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Daidzein as an Antioxidant of Lipid: Effects of the Microenvironment in Relation to Chemical Structure

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Isoflavone daidzein (D, $pK_{a1} = 7.47 \pm 0.02$ and $pK_{a2} = 9.65 \pm 0.07$) was, through a study of the parent compound and its three methyl anisol derivatives 7-methyldaidzein (7-Me-D, $pK_a = 9.89 \pm 0.05$), 4′-methyldaidzein (4′-Me-D, $pK_a = 7.43 \pm 0.03$), and 7,4′-dimethyldaidzein (7,4′-diMe-D), found to retard lipid oxidation in liposomal membranes through two mechanisms: (i) radical scavenging for which the 4′-OH was more effective than the 7-OH group in agreement with the oxidation potentials: 0.69 V for 4′-OH and 0.92 V for 7-OH versus Ag/AgCl in acidic solution and 0.44 V for 4′-O^- and 0.49 V for 7-O^- in alkaline solution and (ii) change in membrane fluidity through incorporation of the isoflavones, in effect hampering radical mobility. The radical scavenging efficiency measured by the rate of the reaction with the $ABTS^{\bullet+}$ in aqueous solution followed the order D > 7-Me-D > 4′-Me-D > 7,4′-diMe-D, as also found for antioxidant efficiency in liposomes when oxidation was initiated with the water-soluble AAPH radical and monitored as the formation of conjugate dienes. For oxidation initiated by the lipid-soluble AMVN radical, the antioxidant efficiency was ranked as 4′-Me-D > D > 7,4′-diMe-D > 7-Me-D, and change in fluorescence anisotropy of fluorescent probes bound to the membrane surface or inside the lipid bilayer confirmed the effects of isoflavones on the membrane fluidity, especially for 7,4′-diMe-D.

KEYWORDS: Daidzein derivative; liposome; radical scavenge; antioxidation

INTRODUCTION

Extensive studies on free-radical biology suggest that reactive oxygen species (ROS), such as $O_2^{\bullet-}$, $^1O_2$, and $^{\bullet}OH$, are involved in the course of aging, development of cancers, and cardiovascular diseases (*1*). ROS may also be responsible for the toxicity of environmental xenobiotics, some antineoplastic agents, as well as radiation damages and may even be involved in the mechanism behind antibiotics (*2*−*7*). The use of antioxidants for the prevention of such effects induced by ROS has great input on advices for human nutrition and health (*8*). Daidzein (D), an isoflavone abundant in soybean and accordingly with a high intake in Asian populations, has been demonstrated to be a potent antioxidant and has been receiving much attention in relation to human health (*9*).

While the antioxidant properties of isoflavones, such as the reducing capacities and the known ability to chelate transition-metal ions (*10*), etc., have been relevant to the health benefits, recent studies have also found other biochemical activities of isoflavones, which may play important physiological roles, such as the possible influence on intracellular redox status (*11*), the interactions with specific proteins central to intracellular-signaling cascades (*12*), the promotion of the cellular level of endogenous antioxidant GSH (reduced glutathione) (*13*), and the specific interactions with nuclear acids (*14*). Most likely, the beneficial health effects of isoflavones derive from different mechanisms, i.e., the antioxidation capacities and specific biochemical interactions, for which the concentration levels relative to other antioxidants have to be taken into account. The present work attempts to investigate the antioxidation properties of daidzein and its derivatives *in vitro*, with an emphasis on the influence from the environmental factors, e.g., the pH, the disposition in lipid bilayer, and the fluidity of the membrane in correlation to the molecular structures of the isoflavones.

The antioxidation effect of isoflavones had been demonstrated in many biologically relevant systems, such as homogeneous solution (*15*), phospholipid bilayers (*16, 17*), low-density lipoproteins (*18, 19*), and whole cells (*20, 21*). Studies of the structure−activity relationship have shown that the B-ring 4′-hydroxyl (4′-OH) is essential in scavenging free radicals, whereas the effect of the A-ring 7-hydroxyl (7-OH) appears to

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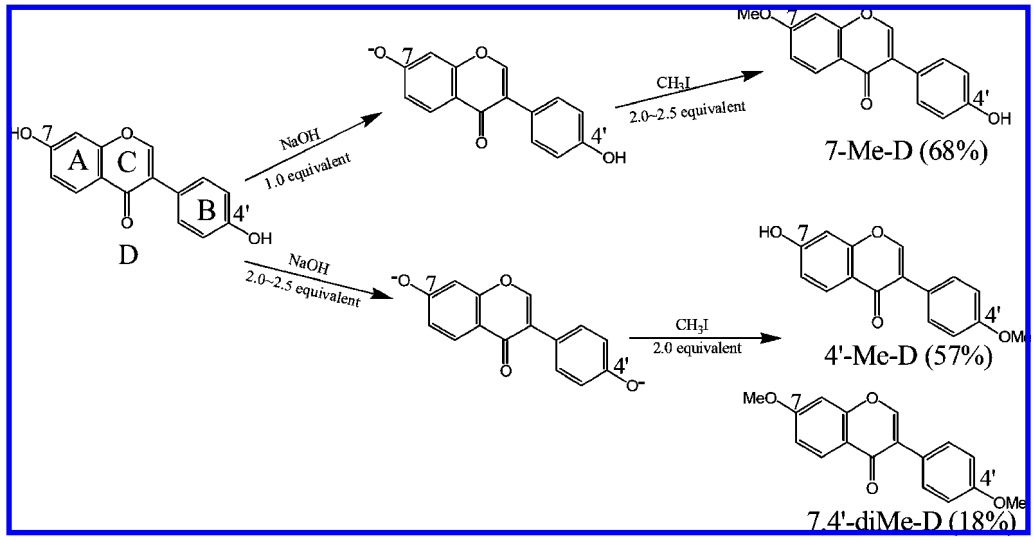
Scheme 1. Synthetic Routes of D Derivatives



be less significant (*22*). In homogeneous solution, the oxidation potentials of (iso)flavones and the lifetimes of their radicals formed by oxidation are important for the antioxidation activities. In cell membranes, liposome, and other structured media, however, additional factors such as the distribution and orientation of (iso)flavone molecules at the surface or inside the membranes together with any changes in the physical properties of the membrane, owing to the incorporation of (iso)flavonoids, also have effects on the antioxidative activity (*23*−*25*). In addition, it had been shown that the effective concentration of an antioxidant may vary over a 1000-fold depending upon the type of target in the oxidation process (*26*). The reactivity of an antioxidant may also vary dramatically depending upon whether the oxidation reaction is induced by a hydrophilic or lipophilic free-radical initiator (*27*). Accordingly, it is important to consider both chemical and physical aspects in any evaluation of antioxidant performance.

We have carried out, in this work, systematic studies of the rather lipophilic D and its methyl anisol derivatives with even more lipophilic properties for their thermodynamic properties (pK_a and oxidation potential), radical scavenging capabilities in homogeneous aqueous solution of varying pH, as well as antioxidation activities in liposome. In addition, the distribution and orientation of D and its derivatives in liposome and their interaction with the local membranal environments were examined. When these properties and the antioxidation performance of D and the derivatives are compared, the structure−activity relationship of these antioxidants with regarding to the interactions with the microenvironment is discussed. It is concluded that both A- and B-ring hydroxyls of D play crucial roles in radical scavenging and antioxidation activities; however, the underlying mechanisms are different depending upon the microenvironments.

MATERIALS AND METHODS

Chemicals. Daidzein (>98%) was purchased from Huike Plant Exploitation, Inc. (Shanxi, China). 8-Anilino-1-naphthalenesulfonic acid (ANS), soybean L-*R*-phosphatidyl choline (PC), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid (ABTS), and 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) were from Sigma-Aldrich Chemical Co. (St. Louis, MO). 2,2′-Azobis(2,4-dimethylvaleronitrile, AMVN) was purchased from Huichang Petrochemical Auxiliary Co. Ltd. (Shandong, China). Methyl iodide (>98%), methanol (analytical grade), potassium persulfate (>99%), ethanol (HPLC grade), and chloroform (>99%) were purchased from Beijing Chemical Plant (Beijing, China). 16-(9-Anthroyloxy) palmitic acid (16-AP) was synthesized from 16-hydroxyhexadecanoic acid and 9-anthracenecarboxylic acid (*28*).

Modification of Daidzein. Modification of D was achieved via the Williamson reaction (*29, 30*). **Scheme 1** shows the synthetic routes. D (0.5 M) mixed with methyl iodide (0.5 or 1.0 M) and sodium hydroxide (0.5 or 1.0−1.25 M) in methanol was stirred for 24 h at 78 °C. The reaction was traced by thin-layer chromatography (TLC). The D derivatives, 7-methyldaidzein (7-Me-D), 4′-methyldaidzein (4′-Me-D), and 7,4′-dimethyldaidzein (7,4′-diMe-D), were purified with TLC using a mixture of dichloromethane and methanol (40:1, v/v) as the eluent and were characterized by nuclear magnetic resonance (NMR) and mass spectroscopies.

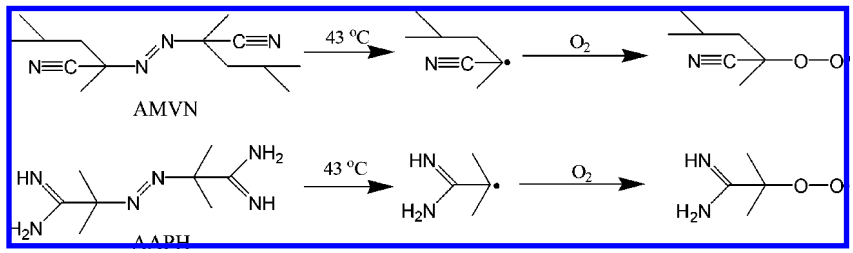
Determination of pK_a. The aqueous solutions of D and derivatives were prepared with Britton−Robinson buffers (1.0×10^{-5} M) in the pH range of 4.0−12.0, for which the ionic strength was controlled at 0.1 using NaCl. The pH was measured with a Mettler-Toledo pH meter (Process Analytics, Urdorf, Switzerland), and the UV−vis absorption spectra were recorded on a Cary 50 spectrophotometer (Varian, Walnut Creek, CA). The solution temperature was controlled at 25 °C using a RTE-110 thermostat (Neslab Instruments, Inc., Newington, NH) during the measurements of pH and optical absorption. The pH was standardized using international activity pH standards, and the so-called "mixed" constant was obtained from the relations, $K_a = a_{H+}(\text{base})/(\text{acid})$ and $pK_a = -\log(K_a)$, where a_{H+} was measured electrochemically and the concentrations of the corresponding acids and bases were measured spectrophotometrically.

For 7-Me-D and 4′-Me-D, the pK_a values were calculated from the experimental data by a linear regression with the relation of $A_{pH} = K_a(A_{base} - A_{pH})/a_{H+} + A_{acid}$. For D, the p$K_{a1}$ and pK_{a2} values were calculated on the basis of the absorbance at 336 nm using the method described in ref *31*.

Determination of Oxidation Potentials. Cyclic voltammetry was performed on a CHI 660A electrochemical analyzer (CH Instruments, Inc., Austin, TX) with a three-electrode configuration (*32*), for which D, 7-Me-D, and 4′-Me-D solutions at a concentration of 1.0×10^{-4} M (ethanol/water = 1:10; 0.5 M Tris buffer with varying pH) were used. The working electrode was a glassy carbon piece (diameter of 4 mm); the reference electrode was of the Ag/AgCl type (KCl saturated); and the counter electrode was a platinum wire. To expel oxygen, the solutions were bubbled with N_2 for 10 min before measurement.

Radical Scavenging Assay. The radical scavenging assay was based on the stoichiometry of the reaction of (iso)flavones with $ABTS^{\bullet+}$ (*33*). Potassium persulphate was added to a solution of 7 mM ABTS (final concentration of 2.45 mM), which was kept at a constant temperature for 12 h in the dark to generate $ABTS^{\bullet+}$ (*34*). The $ABTS^{\bullet+}$ was stable for two days in the dark. The final concentrations of $ABTS^{\bullet+}$ and isoflavone were 2.5×10^{-5} and 1.25×10^{-5} M, respectively. For

Scheme 2. Thermolysis Generation of AMVN and AAPH Radicals



kinetics measurements, $ABTS^{\bullet+}$ solution and antioxidant were filled in each syringe of a stopped-flow spectrometer (Biologic, Claix, France), and the reaction was monitored at the characteristic absorption wavelength of $ABTS^{\bullet+}$ (734 nm). The initial reaction rates under different pH were calculated following the rate equations given in ref *22*.

Evaluation of Antioxidation in Liposome. Liposome was prepared by using an extrusion method (*35*). Briefly, PC (0.68 mg) was dissolved in absolute chloroform (3 mL), and then D or derivatives dissolved in absolute ethanol (45 μL; the concentration of each isoflavone stock solution was 500 μM) were added to reach an isoflavone/PC molar ratio of 3% (the molecular mass of soybean PC was taken as 900). In experiments with the initiation of lipid oxidation in the lipid phase, the lipid-soluble radical generator AMVN in absolute ethanol (25 μL, 64.64 mM) was added. The solvent was then removed under reduced pressure by a rotary evaporator run at a water-bath temperature of 30 °C. Nitrogen gas was introduced to re-establish atmospheric pressure, and the flask was covered with aluminum foil. Then, an oil-free vacuum pump was used to maintain the flask vacuum at <0.5 mmHg for >1 h. The lipid residue was rehydrated with phosphate buffer (5 mL, 10 mM, pH 7.4). The flask was then shaken while being sonicated for 1 min, producing a homogeneous white suspension of multilamellar liposomes. Large unilamellar liposomes were obtained by pushing the multilamellar liposome solution through the polycarbonate membrane with 100 nm sieve pores (Whatman, Maidstone, U.K.) for 5 repeats, and then the water-soluble radical generator AAPH (25 μL, 150 mM) in sodium phosphate buffer (pH 7.4) was added for the initiation of lipid oxidation from the aqueous phase.

Lipid peroxidation was followed by monitoring the formation of conjugated dienes using the absorbance change at 234 nm (A_{234}). The unilamellar liposome suspension (3.5 mL) was pipetted into a quartz cuvette, thermostatted at 43 °C with a RTE-110 thermostat (Neslab Instruments, Inc., Newington, NH), and the change of A_{234} was monitored on a Cary 50 spectrophotometer (Varian, Walnut Creek, CA). The lag phase was determined as the evolution time to the point where a tangent to the propagation phase intercepted that of the initial phase with little or no oxidation (*35*).

Evaluation of Membrane Fluidity. The effects of D and derivatives on membrane fluidity were evaluated using the fluorescent probes 16-AP (*23*) and ANS (*36*). To evaluate the fluidity of membrane surfaces, an isoflavone (4.5 μM) was added to the liposome suspension (150 μM), and ANS in the stock phosphate buffer solution (10 mM, pH 7.4) was added to yield a final concentration of 25 μM. The preparation was incubated at 43 °C for 5 min. Fluorescence spectra (420−600 nm) were measured on a LS-55 luminescence spectrophotometer (Perkin-Elmer, Beaconsfield, U.K.), for which ANS was excited at 400 nm to minimize the isoflavone absorption. To evaluate the influence of D and derivatives on the fluidity of the interior of lipid bilayer, 16-AP was added to the PC solution during the preparation of the liposome (final concentration of 0.5 μM; 16-AP/PC molar ratio of 1:300) and the preparations were then obtained by adding 4.5 μM isoflavone and incubating at 43 °C for 5 min. Fluorescence polarization (P) was determined at 415 nm (excited at 380 nm) according to the relation (*27*)

$$P = (I_{||} - G \cdot I_{\perp})/(I_{||} + G \cdot I_{\perp}) \quad (1)$$

where $I_{||}$ and $I_{\perp}$, respectively, represent the fluorescence intensities measured with the emission polarization parallel and vertical to the excitation polarization and G stands for the instrumental polarization factor, which was determined to be ~1.

Calculation of the Dipole Moments. The geometry optimization of the radicals from AMVN or AAPH (**Scheme 2**) was performed using a density functional theory with Becher's three-parameter hybrid exchange functional (UB3LYP functional) at the 6-31g (d, p) level, and the quantum chemical calculations for the dipole moments of the radicals were performed with the Gaussian 03 program suite (*37*).

RESULTS AND DISCUSSION

pK_a Values and the Oxidation Potentials of Daidzein and Derivatives. The derivatives of D were synthesized by selective functionalization of the 7-O^- or 4′-O^- groups (**Scheme 1**). The difference between the pK_a values of 7-OH (7.47) and 4′-OH (9.65) of D is large enough to allow selective ionization of these two groups, on the basis of which the regioselective, methyl-substituted D derivatives were synthesized (*38, 39*). When 1 equiv NaOH was added, only the 7-OH of D was deprotonated and the reaction of 7-O^- with methyl iodide led to 7-Me-D, whereas when 2 equiv NaOH was added, both hydroxyls of D were deprotonated and 7,4′-diMe-D was obtained as the minor product via methyl substitution, together with 4′-Me-D as the major one.

The pH dependence of the UV−vis spectra of D shown in **Figure 1**A can be accounted for by the two acid/base equilibria of the 7- and 4′-OH groups. The pK_a values determined from **Figure 1**B for 7-Me-D (9.89 ± 0.05) and from **Figure 1**C for 4′-Me-D (7.43 ± 0.03) facilitate the assignment of the two pK_a values of D, i.e., $pK_{a1} = 7.47 \pm 0.02$ to the A-ring 7-OH and $pK_{a2} = 9.65 \pm 0.07$ to the B-ring 4′-OH. Notably, the pK_{a1} of D is almost identical to the pK_a of 4′-Me-D, indicating that the transformation of the B-ring to an anisole does not affect the deprotonation of the A-ring phenol. On the other hand, the pK_{a2} of D is slightly lower than the pK_a of 7-Me-D, suggesting that the negative charge left by the dissociation of the first proton enhances the ionization of the B-ring phenol probably through an extension of conjugation in the fully deprotonated form of D.

The oxidation potentials of D and derivatives in buffer with varying pH were determined as the spiking potentials from their cyclic voltamograms, and the results as are listed in **Table 1**. The oxidation potentials of D at pH 5.0 and 11.5 can be attribute to 4′-OH (0.69 V) and 4′-O^- (0.44 V), respectively, on the basis of a comparison to the oxidation potentials of 7-Me-D having only 4′-OH oxidizable. At pH 8.5, approximately 87% of the 7-OH of D are deprotonated ($pK_{a1} = 7.47$; see the inset of **Figure 1**A), and the oxidation potential of 0.49 V for D at this pH can be attribute to 7-O^- by comparison to the value of 0.50 V for 4′-Me-D at pH 11.5.

The above results prove that, for D at pH 7.4, the 7- and 4′-OH groups are both important as radical scavengers, because about a half of the 7-OH are in anionic form (7-O^-) and the reducing power of 7-O^- (0.49 V) is high enough to compete with that of the 4′-OH ($pK_a = 9.65$; 99% in neutral form with a potential of 0.69 V). Interestingly, for D in acidic form for which both 7-OH and 4′-

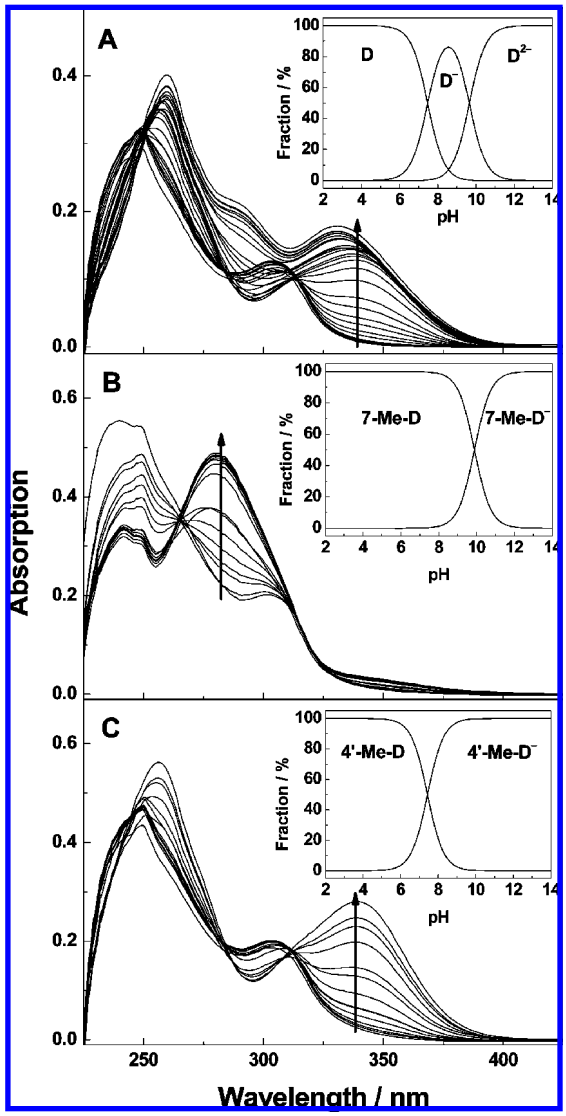


Figure 1. Room-temperature UV−vis spectra of (A) D, (B) 7-Me-D, and (C) 4′-Me-D in aqueous solution with varying pH under an ionic strength of 0.1. Arrows indicate the increase of pH. Acid−base fractional distribution diagrams are shown in the insets.

Table 1. Oxidation Potentials (versus Ag/AgCl; in Aqueous Phase, Ag/AgCl $E^0 = 0.2223$ V/SHE) for D and Derivatives in Aqueous Solution (Water/Methanol = 10:1; 1×10^{-4} M) of Varying pH at 25 °C[a]

	oxidation potential (V)		
pH	D	7-Me-D	4′-Me-D
5.0	0.69 (4′-OH)	0.71 (4′-OH)	0.92 (7-OH)
8.5	0.49 (7-O$^-$)		
11.5	0.44 (4′-O$^-$)	0.46 (4′-O$^-$)	0.50 (7-O$^-$)

[a] Assignments of oxidation potentials to specific hydroxyls are given in parentheses.

OH should be oxidizable, only oxidation of one phenol, i.e., 4′-OH with the lower oxidation potential, could be seen in the cyclic voltammagram. For D^- at pH 8.5, only oxidation of 7-O^- was detected, while for D^{2-} at pH 11.5, only oxidation of 4′-O^- was detected. These observations suggest that charge distribution in the D radical formed by oxidation of one phenol strongly affect the oxidation property of the other. Thus, it may be concluded that the D radical following one-electron transfer reaction becomes strongly stabilized by resonance, and as the result, further oxidation is hampered.

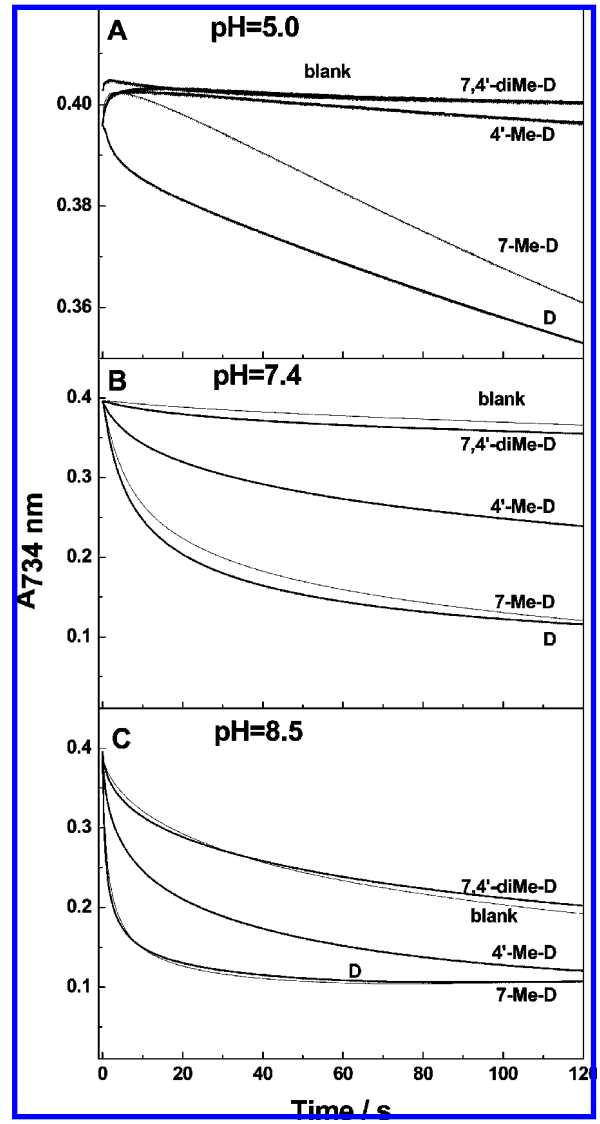


Figure 2. (A−C) Room-temperature stopped-flow kinetics of ABTS$^{\bullet+}$ scavenged by D, 4′-Me-D, 7-Me-D, and 7,4′-diMe-D in aqueous solutions of indicated pH values.

Radical Scavenging and Antioxidant Activities of Daidzein and Derivatives. Scavenging of the metastable ABTS$^{\bullet+}$ by D and derivatives in aqueous solution were evaluated using the stopped-flow technique, and the kinetic traces are shown in **Figure 2**. A comparison of parts A−C of **Figure 2** reveals the effect of pH, i.e., the decay of ABTS$^{\bullet+}$ in the presence of isoflavones became faster with increasing pH, which may be explained by the decreased oxidation potentials of D upon deprotonization (**Table 1**). A similar tendency in the decay kinetics is seen in the absence of isoflavones (blank), which may be due to the reduction of ABTS$^{\bullet+}$ by OH^- in the phosphate buffer at a high pH. The following two reactions, respectively, have been assigned as rate-determining for the radical scavenging by phenols under acidic and basic conditions (*40*):

$$\mathrm{ABTS}^{\bullet+} + \mathrm{D{-}OH} \rightarrow \mathrm{ABTS} + \mathrm{D{-}O}^{\bullet} + \mathrm{H}^+ \quad (2a)$$

$$\mathrm{ABTS}^{\bullet+} + \mathrm{D{-}O}^- \rightarrow \mathrm{ABTS} + \mathrm{D{-}O}^{\bullet} \quad (2b)$$

At pH 5.0 when D or derivatives stay mostly in their neutral forms, the initial rates for the scavenging reactions in **Table 2** show that 7-Me-D (B-ring phenol) has a much higher rate of scavenging than 4′-Me-D (A-ring phenol). At higher pH 7.4

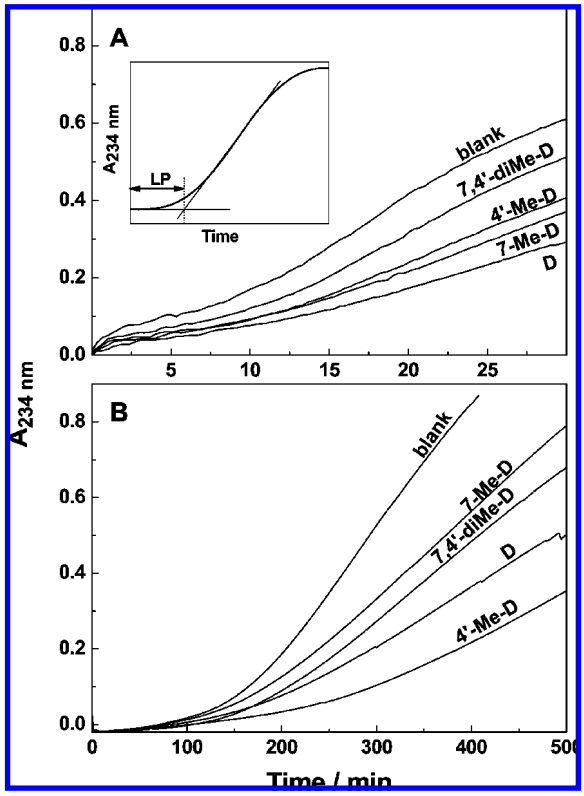


Figure 3. Change in absorbance at 234 nm of liposomes containing 4.5 μM antioxidants with (A) water-soluble AAPH (750 μM) or (B) lipid-soluble AMVN (320 μM) as the initiators of lipid oxidation. The inset in A schematically shows the method of lag-phase (LP) determination.

Table 2. Initial Rates of Scavenging $ABTS^{\bullet+}$ by D, 7-Me-D, 4′-Me-D, and 7,4′-diMe-D in Aqueous Solution of Varying pH as Derived from the Stopped-Flow Kinetics

	rate ($M\ s^{-1}$)		
sample	pH 5.0	pH 7.4	pH 8.5
blank	6.0×10^{-11}	3.5×10^{-8}	1.3×10^{-6}
D	1.1×10^{-7}	1.9×10^{-6}	1.4×10^{-5}
7-Me-D	1.0×10^{-7}	1.5×10^{-6}	1.1×10^{-5}
4′-Me-D	1.4×10^{-8}	5.1×10^{-7}	4.3×10^{-6}
7,4′-diMe-D	1.1×10^{-9}	8.0×10^{-8}	1.5×10^{-6}

(8.5) when the deprotonation fraction of the 7-OH group is 50% (87%), the rate of scavenging of 7-Me-D is still higher than that of 4′-Me-D, although the oxidation potential of 7-O^- is lower than the significantly less deprotonated 4′-OH (**Table 1**). These results lead to the conclusion that, for radical scavenging by D in aqueous solution, the B-ring phenol is more efficient than the A-ring one. It is intriguing to see from **Figure 2** and **Table 2** that the scavenging efficiency of D, despite two hydroxyls, is comparable to that of 7-Me-D, which can be explained by the aforementioned one-electron oxidation mechanism in combination with resonance stabilization of the resulted D radical.

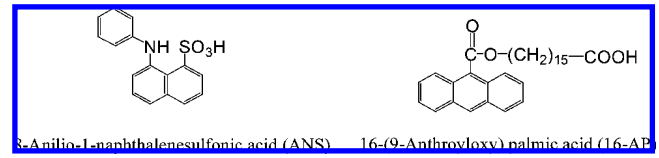
Lipid peroxidation of the PC liposomes at pH 7.4 was induced by the radicals derived from thermolysis of the lipid-soluble AMVN or the water-soluble AAPH (**Scheme 2**). In either case, the lag phase (LP; see the inset of **Figure 3**A) was used to evaluate the antioxidation performance of the isoflavones.

It is seen from **Figure 3**A and **Table 3** for oxidation triggered by AAPH in the aqueous phase that the rank of antioxidation efficiency of D and derivatives is consistent with that of these compounds in scavenging of $ABTS^{\bullet+}$ in aqueous solution (pH 7.4), i.e., D > 7-Me-D > 4′-Me-D > 7,4′-diMe-D. This is rather reasonable in view of the oxidation potentials and the number of hydroxyl groups of the isoflavones. We had confirmed that the antioxidation activities of D and derivatives with the same AAPH concentration but slightly lower isoflavone concentration (3.0 μM), and the same rank held though the differences among the LPs of different isoflavones were smaller. To our surprise, as seen from **Figure 3**B and **Table 3** for the oxidation initiated by AMVN in the lipid phase, 4′-Me-D is the most efficient antioxidant and the rank becomes 4′-Me-D > D > 7,4′-diMe-D > 7-Me-D. Interestingly, 7,4′-diMe-D without any phenolic groups seems a better antioxidant than 7-Me-D, an observation to be explained in terms of the spatial disposition of radicals and antioxidants in the PC bilayer as well as the heterogeneity of the membrane fluidity induced by D and derivatives (*vide infra*).

Table 3. LP Determined by Spectrophotometry of the Conjugated Dienes (cf. **Figure 3**) as a Result of the Oxidation of Soybean Phosphatidylcholine Induced at 43 °C by Radical Initiators in Aqueous Phase (AAPH) or Lipid Phase (AMVN)

	lag phase (min)	
sample	AAPH (water-soluble)	AMVN (lipid-soluble)
blank	13.6	146.8
D	20.1	192.6
7-Me-D	18.9	158.4
4′-Me-D	17.1	251.7
7,4′-diMe-D	15.6	173.3

Scheme 3. Molecular Structures of Fluorescent Probes



8-Anilio-1-naphthalenesulfonic acid (ANS) 16-(9-Anthroyloxy) palmic acid (16-AP)

Interaction between Daidzein or Derivatives and Membrane. To understand the antioxidation behavior of D and derivatives in liposomes, the interactions between antioxidants and membrane must be taken into consideration, because the distribution and orientation of antioxidants in liposome and the modification that they brought to the membrane fluidity would significantly influence their antioxidation reactivities (*23*−*25*). To investigate these effects, we made use of the fluorescent probes ANS and 16-AP (**Scheme 3**), which distribute in different regions of the membrane; i.e., ANS stays preferentially at the surface, whereas the fluorophore of 16-AP locates mainly in the interior of the bilayer.

The membrane surface with higher rigidity incorporates more ANS molecules and, therefore, results in more intense fluorescence from ANS (*41*). As seen from **Figure 4**A, the addition of D and 4′-Me-D to PC liposome enhanced the fluorescence of ANS, whereas little fluorescence change by 7,4′-diMe-D and 7-Me-D was observed. Because ANS was excited at 400 nm, where D or its derivatives have little absorption (**Figure 1**), the ANS fluorescence enhancement must be induced by the increase in ANS molecules incorporated by the membrane. These results suggest that the concentration of D or derivatives in the proximity of the membrane surface follows an order of D > 4′-Me-D > 7-Me-D > 7,4′-diMe-D, a sequence which agrees with that of the increase in the lipophilicity of these isoflavones.

The fluorescence polarization of 16-AP as defined in eq 1 is known to increase with the decrease in membrane fluidity (*27*). The fluorophore of 16-AP is at the 16 position of the fatty acyl chain (**Scheme 3**) and will accordingly locate at the central

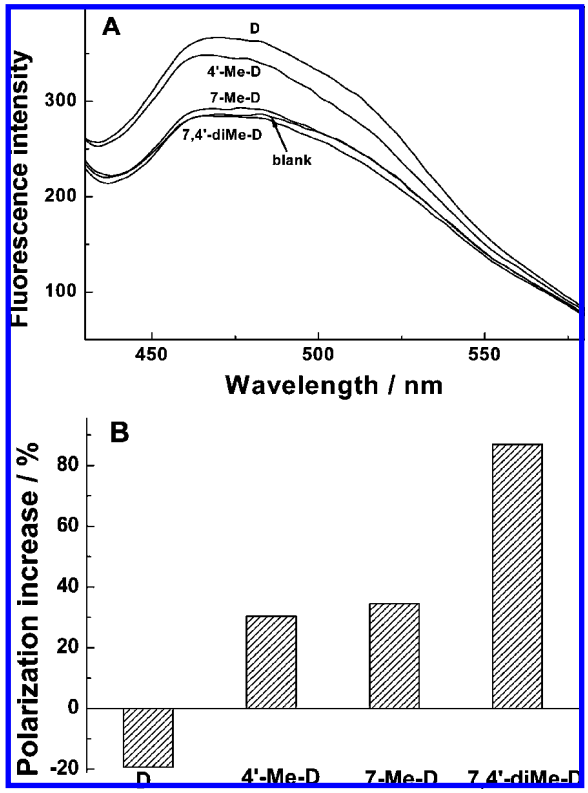


Figure 4. (A) Fluorescence spectra of liposomes suspension containing ANS fluorophore (25 μM) and D or derivatives (4.5 μM). (B) Fluorescence polarization increase of 16-AP (0.5 μM; cf. eq 1) as a function of the addition of D or derivatives (4.5 μM). Liposome preparations containing the fluorescent probes 16-AP were incubated at 43 °C.

region of the PC bilayer. Because D or derivatives incorporated in the hydrophobic region of the PC bilayer would increase the stiffness there, the amplitude of fluorescence polarization of 16-AP thus reflects the concentration of these compounds in its proximity (*42*). As shown in **Figure 4**B, the order of polarization increase induced via isoflavone incorporation follows 7,4′-diMe-D > 7-Me-D > 4′-Me-D > D, reflecting the concentration of these isoflavones at the central domain of the bilayer. These results prove that 7,4′-diMe-D is predominantly in the central region of the bilayer, whereas 7-Me-D locates mainly in the hydrophobic region of PC and less in the membrane surface (**Scheme 4**). This tendency agrees with the aforementioned concentration distribution reported by the ANS probe.

In liposome solution, the pK_a values of the 7-OH of D or 4′-Me-D are both close to 7.4; thus, at this value of pH, about a half amount of 7-OH groups become ionized. As illustrated in **Scheme 4**, the affinity between 7-O$^-$ and the hydrophilic head group of PC makes D-O$^-$ and 4′-Me-D-O$^-$ preferentially adsorb to the liposome surface, creating an environment rich in D and 4′-Me-D. Such an enrichment could limit the access of oxidants to the bilayer and control the rate of propagation of free-radical chain reactions occurring in the center of membranes. D-O$^-$ has the additional polar group 4′-OH and will interact more strongly with the membrane surface than a derivative. Because 4′-Me-D is substituted with a hydrophobic methyl group, it will insert relatively deeper toward the hydrophobic region of the PC bilayer. On the other hand, 7-Me-D and 7,4′-diMe-D are present in the central region, with 7-Me-D interacting relatively stronger with the membrane surface because of its polar 4′-OH group.

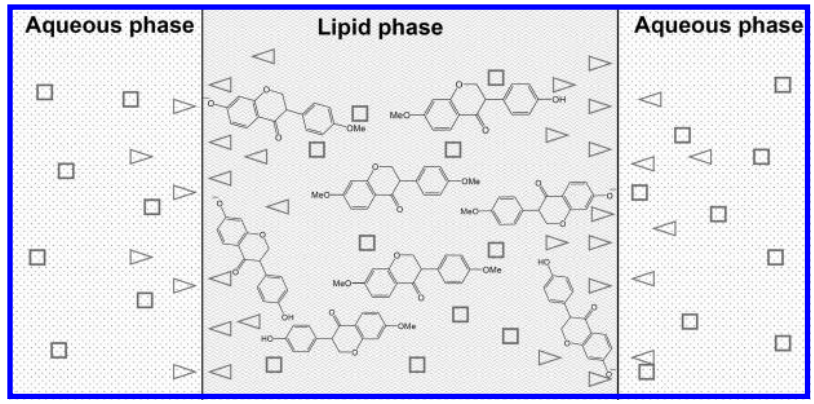
AMVN and AAPH on thermolysis decompose into free radicals inducing lipid oxidation (*43*). The radicals generated from AMVN or AAPH have the dipole moments of 3.71 or 1.91 D, respectively, as obtained from the quantum chemical calculations based on the chemical structures shown in **Scheme 2**. The radical from AMVN with higher polarity is prone to be adsorbed on the liposome surface, whereas that from AAPH with considerably lower polarity may diffuse more freely within the PC bilayer (**Scheme 4**), as also supported by the nearly 10-fold reduced LP compared to the case of AMVN (**Table 3**). With these in mind, we would like to discuss below the mechanisms of retardation of AMVN- and AAPH-induced peroxidation by the isoflavones.

Daidzein, 7-Me-D, and 4′-Me-D have been shown to scavenge ABTS$^{\bullet+}$ with the rates mainly associated with the oxidation potentials and further dependent upon pH. In comparison to the homogeneous solution, the reactivities of D and derivatives in liposome rely on, besides their oxidation potentials, the spatial distribution in the membrane and the membrane fluidity. For AMVN-triggered lipid oxidation, the radical will approach closely the surface of liposomes, where D and 4′-Me-D are concentrated. The 4′-Me-D penetrating relatively deeper into the membrane induces a decrease in the fluidity of the hydrophobic domain of PC and, therefore, restricts the diffusion of AMVN free radical, in effect decreasing lipid oxidation. This mechanism explains why 4′-Me-D acts as the most efficient antioxidant compared to the others under the actual conditions. 7,4′-DiMe-D decreases the fluidity of the central region more than 7-Me-D and, accordingly, behaves as a more efficient antioxidant. The AAPH radical is more uniformly distributed across the membrane, owing to its low polarity; therefore, for lipid oxidation initiated by AAPH, the relative antioxidation efficiency of the flavones in liposome resemble the rank of scavenging ABTS$^{\bullet+}$ in homogeneous solution (D > 7-Me-D > 4′-Me-D > 7,4′-diMe-D).

Guo and co-authors reported, in an electron spin resonance (ESR) study, that D in homogeneous solution with a concentration up to 1 mM exhibited no significant scavenging effects on hydroxyl, superoxide, nitric oxide, galvinoxyl, and lipid-derived radicals initiated with AMVN (*13*). The present work, however, shows that D and derivatives at a rather low concentration (12.5 μM) can efficiently scavenge ABTS$^{\bullet+}$ in aqueous solution and that the protection against AMVN-initiated lipid oxidation functions well at an even lower concentration of D (4.5 μM). Thus, apparent discrepancies exist between our results and Guo and co-authors' with regard to the radical scavenging and antioxidation activities of D. We suggest that, besides the detection sensitivity of different spectroscopic methods, the significant difference in the physicochemical environments between homogeneous solution and the liposome phase may be one of the origins.

Preferential accumulation of D to the interface of aqueous and lipid phases results in local enrichment of D; this may offer effective protection against AMVN-induced lipid peroxidation under a rather low concentration of D (4.5 μM). The same mechanism may also be in operation in biological systems to preserve the structure and function of biological membranes by offering appreciable level of protection against lipid peroxidation induced by external or internal aggressors (oxidants). It was reported that flavonoids could protect neurons against oxidative stress more effectively than ascorbate, even when the latter was kept at a 10-fold higher concentration, and that the antioxidation activity of flavonoids was relevant to their specific structural characteristics, particularly to those defining their reduction potentials and partition coefficients and those affecting their interactions with the microenvironments (*44*). In a dietary

Scheme 4. Schematic Illustration of the Distribution of D and Derivatives and the Radicals Derived from AMVN (Tilted △) or AAPH (□) in the PC Bilayer of the Liposome



intervention study subjected to model animals, consumption of soya protein products containing D led to its mean plasma level of 0.32 μM. In addition, the bioaccumulation of D into low-density lipoprotein (LDL) particles and into lipophilic atheromal plaques associated with the arterial wall was suggested (*9*). Therefore, the antioxidation activity of (iso)flavone, influenced by its redox property and its interaction with the microenvironment, may be of significant physiological importance *in vivo*.

CONCLUSIONS

The present work has demonstrated that the microenvironments of D or derivatives influence the efficiency of these isoflavones as antioxidants. In homogeneous solution, the fractional acid/base forms and the scavenging rate of radicals, such as $ABTS^{\bullet+}$, depend upon pH. The 4′-OH associated with the B-ring of D was found to be the key functional group for radical scavenging, in agreement with its low oxidation potential. In liposome, significant differences are found in the spatial distribution of D and derivatives, which together with the modification of membrane fluidity by the incorporation of these compounds considerably affect their antioxidation efficiency. Deprotonation of 7-OH enables D to interact with the polar head of PC and, therefore, to approach the membrane surface, while the insert of the isoflavone B-ring in the hydrophobic domain of the bilayer restricts the diffusion of free radicals therein because of radical scavenging. In conclusion, the antioxidative effect of D or derivatives as examples of isoflavones comprehensively depends upon the rate with which they may scavenge the free radicals involved in lipid oxidation, their spatial distribution in the lipid bilayer, and the local modification of the membrane fluidity that they induced.

ABBREVIATIONS USED

AAPH, 2,2′-azobis(2-methylpropionamidine) dihydrochloride; $ABTS^{\bullet+}$, radical cation of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic); AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); ANS, 8-anilino-1-naphthalenesulfonic acid; 16-AP, 16-(9-anthroyloxy) palmitic acid; D, daidzein; GSH, reduced glutathione; 7-Me-D, 7-methyl daidzein; 4′-Me-D, 4′-methyl daidzein; 7,4′-diMe-D, 7,4′-dimethyl daidzein; P, polarization; PC, soybean L-*R*-phosphatidyl choline; ROS, reactive oxygen species.

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