little protection against cytocidal action of ROO•. Some of these compounds, which showed little inhibitory action, are well-known scavengers against hydroxyl radical (•OH) (e.g., DMSO, hyaluronic acid, L-methionine), superoxide anion radical ($O_2^{\bullet -}$) (e.g., SOD), or singlet oxygen (e.g., β-carotene, L-methionine) (Tappel, 1973; Halliwell, 1990; Leibovitz *et al.*, 1990; Sato *et al.*, 1992; Akaike *et al.*, 1990) (see Table 1). Therefore, it is most reasonable to conclude that active oxygen species such as •OH, $O_2^{\bullet -}$, and singlet oxygen are not involved *per se* in bactericidal action induced by the reaction of organic peroxides and heme irons as was suggested in our recent paper (Akaike *et al.*, 1992). In addition, diphenylamine, which was reported to be a potent scavenger of alkoxyl radicals (Van der Zee *et al.*, 1989), had no significant suppressive effect on the cytotoxicity of ROO•. This may suggest that RO• may not be the molecular species responsible for generation of bactericidal activity in this reaction system.

Table 1. Antioxidant Activities of Various Substances Determined by a Bioassay Based on Bactericidal Action of ROO•

antioxidant	antioxidant activities (μM) (MIC)[a]		
	t-BuOO•	CumOO•	MeLOO•
α-tocopherol	15.6	31.3	72.0
probucol	31.3	7.8	3.9
L-cysteine	125	250	15.6
glutathione	250	125	7.8
DMPO	250	125	>10000
DPPD	125	125	>2000
L-ascorbate	500	250	>3000
BHA	2000	>2000	31.3
n-propyl gallate	500	60	500
luminol	500	125	125
trolox	500	125	>2000
uric acid	1000	500	1000
BHT	>2000	>2000	31.3
PBN	>10000	>10000	>10000
diphenylamine	>2000	>10000	>10000
hydroquinone	>10000	>2000	>2000
ebselen	>5000	>5000	>5000
L-methionine	>10000	>10000	>10000
β-carotene	>2000	>2000	>2000
hyaluronic acid	>10000	>10000	>10000
DMSO (%)	>20	>20	>20
Cu,Zn-SOD (units)	>10000	>10000	>10000

[a] MIC indicates the minimal concentration needed to inhibit the ROO•-induced cytotoxicity against *S. aureus*.

Although we found that β-carotene showed no appreciable inhibition of the cytotoxic action of ROO•, it was reported previously that β-carotene, a known effective quencher of singlet oxygen, could also trap ROO• and exhibit antioxidant action against ROO• (Burton and Ingold, 1984). However, the β-carotene-derived carbon-centered radical, which is generated via the reaction of ROO• and β-carotene, reacts with molecular oxygen under ambient conditions and produces alkyl peroxyl radical, as observed in the following propagation reaction of lipid peroxidation:

$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet} \quad (6)$$

(R indicates the β-carotene residue containing unconjugated double bonds.) Therefore, it may be that β-carotene showed prooxidant activity becoming cytotoxic ROO•, and thus antioxidant activity was not observed in our cytotoxic system.

Previously, Wayner et al. developed a method of examining peroxyl radical scavenging activities, i.e., the TRAP assay (Wayner *et al.*, 1985). In their reaction system, alkyl radical (R•) was initially generated via thermal decomposition of an azo compound such as 2,2′-azobis(2-amidipropane) hydrochloride, and alkyl peroxyl

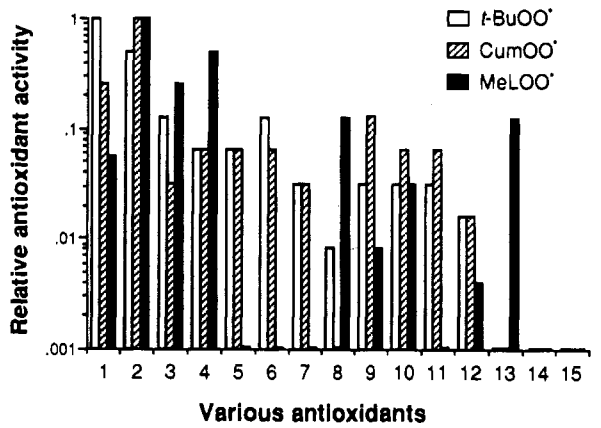

Figure 5. Relative antioxidant activities of 15 substances determined by using three different organic hydroperoxides. Relative antioxidant activity in each system was obtained by calculating ratios of each MIC value to the lowest value (e.g., MICs of α-tocopherol, probucol, or glutathione): 1, α-tocopherol; 2, probucol; 3, L-cysteine; 4, glutathione; 5, DMPO; 6, DPPD; 7, L-ascorbate; 8, BHA; 9, *n*-propyl gallate; 10, luminol; 11, trolox; 12, uric acid; 13, BHT; 14, PBN; 15, diphenylamine.

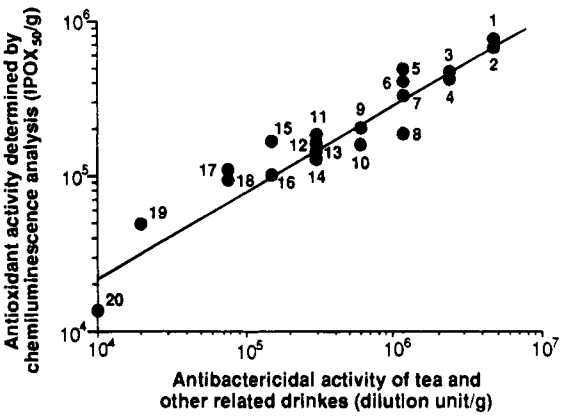

Figure 6. Correlation of antioxidant activity of various natural products determined by chemiluminescence analysis with their antibactericidal potential measured by *t*-BuOOH/metHb/*S. aureus* cytotoxic assay. The antioxidant activity of tea and other related drinks is correlated with their antibactericidal activity. The activity expressed is dilution units per gram of the product, which gives 50% reduction in chemiluminescence response. Each activity of various tea and other nonalcoholic drinks is indicated by number as follows: 1, Yomogi tea (*Artemisia uvineeps*; mugwort); 2, Fukamushi green tea, heavily steamed green tea; 3, Kamairi green tea, lightly roasted green tea; 4, powdered green tea; 5, Shiraori cha, heavily roasted tea; 6, black tea (blended); 7, Gyokuro green tea, powdered; 8, Bancha, heavily roasted tea; 9, Perilla tea; 10, Woolong tea; 11, coffee (mocha); 12, Rooibos tea, African (*Aspalathus linealis*); 13, black tea (Ceylon); 14, coffee (blended); 15, Gen-mai tea, green tea with roasted brown rice; 16, black tea (Darjeeling); 17, Soba cha (*Fagopyrum esculentum*; buckwheat tea); 18, cocoa; 19, Taribu tea, South American (*Tabebuia avellamedae*); 20, roasted barley/wheat tea.

radical (ROO·) was formed by the subsequent reaction of R· with O_2. The antioxidant activity of various substances was tested by analyzing their effects on peroxidation of lipid substrates induced by the azo initiators, which involves ROO· generation. However, this reaction seems to be more complex than our ROO·-generating system, which uses iron-catalyzed decomposition of alkyl hydroperoxides where ROO· is the major product generated directly from alkyl hydroperoxides (Akaike *et al.*, 1992). Thus, the antioxidant activity determined by the trap assay may reflect in part the scavenging effect against other radical species such as R· and RO·. In contrast, the correlation study with ESR spin trapping (Figure 4) indicates that our antioxidant bioassay is more specific for ROO·.

Inhibitory Spectra of Various Antioxidants against Different Alkyl Peroxyl Radicals. Comparison of the antioxidant activity of each substance against three similar cytotoxic systems prepared with different organic hydroperoxides, i.e., *t*-BuOOH, CumOOH, or MeLOOH, showed that the spectra of antioxidant activity of the various compounds were not necessarily similar among the three different systems (Figure 5). Specifically, although the antioxidant activities obtained in the systems with *t*-BuOOH and CumOOH were similar, they seemed to be somewhat different from that of the MeLOOH system. This difference is attributed mainly to the differences in antioxidant activity of DMPO, DPPD, L-ascorbate, trolox, BHA, and BHT. In the case of DMPO, the generation of the DMPO spin adducts of ROO· could be identified clearly by ESR spin trapping in the reaction systems of metHb with *t*-BuOOH and CumOOH (Figure 4) (Taffe *et al.*, 1987; Akaike *et al.*, 1992), but the spin adduct for MeLOO· could not be obtained (data not shown). This result indicates a slower reaction velocity of DMPO with MeLOO·, which possesses longer alkyl side chains than do *t*-BuOO· and CumOO· (see structures shown in Figure 1). Similarly, the differences in the antioxidant activity were obtained with DPPD, trolox, L-ascorbate, BHA, and BHT, although BHT and BHA showed inhibitory potency against cytotoxicity induced only in the system of MeLOOH, which may be also due to the different accessibility of these antioxidants to alkyl peroxyl radicals, because these antioxidant and alkylperoxides were greatly different in their solubility in aqueous system. In view of these different effects obtained with various antioxidant compounds in each reaction system, it would be preferable to use two different organic/lipid peroxyl radical-generating systems such as *t*-BuOO· and MeLOO· to make the method more reliable.

Application of the Antioxidant Bioassay To Determine Antioxidant Activity of Natural Products. We applied our bioassay to examine the antioxidant potential of various natural products. The antioxidant activities of natural products such as tea and other related drinks, which were determined by antibactericidal action induced by ROO·, were compared with those quantitated by luminol-dependent chemiluminescence assay, in which no bacterial cells were included. The antibactericidal potency of these natural products correlated very well with their antioxidant activities ($r = 0.91$) (Figure 6). These results indicate that the antioxidant having ROO·-scavenging potential can give excellent antibactericidal effect induced by ROO· even in the crude reaction system, and our bioassay can be applicable to screening of antioxidant activity in natural foods and other biological materials.

Verification of Specificity of Antioxidant Bioassay Based on Bactericidal Action of ROO·. Regarding the specificity of our bioassay, one may have a concern that ROOH itself could be destroyed directly by the putative antioxidants in samples tested. One way to solve this problem is simply by using an ROOH-sensitive bacteria, *P. aeruginosa* (Akaike *et al.*, 1992); *t*-BuOOH greater than 10 mM killed *P. aeruginosa* completely (Figure 7). We tested ROOH-degrading activity, i.e. the peroxidase-like activity, of all antioxidants as well as some natural products as mentioned above by using a *t*-BuOOH (20 mM)/*P. aeruginosa* cytotoxicity system. In this system, the putative com-

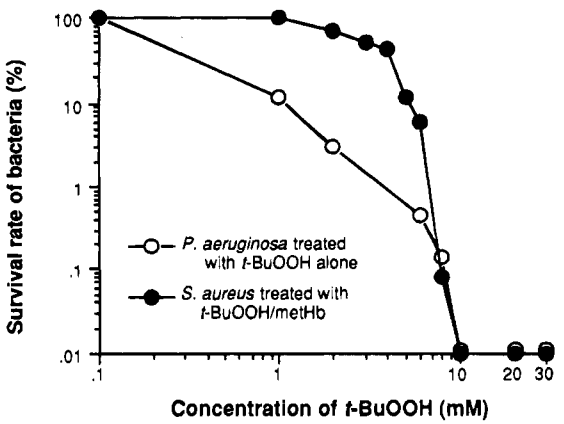

Figure 7. Concentration-dependent profiles of bactericidal effects observed in *t*-BuOOH/metHb/*S. aureus* and *t*-BuOOH/*P. aeruginosa* systems. The survival rate of *S. aureus* treated with *t*-BuOO/metHb in the same manner as in Figure 2 was determined by end point dilution and cultivation method on a 96-well multiplate as described in the text. The viability of *P. aeruginosa* was quantitated similarly to that of *S. aureus* except that *P. aeruginosa* was treated with *t*-BuOOH without any addition of metHb.

pound could show protective effect against direct cytotoxicity induced by *t*-BuOOH only when the ROOH-degrading compound abolished *t*-BuOOH to less than 10 mM. The bactericidal system with *t*-BuOOH/metHb/*S. aureus* also showed a very similar concentration-dependent profile with *t*-BuOOH. Thus, we can assess by using the ROOH/*P. aeruginosa* system whether the ROOH-degrading compound is involved in antibactericidal action of various materials in the ROOH/metHb/*S. aureus* system. No antioxidants tested showed any significant protective effect against ROOH-induced cytotoxicity when *P. aeruginosa* was exposed to 20 mM *t*-BuOOH without metHb in the presence or absence of various antioxidants in the same manner as the *t*-BuOOH/metHb system (data not shown). Therefore, we believe that the antibactericidal activities determined with the present method should reflect essentially the ROO•-scavenging potentials of these antioxidants and natural products.

It is feasible that an iron-sequestering compound exhibits antibactericidal action by inactivating the catalytic potential of heme iron. However, a number of potent iron-chelating agents, such as DTPA, ethylendiaminepentaacetic acid, and deferoxamine, showed neither antibactericidal action nor inhibition of ROO• production in the reaction of the ROOH/metHb system (data not shown).

Conclusions. The method reported here is based on the scavenging reaction of each substance against the radical ROO•, which exhibits strong bactericidal activity, and is highly reproducible. By this method, one can identify the antioxidant potential of various substances such as vegetables and other foodstuffs conveniently and economically without the use of ESR spin trapping, chemiluminescence, or other complex biochemical and chemical procedures.

ABBREVIATIONS USED

ROO•, alkyl peroxyl radical; RO•, alkoxyl radical; R•, alkyl radical; *t*-BuOOH, *tert*-butyl hydroperoxide; CumOOH, cumene hydroperoxide; MeLOOH, methyl linoleate hydroperoxide; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; PBN, *N*-*tert*-butyl-α-phenylnitrone; SOD, superoxide dismutase; DPPD, *N*,*N*-diphenyl-1,4-phenylenediamine; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; DTPA, diethylenetriaminepentaacetic acid; MIC, minimal inhibitory concentration.

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