

Free Radical Interaction Between Vitamin E (alpha-, beta-, gamma- and delta-tocopherol), Ascorbate and Flavonoids

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Abstract. Despite a large number of previous studies, the mechanism of free radical interaction between vitamin E (VE) (alpha-, beta-, gamma- and delta-tocopherol) and ascorbate or flavonoids as coantioxidants remains unclear. VE, particularly alpha-tocopherol, shows less antioxidant activity against peroxy radicals, suggesting that VE possesses functions that are independent of its antioxidant/radical-scavenging activity. The synergistic antioxidant effect of VE or L-ascorbyl 2,6-dibutyrate (ASDB, an ascorbate derivative) with the flavonoids (-)-epicatechin (EC) and (-)-epigallocatechin gallate (EGCG) was investigated using the induction period method in the polymerization of methyl methacrylate initiated by thermal decomposition of benzoyl peroxide (an oxygen-centered radical, PhCOO[•]) under nearly anaerobic conditions. For delta-tocopherol, a synergistic antioxidant effect was observed in the presence of both EC and EGCG, whereas antioxidant activity for alpha-, beta- and gamma-tocopherol was decreased by addition of EC and EGCG. This suggested that the partial regeneration between VE and flavonoids may depend on the chemical structure of VE, i.e., monomethyl, dimethyl, or trimethyl tocol. The regeneration of delta-tocopherol, a monomethyl tocol, by flavonoids may be due to the lower steric effect of tocol. For ASDB, regeneration of vitamin E, which is well-known for a VE/ascorbate mixture, was not observed, possibly due to the anaerobic experimental conditions. The radical interaction between VE and EC, EGCG or ASDB suggests reactivity of VE with biological systems.

Flavonoids, which can be found in fruits, vegetables, wines, spices and herbal medicines, possess beneficial antioxidant, anti-inflammatory and anticancer properties. The synergistic

antioxidant mechanism of alpha-tocopherol with green tea polyphenols, such as EC, EGCG and gallic acid, was recently reported, indicating that green tea flavonoids could reduce the alpha-tocopheroxyl radical to regenerate alpha-tocopherol (1-3). The kinetics of regeneration of VE by a catechol derivative have also been reported (4). Furthermore, the combination of flavonoids and vitamin C was previously reported to produce a synergistic antioxidant effect in an *in vitro* lipoprotein oxidation model (5). Similarly, ascorbate protects (+)-catechin from oxidation under cell-free conditions (6). In the previously reported synergistic antioxidant activity of alpha-tocopherol/flavonoids or ascorbate oxidized by an alkyl radical (a carbon-center radical), it was indicated that partial regeneration of VE occurred in the presence of flavonoids with catechol rings and ascorbate (7). However, our previous study suggested that a totally different reactivity with vitamin E occurred between alkyl and peroxy radicals (8). Alpha-tocopherol was previously reported to have lower reactivity with peroxy radicals (8-10), acting as a pro-oxidant when the concentration was relatively high (9). This compound was recently reported to have cellular functions that are independent of its antioxidant/radical scavenging ability (11).

In the present study, the free radical interaction between vitamin E (alpha-, beta-, gamma- and delta-tocopherol) and ascorbate or flavonoids as coantioxidants was studied. The synergistic antioxidant effect of VE with L-ascorbyl 2,6-dibutyrate (ASDB, an ascorbate derivative) or the flavonoids (-)-epicatechin (EC) and (-)-epigallocatechin gallate (EGCG) was investigated using the induction period method in the polymerization of methyl methacrylate (MMA), initiated by thermal decomposition of benzoyl peroxide (BPO) (an oxygen-centered radical, PhCOO[•]) under nearly anaerobic conditions. The reaction was monitored using the sensitive method of differential scanning calorimetry (DSC) and the model was well able to explain the mechanism of the radical-scavenging activity of these antioxidants. ASDB was used as a representative ascorbate derivative instead of vitamin C which has only limited solubility in MMA.

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Materials and Methods

Reagents. EC and EGCG were obtained from Kurita Kogyo Co., Tokyo, Japan. Benzoyl peroxide (BPO, Osaka, Japan) was obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan, and was recrystallized from methanol/chloroform before use. The chemical structures of the investigated antioxidants are shown in Figure 1.

Experimental procedures. The induction period (IP) and initial rates of polymerization in the presence ($R_{p\text{inh}}$) or absence ($R_{p\text{con}}$) of an antioxidant were determined using the method previously reported (8). In brief, the experimental resin consisted of MMA and BPO in the presence of 0.01 mol% VE (alpha-, beta-, gamma- or delta-tocopherol), EC, EGCG or ASDB. Approximately 10 μl of the experimental resin (MMA: 9.12-9.96 mg) was loaded into an aluminum sample container and sealed by applying pressure. The container was placed in a differential scanning calorimeter (model DSC 3100; MAC Science Co., Tokyo, Japan) maintained at 70°C and the thermal changes induced by polymerization were recorded for the appropriate periods. The heat due to polymerization of MMA was 13.0 kcal/mole in this experiment. The conversion of all samples, as calculated from DSC thermograms, was 92.2-95.0%. Polymerization curves were derived from DSC thermograms using the integrated heat evoked by the polymerization of MMA. Typical time-conversion curves for VE (alpha- and delta-tocopherol) and the EC or EGCG for the BPO system are shown in Figure 2. Polymerization curves break when an inhibitor is consumed. These breaks are sharp and provide a reliable measure of the IP of the inhibitor. The presence of oxygen retards polymerization because oxygen reacts with MMA radicals activated by the initiator and subsequently produces a non-radical product. Thus, polymerization of the control was slightly inhibited, even though the reaction was carried out in a sealed DSC pan, because the pan contained a small amount of oxygen since it had been sealed in air. Tangents were drawn to the polymerization curves at an early stage in the run. The IP of test compounds was determined from the length of time between the zero point on the abscissa and the point of intersection of tangents drawn to the curve at the early stage of polymerization. The IP was calculated from the difference between the induction period of specimens (IP_{observed}) and that of controls (IP_{con}). The initial rates of polymerization in the absence ($R_{p\text{con}}$) and presence ($R_{p\text{inh}}$) of natural and synthetic antioxidants were calculated from the slope of the first linear portion of the plots of the conversion rate of MMA polymerization (tangent drawn at the early polymerization stage). The rate of initiation (R_i) by 0.1 mol% BPO was $1.66 \times 10^{-7} \text{ Ms}^{-1}$.

Computational details. Theoretical calculations of highest occupied molecular orbit (HOMO) and lowest unoccupied molecular orbit (LUMO) energy were carried out by the semi-empirical molecular orbital (MO) method PM3, as implemented in the MOPAC program (CaChe 5.0) (12). In general, the PM3 method cannot give the fine geometry and unpaired electron distribution of a molecule. Therefore, after optimization by CONFLEX (Conflex Co., Tokyo, Japan), the geometry was followed by the PM3 method.

Results and Discussion

VE, ASDB and flavonoids. The radical-scavenging activity of flavonoid antioxidants EC and EGCG with alpha-tocopherol or delta-tocopherol for BPO is shown in Figure

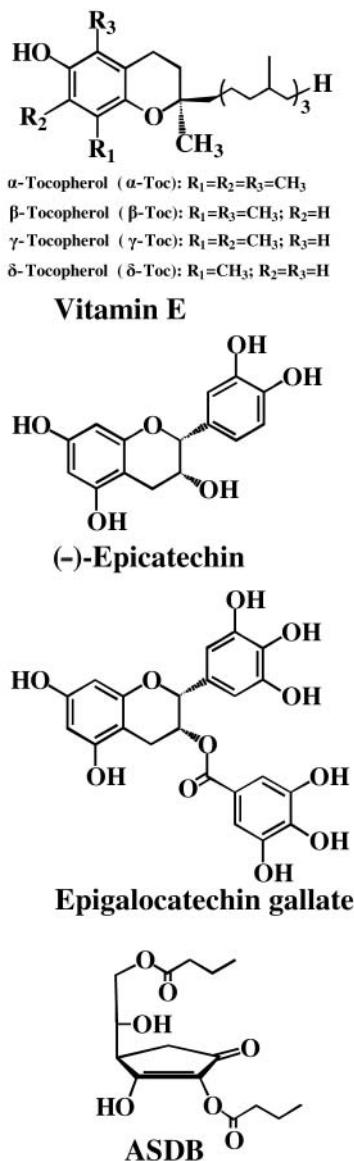


Figure 1. Chemical structures of the investigated antioxidants.

2, and the results for VE, ASDB, EC, EGCG and the VE/EC or EGCG mixture, and the VE/ASDB mixture are presented in Table I. The radical-scavenging activity of antioxidants is characterized by two independent parameters: one is the IP and the other is the initial rate of inhibited polymerization ($R_{p\text{inh}}$). When the initiating radicals are generated at a constant rate by BPO, then the stoichiometric factor (n) is given by Equation 1 (13):

$$n = ([IP] R_i)/[IH] \quad 1$$

where R_i is the rate of chain initiation and $[IH]$ is an inhibitor.

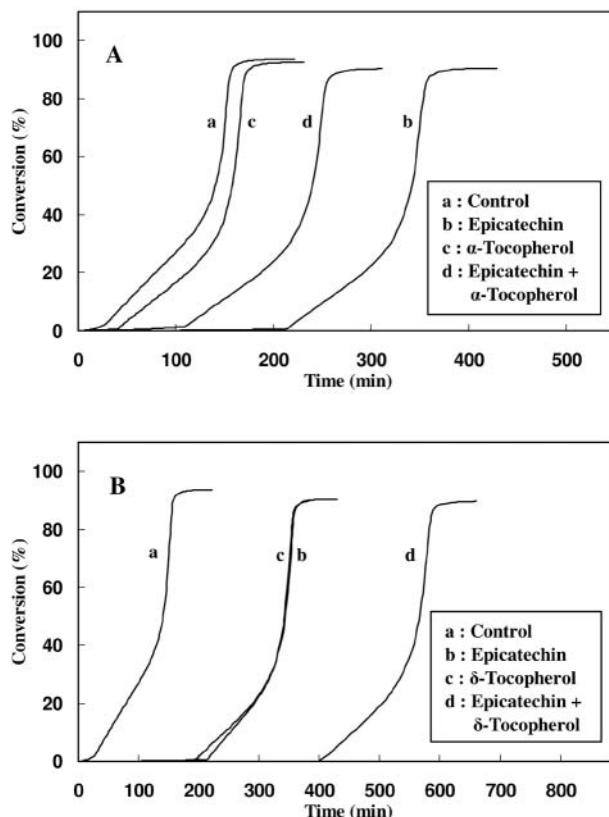


Figure 2. Time-conversion curves for alpha- (A) and delta-tocopherol (B) with or without epicatechin.

As shown in Table I, the IP value for EGCG was greater than that for EC. The *n* value for EGCG and EC was 3.0 and 1.9, respectively. The IP values for VE increased in the order alpha-<beta-<gamma-<delta-tocopherol. The *n* value for alpha-, beta-, gamma- and delta-tocopherol was 0.2, 0.6, 1.1 and 1.7, respectively. ASDB gave the smallest *n* value of 0.1. The IP order was not changed by the addition of ASDB, EC or EGCG. The propagation activity for VE, ASDB, the VE/EC mixture and the VE/ASDB mixture gave $R_{P_{inh}}/R_{P_{con}}$ values ranging from 0.4 for EC or the EGCG/delta-Toc mixtures to 0.9 for ASDB alone. The $R_{P_{inh}}/R_{P_{con}}$ value for delta-tocopherol (0.5) was the low among the tocopherols, suggesting retardation of polymerization. The IPs for mixtures of delta-tocopherol and EC or EGCG were greater than the corresponding calculated IPs, suggesting a synergistic reaction between delta-tocopherol and these flavonoids, in which delta-tocopherol was partially regenerated by the coantioxidants EC and EGCG. In contrast, the IP for EC and EGCG with VE (alpha-, beta- or gamma-tocopherol), was less than the calculated value, suggesting that the antioxidant activity of

Table I. The free radical interaction between vitamin E (alpha-, beta-, gamma- and delta-tocopherol) and L-ascorbyl 2,6-dibutyrate (ASDB, a vitamin C), epicatechin (EC) or epigallocatechin gallate (EGCG).

Additives 0.01 mol%	IP min A	Calculated IP* min B	RP _{inh} /RP _{con}	
			B-A	n
EC	189.52			0.64 1.89
EGCG	300.54			0.49 3.00
ASDB	5.43			0.87 0.08
alpha-tocopherol (TOC)	16.79			0.72 0.17
beta-TOC	55.21			0.70 0.56
gamma-TOC	112.60			0.56 1.12
delta-TOC	168.81			0.50 1.68
ASDB+ alpha-TOC	19.73	22.22	2.48	0.76
ASDB+beta-TOC	48.78	60.64	11.86	0.65
ASDB+gamma-TOC	74.33	118.03	43.70	0.67
ASDB+delta-TOC	138.30	173.24	34.94	0.58
EC+alpha-TOC	82.82	206.31	123.49	0.67
EC+beta-TOC	155.69	244.73	89.04	0.58
EC+gamma-TOC	181.21	302.12	120.09	0.68
EC+delta-TOC	378.32	358.33	-19.99	0.42
EGCG+alpha-TOC	102.76	317.33	214.57	0.58
EGCG+beta-TOC	196.67	355.75	159.08	0.50
EGCG+gamma-TOC	328.30	413.14	84.84	0.49
EGCG+delta-TOC	505.45	469.35	-36.10	0.38

IP, induction period; *n*, stoichiometric factor; RP_{inh} and RP_{con} are initial rate of polymerization with and without an inhibitor, respectively.; BPO, 0.1 mol%; MMA, 9.4 mol/l; * simple sum of IP for VE or ASDB and flavonoid coantioxidant. The IP_{con} and RP_{con} of controls were 22.46 min and 0.42%/min. IP=observed IP – control IP. The values are means for three independent experiments. The computational error was <7%. The procedures are described in the text.

EGCG and EC can be suppressed by the addition of alpha-, beta- or gamma-tocopherol. These findings proved the large length of IP and the great decrease in the RP_{inh} (retardation) of tocopherols to be an important factor for their partial regeneration activity. In order to obtain information about the mechanism of these interactions, we used PM3 calculations to investigate differences in energy level between the HOMO and LUMO of BPO, MMA, EGCG and VE. Possible reaction pathways for EGCG and MMA reacting with benzoate radicals (PhCOO[•]) derived from BPO, and for alpha- or delta-tocopherol and EGCG reacting with PhCOO[•], are proposed in Figure 3. The most favorable reaction occurs between PhCOO[•] and alpha- or delta-tocopherol, rather than with EGCG. Alpha- and delta-tocopherol phenoxyl radicals would be scavenged by EGCG in the BPO system and polymerization of MMA would occur after tocopherol and EGCG were completely consumed. The reaction pathway for EC was similar to that for EGCG (data not shown). This proposed pathway is supported by previous evidence that the reduction potentials of the flavonoid radicals, E₇=0.5-0.7 V, are

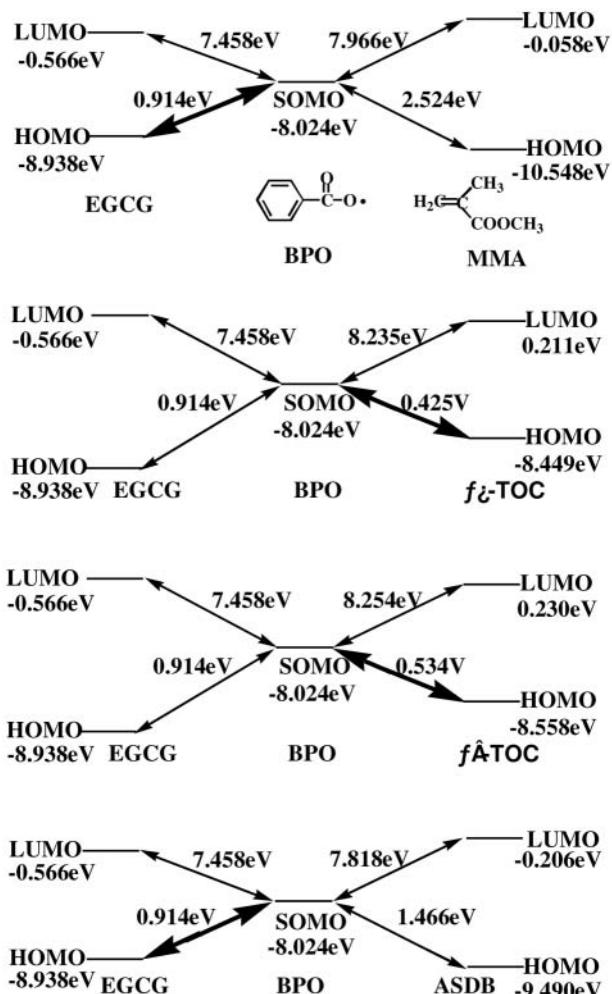


Figure 3. The energy level for SOMO of BPO and the HOMO and LUMO of MMA, VE (alpha- or delta-tocopherol), EGC or ASDB.

higher than that of trolox, a VE derivative, $E_7=0.48$ V, indicating that their reaction with VE is thermodynamically feasible (14). For ASDB, the most favorable reaction possibly occurs between PhCOO \bullet and alpha-tocopherol, rather than with ASDB, since the difference in the energy level between the HOMO of alpha-tocopherol and the singly occupied molecular orbit (SOMO) of BPO is very low (0.534 eV), as calculated using the PM3 method. It is expected that when VE and ascorbates are present in solution in similar amounts, the peroxy radicals will react first with the former to give tocopheroxyl radicals that immediately reacts with ascorbates to regenerate tocopherols (4). However, in the present study, no regeneration of VE with ascorbates was found. This was in disagreement with the behavior reported using the thermally initiated autoxidation of styrene in the presence

of the alpha-tocopherol/vitamin C mixture in an air-saturated chlorobenzene solution (4). This discrepancy may be due to aerobic or anaerobic experimental conditions. The high catalytic effectiveness for ascorbate oxidation may be attributed to the aerobic conditions.

Recently, reduced antioxidant effects of tocopherols were observed using a system containing low oxygen (11). In the present study, delta-tocopherol was a potent radical scavenger and a synergistic antioxidant with flavonoids. In contrast, alpha-tocopherol showed less antioxidant activity. This suggests that alpha-tocopherol may possess functions that are independent of its antioxidant/radical scavenging activity (11, 15).

Cancer cells have anaerobic metabolism (16, 17) and have very poor capability for absorbing adequate amounts of antioxidants. Addition of EGC and EC to alpha-, beta- or gamma-tocopherol oxidized by PhCOO \bullet reduced the antioxidant activity of the vitamin derivatives. VE can react with both alkyl and alkylperoxy radicals during the autoxidation of polyunsaturated lipids. Although the antioxidant/radical scavenging activity of VE showed a difference between alkyl and peroxy radicals (8), flavonoids may act as chemopreventive and anticancer agents. Flavonoids may possess potent anticancer activity (18-20). VE (alpha-, beta-, gamma- and delta-tocopherol) showed less antioxidant activity in the presence of ascorbates under nearly ananearobic conditions. No synergistic antioxidant activity of VE/ascorbate was found. This was in agreement with the results reported previously (7). Taken together, these findings suggest that the interaction between VE and flavonoids or ascorbates is dependent on the type of radical species and the presence of oxygen.

Since biological systems tend to have low oxygen tensions, the effectiveness of antioxidants *in vivo* may differ considerably from that in aerobic systems *in vitro*. However, *in vivo* experiments are too complex to be amenable to simple interpretation, and, therefore, we have undertaken physicochemical studies using the induction period method to study the radical polymerization of MMA in the presence of antioxidants as a biomimetic model for radical-scavenging activity *in vivo*. The free radical interaction between flavonoids and VE or ascorbate may contribute to our knowledge of the potential of these compounds for chemoprevention by induction of cancer cell apoptosis.

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