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Puerarin and Conjugate Bases as Radical Scavengers and Antioxidants: Molecular Mechanism and Synergism with β-Carotene

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The 4′-hydroxyl group of puerarin, a C-glycoside of the isoflavonoid daidzein, was shown, using 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) radical cation and stopped-flow spectroscopy and by comparison with the 7-propylpuerarin (A ring derivative) and 4′-propylpuerarin (B ring derivative), to be a more efficient radical scavenger as compared to the 7-hydroxyl group by a factor of 2, a difference increasing upon deprotonation. The difference in radical scavenging agreed with the oxidation potentials (cyclic voltammetry in acetonitrile, 0.1 M Bu_4NBF_4 at 25 °C): E/mV = 862 ± 3 for puerarin, 905 ± 10 for 7-propylpuerarin, and 1064 ± 2 for 4′-propylpuerarin relative to ferrocene/ferricenium. In aqueous solution, the reduction potential was shown to decrease for increasing pH, and deprotonation of the 4′-hydroxyl group increased radical scavenging more than deprotonation of the 7-hydroxyl group. The 7-hydroxyl was found to be more acidic (pK_{a1} = 7.20 ± 0.01 in puerarin and pK_a = 7.23 ± 0.01 in 4′-propylpuerarin) than the 4′-hydroxyl group (pK_{a2} = 9.84 ± 0.08 in puerarin and pK_a = 9.51 ± 0.02 in 7-propylpuerarin); aqueous solution, ionic strength of 0.1, and 25 °C. In phosphatidyl choline liposome of pH 7.4, puerarin and β-carotene each showed a modest antioxidant activity measured as prolongation of the lag phase for formation of conjugate dienes and using the water-soluble radical initiator APPH with effects of puerarin and β-carotene being additive. For the lipophilic initiator AMVN, the antioxidative effect decreased for puerarin and increased for β-carotene as compared to APPH and showed a clear synergism. A regeneration of β-carotene, effective in the liposome lipid phase as antioxidant, from the cation radical by deprotonated forms of puerarin was demonstrated in 9:1 chloroform/methanol using laser flash photolysis with $k_2 = 2.7 \times 10^4$ L mol^{-1} s^{-1} for the bimolecular process between the cation radical and the puerarin dianion.

KEYWORDS: Puerarin; β-carotene; radical scavenging; antioxidant synergism

INTRODUCTION

A diet rich in fruits and vegetables is known to have positive effects on human health (*1*). The health benefits have often been ascribed to a high content of antioxidants in fruits and vegetables, although evidence for positive effects on biomarkers for lifestyle disease from the antioxidants as such still is lacking (*2*). Antioxidants are also important for food stability and protection of important nutrients in foods during processing and storage (*3*). In relation to the evaluation of antioxidants in foods, it has now been recognized that it should always be made clear prior to selection of evaluation protocols whether or not effects of antioxidants on human health or on food stability are in focus (*4*, *5*). In fruits and vegetables, antioxidants are present in complex mixtures and their interaction may become important for their effects (*6*). A better understanding of synergistic effects between different types of antioxidants seems mandatory in order to obtain better food protection and also for dietary recommendations (*5*).

Antioxidants may be classified according to two criteria, i.e., their nutritive value (vitamin/nonvitamin) and their hydrophilic/lipophilic balance (soluble in water/soluble in lipids). For the resulting four groups of antioxidants, six possibilities for binary interaction become possible as follows: (i) As for the interaction between the two vitamin antioxidants, ascorbate has been shown to regenerate α-tocopherol from its one-electron-oxidized form

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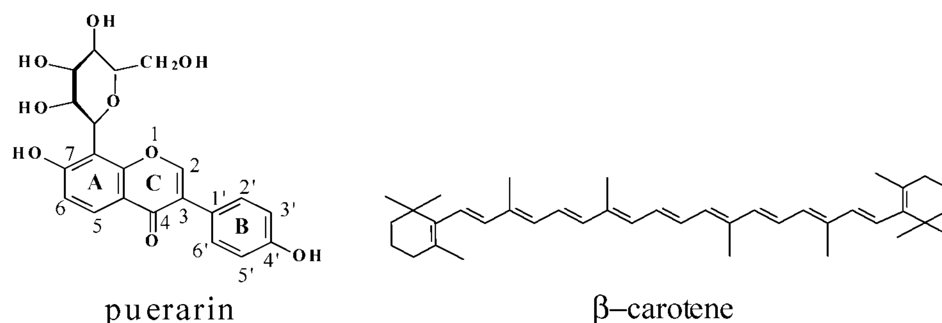
Scheme 1



in a process of physiological importance (*7*). (ii) On the basis of standard reduction potentials, it has been predicted and confirmed by kinetic studies that ascorbate regenerates various plant polyphenols in homogeneous solution from their oxidized forms (*8*). (iii) Ascorbate may at interfaces regenerate carotenoids in similar reactions (*9*). (iv) As for the tocopherols, their interaction with polyphenols depends on subtle differences in thermodynamic properties sensitive to solvent and temperature, and tocopherols have both been found to be regenerated from their oxidized forms by polyphenols and to regenerate polyphenols (*10*, *11*). (v) Tocopherols and carotenoids likewise interact with one-electron transfer under some conditions establishing equilibrium with a thermodynamic preference for regeneration of the carotenoids from their oxidized form (*12*). (vi) The interaction between polyphenols and carotenoids seems, in contrast to the five other combinations of antioxidants, to not have been studied in any detail.

We have accordingly selected a plant polyphenol to be studied in combination with β-carotene for their interaction as antioxidants. Puerarin, an isoflavonoid C-glycoside from the root of pueraria lobata traditionally used in Chinese herbal medicine, was chosen for the study due to its simple structure with only two phenol groups, good solubility properties, and stability in solution; see **Scheme 1** (*13*). The study also included a characterization of the oxidation potential and the acid/base properties of puerarin in aqueous solution in order to understand the influence of the pH on radical-scavenging efficiency of puerarin.

MATERIALS AND METHODS

Sample Preparation. Puerarin crude product (Huike Plant Exploitation Inc., Shanxi, China) was recrystallized twice in a 1:1 mixed solvent of acetic acid (>99.5%, dried and refluxed by the use of P_2O_5 and $KMnO_4$, respectively, Wendaxigui Chemical Plant, Tianjin, China) and methanol (>99.5%, Beijing Chemical Plant, Beijing, China). 7-Propylpuerarin, 4′-propylpuerarin and 7,4′-dipropylpuerarin were prepared and characterized as previously described (*14*). The solution of puerarin$^-$ and puerarin^{2-} was prepared by the addition of 1 equiv of tetramethylammonium hydroxide $(CH_3)_4N^+OH^-$ (97%, Sigma, St. Louis, MO) to puerarin or in excess to puerarin neutral solutions. Tetrabutylammonium tetrafluoroborat was purchased from MP Biochemicals, LLC (Eschwege, Germany). β-Carotene was from the same sources and purified as previously described (*15*, *16*). Methanol [high-performance liquid chromatography (HPLC) grade, Caledon Laboratories, Georgetown, Ontario, Canada] for spectroscopy was used as received. Chloroform (>99.0%, Beijing Chemical Plant) was purified by passing it through an alumina column (Wusi Chemical Reagent Ltd., Shanghai, China) before use.

Determination of pK_a Values. Puerarin, 4′-propylpuerarin, and 7-propylpuerarin in an aqueous solution of 1.2×10^{-4} M were prepared using Britton-Robinson buffers to adjust the varying pH (4.0 < pH < 12) (*17*). The ionic strengths were controlled at 0.1 using NaCl. The pH value of each solution was measured with a Mettler-Toledo pH meter (Mettler-Toledo GmbH, Process Analytics, Urdorf, Switzerland), and the steady-state absorption spectra were recorded on a U-3310 spectrophotometer (Hitachi High-Technologies Corp., Tokyo, Japan). For the pH determination and the absorption spectra measurement, the temperature was controlled at 25 °C using RTE-110 (Neslab Instruments Inc., Newington, NH). The pH was measured relative to international activity pH standards, and accordingly, the so-called "mixed" constant was determined as follows: $K_a = a_{H+}[\text{base}]/[\text{acid}]$; $pK_a = -\log K_a$, in which a_{H+} is measured electrochemically and the concentrations of the corresponding acids and bases are measured spectrophotometrically. For 4′-propylpuerarin and 7-propylpuerarin, the pK_a value was calculated by linear regression of $A_{pH} = K_a(A_{base} - A_{pH})/a_{H+} + A_{acid}$ to the experimental points. For puerarin, we calculated the pK_{a1} and pK_{a2} values based on the absorption value at 336 nm using the method described in the book Ionization Constants of Acids and Bases (*18*).

Determination of Oxidation Potentials. Cyclic voltammetry experiments were carried out with a BAS CV-50 W voltammetric analyzer (Bioanalytical Systems Inc., West Lafayette, IN). All measurements were performed at $T = 25 \pm 0.5$ °C by using a water-jacketed cell vial connected to a thermostated water bath. Solutions were purged for 10 min with nitrogen before recording the voltammograms. Compensation for the voltage drop iR_S (due to solution resistance) was performed electronically with the BAS CV-50 analyzer. The reference electrodes were checked against the ferrocene/ferricinium (Cp_2Fe/Cp_2Fe^+) couple before and after each experiment. A platinum wire was used as the counter electrode. The working electrodes were polished before each scan. Puerarin and its derivatives were added directly to the cell vials to obtain solutions with concentrations of 1 mM and were dissolved by purging with N_2. The corresponding phenolates were prepared in situ by addition of 1 or 2 equiv of tetrabutylammonium hydroxide.

tert-Butyl Alcohol Solutions. The *tert*-butyl alcohol solutions contained 0.1 M tertabutylammonium tetrafluoroborate (Bu_4NBF_4) as the supporting electrolyte. A gold working electrode (1.6 mm diameter) (BAS MF-2014) was used. The nonaqueous reference electrode contained a silver wire in *tert*-butyl alcohol with 0.1 M Bu_4NBF_4 and 0.01 M $AgNO_3$. The standard potential of the ferrocene/ferrocenium couple in *tert*-butyl alcohol/0.1 M Bu_4NBF_4 was −57 mV against the reference electrode.

Acetonitrile Solutions. The acetonitrile contained 0.1 M Bu_4NBF_4 as the supporting electrolyte. A platinum working electrode (1.6 mm diameter) (BAS MF-2013) was used. The nonaqueous reference electrode contained a silver wire in acetonitrile with 0.1 M Bu_4NBF_4 and 0.01 M $AgNO_3$. The standard potential of the ferrocene/ferrocenium couple in acetonitrile/0.1 M Bu_4NBF_4 was 460 mV against the reference electrode.

Laser Flash Photolysis. Submicrosecond time-resolved absorption spectra were obtained at room temperature as described previously (*15*, *16*). The excitation laser pulses at 532 nm with a repetition of 1 Hz were generated from a Nd^{3+}:YAG laser (Tempest 300, New Wave Research). The pump energy was ~ 5 mJ/pulse at the sample cell. The optical path length of the flow cuvette used for laser flash photolysis was 5 mm. The anaerobic condition was achieved by bubbling the solution with high-purity argon for an hour. In all of the measurements, a sample volume of 25 mL was kept in an ice-cooled reservoir and circulated between the reservoir and the sample cell. A setup based on a gated photodiode array detector (model 1420′ EG&G, Princeton, NJ)

was used for measuring the multicolor difference absorption spectra in the visible region. NIR kinetics at individual wavelengths was recorded with a xenon lamp probe (CW, 350 W) and a photodiode detector (S8890-02, Hamamatsu, Hamamatsu City, Japan).

Radical Scavenging Experiments. The blue/green 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) radical cation ($ABTS^{•+}$) chromophore (5 mL) was produced through reaction between 7 mM ABTS (Sigma-Aldrich Chemie GmbH P.O., Steinheim, Germany) and 2.45 mM potassium persulfate (>99.5%, Beijing Chemical Plant) 12 h before use (*19*). The final concentrations of $ABTS^{•+}$ and puerarin or its derivative were 2.5×10^{-5} and 1.25×10^{-5} M, respectively, for the kinetic experiments. Solutions of $ABTS^{•+}$ and puerarin or its derivatives were placed in each syringe of a stopped-flow spectrometer (Bio-logic, Claix, France). The reactions were followed by absorbance measurements at 734 nm ($\epsilon_{ABTS^{•+},734nm} = 1.5 \times 10^4$ M^{-1} cm^{-1} according to ref *20*). The kinetics at pH 5.0 were fitted by using linear regression in the whole time scale, and the kinetics at pH 7.2 and 8.3 within 20 s were fitted nicely by using biexponentials nonlinear regression: $A_{734,t} = a{\cdot}\exp(-k_1t) + b{\cdot}\exp(-k_2t) + c$. The initial reaction rate was calculated according to the following formulas:

$$\text{pH 5, rate}_{t=0} = -dA_{734,t=0}/dt = k$$

$$\text{pH 7.2 and 8.3, rate}_{t=0} = -dA_{734,t=0}/dt = ak_1{\cdot}\exp(-k_1t)_{t=0} + bk_2{\cdot}\exp(-k_2t)_{t=0} = ak_1 + bk_2$$

Antioxidant Evaluation in Liposomes. Liposomes were prepared following the procedure described by Roberts and Gordon (*21*). Briefly, 1 mL of β-carotene in chloroform and/or 1 mL of puerarin or its derivatives in absolute ethanol was added to 2 mL of 0.75 mM chloroform solution of soybean L-α-phosphatidyl choline (PC) (purity 99%, from Sigma-Aldrich Chemic GmbH; the molecular mass of soybean PC was taken as 900). Fifty microliters of 64.64 mM AMVN [2,2′-azobis(2,4-dimethylvaleronitrile)] from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) in absolute ethanol was added to the samples where oxidation was initiated by AMVN. The solvent of the combined soybean PC/antioxidant solution was evaporated on a rotatory evaporator with a water bath set at 30 °C. After evaporation, the atmospheric pressure was re-established by introducing N_2. The residue in the flask was hydrated with 10 mL of 10 mM phosphate buffer (pH 7.4), vortexed for 10 min, and sonicated for 30 s. The final concentration of the antioxidants in the liposome suspension in mol % of the lipid fraction was 1.0. The multilamellar suspension was transferred to an Avestin Liposofast Basic (Avestin Inc., Ottawa, Canada) small volume extrusion device and passed 20 times through a double layer (100 nm pore size polycarbonate membrane) to obtain large unilamellar liposomes. All solvents were supplied by Lab Scan Analytical Science (Dublin, Ireland) and were of HPLC grade.

Lipid peroxidation was followed by measuring the formation of conjugated diene monitored as the change in absorbance at 234 nm (A_{234}) using a Shimadzu UV-2101PC UV−vis scanning spectrophotometer (Shimadzu Corp., Kyoto, Japan). The unilamellar liposome suspension (2.5 mL) was thermostated in a quartz cuvette for 5 min at 43 °C in the spectrophotometer. Twenty-five microliters of 75 mM AAPH in sodium phosphate buffer (pH 7.4) was added to the samples when oxidation was inititated with AAPH [2,2′-azobis(2-amidinopropane) dihydrochloride, from Fluka (Steinheim, Germany)]. A_{234} was measured and recorded continuously for up to 10 h. A sample without soybean PC and antioxidants was used as a blank. The lag phase (LP in min) was determined as the time elapsing to the point where the tangent of the propagation phase and the tangent of the lag phase intercepted and was corrected by subtraction of the lag phase obtained for a sample without antioxidant added. Interactions between antioxidants were studied in liposomes with mixtures of antioxidants each in the same concentration as the liposomes with one antioxidant added. Synergistic effects were observed when

$$LP_{\text{mixed antioxidants (1+2)}} > LP_{\text{antioxidant 1}} + LP_{\text{antioxidant 2}}$$

Calculation of Deprotonation Energy (DE). The geometries of puerarin and its two monodeprotonated forms were optimized by using the B3LYP density functional theory according to the reported methods (*22*, *23*) and were performed by Gaussian 03 code. Single-point energies were evaluated by using a higher 6-311G(d,p) basis set. The gas-phase DE was calculated as the enthalpy difference for the reaction: ArOH → ArO^- + H^+.

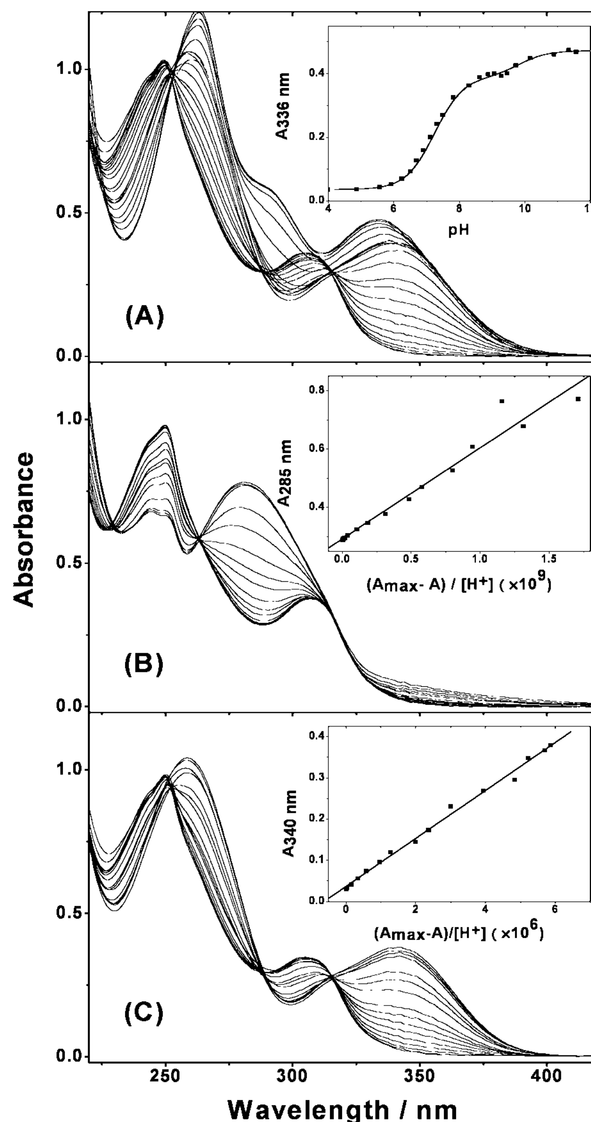


Figure 1. Absorption spectra of puerarin (**A**), 7-propylpuerarin (**B**), and 4′-propylpuerarin (**C**) in aqueous solution of ionic strength 0.1 adjusted with NaCl at 25 °C at varying pH. For puerarin, A_{336nm} for varying pH is shown in the insert with the curve calculated from estimated $pK_{a1} = 7.20 \pm 0.01$ and $pK_{a2} = 9.84 \pm 0.08$ and molar absorptivity of the three acid/base forms (**A**). For the two puerarin derivatives, the dependence of A_{285nm} and A_{340nm} on $(A_{basic} - A_{pH})/[H^+]$, in which A_{basic} is absorption of the basic form at the wavelength used for calculation, is shown as inserts in **B** and **C**, respectively. From the slope obtained by linear regression of a plot in the inserts in **B** and **C**, the values $pK_a = 9.51 \pm 0.02$ for 7-propylpuerarin and $pK_a = 7.23 \pm 0.01$ for 4′-propylpuerarin were calculated.

RESULTS

Puerarin is a glucose C-glycoside of daidzein resistant to hydrolysis and easily soluble in water with a solubility of 10^{-2} M, while the propyl derivatives have a lower solubility of approximately 10^{-3}−10^{-4} M. Puerarin has two phenol groups, and the pH dependence of the UV−visible spectrum could be accounted for by two acid/base equilibria, as may be seen from

Table 1. Anodic Peak Potentials vs the Reversible Ferrocene/Ferricinium Couple (Cp_2Fe/Cp_2Fe^+) Determined by Cyclic Voltammetry at 25 °C for 1 mM Puerarin and Its Derivatives in *tert*-Butyl Alcohol/0.1 M Bu_4NBF_4 and Acetonitrile/0.1 M Bu_4NBF_4

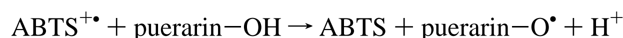
sample	E/mV vs Cp_2Fe/Cp_2Fe^{+a} tert-butyl alcohol	E/mV vs Cp_2Fe/Cp_2Fe^{+a} acetonitrile[b]
puerarin	732 ± 4 (i)	862 ± 3 (i)
7-propylpuerarin	800 ± 2 (i)	905 ± 10 (i)
4'-propylpuerarin		1064 ± 2 (i)

[a] The waves in the cyclic voltammograms are characterized as irreversible (i). [b] For acetonitrile, Cp_2Fe/Cp_2Fe^+ $E^0 = 0.647$ V/SHE (*22*).

Figure 1A. The assignment of the pK_a values to the two phenol groups was based on a comparison with the pK_a values determined for 7-propylpuerarin and 4′-propylpuerarin, the two ethers of the puerarin previously characterized (*14*). 7-Propylpuerarin (**Figure 1B**) was found to have a pK_a = 9.51 ± 0.02 at 25 °C in an aqueous solution of ionic strength 0.1, and 4′-propylpuerarin (**Figure 1C**) had a pK_a = 7.23 ± 0.01. Accordingly, the pK_{a1} = 7.20 ± 0.01 obtained for puerarin was assigned to the A ring phenol (**Scheme 1**), while pK_{a2} = 9.84 ± 0.08 was assigned to the B ring phenol. Notably, the pK_{a1} of the puerarin is identical to the pK_a of 4′-propylpuerarin showing that transformation of the B ring phenol to an anisole does not affect the dissociation of the A ring phenol, while the pK_{a2} of puerarin is higher than the pK_a of 7-propylpuerarin showing that the negative charge developed by the dissociation of the first proton of puerarin to some degree hampers the dissociation of the second proton in the B ring. The assignment of the two pK_a values was quantitive in agreement with the results of the calculated DE, 314.5 and 343.6 kcal/mol for 7-hydroxyl and 4′-hydroxyl, respectively.

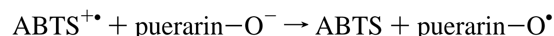
Puerarin may, like other phenols, be oxidized, and the ease of oxidation of puerarin was determined by cyclic voltammetry. Attempts to determine redox potentials of any acceptable accuracy in aqueous solution were unsuccessful, but it was concluded from the cyclic voltammograms of puerarin, 7-propylpuerarin, and 4′-propylpuerarin that the oxidation potential, *E*, decreased as the pH increased, as would be expected. An approximated value of 1.0 V could be estimated for the standard redox potential, *E*°, at pH 7.2 (data not shown). For puerarin and the two propyl derivatives, the oxidation potential could be determined in acetonitrile as the anodic peak potentials and are presented in **Table 1**. For all of the potentials reported, the waves in the cyclic voltammograms were characterized as irreversible. Puerarin is the more reducing, and notably, 7-propylpuerarin is more reducing than 4′-propylpuerarin, indicating that the B ring phenol is more easily oxidized than the A ring phenol. For comparison, *E* was also determined in *tert*-butyl alcohol for puerarin and 7-propylpuerarin. In this solvent with a high hydrogen bond-accepting ability, the difference in *E* became larger. The values of *E* of **Table 1** may be converted relative to the potential of the standard hydrogen electrode by addition of 0.647 V yielding *E* = 1.509 V for puerarin in acetonitrile (*24*). This value is comparable to redox potentials in other nonaqueous solvents for other plant phenols (*25*).

Puerarin, 7-propylpuerarin, and 4′-propylpuerarin were found to scavenge the semistable radical $ABTS^{\bullet+}$ in aqueous solution and with increasing rate for increasing pH, as may be seen from **Figure 2**. In acidic solution, hydrogen atom transfer is the most likely mechanism:



$$ABTS^{+\bullet} + \text{puerarin}-OH \rightarrow ABTS + \text{puerarin}-O^{\bullet} + H^{+}$$

In contrast, electron transfer dominates under neutral and alkaline conditions:



$$ABTS^{+\bullet} + \text{puerarin}-O^{-} \rightarrow ABTS + \text{puerarin}-O^{\bullet}$$

For a comparison between the different phenolic compounds and pH conditions, the initial rate for scavenging was deter-

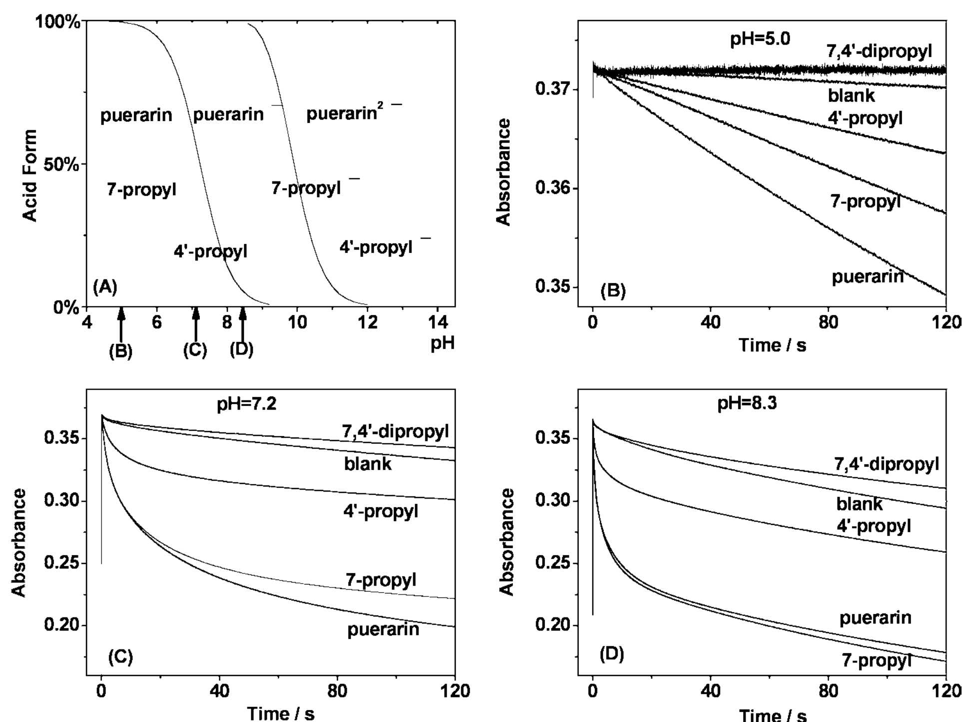


Figure 2. Rate of scavenging of $ABTS^{\bullet+}$ radical by puerarin, 4′-propylpuerarin (4′-propyl), 7-propylpuerarin (7-propyl), and 7,4′-dipropylpuerarin (7,4′-dipropyl) in aqueous solution of increasing pH. Rates given in **Table 2** were calculated as $(dA_{734nm}/dt)_{t=0}$. (**A**) Bjerrum diagram for distribution between acid/base forms. (**B**) pH 5.0 with acidic forms dominating. (**C**) pH 7.2 with 48% 4′-propylpuerarin and 0.5% of 7-propylpuerarin on anionic forms, respectively. (**D**) pH 8.3 with 93% of 4′-propylpuerarin and 6% of 7-propylpuerarin on anionic forms, respectively.

Table 2. Initial Rate for Scavenging of the ABTS•+ by Puerarin, 7-Propylpuerarin, and 4′-Propylpuerarin in Aqueous Solution of Increasing pH As Determined by Stopped-Flow Absorption Spectroscopy

sample	c (× 10^{-5} M)	rate (s^{-1})		
		pH 5.0	pH 7.2	pH 8.3
blank		1.3×10^{-5}	4.3×10^{-3}	5.5×10^{-3}
7,4′-propylpuerarin	1.25	-1.3×10^{-6}	3.8×10^{-3}	4.1×10^{-3}
4′-propylpuerarin	1.25	7.3×10^{-5}	1.3×10^{-2}	4.5×10^{-2}
7-propylpuerarin	1.25	1.2×10^{-4}	3.2×10^{-2}	9.7×10^{-2}
puerarin	1.25	1.9×10^{-4}	3.3×10^{-2}	1.2×10^{-1}

Table 3. Lag-Phase Found by Spectrophotometric Measurement of Conjugated Dienes in Soybean Phosphatidylcholine Liposomes with Free Radical Initiation of Oxidation in the Aqueous Phase (APPH) with pH 7.4 or in the Lipid Phase (AMVN) at 43 °C[a]

sample	lag phase (min)	
	AAPH (water soluble)	AMVN (lipid soluble)
control	3 ± 0.3	5 ± 1
β-carotene	15 ± 2	38 ± 3
puerarin	15 ± 1	8 ± 0.5
β-carotene + puerarin	32 ± 2	65 ± 0.4
7,4′-dipropylpuerarin	6 ± 0.1	2 ± 0.4
7-propylpuerarin	5 ± 0.4	11 ± 0.4
4′-propylpuerarin	5 ± 0.4	8 ± 0.7

[a] [Phosphatidyl choline] = 0.15 mM, [AAPH] = 0.75 mM, [AMVN] = 0.32 mM, and [antioxidant] = 1 mol % of the lipid fraction.

mined, including 7,4′-dipropylpuerarin, which was not expected to be a scavenger. The rates presented in **Table 2** for pH 5 show that the phenolic group in the B ring has a higher scavenging ability than the phenolic group in the A ring, since the 4′-propylpuerarin is much slower in scavenging than 7-propylpuerarin. For pH 7.2, the ability of radical scavenging increases for puerarin and the two derivatives. For 7-propylpuerarin, although as little as 0.5% is deprotonated, the increase in scavenging is remarkable and much more significant than for 4′-propylpuerarin with 48% on the deprotonated form. Deprotonation of the A ring phenol is accordingly concluded to have less effect on radical scavenging than deprotonation of the B ring phenol, most likely due to involvement of hydrogen bonding from the hydroxyl groups from the sugar moiety. Puerarin is still the most efficient scavenger due to deprotonation of both the A ring phenol (50%) and the B ring phenol (0.2%). For pH 8.3, although puerarin is the most efficient scavenger at initial time (cf. **Table 2**), the 7-propylpuerarin shows higher scavenging than puerarin, especially after 20 s, due to a larger deprotonation of the B ring phenol (6%) as compared to puerarin (3%).

The antioxidant efficiency was tested in a liposome model with initiation of oxidation either in the aqueous phase or in the lipid phase and, in both cases, with measurement of the lag phase for formation of conjugated dienes. The temperature was raised to 43 °C in order to obtain an appropriate rate of initiation. From the results as presented in **Table 3**, it is seen that for initiation in the aqueous phase, only β-carotene and puerarin are effective in prolonging the lag phase significantly. Upon combining the two antioxidants, an additive effect is seen. For initiation in the lipid phase, it is seen that 7-propylpuerarin is a better antioxidant than 4′-propylpuerarin, confirming the findings for the radical scavenging abilities with the B ring phenol being a better antioxidant. Puerarin is less efficient than 7-propylpuerarin, and β-carotene located in the lipid phase is also better

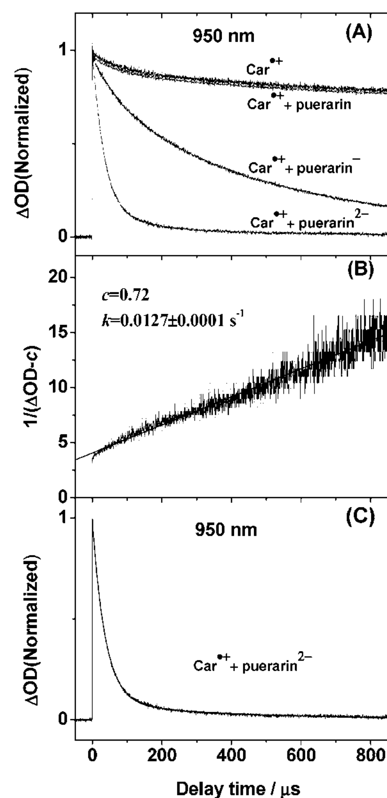


Figure 3. (**A**) Time traces of β-carotene radical cation measured by transient absorption spectroscopy following a laser flash of solutions of β-carotene (5×10^{-5} M) without or with puerarin (9×10^{-4} M) or puerarin deprotonated with one or two equivalents of $(CH_3)_4N^+OH^-$. (**B**) Second-order plot for decay of β-car•+ in the absence of puerarin anions. (**C**) Kinetic analysis of a time trace of β-carotene radical cation decay in the presence of puerarin dianion. The biexponential fit showed a major contribution (91%) with a time constant of 37 μs and a minor component (9%) with a time constant of 380 μs.

than puerarin. The most notable result is, however, the clear synergism seen for the combination of puerarin and β-carotene. The pH of the liposomes was between 7 and 8 for which values the anionic forms are present in significant fractions.

To investigate this synergism in more detail and obtain a mechanistic explanation, a mixture of chloroform and methanol was selected as an electron-withdrawing solvent in which puerarin could also dissolve. Upon irradiation with near-UV light, β-carotene will form cation radicals, the decay of which may be followed in real time by laser flash photolysis with near-infrared detection (*15*, *16*). As may be seen from **Figure 3**, the decay rate of β-carotene cation radical is not affected by the presence of puerarin. The decay of the cation radical may under the actual conditions be described as a second-order reaction (**Figure 3B**). However, the presence of the dianion of puerarin clearly increased the decay rate, and under the pseudo-first-order condition used with 20-fold excess of puerarin, the second-order rate constant was calculated from the exponential decay to have the value 2.7×10^4 L mol^{-1} s^{-1}. For the monodeprotonated form of puerarin, the kinetics becomes complicated due to the simultaneous presence of many species; cf. **Figure 2**. The conclusion is, however, clear; puerarin is not capable of reducing the β-carotene cation radical, the monoanion form of puerarin is, and the diprotonated form is even better.

Except for the long-lived bleaching of ground-state molecules, which were almost unchanged for 2 ms, the transient absorption spectral changes in the visible region in the absence of puerarin

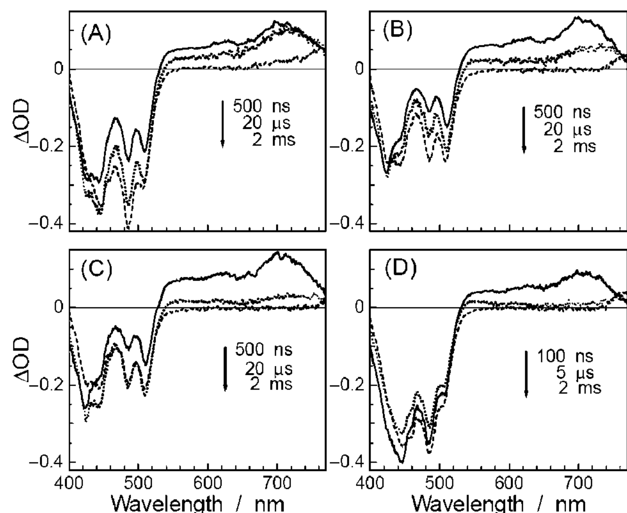


Figure 4. Transient absorption spectra of β-carotene (7.5×10^{-5} M) in the absence of puerarin (**A**), in the presence of puerarin (9×10^{-4} M) and $(CH_3)_4NOH$ (**B**, 9×10^{-4} M; and **C**, 1.8×10^{-3} M), and only in the presence of $(CH_3)_4NOH$ (**D**, 9×10^{-4} M) in chloroform:methanol (9:1) at an indicated delay time upon 532 nm excitation.

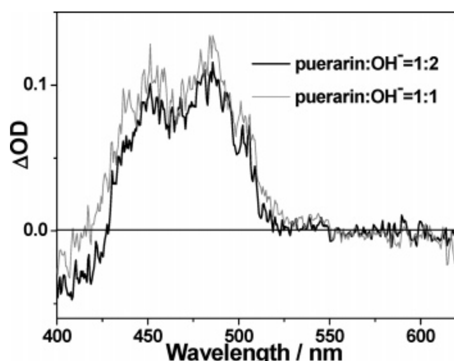


Figure 5. Difference spectra between the transient absorption spectra in **Figure 4** (B-A for puerarin:$OH^- = 1{:}1$, C-A for puerarin:$OH^- = 1{:}2$) at 2 ms.

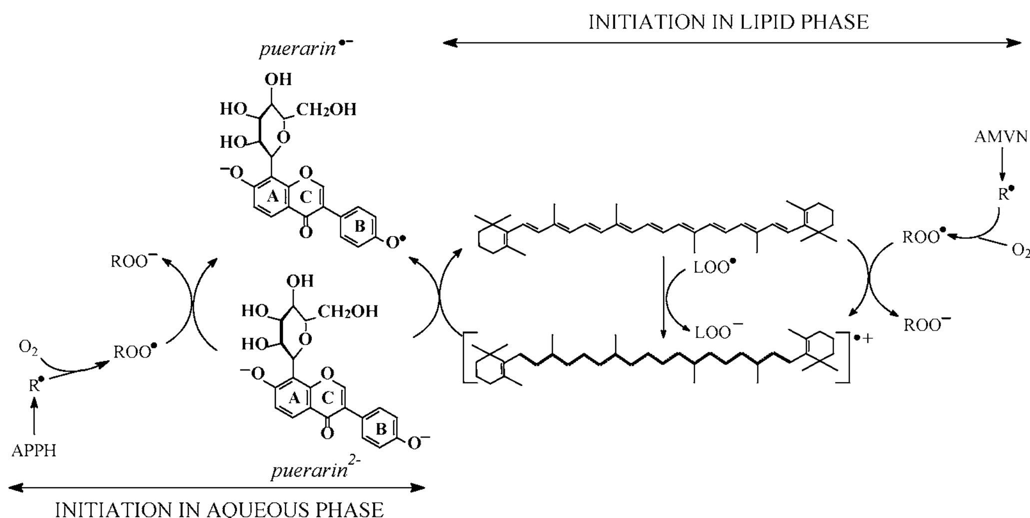
(**Figure 4A**) and the presence of puerarin mono-/dianion (**Figure 4B**/**C**) or in the presence of $(CH_3)_4NOH$ (**Figure 4D**) could be ascribed to the precursor of carotenoid radical cation with an absorption maximum at 700 nm (*26*). In the presence of anionic puerarin (**Figure 4B**,**C**), the bleaching of β-carotene is quite different from solutions with only β-carotene present (**Figure 4A**), especially for the spectra at 20 μs. Although $(CH_3)_4NOH$ can also accelerate the decay of β-carotene$^{\bullet+}$, which is due to formation of carotene hydroxyl adduct radical (*27*), the bleaching was stable for at least 2 ms. Difference spectra from transient absorption at 2 ms (**Figure 5**) between samples with and without mono-/dianion in the visible region provided evidence for the formation of the puerarin radical, based on spectral similarity with well-documented spectra of flavonoid radicals (*28*, *29*).

DISCUSSION

Plant phenols are chain-breaking antioxidants in some lipophilic solvents and retarders of liquid oxidation in solvents with high hydrogen bond-accepting properties (*30*). Puerarin has been shown in the present study to inhibit lipid oxidation in liposomes when oxidation was initiated in the aqueous phase. The effect was comparable to the effect of β-carotene, and only a modest prolongation of the lag phase for the formation of conjugated dienes, an indicator of peroxide formation, was seen for both antioxidants. In water, which has a high hydrogen bond-accepting ability, puerarin must accordingly be classified as a lipid oxidation retarder. As for initiation in the lipid phase, puerarin has an even less effect, which may be understood on the basis of the phase separation between the lipid radicals and the potential antioxidant. In contrast, β-carotene, in close contact with the lipid and upon initiation of oxidation with the lipid radicals, shows a more significant effect. 7-Propylpuerarin is under these conditions an even better antioxidant than puerarin, which may be understood on the basis on the higher lipophilicity of the propyl derivative. 4′-Propylpuerarin is comparable with puerarin, confirming the more efficient radical scavenging by the B ring phenol as compared to the A ring phenol.

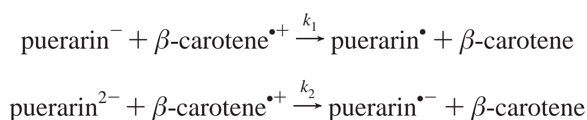
The most significant finding is, however, the synergism seen between puerarin and β-carotene for lipid oxidation in the liposome system when oxidation is initiated in the lipid phase. The following mechanism is suggested and outlined in **Scheme 2**. β-Carotene, in close contact with the membrane lipid in the liposome, scavenges the initiating radicals or the lipid radicals forming cation radicals, in effect halting the formation of conjugated dienes until β-carotene is consumed. In the presence of puerarin, which in the aqueous phase of the liposomes will be present in a significant concentration as the anionic form, β-carotene cation radicals will to some extent be regenerated at the interface between the aqueous phase and the membrane.

Scheme 2



Puerarin is not an efficient antioxidant by itself when initiation occurs in the lipid phase, but through the regeneration of β-carotene, it becomes active.

Two mechanisms, hydrogen atom transfer or electron transfer, may account for the antioxidant activity of phenols. For neutral and alkaline solutions, the deprotonated phenols react faster, as also seen in the present study, in favor of an electron-transfer mechanism. The mechanism described in **Scheme 2** for puerarin^{2-} as the most reactive form even at physiological pH draws support from the regeneration of β-carotene by the anionic forms of puerarin demonstrated by laser flash transient absorption spectroscopy. The decay of the β-carotene cation radical was enhanced in the presence of the anionic forms of puerarin, and notably, the product of the reaction regenerating β-carotene was a phenoxyl radical as shown by transient absorption spectroscopy. The phenoxyl radical formed may be identified as a puerarin radical:



$$\text{puerarin}^{-} + \beta\text{-carotene}^{\bullet+} \xrightarrow{k_1} \text{puerarin}^{\bullet} + \beta\text{-carotene}$$

$$\text{puerarin}^{2-} + \beta\text{-carotene}^{\bullet+} \xrightarrow{k_2} \text{puerarin}^{\bullet-} + \beta\text{-carotene}$$

The exact nature of puerarin$^{\bullet-}$/puerarin$^{\bullet}$ is now being further studied; however, the rate constant for the bimolecular reaction regenerating β-carotene by puerarin^{2-} was found to have an approximately value of $k_2 = 2.7 \times 10^4$ L mol^{-1} s^{-1}. Electrochemical data are not available for puerarin$^{\bullet}$/puerarin and β-carotene$^{\bullet+}$/β-carotene couples in the same solvent for calculation of the driving force. In dichloromethane, β-carotene$^{\bullet+}$/β-carotene has the standard potential $E° = +0.82$ V vs SHE (*16*), while in water the value is $+1.1$ V vs SHE (*31*). Puerarin$^{\bullet}$/puerarin has in the present study been determined in acetonitrile to have $E° = +1.5$ V vs SHE (**Table 1**). A crude estimate for puerarin$^{\bullet}$/puerarin in aqueous solution with pH 7.2 is $E° = +1.0$ V based on the cyclic voltammograms for aqueous solutions mentioned in the Results section. A difference of 0.5 V between acetonitrile and water is realistic as seen from a comparison with $E°$ values for plant phenols determined in other aprotic solvents and water (*11*). The small difference in the redox potentials for the couple corresponds to a small driving force, which further may explain the modest rate of regeneration of β-carotene by the anionic forms of puerarin. The acidic form of puerarin may even be less reducing than β-carotene and the regeneration reaction being reversed.

In conclusion, antioxidant radical interactions between a plant phenol and a carotenoid have been demonstrated using real-time kinetic methods. Puerarin, an isoflavonoid C-glycoside, is in its anionic form capable of regeneration of β-carotene from β-carotene cation radical, a generation that manifests itself as antioxidant synergy in a liposome system where lipid oxidation is initiated in the lipid phase.

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