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A Novel Method of Measuring Hydroxyl Radical-Scavenging Activity of Antioxidants Using γ-Irradiation

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A new method using ESR spin trapping was proposed for measuring the scavenging activity of antioxidants for the hydroxyl (OH) radical. (-)-Epigallocatechin gallate (EGCg) and 5,5-dimethyl-1-pyrroline N-oxide (DMPO) were used as the antioxidant and spin trapping agent, respectively. The conventional method using a Fenton reaction had problems associated with the estimation of activity, because the antioxidant disturbs the system for generating OH radical by coordinating on Fe^{2+} and by consuming H_2O_2, besides scavenging the spin adduct (DMPO-OH). Intense γ-irradiation was therefore used to generate OH radicals, and the intensity decrease in DMPO-OH after irradiation was followed to obtain the rate constant for the scavenging of DMPO-OH by EGCg. The intensities were extrapolated to zero time to estimate the quantity of DMPO-OH formed during γ-irradiation. By using these values, the reaction rate constant between OH radical and EGCg was calculated as a ratio to that of DMPO. It was shown that this method is useful for comparing the OH radical-scavenging activity of various antioxidants.

Keywords: Spin trapping method, hydroxyl radical scavenging, (-)-epigallocatechin gallate (EGCg), 5,5-dimethyl-1-pyrroline N-oxide (DMPO), γ-irradiation

INTRODUCTION

Reactive oxygen species play an important role in inducing various lesions in living organisms. It is, therefore, important in terms of preventive medicine to search for substances which scavenge them. We have been focusing on green tea percolate and its constituents,[1–6] because they are ingested daily and have been reported to show various pharmacological effects including anti-mutagenic or anti-carcinogenic activity.[7–12] Previously,[1,2] we revealed that tea percolate and a main constituent (-)-epigallocatechin gallate

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(EGCg) have a protective effect against radiation-induced scission of DNA, and the effect was considered to be derived from the scavenging of the hydroxyl (OH) radical formed by γ- and β-irradiations. As OH radical is the most reactive of the reactive oxygen species and seriously damages biomolecules, it is necessary to examine the OH radical-scavenging activity of antioxidants. The spin trapping method is useful for this purpose,[13–16] but the conventional method had several defects and, as a result, was unreliable. We propose a new method which is able to overcome such defects and applicable to evaluate unambiguously the OH radical-scavenging activity of various antioxidants.

EXPERIMENT

(-)-Epigallocatechin gallate (EGCg, supplied by Mitsui Norin Co., Ltd.,) and 5,5-dimethyl-1-pyrroline N-oxide (DMPO, Labotec Co., Ltd.,) were used as a tea catechin and a spin trapping agent, respectively. These reagents were of the highest grade and, therefore, used without further purification. Aqueous solutions containing 0.05 M DMPO and various amounts of EGCg were sucked into capillary glass tubes of about 0.8 mm i.d. (75-μl micropipette, Drummond), and both ends of which were sealed by quickly fusing the glass. The samples were γ-irradiated at room temperature for two minutes by using an intense ^{60}Co source (dose rate, 186 Gy/min) established at the Research Reactor Institute, Kyoto University and were rapidly frozen just after the irradiation to stop the reaction and carry them to ESR spectrometer. Each sample was then quickly melted just before ESR measurement. Spectra were measured repeatedly as a function of time after melting the sample at room temperature using a JEOL RE.3X spectrometer, and the relationship between the intensity of the spin adduct (DMPO-OH) and time was examined.

RESULTS AND DISCUSSION

As the spin trapping method is relatively simple and with it one is able to obtain directly information about a radical, it has been used to examine the scavenging activity of OH and superoxide anion radicals of antioxidants.[13] An appropriate radical-generating system must be adopted for this purpose. The Fenton reaction ($Fe^{2+} + H_2O_2$) is generally used for generating OH radicals, and DMPO and an antioxidant are mixed with it. A fraction of the OH radicals generated are trapped with DMPO forming the adduct DMPO-OH, but some fraction is scavenged by the antioxidant. It is, therefore, possible to calculate the OH radical-scavenging activity of an antioxidant by measuring the ratio of the intensity of DMPO-OH of the sample containing antioxidant to that of the sample without antioxidant. However, this method has the following problems:

1. The OH radical generating system is perturbed by the chelating of the antioxidant to Fe^{2+} ion. That is to say, the quantity and the rate of OH generation might be changed by the coordination of the antioxidant. This is understandable because it is known that the redox potential of Fe^{2+} is strongly affected by the ligands. To solve this problem, a chelating agent like diethylenetriaminepentaacetic acid (DETAPAC) was usually added to the solution. However, antioxidants like EGCg have strong chelating activity, so the effect of the addition of DETAPAC is doubtful. In addition, antioxidants may consume H_2O_2. Actually, it is known that EGCg scavenges H_2O_2. This action also perturbs the OH generating system.
2. DMPO-OH is also scavenged by antioxidant. DMPO-OH is a radical, not as reactive as the OH radical but more reactive than the nitroxide radical, and is scavenged by reducing agents.

In some cases, therefore, results were obtained as if the antioxidant showed scavenging activity, nevertheless, OH radical was not really scavenged.

To solve problem (1), we thought to use γ-irradiation in place of the Fenton reaction. As the OH radical is formed from the decomposition of a water molecule by γ-irradiation, the quantity of the radical generated might not be affected by the presence of the solute molecule, namely the antioxidant. On the other hand, it seems to be difficult to overcome problem (2), because DMPO-OH and the antioxidant molecule coexist in solution and it is impossible to stop their collision. Therefore, we considered estimating the quantity of DMPO-OH scavenged by the antioxidant from the decreasing curve of intensity.

The change in the intensity of DMPO-OH was simulated and is shown in Figure 1, which will be explained later. Samples containing a definite amount of DMPO and various amounts of EGCg were γ-irradiated for a short period (t_0') at a constant dose rate (referred to Process 1). Then, the ESR intensity of DMPO-OH was measured as a function of time after γ-irradiation (Process 2).

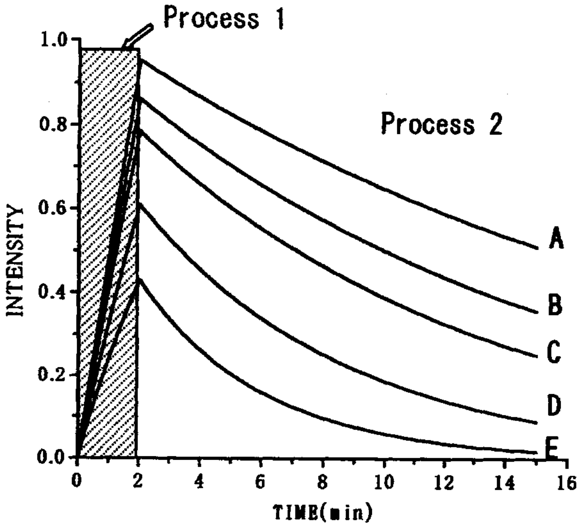

FIGURE 1 Schematic Intensity Change in DMPO-OH during (Process 1) and after (Process 2) γ-Irradiation. Each curve was simulated using the values; $[\mathrm{DMPO}] = 0.05\,\mathrm{M}$, $k_{UNK} = 0.048\,\mathrm{min}^{-1}$, $k_{SC} = 40\,\mathrm{M}^{-1}\cdot\mathrm{min}^{-1}$, $k_{EGCg}/k_{DMPO} = 8.3$, $[\mathrm{EGCg}] = 0$ (A), 0.5 (B), 1.0 (C), 2.5 (D), 5.0 (E) $\times 10^{-3}\,\mathrm{M}$.

In Process 1, OH radical is formed constantly by γ-irradiation and is trapped by DMPO forming DMPO-OH in parallel with being scavenged by EGCg. Scavenging of DMPO-OH by EGCg proceeds simultaneously. However, only the scavenging of DMPO-OH proceeds in Process 2. The scavenging of DMPO-OH in Process 1 should be minimized to obtain reliable data on the scavenging of OH radical with EGCg, because if this factor has a serious effect, the ESR signal becomes too small to measure. For this reason, a stronger and shorter γ-irradiation was better. We, therefore, used the ^{60}Co source (dose rate, 186 Gy/min) at the Research Reactor Institute, Kyoto University and a 2-min irradiation.

The spectra of all the samples showed the typical 4-line absorption characteristic of DMPO-OH overlapping the absorptions of the other weak impurity radicals and a broad background absorption due to γ-irradiated glass sample tube, as shown in Figure 2. Figure 3 shows the intensity change in DMPO-OH for all the samples as a function of time. It decreased almost exponentially with time in each case, showing that DMPO-OH was scavenged by EGCg and unknown processes.

We tried to analyze kinetically the change in DMPO-OH assuming that the following reaction processes are involved.

1. As the sample is γ-irradiated at a constant dose rate, OH radical is generated at a constant rate of g mole/min during γ-irradiation.
2. OH radical is trapped by DMPO forming DMPO-OH or scavenged by EGCg, other processes which might quench OH radical were neglected. These two processes proceed competitively and the fractions of OH radicals trapped and scavenged are proportional to their second order reaction rate, namely $k_{DMPO}[\mathrm{DMPO}][\mathrm{OH}]$ and $k_{EGCg}[\mathrm{EGCg}][\mathrm{OH}]$. Therefore, the rate of OH radical trapping is written as, $g \cdot k_{DMPO}[\mathrm{DMPO}][\mathrm{OH}] / \{k_{DMPO}[\mathrm{DMPO}][\mathrm{OH}] + k_{EGCg}[\mathrm{EGCg}][\mathrm{OH}]\}$.

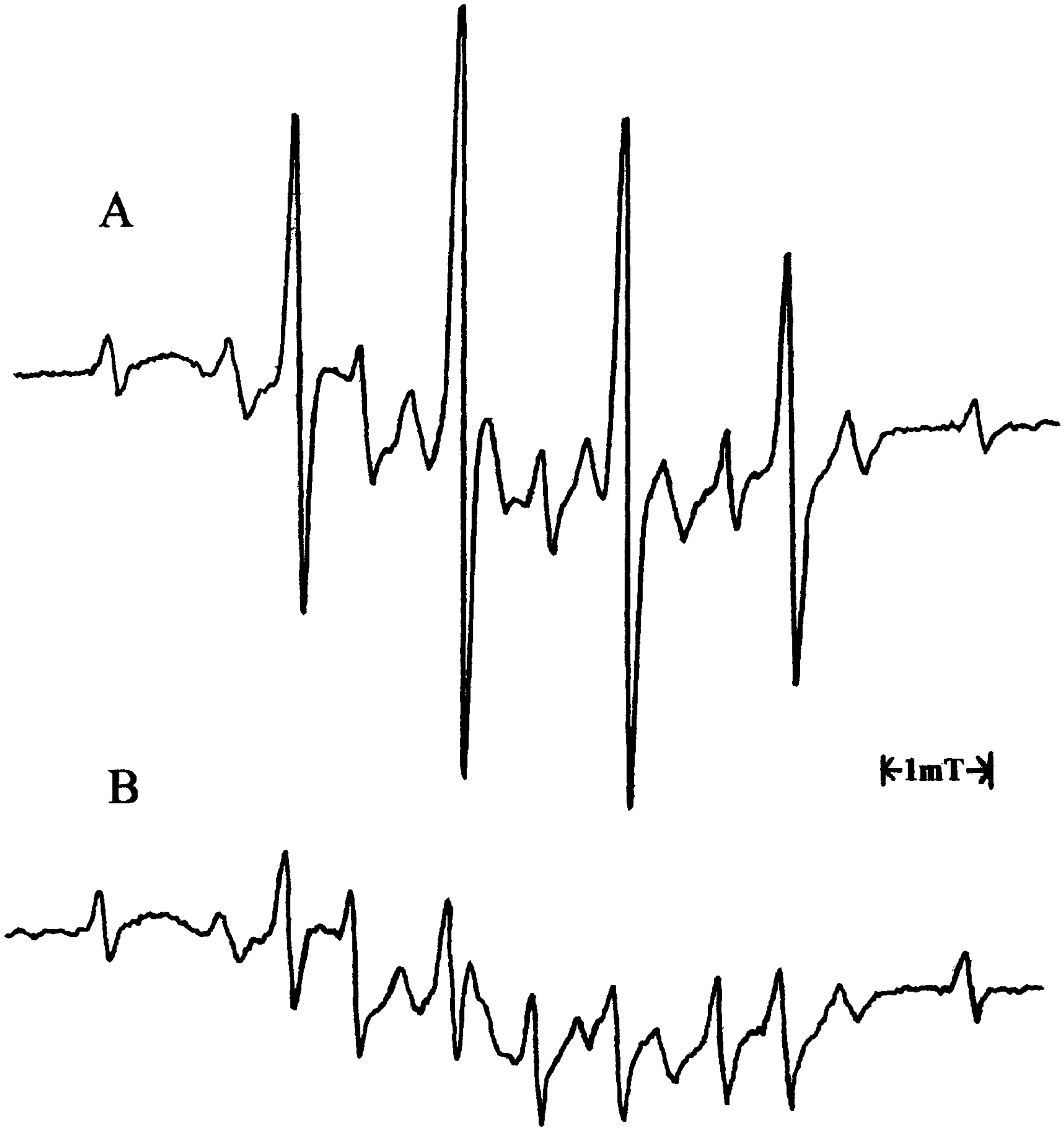

FIGURE 2 ESR Spectra of the γ-Irradiated Sample Containing only DMPO (0.05 M). Spectra were measured at 1 (A) and 20 (B) min after γ-irradiation. Measuring conditions: magnetic field, 334 mT; microwave power, 10 mW; modulation frequency, 100 kHz; modulation width, 0.07 mT; time constant, 0.3 sec; sweep time, 4 min; sweep width, ± 4 mT; gain, 1 × 100.

As the generation rate for DMPO-OH is equal to the rate of trapping of OH radical, this formulae also gives the rate of generation of DMPO-OH. However, one should pay attention to whether recombination of OH radical to form H_2O_2 contributes to the consumption of the radical. It is possible to compare the rates of recombination and trapping by using the rate constants, 5.5×10^9 and 4.3×10^9 ($M^{-1}sec^{-1}$), from Ref. 17. Assuming $[OH] = 10^{-4}$ M, the trapping rate was three orders of magnitude larger than that of recombination, suggesting that the above assumption was reasonable.

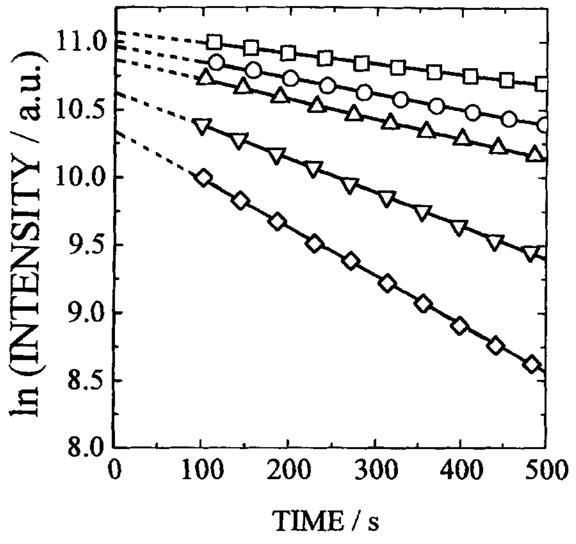

FIGURE 3 Experimental Intensity Change in DMPO-OH after γ-Irradiation. [EGCg]; 0 (□), 0.5 (○), 1.0 (△), 2.5 (▽), 5.0 (◇) × 10^{-3} M [DMPO]; 0.05 M.

3. DMPO-OH disappears through unknown mechanisms, which proceed nearly like a first-order reaction, as shown in Figure 3. Therefore, the reaction formulae of this process is describable as k_{UNK}[DMPO-OH].
4. DMPO-OH is scavenged by EGCg in accordance with the equation, k_{SC}[DMPO-OH] · [EGCg].

Here, [OH], [DMPO], [EGCg] and [DMPO-OH] are the concentrations of OH radical, DMPO, EGCg and DMPO-OH, respectively. The rate constants, k_{DMPO}, k_{EGCg}, k_{UNK}, and k_{SC}, are understood from the above equations.

The change of DMPO-OH in Process 1 is described as follows (here t' is the time from the starting point of γ-irradiation).

$$\begin{aligned} &\mathrm{d[DMPO\text{-}OH]}/\mathrm{d}t' \\ &= g\cdot k_{DMPO}[\mathrm{DMPO}][\mathrm{OH}]/\{k_{DMPO}[\mathrm{DMPO}][\mathrm{OH}] \\ &\quad + k_{EGCg}[\mathrm{EGCg}][\mathrm{OH}]\} - k_{UNK}[\mathrm{DMPO\text{-}OH}] \\ &\quad - k_{SC}[\mathrm{DMPO\text{-}OH}][\mathrm{EGCg}] \\ &= g/\{1 + k_{EGCg}[\mathrm{EGCg}]/k_{DMPO}[\mathrm{DMPO}]\} \\ &\quad - \{k_{UNK} + k_{SC}[\mathrm{EGCg}]\}[\mathrm{DMPO\text{-}OH}] \qquad (1) \end{aligned}$$

Initial concentrations of DMPO and EGCg are large compared with the OH formed during γ-irradiation, so it was possible to assume that [DMPO] and [EGCg] remain at the initial value and are constant. The value of [OH] was in the order of 10^{-5} M. Comparison of the intensity of ESR with that of TEMPOL solution of known concentration suggested that this assumption was reasonable. Therefore, substituting as,

$$g/\{1 + k_{EGCg}[\mathrm{EGCg}]/k_{DMPO}[\mathrm{DMPO}]\} = \mathbf{a} \qquad (2)$$

$$k_{UNK} + k_{SC}[\mathrm{EGCg}] = \mathbf{b} \qquad (3)$$

and integrating Equation (1),

$$[\mathrm{DMPO\text{-}OH}] = (\mathbf{a}/\mathbf{b})\{1\text{-}\exp(\text{-}\mathbf{b}t')\} \qquad (4)$$

Therefore, the concentration of DMPO-OH just after 2-min irradiation is

$$[\mathrm{DMPO\text{-}OH}]_{t'=2} = (\mathbf{a}/\mathbf{b})\{1\text{-}\exp(\text{-}2\mathbf{b})\} \qquad (5)$$

On the other hand, the change of [DMPO-OH] in Process 2 is simply described as follows, because **a** is zero in this case:

$$[\mathrm{DMPO\text{-}OH}] = [\mathrm{DMPO\text{-}OH}]_{t=0}\cdot \exp(\text{-}\mathbf{b}\mathrm{t}) \qquad (6)$$

Here, zero time was reset at the end of γ-irradiation, therefore $[\mathrm{DMPO\text{-}OH}]_{t'=2}$ is equal to $[\mathrm{DMPO\text{-}OH}]_{t=0}$.

We prepared five samples containing various amounts of EGCg, and obtained the values of **a** and **b** for each sample experimentally. First, **b** values were obtained from the slopes of the lines in Figure 3, then $[\mathrm{DMPO\text{-}OH}]_{t=0}$ was obtained by extrapolating these lines to time zero. In this case, the unit of **a** and [DMPO-OH] could be selected arbitrarily, because we needed only the ratio of them between samples as recognized from Equation (7). Therefore, we used ESR intensity directly. By using these **b** and $[\mathrm{DMPO\text{-}OH}]_{t=0}$ values, **a** is calculated from Equation (5).

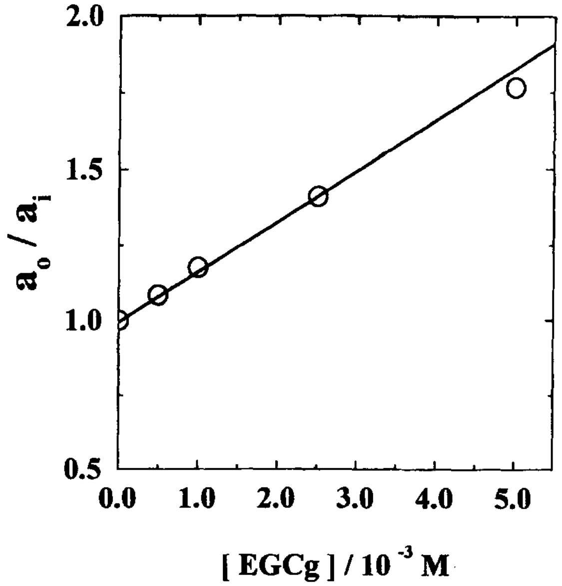

FIGURE 4 Relationship between $\mathbf{a}_0/\mathbf{a}_i$ and [EGCg].

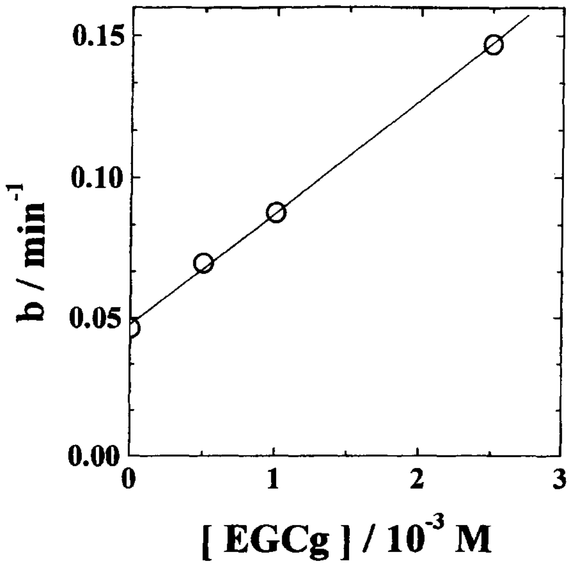

FIGURE 5 Relationship between **b** values and [EGCg].

The relationship between **a** and [EGCg] was shown in Equation (2), so the following equation is reduced;

$$\mathbf{a}_0/\mathbf{a}_i = 1 + \{k_{EGCg}/k_{DMPO}[DMPO]\}[EGCg]_i \quad (7)$$

Here, $\mathbf{a}_0$ and $\mathbf{a}_i$ are the **a** values of the samples containing no EGCg and of the *i*-th sample containing a certain amount of EGCg, respectively. The ratio $\mathbf{a}_0/\mathbf{a}_i$ was plotted against [EGCg] as shown in Figure 4, where satisfiable linearity was obtained. As the slope is k_{EGCg}/k_{DMPO} [DMPO] and [DMPO] is known, it is possible to calculate the ratio of the reaction constants, k_{EGCg}/k_{DMPO}. The ratio k_{EGCg}/k_{DMPO} was 8.3, indicating that EGCg has 8.3-fold more reactivity with OH radical than the typical spin trapping agent, DMPO. This result suggests that EGCg really acts as a OH radical scavenger. We have already calculated this value at −70 °C to be 16, using the solid state spin trapping method.[6] The difference between the values was not so large, nevertheless they were measured at very different temperatures and in different phases. It is also possible to calculate k_{EGCg} as 3.6×10^{10} ($M^{-1}sec^{-1}$) by using the value of $k_{DMPO} = 4.3 \times 10^9$ ($M^{-1}sec^{-1}$).[17] These large values suggest that both reactions are diffusion-controlled and, therefore, the difference between them comes from the difference of collision cross sections. That is to say, EGCg has a size larger than that of DMPO, in addition to the many reactive points on the surface, namely hydroxyl groups.

Equation (3) shows that **b** should be linear to [EGCg]. The plot of **b** against [EGCg] is shown in Figure 5, and good linearity was observed between them, showing that this analysis is substantially reasonable.

Finally, the intensity change in DMPO-OH was simulated using the values obtained experimentally. They are shown in Figure 1, where value 1 on the ordinate was adjusted to the quantity of OH radical generated for 2 min. From the line shape in Process 1, it is understood that the quantity of DMPO-OH scavenged during γ-irradiation is not so large compared with that of OH radical scavenged by EGCg. This was the reason why we used intense γ-irradiation.

From these results and the discussion, it is clear that this method is useful for measuring the OH radical-scavenging activity of various antioxidants. We are now applying it to other antioxidants.

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