

Baicalin in Radical Scavenging and Its Synergistic Effect with β -Carotene in Antilipoxidation

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The lipophilic flavonoid glycoside baicalin from the traditional oriental herb *Scutellaria baicalensis* Georgi ($\log P = 1.27$, $pK_{a1} = 7.6$, $pK_{a2} = 10.1$ as determined at 25 °C in 0.1 M NaCl) is found to be as reducing (0.39 V vs NHE, reversible two-electron oxidation by CV at pH 7.4) as other catechol flavonoids but a poor radical scavenger (TEAC = 1.12, pH 7.4) and a poor antioxidant against free radical initiated lipid oxidation in liposomes. However, this compound is able to regenerate β -carotene (β -Car) from β -Car^{•+} with a second-order rate constant of $(5.6 \pm 0.5) \times 10^9 \text{ L mol}^{-1} \text{ s}^{-1}$ in the methanol/chloroform binary solvent (1:9, v/v) and, more importantly, to exhibit a prominent synergistic effect with β -Car against the lipoxidation induced by AMVN-derived peroxy radical in liposomal membrane. Thus, baicalin by itself is not an effective antioxidant, but it becomes one via interaction with β -Car. The radical scavenging and antilipoxidation properties of baicalin are discussed in terms of its physicochemical properties and molecular structures.

KEYWORDS: Baicalin; β -carotene; antioxidation; radical scavenging; synergism

INTRODUCTION

Flavonoids are a group of polyphenolic compounds presenting ubiquitously in plants. Much epidemiological research has shown that a high intake of flavonoids via the consumption of fruits and vegetables is inversely correlated to the risk of cardiovascular diseases and cancers (1, 2). One of the flavonoids currently drawing much research interest is baicalin (β -D-glucopyranosiduronic acid, 5,6-dihydroxy-4-oxo-2-phenyl-4H-1-benzopyran-7-yl; **Scheme 1**) extracted from the radix of *Scutellaria baicalensis* Georgi (3), a traditional oriental herbal medicine with anti-inflammatory properties and also used for the treatment of hepatitis, tumors, and diarrhea.

Baicalin, the 7-O-glucuronide of baicalein, is known to be a poor superoxide radical scavenger and xanthine oxidase inhibitor (4, 5). In contrast, baicalein, an initial metabolite of baicalin formed by losing the glycoside moiety (6), possesses three adjacent hydroxyl groups and exhibits high activities in the inhibition of xanthine oxidase, radical scavenging, and metal ion chelation, as well as in antioxidation (5, 7). On the other hand, baicalin was shown to be a non-nucleoside reverse transcriptase inhibitor, as well as an effective anti-HIV agent inhibiting the infectivity and replication of the virus (8–10). It was recently found that baicalin can protect DNA against chemically induced damage (11–13), which is especially interesting from the pharmaceutical point of view.

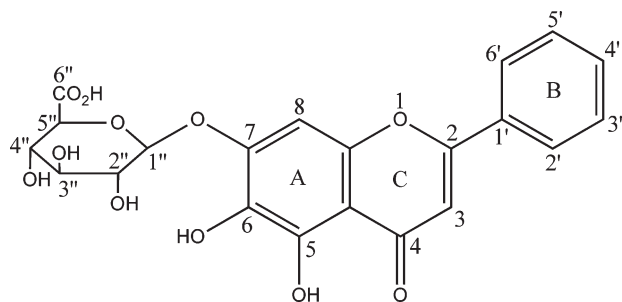
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This work is intended to elucidate the structure–activity relationship of baicalin as a potential natural antioxidant and to examine its possible interaction with the lipophilic antioxidant β -carotene (β -Car) by means of the experimental and computational methods recently developed for the study of the isoflavone C-glycoside puerarin (14–16). To this end the physicochemical properties of baicalin such as pK_a , \log_{10} partition coefficient, and oxidation potential and its radical scavenging activities in aqueous solution were investigated systematically. On the basis of these results the interaction of baicalin with β -Car both in liposome and in solution was further studied. It is shown that baicalin by itself is rather ineffective in antilipoxidation; however, when combined with β -Car, it shows a significant synergistic antilipoxidation effect.

MATERIALS AND METHODS

Chemicals and Baicalin Sample Preparation. Baicalin (>98%) was purchased from the China National Institute for the Control of Pharmaceutical and Biological Products. β -Car (>95%), 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2'-azobis(2-methylpropionamide) dihydrochloride (AAPH), and 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN) were from Sigma (St. Louis, MO). Potassium persulfate was supplied by Beijing Chemical Plant (Beijing, China). Methanol (HPLC grade, Caledon Laboratories Ltd., Ontario, Canada) and *n*-octanol (AR; Beijing Chemical Plant, China) were used as received, whereas chloroform (AR; Beijing Chemical Plant) was purified by distillation before use. To increase the solubility in aqueous solution, baicalin predissolved in dimethyl sulfoxide (DMSO, AR; Beijing Chemical Plant) was added to ion-exchanged water from a Milli-Q device (Millipore Corp., Billerica, MA). For laser flash photolysis, baicalin conjugated bases, baicalin⁻ or baicalin²⁻, dissolved in methanol/chloroform binary solvents were prepared by adding 1 equiv

Scheme 1. Chemical Structure of Baicalin



or an excess amount of tetramethylammonium hydroxide ($\text{Me}_4\text{N}^+\text{OH}^-$, 97%, Sigma).

Molar Extinction Coefficient, \log_{10} Partition, and $\text{p}K_a$. To determine the molar extinction coefficient (ϵ), baicalin samples weighed with an accuracy of 0.01 mg using an AB-135S balance (Mettler Toledo, Urdorf, Switzerland) were predissolved in 0.5 mL of DMSO and then diluted with ion-exchanged water to 100 mL. UV-visible spectra of the preparations with various baicalin concentrations were recorded with a standard quartz cuvette (optical path length, 1 cm) on a Cary50 spectrophotometer (Varian Inc., Palo Alto, CA), and ϵ was determined on the basis of the Lambert–Beer law by linear regression fitting to the data of absorbance versus absolute sample concentration.

UV-visible absorption spectra of baicalin in *n*-octanol were measured before and after the extraction with aqueous phase, and the \log_{10} partition coefficient ($\log P$) was obtained using the relationship

$$\log P = \log \frac{[\text{B}]_{\text{org}}}{[\text{B}]_{\text{aq}}} = \log \frac{[\text{B}]}{[\text{B}]_0 - [\text{B}]} \quad (1)$$

where subscripts org and aq stand for the organic and aqueous phases respectively, and $[\text{B}]_0$ and $[\text{B}]$ for the concentration of baicalin in the organic phase before and after the aqueous extraction, respectively.

To determine $\text{p}K_a$, baicalin (5.0×10^{-5} M) in Britton–Robinson buffer (0.1 M) was prepared over a pH range of 2.0–12.0 with the ionic strength controlled at 0.1 M using NaCl (17). During the spectroscopic and pH measurements samples were regulated at 25 °C by the use of a water-flow type thermostat (RTE-100, Neslab Instruments Inc., Newington, NH). The $\text{p}K_a$ values were determined by fitting the data of absorbance (at 420 nm) against pH to the relationship derived from the acid–base equilibrium (18)

$$A = c_1 \frac{[\text{H}^+]^2}{[\text{H}^+]^2 + [\text{H}^+]K_1 + K_1K_2} + c_2 \frac{[\text{H}^+]K_1}{[\text{H}^+]^2 + [\text{H}^+]K_1 + K_1K_2} + c_3 \frac{K_1K_2}{[\text{H}^+]^2 + [\text{H}^+]K_1 + K_1K_2} \quad (2)$$

where K_1 and K_2 are the two dissociation constants and c_1 , c_2 , and c_3 , respectively, are fitting parameters related to the molar absorptivity at 420 nm for baicalin, baicalin⁻, and baicalin²⁻.

Electrochemistry. Before being subjected to cyclic voltammetry, baicalin (3.6×10^{-5} M) in Britton–Robinson buffer (0.1 M, pH 2.0–12.0) at controlled ionic strength was bubbled with high-purity nitrogen for 15 min, and the measurements were performed at 25 °C on an electrochemical workstation (Epsilon BAS C3, Bioanalytical Systems Inc., West Lafayette, IN) in a three-electrode configuration with a glassy carbon working electrode (diameter, 5 mm), a platinum counter electrode, and a standard Ag|AgCl|KCl (1.0 M) reference electrode (0.2224 V vs standard hydrogen electrode (NHE)).

Trolox Equivalent Antioxidant Capacity (TEAC). ABTS⁺ solution was prepared by dissolving 20 mg of ABTS in 5.0 mL of deionized water and subsequent oxidization with 3.5 mg of potassium persulfate (19). The final concentrations of baicalin and ABTS⁺ were 1.0×10^{-5} and 5.0×10^{-6} – 6.0×10^{-5} M, respectively. The reaction kinetics of scavenging ABTS⁺ were followed by monitoring its characteristic absorption at 734 nm ($\epsilon_{734} = 1.5 \times 10^4$ L mol⁻¹ cm⁻¹) (20). The absorbance difference before and after reaction (ΔA_{734}) was plotted against the ABTS⁺

concentration c and was fitted to the relationship $\Delta A_{734} = a(1 - e^{-bc})$. The TEAC of baicalin was then obtained as $\text{TEAC} = a/(\epsilon_{734}c_{\text{baicalin}} \times 1.9)$, where 1.9 was the antioxidant capacity of Trolox (19).

Antilipoxidation Activity of Baicalin and Its Synergistic Interaction with β -Car. Liposome was prepared following the method of ref 21 with certain modifications. Briefly, soybean lecithin (PC), L- α -phosphatidylcholine (Sigma) in CHCl_3 (0.75 mM), was mixed with methanol, and AMVN in ethanol was added when needed. Solvents were removed under reduced pressure by using a rotary evaporator at the bath temperature of 30 °C. Afterward, high-purity nitrogen was introduced to re-establish the atmospheric pressure. The flask was covered with aluminum foil and kept under vacuum for 1.5 h.

The lipid residues were rehydrated with 0.01 M sodium phosphate buffer (pH 7.40) under sonication, and then an aqueous solution of baicalin (1.0×10^{-4} M) or the DMSO solution of β -Car (1.0×10^{-4} M) was added. AAPH in sodium phosphate buffer was added when needed. The preparation was passed through hydrophilic polyether sulfone membranes (200 nm pore size) for 20 repeats with Acrodisc syringe filters (Pall Corp., East Hills, NY). The final concentration of lecithin was 0.15 mM, and those of AMVN and AAPH were 0.32 and 0.20 mM, respectively. The final β -Car or baicalin concentration was 1% of the molar fractions of lecithin.

Following ref 22, the time evolution profiles of AMVN- or AAPH-induced lipid peroxidation were traced by the absorbance of conjugated diene at the characteristic wavelength of 234 nm, and 3.0 mL of the suspension in a 1 cm quartz cuvette was thermostated at 43 °C during the measurements. Liposomal preparation without antioxidant was used as a reference. The lag phase time (LP, in min) was determined as the time elapsed to the intersection of the tangent of the propagation phase and that of the lag phase and was corrected by subtracting the LP of the reference. The interaction between baicalin and β -Car with equal concentration was studied, and the synergistic effect could be confirmed when the relationship $\text{LP}_{\text{baicalin}+\beta\text{-Car}} > \text{LP}_{\text{baicalin}} + \text{LP}_{\beta\text{-Car}}$ was satisfied.

Laser Flash Photolysis. The experimental setup has been described in detail elsewhere (14, 23). Briefly, the pump laser pulse with 10 ns duration at 532 nm was supplied by a Nd:YAG laser operated at a repetition rate of 10 Hz (Quanta-Ray Pro-Series, Spectra Physics Lasers Inc., Mountain View, CA), and the pulse energy to excite sample was ~ 5 mJ. β -Car and baicalin or baicalin⁻ dissolved in the methanol/chloroform binary solvent (1:9, v/v) were kept in quartz cuvettes (optical path length, 1 cm) and stirred by the use of a magnetic stirrer. Near-infrared kinetics probed at 950 nm for β -Car radical cation ($\beta\text{-Car}^{*+}$) was detected with an avalanche photodiode (model C5460, Hamamatsu Photonics, Hamamatsu, Japan) attached to a TriVista spectrograph (Princeton Instruments, Trenton, NJ), and the kinetics traces were stored and averaged with a digital oscilloscope (bandwidth 200 MHz; model DSI202CA, Rigol Technologies Inc., Beijing, China) connected to a personal computer. For kinetics analyses, the time evolution profiles of absorbance change (ΔA) upon pulsed excitation were fit to a monoexponential decay function, $\Delta A(t) = a + b \times \exp(-kt)$, where a is the baseline parameter and k is the pseudo-first-order rate constant. The program of least-squares curve fitting was coded on the basis of Matlab 5.2 (Mathworks Inc., Natick, MA), and the fitting goodness was evaluated by the use of χ^2 statistics.

Quantum Chemical Calculations. The molecular geometries of baicalin and its conjugated bases were optimized with the B3LYP density functional theory in conjunction with the 6-31G(d,p) basis set by the use of the Gaussian 03 package (24). Gas-phase deprotonation enthalpy (DE) and bond dissociation enthalpy (BDE) were calculated, respectively, as the enthalpy differences of the processes $\text{ArOH} \rightarrow \text{ArO}^- + \text{H}^+$ and $\text{ArOH} \rightarrow \text{ArO}^\bullet + \text{H}^\bullet$ (14).

RESULTS AND DISCUSSION

Physicochemical Properties. Figure 1A shows the change of absorption spectra of baicalin aqueous solution as a function of pH. The absorption spectra vary little for pH below 7.0, all of which show two maxima at 276 and 317 nm. For pH above 7.0, the 276 nm band and the 317 nm one undergo red and blue shifts, respectively, and both experience profound absorptivity variation. In addition, a new band appears around 420 nm, which may originate from the absorptive transition with intramolecular

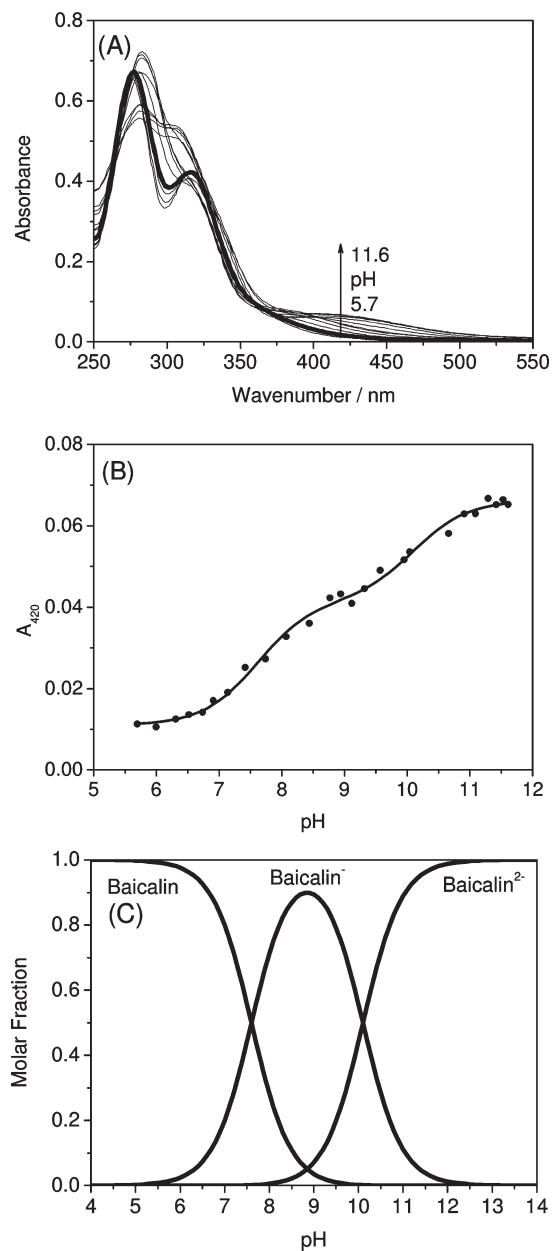


Figure 1. (A) Selected UV–visible absorption spectra of baicalin in aqueous solutions of various pH values (3.0×10^{-4} M; ionic strength, 0.1 mol kg^{-1}). Arrow shows the direction of pH increase. Bold line indicates the spectrum at pH 7.0. (B) Change of absorbance at 420 nm (A_{420}) as a function of pH. Solid curve is the result of least-squares fitting to eq 2. (C) Molar fractions of baicalin and its conjugated bases in aqueous solutions of various pH values.

charge transfer character owing to the electron-donating properties of phenolates. With increasing pH, the spectral changes in the region of 250–350 nm are due to the transformation of acidic baicalin into its conjugated bases, that is, baicalin[−] (6-O[−]) or baicalin^{2−} (5-O[−], 6-O[−]) (vide infra).

The plot of absorbance change at 420 nm against pH in **Figure 1B** allows us to determine the dissociation equilibrium constants (pK_a) of the A-ring phenolic hydroxyls (eq 2), and two values, $pK_{a1} = 7.6 \pm 0.1$ and $pK_{a2} = 10.1 \pm 0.1$, thus obtained are assigned to 6-OH and 5-OH, respectively, on the basis of the following considerations. (i) Wolniak and coauthors have recently proven by the use of 2D NMR spectroscopy that, for baicalin in solid state or in DMSO solution, an intramolecular

hydrogen bond 5—OH...O=4 exists (25), meaning that the 5-OH in acidic form is stabilized by this specific interaction. (ii) Our quantum chemical calculations show that the DE of 5-OH is $\sim 28 \text{ kJ mol}^{-1}$ higher than that of 6-OH.

At pH 5.7 the fraction of baicalin with both A-ring hydroxyls in acidic form is $\sim 99\%$ (**Figure 1C**), and the corresponding absorption spectrum is almost identical to that at pH 7.0. The molar extinction coefficients of the two absorption maxima are $\epsilon_{276} = 2.2 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$ and $\epsilon_{317} = 1.4 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$, respectively. Here, we note that even at pH 5.7, the 7-glucuronic acid could be ionized at the 6''-carboxyl because the pK_a of this type of carboxyl is ~ 5.0 (26). However, this ionization would not affect the spectrum of UV–visible absorption originating from the conjugated ring system, as also confirmed by a comparison to the spectrum recorded at pH 2.0 when the 6''-carboxyl was in acidic form.

Figure 2A shows the cyclic voltammograms of baicalin in aqueous solutions with various pH values, and the cathodic and anodic peak potentials determined from the voltammetric peaks are plotted against pH in **Figure 2B**. The presence of both oxidation and reduction peaks suggests the reversibility of electrode reactions. It is seen in **Figure 2B** that as the pH increases, both the oxidation and the reduction peak potentials decrease linearly. Linear regression of the Nernst equation to the data derives the relations $0.629 - 0.059\text{pH}$ for the reductive electrode reaction and $0.610 - 0.061\text{pH}$ for the oxidative one (vs Ag|AgCl|KCl (1.0 M)), both of which show a slope of $\sim 59 \text{ mV}$. This behavior indicates the attendance of H⁺ in the reactions, which is commonly seen for a normal proton-dependent electrode reaction, for example, the oxygen electrode. For pH > 9.0, the difference between the oxidation and the reduction potentials slightly increases, probably owing to the more easily oxidizable phenolic hydroxyls and/or the less reversible electrode reactions. At low pH when baicalin is mostly in acidic form the oxidation peak is seen as single band (**Figure 2A**), and the difference in peak potentials becomes smaller (**Figure 2B**). The approximate 28 mV separation of oxidative and reductive peaks suggests that the two A-ring hydroxyls are oxidized or reduced simultaneously (two-electron transfer mechanism), which may be understandable in view of the *o*-diphenols substituting in the same A-ring. Here, it is worth noting that the tendency of pH independence above the pH of pK_a observed for a variety of para-substituted phenols (27) driven by one-electron electrode reaction is not seen in the present case of baicalin, where the reduction or oxidation potentials hold a linear relationship over the entire pH range even for pH values above pK_{a2} .

Baicalin ABTS^{•+}-Scavenging Capacity and Its Synergistic Interaction with β -Car against Lipid Peroxidation. The TEAC of baicalin in aqueous solution was determined to be 1.12 ± 0.06 (pH 7.4), indicating that its ABTS^{•+}-scavenging capacity is rather low. Compared to baicalin, baicalein with an A-ring hydroxyl 7-OH instead of a 7-O-glycoside shows a much higher TEAC of 2.0 ± 0.1 (pH 7.4). The different radical scavenging capacities may be partially due to structural reasons. For baicalin the steric hindrance of the 7-O-glycoside moiety may prevent ABTS^{•+} from accessing the active sites, whereas for baicalein an additional hydroxyl (7-OH) tends to enhance its radical scavenging capacity because flavonoids with more phenolic hydroxyls are known to be better radical scavengers (28).

The antioxidant activity of baicalin and its synergism with β -Car were evaluated by monitoring the time evolution profiles of diene absorption (**Figure 3**), and the LP thus derived are listed in **Table 1**. The results show that, for lipoxidation induced by AAPH from the aqueous phase, baicalin or β -Car alone as well as their combination shows little activity. For lipoxidation induced by

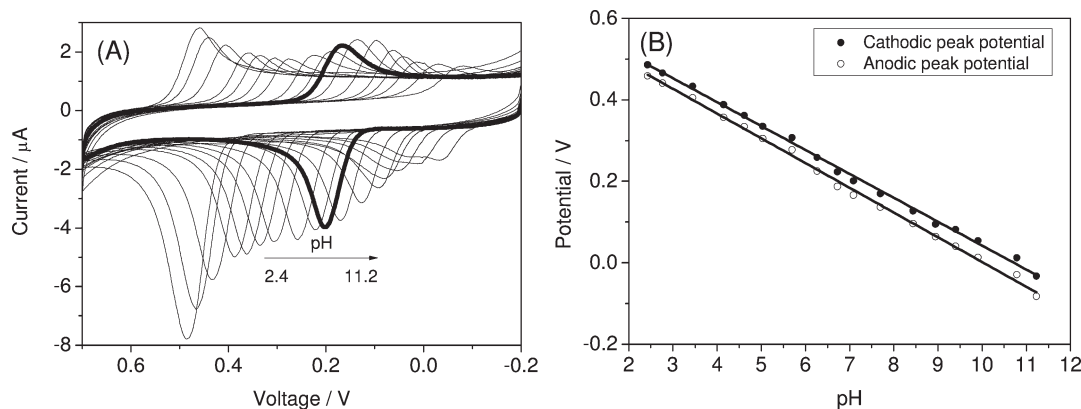


Figure 2. (A) Cyclic voltammograms of baicalin in Britton–Robinson buffer with various pH values. Bold line shows the waveform at pH 7.0. Scans started from -0.2 to 0.7 V with negative peaks correspond to oxidation, whereas the reverse scans with positive peaks to reduction. The scan rate was set to 100 mV/s. (B) Changes in the cathodic peak (solid circle) and the anodic peak (open circle) potentials (vs Ag|AgCl|KCl (1.0 M)) as a function of pH. Solid lines are the results of linear regression fitting to the Nernst equation ($R^2 = 0.99$, see text for details).

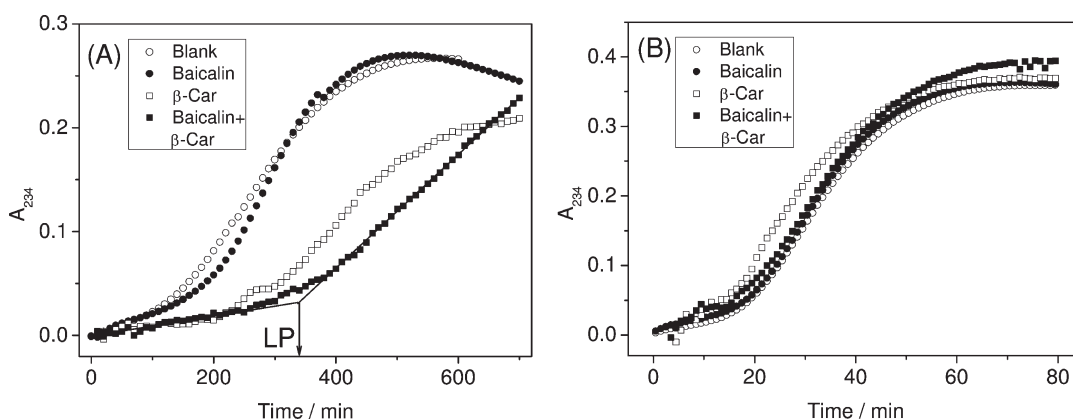


Figure 3. Time evolution profiles of diene absorption at 234 nm (A_{234}) arising from the lipoxidation initiated by AMVN inside membrane (A) and by AAPH in aqueous phase (B): liposomes without antioxidants (\circ), with baicalin only (\bullet), with β -Car only (\square), and with a combination of baicalin and β -Car (\blacksquare). Thermolysis temperature was 43 °C. Method of determination of lag phase time (LP) is illustrated in A.

Table 1. Lag Phase Times (LP) of Different Antioxidant-Adopted Liposome Preparations^a

liposome preparation	LP/min	
	AAPH (water-soluble)	AMVN (lipid soluble)
blank	0	0
baicalin	2	55
β -Car	–4	123
baicalin + β -Car	2	202

^aLipid peroxidation was induced by AMVN- or AAPH-derived peroxy radicals at 43 °C (pH 7.40).

AMVN in liposomal membrane, the effect of baicalin is appreciable ($LP_{\text{baicalin}} = 55$ min), and β -Car acts much more efficiently ($LP_{\beta\text{-Car}} = 123$ min). Here, it is intriguing to see the prominent synergistic effect for the combination of baicalin and β -Car ($LP_{\text{baicalin}+\beta\text{-Car}} = 202$ min), which may be further quantified by a synergistic effect of 13% as calculated by $(LP_{\text{baicalin}+\beta\text{-Car}} - (LP_{\text{baicalin}} + LP_{\beta\text{-Car}})) / (LP_{\text{baicalin}} + LP_{\beta\text{-Car}})$. We have recently reported a higher synergistic effect of $\sim 30\%$ for the combination of puerarin and β -Car and proposed that β -Car^{•+} formed during scavenging prooxide radical is reduced by the conjugated bases of puerarin at the water–lipid interface via electron transfer reaction (14).

We have also examined the activities of baicalin and its combination with β -Car against lipoxidation induced by the AMVN-derived peroxy radical in lipid phase. The antioxidation activity of baicalin was comparable to that of baicalin; however, the activity of the baicalin/ β -Car combination became lower than the case of β -Car alone, although it was still higher than the case of baicalin alone. Here, the prooxidation effect that baicalin brings to β -Car may be partially due to the higher lipophilicity of baicalin ($\log P \sim 1.79$) with respect to baicalin (vide infra), although the detailed mechanism needs to be further studied. It is interesting to correlate this observation to Kim et al.'s recent study (6), in which the metabolites of herbal constituents (e.g., baicalin) were found to be generally more cytotoxic against tumor cells than their nonmetabolized counterparts (e.g., baicalin).

The $\log P$ of baicalin important for antilipoxidation or other bioactivities was determined to be 1.27 (pH 7), indicating that the fraction of baicalin in organic phase is much higher than that in aqueous phase. The relatively high lipid affinity may be due to the lack of any hydrophilic substituent to the B-ring despite the presence of the hydrophilic 7-*O*-glycoside and hydroxyls in the A-ring (Scheme 1). Similar to the case of baicalin, the activity against AAPH-induced lipoxidation for more lipophilic daidzein ($\log P \sim 1.75$) is a few 10-fold lower than that against AMVN-induced lipoxidation (29). In contrast, hydrophilic puerarin ($\log P \sim -0.35$) shows a 4-fold higher activity

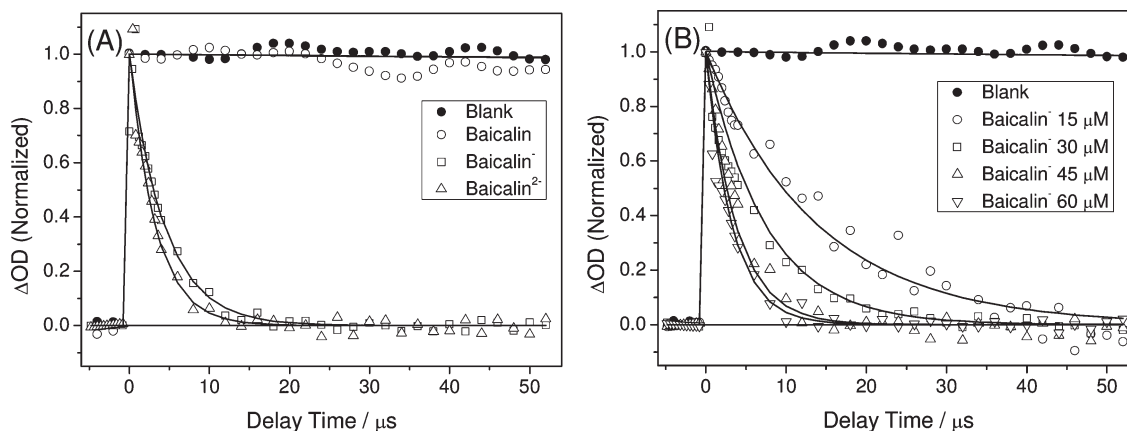


Figure 4. (A) Decay kinetics of $\beta\text{-Car}^{\bullet+}$ in methanol/chloroform binary solvent (1:9, v/v) with the addition of baicalin or its conjugated bases ($75\ \mu\text{M}$). (B) Dependence of decay kinetics of $\beta\text{-Car}^{\bullet+}$ on baicalin $^-$ concentration. Excitation and probing wavelengths were 532 and 950 nm, respectively. Solid lines are monoexponential fitting curves.

Table 2. Pseudo-First-Order Decay Rate Constants of $\beta\text{-Car}^{\bullet+}$ in Methanol/Chloroform Binary Solvent (1:9, v/v) in the Presence of $75\ \mu\text{M}$ Baicalin or Its Conjugated Bases (Left Two Columns) and Those with the Addition of Baicalin $^-$ in Various Concentrations (Right Two Columns)

solution	rate constant/ s^{-1}	baicalin $^-$ concn/ μM	rate constant/ s^{-1}
$\beta\text{-Car}$ (blank)	$(2.7 \pm 0.1) \times 10^2$	15	$(5.7 \pm 0.9) \times 10^4$
$\beta\text{-Car/baicalin}$	$(2.3 \pm 0.1) \times 10^2$	30	$(1.4 \pm 0.1) \times 10^5$
$\beta\text{-Car/baicalin}^-$	$(2.3 \pm 0.2) \times 10^5$	45	$(2.3 \pm 0.2) \times 10^5$
$\beta\text{-Car/baicalin}^{2-}$	$(3.1 \pm 0.3) \times 10^5$	60	$(3.1 \pm 0.3) \times 10^5$

against AAPH-induced lipoxidation than against AMVN-induced lipoxidation (14). Apparently, the antilipoxidation activities of (iso)flavonoids depend on their lipid affinities as well as on the properties of oxidative peroxy radicals, and an (iso)flavonoid with lower log P seems to correlate to its higher synergistic activity with $\beta\text{-Car}$ against lipid peroxidation. Nevertheless, the present work proves that baicalin by itself is not an effective antioxidant, but it becomes one via the synergistic interaction with $\beta\text{-Car}$.

Regeneration of $\beta\text{-Car}$ from Its Radical Cation in Solution. To gain a deeper insight into the mechanism for the synergistic interactions between baicalin and $\beta\text{-Car}$, regeneration of $\beta\text{-Car}$ from $\beta\text{-Car}^{\bullet+}$ by baicalin was investigated by means of laser flash photolysis. A methanol/chloroform binary solvent was used for the rapid photoinduced formation of $\beta\text{-Car}^{\bullet+}$, as well as for a reasonable solubility of baicalin. **Figure 4A** shows the kinetics traces of $\beta\text{-Car}^{\bullet+}$ recorded with the addition of baicalin or its conjugated bases, whereas **Figure 4B** presents those with the addition of various amounts of baicalin $^-$. Decay rate constants of $\beta\text{-Car}^{\bullet+}$ obtained by fitting the kinetics to a monoexponential model function are listed in **Table 2**.

The results show that baicalin in full acidic form is not able to regenerate $\beta\text{-Car}$, but its conjugated bases can (**Figure 4A**; **Table 2**), and that the decay of $\beta\text{-Car}^{\bullet+}$ kinetics becomes progressively faster when the baicalin $^-$ concentration is increased (**Figure 4B**; **Table 2**). A second-order rate constant of $(5.6 \pm 0.5) \times 10^9\ \text{L mol}^{-1}\ \text{s}^{-1}$ was obtained by linear regression fitting to the data of the pseudo-first-order rate constant versus baicalin $^-$ concentration shown in the right two columns of **Table 2**. The observation that baicalin with two A-ring hydroxyls has no quench effect on the $\beta\text{-Car}^{\bullet+}$ kinetics is in agreement with its low activity in scavenging $\text{ABTS}^{\bullet+}$ as measured by TEAC. Importantly, the kinetics results prove that it is the conjugated

bases of baicalin that contribute to the baicalin-to- $\beta\text{-Car}$ synergism against lipid peroxidation, a mechanism which is similar to that proposed for the puerarin- $\beta\text{-Car}$ synergism (14).

Flavone Backbone Conformation and Effect of the 7-*O*-Glycoside Moiety of Baicalin. The geometries of baicalin and its conjugated bases are optimized following the methods of refs 15 and 16. The BDEs are 391.2 and 357.7 kJ mol^{-1} , and the DEs are 1624.2 and 1593.8 kJ mol^{-1} for 5-OH and 6-OH, respectively. The AC-B dihedral angle (α) of fully acidic baicalin (neutral form) is -20.6° , whereas for both of the conjugated bases (anionic forms) the flavone backbones are rather coplanar, that is, the dihedral angles are $+3.8^\circ$ and $+0.4^\circ$ for baicalin $^-$ and baicalin $^{2-}$, respectively. Interestingly, for baicalin deprotonated only at the 6'-carboxyl in the 7-*O*-glycoside moiety, the AC and B rings are also coplanar ($\alpha = +0.4^\circ$). We have further examined baicalin neutral radicals in the form of 5- O^\bullet or 6- O^\bullet , and the dihedral angles are -19.8° or -19.7° , respectively. Nevertheless, the flavone backbone keeps high planarity whenever the baicalin molecule is negatively charged. However, when it is neutral irrespective of fully acidic or radical forms, the B-ring rotates around C2-C1' for $\sim 20^\circ$ with respect to the AC plane.

The theoretical results suggest that, depending on the charge status of baicalin, the 7-*O*-glycoside moiety may induce structural deformation of the conjugated backbone. As seen in **Figure 1A**, when the pH increases from 5.7 to 9.0, the shorter wavelength band shows an appreciable absorptivity increase along with a red shift, which may be explained by invoking the change of deformed conjugated backbone of the acidic baicalin ($\alpha \sim 20^\circ$) to the coplanar geometry of baicalin $^-$ ($\alpha \sim 3.8^\circ$). Further increase of pH leads baicalin $^-$ to transform into baicalin $^{2-}$ having two phenolates, 5- O^- and 6- O^- , and accordingly more profound spectral changes are seen. The influence of 7-*O*-glycoside on the backbone geometry also draws support from a recent 2D NMR study of neutral baicalin showing the possible C6-OH \cdots O-C2'' hydrogen bond interaction (25). On the other hand, we have confirmed that, with the 7-*O*-glycoside moiety of baicalin replaced by 7-OH, baicalin always holds high backbone coplanarity irrespective of its acidic, basic, or radical forms. We have also calculated the dipole moments for baicalin and baicalein in neutral forms, which are found to be 7.9 and 2.9 D, respectively, and those of baicalin anions (> 11.0 D) and baicalein anions (> 13.0 D) are even larger. As a microscopic property, a low value of the dipole moment seems better correlated with the efficiency of antioxidation compared to the partition coefficient (log P) as a

macroscopic property (29). Taken together, the differences in chemical structures between baicalin and baicalein, the effects of 7-*O*-glycoside moiety on the conformation of flavones backbone, and its steric hindrance against oxidative peroxy radicals may all account for the difference in the radical scavenging activities between these two flavonoids.

In summary, we have systematically investigated the physicochemical properties, the radical scavenging, and antilipoxidation activities of baicalin, as well as the kinetics of regenerating β -Car from β -Car⁺⁺ by baicalin. Baicalin as a glycoside-bearing flavonoid is shown to be lipophilic and very reducing, but it is of rather low radical scavenging activity and is almost ineffective in the retardation of AAPH- or AMVN-induced lipid peroxidation. In homogeneous solution the conjugated bases of baicalin are found to be capable of regenerating β -Car, whereas neutral baicalin cannot. The present work has proven that baicalin by itself shows little antilipoxidation activity; however, it becomes very effective when combined with β -Car owing to the synergistic interaction between its conjugated bases and β -Car in liposomal membrane. The influence of the 7-*O*-glycoside moiety on the radical scavenging activities of baicalin has been discussed with reference to its metabolite baicalein, and the radical scavenging and antilipoxidation properties of baicalin have been discussed in terms of its physicochemical properties and molecular structures.

ABBREVIATIONS USED

AAPH, 2,2'-azobis(2-methylpropionamide) dihydrochloride; AMVN, 2,2'-azobis(2,4-dimethylvaleronitrile); ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); β -Car, β -carotene; DMSO, dimethyl sulfoxide; TEAC, Trolox equivalent antioxidant capacity; PC, L- α -phosphatidylcholine.

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