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ARTICLE

Glycosylation of Dietary Flavonoids Decreases the Affinities for Plasma Protein

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The interaction between flavonoids and proteins has attracted great interest among researchers. However, few reports have focused on the structure−affinity relationship of flavonoids binding to proteins. This work mainly concerns the effect of glycosylation of flavonoids on the affinities for BSA. Four flavonoid aglycones (baicalein, quercetin, daidzein, and genistein) and their monoglycosides (baicalin, quercitrin, daidzin, and puerarin, genistin) and another poly glycoside (rutin) are studied for their affinities for BSA. The glycosylation of flavonoids significantly affects the quenching/binding process. In general, the glycosylation of flavonoids lowers the affinity for BSA by 1 to 3 orders of magnitude depending on the conjugation site and the class of sugar moiety. The glucopyranosylation (daidzin and genistin) of flavonoids lowered the affinity for BSA by 5−10 times. Rhamnosylation (quercitrin) of flavonoids, however, lowered the affinity for BSA by 5600 times. This result partly supports that flavonoid aglycones are more easily absorbed than flavonoid glycosides. Higher binding affinities for BSA are associated with higher antioxidant activities for flavonoids. Glycosylation also decreases the hydrophobicity of flavonoids, and hydrophobic interaction may play an important role in binding flavonoids to proteins.

KEYWORDS: Affinity; flavonoid glycosides; glycosylation; plasma protein

INTRODUCTION

Flavonoids are important dietary polyphenols in many foods, such as fruits, vegetables, nuts, and tea (*1*−*4*). Over 6,000 flavonoids have been separated and identified from plants and most of which can be classified to flavone, flavonol, isoflavone, catechin, and so on according to the different structures. The structural difference of flavonoids also strongly affects their binding process with plasma proteins. The interaction between flavonoids and proteins has attracted great interest among researchers (*5*−*17*). The interaction between proteins and flavonoids results in forming stable complexes, which may be considered as a model for gaining general fundamental insights into flavonoid−protein interaction (*9*−*14*). Most of the reports only focused on the binding process between flavonoids and serum albumins, such as the forces involving in binding (such as hydrogen bonding, hydrophobic forces, and electrostatic forces), binding distance, energy transfer, and molecular modeling. Few reports, however, have focused on the structure−affinity relationship of flavonoids on binding to proteins.

The dietary flavonoids in nature exist almost always as β-glycosides (*18*). The flavonols are found mainly as the 3-*O*-glycoside, although the 7 and 4′ positions may also be glycosylated in some plants (*19*). Other classes of favonoids are found mainly glycosylated in the 7 position (*18*). However, puerarin is an isoflavone-8-*C*-glucose (**Figure 1**).

Flavonoid glycosides in most cases are hydrolyzed to their aglycones to produce effects in the body (*20*). Flavonoids in general are absorbed as their aglycones after prior hydrolysis of the glycosides along the aerodigestive tract. Small amounts of aglycones can also be present in the diet (*20*, *21*). The different structures of flavonoids significantly affect the absorption, metabolism, bioactivities in vivo, and the binding process with plasma proteins (*21*).

We have reported the influence of B-ring hydroxylation on binding flavonols to BSA (*22*). Dufour and Dangles (*5*, *23*) determined the binding constants between the flavonol (quercetin) and its 3-position glycosides with serum albumins and concluded that glycosylation of flavonoids could lower the affinity to albumins by 1 order of magnitude depending on the conjugation site. However, it is not reasonable to reach this conclusion because the structural difference between the different flavonoid classes was not taken into account. Recently, we simply discussed the influence of glycosylation in ring A of soybean isoflavones on interaction with BSA in a short communication (*24*). The sugar moieties are in 3,6,7,8-positions of flavonoids. Four flavonoid aglycones (baicalein, quercetin, daidzein, and genistein) and their monoglycosides (baicalin, quercitrin, daidzin and puerarin, and genistin), and another disaccharide glycosides (rutin) (**Figure 1**) are evaluated in this study. This

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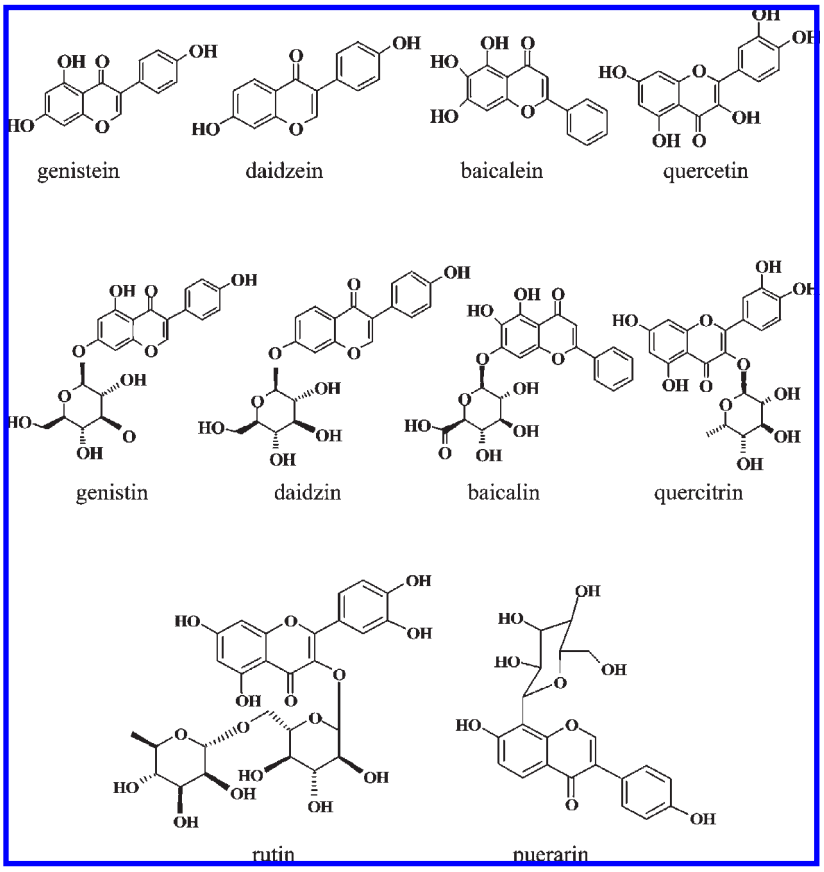

Figure 1. Structures of flavonoids in this study.

article also concerns the relationship between the affinities and the number of binding sites, the relationship between the affinities and the half-wave potential, and the relationship between the affinities and the bioactivities of flavonoids.

MATERIALS AND METHODS

Apparatus and Reagents. Fluorescence spectra were recorded on a JASCO FP-6500 spectrofluorometer (Tokyo, Japan). UV−vis spectra were recorded on a Shimadzu UV-2450 spectrophotometer (Kyoto, Japan). The pH measurements were carried out on a Cole-Parmer PHS-3C Exact Digital pH meter (IL, USA). BSA (fraction V), daidzin, and genistin were purchased from Sigma Co. (MO, USA) and were used without further purification. Baicalein, rutin, genistein, daidzein, quercetin, baicalin, puerarin, and quercitrin were obtained commercially from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) and were used without further purification. A working solution of flavonoids (1.0×10^{-4} mol/L) was prepared by dissolving each flavonoid in a methanol−water solution (2:8, v/v) (This solution did not affect the fluorescence of BSA). Tris-HCl buffer (0.20 mol/L, pH 7.4) containing 0.10 mol/L NaCl was selected to keep the pH value and maintain the ionic strength of the solution. The working solution of BSA (1.0×10^{-5} mol/L) was prepared with the Tris-HCl buffer and stored in a refrigerator for one month prior to use. All other reagents and solvents were of analytical reagent grade and were used without further purification unless otherwise noted. All aqueous solutions were prepared using newly double-distilled water.

Fluorescence Spectra. An appropriate quantity of flavonoid solution was transferred to a 10.0 mL flask, and then 1.0 mL of BSA solution was added and diluted to 10.0 mL with Tris-HCl buffer. The concentrations of baicalein, baicalin, quercitrin, and genistein were from 1.00 to 10.00 μmol/L. The concentrations of daidzein, daidzin, and genistin were from 2.50 to 25.00 μmol/L. The concentrations of quercetin were from 0.20 to 2.00 μmol/L, and the concentrations of rutin were from 2.50 to 40.00 μmol/L. The resultant mixture was subsequently incubated at 37 °C for 0.5 h. The solution was spectrofluorometrically scanned with the range of 290−500 nm. Each fluorescence intensity determination was repeated 3 times. The bandwidths for excitation and emission were 3 and 5 nm, respectively. The fluorescent intensity at 340 nm was determined with an excitation wavelength of 280 nm.

The relationship between fluorescence quenching intensity and the concentration of flavonoids can be described by the binding constant formula (*25*):

$$\log(F_0 - F)/F = \log K_a + n\log[Q] \quad (1)$$

where F_0 and F represent the fluorescence intensities in the absence and in the presence of flavonoid, and $[Q]$ is the concentration of flavonoid. K_a is the binding constant, and n is the number of binding sites per protein. After the fluorescence quenching intensities on protein at 340 nm were measured, the double-logarithm algorithm was assessed by eq 1.

RESULTS AND DISCUSSION

Effect of Flavonoids on BSA Fluorescence Spectra. These 10 flavonoids remarkably decreased the fluorescence intensity of BSA with the addition of flavonoids. **Figure 2** showed the representative fluorescence spectra (quercitrin, quercetin, and rutin). Quercetin can quench BSA fluorescence proportionally. Quercitrin, however, quenched BSA fluorescence nonproportionally. Quercetin quenched more BSA fluorescence than quercitrin even though the concentration of quercetin was lower than that of quercitrin. Experiments with quercitrin (10.00 μmol/L) only quenched 30% of BSA fluorescence, while quercetin (2.00 μmol/L) can quench more than 75% of BSA fluorescence. Rutin (40.00 μmol/L) only can quench about 70% of BSA fluorescence. From this point, the glycosylation of flavonoids affected the quenching effect on BSA fluorescence.

When considering the effect of flavonoids on the fluorescence spectra of BSA, there were weak blue shifts of λ_{em} for baicalein (5.4 nm) (as described in ref *24*), quercetin (6 nm), and rutin

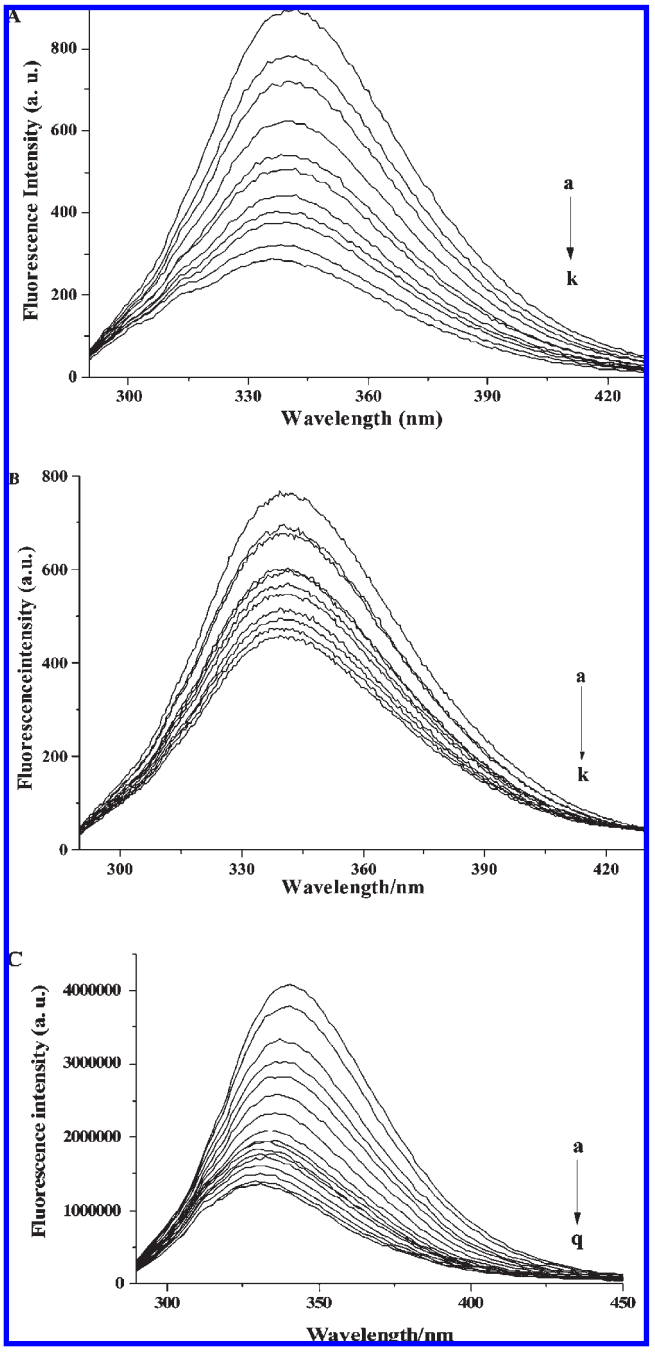

Figure 2. Quenching effect of flavonoids on BSA fluorescence. λ_{ex} = 280 nm; BSA, 1.0×10^{-6} mol/L; a−k, 0.00, 2.00... 20.00 ($\times 10^{-7}$ mol/L) of quercetin (**A**); 0.00, 1.00, ... 10.00 ($\times 10^{-6}$ mol/L) of quercitrin (**B**); (a−q) 0.00, 0.25... 4.00 ($\times 10^{-5}$ mol/L) of rutin (**C**).

(9.4 nm). These results suggested that there was a change in the immediate environment of the tryptophan and tyrosine residues and the fact that the flavonoids were situated at close proximity to the tryptophan and tyrosine residues for the quenching to occur. The fluorescence of BSA is primarily from the 2 tryptophan and 18 tyrosine residues. When the excitation wavelength is 280 nm, the fluorescence emission shows the characteristic of tyrosine and tryptophan residues. In present study, the information about other amino acid residues was not understood. This means that the molecular conformation of the protein was affected, which is in agreement with recent studies that have shown that the tertiary structure of proteins changes upon binding to phenols (*6*−*17*). The fluorescence of these flavonoids with the excitation

Table 1. Binding Parameters for Flavonoid−BSA Systems (310.15 K)

flavonoids	type	log K_a	*n*	*R*
quercetin	flavonol	7.5626	1.291	0.9946
quercitrin	glycoside	3.8107	0.814	0.9835
rutin	glycoside	5.65	1.19	0.9965
baicalein	flavone	7.8209	1.333	0.9934
baicalin	glycoside	6.2128	1.173	0.9961
genistein	isoflavone	5.9243	1.015	0.9979
genistin	glycoside	5.1580	1.170	0.9917
daidzein	isoflavone	4.7336	1.037	0.9975
daidzin	glycoside	3.7464	0.878	0.9817
puerarin	glycoside	3.1987	0.73	0.9929

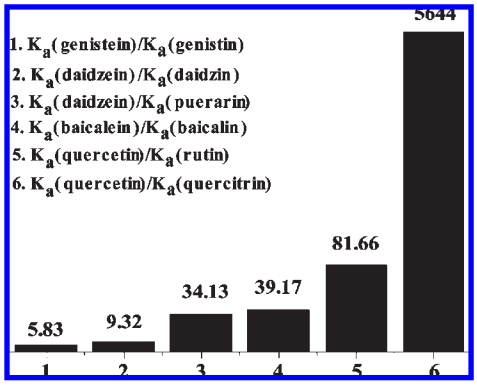

Figure 3. Glycosylation lowers the affinities of flavonoids for BSA by 1−3 orders of magnitude.

wavelength of 280 nm did not affect the fluorescence spectra of BSA (data is not shown here).

Binding Constants and Structure−Affinity Relationship. Table 1 gives the binding parameters for flavonoid−BSA systems according to eq 1. The binding constants (K_a) between flavonoids and BSA decrease after glycosylation. Glycosylation of flavonoids can lower the affinities for BSA by 1 to 3 orders of magnitude depending on the conjugation site and the class of sugar (**Figure 3**). The affinity of quercetin for BSA is about 5600-fold and 82-fold higher than those of quercitrin and rutin, respectively, but the affinity of genistein for BSA is only 5 times higher than that of genistin. The sugar moieties are glucopyranose, glucuronic acid, rhamnose, rutinose, and glucose−rhamnose. As shown in **Figure 3**, the glucopyranosylation (daidzin and genistin) of isoflavones lowered the affinity for BSA by 5−10 times. Rhamnosylation (quercitrin) of quercetin, however, lowered the affinity for BSA by 5600 times. Compared with the affinity of daidzein for BSA, the affinity of puerarin (daidzein-8-*C*-glucose) for BSA decreased 34 times.

The decreasing affinity for BSA after glycosylation may be caused by the increasing molecular size and polarity, and transfer to the nonplanar structure. After the hydroxyl group is substituted by a glycoside, steric hindrance may take place, which weakens the affinity for BSA. Another possible explanation is that the glycosylation decreases the hydrophobicity of flavonoids. Many papers reported that the hydrophobic interaction plays an important role in binding small molecules to proteins (*26*−*29*).

Our results partly support the hypothesis that flavonoid aglycones are more easily absorbed than flavonoid glycosides (*21*). The fairly large and highly polar flavonoid glycosides cannot be absorbed after oral ingestion but are hydrolyzed to their aglycones by bacterial enzymes in the lower part of the intestine (*20*, *21*). The binding of small molecules to plasma proteins is a very important parameter for drug metabolism

and pharmacokinetic studies. If a molecule is lowly bound to plasma proteins, the amount of drug available to diffuse into the target tissue may be significantly reduced, and the efficacy of the drug may consequently be poor. Determining the level of binding, therefore, is critical and will directly correlate with the in vivo efficacy of the molecule.

Martini et al. (*30*) applied NMR methodology to investigate the interaction between quercetin and quercetin 3-*O*-*β*-D-glucopyranoside with BSA. The calculated values of the affinity indexes and thermodynamic equilibrium constants suggested a much stronger capacity of quercetin to interact with BSA when compared with its glucosylated derivative.

There are controversies about which form of flavonoids is actually absorbed. Hollman et al. (*31*) found that both quercetin and its glycosides can be absorbed. Walle et al. (*32*), however, did not find any quercetin glycosides in the ileostomy fluid, whereas plentiful amounts of quercetin aglycones were identified. This illustrates that glycosides were hydrolyzed to quercetin and then absorbed. Many flavonoid glycosides are not absorbed because of efficient efflux transport by multidrug resistance-associated protein (*33*).

Flavonoid glycosides are generally hydrophilic in nature and thus cannot be transported across membranes by passive diffusion. Upon hydrolysis by the enzymes released by enterobacteria, the sugar moiety of flavonoid glycosides is cleaved, resulting in more lipophilic aglycones. These become permeable through the gut wall. Studies showed that quercetin glucosides from onions were absorbed more efficiently than those from apples or quercetin glycoside supplements (*34*).

Relationship between the Affinities and the Number of Binding Sites. Most reports only focused on the thermodynamics and the mechanism of binding flavonoids to proteins. In this work, the effect of sugar moieties in flavonoids on the affinities for BSA is discussed. In total, 10 flavonoids are tested. The binding constants (K_a) and the number of binding sites (n) between flavonoids and BSA are listed in **Table 1**.

Many models by means of fluorescence quenching of proteins have been used to study the interaction between small molecules and proteins, such as the Stern−Volmer equation, double logarithm equation, Levine equation, Lineweaver−Burk curve, Benesi−Hildebrand equation, and Scatchard equation. However, the binding constants and the number of binding sites obtained were different when calculated using different models or equations. For example, several reports based on fluorescence quenching studied the interaction between quercetin and BSA by means of the Johansson equation (*35*), Scatchard equation (*5*, *23*), and the double logarithm regression curve (*36*, *37*). The binding parameters of quercetin and BSA obtained by these models are quite different from each other. The numbers of binding sites (n) were determined as 0.89, 1.77, 1.19, 1.01, and 0.95. In present work, however, the number of binding sites between quercetin and BSA was determined as 1.29 (double-logarithm equation).

The obtained n values (0.73−1.333) in this study correspond to the binding sites with high affinity; however, the existence of the low affinity sites was not studied in this work. Recently, Berezhkovskiy illustrated that the calculated number of binding sites increased with the increase of compound concentration using the measured values of the unbound drug fraction (*38*). The number of binding sites (n) is different from the number of molecules actually bound to the sites (*38*, *39*). The number of molecules bound to the binding sites of a biomacromolecule follows a binomial distribution, if the number of binding sites is fixed (*39*). When binding to the receptor with n sites of the same reaction is considered, and $K_d = 1/K_a$ is the dissociation binding constant

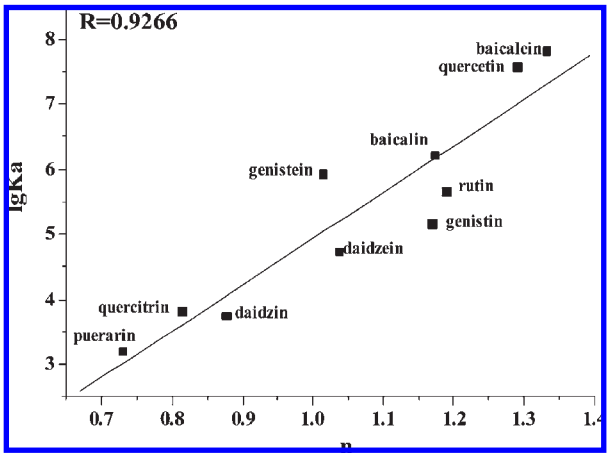

Figure 4. Relationship between log K_a and the number of binding sites (n) between flavonoids and BSA.

(affinity of the binding site), it is necessary to have the ligand concentration roughly equal to $10 \cdot K_d$ to occupy 90% of the binding sites. The low affinity sites (K_d about or greater than 1000 μM^{-1}) were not practically occupied (compared to the binding sites with high affinity) and thus were not detected at the low concentration of flavones used in the experiments. If the number of low affinity sites is significant (for instance 10 with $K_d = 1000\ \mu M^{-1}$), binding to them will be comparable to binding to a single high affinity site. This is because the increase of the quantity of sites leads to the increase of ligand bound to them. Therefore, the number of binding sites increasing with increasing binding constant can be considered as one theory to evaluate these models. The relationship between log K_a and the number of binding sites (n) between flavonoids and BSA is shown in **Figure 4**. The values of log K_a are proportional to the number of binding sites (n). This result confirms that the method used here is suitable to study the interaction between flavonoids and BSA.

Relationship between the Affinities and the Hydrophobicity of Flavonoids. The partition coefficient (P) is the ratio of the concentrations of un-ionized compound between the two solutions. The logarithm of the ratio of the concentrations of the un-ionized solute in the solvents is called log P. The partition coefficient serves as a quantitative descriptor of lipophilicity and is one of the key determinants of pharmacokinetic properties. The log P values are important to many industries and areas of research in determining how to deliver chemical substances to specific sites. It is also crucial for eliminating chemicals from others, as well as limiting unwanted dispersal of chemicals through the environment.

The lipophilicity of these flavonoids was assessed by their partition coefficient values (P) according to Yang et al. (*40*) and Lee and Row (*41*). The log P values of flavonoid aglycones decreased after glycosylation. In this work, we found that the log P values of flavonoids with the same aglycone are inversely proportional to the affinities for BSA (**Figure 5**). This result confirmed that the glycosylation decreases the hydrophobicity of flavonoids and that hydrophobic interaction plays an important role in binding flavonoids to proteins.

Relationship between the Affinities and the Half-Wave Potential of Flavonoids. Yang et al. (*40*) used the half-wave potential values ($E_{1/2}$) to estimate the antioxidant activities of flavonoids. The $E_{1/2}$ values of flavonols are inversely proportional to the number of hydroxyl groups in the B-ring, and lower $E_{1/2}$ values are associated with higher antioxidant and free radical scavenger activities for flavonoids (*40*). In this work, we found that the $E_{1/2}$ values of

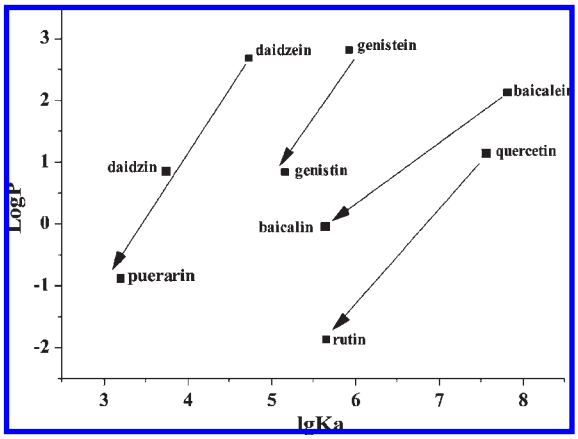

Figure 5. Relationship of binding constants (log K_a) with partition coefficient (log P) of flavonoids. The partition coefficient values (P) were taken from Yang et al. (*40*) and Lee and Row (*41*).

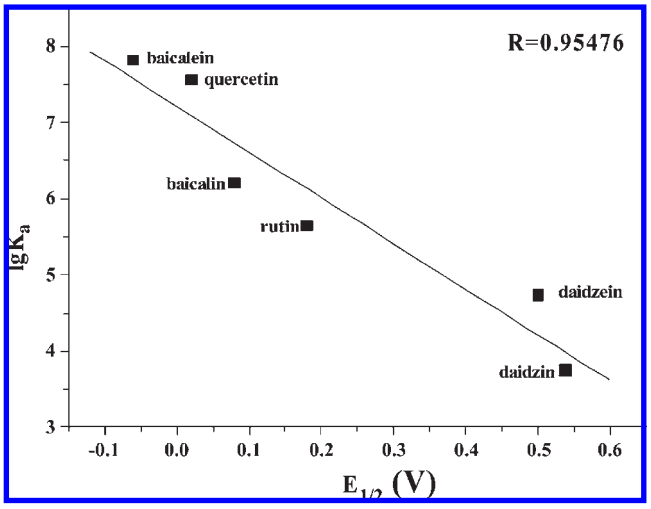

Figure 6. Relationship between log K_a and the half-wave potential ($E_{1/2}$) of the flavonoids tested. The half-wave potential values ($E_{1/2}$) were taken from Yang et al. (*40*).

flavonoids are inversely proportional to the binding constants with BSA (only 6 flavonoids with $E_{1/2}$ values are in this study). Higher binding affinities with BSA are associated with higher antioxidant and free radical scavenger activities for flavonoids (**Figure 6**).

Relationship between the Affinities and the Bioactivities of Flavonoids. Plants usually glycosylate metabolites to enhance their solubility and improve sequestration into specific cellular compartments. Deglycosylation normally generates the active form of the compound. Thus, glycosylation not only will mask one of the hydroxyl groups but also will make the flavanoid less planar; both of these conditions could be expected to impact binding to a protein.

Many researchers have proved that flavonoid aglycones have stronger bioactivity than their glycosides in vivo. Chow et al. (*42*) found that quercetin, but not its glycoside rutin and quercitrin, prevented H_2O_2-induced apoptosis in macrophages. Lin et al. (*43*) found flavonoids without glycosides exhibited more significant inhibitory effects on LPS-induced NO and PGE2 production than the respective glycosylated flavonoids via HO-1 induction. Only flavonoid glycosides with OH moieties at specific positions, such as the 3′,4′-positions, possess remarkable biological activities (e.g., rutin and quercitrin) (*44*).

Amakura et al. (*45*) studied in vitro aryl hydrocarbon receptor (AhR)-inducing potencies of 95 plant constituents by the CALUX assay, which measures the ability of the chemical to activate AhR-dependent gene expression in cells in culture. The glycosides (daidzin, baicalin, and genistin) showed lower AhR responses than aglycones (daidzin, baicalein, and genistin). From the structural differences of glycosides and aglycones, Amakura et al. (*45*) concluded that the differences in activity may be ascribed to the increasing molecular size and polarity and to the transfer to the nonplanar structure produced by glycosylation. Their conclusion is confirmed in this study. It was found that the glycosylation of flavonoids decreased the affinity for serum albumin. The glycosylation of flavonoids may also decrease the affinity for proteins such as the aryl hydrocarbon receptor.

Takano-Ishikawa et al. (*46*) reported the structure−activity relationship of inhibitory effects of various flavonoids on lipopolysaccharide-induced prostaglandin E2 production in rat peritoneal macrophages. The 50% inhibitory concentrations (IC_{50}) of baicalein, quercetin, daidzein, and genistein for the inhibition of PGE2 biosynthesis were determined as 2.5, 13.9, 37.6, and 7.2 μmol/L. However, the IC_{50} values of baicalin, quercitrin, daidzin, genistin, and rutin for the inhibition of PGE2 biosynthesis were all higher than 1000 μM. For a flavonoid aglycone and its corresponding glycoside, higher binding affinities for BSA are associated with a higher inhibitory effect on PGE2 biosynthesis.

Pairoba et al. described that the glycosylation of hydroxyl groups on the flavonoids weakened the inhibitory effects on the activity of PEP carboxylase and NADP malic enzyme (*47*). IC_{50} values for the inhibition of these two enzymes were determined as quercetin < quercitrin < rutin. The decreased inhibitory effects on these enzymes caused by glycosylation may be due to the weak affinities for these enzymes. The affinity−activity relationship of flavonoids for the inhibition of enzymes is an interesting topic, which may explore the inhibitory nature.

Kim et al. (*48*) reported that puerarin and daidzin are metabolized to daidzein by human intestinal microflora and that the metabolite daidzein exhibited the more potent antioxidant and antitumor cytotoxic effects. Park et al. (*49*) found that the estrogenic effect of *Pueraria thunbergiana* may be dependent on the metabolism of its isoflavone glycosides, and the metabolite daidzein has been reported to exhibit potent biological effects such as cytotoxic, antiallergic, and antioxidant effects. For isoflavones, the increasing affinities for plasma protein maybe one of the explanations for the increased pharmacological effects of isoflavone aglycones.

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