Factors affecting the rate constants of radical scavenging by antioxidants.

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Summary

Second order rate constants (k_2) of the scavenging reaction of various radicals with tea catechins and related polyphenols were measured by ESR spectroscopy. For the measurement of the decrease in the ESR intensity of DPPH, an ESR stopped-flow apparatus combining syringe pump, switching bulb and 4-jet mixer, was constructed, which allowed to follow the reaction completed for sub-seconds. The k_2 values of catechins changed from the order of 10^9 (OH radical) to 10^{-2} $M^{-1}s^{-1}$ (nitroxide radical), depending on the reactivity of radicals. Difference of the k_2 values for scavenging OH radical was not so large among various polyphenols, showing that each phenolic hydroxyl group (ϕ -OH) acted similarly for the scavenging. In the case of DPPH, the values of resorcinol and phloroglucinol were far smaller than those of catechol and tea catechins, which meant that more than two ϕ -OHs locating ortho- or para- positions were necessary for the strong scavenging activity. Neighboring tri-hydroxyl structure included in the structures of gallocatechins or gallates such as EGC, ECg and EGCg was required for the strong scavenging of stable nitroxide radicals. These result showed that k_2 value and relative reactivity depended both on the structures of radicals and antioxidants.

Keywords

Tea catechin, Polyphenol, ESR, Rate constant, Stopped-flow.

Introduction

It seems to be very important to study the radical scavenging reaction of tea catechins, because most of the pharmacological activities of tea catechins are attributable to the scavenging of reactive oxygen species. However, physicochemical studies analyzed from a kinetic point of view are not so many except for those by pulse radiolysis. Radical-scavenging reaction by antioxidants usually accompanies electron-transfer from the antioxidant to radical. Therefore, the reaction rate depends on various factors such as redox potential of both radical and antioxidant, the steric structure, the location of them in complex system like biomembrane etc. We, therefore, examined how reaction rate constants of tea catechins and the relative rates among them change depending on the kind of radical. For this experiment, we constructed an ESR stopped-flow apparatus combining syringe pump, switching bulb and 4-jet mixer, which allowed to follow the reaction completed for sub-seconds. The rates of scavenging 1,1-diphenyl-2-picrylhydrazyl (DPPH) were measured both in a uniform solution and in a liposome system.

Experimental

Materials. (−)-Epicatechin (EC), (−)-epicatechin gallate (ECg), (−)-epigallocatechin (EGC) and (−)-epigallocatechin gallate (EGCg) were purchased from Nakahara Kagaku Co. Dioleoylphosphatidylcholine (DOPC) was gifted from Nichiyu Liposome Co. 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) and 4-carboxy-tetramethyl-1-piperidinyloxy (4-carboxy-TEMPO) were purchased from Labotec Co. and Aldrch Co. respectively. Other chemicals were obtained from Wako Pure Chemicals Co. and were of guaranteed grade.

Method. The method for measuring the rate constant of OH radical scavenging will be stated in this proceedings "Measurement of OH Radical-Scavenging Activity of tea catechins using gamma-irradiation" (Ⅲ-P-51).

The reactions of DPPH were performed in two systems. As a solvent, mixture of ethanol and water (2:1) was used in EtOH-H_2O system. The reactions in liposome system were carried out using water as a solvent, and DPPH were in liposomal membrane.

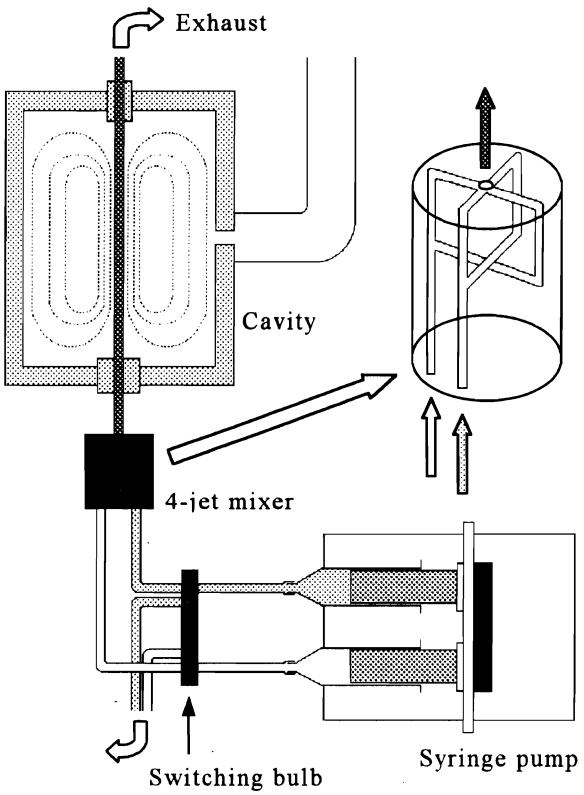

Fig. 1. A Schematic Structure of the ESR Stopped-Flow Apparatus.

Figure 1 shows the ESR stopped-flow apparatus constructed for this experiment. The radical and the antioxidant solutions were put into two syringes, respectively. These syringes were set on a syringe pump and flowed at a constant rate to the 4-jet mixer set in front of the cavity. The paths of each solution were divided and the resulting four solutions were crashed at a point at high speed to mix solutions thoroughly. The mixed solution was flowed in a quartz capillary tube set in the center of the cavity. If the flow rate is large and the time necessary to flow from the mixing point in the 4-jet mixer to the area where ESR was measured is short enough compared with the time of radical scavenging, the ESR intensity does not change with that of the original solution.

For the measurement of the decrease of the intensity, the flow of the solution must be stopped instantly, and it had better to do this operation after the mixing point of the solution, but it induced various difficulties. We adopted a duplex switching bulb used usually for HPLC. The switching bulb was set between the syringe pump and the 4-jet mixer. The paths of two solutions were changed rapidly and

simultaneously to exhaust line and, as a result, the flow into the cavity was stopped instantly.

Results and Discussion

Figure 2 shows the intensity change of DPPH radical mixed with the EGCg solution. The line A is the change in the case mixed with the solution containing no EGCg. Intensity kept a constant value showing that the flow rate of two solutions were uniform during the measurement. The line B is the intensity change of the DPPH solution (2.5×10^{-4} M) mixed with an EGCg solution (7.5×10^{-4} M). The intensity before the flow of the solution was stopped was same as that of the line A. This means that the time of the flow from the mixing to the ESR measuring points is short enough and the quantity of DPPH scavenging during the process of this flow was negligible. The intensity began to decrease exponentially just after the flow was stopped. This also means that the switching bulb acted satisfactorily.

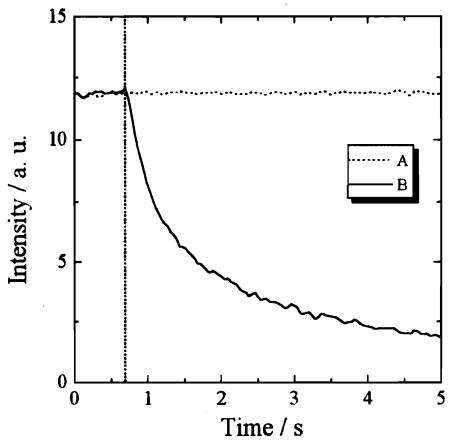

Fig. 2. ESR Intensity Change of DPPH Solution after Mixed with EGCg Solution in Water system.
[DPPH] = 2.5×10^{-4} M,
A: [EGCg] = 0 M,
B: [EGCg] = 7.5×10^{-4} M

The results were analyzed according to the assumption that the radical scavenging occurs as a first order to the radical and the antioxidant concentrations and a second order as a whole. The second order rate constants (k_2) were obtained by the fitting according to the following equation.

$$Y = \{1/(a-b)\}\cdot\ln\{b(a-x)/(a-x)\} = k_2 t \qquad (1)$$

Here, a and b are the initial concentrations of the antioxidant and the radical. The x is the concentration of the radical at time t. Figure 3 shows the points plotted according to the equation (1).

Table 1 shows the results obtained by the method stated above as well as those cited from the references. The k_2 values of catechins changed from the order of 10^9 (OH radical) to 10^{-2} $M^{-1}s^{-1}$ (nitroxide radical), depending on the reactivity of radicals. Difference of the k_2 values for scavenging OH radical was not so large among various polyphenols, showing that each phenolic hydroxyl group (ϕ -OH) acted similarly for the scavenging. This large values suggests that the scavenging of the OH radical is diffusion-controlled, and the reaction of the OH radical with ϕ -OH always occurs when they collides. Therefore, the difference among various ϕ -OH is not apparent.

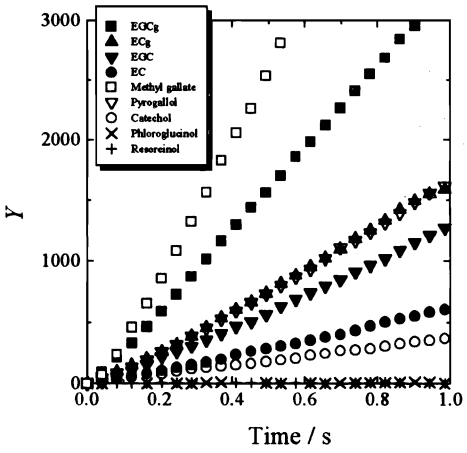

Fig. 3. Y vs. Time.
[DPPH] = 2.5×10^{-4} M,
[Antioxidant] = 7.5×10^{-4} M
Reaction system: EtOH-H_2O

In the case of DPPH, the values are seven figures smaller than those of the OH radical. In addition, the difference among polyphenols becomes large; the values of resorcinol and phloroglucinol were far smaller than those of catechol and tea catechins, which meant that more than two ϕ-OHs locating ortho- or para- positions were necessary for the strong scavenging activity. Therefore, the scavenging reaction needs activation energy and depends on the structure of polyphenols in this case. EC or catechol did not show strong activity to the stable nitroxide radicals compared with ECg, EGC and EGCg. This means that neighboring tri-hydroxyl structure included in the structures of gallocatechins or gallates such as EGC, ECg and EGCg was required for the strong scavenging.

Relative scavenging activity in a liposome system was different from that in a uniform EtOH-H_2O system. DPPH is lipophilic and is thought to exist almost in the lipid membrane. On the other hand, catechins are hydrophilic and dissolve mainly in water but have the possibility to enter into the membrane. Therefore, two factors affect on the k_2 value. One is the scavenging activity of the antioxidant molecule itself in the membrane. This might be different from that in a uniform solution because of the complex structure of the membrane. The other is the rate to enter into the membrane from water. Although the k_2 value of methyl gallate in EtOH-H_2O system was large, that in lipid system became significantly small. But in the case of pyrogallol, k_2 in liposome system did became not so small. Therefore, it was considered that the structure of pyrogallol is effective enough to scavenge the radical in lipid membrane. We have scarcely any information on these matters, so a detailed analysis is a future problem.

From these results and discussion, it was revealed that the k_2 value and the relative reactivity of radical scavenging depended both on the structures of radicals and antioxidants.

Table 1. The k_2 values of Tea Catechins to Various Radicals.

Radical	OH radical	4-carboxy-TEMPO	DPPH	DPPH
Solvent (System)	Phosphate buffer	Phosphate buffer	(EtOH-H_2O)	(Liposome)
pH	7.4	7.4		
Temp.	30 ℃	25 ℃	21 ℃	21 ℃
	k_2 / 10^9 M^{-1} s^{-1}	k_2 / 10^{-2} M^{-1} s^{-1}	k_{app} / 10^3 M^{-1} s^{-1}	k_2 / 10^3 M^{-1} s^{-1}
Resorcinol	--	--	0.0031 ± 0.0007	0.0040 ± 0.0004
Phloroglucinol	--	--	0.012 ± 0.002	0.025 ± 0.003
Catechol	--	--	0.40 ± 0.07	0.13 ± 0.01
Pyrogallol	--	--	1.7 ± 0.1	0.86 ± 0.06
Methyl Gallate	--	--	2.8 ± 0.6	0.67 ± 0.05
EC	0.63 ± 0.04	0.090 ± 0.012	0.67 ± 0.12	0.23 ± 0.03
EGC	0.96 ± 0.3	2.3 ± 0.3	1.4 ± 0.1	0.74 ± 0.01
ECg	1.3 ± 0.5	2.0 ± 0.2	1.7 ± 0.2	0.24 ± 0.03
EGCg	1.9 ± 0.5	5.1 ± 0.1	2.5 ± 0.1	2.3 ± 0.5