



A New Method for Measuring Scavenging Activity of Antioxidants to the Hydroxyl Radical Formed by Gamma-Irradiation

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Abstract—A new method using ESR spin trapping was proposed for measuring scavenging activity of antioxidants to the hydroxyl (OH) radical. (-)-epigallocatechin gallate (EGCg) and 5,5-dimethyl-1-pyrroline N-oxide (DMPO) were used as an antioxidant and a spin trapping agent, respectively. Conventional method using a Fenton reaction had some defects on the estimation of the activity, because antioxidant disturbed the generating system of OH radical besides it scavenged the spin adduct (DMPO-OH). This method used intense γ -irradiation as OH radical generating system, and the intensity decrease of DMPO-OH after the end of the irradiation was followed to obtain the rate constant of the scavenging of DMPO-OH with EGCg and 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 to compare precisely the OH radical scavenging activity of various antioxidants.

Introduction

It has been known that reactive oxygen species play an important role in inducing various lesions in living organisms. It is, therefore, important to search for the substances which scavenge them, from a viewpoint of preventive medicine. We have been focusing our attention on the green tea percolate and the constituents,¹⁻⁴⁾ because they are intaked daily and it has been reported that they show various pharmacological effects such as anti-mutagenic or anti-carcinogenic effects.⁵⁻¹⁰⁾ There, we revealed that the tea percolate and a main constituent (-)-epigallocatechin gallate (EGCg) show a protecting effect against radiation-induced scission of DNA, and the effect was derived from the scavenging of the hydroxyl (OH) radical formed by γ - and β -irradiations. As OH radical is the most reactive species among reactive oxygen species and affects seriously on biomolecules, it is necessary to examine the OH radical scavenging activity of antioxidants. Spin trapping method is very useful for this purpose,¹¹⁻¹⁴⁾ but the conventional method has a few defects and, as the result, the scavenging activity calculated were unreliable. We propose a new method which is able to overcome such defects and are applicable to evaluate unambiguously the OH radical scavenging activity of various antioxidants.

Experimental

(-)-epigallocatechin gallate (EGCg, supplied from 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. The phosphate buffer solutions containing 0.05M DMPO and various amount of EGCg were gamma-irradiated for two min using ^{60}Co source (dose rate, 186 Gy/min), and were rapidly frozen just after completing irradiation. Each samples were then melted, put into a flat-cell and the ESR spectra were measured at room temperature using a JEOL RE.3X spectrometer. The relationship between the intensity of the spin adduct (DMPO-OH) and time after melting the sample was examined.

Results and Discussion

As the spin trapping method is relatively simple and is able to obtain directly the information about the radical, it has been used as one of the methods to examine the scavenging activity of OH and superoxide anion radicals. An appropriate radical-generating system must be adopted for this purpose. Fenton reaction ($\text{Fe}^{2+} + \text{H}_2\text{O}_2$) is generally used for generating OH radical, and DMPO and an antioxidant are mixed with it. A fraction of OH radical generated is trapped with DMPO forming the adduct DMPO-OH, but some fraction is scavenged by the antioxidant. It is, therefore, possible by examining the intensity decrease of DMPO-OH to calculate the OH radical scavenging activity of any antioxidants, compared with the intensity of the sample without the antioxidant. However, this method has following two problems; 1) OH radical generating system is perturbed by the chelating of the antioxidant to Fe^{2+} ion. 2) DMPO-OH is also scavenged by the antioxidant. In some cases, therefore, results were obtained as if the antioxidant showed the scavenging activity, nevertheless OH radical was not really scavenged. To solve the problem 1), we thought that γ -irradiation is usable 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 antioxidant. On the other hand, it seems to be difficult to overcome the problem 2), because DMPO-OH and the antioxidant molecule coexist in a solution and are impossible to avoid their collision. Therefore, we considered to estimate the quantity of DMPO-OH scavenged by the antioxidant.

The samples containing definite amount of DMPO and various amount of EGCg was γ -irradiated for a short period (t_0) at a constant dose rate (called as process 1). Then, the ESR Intensity of DMPO-OH was measured as a function of the time after the end of γ -irradiation (process 2). In the process 1, trapping of the OH radical forming DMPO-OH and the scavenging of DMPO-OH proceed simultaneously. However, only the scavenging of DMPO-OH proceeds in the process 2. The scavenging of DMPO-OH in the process 1 should be minimized to obtain reliable data on the scavenging of OH radical with EGCg. For this purpose, stronger and shorter γ -irradiation was better. We used ^{60}Co source (dose rate, 186 Gy/min) at Kyoto University Research Reactor Institute and 2-minute irradiation was applied.

The spectra of all the samples showed the typical 4-line absorption characteristic to DMPO-OH. The intensity of DMPO-OH decreased almost exponentially with time in each case, showing that it

was scavenged by EGCg and unknown processes as shown in Fig. 1. We tried to analyze kinetically the change of DMPO-OH assuming that following processes are involved

1. OH radical is generated at a constant rate of g molecules /sec during γ -irradiation.
2. OH radical is trapped with DMPO forming DMPO-OH or scavenged with EGCg, and 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

$$k_{\text{DMPO}}[\text{DMPO}][\text{OH}] \quad \text{and} \quad k_{\text{EGCg}}[\text{EGCg}][\text{OH}]$$

- 3 DMPO-OH disappears through unknown mechanisms, which proceed nearly like a first- order reaction, as shown in Fig. 1. Therefore, the reaction formulae of this process is describable as $k_{\text{UNK}}[\text{DMPO-OH}]$.

- 4 DMPO-OH is scavenged with EGCg in accordance with the equation, $k_{\text{SC}}[\text{DMPO-OH}] [\text{EGCg}]$. Here, $[\text{OH}]$, $[\text{DMPO}]$, $[\text{EGCg}]$ and $[\text{DMPO-OH}]$ 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 above equations.

The change of DMPO-OH in the process 1 is described as follows;

$$\begin{aligned} d[\text{DMPO-OH}] / dt &= g \cdot k_{\text{DMPO}}[\text{DMPO}][\text{OH}] / \{k_{\text{DMPO}}[\text{DMPO}][\text{OH}] + k_{\text{EGCg}}[\text{EGCg}][\text{OH}]\} \\ &\quad - k_{\text{UNK}}[\text{DMPO-OH}] - k_{\text{SC}}[\text{DMPO-OH}][\text{EGCg}] \\ &= g / \{1 + k_{\text{EGCg}}[\text{EGCg}] / k_{\text{DMPO}}[\text{DMPO}]\} \\ &\quad - \{k_{\text{UNK}} + k_{\text{SC}} [\text{EGCg}]\}[\text{DMPO-OH}] \end{aligned} \quad (1)$$

Initial concentrations of DMPO and EGCg are large enough compared with DMPO formed during γ -irradiation, so it was possible to assume that $[\text{DMPO}]$ and $[\text{EGCg}]$ remain at the original value and are constant. Therefore, substituting as,

$$g / \{1 + k_{\text{EGCg}}[\text{EGCg}] / k_{\text{DMPO}}[\text{DMPO}]\} = a \quad (2)$$

$$k_{\text{UNK}} + k_{\text{SC}} [\text{EGCg}] = b \quad (3)$$

equation (1) is expressed simply as follows,

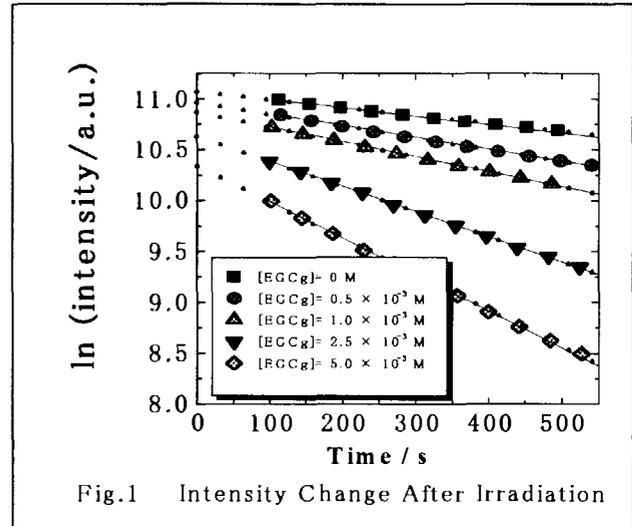


Fig.1 Intensity Change After Irradiation

$$dx / dt' = a - bx \quad (4)$$

Here $[EGCg] = x$. Integrating above equation,

$$x = (a / b)\{1 - \exp(-bt')\} \quad (5)$$

Therefore, the quantity of DMPO-OH (x_0) just after 2-minute irradiation is

$$x_0 = (a / b)\{1 - \exp(-2b)\} \quad (6)$$

On the other hand, the change of DMPO-OH in the process 2 is simply described as follows, because a is zero in this case;

$$Dx / dt = -bx \quad (7)$$

So,

$$x = x_0 \cdot \exp(-bt) \quad (8)$$

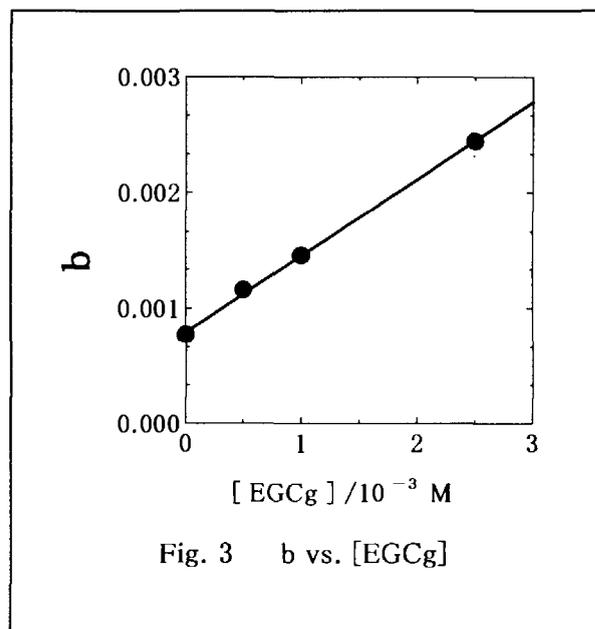
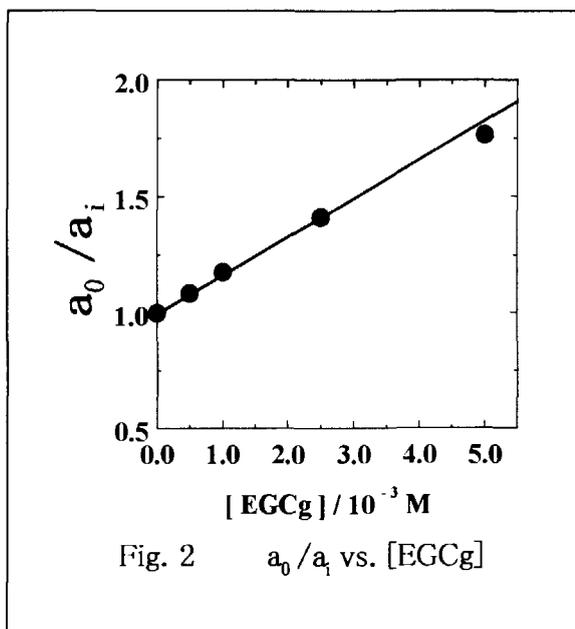
Here, zero time was reset at the end of γ -irradiation.

We prepared five samples containing various amount of EGCg, and the values of a and b were obtained in each sample. First, b value is obtained from the slopes in Fig. 1, then x_0 is obtained by extrapolating these lines to time zero. In this case, the unit of a and x were possible to select arbitrarily, because we needed only the ratio of them as recognized from equation 9. Therefore, we used ESR intensity directly. By using these b and x_0 values, a is calculated from equation (6).

The relationship between a and $[EGCg]$ was shown in equation 2, so a following equation is reduced;

$$a_0 / a_i = 1 + \{k_{EGCg} / k_{DMPO}[DMPO]\}[EGCg]_i \quad (9)$$

Here, a_0 and a_i are the a values of the samples containing no EGCg and of i -th sample containing a certain amount of EGCg, respectively. The ratio a_0/a_i was plotted against $[EGCg]$ as shown in Fig. 2. 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 larger reactivity with OH radical than the typical spin trapping agent DMPO. This suggests that EGCg really act as a OH radical scavenger. We have already calculated this value at -70 °C to be 16, using solid state spin trapping method.³⁾ The result that the difference between them measured at different temperatures was not so large is explainable as follows. As known by Arrhenius equation, a rate constant is written by the product of a frequency factor and a term, $\exp(-E/RT)$. In the case of the reaction of OH radical and EGCg or DMPO, the activation energy must be small and $\exp(-E/RT)$



is nearly 1. If this is the case, $k_{\text{EGCg}}/k_{\text{DMPO}}$ is determined by the ratio of their frequency factors and, as the result, does not depend largely on the temperature.

The equation 3 shows that b should be linear to [EGCg]. The plot of b against [EGCg] is shown in Fig. 3, and a sufficient linearity was observed between them, showing that this analysis is substantially reasonable.

From these results and discussion, it was revealed that this method is useful to measure precisely the OH radical scavenging activity of various antioxidants.

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