Kinetic Study of Free-Radical-Scavenging Action of Flavonoids in Homogeneous and Aqueous Triton X-100 Micellar Solutions

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Kinetic study of free-radical-scavenging action of six kinds of flavonoids (flavone, chrysin, flavonol, apigenin, rutin, and quercetin) has been performed. The second-order rate constants for the reaction of flavonoids with aroxyl ($ArO^{\bullet}$) (k_s) and 5,7-diisopropyltocopheroxyl ($Toc^{\bullet}$) radical (k_r) have been measured in ethanol, 2-propanol/water (5:1, v/v), and aqueous Triton X-100 micellar solution (5.0 wt %). The rate constants (k_s and k_r) observed for flavone, chrysin, and flavonol are very slow, indicating that the reactivities of 5- and 7-OH groups at A-ring and 3-OH group at C-ring are very weak and almost negligible. Rutin and quercetin with 3′- and 4′-OH groups at B-ring showed high reactivity, indicating that the *o*-dihydroxyl (catechol) structure in the B-ring is the obvious radical target site for flavonoids. The rate constants (k_s and k_r) obtained in micellar solution showed notable pH dependence. For instance, both the k_s and k_r values of rutin increased with increasing pH value from 4 to 11. Rutin is a tetrabasic acid and can exist in five different molecular forms, depending on the pH value. By comparing the k_s values with the mole fraction (f) of each molecular form of rutin, the reaction rate k_{s1} for undissociated form (RuH_4), k_{s2} for monoanion (RuH_3^-), k_{s3} for dianion (RuH_2^{2-}), and k_{s4} for trianion (RuH^{3-}) were determined; the values are $9.5 \times 10\ M^{-1}\ s^{-1}$, $4.0 \times 10^2\ M^{-1}\ s^{-1}$, $3.8 \times 10^3\ M^{-1}\ s^{-1}$, and $4.0 \times 10^3\ M^{-1}\ s^{-1}$, respectively. The reaction rates (k_{si}) increase remarkably with increasing the anionic character of rutin, that is, the electron-donating capacity of rutin. It was found that quercetin and rutin have high activity in vitamin E regeneration.

Introduction

Flavonoids are phenol derivatives widely distributed in plants. Flavonoids possess high antioxidant and free-radical-scavenging activity in foods and plants.[1−3] For instance, flavonoids have been compared in a dose-response manner with vitamin C and E and β-carotene and found to be powerful antioxidants using an in vitro lipoprotein oxidation model.[4,5] Several kinetic studies have been performed for the reaction of flavonoids with active free radicals, such as $N_3^{\bullet}$, $HO^{\bullet}$, $O^{2\bullet-}$, *t*-$BuO^{\bullet}$, and $LOO^{\bullet}$, by the pulse radiolysis technique.[6−9] It has been reported that flavonoids may act as efficient scavengers of these active free radicals. However, the above kinetic studies have been generally performed at relatively high pH region (pH = 11.5 and 10). Owing to the various dissociable phenolic hydroxyl groups in flavonoids, it is expected that the reaction rates between flavonoids and active free radical show notable pH dependence.[9] Therefore, it is necessary to measure the free-radical-scavenging rates of flavonoids at various pH in aqueous solution and in organic solvents, in order to discuss the antioxidant activity of the flavonoids in foods and biological systems. It was reported that quercetin, epicatechin, and epicatechin gallate can act as a chain-breaking antioxidant in the hydroperoxidation of methyl linoleate and their peroxyl-radical-scavenging rates (k_{inh}) are 5−20 times smaller than that of α-tocopherol in *n*-hexane/2-propanol (1:1, v/v) solution.[10,11] However, it is still obscure how these flavonoids contribute to the inhibition of lipid peroxidation.

In previous works, we measured the reaction rates (k_s) of α-, β-, γ-, and δ-tocopherols (TocH's) with 2,6-di-*tert*-butyl-4-(4-methoxyphenyl)phenoxyl ($ArO^{\bullet}$ (abbreviated to aroxyl), see Figure 1) in ethanol solution (reaction 1), using stopped-flow spectrophotometry:[12,13]

$$ArO^{\bullet} + TocH \xrightarrow{k_s} ArOH + Toc^{\bullet} \quad (1)$$

$$LOO^{\bullet} + TocH \xrightarrow{k_{inh}} LOOH + Toc^{\bullet} \quad (2)$$

The second-order rate constants (k_s) obtained are 5.12×10^3 (α-TocH), 2.24×10^3 (β-TocH), 2.42×10^3 (γ-TocH), and 1.00×10^3 (δ-TocH) $M^{-1}\ s^{-1}$ in ethanol at 25.0 °C. The relative rates (α:β:γ:δ = 100:44:47:20) agree well with those obtained from studies of the reactivities of tocopherols toward poly(peroxystyryl)peroxyl radicals (100:41:44:14) by the O_2 consumption method (reaction 2).[14,15] The result suggests that the relative reactivities of tocopherols in solution probably do not depend on the kinds of oxyradicals ($ArO^{\bullet}$ and $LOO^{\bullet}$) used.[12,13]

Recently, Foti et al.[16] measured the reaction rates between the phenoxyl radical and some natural phenolic antioxidants including α-, γ-, and δ-tocopherol (reaction 3).

The phenoxyl radical has been found to be roughly 100−300 times more reactive than peroxyl radicals, i.e., $k_3 \sim [(1-3) \times 10^2]k_{inh}$ for the same phenolic antioxidants. The relative reactivities obtained for tocopherols were α:β:γ = 100:29:6 in CH_3CN at 20 °C. Such a high reactivity of phenoxyl radical is interesting, because, for example, the reactivity of the phenoxyl radical is expected to be similar to that of the tyrosyl radical, as they described.

$$PhO^{\bullet} + TocH \xrightarrow{k_3} PhOH + Toc^{\bullet} \quad (3)$$

In the present work, in order to clear the structure−activity relationship in the scavenging reaction of free radical by

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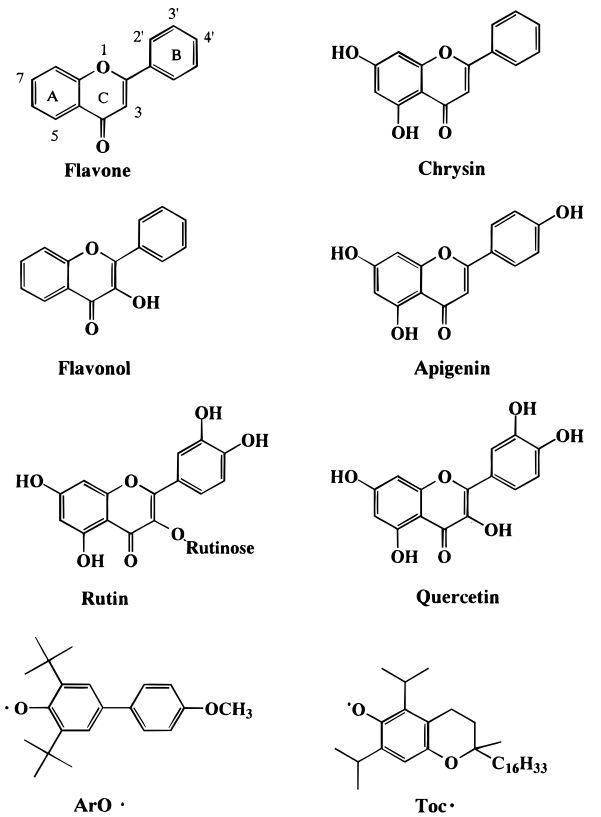

Figure 1. Molecular structures of six kinds of flavonoids, aroxyl radical (ArO•) and 5,7-diisopropyltocopheroxyl (Toc•).

flavonoids, we have measured the second-order rate constants (k_s and k_r) for the reaction of six kinds of flavonoids (flavone, chrysin, flavonol, apigenin, rutin, and quercetin) with aroxyl radical (ArO•) and 5,7-diisopropyltocopheroxyl (Toc•) radicals (see Figure 1) in ethanol, 2-propanol/water (5:1, v/v), and aqueous Triton X-100 micellar solution (5.0 wt %; reactions 4 and 5).[17,18] The rate constants obtained in micellar solution were pH dependent because of the dissociation of various phenolic hydroxyl groups in the flavonoids. The observed rates, k_s and k_r, were compared to those of α-tocopherol, ubiquinol-10, and vitamin C (L-ascorbic acid), which are well-known as most popular biological antioxidants.

$$\mathrm{ArO}^{\bullet} + \text{flavonoid} \xrightarrow{k_s} \mathrm{ArOH} + \text{flavonoid}^{\bullet} \qquad (4)$$

$$\mathrm{Toc}^{\bullet} + \text{flavonoid} \xrightarrow{k_r} \mathrm{TocH} + \text{flavonoid}^{\bullet} \qquad (5)$$

Experimental Section

Flavone, chrysin, flavonol, apigenin, rutin, and quercetin are commercially available. Aroxyl radical (ArO•) was prepared according to the method of Rieker et al.[19] The 5,7-diisopropyltocopheroxyl (Toc•) radical is fairly stable and was prepared by PbO_2 oxidation of the corresponding 5,7-diisopropyltocol in ethanol or in 2-propanol/water (5:1, v/v) solutions under a nitrogen atmosphere.[20,21] In the case of the reaction in micellar solution, Toc• radical was prepared by the reaction between the ArO• radical and 5,7-diisopropyltocol in aqueous Triton X-100 micellar solution (5.0 wt %) at 25 °C and was reacted immediately with Triton X-100 micellar solution (5.0 wt %) of flavonoids.[17,18]

The kinetic data were obtained with a Shimadzu UV-2100S spectrophotometer by mixing equal volumes of ethanol (or 2-propanol/water (5:1, v/v), or aqueous 5.0 wt % Triton X-100 micellar) solutions of flavonoids and ArO• (or Toc•) under nitrogen atmosphere. If the reaction rates (k_s and k_r) were faster than 1 M^{-1} s^{-1}, the measurements of rate constant were performed by using an Unisoku Model RS-450 stopped-flow spectrophotometer. All measurements were performed at 25.0 ± 0.5 °C.

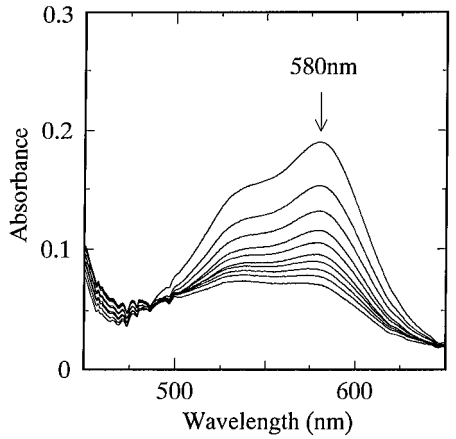

Figure 2. Change in electronic absorption spectrum of aroxyl radical (ArO•) during reaction of ArO• with rutin in ethanol solution at 25.0 °C. $[ArO^{\bullet}]_{t=0}$ = ~0.049 mM, and $[rutin]_{t=0}$ = 0.388 mM. The spectra were recorded at 7550 ms intervals. The arrow indicates a decrease in absorbance with time.

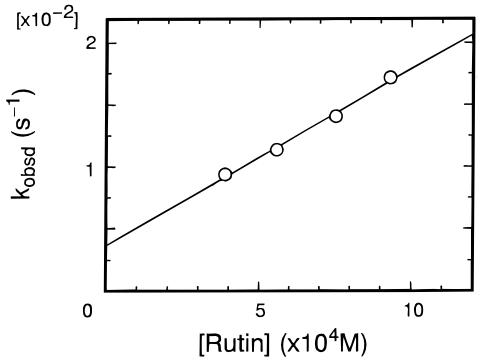

Figure 3. Dependence of pseudo-first-order rate constant (k_{obsd}) on concentration of rutin in ethanol.

Results

Aroxyl-Radical-Scavenging Rate (k_s) of Flavonoids in Ethanol Solution. Measurements of the rate constant (k_s) for the reaction of aroxyl radical (ArO•) with flavonoids were performed in ethanol solution at 25.0 °C. Figure 2 shows an example of the interaction between ArO• (~0.049 mM) and rutin (0.388 mM) in ethanol solution. The pseudo-first-order rate constant (k_{obsd}) was obtained by following the decrease in absorbance at 580 nm of the ArO• radical. The details of the experiments were reported in a previous paper.[12] ArO• radical shows a slow natural decay in ethanol solution. Therefore, the rate constant (k_{obsd}) for ArO• bleaching is given by

$$k_{obsd} = k_0 + k_s[\text{rutin}] \qquad (6)$$

where k_0 is the rate constant for the natural decay of ArO• in the medium, and k_s is the second-order rate constant for the reaction of ArO• with rutin. These parameters are obtained by plotting k_{obsd} against [rutin] (see Figure 3).[22] The second-order rate constant (k_s) obtained for rutin is 1.42 × 10 M^{-1} s^{-1}.

Similar measurements were performed for the reaction of ArO• with flavonoids in ethanol solution. The values of k_s obtained are listed in Table 1, together with those obtained for

TABLE 1: Second-Order Rate Constants (k_s and k_r) for the Reaction of ArO• and Toc• with Flavonoids in Ethanol and 2-Propanol/Water (5:1, v/v) Solutions at 25.0 °C

	k_s (M^{-1} s^{-1})[e]	k_r (M^{-1} s^{-1})[e]	
	ArO•	Toc•	
flavonoids	ethanol	ethanol	^{i}PrOH/H_2O (5:1, v/v)
flavone	$<10^{-2}$	$<10^{-2}$	$<10^{-2}$
chrysin	$\sim10^{-2}$	$\sim10^{-1}$	$\sim10^{-2}$
flavonol	5.60×10^{-2}	6.83×10^{-1}	6.25×10^{-1}
apigenin	4.73×10^{-1}	*a*	*a*
rutin	1.42×10	1.03×10	7.40
quercetin	*b*	2.98×10^{2}	2.93×10^{2}
BHT	3.5×10		
α-tocopherol	5.12×10^{3} [c]		
ubiquinol-10	5.19×10^{3}	3.64×10^{4} [d]	5.33×10^{4} [d]
Na^+AsH^-	insoluble	insoluble	6.32×10^{4}
methyl linoleate		1.26×10^{-2}	

[a] Absorption spectrum of Toc• radical overlaps with that of apigenin aroxyl radical. [b] Absorption spectrum of ArO• radical overlaps with that of quercetin aroxyl radical (see refs 7 and 8). [c] See ref 20. [d] See ref 18. [e] Experimental errors $<\pm7\%$.

α-tocopherol, ubiquinol-10, and BHT, which are well-known as popular lipid-soluble antioxidants. However, in the case of quercetin, the absorption spectra of ArO• ($\lambda_{max} = 580$ nm) and quercetin aroxyl radical ($\lambda_{max} = 530$ nm) which is produced by the reaction between ArO• and quercetin overlap each other,[7] and we were unsuccessful in determining the reaction rate.

As is clear from the k_s values listed in Table 1, the rate of the scavenging reaction of ArO• with the above flavonoids increases in the order of flavone < chrysin < flavonol < apigenin < rutin. Rutin has the highest scavenging activity among these flavonoids. The rate constant of rutin ($k_s = 1.42 \times 10$ M^{-1} s^{-1}) is similar to that of BHT ($k_s = 3.5 \times 10$ M^{-1} s^{-1}). However, the k_s value of rutin is approximately 2 orders of magnitude smaller than that of α-tocopherol ($k_s = 5.12 \times 10^3$ M^{-1} s^{-1}), which has the highest free-radical-scavenging activity among natural phenolic antioxidants.[15]

Rates (k_r) of Vitamin E Regeneration Reaction with Flavonoids in Ethanol and 2-Propanol/Water (5:1, v/v) Solutions. Measurements of the rate constant (k_r) for the reaction of Toc• with flavonoids were performed in ethanol and ^{i}PrOH/H_2O (5:1, v/v) solutions at 25 °C. The rate was measured by following the decrease in absorbance at 420 nm of the Toc• radical.[17,18,21] In the case of quercetin, visible absorption of Toc• at 420 nm overlaps with that of quercetin and quercetin aroxyl radical,[7] and thus the rate constant was determined by analyzing the increase in absorbance at 560 nm of quercetin aroxyl radical which is produced by the reaction of Toc• with quercetin. The k_r values obtained are summarized in Table 1, together with those obtained for ubiquinol-10 and sodium ascorbate anion (Na^+AsH^-). The reactions of α-tocopheroxyl with vitamin C (ascorbate) and ubiquinol-10 are well-known as the usual tocopherol regeneration reaction in biomembrane systems.[17,18,24−30]

The k_r values of flavonoids obtained in ethanol are similar to corresponding those obtained in ^{i}PrOH/H_2O (5:1, v/v) solution. As observed for the reaction between ArO• and flavonoids, the k_r values increase in the order of flavone < chrysin < flavonol < apigenin < rutin < quercetin in both the solutions. Quercetin has the highest scavenging activity among these flavonoids. However, the rate constant (k_r) of quercetin is approximately 2 orders of magnitude smaller than those of sodium ascorbate and ubiquinol-10.

It was reported that the hydrogen abstraction reaction (reaction 7) from unsaturated lipids, such as methyl linoleate and methyl linolenate, by α-tocopheroxyl radical may relate to the prooxidant effect of α-tocopherol:[21,31−33]

$$\text{Toc}^\bullet + \text{LH} \xrightarrow{k_p} \text{TocH} + \text{L}^\bullet \qquad (7)$$

The reaction rate constant (k_p) between Toc• and methyl linoleate is 1.26×10^{-2} M^{-1} s^{-1} in ethanol. The k_r values of rutin and quercetin are 1.03×10 and 2.98×10^2 M^{-1} s^{-1}, respectively, and are about from 3 to 4 orders of magnitude larger than that (k_p) of methyl linoleate. The result suggests that rutin and quercetin may contribute to a tocopheroxyl-radical-scavenging reaction in biological systems. In fact, suppression of the α-tocopherol consumption by flavonoids was previously reported in the case of oxidative modification of human low-density lipoprotein treated with macrophage or metal ion.[34,35] Further, the flavonoids in wine are hypothesized to act synergistically with tocopherol to inhibit lipid peroxidation.[36]

pH Dependence of the Aroxyl- and Tocopheroxyl-Radical-Scavenging Rates (k_s and k_r) of Flavonoids in Aqueous Triton X-100 Micellar Solution. Measurements of the rate constant (k_s) for the reaction of ArO• with flavonoids were performed at various pH values in aqueous Triton X-100 micellar solution (5.0 wt %). The problem with flavonoid aglycones is their poor solubility in aqueous Triton X-100 micellar solution (see Table 2). Flavone is insoluble in Triton X-100 micellar solution. Chrysin and flavonol are also insoluble in Triton X-100 micellar solution at pH <10 and <11, respectively. These compounds are soluble at higher pH region, because of the dissociation of phenolic hydroxyl groups in the molecule. The k_s values of chrysin are $<10^{-2}$ M^{-1} s^{-1} at pH 10 and 11, and the k_s value of flavonol is 4.89×10^{-1} M^{-1} s^{-1} at pH 11. The aroxyl-radical-scavenging rates of chrysin and flavonol are very slow in micellar solution, as observed for the reaction in homogeneous solution. Quercetin having five OH groups is also insoluble in micellar solution at pH = 7, and soluble at pH 8 increasing the ionic character of the molecule at higher pH region. However, the absorption spectra of ArO• ($\lambda_{max} = 580$ nm) and quercetin aroxyl radical ($\lambda_{max} = 530$ nm) which is produced by the reaction between ArO• and quercetin overlap each other,[7] and we were unsuccessful in determining the reaction rate.

On the other hand, rutin having sugar substituent at 3-position is soluble in water, and we have succeeded in measuring the rate constant (k_s) at wide pH region between pH 3.5 and 11. The k_s values obtained are summarized in Tables 2 and 3, together with those obtained for α-tocopherol, ubiquinol-10, and BHT. Where pH is <3 or >11, ArO• radical and/or flavonoids are unstable, and the measurement of the k_s value was unsuccessful. The pH dependence of the second-order rate constants (k_s) of rutin is shown in Figure 4. By increasing pH values, the k_s of rutin remains constant ($k_s = 9.5 \times 10$ M^{-1} s^{-1}) between pH 4.0 and 5.0, increases gradually from 1.17×10^2 M^{-1} s^{-1} at pH 6.0 to 4.14×10^2 M^{-1} s^{-1} at pH 8.0, and then increases rapidly from 5.32×10^2 M^{-1} s^{-1} at pH 8.25 to 3.89×10^3 M^{-1} s^{-1} at pH 11.0 (see Figure 4 and Table 3). This pH dependence reflects a complex mechanism that will be discussed later. Similarly, the pH effect on the reaction between flavonoids and Toc• radical was studied. As listed in Table 2, the tocopheroxyl-radical-scavenging rates (k_r) of chrysin and flavonol obtained at pH = 11 are very slow. As shown in Figure 5, the reaction rate (k_r) of rutin also increases with increasing pH value from 3.5 to 9.0, as observed for the reaction between rutin and ArO• radical. The k_r value at pH 9.0 ($k_r = 3.02 \times 10^3$ M^{-1} s^{-1}) is about 3 orders of magnitude larger than that at pH 3.5 ($k_r = 3.60$ M^{-1} s^{-1}), as listed in Table 3. Visible absorption of Toc• ($\lambda_{max} = 417$ nm) overlaps with that of

TABLE 2: Second-Order Rate Constants (k_s and k_r) for the Reaction of ArO• and Toc• with Flavonoids in Triton X-100 Micellar Solution (5.0 wt %) at 25.0 °C

	k_s (M^{-1} s^{-1})[c] (ArO•)				
flavonoids	pH 7	pH 8	pH 9	pH 10	pH 11
chrysin	insoluble	insoluble	insoluble	$<10^{-2}$	$<10^{-2}$
flavonol	insoluble	insoluble	insoluble	insoluble	4.89×10^{-1}
rutin	2.28×10^{2}	4.14×10^{2}	2.65×10^{3}	2.94×10^{3}	3.89×10^{3}
quercetin	insoluble	*a*	*a*	*a*	*a*
BHT	8.62×10^{3}				
α-tocopherol	4.53×10^{5}				
ubiquinol-10	1.25×10^{5}				
	k_r (M^{-1} s^{-1})[c] (Toc•)				
chrysin	insoluble	insoluble	insoluble	$\sim 10^{-2}$	$\sim 10^{-2}$
flavonol	insoluble	insoluble	insoluble	insoluble	~3
rutin	5.48×10^{2}	1.64×10^{3}	3.02×10^{3}	*b*	*b*
quercetin	insoluble	3.73×10^{4}	1.14×10^{5}	3.38×10^{5}	
ubiquinol-10	9.24×10^{5}				
ascorbic acid	2.49×10^{3}	2.56×10^{3}	2.47×10^{3}	2.30×10^{3}	
methyl linoleate	2.40×10^{-2}				

[a] Absorption spectrum of ArO• radical overlaps with that of quercetin aroxyl radical (see refs 7 and 8). [b] Absorption spectrum of Toc• radical overlaps with that of rutin aroxyl radical. [c] Experimental errors $<\pm 7\%$.

TABLE 3: pH Dependence of the Second-Order Rate Constants (k_s and k_r) for the Reaction of Flavonoids with Aroxyl (ArO•) and Tocopheroxyl (Toc•) Radicals in Triton X-100 Micellar Solution at 25.0 °C

	k_s (M^{-1} s^{-1})	k_r (M^{-1} s^{-1})		
	ArO•	Toc•		
pH	rutin	rutin	quercetin	AsA
3.5		3.60		
4.0	9.48×10	4.33		7.98×10^{2}
4.5	1.05×10^{2}	5.04		
4.75	8.92×10			
5.0	9.43×10	8.27		1.56×10^{3}
6.0	1.17×10^{2}	7.36×10		2.26×10^{3}
6.5	1.58×10^{2}	1.43×10^{2}		
6.75		1.70×10^{2}		
6.8		4.45×10^{2}		
6.9		5.82×10^{2}		
7.0	2.28×10^{2}	5.48×10^{2}		2.49×10^{3}
7.25		5.89×10^{2}		
7.3		8.07×10^{2}		
7.4		1.16×10^{3}		
7.5	3.99×10^{2}			
8.0	4.14×10^{2}	1.64×10^{3}	3.73×10^{4}	2.56×10^{3}
8.25	5.32×10^{2}			
8.5	9.69×10^{2}	2.04×10^{3}	1.01×10^{5}	
9.0	2.65×10^{3}	3.02×10^{3}	1.14×10^{5}	2.47×10^{3}
9.25	2.82×10^{3}			
9.5	2.82×10^{3}		2.67×10^{5}	
9.75	3.15×10^{3}			
10.0	2.94×10^{3}		3.38×10^{5}	2.30×10^{3}
10.25	3.15×10^{3}			
10.5	3.73×10^{3}			
11.0	3.89×10^{3}			

quercetin and quercetin aroxyl radical,[7,8] and thus the k_r value for quercetin was determined by analyzing the increase in absorbance at 560 nm of quercetin aroxyl radical. The second-order rate constants (k_r) of quercetin also increased with increasing pH value from 8 to 10 (see Figure 6).

Discussion

Structure−Activity Relationship of the Free-Radical-Scavenging Reaction by Flavonoids in Homogeneous Solution. As listed in Table 1, the k_s values obtained are less than 10^{-2} M^{-1} s^{-1} for flavone, about 10^{-2} M^{-1} s^{-1} for chrysin, and 5.60×10^{-2} M^{-1} s^{-1} for flavonol. Flavone having no OH substituent shows no reactivity. The reactivity of chrysin with two OH groups at A-ring is very weak and almost negligible.

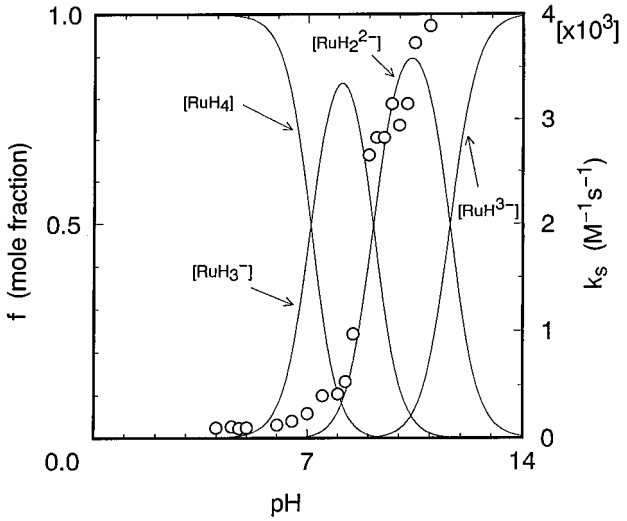

Figure 4. Plots of second-order rate constant (k_s) for rutin (open circle) versus pH and of mole fraction (f) of four rutin species (RuH_4, RuH_3^-, RuH_2^{2-}, and RuH^{3-}) versus pH (solid line).

Flavonol with 3-OH group at the C-ring shows weak reactivity. These results clearly indicate that the 5- and 7-OH groups at the A-ring and 3-OH group at the the C-ring do not contribute to the free-radical-scavenging action by flavonoids. The reaction rate of apigenin with 4′-OH group at B-ring is 4.73×10^{-1} M^{-1} s^{-1}. This value is about 1 order of magnitude larger than that of flavonol, suggesting that the 4′-OH group at the B-ring contributes to the free-radical scavenging. On the other hand, the k_s value obtained for rutin which has 3′-OH and 4′-OH groups at the B-ring is 30 times larger than that of apigenin. The result indicates that the *o*-dihydroxyl (catechol) structure in the B-ring is the obvious radical target site for rutin.

Similar behavior was observed for the reaction between Toc• and flavonoids in ethanol and ^{i}PrOH/H_2O (5:1, v/v) solutions (see Table 1), indicating that the existence of catechol structure in B-ring is essential for the free-radical-scavenging of the flavonoids.

Both quercetin and rutin, which is quercetin rutinoside at 3-position, have OH substituents at 3′-, 4′-, 5-, and 7-positions, and we can expect similar rate constants (k_r) for these flavonoids. However, the k_r values of rutin are 30−40 times smaller than those of quercetin in homogeneous and micellar solutions as listed in Tables 1 and 2. The π conjugation between the B-

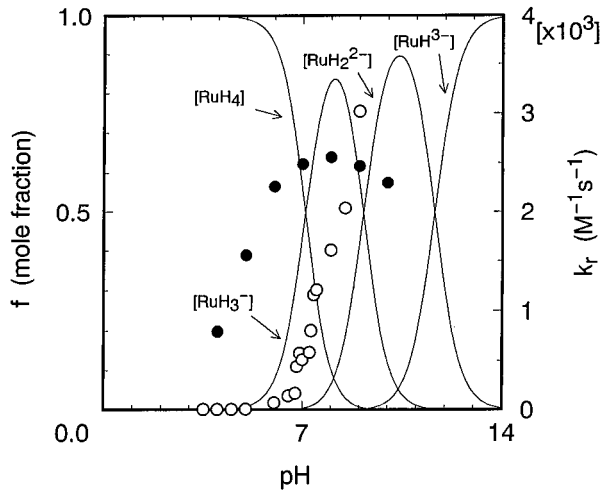

Figure 5. Plots of second-order rate constant (k_r) for the reaction of 5,7-diisopropyltocopheroxyl with rutin (open circle) and ascorbic acid (closed circle) versus pH and of mole fraction (f) of four rutin species versus pH (solid line).

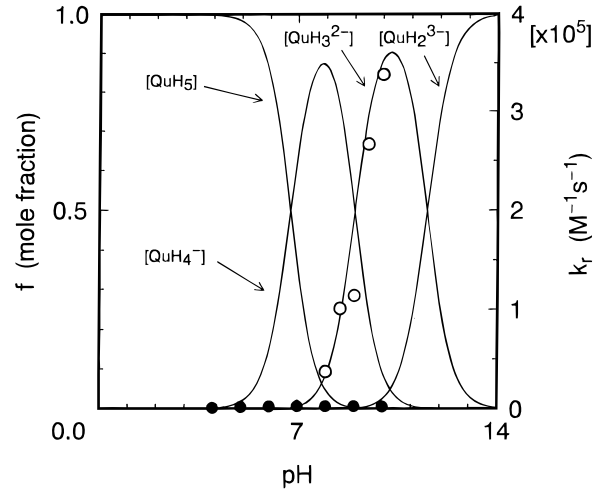

Figure 6. Plots of second-order rate constant (k_r) for the reaction of 5,7-diisopropyltocopheroxyl with quercetin (open circle) and ascorbic acid (closed circle) versus pH and of mole fraction (f) of four quercetin species versus pH (solid line).

and C-rings in quercetin will decrease in rutin, because the B-ring of rutin is considered to twist much more than that of quercetin by the steric repulsion between the 6′- (or 2′-) ring proton at the B-ring and rutinose group. In such a case, the oxidation potential of rutin increases and thus the k_r value will decrease, as observed for the reaction of ArO$^\bullet$ radical with many phenolic antioxidants.[13,20] In fact, the value of the peak oxidation potential (E_p) of rutin (180 mV vs SCE) is higher than that of quercetin (30 mV).[37]

Bors et al.[6−8,38] measured the second-order rate constants for the reaction of flavonoids with HO$^\bullet$, $N_3^\bullet$, and ^{t}BuO$^\bullet$ radicals at pH 11.5, using pulse radiolysis technique, and reported that three structural groups in flavonoids are important determinants for radical scavenging: (i) the 3′- and 4′-OH groups (catechol structure) in the B-ring, which are the obvious radical target site for all flavonoids; (ii) the 2,3-double bond in conjugation with a 4-oxo function, which is responsible for electron delocalization from the B-ring; (iii) the existence of both 3- and 5-OH groups for maximal radical-scavenging activity.

Owing to the various dissociable phenolic hydroxyl groups in flavonoids, the above active free radicals (HO$^\bullet$, $N_3^\bullet$, and ^{t}BuO$^\bullet$) react with phenolate anions at pH 11.5 rather than with undissociated phenols. The k_s and k_r values obtained by the measurements in micellar solution increase in the order of chrysin < flavonol ≪ rutin < quercetin independent of pH value, as listed in Table 2. On the other hand, in ethanol, flavonoids will take undissociated forms, differing from those in aqueous solution. However, the k_s and k_r values of flavonoids observed in ethanol solution also increase in the order of flavone < chrysin < flavonol < apigenin < rutin < quercetin. These results indicate that the above determinants (i) and (ii) reported by Bors et al. are important for free-radical scavenging in flavonoids independent of both pH value and solvent system. It seems that the existence of the 3-OH group at the C-ring and the 5- and 7-OH groups at the A-ring is not necessary for radical-scavenging, if the transition-metal ions such as Cu^+ and Fe^{2+} ions are not included in the reaction system. The ability of flavonoids to form complexes with metal ions relates to the antioxidant action of flavonoids.[39,40] Chelation of metal ions renders them catalytically inactive. Recently, similar results were obtained from the investigation of peroxyl-radical-scavenging activities of quercetin and its monoglucosides at the 3-, 7-, and 4′-positions in *n*-hexane/2-propanol (1:1, v/v) solution.[11]

Recently, Hendrickson et al.[41] found that the effect of flavonoids on microsomal phenol hydroxylase activity correlates well with the oxidation potential (E_p) for flavonoid aglycones; the flavonoids which have smaller E_p values show higher inhibitions of phenol hydroxilase activity. Further, the correlation between the E_p values of the flavonoids and their log IC_{50} of the doxorubicin induced lipid peroxidation has been reported by Acker et al.[37] As reported in previous works,[13,20,42] the rate constants of scavenging of ArO$^\bullet$ (k_s) by phenolic antioxidants increased as the total electron donating capacity of the methyl groups at the aromatic ring increased. A plot of log k_s vs peak oxidation potential (E_p) was found to be linear and the slope was negative. As described above, the k_s and k_r values of flavonoids increase in the order of chrysin < flavonol < apigenin < rutin < quercetin independent of both pH value and solvent system. On the other hand, the E_p values of the above flavonoids reported by Hendrickson et al.[41] and Acker et al.[37] decrease in the order of chrysin > apigenin > flavonol > rutin > quercetin. The result suggests that the flavonoids which have smaller E_p values show higher free-radical-scavenging activity and thus higher biological activity.

Structure−Activity Relationship of the Free-Radical-Scavenging Reaction by Flavonoids in Aqueous Micellar Solution. In a previous work, a kinetic study of the reaction between vitamin C (L-ascorbic acid, AsA) and a tocopheroxyl radical (7-*tert*-butyl-5-isopropyltocopheroxyl) in aqueous Triton X-100 micellar solution has been performed using stopped-flow spectrophotometry.[17] The second-order rate constants k_r obtained showed a notable pH dependence with a broad maximum around pH 8. A good correlation between the rate constants and the mole fraction of ascorbate monoanion was observed, showing that ascorbate can regenerate the tocopherol from tocopheroxyl in biological systems. Furthermore, the results indicated that the undissociated form of ascorbic acid does not have the ability to regenerate the tocopherol in aqueous solution.

In this work, the rates of reaction (k_s and k_r) of rutin with ArO$^\bullet$ and Toc$^\bullet$ in Triton X-100 micellar solutions have been measured by varying pH value. The observed second-order rate constants (k_s and k_r) increased with increasing pH value (see Figures 4 and 5).

Rutin is tetrabasic (see Figure 1) and can exist in five different molecular forms, i.e., undissociated form (RuH_4), monoanion

(RuH_3^-), dianion (RuH_2^{2-}), trianion (RuH^{3-}), and tetraanion (Ru^{4-}), depending on the pH value. The equilibrium reactions have the form:

$$RuH_4 \overset{K_{a1}}{\rightleftharpoons} RuH_3^- \overset{K_{a2}}{\rightleftharpoons} RuH_2^{2-} \overset{K_{a3}}{\rightleftharpoons} RuH^{3-} \overset{K_{a4}}{\rightleftharpoons} Ru^{4-} \quad (8)$$

The pK_{a1}, pK_{a2}, and pK_{a3} values of rutin have been reported by Jovanovic et al.[9] The values are $pK_{a1} = 7.1$, $pK_{a2} = 9.15$, and $pK_{a3} = 11.65$. Therefore, the mole fractions (f) present as the RuH_4 molecule and the RuH_3^-, RuH_2^{2-}, and RuH^{3-} ions were calculated as a function of pH.[17] The analytical concentration (C_a) is given in

$$C_a = [RuH_4] + [RuH_3^-] + [RuH_2^{2-}] + [RuH^{3-}] \quad (9)$$

The contribution from tetraanion (Ru^{4-}) was neglected in eq 9, because the pK_{a4} value is not reported. Mole fractions (f) present as RuH_4, RuH_3^-, RuH_2^{2-}, and RuH^{3-} are shown as functions of pH in Figure 4. The result suggests that the reaction rates k_s increase with increasing the degree of dissociation in rutin.

If we assume that the k_{s1}, k_{s2}, k_{s3}, and k_{s4} are the reaction rates for undissociated (RuH_4), monoanion (RuH_3^-), dianion (RuH_2^{2-}) and trianion (RuH^{3-}) form of rutin, respectively, the total rate k_s will be expressed as

$$k_s = k_{s1}f(RuH_4) + k_{s2}f(RuH_3^-) + k_{s3}f(RuH_2^{2-}) + k_{s4}f(RuH^{3-}) \quad (10)$$

By comparing the observed pH dependence of k_s with the pH dependence of mole fraction, the values of k_{si} were determined: for instance, at pH 4.0 only the undissociated form of rutin exists in solution, that is, $f(RuH_4) = 1$, and we can immediately determine the k_{s1} value. At pH 6.5, both the undissociated and mono anion form exist in solution, and the mole fractions are $f(RuH_4) = 0.799$ and $f(RuH_3^-) = 0.201$. Consequently, we can determine the k_{s2} value, using eq 10. Similarly, the k_{s3} value was determined. The k_{s1}, k_{s2}, and k_{s3} values obtained for three molecular forms of rutin are 9.5×10, 4.0×10^2, and 3.8×10^3 M^{-1} s^{-1}, respectively. The k_{s3} value is 40 times larger than the k_{s1} value. The result indicates that the reaction rate k_{si} increases by increasing the anionic character of rutin, that is, the electron-donating capacity of rutin. By using these k_{s1}, k_{s2}, and k_{s3} values and by varying the k_{s4} value, we simulated the experimental data. As shown in Figure 7, good accordance between the observed rate constants k_s and theoretical curve was obtained for the k_{s4} value of 4.0×10^3 M^{-1} s^{-1}, suggesting that each reaction rate estimated is reasonable.

As is clear from the results shown in Figure 4 and listed in Table 3, the k_s of rutin increases rapidly from 5.32×10^2 M^{-1} s^{-1} at pH 8.25 to 3.89×10^3 M^{-1} s^{-1} at pH 11.0. A good correlation between the rate constants (k_s) and mole fraction (f) of dianion form (RuH_2^{2-}) was observed. The result shows that the dianion (RuH_2^{2-}) of rutin mainly contributes to the scavenging of free radical at this pH region (pH 8−11). On the other hand, at lower pH region ($7 < pH < 8$) the undissociated form (RuH_4) and the monoanion (RuH_3^-) also contribute to the scavenging of free radical.

The pK_a values of several flavonoids and a series of substituted catechols were reported by Slabbert.[43] By comparing the pK_a values of flavonoids with those of substituted catechols, the pK_a^A values of 7- and 5-OH groups at the A-ring in flavonoids are estimated to be 6.74−7.07 and 11.55, respectively. Further, the pK_a^B values of 3′- and 4′-OH groups at the B-ring are reported to be 8.77−9.02 and 13.20−13.25, respectively. Therefore, as shown in Figure 8, the dissociation of rutin is considered to proceed in the order of 7-OH, 3′-OH, 5-OH, 4′-OH, by increasing the pH value. The *o*-dihydroxyl (catechol) structure in the B-ring is the obvious radical target site for rutin.[6−9,11] It is reasonable that the k_{s3} value for RuH_2^{2-} form is about 10 times larger than k_{s2} value for RuH_3^- form, as the dissociation in 3′-OH group at the B-ring proceeds at this stage. Further, it is also reasonable that RuH_2^{2-} and RuH^{3-} forms show rate constant similar to each other, because the B-ring takes the same ionic structure in both the forms.

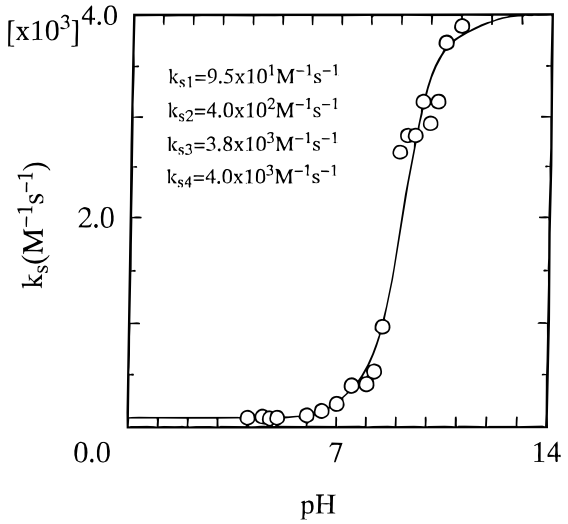

Figure 7. Plots of second-order rate constant (k_s) for rutin (open circle) versus pH and simulation curve (solid line).

A similar pH dependence of k_r value was observed for the reaction between tocopheroxyl and rutin, as shown in Figure 5. At pH 3.5, only the undissociated form (RuH_4) of rutin exists in solution, and the k_{r1} value was immediately estimated to be 3.6 M^{-1} s^{-1}. However, as is clear from the results shown in Figure 5, the rapid increase of k_r value does not correlate well with the mole fraction (f) of the dianion (RuH_2^{2-}) of rutin. If each pK_{ai} value of rutin decreases by about 1 in this reaction, a good accordance between the increase of k_r and the increase of mole fraction (f) of dianion form of rutin will be observed. The reason for such a deviation is not clear at present.

As shown in Figure 6, the rate constant (k_r) of quercetin also showed notable pH dependence. Using the values of $pK_{a1} = 6.74$, $pK_{a2} = 9.02$, and $pK_{a3} = 11.55$ for quercetin,[9,44] the mole fractions (f) of QuH_5, QuH_4^-, QuH_3^{2-}, and QuH_2^{3-} forms of quercetin were calculated as a function of pH. A good correlation between the rate constants and the mole fraction (f) of dianion (QuH_3^{2-}) of quercetin was observed, as shown in Figure 6. The result shows that dianion form of quercetin can regenerate the tocopherol from tocopheroxyl, as found for the reaction between $ArO^\bullet$ and rutin. The reaction rate (k_{r3}) for dianion form (QuH_3^{2-}) of quercetin was estimated to be about 4.0×10^5 M^{-1} s^{-1}, assuming that the k_{r1} and k_{r2} values are small compared to k_{r3} and negligible, and k_{r3} equals k_{r4} for trianion form (QuH_2^{3-}).

Comparison between Rates of Vitamin E Regeneration Reaction with Flavonoids and Vitamin C in Aqueous Triton X-100 Micellar Solution. To compare the reaction rates (k_r) obtained for rutin and quercetin with those for vitamin C (L-ascorbic acid), pH dependence of tocopheroxyl-radical-scavenging rate (k_r) of vitamin C has been measured in aqueous Triton X-100 micellar solution (5.0 wt %). The second-order rate constants (k_r) obtained showed notable pH dependence with a broad maximum around pH 8, as shown in Figure 5.[17] For instance, the k_r values obtained are 7.98×10^2 M^{-1} s^{-1} at pH

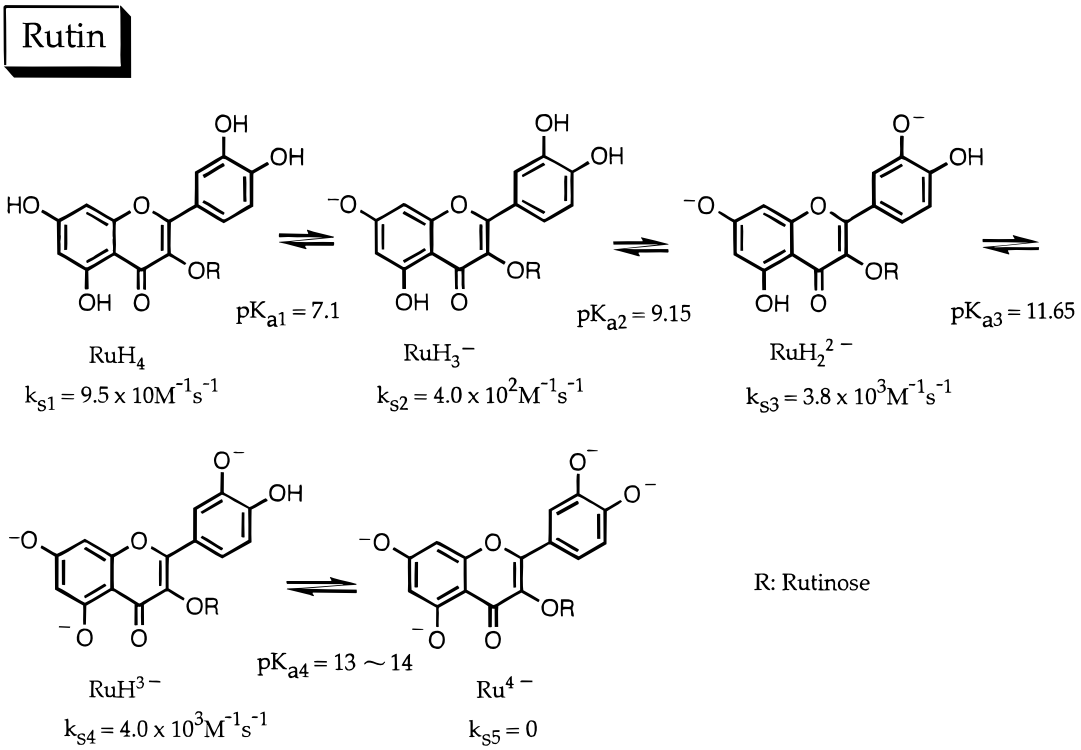

Figure 8. Five different molecular forms (RuH_4, RuH_3^-, RuH_2^{2-}, RuH^{3-}, and Ru^{4-}) of rutin in aqueous solution and their reaction rates, k_{si}.

4.0, 2.49×10^3 M^{-1} s^{-1} at pH 7.0, and 2.45×10^3 M^{-1} s^{-1} at pH 9.0. As shown in Figure 5 and as listed in Table 3, the rates of vitamin E regeneration reaction with rutin are much slower than corresponding those of ascorbic acid at the low-pH region (pH 4−6). However, the rate is similar to each other at around pH 7−9. Further, the rate constants (k_r) obtained for quercetin are 15 and 150 times larger than those of ascorbic acid at pH 8.0 and 10.0, respectively, as shown in Figure 6 and as listed in Table 3. The reaction rate (k_p) between Toc$^\bullet$ and methyl linoleate (reaction 6) is 2.4×10^{-1} M^{-1} s^{-1} in micellar solution. The k_r values of rutin and quercetin are much faster than that (k_p) of methyl linoleate. Especially, quercetin has high activity in vitamin E regeneration. However, the tocopheroxyl-radical-scavenging rates (k_r) of rutin and quercetin are smaller than that ($k_r = 9.24 \times 10^5$ M^{-1} s^{-1} at pH = 7.0) of ubiquinol-10, which is also well-known as vitamin E regeneration compounds in biomembrane systems.[18,27−30]

Flavonoids are found in high concentration in fruits, fruit juices, and wines. There exists a significant inverse association between flavonoid intake (especially wine consumption in various countries) and heart disease mortality.[45,46] Of special importance to heart disease is low-density lipoprotein (LDL) oxidation. It has been reported that rutin and quercetin exhibit lipoprotein-bound antioxidant activity using an in vitro oxidation model for heart disease.[4,5] The results of the present kinetic study suggest that quercetin and rutin may function as vitamin E regeneration compounds in biological systems. In fact, suppression of the α-tocopherol consumption by flavonoids was previously reported in the case of oxidative modification of human LDL treated with macrophage or metal ion.[34,35] Further, the flavonoids in wine are hypothesized to act synergistically with tocopherol to inhibit lipid peroxidation.[36]

Conclusion

Detailed kinetic studies have been performed for the reaction of aroxyl (ArO$^\bullet$) and 5,7-diisopropyltocopheroxyl (Toc$^\bullet$) radicals with several flavonoids in homogeneous and micellar solutions. The structure−activity relationship in the scavenging reaction of free radical by flavonoids in solution has been discussed. It has been found that quercetin and rutin have high activity in vitamin E regeneration. The results of the present kinetic study should provide a foundation for the interpretation of reactions of flavonoids with active free radicals, such as lipid peroxyl and α-tocopheroxyl radicals in more complex biological systems.

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