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ALKYL PEROXYL RADICAL-SCAVENGING ACTIVITY OF CATECHINS

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Key Word Index—alkyl peroxyl radical; antioxidant; bactericidal action; radical scavenger; catechins.

Abstract—Alkyl peroxyl radical (ROO•) generated from the reaction between 20 mM *t*-butyl hydroperoxide (*t*-BuOOH) and 200 μM hematin could kill *E. coli*. The minimum concentrations of catechins sufficient to rescue the bacteria treated with ROO• were found to be 70 μM for (−)-epicatechingallate, 100 μM for (−)-epicatechin and 125 μM for (+)-catechin. These values were comparable with the value of α-tocopherol, a typical ROO• scavenger. On the other hand, L-ascorbate and β-carotene revealed about one tenth the scavenging activity of catechins. No scavenging activity was found for superoxide dismutase even at 86 mM. These facts indicate that catechins have high ROO• scavenging activity.

INTRODUCTION

Catechins, the basic structural unit of condensed tannins, belong to flavan-3-ol derivatives, and are found in a wide variety of plant sources such as vegetables, herbs, and teas [1]. Various pharmacological functions of catechins have been proven to be antibacterial, antiviral or anticancer [2, 3]. Catechins are also found to be scavengers of active oxygen such as the superoxide anion radical ($O_2^{\bullet -}$), singlet oxygen ($^1O_2$) and hydroxyl radical (OH•) [4–6]. In contrast to these active oxygen species, alkyl peroxyl radical (ROO•) and alkoxyl radical (RO•) are soluble in membranes and considered to be the major generators of lipid peroxides [7], which cause many kinds of disease, including liver injury and cancer [8]. Akaike *et al.* found that ROO• alone was generated by heme-iron-catalyzed decomposition of *t*-BuOOH, and that ROO•-scavenging activity of various antioxidants could be estimated by electron spin resonance spectroscopy and chemiluminescence [9, 10]. Recently, they also found that ROO• generated from *t*-BuOOH has potent bactericidal action and that the radical-scavenging activity of various antioxidants can be quantitatively estimated on the basis of the inhibitory activity against the ROO•-induced cytotoxicity toward *Staphylococcus aureus* [9, 11]. In the presence of ROO•, RO• and OH• the radical-scavenging activity of catechins was detected by the chemiluminescence method [12]. However, there are no reports on the estimation of scavenging activity of catechin against ROO• or RO• alone. For pharmacological purposes, the bioassay is valuable because catechins have bactericidal activity, unlike other antioxidants such as L-ascorbate, α-tocopherol and β-carotene. In the present experiments, we determined the ROO•-scavenging activity of several antioxidants, including catechins, by the bioassay and found high antioxidant activity of catechins.

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RESULTS AND DISCUSSION

Bactericidal action against E. coli *by reaction of* t-*BuOOH and heme iron*

The estimation of ROO•-scavenging activity in the bioassay was reported to be based on two prerequisites [9]. First, in the absence of antioxidants the bacteria must be killed under the mildest conditions possible, such as the minimum level of ROO•. Second, the ROO•-scavenging activity is estimated from the minimum concentration of antioxidant that can rescue the bacteria treated with ROO•. *Staphylococcus aureus* has been used for the

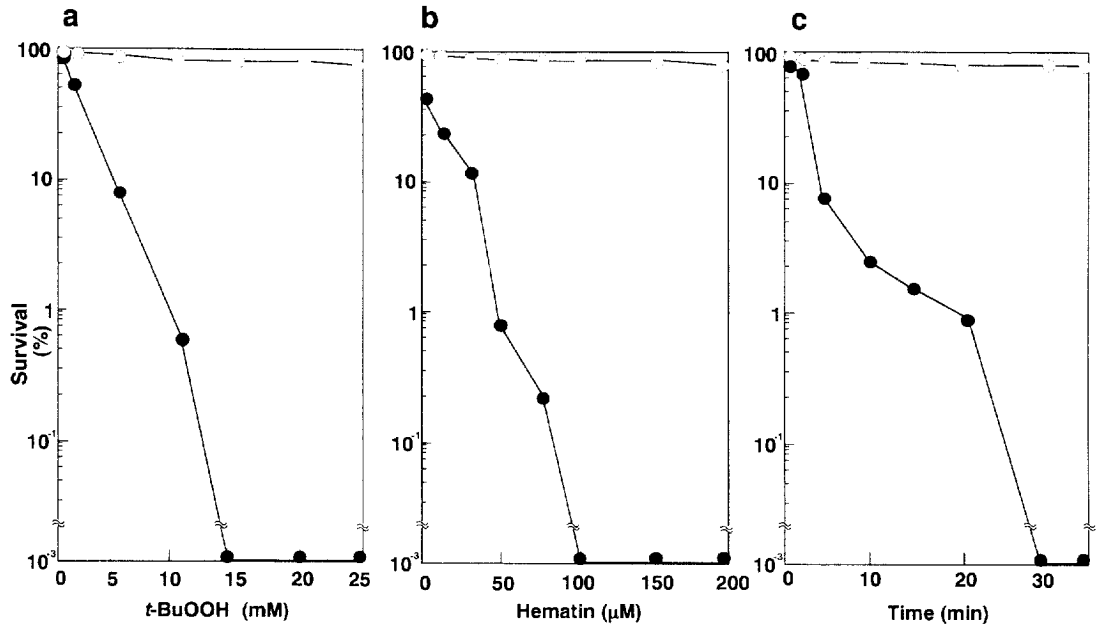

Fig. 1. Effects of *t*-BuOOH, hematin and incubation time on cytocidal activity against *E. coli*. (a) *E. coli* was incubated in medium containing various concentrations of *t*-BuOOH supplemented with 200 μM hematin (●) or without hematin (○). (b) The bacteria were incubated in medium containing various concentrations of hematin supplemented with 20 mM *t*-BuOOH (●) or without *t*-BuOOH (○). (c) The bacteria were incubated in medium containing 20 mM *t*-BuOOH and 200 μM hematin (●) or 20 mM *t*-BuOOH alone (○) for various incubation periods. The number of viable bacteria was measured by the colony formation assay.

assay, because it has much higher sensitivity to ROO·, compared with *Escherichia coli* [9]. In the present experiment, we wanted to know the response of catechins to high level of ROO·, and therefore used *E. coli* instead of *S. aureus*. First, the optimal conditions of the bioassay were determined (Fig. 1). *E. coli* was incubated in a reaction mixture containing 200 μM hematin and various concentrations of *t*-BuOOH for 35 min at 37°. Then, the number of viable bacteria was counted by the colony formation assay (Fig. 1(a)). The bactericidal activity was detected at higher concentration than 15 mM *t*-BuOOH. In contrast, *t*-BuOOH alone had little bactericidal effect. The effect of hematin with or without *t*-BuOOH on bactericidal activity was examined (Fig. 1(b)). In the presence of *t*-BuOOH, 100 μM hematin killed the bacteria while hematin alone had little effect. The effect of incubation time on bactericidal activity was examined (Fig. 1(c)). The bacteria survived after incubation for less than 5 min, but were killed completely by incubation for more than 30 min. Considering these results, in the following experiments *E. coli* was incubated with 20 mM *t*-BuOOH, 200 μM hematin and various concentrations of antioxidants for 35 min.

Estimation of ROO·-scavenging activity of catechins based on bactericidal action by ROO·

The ability of (+)-catechin, (−)-epicatechin and (−)-epicatechingallate to inhibit the bactericidal activity of ROO· against *E. coli* was investigated (Fig. 2). The minimum concentrations of catechins sufficient to rescue the bacteria were 70 μM (−)-epicatechingallate, 100 μM (−)-epicatechin and 125 μM (+)-catechin. Figure 2 also shows that the

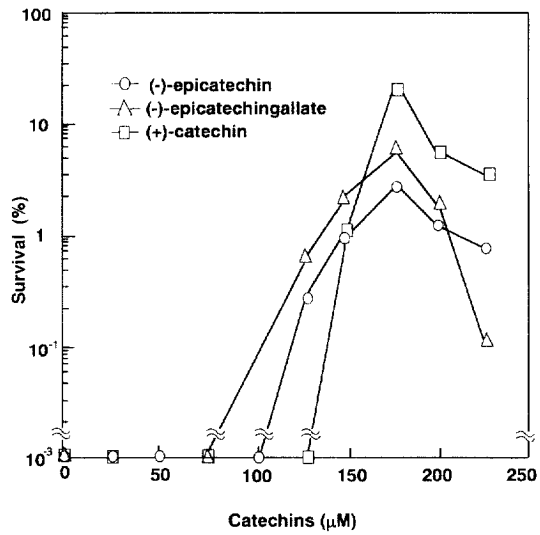

Fig. 2. Effect of catechins on the cytocidal activity induced by ROO·. *E. coli* was incubated in a reaction mixture containing 20 mM *t*-BuOOH, 200 μM hematin, and (−)-epicatechin (○), (−)-epicatechingallate (△) or (+)-catechin (□) at 37°C for 35 min. The number of bacteria was measured by the same method described in Fig. 1.

Table 1. ROO·-scavenging activities of antioxidants

Antioxidant	ROO·-scavenging activities (μM)*
α-tocopherol	90 (1.00)
(±)-epicatechingallate	70 (1.25)
(±)-epicatechin	100 (0.90)
(+)-catechin	125 (0.72)
L-ascorbate	600 (0.15)
β-carotene	> 1500 (< 0.06)
SOD	> 85600 (< 0.009)

*ROO·-scavenging activities are indicated by the minimal concentration (μM) needed to inhibit the ROO·-induced cytotoxicity against *E. coli*. The value in parentheses shows the inverse ratio of each antioxidant (μM) to α-tocopherol (90 μM).

scavenging activities of catechins were decreased at concentrations higher than 200 μM. Catechins were reported to have cytotoxicity at concentrations higher than 500 μM in rat cells [13]. In our experiments catechin alone did not show bactericidal effects even at 500 μM (data not shown). During the ROO·-scavenging reaction by β-carotene, the β-carotene-derived radical was reported to be generated transiently [10]. Further experiments will be required to determine whether the decrease in scavenging activity at higher concentrations of catechins results from cytocidal action of the catechin-derived radical.

Table 1 shows the comparison of ROO·-scavenging activities of catechins with those of other antioxidants. α-tocopherol works as an inhibitor of auto-oxidative peroxidation via the free radical chain reaction [14], L-ascorbate is a potent scavenger against OH· and $O_2^{\cdot -}$ [15], β-carotene and SOD are potent scavengers against $^1O_2$ and $O_2^{\cdot -}$, respectively [16]. As shown in Table 1, the ROO·-scavenging activities of the three catechins were similar to the activity of α-tocopherol, while the activities of β-carotene and L-ascorbate were much lower than those of catechins. SOD did not affect the cytocidal activity even at 86 mM. These results indicate that the three catechins have high ROO·-scavenging activity.

Akaike *et al.* [9] suggested that during the reaction of *t*-BuOOH and hematin, ROO· alone was generated, and no other active oxygen species such as OH·, $O_2^{\cdot -}$ or $^1O_2$ were generated. The lack of effect of SOD against the cytocidal action of ROO· (Table 1) also support the likelihood that ROO· alone is generated in our experimental conditions. In the present experiments, the level of ROO· was expected to be higher than that in the assay using *S. aureus* [11]. Nevertheless, the relative scavenging activities of α-tocopherol, β-carotene and L-ascorbate (Table 1) were consistent with those in the bioassay using *S. aureus* [11]. These facts also support the conclusion that catechins have high ROO·-scavenging activity. This property is distinct from other oxidative effects of catechins against $O_2^{\cdot -}$, OH· and $^1O_2$, because ROO· is a lipophilic radical. The ability of the ROO·-scavenging reaction relate to the accessibility of antioxidants to the lipid radical, therefore antioxidants being preferentially localized in the aqueous phase are least effective [17, 18].

In the present study, the catechins had the property of hydrophilicity and lipophilicity, and ROO·-scavenging activity was greater than the L-ascorbate, being more hydrophilic. ROO· and RO· generated by the process of the radical chain reaction further induce lipid peroxide and attack another components of the organism [19]. It is indicated that the scavenger of ROO· of catechins blocks the radical chain reaction and prevents the peroxidation process. Due to technical limitation, in the bioassay for ROO·-scavenging activity, ROO· is generated in the liquid phase. To determine the biological functions of catechins, it must be ascertained whether the catechins can scavenge ROO·-derived radicals in the bacterial membranes.

EXPERIMENTAL

Reagents

tert-butyl hydroperoxide (*t*-BuOOH) and (−)-epicatechingallate were supplied by Sigma. (+)-catechin, α-tocopherol and hematin from bovine blood were purchased from Nacalai Tesque. β-carotene, dimethyl sulfoxide (DMSO) and superoxide dismutase (SOD) from bovine erythrocytes were obtained from Wako Pure Chemical Industry.

Standard assay conditions for catechins based on bactericidal action of ROO·

Escherichia coli (DH5α, MCR) was cultured overnight in LB medium and was washed 3 times with 0.01 M phosphate-buffered 0.15 M saline (PBS; pH 7.3) before use in the cytotoxicity assay. We first determined the appropriate conditions for killing *E. coli* by ROO· as follows. The reaction mixture (1 ml) contained the bacteria (1×10^6 CFU/ml), *t*-BuOOH, hematin and one seventh strength of PBS. The reaction was started by addition of *t*-BuOOH and carried out for a specified time at 37°. Then the bacterial suspension was diluted 10^2–10^4 times with PBS, and 10 μl of the suspension was spread on LB agar. After incubation overnight at 37°, the number of viable bacteria was counted. Appropriate conditions for killing the bacteria by ROO· were determined to be the incubation of bacteria with 20 mM *t*-BuOOH and 200 μL hematin at 37° for 35 min, and these conditions were used in the assay for antioxidant activity. Various concentrations of antioxidants were included in the reaction medium described above. For the assay of lipid-soluble components such as β-carotene, (−)-epicatechingallate, (−)-epicatechin, (+)-catechin and α-tocopherol, 2% DMSO was included in the reaction medium. The ROO·-scavenging activities

of antioxidants were estimated from the minimum concentration of antioxidant required to inhibit ROO·-induced cytotoxicity.

REFERENCES

1. Wollenweber, E. and Dietz, V. H., *Phytochemistry*, 1981, **20,** 869.
2. Salvayre, R., Braquet, P., Perruchot, T. and Douste-Blazy, L., in *Flavonoids and Bioflavonoids*, eds L. Farkas, M. Gabor and F. Kallay. Elsevier, Amsterdam, 1982, p. 437.
3. Ohomori, Y., Ito, M., Kishi, M., Mizutani, H., Katada, T. and Konishi, H., *Biological and Pharmaceutical Bulletin*, 1995, **18,** 683.
4. Scott, B. C., Butler, J., Halliwell, B. and Aruoma, O. I., *Free Radical Research Communications*, 1993, **19,** 241.
5. Tournaire, C., Croux, S., Maurette, M. T., Beck, I., Hocquaux, M., Braun, A. M. and Oliveros, E., *Journal of Photochemistry and Photobiology*, 1993, **19,** 205.
6. Nakayama, T., Yamada, M., Osawa, T. and Kawakishi, S., *Biochemical Pharmacology*, 1993, **45,** 265.
7. Watabe, T., Tsubaki, A., Isobe, M., Ozawa, N. and Hiratsuka, A., *Biochimica et Biophysica Acta*, 1984, **795,** 60.
8. Fraga, C. G. and Tappel, A. L., *The Biochemical Journal*, 1988, **252,** 893.
9. Akaike, T., Sato, K., Ijiri, S., Miyamoto, Y., Kohno, M., Ando, M. and Maeda, H., *Archives of Biochemistry and Biophysics*, 1992, **294,** 55.
10. Maeda, H., Katsuki, T., Akaike, T. and Yasutake, R., *Japanese Journal of Cancer Research*, 1992, **83,** 923.
11. Akaike, T., Ijiri, S., Sato, K., Katsuki, T. and Maeda, H., *Journal of Agricultural and Food Chemistry*, 1995, **43,** 1864.
12. Yoshiki, Y., Kahara, T. and Okubo, K., *Journal of Bioluminescence and Chemiluminescence*, 1996, **11,** 131.
13. Mitsui, T., Yamada, K., Yamashita, K., Matsuo, N., Okuda, A., Kimura, G. and Sugano, M., *International Journal of Oncology*, 1995, **6,** 377.
14. Terao, J., Sugino, K. and Matsushita, S., *Journal of Nutritional Science and Vitaminology*, 1985, **31,** 499.
15. McCay, P. B., *Annual Review of Nutrition*, 1985, **5,** 323.
16. Van der Zee, J., Steveninck, J. V., Koster, J. F. and Dubbelman, T. M. A. R., *Biochimica et Biophysical Acta*, 1989, **980,** 175.
17. Miura, S., Watanabe, J., Sano, M., Tomita, T., Osawa, T., Hara, Y. and Tomita, I., *Biological and Pharmaceutical Bulletin*, 1995, **18,** 1.
18. Rice-Evans, C., Miller, N. J. and Paganga, G., *Trends in Plant Science*, 1997, **4,** 152.
19. Bielski, B. H., Arudi, R. L. and Sutherland, M. W., *The Journal of Biological Chemistry*, 1983, **258,** 4759.