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Antioxidative activity of quercetin and quercetin monoglucosides in solution and phospholipid bilayers

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Abstract

The antioxidative effect of quercetin, quercetin 3-*O*-β-D-glucopyranoside (Q3G), quercetin 4′-*O*-β-D-glucopyranoside (Q4′G) and quercetin 7-*O*-β-D-glucopyranoside (Q7G) was examined in solution and liposomal phospholipid suspension. First, their peroxyl radical-scavenging activities were investigated by measuring the inhibition of hydroperoxidation of methyl linoleate initiated by a radical initiator, 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN). Quercetin exhibited the highest peroxyl radical-scavenging activity judging from the rate of hydroperoxidation during the induction period (R_{inh}) and the length of induction period (t_{inh}). Although Q7G showed an induction period, its R_{inh} was higher and its t_{inh} was lower than that of quercetin. Neither Q3G nor Q4′G gave a clear induction period in the curve of hydroperoxide formation. The rate of hydroperoxidation in the presence of Q3G was higher than R_{inh} of quercetin and the oxidative loss of Q3G was much slower than quercetin or Q7G when exposed to AMVN in solution. Q4′G exerted little inhibition compared to Q3G or Q7G. Next, the antioxidative activity of quercetin and its monoglucosides in phospholipid bilayers was examined by measuring the inhibition of lipid peroxidation in large unilamellar vesicles composed of egg yolk phosphatidylcholine (PC) and a water-soluble radical initiator. They retarded the accumulation of PC-hydroperoxides and the induction period increased in the order of Q4′G < Q3G ~ Q7G < quercetin. It is therefore concluded that quercetin acts as an antioxidant more efficiently than its monoglucosides when phospholipid bilayers are exposed to aqueous oxygen radicals.

Keywords: Quercetin; Quercetin monoglucoside; Flavonoid; Antioxidative activity; Lipid peroxidation

1. Introduction

Flavonoids are widely distributed in plant foods and beverages such as tea and wine [1,2]. They have been well recognized to act as antioxidants by scavenging oxygen radicals [3–11]. In recent years, their physiological potentials have attracted much attention in relation to their role in the cellular and extracellular antioxidant defenses against oxygen radicals [12–18]. An epidemiological study [19] demonstrated that consumption of flavonoids is helpful in lowering the risk of coronary heart disease. On the other hand, dietary flavonoids mainly exist in the form of glycoside [20]. It is suggested that glucosidase found in human fecal and salivary cultures [21] and intestinal bacteria [22] can liberate the sugar moiety from flavonoid glycoside resulting in flavonoid aglycone. It is therefore of interest to compare the antioxidant activities of flavonoid glycosides and those of aglycones. Considerable works have been carried out on the antioxidant activity of flavonols and their glycosides occurring in nature in photohemolysis of human erythrocytes [3], photoperoxidation of chloroplast [23], autoxidation of linoleic acid in micelles [8,24], lipid peroxidation of rat liver microsomes [25] and UV irradiation of low-density lipoprotein [10]. However, little is known about the relationship between the position where the sugar moiety is attached and the antioxidant activity in peroxyl radical-driven lipid peroxidation.

Here we selected quercetin as a typical flavonol widespread in plant foods and synthesized three glycosides

Abbreviations: HPLC, high performance liquid chromatography; PC, phosphatidylcholine; PC-OOH, phosphatidylcholine hydroperoxides; AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; Q3G, quercetin 3-*O*-β-D-glucopyranoside; Q4′G, quercetin 4′-*O*-β-D-glucopyranoside; Q7G, quercetin 7-*O*-β-D-glucopyranoside.

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from quercetin, that is, quercetin 3-*O*-β-D-glucopyranoside (Q3G), quercetin 4′-*O*-β-D-glucopyranoside (Q4′G) and quercetin 7-*O*-β-D-glucopyranoside (Q7G) (Fig. 1). Their activities as chain-breaking antioxidants were estimated by the inhibition of peroxyl radical-driven hydroperoxidation of methyl linoleate in solution. We also examined the antioxidant effect of quercetin and its monoglucosides by exposing aqueous oxygen radicals to phospholipid bilayers. The results strongly suggest that antioxidant effect of flavonol in glycoside form is lower than that of flavonol aglycone.

2. Materials and methods

2.1. Materials

Quercetin (3,3′,4′,5,7-pentahydroxyflavone) was obtained from Tokyo Kasei Organic Chemicals (Tokyo, Japan) and purified by silica gel column chromatography with eluting benzene and acetone. Q3G, Q7G, and Q4′G were chemically synthesized from quercetin and 2,3,4,6-tetra-*O*-acetyl-α-glucopyranosyl bromide using the Koenigs-Knorr reaction [26]. The detailed procedures were published elsewhere [27]. All products were eluted as single fraction in HPLC after purification. D-α-Tocopherol was kindly supplied by Eisai Company (Tokyo, Japan). 2,2′-Azobis(2,4-dimethylvaleronitrile) (AMVN) and 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) were purchased from Wako Pure Chemicals (Osaka, Japan). Methyl linoleate (99% grade) were obtained from Sigma (St. Louis, MO, USA) and further purified by Florisil column chromatography to remove contaminant hydroperoxides [28]. Egg yolk phosphatidylcholine (PC; 99% grade, Sigma) was also purified by reversed phase column chromatography as described previously [29].

2.2. Measurement of peroxyl radical-scavenging activity in solution

The peroxyl radical-scavenging activities of quercetin and its monoglucosides in solution were determined by the inhibition of hydroperoxidation of methyl linoleate induced by an azo compound. The principal procedures were the same as those published in the preceding paper [11]. Briefly, an appropriate amount of quercetin or its monoglucosides in ethanol solution was added to a test tube and then evaporated in vacuo. The residue was mixed with the solution of methyl linoleate (2 ml, n-hexane/2-propanol = 1:1, v/v). After preincubation at 37°C for 5 min, oxidation of methyl linoleate was started by the addition of AMVN in n-hexane and 2-propanol solution (1:1, v/v, 0.1 ml). Final concentrations of methyl linoleate and AMVN were 80.7 mM and 8.5 mM, respectively. The reaction mixture was incubated in the dark at 37°C and the amount of methyl linoleate hydroperoxides was determined by HPLC at regular interval [30]. The kinetic parameters for the inhibition of peroxidation were calculated by the method described in the preceding paper [10] by assuming that antioxidant mechanism of quercetin and its monoglucosides was the same as that of α-tocopherol and other phenolic compounds [31].

2.3. Determination of quercetin and quercetin monoglucosides

Quercetin and its monoglucosides in the reaction mixture with or without methyl linoleate were determined by reversed phase HPLC using 655 Hitachi liquid chromatograph (Hitachi, Tokyo, Japan) with an Inertsil ODS-80A column (4.6 × 150 mm, GL Science, Tokyo, Japan). Hitachi UV detector 638-41 was used to monitor the absorbance at 350 nm. The eluting solvent was composed of

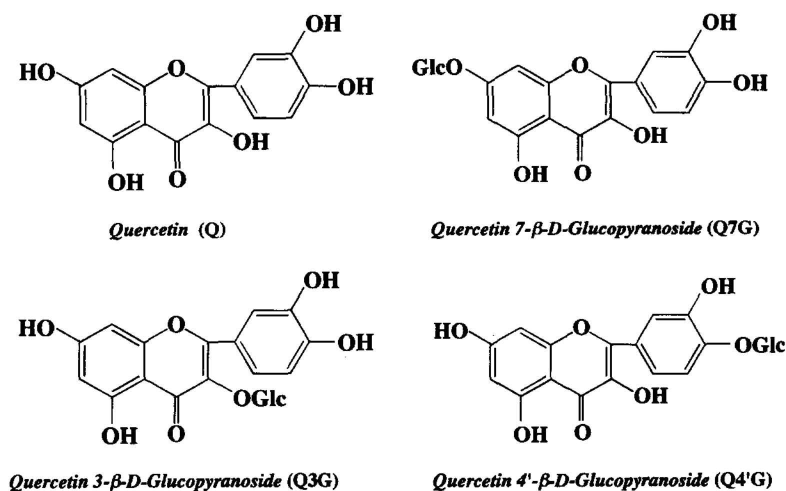

Fig. 1. Structure of quercetin and quercetin monoglucosides. Glc, glucose.

methanol, water and acetic acid (55:45:1, v/v, for monoglucosides and 50:50:1, v/v, for quercetin). The flow rate was set at 1.0 ml/min.

2.4. Lipid peroxidation in unilamellar liposomes

Large unilamellar vesicles containing quercetin or its monoglucosides were prepared by the extrusion method described previously [11]. Briefly, egg yolk PC and quercetin or its monoglucoside were suspended in 0.7 ml of Tris-HCl buffer (10 mM, pH 7.4) containing diethylenetriaminepentaacetic acid (0.5 mM), which was used for preventing the prooxidant effect of contaminant metal ions, and mixed with Vortex mixer for 1 min followed by ultrasonication for 30 s. The suspension was passed through a polycarbonate membrane (pore size 100 nm) and diluted with the same volume of Tris-HCl buffer. After the resulting unilamellar liposomal suspension (1.0 ml) were preincubated for 5 min at 37°C, the peroxidation was started by the addition of 0.1 ml AAPH solution. The final concentrations in the incubation mixture were as follows; PC 5 mM, quercetin or quercetin monoglucosides 20 μM, AAPH 10 mM. The amount of PC-hydroperoxides (PC-OOH) was determined by reversed phase HPLC according to the method described previously [32].

3. Results

3.1. Peroxyl radical-scavenging activity of flavonoids in solution

Peroxyl radical-driven peroxidation of methyl linoleate was carried out in a solution of n-hexane and 2-propanol

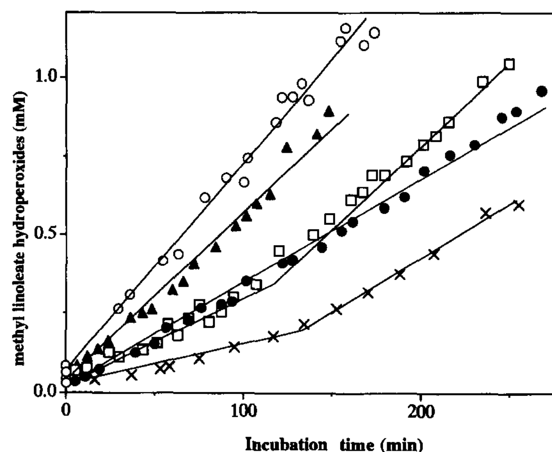

Fig. 2. Effect of quercetin and quercetin monoglucosides on AMVN-initiated oxidation of methyl linoleate in solution. The reaction system consisted of methyl linoleate (80.7 mM), quercetin or quercetin monoglucoside (80 μM) and AMVN (8.5 mM) in the solution of n-hexane and 2-propanol (1:1, v/v). Q4′G (▲), Q7G (□), Q3G (●), quercetin (×), no addition (○).

using a lipophilic radical generator, AMVN. Fig. 2 shows the results of the inhibition of peroxidation by quercetin and quercetin monoglucosides at the concentration of 80 μM (0.1 mol% to methyl linoleate). Quercetin and Q7G gave a clear induction period followed by accelerated hydroperoxidation. The induction period by Q7G was shorter than that by quercetin. However, no apparent induction period was observed during the incubation period in the case of Q3G and Q4′G. Q4′G was less effective than quercetin and other monoglucosides in the suppression of hydroperoxidation through the incubation period. Table 1 shows the kinetic parameters of the inhibition for quercetin and quercetin monoglucosides at the concentrations rang-

Table 1
Inhibition of radical chain oxidation of methyl linoleate in solution by quercetin, quercetin monoglucosides and α-tocopherol [ab]

Antioxidant	[InH] [c] (M) ($\times 10^6$)	R_{inh} (M s^{-1}) ($\times 10^8$)	R_p (M s^{-1}) ($\times 10^8$)	t_{inh} (s) ($\times 10^{-3}$)	k_{inh}/k_p	n
None			10.0 ± 0.3			
Q3G [d]	40	6.2 ± 0.5				
	80	4.7 ± 1.1				
	160	3.5 ± 0.3				
Q7G	40	5.6 ± 1.3	9.3 ± 0.8	4.1 ± 0.3	380 ± 130	2.7 ± 0.2
	80	4.0	7.7	5.7 ± 0.5	320 ± 40	1.9 ± 0.1
	160	2.1 ± 0.5	5.3 ± 0.4	9.3 ± 0.4	430 ± 80	1.5 ± 0.1
Q4′G [d]	40	8.4 ± 2.7				
	80	9.3 ± 0.2				
	160	5.7 ± 1.0	8.3 ± 0.3	8.0 ± 0.6	180 ± 40	1.3 ± 0.1
Quercetin	40	3.5 ± 0.3	7.1 ± 0.4	5.4 ± 0.2	430 ± 50	3.5 ± 0.2
	80	2.9 ± 0.2	6.1 ± 0.6	10.1 ± 1.1	280 ± 30	3.3 ± 0.3
	160	1.7 ± 0.2	3.5	14.1 ± 1.2	340 ± 60	2.3 ± 0.2
α-Tocopherol	80	0.5 ± 0.1	12.0 ± 0.1	6.1	2770 ± 760	2.0

The reaction conditions were the same as those described in Fig. 2.
[a] Concentration of methyl linoleate, [LH], was $80.7 \cdot 10^{-3}$ M in each experiment.
[b] Average values ± S.D. for three experiments except for R_p without antioxidant (sixteen experiments).
[c] Concentration of antioxidant.
[d] The parameters other than R_{inh} were not obtained in the case of Q4′G at 40 and 80 μM and Q3G at the three concentrations, because no induction period appeared in the reaction curves.

ing from 40 to 160 μM. α-Tocopherol at the concentration of 80 μM was used to calculate the rate of chain initiation by AMVN (R_i) by assuming that α-tocopherol traps two chain-propagating peroxyl radicals [33] ($R_i = (2 \times 80 \cdot 10^{-6})/(6.1 \cdot 10^3) = 2.6 \cdot 10^{-8}$ M s^{-1}). The rate of chain propagation of methyl linoleate hydroperoxides (R_p) without antioxidants was $(10.0 \pm 0.3) \cdot 10^{-8}$ M s^{-1} and the kinetic chain length (kcl $= R_p/R_i$) [33] was calculated to be 3.8. In the case of quercetin and Q7G, the rate of hydroperoxide formation during the induction period (R_{inh}) and that of accelerated hydroperoxidation after induction period (R_p) were determined on the bases of the slope during the induction period and the slope at the period of accelerated hydroperoxidation. The length of induction period (t_{inh}) was determined from the intersection of the line for R_{inh} and R_p. The rate of hydroperoxidation in the presence of Q4′G except for 160 μM and Q3G was calculated by the slope during the whole incubation period and also expressed by R_{inh} because no clear induction period appeared in the reaction curve. In Table 1, R_{inh} of each antioxidant was lowered by increasing its concentration and the order at 80 μM was as follows; Q4′G > Q3G ~ Q7G > quercetin > α-tocopherol. Interestingly, R_p of Q7G and quercetin were lowered by elevating their concentrations and these values were significantly lower than that without antioxidants. Thus, quercetin and Q7G still exhibited some inhibitory effect after the induction period was terminated. Though R_{inh} of quercetin was rather high as compared with that of α-tocopherol, t_{inh} of quercetin was larger than that of α-tocopherol at 80 μM. In quercetin and Q7G, the ratio of the rate constant for inhibition (k_{inh}) to that for chain propagation (k_p) was calculated according to the equation [33],

$$k_{inh}/k_p = [LH]/(t_{inh} \times R_{inh})$$

in which [LH] is the concentration of methyl linoleate. These values of Q7G and quercetin were not significantly verified in each concentration in the range from 300–400 and were about 9-times lower than that of α-tocopherol at the concentration of 80 μM as shown in Table 1. On the other hand, stoichiometric number (n) of radicals trapped by antioxidant was determined by the following equation [33];

$$n = (R_i \times t_{inh})/[InH]$$

in which [InH] is the concentration of antioxidant. The n value of Q7G was lower than that of quercetin because of its lower t_{inh} (Table 1). In addition, Q4′G at 160 μM also gave lower k_{inh}/k_p and lower n value than quercetin.

3.2. Reactivity of quercetin and its monoglucosides to peroxyl radicals in solution

Fig. 3 shows the loss of quercetin and quercetin monoglucosides by the exposure to AMVN in the solution of n-hexane and 2-propanol (1:1, v/v) in the presence or absence of methyl linoleate. Quercetin and its monoglucosides were decreased by the reaction with peroxyl radicals generated by AMVN in the absence of methyl linoleate (Fig. 3B), whereas they were decreased by the reaction with both chain-initiating peroxyl radicals generated by AMVN and chain-propagating lipid-peroxyl radicals in the presence of methyl linoleate (Fig. 3A). It was found that Q3G was the most resistant to the reaction with these peroxyl radicals. In both reaction systems, the rates of loss of quercetin and that of Q7G were similar and were much faster than that of Q3G regardless of methyl linoleate. Although Q4′G decreased fast in the initial stage, its rate was slow in the later stage.

3.3. Inhibition of liposomal phospholipid peroxidation by quercetin and its monoglucosides

Fig. 4 shows the hydroperoxide formation from liposomal PC by exposing to a water-soluble radical initiator, AAPH. The effects of quercetin and its monoglucosides on this reaction system were measured at the concentration of 20 μM (0.4 mol% to PC). Quercetin retarded the accelerated formation of PC-OOH for the longest period among the compounds tested. In contrast, Q4′G had little in-

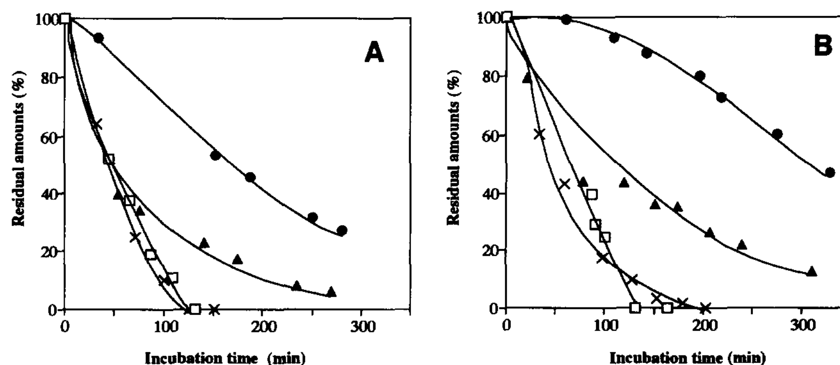

Fig. 3. Decrease of quercetin and quercetin monoglucosides by the exposure to AMVN in the presence of methyl linoleate (A) and in the absence of methyl linoleate (B). The reaction system consisted of quercetin or quercetin monoglucosides (80 μM) and AAPH (10 mM) with or without methyl linoleate (80.7 mM) in the solution of n-hexane and 2-propanol (1:1, v/v). Q4′G (▲), Q7G (□), Q3G (●), quercetin (×).

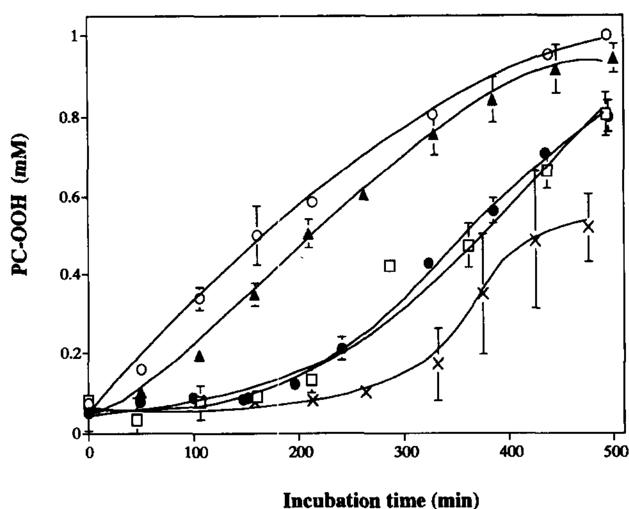

Fig. 4. Inhibition by quercetin and quercetin monoglucosides of AAPH-initiated peroxidation of PC liposomes. The reaction system consisted of quercetin or quercetin monoglucoside (20 μM), AAPH (10 mM), egg yolk PC (5 mM) and diethylenetriaminepentaacetic acid (0.5 mM) in 10 mM Tris-HCl buffer (pH 7.4). Q4′G (▲), Q7G (□), Q3G (●), quercetin (×), no addition (○). Triplicate experiments were challenged and mean value ± S.D. are presented.

hibitory effect on the oxidation of liposomal phospholipids. Antioxidative activities of Q3G and Q7G were indistinguishable to each other in this membrane system.

4. Discussion

In plant foods, quercetin and the other flavonols are mainly distributed in the form of glycosides in which sugar is attached to the 3-OH position, much less frequently the 7-OH position and only in the rare case the 3′-, 4′- or 5-OH position [20]. Quercetin glycosides synthesized here are also present in nature, as Q3G, Q4′G and Q7G are found in tea [34], shallot [2] and asian cotton [35], respectively. We already found that quercetin can act as a chain-breaking antioxidant in the hydroperoxidation of methyl linoleate and its peroxyl radical-scavenging activity is much lower than that of α-tocopherol, a well known chain-breaking antioxidant [11]. Lower k_{inh}/k_p of quercetin in this study confirms that quercetin is inferior to α-tocopherol in peroxyl radical-scavenging activity. Nevertheless, higher n and lower R_p value of quercetin indicates that the antioxidant effect of quercetin lasts longer than α-tocopherol. The phenomenon that R_p of quercetin is significantly lower than that of chain propagation without antioxidants suggests that its antioxidant mechanism somewhat differs from that of α-tocopherol.

It is apparent that the antioxidative effect of quercetin is much higher than that of quercetin glucosides in solution, judging from its lowest R_{inh} and largest t_{inh} at the different concentrations among quercetin and quercetin monoglucosides tested. The binding of glucose moiety to hydroxyl group of quercetin lowers the peroxyl radical-scavenging activity irrespective of the position of hydroxyl group. However, both of the activity and the mechanism for antioxidant action seem to be determined by the position where glucose is bound. The binding of glucose to the 7-OH position lowers the antioxidant activity in solution because Q7G showed higher R_{inh} and lower t_{inh} than quercetin. However, it is likely that Q7G and quercetin possess similar reactivity toward peroxyl radicals in solution as shown in Fig. 3. The efficiency of chain-breaking antioxidants depends on the reactivity with the chain-propagating peroxyl radicals and the stability of the trapped radicals to give a termination reaction with another peroxyl radicals [36]. Therefore, the 7-OH position seems to affect the stability of trapped radicals resulting in the decrease of chain-breaking activity.

On the other hand, the binding of glucose to the 3-OH position may influence the antioxidant mechanism of quercetin, because Q3G gave no induction period and its reactivity toward peroxyl radicals in solution was much lower than that of quercetin (Fig. 3). Bors et al. [37] suggested that the hydroxyl groups at both 3-position and 5-position are required for maximal radical scavenging potential and strongest radical absorption in the antioxidative activity of flavonols. Our results strongly suggest that hydroxyl group at the 3-position is helpful to elevate the reactivity toward peroxyl radicals in the inhibition of peroxyl radical-mediated lipid peroxidation. That is to say, loss of hydroxyl group at this position increases the stability of flavonols against the peroxidative attack, although the antioxidant activity itself is lowered. It can be said that quercetin loses its antioxidant activity substantially by the binding of glucose to the 4′-OH position. It has been suggested that the o-dihydroxyl structure at the 3′- and 4′-OH position is most responsible for the peroxyl radical-scavenging activity of flavonols [37–39]. Our results also supports the idea that the o-dihydroxyl structure in the B ring is essential for exhibiting the antioxidant activity of flavonols in peroxyl radical-driven lipid peroxidation.

We have also investigated the effectiveness of quercetin and its monoglucosides on the peroxidation of unilamellar vesicles of phospholipids initiated by AAPH, because phospholipid bilayers are major target of oxygen radicals in cellular systems. The water-soluble azo compound, AAPH, is frequently used to know the effectiveness of antioxidants against the attack of oxygen radicals to biomembranes from aqueous phase [11,40]. The fact that quercetin was the most effective antioxidant among compounds tested in this membrane model can be explained by its highest peroxyl-radical scavenging activity as described above. It is reasonable that Q4′G has the lowest effect because of little peroxyl radical-scavenging activity. An alternative for the superiority of quercetin may be derived from the different polarity between glycoside and its aglycone. Quercetin is rather lipophilic antioxidant as compared with its glucosides and seems to interact with the

polar head of phospholipid bilayers by locating near the surface of membranes [41]. This location may be favorable for the trap of peroxyl radicals originating from the aqueous phase. It is reported that the introduction of glucosyl group to flavonoid increases its solubility in water [42]. Quercetin glysosides are more hydrophilic and likely to be distributed in water phase, although further studies are required to know the exact distribution of quercetin and quercetin glycosides in membraneous systems.

In conclusion, quercetin acts as a chain-breaking antioxidant more effectively than quercetin monoglucosides, Q3G, Q7G and Q4′G, and the antioxidant effect of quercetin is higher than that of quercetin monoglucosides on the oxidation of phospholipid bilayers induced by aqueous oxygen radicals. The liberation of aglycone from flavonol glycosides may be an essential step to elevate the antioxidant activity of flavonols occurring in plant foods.

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