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Antioxidative Effects of Flavonols and Their Glycosides against the Free-Radical-Induced Peroxidation of Linoleic Acid in Solution and in Micelles

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Abstract: The antioxidative effect of flavonols and their glycosides against the peroxidation of linoleic acid has been studied in homogeneous solution (*t*BuOH/H_2O, 3:2) and in sodium dodecyl sulfate and cetyl trimethylammonium bromide micelles. The peroxidation was initiated thermally by the water-soluble initiator 2,2′-azobis(2-methylpropionamidine) dihydrochloride, and the reaction kinetics were studied by monitoring the formation of linoleic acid hydroperoxides. The synergistic antioxidant effect of the flavonols with α-tocopherol (vitamin E) was also studied by following the decay kinetics of α-tocopherol and the α-tocopheroxyl radical. Kinetic analysis of the antioxidative process demonstrates that the flavonols are effective antioxidants in solution and in micelles, either alone or in combination with α-tocopherol. The antioxidative action involves trapping the initiating radicals in solution or in the bulk-water phase of the micelles, trapping the propagating lipid peroxyl radicals on the surface of the micelles, and regenerating α-tocopherol by reducing the α-tocopheroxyl radical. It was found that the antioxidant activity of the flavonols and their glycosides depends significantly on the position and number of the hydroxy groups, the oxidation potential of the molecule, and the reaction medium. The flavonols bearing *ortho*-dihydroxy groups possess significantly higher antioxidative activity than those without such functionalities, and the glycosides are less active than their parent aglycones. The activity of the flavonols is higher in micelles than in solution, while the activity of α-tocopherol is lower in micelles than in solution. This is because the predominant factor for controlling the activity is the hydrogen-bonding interaction of the antioxidant with the micellar surface in the case of hydrophilic flavonols, while it is the inter- and intramicellar diffusion in the case of lipophilic α-tocopherol.

Keywords: antioxidation · flavonols · peroxides · radical reactions · reaction mechanisms

Introduction

Epidemiological, biological, and clinical studies have provided various lines of evidence in the past decade to indicate that free-radical-induced oxidative damage of cell membranes, DNA, and proteins might play a causative role in aging and several degenerative diseases, such as cancer, atherosclerosis, and cataract formation, and that antioxidants, such as α-tocopherol (vitamin E), L-ascorbic acid (vitamin C), and β-carotene, might have beneficial effects in protecting against these diseases.[1] Therefore, inhibition of free-radical-induced oxidative damage by supplementation of antioxidants has become an attractive therapeutic strategy to reduce the risk of these diseases.[2] Flavonoids, such as flavones, flavanone, flavonols, flavanols, and isoflavones, are naturally occurring polyphenolic compounds present in vegetables, fruits, tea, and red wine and possess a wide range of biological activities,[3] of which antioxidation has been extensively explored.[4] One interesting example of flavonoid activity is the so-called "French paradox",[5] that is, despite high fat intake, mortality from coronary heart disease is lower in some regions of France, a fact attributed to the regular drinking of red wine which contains high levels of flavonoids (approximately 200 mg per glass)[3a,6] and resveratrol (0.1–15 mg L^{-1}).[7] These compounds have been proved to be good antioxidants against low-density lipoprotein (LDL) peroxidation,[3b,4a] a process believed to be critical in the risk of human atherosclerosis,[1h,8] as well as to possess cancer chemopreventive activity.[3b,9] We have recently found that flavanols isolated from green-tea leaves are good antioxidants against free-radical-initiated lipid peroxidation in solution,[10] in micelles,[11] in human red blood cells,[12] in human low-density lipoprotein,[13] and in rat liver microsomes,[14] and that the antioxidant activity of these fla-

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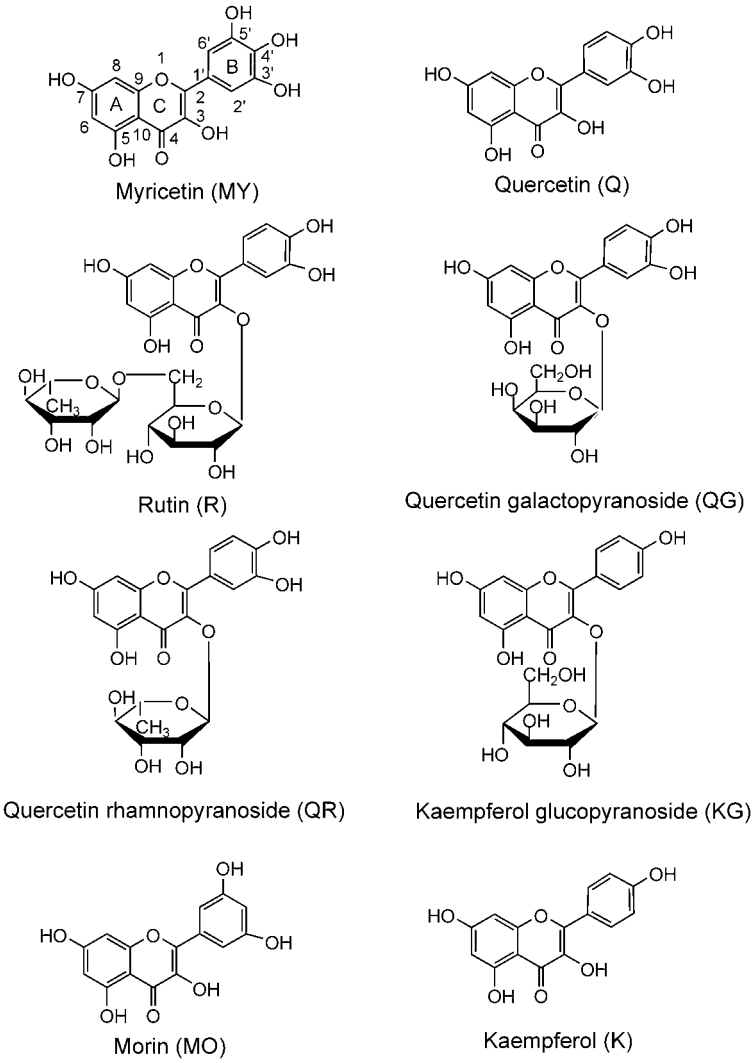
vanols depends significantly on the structure of the molecules and the initiation conditions.[10–14] It was also found that these green-tea flavanols and resveratrol might interact with α-tocopherol (vitamin E) synergistically to enhance the antioxidant activity.[15,16] Therefore, it is of interest to extend this research and study the structure–activity relationships of other dietary flavonoids and their glycosides, since many dietary flavonoids exist in the form of glycosides.[17] We report herein a quantitative kinetic study of the antioxidative behavior of a set of typical flavonols and their glycosides against linoleic acid peroxidation in solution and in micelles, with emphasis placed on the structure–activity relationships and the mechanistic details of the antioxidation, including the synergistic interaction between these flavonols and α-tocopherol (vitamin E). The flavonols studied were myricetin (MY), quercetin (Q), quercetin galactopyranoside (QG), quercetin rhamnopyranoside (QR), rutin (R), morin (MO), kaempferol (K), and kaempferol glucoside (KG). The peroxidation was thermally initiated at physiological temperature by a water-souble azo initiator, 2,2′-azobis(methylpropionamidine) dihydrochloride (AAPH), and conducted either in *tert*-butyl alcohol/water (3:2) solution or in sodium dodecyl sulfate (SDS) and cetyl trimethylammonium bromide (CTAB) micelles to study the effect of microenvironment on the reaction. The interaction of these compounds with α-tocopherol (TOH, vitamin E) was also investigated.



Results

Inhibition of linoleic acid peroxidation by flavonols and their glycosides in solution: Peroxidation of linoleic acid or its esters gives different hydroperoxides depending on the reaction conditions.[18] Hydroperoxide substitution at the C-9 or C-13 positions produces either *trans,trans*- or *cis,trans*-conjugated dienes, which are the major products in the absence of antioxidants or in the presence of small amount of antioxidants, for example, millimolar concentrations of α-tocopherol.[18a,b] It was found recently that these conjugated dienes were formed from the rapid β scission of the primarily formed bisallylic 11-peroxyl radical,[18c,d] and the kinetically controlled product, that is, the nonconjugated 11-substituted hydroperoxide, might become the major product in the presence of high concentrations of antioxidant, for example, molar concentrations of α-tocopherol.[18d] These experimental observations have been rationalized recently by theoretical calculations.[19] The present experiment used very small amounts of the antioxidants (micromolar α-tocopherol and/or flavonols and their glycosides), hence the production of the nonconjugated 11-hydroperoxide should be negligible, and the conjugated hydroperoxides were the predominant products. The latter showed a characteristic ultraviolet (UV) absorption at 235 nm[20] that was used to monitor the formation of the total hydroperoxides formed during the peroxidation after separation of the reaction mixture by high-performance liquid chromatography (HPLC).

A set of representative kinetic curves of the total hydroperoxide formation during the peroxidation of linoleic acid in *t*BuOH/H_2O (3:2) solution is shown in Figures 1 and 2. It can be seen from the figures that,

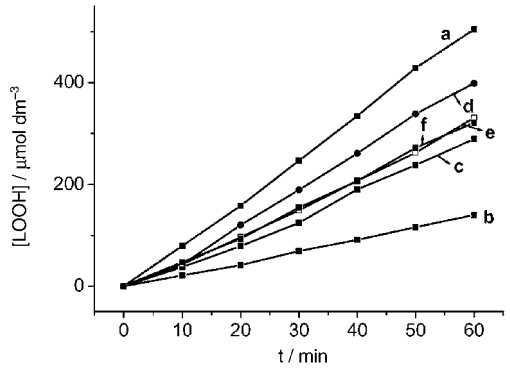


Figure 1. Representative kinetic curves for the formation of total hydroperoxides (LOOH) during the peroxidation of LH in *tert*-butyl alcohol/water (3:2) solution at 37 °C, initiated with AAPH and inhibited with flavonols and their glycosides (FOHs). $[LH]_0 = 0.1\ mol\,L^{-1}$, $[AAPH]_0 = 10\ mmol\,L^{-1}$, $[FOHs]_0 = 20\ \mu mol\,L^{-1}$. a) Uninhibited reaction, b) reaction inhibited with MY, c) reaction inhibited with Q, d) reaction inhibited with QG, e) reaction inhibited with MO, f) reaction inhibited with K. Curves of other FOHs are not shown for clarity.

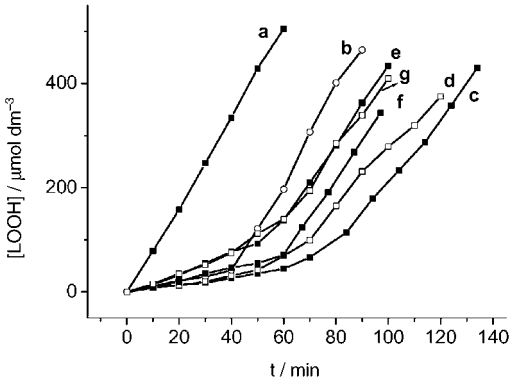


Figure 2. Representative kinetic curves for the formation of total hydroperoxides (LOOH) during the peroxidation of LH in *tert*-butyl alcohol/water (3:2) solution at 37 °C, initiated with AAPH and inhibited with TOH and FOHs. $[LH]_0 = 0.1\ mol\,L^{-1}$, $[AAPH]_0 = 10\ mmol\,L^{-1}$, $[TOH]_0 = 20\ \mu mol\,L^{-1}$, $[FOHs]_0 = 20\ \mu mol\,L^{-1}$. a) Uninhibited reaction, b) reaction inhibited with TOH, c) reaction inhibited with MY and TOH, d) reaction inhibited with Q and TOH, e) reaction inhibited with QG and TOH, f) reaction inhibited with MO and TOH, g) reaction inhibited with K and TOH. Curves of other FOHs are not shown for clarity.

upon AAPH initiation, the concentration of linoleic acid hydroperoxides increased quickly and linearly with time in the absence of antioxidants (line a in Figures 1 and 2), a result indicating the fast peroxidation of the substrate. The slope of the line corresponds to the rate of propagation, R_p, of the peroxidation. The formation of the hydroperoxides was remarkably inhibited by the addition of α-tocopherol (TOH) during the so-called "inhibition period" or "induction period" (line b in Figure 2). During the inhibition period, the concentration of the hydroperoxides also increased approximately linearly with time, and the slope of this line was designated as R_{inh}, the value of which reflects the antioxidative potential of the antioxidant. After the inhibition period, the rate of hydroperoxide formation increased to close to the original rate of the propagation, a result indicating the exhaustion of the antioxidant. The turning point from the inhibition period to the restoration of fast peroxidation relates to the inhibition time, t_{inh}, which is also an indication of the efficacy of the antioxidants. Addition of only the flavonols and their glycosides (FOHs) to the solution decreased the rate of hydroperoxide formation from R_p to R_{inh}, but no inhibition period was observed (lines b–f in Figure 1). The antioxidant activity can be expressed by the percentage inhibition of the peroxidation, $P_{inh} = (R_p - R_{inh})/R_p \times 100\,\%$, which follows the sequence $MY > Q > K \approx MO > QG \approx QR > R \approx KG$ (see Table 1 below).

Figure 2 shows the antioxidative effect of representative FOHs in the presence of α-tocopherol (TOH) in solution. The addition of TOH produced a typical kinetic curve for a chain-breaking antioxidative reaction, with a clear inhibition period (line b in Figure 2) as reported previously.[10] Interestingly, the addition of FOHs, which showed no inhibition period in the absence of TOH, remarkably prolonged the inhibition period of TOH (lines c–g in Figure 2). This demonstrates a synergistic antioxidation effect of FOHs and TOH (see below).

Inhibition of linoleic acid peroxidation by flavonols and their glycosides in micelles: It has been recognized that antioxidant activity in homogenous solutions may not parallel that in heterogeneous media, let alone the activity in vivo.[21] To bridge the gap between chemical and biological activities, it is essential to understand and evaluate the dependence of the antioxidant activity upon the microenvironment of the reaction media. A general methodology for this is to carry out the reaction in membrane mimetic systems, such as micelles and artificial bilayers.[22] Therefore, the antioxidative effect of FOHs was investigated in sodium dodecyl sulfate (SDS) and cetyl trimethylammonium bromide (CTAB) micelles, as shown in Figures 3 and 4, respectively. It can be

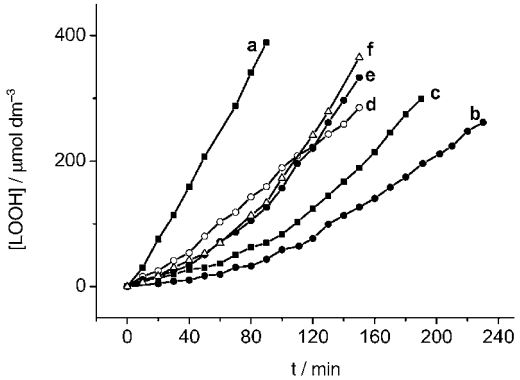


Figure 3. Representative kinetic curves for the formation of total hydroperoxides (LOOH) during the peroxidation of linoleic acid (LH) in SDS micelles ($0.1\ mol\,L^{-1}$) at pH 7.4 and 37 °C, initiated with AAPH and inhibited by flavonols and their glycosides (FOHs). $[LH]_0 = 15.2\ mmol\,L^{-1}$, $[AAPH]_0 = 6.3\ mmol\,L^{-1}$, $[FOHs]_0 = 10\ \mu mol\,L^{-1}$. a) Uninhibited peroxidation, b) reaction inhibited with MY, c) reaction inhibited with Q, d) reaction inhibited with QG, e) reaction inhibited with MO, f) reaction inhibited with K. Curves of other FOHs are not shown for clarity.

seen from Figure 3 that the antioxidative behavior of the flavonols in SDS micelles is distinctly different from that in the homogeneous solution. All of the FOHs, which showed no inhibition period in solution, exhibited clear inhibition periods in SDS micelles and behaved well as chain-breaking antioxidants. Similar results were obtained in CTAB micelles (Figure 4), but the kinetic parameters in the two micelles were appreciably different. The details will be discussed in the following sections.

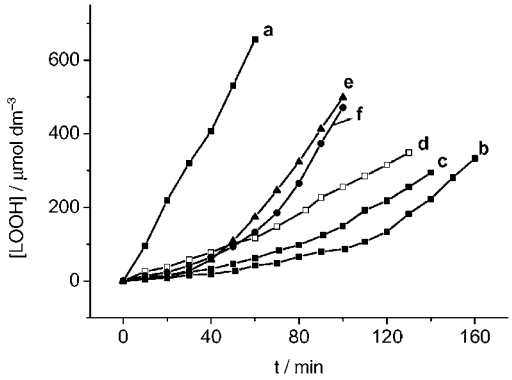


Figure 4. Representative kinetic curves for the formation of total hydroperoxides (LOOH) during the peroxidation of linoleic acid (LH) in CTAB micelles (15 mmol L^{-1}) at pH 7.4 and 37 °C, initiated with AAPH and inhibited by flavonols and their glycosides (FOHs). $[LH]_0$ = 15.2 mmol L^{-1}, $[AAPH]_0$ = 6.3 mmol L^{-1}, $[FOHs]_0$ = 10 μmol L^{-1}. a) Uninhibited peroxidation, b) reaction inhibited with MY, c) reaction inhibited with Q, d) reaction inhibited with QG, e) reaction inhibited with MO, f) reaction inhibited with K. Curves of other FOHs are not shown for clarity.

Figures 5 and 6 show the antioxidative effect of FOHs in the presence of TOH in SDS and CTAB micelles, respectively. A comparison of Figures 5 and 6 with Figure 2 clearly indicates that the antioxidant synergism of FOHs with TOH

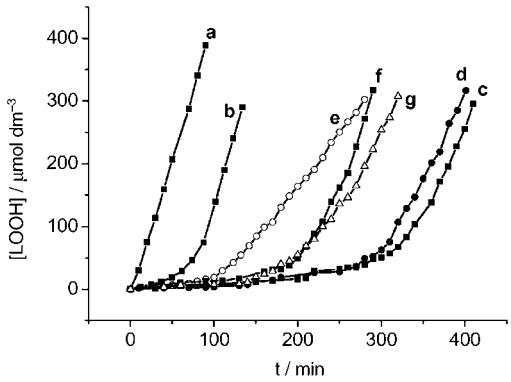


Figure 5. Representative kinetic curves for the formation of total hydroperoxides (LOOH) during the peroxidation of linoleic acid (LH) in SDS micelles (0.1 mol L^{-1}) at pH 7.4 and 37 °C, initiated with AAPH and inhibited with TOH and FOHs. $[LH]_0$ = 15.2 mmol L^{-1}, $[AAPH]_0$ = 6.3 mmol L^{-1}, $[FOHs]_0$ = 10 μmol L^{-1}, $[TOH]_0$ = 7.5 μmol L^{-1}. a) Uninhibited peroxidation, b) reaction inhibited with TOH, c) reaction inhibited with MY and TOH, d) reaction inhibited with Q and TOH, e) reaction inhibited with QG and TOH, f) reaction inhibited with MO and TOH, g) reaction inhibited with K and TOH. Curves of other FOHs are not shown for clarity.

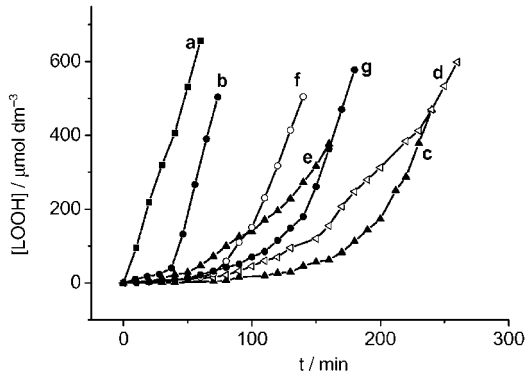


Figure 6. Representative kinetic curves for the formation of total hydroperoxides (LOOH) during the peroxidation of linoleic acid (LH) in CTAB micelles (15 mmol L^{-1}) at pH 7.4 and 37 °C, initiated with AAPH and inhibited with TOH and FOHs. $[LH]_0$ = 15.2 mmol L^{-1}, $[AAPH]_0$ = 6.3 mmol L^{-1}, $[FOHs]_0$ = 10 μmol L^{-1}, $[TOH]_0$ = 10 μmol L^{-1}. a) Uninhibited peroxidation, b) reaction inhibited with TOH, c) reaction inhibited with MY and TOH, d) reaction inhibited with Q and TOH e) reaction inhibited with QG and TOH, f) reaction inhibited with MO and TOH, g) reaction inhibited with K and TOH. Curves of other FOHs are not shown for clarity.

is much more pronounced in the micelles than in the homogeneous solution, especially in the case of Q and MY, which almost completely inhibited the peroxidation for a very long time. For example, the inhibition period produced by TOH and Q in combination in SDS micelles was 293 min, which is much longer than the sum of the inhibition periods produced by TOH (78 min) and Q (90 min) when they were used individually under the same experimental conditions. The mechanistic details of this antioxidant synergism will be discussed in the following sections.

Decay kinetics of α-tocopherol and the α-tocopheroxyl radical: To rationalize the mechanism of the antioxidant synergism of TOH and the FOHs, the decay kinetics of TOH and the α-tocopheroxyl radical (TO$^{\bullet}$) in the absence and presence of quercetin (Q) were studied. The decay of TOH was determined by HPLC separation of the reaction mixture, followed by electrochemical determination of the amount of remaining TOH. Representative results are illustrated in Figures 7 and 8. It was found that the decay of TOH was approximately linear in the absence of Q in the solution and in SDS and CTAB micelles (line a in Figure 7, lines a and c in Figure 8), in accordance with the kinetic demand for chain-breaking antioxidation reactions (see Equation (8) below). The decay rates were 9.0×10^{-9}, 1.6×10^{-9}, and 4.1×10^{-9} mol L^{-1} in solution and in SDS and CTAB micelles, respectively, due to the different rates of initiation in these media (see below). On the other hand, the decay of TOH in the presence of Q was different in the solution and in the micelles. In the solution the decay of TOH was only slightly reduced by the coexistent Q (line b in Figure 7), while it was significantly diminished in the micelles, especially in SDS micelles (line b in Figure 8). The decay rates of TOH in the presence of Q were determined to be 7.5×10^{-9}, 0.5×10^{-9},

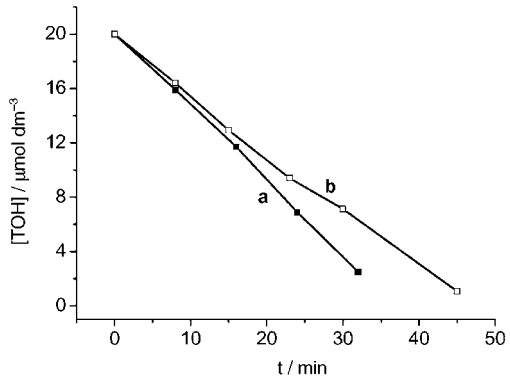


Figure 7. Decay of α-tocopherol (TOH) during the inhibition of linoleic acid peroxidation in *tert*-butyl alcohol/water (3:2) solution at 37 °C, initiated with AAPH and inhibited with TOH in the absence (a) and presence (b) of quercetin (Q). $[LH]_0 = 0.1\ mol\,L^{-1}$, $[AAPH]_0 = 10\ mmol\,L^{-1}$, $[TOH]_0 = 20\ \mu mol\,L^{-1}$, $[Q]_0 = 20\ \mu mol\,L^{-1}$.

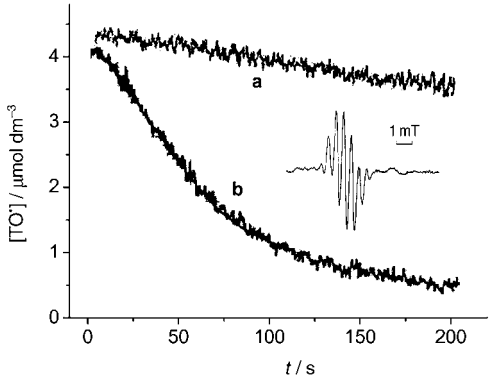


Figure 9. Decay of α-tocopheroxyl radical (TO•) in SDS micelles ($0.2\ mol\,L^{-1}$) at pH 7.4 and room temperature under air in the absence (a) and presence (b) of quercetin ($0.21\ mmol\,L^{-1}$). The inset shows the EPR spectrum of TO• obtained under fast flow.

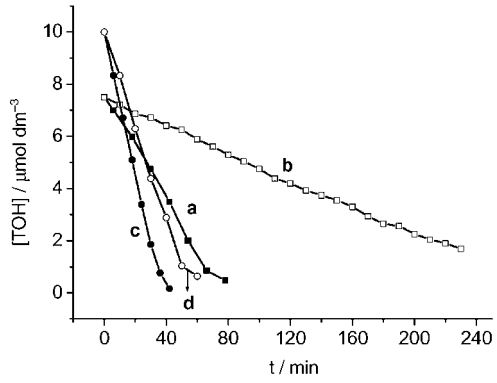


Figure 8. Decay of α-tocopherol (TOH) during the inhibition of linoleic acid peroxidation in micelles at 37 °C, initiated with AAPH and inhibited with TOH and quercetin (Q). a) Decay of TOH in the absence of Q in SDS micelles ($0.1\ mol\,L^{-1}$), b) decay of TOH in the presence of Q in SDS micelles ($0.1\ mol\,L^{-1}$), c) decay of TOH in the absence of Q in CTAB micelles ($15\ mmol\,L^{-1}$), d) decay of TOH in the presence of Q in CTAB micelles ($15\ mmol\,L^{-1}$). $[LH]_0 = 15.2\ mmol\,L^{-1}$, $[AAPH]_0 = 6.3\ mmol\,L^{-1}$, $[TOH]_0 = 7.5\ \mu mol\,L^{-1}$ (SDS), $[TOH]_0 = 10\ \mu mol\,L^{-1}$ (CTAB), $[Q]_0 = 10\ \mu mol\,L^{-1}$.

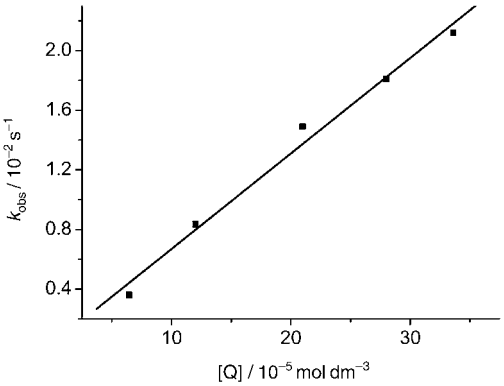


Figure 10. Plot of the pseudo-first-order rate constants, k_{obs}, of the decay of TO• versus the initial concentration of quercetin in SDS micelles ($0.2\ mol\,L^{-1}$).

and $3.0 \times 10^{-9}\ mol\,L^{-1}$ in solution and in SDS and CTAB micelles, respectively.

The α-tocopheroxyl radical (TO•) was produced by oxidizing TOH with PbO_2, and its decay kinetics were determined by stopped-flow electron paramagnetic resonance (EPR) spectroscopy as described previously.[15,16] TO• is much more persistent in micelles than in homogenous solutions,[16,23] a fact that makes it easy to determine the reaction kinetics of the radical by EPR spectroscopy at ambient temperatures in micelles. As shown in Figure 9, TO• decayed fairly slowly in SDS micelles with a rate constant of $75\ M^{-1}\,s^{-1}$. Addition of Q remarkably increased the decay of TO•, the kinetics of which were found to be pseudo first order in the presence of a large excess of Q (line b in Figure 9). Plotting this first-order rate constant, k_{obs}, against the concentration of Q gave a straight line (Figure 10), from which the bimolecular rate constant between TO• and Q, that is, the rate for the α-tocopherol regeneration reaction (see Equation (12) below), was obtained as $64\ M^{-1}\,s^{-1}$.

Discussion

Reaction kinetics of lipid peroxidation: It has been proven that the reaction kinetics of lipid peroxidation in micelles and biomembranes follow the same rate law as those in homogenous solutions;[22,24] therefore, the same rate law was accepted in solution and micelles. The kinetics of linoleic acid (LH) peroxidation initiated by azo compounds and its inhibition by a chain-breaking antioxidant (AH) have been discussed in detail previously.[10,11a,22,24] The rate of propagation (R_p) and the rate of peroxide formation in the inhibition period (R_{inh}) are given by Equations (1) and (2), respectively.

$$d[LOOH]/dt = R_p = [k_p/(2k_t)^{1/2}]R_i^{1/2}[LH] \quad (1)$$

$$R_{inh} = k_p R_i [LH]/(n\,k_{inh}[AH]) \quad (2)$$

Here k_p, k_t, and k_{inh} are rate constants for the chain propagation [Eq. (3)], chain termination [Eq. (4)] and chain inhibition by the antioxidant [Eq. (5)], respectively, R_i is the apparent rate of chain initiation [Eq. (6)], and n is the stoichiometric factor (see below).

$$LOO^{\cdot} + LH \xrightarrow{k_p} LOOH + L^{\cdot} \quad (3)$$

$$2\,LOO^{\cdot} \xrightarrow{2k_t} \text{molecular products} \quad (4)$$

$$LOO^{\cdot} + AH \xrightarrow{k_{inh}} LOOH + A^{\cdot} \quad (5)$$

$$R_i = 2k_g e[R{-}N{=}N{-}R] \quad (6)$$

Although the radical generation rate, R_i, of AAPH is known as $(1.4\pm0.2)\times10^{-6}$[AAPH] s^{-1} at 37 °C for protein-containing solutions and liposomal dispersions,[25,26] the cage effect parameter, e, varies appreciably depending on the medium and the concentrations of the antioxidant and the initiator.[25] Therefore, the R_i value is generally determined by the inhibition period and/or by the decay rate of TOH [Eqs. (7) and (8), respectively].

$$R_i = n[AH]_0/t_{inh} \quad (7)$$

$$R_i = -n\,d[AH]/dt \quad (8)$$

Here, n is the stoichiometric factor that designates the number of peroxyl radicals trapped by each antioxidant molecule. Since the n value of α-tocopherol is generally assumed to be 2,[22,24] the R_i value can be determined from the inhibition period and/or the decay rate of α-tocopherol.

The kinetic chain length (*kcl*) defines the number of chain propagations initiated by each initiating radical and is given by Equations (9) and (10) for the uninhibited and inhibited peroxidations, respectively. The kinetic parameters deduced from Figures 1–6 are listed in Tables 1–3.

$$kcl_p = R_p/R_i \quad (9)$$

$$kcl_{inh} = R_{inh}/R_i \quad (10)$$

Micellar effects on the initiation and antioxidation: It can be seen from Figures 1–8 and Tables 1–3 that the reaction medium exerts significant effects on the rate of initiation, the rate of propagation, and the antioxidant activity of α-tocopherol (TOH) and flavonols and their glycosides (FOHs).

The R_i values calculated from the inhibition periods [Eq. (7)] are 1.6×10^{-8}, 3.1×10^{-9}, and 8.3×10^{-9} mol L^{-1} in solution and in SDS and CTAB micelles, respectively. These values are in reasonable agreement with the values of 1.8×10^{-8}, 3.2×10^{-9}, and 8.2×10^{-9} mol L^{-1}, respectively, that were obtained from the decay of TOH [Eq. (8)]. The values in solution and in CTAB micelles are very close to the value of $(1.4\pm0.2)\times10^{-6}$[AAPH] s^{-1} reported previously for liposomal dispersions,[25,26] with the concentrations of AAPH taken as 10 and 6.3 mmol L^{-1} in solution and in the micelles, respectively, in the present experiments. However, the R_i value of AAPH in SDS micelles is significantly smaller than that in CTAB micelles. This can be understood because AAPH is positively charged, hence it is prone to being adsorbed onto the surface of the SDS micelles; this, in turn, reduces the effective initiation, due to the cage effect.

The inhibition rate constant, k_{inh}, for the antioxidation reaction [Eq. (5)] by TOH in *tert*-butyl alcohol/water solution was calculated to be 4.9×10^5 $M^{-1}s^{-1}$ by taking $k_p = 100$ $M^{-1}s^{-1}$ at 37 °C.[27] This value is close to the value obtained in *tert*-

Table 1. Inhibition of AAPH-initiated peroxidation of linoleic acid by flavonols and their glycosides (FOHs) and α-tocopherol (TOH) in solution.[a,b]

Antioxidant	$R_p/10^{-8}$ [mol $L^{-1}s^{-1}$]	$R_{inh}/10^{-8}$ [mol $L^{-1}s^{-1}$]	$t_{inh}/10^2$ [s]	k_{inh}[c]$/10^5$ [$L\,mol^{-1}s^{-1}$]	kcl_p	kcl_{inh}	P_{inh} [%]	*SE*% [%]
none	14.3				9.0			
MY		3.8				2.4	73	
Q		8.2				5.1	43	
R		12.4				7.7	13	
QG		11.4				7.1	20	
QR		11.7				7.3	18	
MO		9.2				5.8	36	
K		9.1				5.7	36	
KG		12.6				7.9	12	
TOH	14.8[d]	1.7	25.2	4.9	9.3	1.1		
MY + TOH	10.3[d]	1.2	45.0	3.1	6.5	0.8		78
Q + TOH	9.0[d]	1.3	37.2	3.6	5.7	0.8		48
R + TOH	12.2[d]	1.8	28.7	3.1	7.7	1.1		14
QG + TOH	12.4[d]	3.2	31.5	1.5	7.8	2.0		25
QR + TOH	16.5[d]	1.8	29.9	2.9	10.4	1.2		19
MO + TOH	12.3[d]	2.0	34.8	2.5	7.7	1.2		38
K + TOH	11.4[d]	3.1	33.6	1.9	7.2	1.9		33
KG + TOH	11.4[d]	2.2	27.3	2.8	7.2	1.4		8

[a] In *t*BuOH/H_2O (3:2) mixed solvent at 37 °C initiated with AAPH (10 mmol L^{-1}). The initial concentrations of linoleic acid, FOHs and TOH were 0.1 mol L^{-1}, 20 μmol L^{-1}, and 20 μmol L^{-1}, respectively. Data are the averages of three determinations with a deviation of less than ±10%. [b] The R_i value was taken as 1.6×10^{-8} mol $L^{-1}s^{-1}$; see text for further details. [c] Calculated from Equation (2) by taking the k_p value as 100 $L\,mol^{-1}s^{-1}$.[27] [d] Rate of propagation after exhaustion of the antioxidants.

Table 2. Inhibition of AAPH-initiated peroxidation of linoleic acid by flavonols and their glycosides (FOHs) in micelles.[a,b]

Micelle	FOHs	$R_p/10^{-8}$ [mol L^{-1} s^{-1}]	$R_{inh}/10^{-8}$ [mol L^{-1} s^{-1}]	$t_{inh}/10^3$ [s]	k_{inh}[c]$/10^4$ [L mol^{-1} s^{-1}]	n	kcl_p	kcl_{inh}	P_{inh} [%]
SDS	none	7.1					22.9		
SDS	MY	2.8[d]	0.7	5.5	2.5	1.7	9.0	2.3	75
SDS	Q	3.9[d]	1.1	5.4	1.7	1.7	12.6	3.5	72
SDS	R	3.3[d]	2.2	2.9	1.4	0.9	10.6	7.1	33
SDS	QG	3.4[d]	2.2	2.4	1.6	0.7	11.0	7.1	35
SDS	QR	3.7[d]	2.4	2.4	1.5	0.7	11.9	7.7	35
SDS	MO	5.8[d]	1.6	4.5	1.6	1.4	18.7	5.1	72
SDS	K	6.3[d]	1.9	4.7	1.1	1.5	20.3	6.1	70
SDS	KG	5.9[d]	2.9	1.6	1.8	0.5	19.0	9.4	51
CTAB	none	18					21.7		
CTAB	MY	8.2[d]	1.2	6.3	1.4	5.2	10.0	1.4	85
CTAB	Q	5.7[d]	1.5	4.7	1.4	3.9	6.9	1.8	74
CTAB	R	4.8[d]	4.1	2.8	0.8	2.3	5.8	4.9	15
CTAB	QG	5.1[d]	3.3	3.5	0.8	2.9	6.1	4.0	35
CTAB	QR	6.3[d]	3.1	3.2	0.8	2.7	7.6	3.7	51
CTAB	MO	13.6[d]	1.5	2.6	1.8	2.2	16.4	1.8	89
CTAB	K	16.1[d]	2.6	3.6	1.1	3.0	19.4	3.1	84
CTAB	KG	16.4[d]	7.9	1.4	0.8	1.2	19.8	9.5	52

[a] The reaction conditions and the initial concentration of substrates are the same as those described in the legends of Figures 3 and 4 for reactions conducted in SDS and CTAB micelles, respectively. The calculations were based on the total reaction volume. Data are the averages of three reproducible determinations with a deviation of less than $\pm 10\%$. [b] The R_i values were taken as 3.1 and 8.3 nmol L^{-1} s^{-1} in SDS and CTAB micelles, respectively; see the text for further details. [c] Calculated from Equation (2) by taking the k_p value as 37 L mol^{-1} s^{-1}.[30] [d] Rate of propagation after exhaustion of the antioxidants.

Table 3. Inhibition of AAPH-initiated peroxidation of linoleic acid by flavonols and their glycosides (FOHs) and α-tocopherol (TOH) in micelles.[a,b]

Micelle	Antioxidant	$R_p/10^{-8}$ [mol L^{-1} s^{-1}]	$R_{inh}/10^{-8}$ [mol L^{-1} s^{-1}]	$t_{inh}/10^3$ [s]	k_{inh}[c]$/10^4$ [L mol^{-1} s^{-1}]	n'[d]	kcl_p	kcl_{inh}	$SE\%$ [%]
SDS	TOH	7.8[e]	0.6	4.7	3.6	2.0	24	1.9	
SDS	MY + TOH	5.1[e]	0.2	19.5	3.1	3.0	16.5	0.6	91
SDS	Q + TOH	4.3[e]	0.2	17.6	3.7	2.7	13.9	0.6	75
SDS	R + TOH	2.7[e]	0.3	10.1	2.9	1.8	8.7	1.0	33
SDS	QG + TOH	2.8[e]	0.3	6.9	4.9	1.1	9.0	1.0	0
SDS	QG + TOH	4.3[e]	0.4	6.8	4.2	1.1	13.9	1.3	0
SDS	MO + TOH	5.7[e]	0.3	12.8	2.7	2.0	18.4	1.0	40
SDS	K + TOH	4.5[e]	0.2	12.6	3.9	2.0	14.5	0.6	34
SDS	KG + TOH	7.0[e]	0.4	6.2	4.3	1.0	22.6	1.3	0
CTAB	TOH	22[e]	1.8	2.3	2.0	2.0	27	2.1	
CTAB	MY + TOH	12.3[e]	0.3	11.0	3.0	4.6	14.8	0.4	28
CTAB	Q + TOH	6.8[e]	0.4	8.5	2.7	3.5	8.2	0.5	21
CTAB	R + TOH	4.8[e]	0.9	4.6	2.2	1.9	5.7	1.1	0
CTAB	QG + TOH	6.9[e]	1.3	5.2	1.6	2.2	8.3	1.6	0
CTAB	QR + TOH	6.5[e]	1.1	5.1	1.9	2.1	7.8	1.3	0
CTAB	MO + TOH	14.7[e]	0.4	5.2	5.0	2.2	17.7	0.5	0
CTAB	K + TOH	16.8[e]	0.4	7.6	3.1	3.2	20.2	0.5	29
CTAB	KG + TOH	15.0[e]	0.4	3.2	6.8	1.3	18.1	0.5	0

[a] The reaction conditions and the initial concentration of substrates are the same as those described in the legends of Figures 5 and 6 for reactions conducted in SDS and CTAB micelles, respectively. The calculations were based on the total reaction volume. Data are the averages of three reproducible determinations with a deviation of less than $\pm 10\%$. [b] The R_i values were taken as 3.1 and 8.3 nmol L^{-1} s^{-1} in SDS and CTAB micelles, respectively; see the text for further details. [c] Calculated from Equation (2) by taking the k_p value as 37 L mol^{-1} s^{-1}.[30] [d] $n' = R_i t_{inh}/([FOH]_0 + [TOH]_0)$. [e] Rate of propagation after exhaustion of the antioxidants.

butyl alcohol (5.1×10^5 M^{-1} s^{-1}) that was reported previously[26] but is remarkably smaller than that obtained in chlorobenzene (3.2×10^6 M^{-1} s^{-1}).[23] This decrease of the k_{inh} value in the polar solvent is expected since the strong hydrogen-bonding ability of *tert*-butyl alcohol with TOH would make the antioxidation reaction more difficult.[27–29] On the other hand, the k_{inh} value in micelles (3.6×10^4 and 2.0×10^4 M^{-1} s^{-1} in SDS and CTAB micelles, respectively, for $k_p = 37$ M^{-1} s^{-1}[30]) was more than one order of magnitude smaller than that in *tert*-butyl alcohol. Obviously, this lower reactivity of TOH in micelles cannot be explained by the hydrogen-bonding interaction of water with TOH since water is not as good a hydrogen-bond acceptor as *tert*-butyl alcohol. The solute hydrogen-bond basicity parameters, β^H_2, of water and

tert-butyl alcohol were reported to be 0.38 and 0.49, respectively.[31] Therefore, it must be a consequence of the physical separation and the slow diffusion rate of the lipid peroxyl radicals and TOH in the interior of the micelles.[32,33] Castle and Perkins[32] and we[33] have proven previously that inter- and intramicellar diffusions are the rate-limiting steps for the antioxidation reaction conducted in micelles. Indeed, the k_{inh} value of TOH in SDS micelles ($3.6\times10^4\,M^{-1}s^{-1}$) is about two times larger than that in CTAB micelles ($2.0\times10^4\,M^{-1}s^{-1}$) because the microviscosity in the interior of CTAB micelles is 2.6 times larger than that in SDS micelles;[34] this makes the intramicellar diffusion slower in CTAB micelles. In addition, since lipid peroxyl radicals are polar (dipole moment of approximately 2.6 Debye) and electrophilic,[24] they should move to the surface of micelles and be subject to intermicellar diffusion[32,33] more quickly in SDS than in CTAB micelles, so as to react with TOH whose reactive phenoxyl functional group resides on the surface of the micelle.[32]

Antioxidative activity of the flavonols and their glycosides: It cab be seen from Figure 1 and Table 1 that the addition of flavonols and their glycosides (FOHs) decreased the rate of hydroperoxide formation and the kinetic chain length, but no inhibition period was observed in the homogeneous solution. This indicates that, in solution, FOHs can trap the initiating radicals (ROO$^\bullet$) derived from the thermal decomposition of AAPH but are unable to trap the propagating linoleic acid peroxyl radicals (LOO$^\bullet$), probably because the latter reaction is too slow to compete with the former reaction in solution. This behavior is similar to that reported previously for green-tea polyphenols in homogeneous solution.[10] The antioxidative efficacy can be assessed by the percentage inhibition (P_{inh}) or the kinetic chain length in the inhibition period (kcl_{inh}) and follows the sequence MY > Q > K ≈ MO > QG ≈ QR > R ≈ KG.

It is worth noting that the antioxidation behavior of FOHs in micelles is distinctly different from that in homogeneous solution. In SDS and CTAB micelles all the flavonols and their glycosides showed clear inhibition periods in which the rate of propagation and the kinetic chain length are remarkably reduced. This indicates that these flavonols and their glycosides are able to trap the propagating linoleic acid peroxyl radicals in micelles and behave well as chain-breaking antioxidants. This might be due to the fact that these flavonols and their glycosides can bind to the Stern layer of the micelles by hydrogen-bonding interactions, concentrated on the micellar surface, hence facilitating their interaction with the lipid peroxyl radical whose polar phenoxyl group also resides in the surface of the micelle.[32] It can be seen from Table 2 that the rate constant for trapping the propagating peroxyl radicals, k_{inh}, of these flavonols and their glycosides is in the range of $0.8–2.5\times10^4\,M^{-1}s^{-1}$ in both SDS and CTAB micelles. These values are comparable to those of TOH (3.6×10^4 and $2.0\times10^4\,M^{-1}s^{-1}$ in SDS and CTAB micelles, respectively; see Table 3), to those of resveratrol and its analogues ($0.5–3.1\times10^4\,M^{-1}s^{-1}$ in micelles),[16] and to those of green-tea polyphenols ($0.3–3.7\times10^4\,M^{-1}s^{-1}$ in micelles).[11a]

The antioxidant efficacy of these FOHs in micelles can be assessed by comparing their inhibition times (t_{inh}) and/or kcl_{inh} values. This comparison gives the efficacy sequence MY > Q > K ≈ MO > QG ≈ QR ≈ R > KG, which is similar to the sequence in the homogeneous solution.

Antioxidant synergism of flavonols and their glycosides with α-tocopherol: It can be seen from Figures 1–6 and Tables 1 and 3 that addition of flavonols and their glycosides (FOHs) together with α-tocopherol (TOH) significantly increased the inhibition period of the latter, even if no inhibition period could be observed when the FOHs was used alone. This antioxidant synergism can be quantified by the percentage increment of the inhibition period when the two antioxidants are used in combination with reference to the sum of the inhibition periods when the two antioxidants are used individually; this vale is termed the synergistic efficiency, *SE*% [Eq. (11)].[35]The antioxidant synergism of α-tocopherol (TOH) with coexistent antioxidants, such as L-ascorbic acid (vitamin C),[26] green-tea polyphenols,[11,15] and resveratrol analogues,[16] has been extensively studied and rationalized as due to the reduction of the α-tocopheroxyl radical (TO$^\bullet$) by the coexistent antioxidant to regenerate TOH. The prolonged decay of TOH in the presence of Q (Figures 7 and 8) and, especially, the stopped-flow EPR spectroscopy experiments (Figures 9 and 10) demonstrate clearly that Q can also reduce TO$^\bullet$ to regenerate TOH [Eq. (12)], hence providing a rationale for the antioxidant synergism.

$$SE\% = \{t_{inh}(TOH + FOH) - [t_{inh}(TOH) + t_{inh}(FOH)]\}/[t_{inh}(TOH) + t_{inh}(FOH)] \times 100 \quad (11)$$

$$TO^\bullet + FOH \xrightarrow{k_{reg}} TOH + FO^\bullet \quad (12)$$

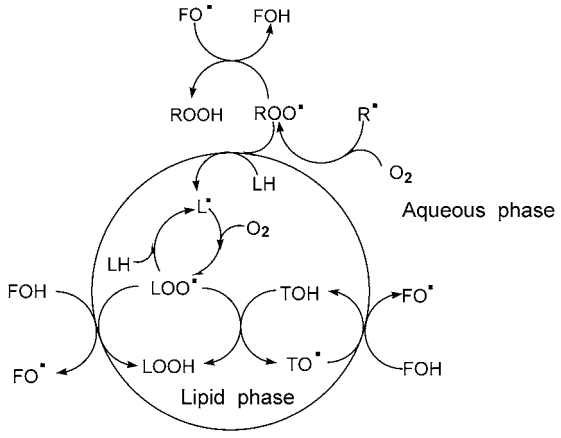
It should be pointed out in this context, however, that this α-tocopherol regeneration reaction may not be adequate to explain the antioxidant synergism. The steady-state concentration of TO$^\bullet$ during the antioxidation reaction in SDS micelles can be estimated as 0.06 μmol L^{-1};[36] hence the rate of TOH regeneration by Q is calculated to be 0.04×10^{-9} mol L^{-1}s^{-1} if the k_{reg} value [Eq. (12), the rate for the α-tocopherol regeneration] of Q in SDS micelles is taken as 64 $M^{-1}s^{-1}$ (see above) and the initial concentration of Q as 10 μmol L^{-1}. Obviously, this rate of regeneration can only account for approximately 4% of the rate decrease of the decay of TOH (from 1.6×10^{-9} mol L^{-1}s^{-1} in the absence of Q to 0.5×10^{-9} mol L^{-1}s^{-1} in the presence of Q; Figure 8), hence the increase of the inhibition period [Eqs. (7) and (8)]. Therefore, there must be an additional mechanism responsible for the remarkable antioxidant synergism of Q with TOH.

It can be seen from Figures 1 and 2 and Table 1 that although the FOHs did not produce inhibition periods in solution, they significantly prolonged the inhibition period of

TOH when the FOHs were used together with the latter. Comparison of the percentage inhibition, P_{inh}, with the synergistic efficiency, *SE*%, demonstrates that these two parameters are almost the same within the experimental deviation for every FOH. Since the inhibition period is inversely proportional to the initial concentration of AAPH,[22] that is, the rate of initiation, the reduced rate of the initiation due to the trapping of initiating radicals by the FOHs must be the predominant factor for the antioxidant synergism in the solution.

The same mechanism should also play an important role in the antioxidant synergism of TOH with FOHs in micelles. In the case of Q in SDS micelles, for example, the reduced initiation contributes 72% of the antioxidant synergism, while the TOH regeneration contributes only 4%. This is in good agreement with the 75% synergistic efficiency of TOH and Q calculated from the inhibition period (Tables 2 and 3). Other FOHs showed similar results, as listed in Tables 2 and 3. It should be pointed out, however, that if the FOH itself can produce an inhibition period, the diminished rate of propagation in the inhibition period, R_{inh}, would be caused not only by the reduced initiation but also by the inhibited propagation [Eq. (5)]. In this case the calculated P_{inh} value would no longer reflect the true value of the reduced inhibition; hence, in some cases the value of P_{inh} is appreciably larger than the value of *SE*%.

Antioxidation mechanism of flavonols and their glycosides: Based on the above discussions the antioxidation mechanism of flavonols and their glycosides (FOHs) in micelles might involve trapping the initiating radical (ROO•) that reduces the effective initiation, trapping the propagating linoleic acid peroxyl radical (LOO•) that produces the inhibition period, and reducing the α-tocopheroxyl radical (TO•) that regenerates TOH, therby prolonging the inhibition period, as depicted in Scheme 1. On the other hand, in homogeneous solutions the FOHs can only trap the initiating radicals

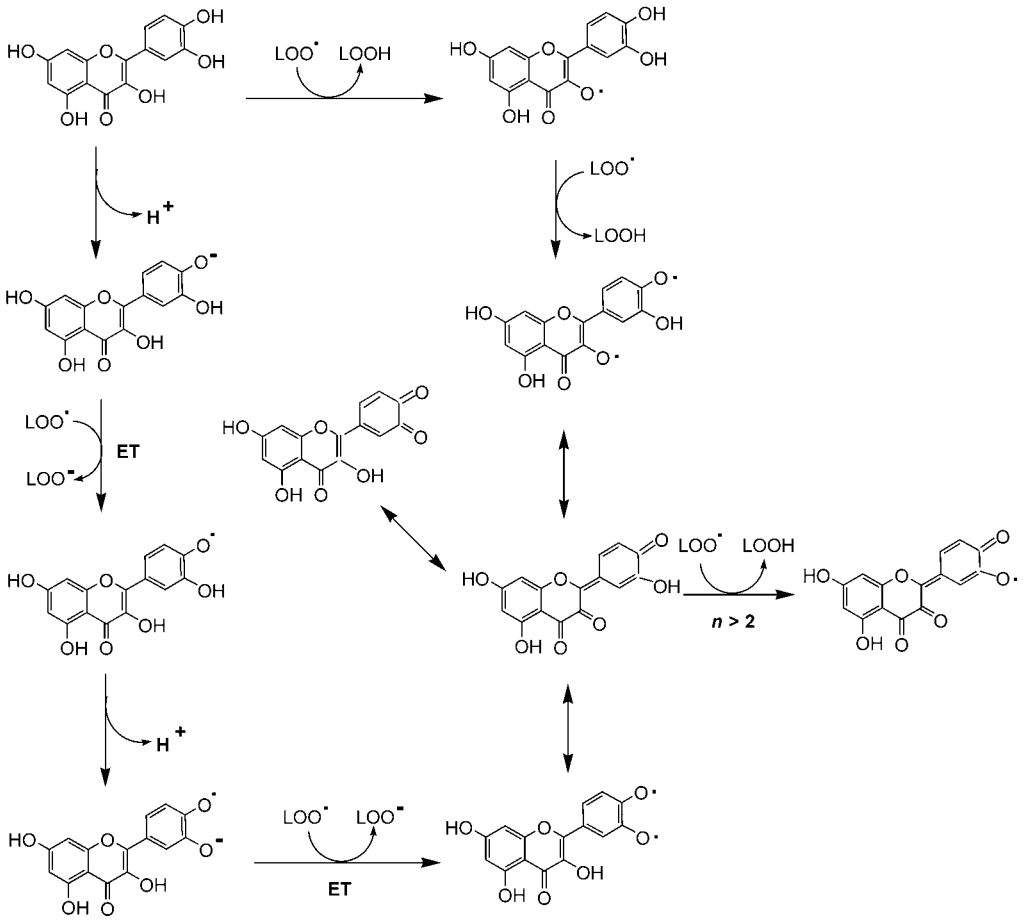


Scheme 1. Antioxidative and TOH-regeneration reactions of FOH in micelles.

to reduce the effective initiation. This, in turn, reduces the rate of propagation and decreases the kinetic chain length. The antioxidant synergism with TOH is caused by the reduction of the rate of initiation and/or by the regeneration of TOH, depending on the reaction medium and the structure of the FOH, as discussed in the previous section.

Structure–activity relationship: It can be seen from the results listed in Tables 1–3 that the antioxidative activity of MY and Q is appreciably higher than MO and K. That is, the molecules bearing an *ortho*-diphenoxyl functionality possess higher activity than those bearing no such functionality. The higher activity of flavonoids bearing *ortho*-diphenoxyl functionality on the B ring has been reported previously.[4b,i,m,n] This can be understood because the oxidation intermediate of MY and Q, the *ortho*-hydroxy phenoxyl radical, is more stable due to the intramolecular hydrogen-bonding interaction, as evidenced recently from both experiments[4b,37] and theoretical calculations.[38] The theoretical calculation showed that the hydrogen bond in the *ortho*-hydroxy phenoxyl radical is approximately 4 kcal mol^{-1} stronger than that in the parent catechol and that the bond dissociation energy (BDE) of catechol is 9.1 kcal mol^{-1} lower than that of phenol and 8.8 kcal mol^{-1} lower than that of resorcinol.[38a] In addition, it should be easier to further oxidize the *ortho*-hydroxy phenoxyl radical and/or *ortho*-semiquinone radical anion to form the *ortho*-quinone intermediate and/or product.[37,39] (Scheme 2). The fact that the stoichiometric factor, *n*, of MY and Q in micellles is larger than one (Table 2) suggests that the second peroxyl radical must be involved in the antioxidation reaction that leads to the formation of the corresponding *ortho*-quinones, as shown in Scheme 2.

It can also be seen that the flavonol glycosides (R, QG, QR, KG) possess appreciable lower antioxidant activity than their aglycones (Q and K) in solution and in micelles. It has been reported that the hydroxy group at position 3 is required for the maximal radical scavenging activity of flavonols.[40] Theoretical calculations also indicated that the O–H bond with the lowest BDE value in these flavonols (MY, Q and K) is that of the 3-OH group on ring C.[8d] For example, the BDE values of the O–H bond at positions 3, 4′, 3′, 7, and 5 in myricetin (MY) were calculated to be 26.9, 29.1, 33.2, 42.4, and 44.9 kcal mol^{-1}, respectively; thus, the order of preference of the position from which a hydrogen atom is most likely to be abstracted in the MY molecule should be 3, 4′, 3′, 7, and 5.[8d] In addition, the 3-OH group of quercetin is highly effective in stabilizing the reaction intermediate radical anion and allows the formation of the *p*-quinonoid structure[41] as exemplified in Scheme 2. Obviously, blocking position 3 by binding with a sugar residue will decrease the activity of the molecule. Introduction of a sugar to the flavonols would make them more hydrophilic,[41a] and hence inhibit them from reaching the micellar surface and reacting with the lipophilic peroxyl radicals residing on the interior of the micelle. It has been pointed out that the partition between the water and oil phases is an important factor for



Scheme 2. Antioxidation reactions of quercetin (Q) by hydrogen abstraction and electron transfer.

the antioxidant activity of flavonoids in membranes and in lipid bilayers, and the flavonoid partitioned more in the water phase possesses less activity.[41b–d] The lower activity of QG than Q in phospholipid bilayers has been reported previously.[4j] Our recent results also show that flavonol glycosides are less active than their aglycones in the inhibition of lipid peroxidation of human low-density lipoprotein[42] and erythrocyte ghosts.[43]

It is also noticeable that the antioxidative activity of FOHs is correlated with the electrochemical behavior of the molecule. The oxidation peak potential, E_{pa}, was reported to be 0.02, 0.11, 0.17, and 0.20 V (versus a saturated calomel electrode (SCE)) for MY, Q, K, and MO, respectively,[4h] and the introduction of a sugar to flavonoids increases their oxidation potential. For example, the oxidation potentials at pH 7, E_7, of Q and R were reported to be 0.33[44] and 0.6 V[45] (versus a normal hydrogen electrode (NHE)), respectively, and their half-peak oxidation potentials, $E_{p/2}$ are 0.03 and 0.18 V (versus an SCE), respectively.[46] Therefore, the increase of the oxidation potential correlates well with the decrease of the antioxidative activity in the sequence of MY > Q > K > MO > R. This correlation suggests that electron-transfer antioxidation might take place simultaneously with the direct hydrogen-abstraction reaction, as exemplified in Scheme 2. It is well known that phenoxides undergo electron-transfer oxidation more easily to produce relatively stable phenoxide radical anions in alkaline media. The acid dissociation constants, pK_1, of Q, K and, R were reported to be 6.7, 8.2, and 7.1, respectively.[45] This demonstrates that these FOHs can partially dissociate under our experimental conditions (pH 7.4) and the electron-transfer reaction is therefore feasible. Cooperation between hydrogen-abstraction and electron-transfer processes in antioxidation reactions by phenolic antioxidants has recently been discussed.[38a,47] It has been pointed out that the relative contribution of hydrogen abstraction and electron transfer in the antioxidation of flavanols depends on the experimental conditions, such as the character of the attacking radical, the pH value, and the stability of the intermediate radical species.[47] In the present case the hydrogen abstraction might take place first from the 3-OH group because this O–H bond is the weakest O–H bond in the flavonols,[8d] and the electron transfer might take place first from the 4′-phenolate of the B ring because the 4′-OH group is the most acidic in the molecule.[47] The proposed mechanism is depicted in Scheme 2.

Conclusion

Flavonols and their glycosides (FOHs), that is, MY, Q, MO, K, R, QG, QR, and KG, are effective antioxidants against linoleic acid peroxidation in solution and micelles. The reaction medium exerts significant influence on the antioxidant activity of FOHs and the synergistic antioxidation mechanism between TOH and the FOHs. The activity of flavonols is higher in micelles than in solution, while the activity of α-tocopherol is lower in micelles than in solution. This is because the predominant factor of controlling the activity is the hydrogen-bonding interaction of the antioxidant with the micellar surface in the case of hydrophilic flavonols, while it is the inter- and intramicellar diffusion in the case of lipophilic α-tocopherol. The observation that flavonols and their glycosides bearing *ortho*-diphenoxyl and 3-hydroxy functionalities possess remarkably higher antioxidant activity than those without such functionalities gives us useful information for antioxidant drug design.

Experimental Section

Materials: QR, QG, and KG were isolated from apple peels and green-tea leaves, respectively, by consecutive extraction with methanol, water, and ethyl acetate and chromatographic separation on a Sephadex LH-20 column, with reference to procedures reported previously.[48] Their structures and purity were confirmed by ^{1}H and ^{13}C NMR spectra and HPLC. MY and Q (from Sigma), R (from Aldrich), MO (from Tokyo Kaset Kogyo), K (from Fluka), TOH (from Merck), and linoleic acid (from Sigma) were purchased with the highest purity available and used as received. 2,2′-Azobis(2-methylpropioamidine) dihydrochloride (AAPH; from Aldrich) was used as received. The surfactants SDS and CTAB were recrystallized from ethyl alcohol and acetone/water (9:1), respectively.

Determination of linoleic acid hydroperoxide quantities: Aliquots of the reaction mixture were taken out of an open vessel at appropriate time intervals and subjected to HPLC analysis on a Gilson liquid chromatograph with a ZORBAX ODS reversed-phase column (6×250 mm, Du Pont Instruments), then eluted with methanol/water (9:1), for the experiments conducted in homogeneous solution, or with methanol/water (5:1) for the experiments conducted in micelles. The flow rate was set at 1.0 mL min^{-1}. A Gilson 116 UV detector was used to monitor the total linoleic acid hydroperoxides at 235 nm. Every determination was repeated three times and the experimental deviations were within ±10%.

Determination of α-tocopherol quantities: The procedure was the same as that described above for the determination of linoleic acid hydroperoxides, except that a Gilson 142 electrochemical detector set at +700 mV (versus an SCE) was used for monitoring TOH. The column was eluted with methanol/formic acid (99:1) containing sodium perchlorate (50 mmol L^{-1}) as the supporting electrolyte for the experiment conducted in homogeneous solution or with methanol/propan-2-ol/formic acid (80:20:1) for the experiment conducted in micelles.

Determination of α-tocopheroxyl radical quantities: EPR spectroscopy measurements were carried out on a Bruker ER200D spectrometer operated in the X-band with 100 kHz modulation, modulation amplitude of 0.25 mT, time constant of 0.2 s, and microwave power of 25 mW. A flat quartz flow cell (0.4×5.5×60 mm) was used for the stopped-flow determination of the reaction kinetics as described previously.[33] The α-tocopheroxyl radical was generated by vigorously stirring α-tocopherol (1 mmol L^{-1}) and excess lead oxide with a Vortex mixer for 3 min in SDS (0.2 mol L^{-1}) micelles at pH 7.4 and room temperature.

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