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Original Contribution

SCAVENGING MECHANISMS OF (-)-EPIGALLOCATECHIN GALLATE AND (-)-EPICATECHIN GALLATE ON PEROXYL RADICALS AND FORMATION OF SUPEROXIDE DURING THE INHIBITORY ACTION

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Abstract—The scavenging effects of (-)-epigallocatechin gallate (EGCG) and (-)-epicatechin gallate (ECG) on peroxyl radicals and their mechanisms were studied by investigating the products formed during the first stages by 2,2′-azobis(2-aminopropane) hydrochloride (AAPH)-induced oxidation, without any isolation, using LC/MS, spectrophotometry, chemiluminescence analyses, and semiempirical molecular orbital (MO) calculations. The results show that EGCG can be converted to an anthocyaninlike compound followed by cleavage of the gallate moiety by oxidation. On the other hand, ECG can be converted to an anthocyaninlike compound after cleavage of the gallate moiety. The calculated C-H bond dissociation enthalpies (BDEs) for EGCG and ECG at the C-2 position were quite low (62.7 and 66.8 kcal/mol, respectively) compared with O-H BDEs at the phenolic sites (ca. 70 kcal/mol), suggesting that the C-2 hydrogen can be abstracted by free radicals. The addition of superoxide dismutase (SOD) decreased the chemiluminescence in EGCG by one-half during the inhibitory action. Active oxygen including superoxide (O_2^-) would be produced in EGCG, but not in ECG. The authors proposed the antioxidative mechanisms of EGCG and ECG depending on the experimental results and theoretical calculations. © 1999 Elsevier Science Inc.

Keywords—Catechins, APCI-LC/MS, Photodiode array, MO calculation, Superoxide, Chemiluminescence, Free radicals

INTRODUCTION

Catechins are a group of polyphenolic compounds, which have the diphenylpropane ($C_6C_3C_6$) skeleton, ubiquitously found in green tea. The main polyphenolic components in green tea are (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), and (-)-epigallocatechin gallate (EGCG). It is well known that flavonoids such as catechins can be protective against cancer and inflammatory and cardiovascular diseases [1–4]. These protective effects are largely due to their inhibition of some enzymes and antioxidative activities by scavenging free radicals. Epidemiological studies have demonstrated that the consumption of catechins and other flavonoids is effective in lowering the risk of coronary heart disease [1,2,5,6]. This suggests that catechins as well as other flavonoids can play an important role in scavenging free radicals in living systems. Numerous researchers have described the effectiveness of catechins in suppressing the formation of lipid peroxidation in biological tissues and subcellular fractions such as microsomes and low-density lipoprotein (LDL) [7–11]. Recently, however, negative results of catechins on resistance of LDL to oxidation in humans have been reported by van het Hof et al. [12].

Unno et al. [13,14] and Nakagawa et al. [15] have reported interesting results concerning the absorption of EGCG in rats and human. They demonstrated that the concentration of EGCG in human plasma was in the range of 0.3–4.0 μM. The fact shows catechins can function as antioxidative agents at a lower concentration than expected.

On the other hand, some antioxidants such as ascorbic acid can act as pro-oxidants. Metodiewa et al. [16] have reported that quercetin may act as a cytotoxic prooxidant. This suggests that the biologic action of catechins for

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free radicals may not be very simple. In addition, little is known about the scavenging mechanisms of catechins, despite the fact that much attention has been paid to the inhibitory effect of catechins on lipid peroxidation.

Electron spin resonance (ESR) and pulse radiolysis studies are often used for free radical research, and their results have provided much valuable information [17–21]. There are several reports using ESR for mechanistic studies of catechins against radical oxidation [22–24]. Recently, Gardner et al. [25] have reported the antioxidant potentials of tea catechins in aqueous and organic media based on an ESR study. The scavenging effect of catechins and their acetylated derivatives were shown by Nanjo et al. [26], indicating an interesting result that the 3-, 5-, and 7-hydroxyl groups did not influence the scavenging effect of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals. However, these reports did not give any positive evidence about the reaction mechanisms of catechins. Therefore, it is very important to clarify the scavenging mechanisms in understanding the potential of catechins in biological fluids.

Recently, we have shown that LC/MS and a photodiode array detector (PDA) in combination with semiempirical molecular orbital (MO) calculations are very useful for the structural confirmation of reaction intermediates and have reported the scavenging effects and mechanisms of EC and EGC [27]. The effect and mechanisms of catechins esterified with gallic acid at the C-3 position have not yet been elucidated.

In the present study, we tried to elucidate the scavenging mechanisms of EGCG and ECG by investigating the products formed during the first stages of oxidation without any isolation because a large number of inseparable products were produced by oxidation. Using the chemiluminescence (CL) method in addition to LC/MS, PDA, and semiempirical MO calculations, we show here the scavenging effects and mechanisms, and the formation of superoxide from four catechins during the antioxidative action.

MATERIALS AND METHODS

Reagents

EC, EGC, ECG, and EGCG were from Kurita Industry Company (Tokyo, Japan). 2,2′-Azobis (2-aminopropane) hydrochloride (AAPH), methyl gallate (MG), luminol, Cu, Zn-superoxide dismutase (SOD), and phosphate buffer were from Wako Pure Chemicals (Osaka, Japan). Diethylenetriamine-N, N, N′, N′′, N′′-pentaacetic acid (DTPA) was purchased from Dojindo Laboratories (Kumamoto, Japan). All other chemicals were of analytical grade. All chemicals were used without further purification.

Determination of PC-hydroperoxides (PCOOHs)

PC-hydroperoxides (PCOOHs) were determined by normal-phase high-pressure liquid chromatography (HPLC) according to the method of Miyazawa et al. [28]. PCOOHs were detected at 234 nm using a molar absorption coefficient of 27,000. Detailed experimental conditions were described previously [27].

Mass spectrometric analyses

An LCQ mass spectrometer (Thermoquest, Manchester, UK) equipped with an atmospheric pressure chemical ionization (APCI) source and coupled to a Hitachi L-7000 series (Tokyo, Japan) HPLC (APCI-LC/MS) was used in a positive ionization mode for mass spectral confirmation of the reaction intermediates of catechins. The column was TSK-GEL ODS-80Ts (2.0 $\times$ 150 mm, Toso, Tokyo, Japan). A mixture of methanol and water containing 1% acetic acid (2:8) was used as an eluent, and the flow rate was 0.2 ml/min. The further procedure was principally performed as described for PC in the previous paper [27]. Briefly, 70 μM of catechins and 20 mM of AAPH were used in the LC/MS analyses. After oxidation was initiated by AAPH, aliquots of the reaction mixture (10 μl) were withdrawn at intervals and then injected onto the HPLC column, and the structures of the reaction intermediates were analyzed by liquid chromatography equipped with mass spectrometer (LC/MS) with a UV detector (280 nm). MS/MS analyses were achieved using helium as a collision gas at a pressure of 0.1 Pa (10^{-3} Torr).

Spectrophotometric analyses

Spectrophotometric data were obtained using a Toso HPLC system (Tokyo, Japan) with a photodiode array detector (MCPD-3600, Otsuka Electronics Co., Osaka, Japan). The column was TSK-GEL ODS-80Ts (4.6 $\times$ 150 mm). Aliquots of the reaction mixture (10 μl) were withdrawn and injected onto the column.

Effects of SOD on catechins during the antioxidative action

Luminol-dependent chemiluminescence was measured using a Bio-Orbit Luminometer 1251 (Turku, Finland). One micromolar of catechins (final concentration) and 10 μL of Cu, Zn-SOD (70 U/ml) was preincubated in 130 μM of luminol at 37°C for 5 min in the absence and presence of SOD, and then AAPH was added to the mixtures by a Bio-Orbit Dispenser model SVD. Chemiluminescence was measured in a light-tight housing thermostatically controlled at 37°C for 15 min. Continuous

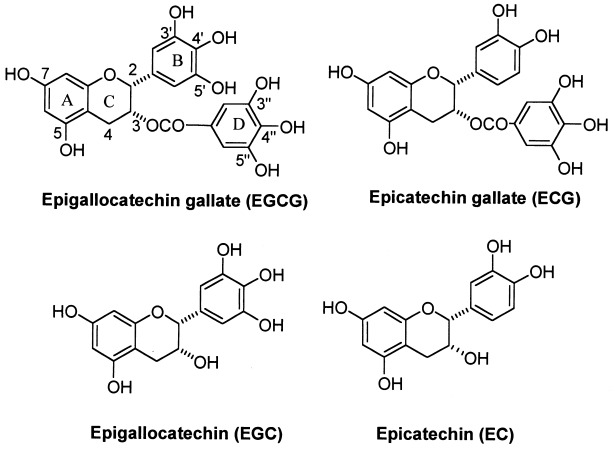

Fig. 1. Structures of catechins tested.

mixing was achieved by rotating the cuvette slowly and changing the direction every 0.5 s. The same volume of luminol alone in phosphate buffer was added to the control in the cuvette, and the obtained background chemiluminescence was subtracted from each sample chemiluminescence. The amount of active oxygen was obtained by the integral of chemiluminescence over 15 min.

Semiempirical MO calculation

The program SPARTAN (ver. 3.1, Wavefunction Inc., Irvine, USA) was used for all MO calculations described in this paper. First, the structures of catechins and catechin radicals were optimized by a conformation search (MacroModel ver.5.5; Monte Carlo Method, MM2* force field, Schrödinger Inc., Jersey City, NJ, USA) and then the bond dissociation enthalpies of their structures (BDEs) were calculated using the semiempirical PM3 and AM1 methods [27,29].

RESULTS

Inhibitory effects of EGCG and ECG on peroxyl radicals in the liposomal and aqueous systems

The structures of four catechins used in this study are listed in Fig. 1. As described previously, EGCG and ECG were more effective than EC and EGC against lipid peroxidation. In Table 1, the inhibition period (t_{inh}) is the period in which the formation of PCOOH was inhibited by catechin and the slope during this time is expressed as the inhibition rate (R_{inh}). The ration of the rate constant for inhibition (k_{inh}) for cahin propagation (k_p) was determined by the equation

$$k_{inh} / k_p = [LH] / R_{inh} \cdot t_{inh}$$

Table 1. Effect of Catechins Against the Peroxidation of Soybean PC

	t_{inh} (s)[a]	R_{inh} ($M \cdot s^{-1}$)[a]	k_{inh}/k_p[b]	Inhibition, %[c]	n[d]
EGCG	4980	1.6×10^{-9}	628	75	1.7
ECG	—	—	—	79	—
EC	9360	13.3×10^{-9}	41	68	3.1
EGC	3420	6.3×10^{-9}	232	26	1.1

[a] t_{inh} and R_{inh}, the inhibition period and rates in the presence of catechins, respectively.
[b] k_{inh}/k_p, the ratio of the rate constant for inhibition to that for chain propagation.
[c] Inhibition ratio (%) when control is 0 (%).
[d] n, stoichiometric number of radicals trapped by antioxidant.

where [LH] is the concentration of PC. The inhibition period (t_{inh}) of EGC (3420) and EGCG (4980) was shorter than that of EC (9360) as shown in Table 1. However, the values of k_{inh} / k_p for EGC (232) and EGCG (628) were higher than that of EC (41). These kinetic parameters of ECG were not obtained because of the linear formation of PCOOH during the experiment. Although a distinct increase in PCOOH was observed after the inhibition period (t_{inh} = 4980 s) in EGCG, but not in ECG as shown in Fig. 2. Therefore, ECG was more effective than EGCG in reducing the formation of PCOOH during the experiment (79 and 75% inhibition, respectively). The inhibitory effect in the aqueous system also showed that EGCG was less effective in scavenging peroxyl radicals than ECG (Fig. 3).

Reaction intermediates generated from EGCG and ECG by AAPH-induced radical oxidation

The reaction intermediates from EGCG and ECG were investigated using APCI-LC/MS. Because Electro-Spray Ionization (ESI)-LC/MS did not give positive evidence for the structural confirmation of the intermidiates

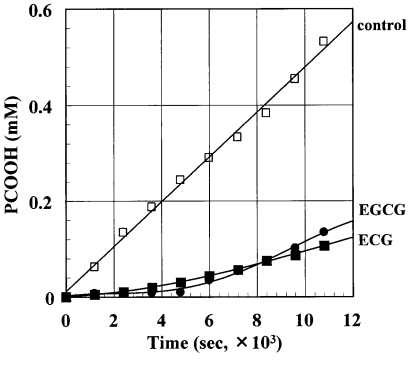

Fig. 2. Effect of EGCG and ECG against the peroxidation of soybean PC liposomes. The reaction system consisted of soybean PC (5 mM) and catechins (5 μM) in 10 mM Tris-HCl buffer (pH 7.4) containing 0.5 mM DTPA. Control (□), ECG (■), EGCG (●).

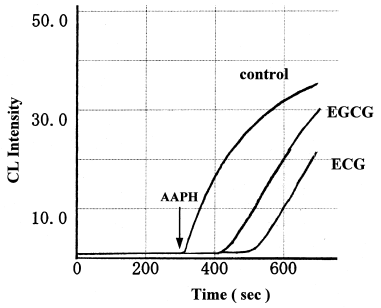

Fig. 3. Effect of EGCG and ECG against radical oxidation in aqueous media. EGCG and ECG (1 μM) were oxidized by the addition of AAPH (2 mM) in the presence of luminol. Chemiluminescence was recorded for 10 min after preincubation at 37°C for 5 min.

from EGCG or ECG, an APCI source was used in this experiment.

In Fig. 4, chromatograms monitored using the ultraviolet (UV) detector (280 nm) (A, C) and total ion chromatograms (TIC) (B, D) of the reaction mixtures produced from EGCG (left) and ECG (right) are shown. One peak was observed from EGCG at the retention time of 2.70 min (peak A) immediately after AAPH-induced oxidation was started. However, in the case of ECG, two peaks (peaks B and X) were formed after oxidation. The mass spectra of peaks A and B resembled each other (data not shown). The pseudomolecular ion of the two peaks was shown at *m/z* 154.

To confirm the structure of their peaks in LC/MS, we have also examined the reaction intermediates generated from methyl gallate, which have a common partial structure for both EGCG and ECG. Peak C was produced by the addition of AAPH to methyl gallate. Figure 5 illustrates the TIC of the reaction mixtures containing methyl gallate (left) and the mass spectrum of the compound (peak C) generated from methyl gallate (right). The mass spectrum was identical with those of peaks A and B in Fig. 4. LC/MS/MS analyses for three peaks (*m/z* 154) gave *m/z* 139, suggesting the structure shown in Fig. 5 for three peaks. No significant information on the counterpart of the gallate moiety was obtained in the LC/MS analyses.

Spectrophotometric analyses for the reaction intermediates

In Fig. 6, the UV spectra of peaks A, B, C, and methyl gallate were obtained using PDA that is effective for structural analyses as with LC/MS. Spectra I (peak A), II (peak B), and III (peak C) were definitely the same. Spectrum IV (methyl gallate) shows a large absorption at 280 nm due to the benzene structure coupled with the carbonyl group. In the other three spectra, the strong absorption at 280 nm decreased. This indicates that the carbonyl group conjugated with an aromatic ring disappeared. The spectrum of peak X is illustrated in Fig. 7, showing that peak X has the stronger absorption at 280 nm than EC.

BDEs for EGCG and ECG

To clarify the scavenging mechanisms of EGCG and ECG, BDEs were evaluated using PM3 semiempirical MO calculations. All MO calculations for catechins and catechin radicals were carried out after the structural optimization by the conformation search. As shown in Fig. 8, C-H BDEs at the C-2 position of EGCG and ECG were much lower (62.7 and 66.8 kcal/mol, respectively) than the BDEs for the phenolic O-H bond (ca. 70 kcal/mol), suggesting that the C-2 proton would easily be subtracted by free radicals in the esterified catechin. In addition, the O-H BDEs of 4′-OH in EGCG (67.7 kcal/mol) and 4′′-OH in ECG (69.1 kcal/mol) were also low.

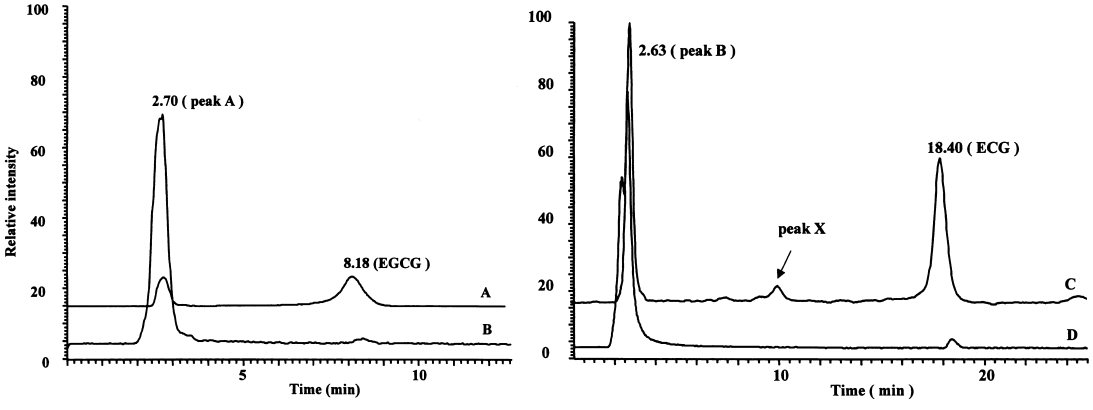

Fig. 4. Chromatograms monitored using the UV detector (280 nm) (A, C) and total ion chromatograms (TIC) (B, D) of the reaction mixtures produced from EGCG (left) and ECG (right). Oxidation was initiated by the addition of AAPH at 37°C. Reaction mixtures containing EGCG or ECG were withdrawn at intervals and then injected onto column of LC/MS.

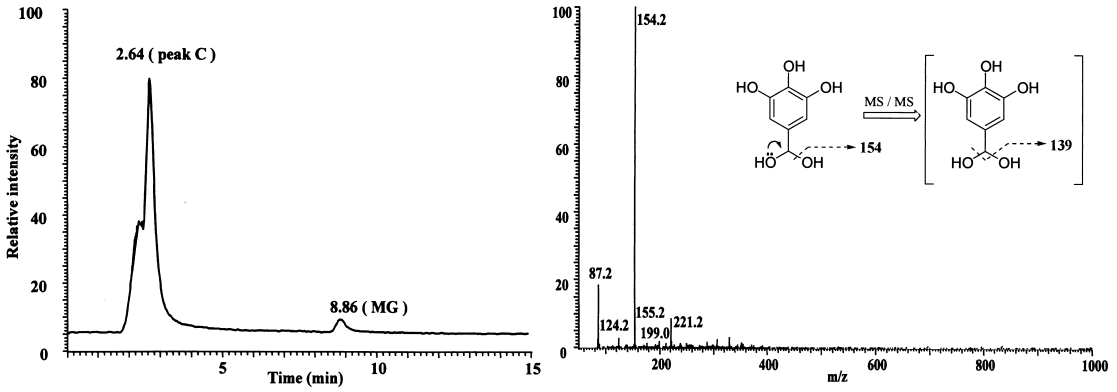

Fig. 5. Total ion chromatogram of the reaction mixtures containing methyl gallate and the mass spectrum of peak C generated from methyl gallate. Reaction conditions were described under the Materials and Methods section.

There is no difference between the PM3 and AM1 method in calculating the BDEs of catechins.

Formation of superoxide from catechins during the antioxidative action

Superoxide formation from four catechins during the antioxidative action was investigated in the absence and presence of SOD using the luminol-dependent chemiluminescence method. As shown in Fig. 9, SOD has little effect on the chemiluminescence of EC and ECG during the action. On the other hand, SOD reduced the sharp increase in the chemiluminescence in both EGC and EGCG by one-half. This result indicates the involvement of superoxide during the inhibitory action.

DISCUSSION

The antioxidative mechanisms of EGCG and ECG were studied using APCI-LC/MS, spectrophotomeric

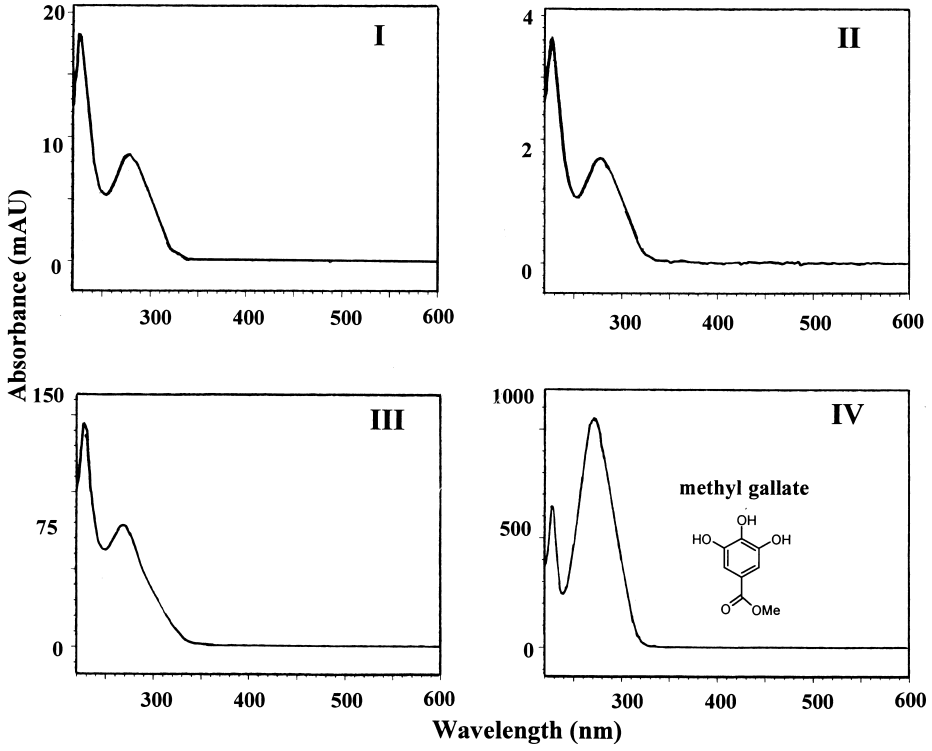

Fig. 6. Spectra of peaks A, B, C, and methyl gallate. All spectra were measured by HPLC with the photodiode array detector (PDA). (I) Peak A. (II) Peak B. (III) Peak C. (IV) Methyl gallate.

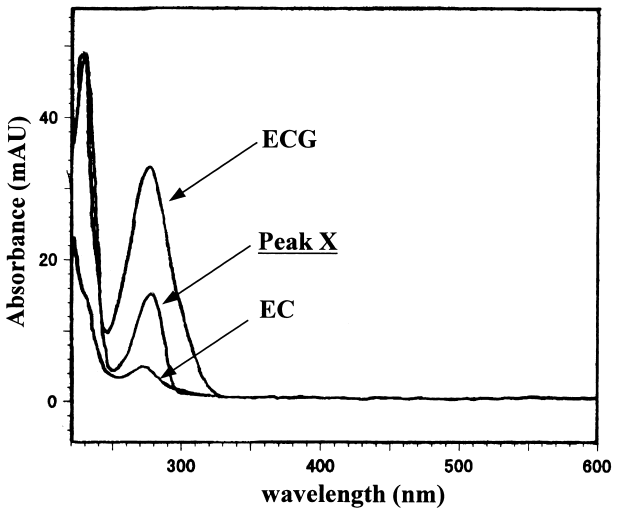

Fig. 7. Spectra of peak X (minor peak), ECG, and EC. All spectra were measured by HPLC and PDA.

studies, chemiluminescence analyses, and semiempirical MO calculations. AAPH, which generates peroxyl radicals by its reaction with oxygen, was used as an initiator of lipid peroxidation in the liposomal system and of radical oxidation in the aqueous system.

Bors et al. [30] proposed the three criteria for effective radical scavenging, showing that quercetin satisfies all three criteria and seems to be a more effective antioxidant than catechins, which lack two out of the three determinants.

In the previous study, we clarified that abstraction of a hydrogen atom at the C-2 positions in EC and EGC play an important role in the antioxidative effect. This is why catechins, which have a benzylic proton, exerted inhibitory effects greater than the other flavonoids such as quercetin, which have a lower redox potential [27]. Recently, Saint-Cricq de Gaulejac et al. [31] have reported that the scavenging ability of procyanidin A-2, which lacks one out of two C-2 proton, is much lower than procyanidin B-2, which has two C-2 proton. This report indicates the importance of the C-2 proton in catechins.

In this study, we examined the effects of EGCG, ECG, and gallate esters of EGC and EC on peroxyl radicals and their mechanisms. The effects were investigated in the liposomal and aqueous systems. ECG was more effective than EGCG against lipid peroxidation during the experiments despite the fact that EGCG has a trihydroxy structure in the B ring to scavenge free radicals more effectively (Fig. 2). The lower effectiveness of EGCG than ECG was obtained from the results in the aqueous system (Fig. 3). This suggests that antioxidant activity may not simply correlate with the number of phenolic O-H group.

The LC/MS result of the intermediates generated from EGCG, ECG, and methyl gallate showed that a common compound was produced from these three compounds by oxidation (Figs. 4 and 5). The UV spectrum of this compound lacks the strong absorption at 280 nm, indicating the disappearance of a carbonyl group coupled with an aromatic ring (Fig. 6). Furthermore, the difference in heat of formation between this compound and gallic acid is 7.0 (PM3) ~15.0 (AM1) kcal/mol. The formation of this compound would be thermodynamically favored. The compound would be more stabilized in water. These results from the LC/MS and spectrophotomeric studies suggest that cleavage of the gallate moiety at the C-3 position of EGCG and ECG can occur by peroxyl radicals generated from AAPH during the first stage.

In general, antioxidative activity has been considered to be the potential to scavenge free radicals by donating a hydrogen atom from the phenolic O-H. Recently, Wright et al. [32] have reported the theoretical calculation of phenolic O-H BDEs of simple phenolic antioxidants such as phenol and substituted phenols. They described the O-H bond weakening effect of the *o*-substituted phenol. EC and quercetin have a common *o*-dihydroxyl structure in the B ring. Quercetin has a

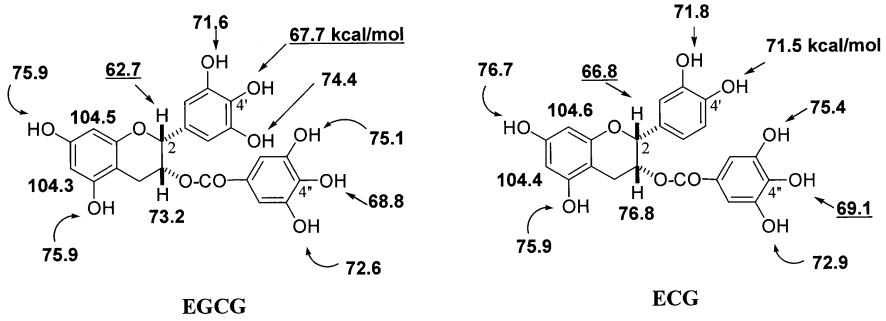

Fig. 8. Calculated bond dissociation enthalpies (BDEs) for EGCG and ECG. All calculation was carried out using the SPARTAN program (PM3).

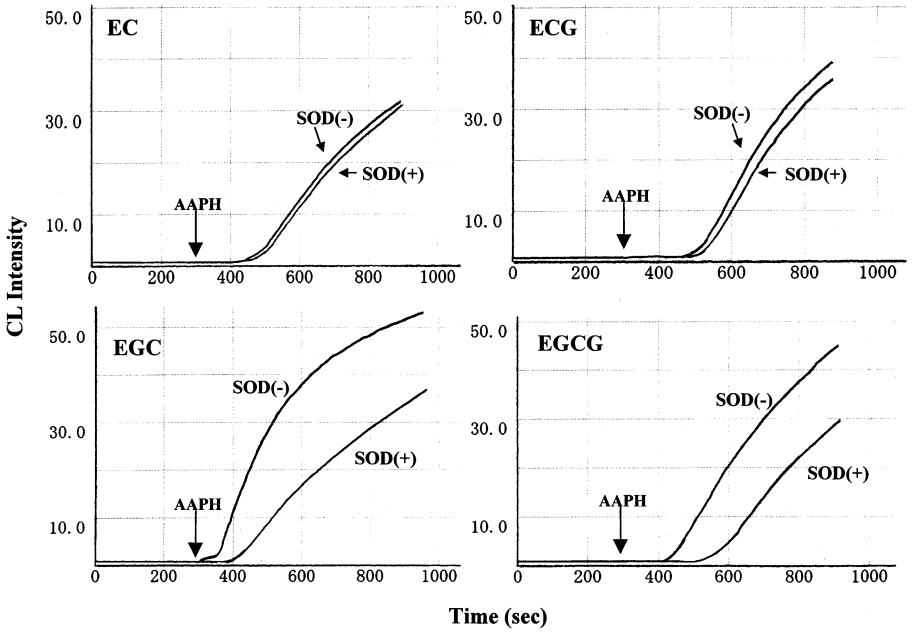

Fig. 9. Effect of SOD on catechins during the antioxidative action. The inhibitory effect of catechins on radical oxidation was examined in the absence (SOD [−]) and the presence of (SOD [+]) of Cu, Zn-SOD. Chemiluminescence was monitored at 37°C for 15 min. Oxidation was initiated by AAPH after preincubation.

lower redox potential than EC. However, the effect of EC against free radicals, in many cases, is the same as that of quercetin or greater. This suggests the involvement of hydrogen atoms other than the phenolic site in case of catechins.

All the O-H and C-H BDEs of EGCG and ECG were then calculated. The PM3 semiempirical MO calculations have shown that C-H BDE at the C-2 position and O-H BDE of the C-4′ are quite low in EGCG (62.7 and 67.7 kcal/mol, respectively) and that the BDEs at the C-2 and C-4″ are also low in ECG (66.8 and 69.1 kcal/mol, respectively) (Fig. 8). These results suggest that oxidation of the B and D rings in EGCG and the D ring in ECG would first occur.

We have also examined the structures of the minor peaks. The UV spectrum of peak X generated from ECG (Fig. 7) showed that the absorption at 280 nm was stronger than EC but weaker than ECG, indicating that peak X may be the oxidized flavan-3-ol structure, which is the counterpart of the gallate moiety. In addition, judging from polarity of the compound based on the retention time, the structure of peak X can be compound 8 shown in Fig. 10. In the case of EGCG, a very small peak with almost the same UV spectrum as EGCG itself was observed just before peak A, suggesting that the peak retains the gallate moiety (data not shown). The structure of the small peak may be intermediate 2a shown in Fig. 10. Namely, EGCG and ECG would be changed to anthocyaninlike compounds (compounds 4 in EGCG and 8 in ECG, respectively) by subtraction of the C-2 proton and the C-4′ phenolic hydrogen accompanied with cleavage of the gallate moiety (Fig. 10).

Although there are several reports on the pro-oxidant action of flavonoids, its mechanism is still unclear [33–35]. To further investigate the reaction mechanisms of catechins, we have studied the involvement of superoxide during the inhibitory action using the luminol-dependent chemiluminescence method (Fig. 9). In EGC and EGCG, the chemiluminescence was remarkably inhibited by SOD whereas the chemiluminescence of EC and ECG remained unchanged, suggesting that EGC and EGCG, which have the pyrogallol structure in the B ring, generate superoxide during the action. On the other hand, gallate moiety, which has the trihydroxy structure coupled with the carbonyl group, may not produce superoxide because of the stability of its radical. The low effectiveness of EGC and the shorter inhibition period of EGCG than that of EC against lipid peroxidation can be attributed to the reasons described above. The results in the present work are in agreement with those in LDL by Miura et al. [7].

Auroma et al. [33] previously reported the pro-oxidant action of gallic acid and its derivatives in the presence of ferric-ethylenediaminetetraacetate (EDTA) and H_2O_2. However, transition metals and EDTA do not exist in our system.

In addition to the previous study, the structural analysis by LC/MS for the compound formed from the gallate moiety by oxidation (compound 3), the results from spectroscopic studies of the minor peaks, the involve-

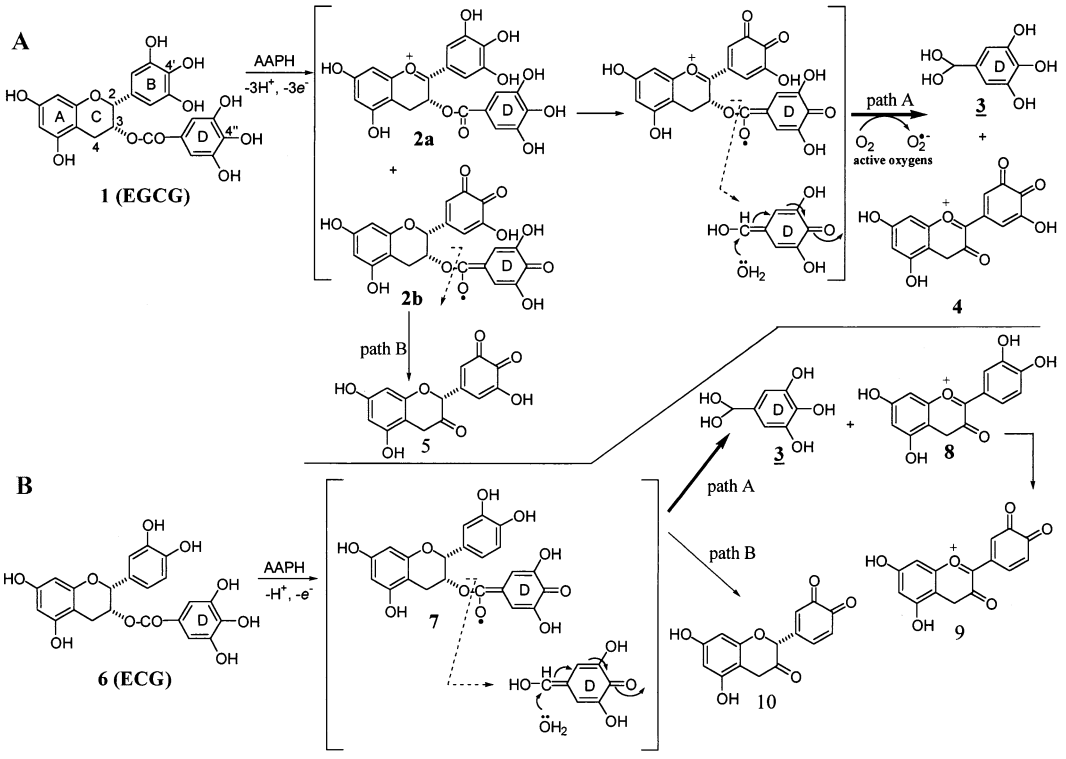

Fig. 10. Proposed mechanisms of EGCG and ECG in radical oxidation.

ment of superoxide during the antioxidative action, and the BDEs for EGCG and ECG provided sufficient evidence to consider the scavenging mechanisms of EGCG and ECG (Fig. 10).

EGCG would be converted to compounds 3 and 4 via intermediates 2 by subtraction of the C-2 proton and the C-4′ phenolic hydrogen accompanied with oxidation of the D ring at the C-4′′ position In this case, an active oxygen such as superoxide may be produced because of a lower redox potential of intermediates 2. On the other hand, ECG would be transformed to compounds 3 and 8 after oxidative cleavage of the gallate moiety. Intermediate 7 and compound 8 may not have electron donating ability to reduce molecular oxygen. Consequently, ECG is more effective than EGCG.

We also carried out the same experiment using EGCG and ECG in an organic solvent, ethanol. However, compound 3 was not detected. The antioxidative mechanisms illustrated in Fig. 10 are supported by this result. This also suggests that the antioxidative activity and mechanism are affected by solvent system used and perhaps radical species. Gardner et al. [25] reported that the activity of catechins, especially EGC and EGCG that have the trihydroxyl structure in the B ring, was lower in water than in ethanol.

To summarize the present work, EC, EGCG, and ECG exert a strong inhibitory effect, whereas EGC and EGCG with the trihydroxyl structure in the B ring produce an active oxygen such as superoxide. Consequently, the effect of EGCG and EGC cannot last longer than EC, although EGCG and EGC can scavenge peroxyl radicals very quickly.

Recently, there is a report on the modulation of arachidonic acid metabolisms by simple phenols [36]. Research should be expanded not only to the antioxidant action for free radicals but also to the modulation of chemical mediators such as leukotrienes and redox signaling [37] in order to clarify the biological activities of catechins and other polyphenolic compounds.

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ABBREVIATIONS

AAPH—2,2′-azobis(2-aminopropane) hydrochloride
EGCG—(-)-epigallocatechin gallate
ECG—(-)-epicatechin gallate
EC—(-)-epicatechin
EGC—(-)-epigallocatechin
MG—methyl gallate
MO—molecular orbital
BDE—bond dissociation enthalpy
APCI—atmospheric pressure chemical ionization
PDA—photodiode array detector
SOD—superoxide dismutase