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Noncovalent Interaction of Dietary Polyphenols with Bovine Hemoglobin in Vitro: Molecular Structure/Property−Affinity Relationship Aspects

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ABSTRACT: The relationship between the structural properties of selected dietary polyphenols and their affinities for bovine hemoglobin (bHB) was investigated by fluorescence analysis. The presence of an additional methoxy group on flavonoids weakened the affinities for bHB by 1.15−13.80 times. Hydroxylation on rings A, B, and C also significantly affected the affinity for bHB. The glycosylation of flavonoids decreased the affinities for bHB by 1 order of magnitude depending on the conjugation site and the class of sugar moiety. Hydrogenation of the C2═C3 double bond also decreased the binding affinity. The galloylated catechins exhibited higher binding affinities for bHB than nongalloylated. The glycosylation of resveratrol increased its affinity for bHB. The binding process with bHB was strongly influenced by the structural differences of dietary polyphenols. The affinities for bHB increased with increasing partition coefficients and decreased with increasing hydrogen bond donor and acceptor numbers of polyphenols, which suggested that the binding interaction was mainly caused by the hydrophobic force.

KEYWORDS: structure−affinity relationship, property−affinity relationship, polyphenols, bovine hemoglobin, protein binding

INTRODUCTION

Dietary flavonoids and stilbenes are important polyphenols in foods, such as fruits, vegetables, nuts, and tea,[1−5] as they are of great interest for their bioactivities, which are basically related to their antioxidative properties.[5−8] The structural differences between the various classes concern the chemistry of ring C, as well as the number and distribution of hydroxyl groups and their substitutions on rings A and B. These differences significantly affect their absorption, metabolism, and bioactivities in vivo. For instance, methylation of the free hydroxyl groups in the flavones dramatically increased their intestinal absorption and metabolic stability by preventing the formation of glucuronic acid and sulfate conjugates.[9,10] Walle concluded that methylation appears to be a simple and effective way to increase the metabolic resistance and transport of flavonoids.[11] Courts and Williamson found that the *C*-glycoside aspalathin was methylated and glucuronidated in vivo in an intact form in humans.[12] The flavonol moiety, that is, the 2,3-double bond in conjugation with a 4-oxo group and a 3-hydroxyl group, and the 5,7-dihydroxylation at the A-ring have been found to be important structural features for significant antioxidant activity.[13] In addition to −OH moieties in the structural arrangements of flavonoids, the resonance of electrons between rings A and B was reported to be essential for the antioxidant and biological activities of the compounds.[14]

Bovine hemoglobin (bHB) is the iron-containing oxygen-transport metalloprotein in the red blood cells. Hemoglobin in the blood transports oxygen from the lungs or gills to the rest of the tissues, where it releases the oxygen for cell use and collects carbon dioxide to bring it back to the lungs. Hemoglobin is made up of four protein molecules (globulin chains) that are connected together. Recently, the interactions between polyphenols and hemoglobin have attracted great interest.[15−19] The distribution, metabolism, and efficacy of many drugs are correlated with their affinities toward hemoglobin.[18] Studies of the interaction of small molecules with hemoglobin are imperative and of fundamental importance. Few papers, however, have focused on the structure−affinity relationship of polyphenols on the affinities for bHB. The present work concerns the relationship between the molecular properties of dietary polyphenols and their affinities for bHB. Thirty-eight polyphenols (Table 1) were studied.

MATERIALS AND METHODS

Apparatus and Reagents. The fluorescence spectra were recorded on a JASCO FP-6500 fluorometer (Tokyo, Japan). The pH measurements were carried out on a Cole-Parmer PHS-3C Exact Digital pH-meter (Vernon Hills, IL). bHB (∼99%, lyophilized powder) and 7-hydroxyflavone (99.5%) were purchased from Sigma Chemical Co. (St. Louis, MO). Biochanin A, genistein, apigenin, puerarin, catechin (C), epicatechin (EC), and luteolin (99.0%) were purchased from Aladin Co. Ltd. (Shanghai, China). Flavone, chrysin, and baicalein (99.5%) were obtained commercially from Wako Pure Chemical Industries (Osaka, Japan). Kaempferide, kaempferol, tangeretin, nobiletin, quercetin, myricetin, daidzein, baicalin, wogonin, kaempferitrin, galangin, fisetin, genistin, dihydromyricetin, (−)-epigallocatechin (EGC), (−)-epigallocatechin gallate (EGCG), gallocatechin gallate (GCG), tectorigenin, rutin, polydatin, resveratrol, narirutin, naringin, naringenin, hesperitin, quercitrin, and formononetin (>98.0%) were obtained commercially from Shanghai Tauto Biotech Co., Ltd. (Shanghai, China). The working solutions of the polyphenols (1.0×10^{-3} mol/L) were prepared by dissolving each polyphenol with methanol. Tris-HCl buffer (0.20 mmol/L, pH 7.4) containing 0.10 mol/L NaCl was selected to keep the

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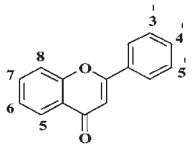
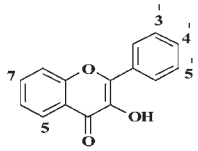
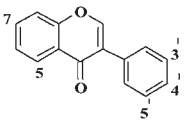
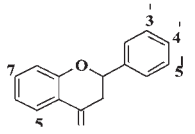
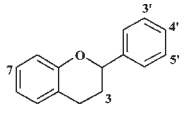
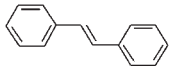
Table 1. Chemical Structures of the Various Polyphenols and Their Affinities for Bovine Hemoglobin in Vitro

Subclass	Name	Substitutions			lgK_a	n
		OH	OCH_3	others		
Flavones	Flavone				4.68	1.031
	7-OHflavone	7			4.92	1.041
	Chrysin	5,7			5.02	1.044
	Baicalein	5,6,7			4.83	0.982
	Baicalin	5,6		7-β-D-glucuronide	4.46	0.941
	Apigenin	5,7, 4'			4.96	1.029
	Luteolin	5,7,3', 4'			4.16	0.897
	Wogonin	5,7	8		3.88	0.853
	Tangeretin		5,6,7,8,4'		5.26	1.066
	Nobiletin		5,6,7,8,4',5'		4.67	0.953
Flavonols	Galangin	3,5,7			4.44	0.924
	Kaempferide	3,5,7	4'		4.10	0.894
	Kaempferol	3,5,7, 4'			4.29	0.904
	Kaempferitrin	5,4'		3,7-dirhamnoside	4.28	0.932
	Quercetin	3,5,7,3', 4'			4.65	0.983
	Quercitrin	5,7,3', 4'		3-o-β-D-glucoside	3.55	0.761
	Myricetin	3,5,7,3', 4', 5'			3.61	0.801
	Fisetin	3,7,3', 4'			4.12	0.899
	Rutin	5,7,3', 4'		3-α-L-Rham-1,6-D-Glc	3.79	0.845
Isoflavones	Formononetin	7	4'		4.35	1.028
	Genistein	5,7,4'			4.08	0.929
	Daidzein	7,4'			4.62	1.072
	Genistin	5,4'		7-glucoside	3.13	0.776
	Biochanin A	5,7	4'		4.48	0.959
	Tectorigenin	5,7,4'	6		4.02	0.871
	Puerarin	7,4'		8-C-glucose	4.23	0.976
	Hesperitin	5,7, 3'	4'		3.90	0.862
Flavanone	Naringenin	5,7, 4'			3.58	0.820
	Naringin	5,4'		7-neohesperidose	3.20	0.713
	Narirutin	5,4'		7-α-L-Rham-1,6-D-Glc	3.20	0.754
	Dihydromyricetin	3,5,7,3', 4', 5'			3.39	0.781
Flavanonol	GCG (2,3-trans)	5,7,3',4',5'		3-gallate	3.31	0.753
	EGCG (2,3-cis)	5,7,3',4',5'		3-gallate	3.44	0.813
	EC (2,3-cis)	3,5,7,4',5'				
	EGC (2,3-cis)	3,5,7,3',4',5'				
	C (2,3-trans)	3,5,7,4',5'				
Stilbene	Resveratrol	3,5,4'			4.44	0.967
	Polydatin	5,4'		3-glucoside	4.71	1.015

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pH value and maintain the ionic strength of the solution. The working solution of bHB (1.0×10^{-5} mol/L) was prepared with Tris-HCl buffer and stored in a refrigerator prior to use. All other reagents and solvents were of analytical grade, and all aqueous solutions were prepared using newly double-distilled water.

Fluorescence Spectra. Three milliliters of a working solution of bHB (1.0×10^{-5} mol/L) was transferred to a 1.0 cm quartz cell and then titrated with successive additions of 3.0 μL of polyphenol solution (1.0×10^{-3} mol/L). Titrations were performed manually by using trace syringes. In each titration, the fluorescence spectrum was collected with the working solution of bHB (1.0×10^{-5} mol/L). The fluorescence spectra were recorded in the wavelength range of 310−450 nm upon excitation at 280 nm when bHB was titrated with polyphenols. Slit widths, scan speed, and excitation voltage were kept constant within each data set, and each spectrum was the average of three scans. The results of the time course experiments for the equilibration are not given here. The polyphenols were stable during the fluorescence measurements, as shown by HPLC analyses (not given here). Each fluorescence intensity determination was repeated three times and found to be reproducible within experimental error (<3%).

RESULTS AND DISCUSSION

Binding Constants (K_a) and Number of Binding Sites (n). As representative examples, the fluorescence spectra of bHB with the addition of genistein and genistin are shown in Figure 1 (The fluorescence spectra of bHB quenched by other polyphenols are not given here.) In these and all other cases, the fluorescence intensities of bHB decreased remarkably with increasing concentration of polyphenols. Obvious blue shifts of the maximum λ_{em} of bHB fluorescence were observed for genistein and genistin (The λ_{max} values of the first and last spectrum are given in Figure 1.) The molecular conformation of bHB was affected, which is in good agreement with the data of recent similar studies.[20−25]

The binding constants were calculated according to the double-logarithm equation[20−25]

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

where F_0 and F represent the fluorescence intensities of bHB in the absence and in the presence of polyphenols, respectively, K_a is the binding constant, n is the number of binding sites per bHB, and [Q] is the concentration of polyphenols. According to Fluorescence Spectra, the values of $(F_0 - F)/F$ can be obtained in each [Q]. Then, the linear regression equation between the $\lg[(F_0 - F)/F]$ values and lg[Q] values was obtained on Origin 7.5 software. The slope factor means n and the intercept refer to $\lg K_a$. Table 1 summarizes the calculated results according to eq 1. The values of $\lg K_a$ are proportional to the number of binding sites (n) (Figure 2), which indicates that the eq 1 used here is suitable for the study of the interaction between polyphenols and bHB.[26,27] The magnitudes of apparent binding constants for bHB were almost all in the range of 10^3−10^5 L/mol, which were similar to recent reports for bHB.[15−19] However, these data were much smaller than the affinities of polyphenols for bovine serum albumin (BSA) and human serum albumin (HSA) from our previous studies (10^4−10^8 L/mol).[21−24]

Influence of an Additional Methoxy Group in Flavonoids on the Affinities for bHB. As shown in Figure 3, the presence of an additional methoxy group tended to decrease the affinity for bHB by 1.15−13.80 times, most notably for wogonin compared to chrysin. On the other hand, the presence of an additional methoxy group on genistein or kaempferol little affected the affinity for bHB (Figure 3).

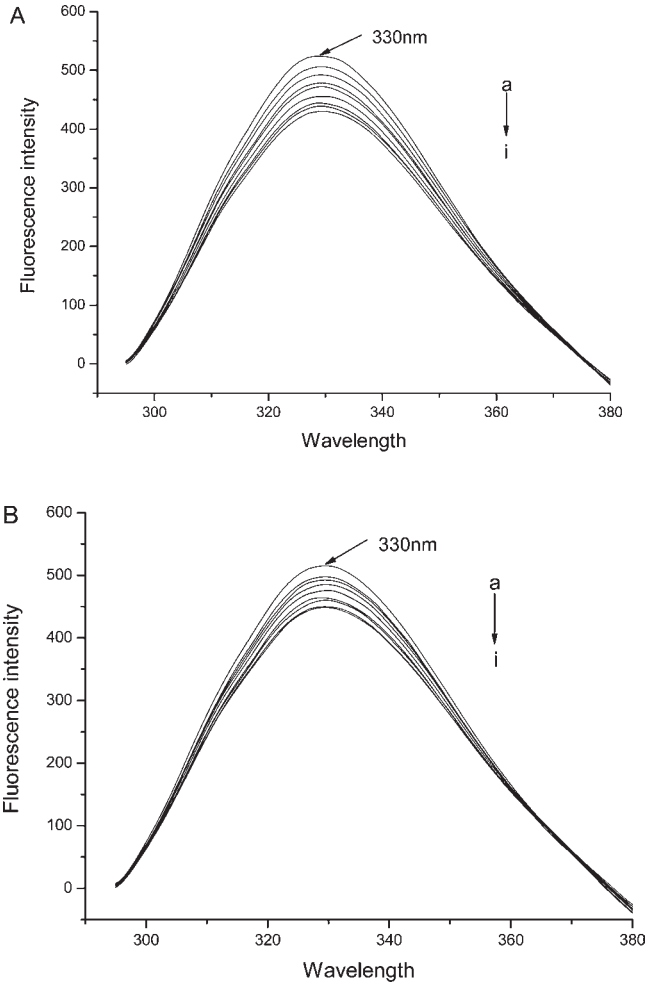

Figure 1. Quenching effect of genistein (A) and genistin (B) on bHB fluorescence spectra at 300.15 K. λ_{ex} = 280 nm; HB, 10.00 μmol/L. a−i: 0.00, 1.00, 2.00, ..., 9.00 ($\times 10^{-6}$ mol/L) of polyphenols.

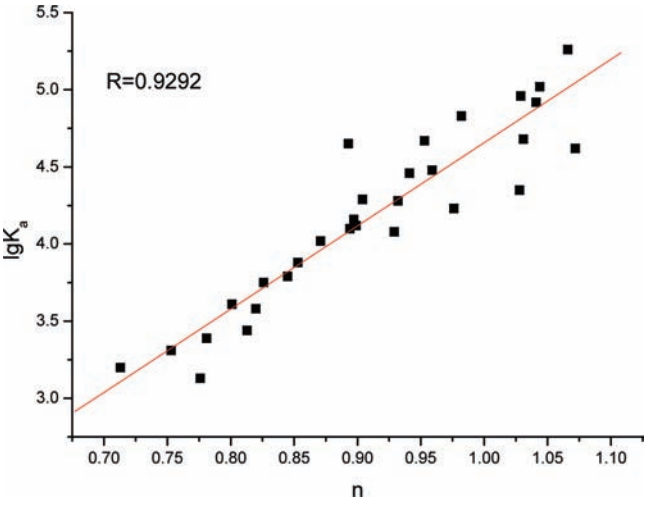

Figure 2. Relationship between the affinities ($\lg K_a$) and the number of binding sites (n) between polyphenols and bHB.

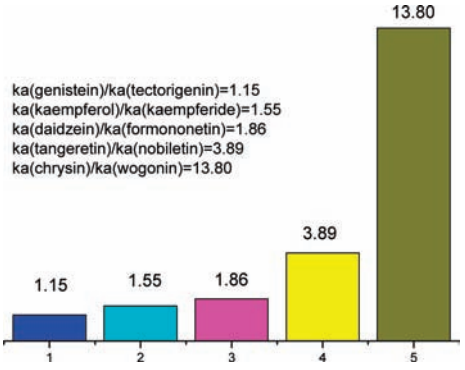

Figure 3. Influence of an additional methoxy group in flavonoids on the affinities for bHB.

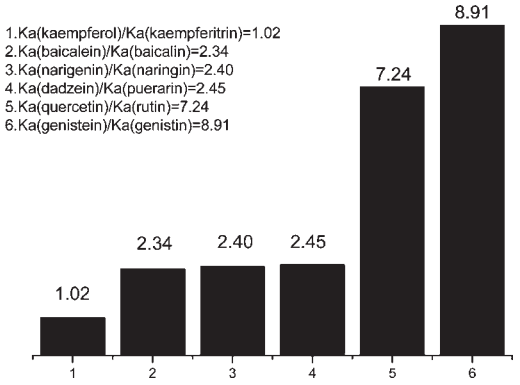

Figure 4. Glycosylation decreases the affinity of the polyphenols for bHB.

Table 2. Effects of Hydroxylation of Flavonoids on the Affinities for Bovine Hemoglobin in Vitro

class	ring	position	example	effect (times)
flavone	A	7H → OH	flavone → 7-OH-flavone	↑(1.74)
		5H → OH	7-OH-flavone → chrysin	↑(1.26)
		6H → OH	chrysin → baicalein	↓(1.55)
	B	4′H → OH	chrysin → apigenin	↓(1.15)
		3′H → OH	apigenin → luteolin	↓(6.31)
	C	3H → OH	chrysin → galangin	↓(3.80)
			apigenin → kaempferol	↓(4.68)
			luteolin → quercetin	↑(3.09)
flavonol	A	5H → OH	fisetin → quercetin	↑(3.39)
	B	3′H → OH	kaempferol → quercetin	↑(2.29)
		4′H → OH	galangin → kaempferol	↓(1.41)
		5′H → OH	quercetin → myricetin	↓(10.96)
isoflavone	A	5H → OH	daidzein → genistein	↓(3.47)
			formononetin → biochanin A	↑(1.35)

Effects of Hydroxylation of Flavonoids on the Affinities for HB. Table 2 shows the effects of hydroxylation of flavonoids on the affinities for bHB in vitro. As seen from the data, hydroxylation on rings A, B, and C of flavonoids significantly affected the binding affinities for bHB.

Hydroxylation on Ring A of Flavones. As illustrated in Table 2, it appears that the optimal hydroxyl groups introduced to ring A of flavones are at the 5,7-position, as the highest binding was observed with chrysin (containing two hydroxyl groups). When an additional hydroxy group was introduced to the C-6 position of chrysin, its affinity for bHB slightly decreased (baicalein).

Hydroxylation on Ring B of Flavones. As shown in Table 2, the apparent binding constants (K_a) between flavones and bHB reduced with the increasing number of hydroxyl groups on the B-ring. Extremely, the affinity of luteolin (5,7,3′,4′) for bHB was found to be 6.31 times lower than that of apigenin (5,7,3′) (Figure 3). On the other hand, the affinity of apigenin (5,7,3′) for bHB was only 1.15 times lower than that of chrysin (5,7).

Hydroxylation on Ring C of Flavones. As shown in Table 2, the affinities of kaempferol (3,5,7,3′) and galangin (3,5,7) for bHB are 4.68 and 3.80 times lower than those of apigenin (5,7,3′) and chrysin (5,7). In contrast, the affinity of quercetin (3,5,7,3′,4′) for bHB is 3.09 times higher than that of luteolin (5,7,3′,4′).

Hydroxylation of Rings A and B of Flavonols. In an earlier study we reported that the binding constants (K_a) and the number of binding sites (n) between flavonols and BSA increased with increasing hydroxyl groups on ring B.[23] In this study, however, it was found that the hydroxylation on position 3′ of flavonol improves the binding affinity for bHB but that hydroxylation on position 5′ of flavonol significantly reduced their binding affinities for bHB. The affinity of quercetin (3′,4′) for bHB was 2.29 times higher than that of kaempferol (4′). The affinities of kaempferol (3,5,7,4′) and myricetin (3′,4′,5′) for bHB were 1.41 and 10.96 times lower than those of galangin (3,5,7) and quercetin (3′,4′). Flavonols are the most prominent flavonoids in plants. The most prominent flavonols, such as quercetin and kaempferol, in foods exist with 5,7-dihydroxyl groups on ring A. Here, it was found that hydroxylation on position 5 of flavonol slightly enhances the binding affinity for bHB by 3.39 times.

Hydroxylation on Ring A of Isoflavones. As shown in Table 2, the affinity of genistein (5,7,4′) for bHB was 3.47 times lower than that of daidzein (7,4′). The affinity of genistin (5,7,4′) for bHB was 30.9 times lower than that of daidzein (7,4′). Conversely, the affinity of biochanin A (5,7) for bHB was 1.35 times higher than that of formononetin (7).

Comparing the Affinities of Flavonoid Isomers with bHB. Previously, we have compared the affinities of flavonoid isomers with BSA and found the apparent binding constants (K_a) were determined as flavone > isoflavone ≈ flavonol.[28] In this study, we can compare two isomer groups (apigenin, baicalein, genistein and luteolin, kaempferol). The binding constants (K_a) were determined as apigenin > baicalein > genistein and luteolin ≈ kaempferol. The current results are in good agreement with data reported previously about the interaction between flavonoid isomers with BSA and HSA.[28,29]

Effects of Glycosylation of Dietary Flavonoids on the Affinities for HB. The dietary flavonoids in nature occur mostly as β-glycosides. The flavonols are found mainly as the 3- and 7-*O*-glycosides, although the 4′-position may also be glycosylated in some plants.[24] Most recently, we have reported that glycosylation of flavonoids lowered the affinity for BSA by 1−3 orders of magnitude depending on the conjugation site and the class of sugar moiety.[24] Herein, the effect of glycosylation of dietary flavonoids on the affinities for bHB was investigated. The sugar moieties are in the 3- or 7 -position of flavonoids. In our present study, the glycosylation of flavonoids lowered the affinity for

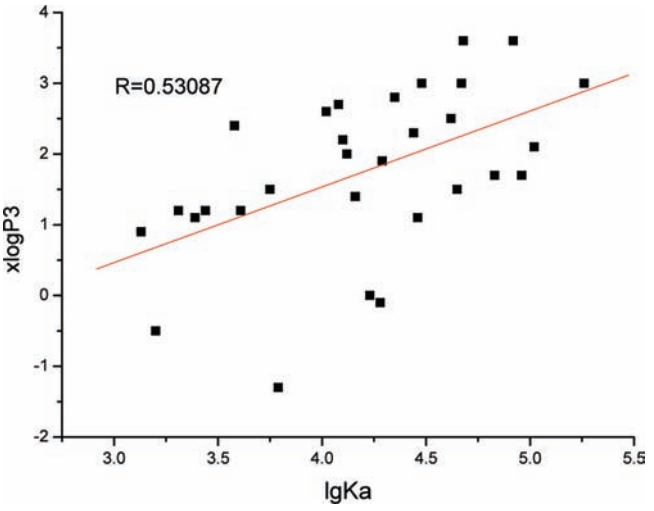

Figure 5. Relationship between $XlogP_3$ values and lg K_a values of polyphenols for bHB.

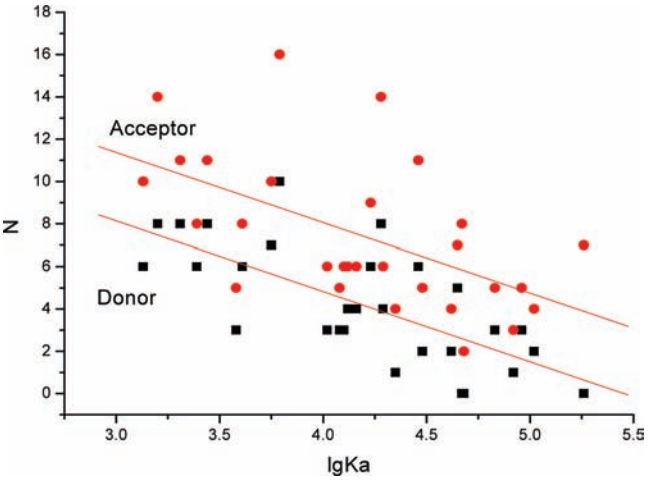

Figure 6. Relationships of the hydrogen bond acceptor/donor number of polyphenols (N) with the affinities for bHB. The hydrogen bond acceptor/donor numbers were taken from PubChem Public Chemical Database.[33].

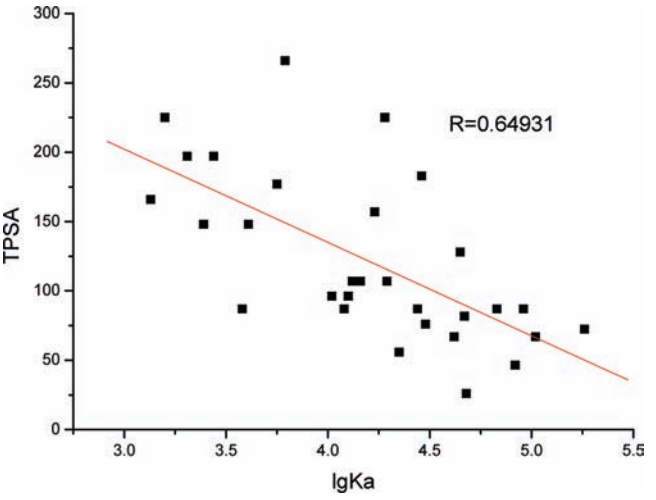

Figure 7. Relationship between TPSA values and lg K_a values of polyphenols for bHB.

bHB by 1 order of magnitude (Figure 4). The affinity of genistin for bHB was 8.91-fold lower than that of genistein, but the affinity of kaempferitrin for bHB is approximately 1.02 times higher than that of kaempferol. The affinities of rutin, puerarin (8-*C*-glycoside), naringin, and baicalin for bHB were about 7.24, 2.45, 2.40, and 2.34 times higher than that of quercetin, daidzein, narigenin, and baicalein, respectively. The decreasing affinity for bHB after glycosylation may be caused by the nonplanar structure. Steric hindrance and hydrophilicity are other possible causes for the decreased affinity.[24]

Effects of Hydrogenation of the C2═C3 Double Bond of Dietary Flavonoids on the Affinities for bHB. The C2═C3 double bond in conjugation with a 4-oxo group plays a very important role for the affinity for HB. It was found that hydrogenation of the C2═C3 double bond of flavonoids decreased the binding affinities for bHB. As shown in Table 1, the affinities of apigenin and myricetin for bHB were about 23.99 and 1.65 times higher than those of naringenin and dihydromyricetin, respectively. Previously, we have investigated the effect of hydrogenation of the C2═C3 double bond in flavonoids on the affinities for BSA, bovine γ-globulin, and HSA.[29−31] Hydrogenation of the C2═C3 double bond for many flavonoids decreased the binding affinities for BSA and HSA by 2−4 orders of magnitude. Planarity of the C ring in flavonoids may be important for binding interaction with proteins, as the molecules with saturated C2—C3 bonds (flavanones and certain others) permit more twisting of the B-ring with reference to the C-ring. A C2═C3 double bond increases the π-conjugation of the bond linking the B- and C-rings, which favors near-planarity of the two rings.[32] Molecules with near-planar structure more easily enter the hydrophobic pockets in proteins.

Catechins. Catechins are the major polyphenols in green tea leaves. The major catechins of green tea extract are C, EC, EGC, (−)-epicatechin gallate (ECG), EGCG, and GCG. Recent studies have suggested that the catechins form complexes with hemoglobin for transport in human blood, and their binding affinity for albumin is believed to modulate their bioavailability. Here, we determined the affinities between catechins and bHB by fluorescence quenching method with a double-logarithm regression curve. The binding constants (lg K_a) between EGCG and GCG for bHB were 3.44 and 3.31, respectively. However, EC, EGC, and C hardly quenched the fluorescence of bHB. This illustrated that the galloylated catechins have higher binding affinities with bHB than nongalloylated catechins. The presence of the galloyl moiety is the most decisive factor that increases the affinity for bHB. In our present study, the affinity of the catechin with a 2,3-trans structure (GCG) for bHB was lower than that of the catechin with a 2,3-cis structure (EGCG).

Stilbenes. Stilbenes are important polyphenols with a $C_6-C_2-C_6$ structure. The typical natural stilbenes are resveratrol and its 3-glucoside, polydatin. The glycosylation of resveratrol obviously enhanced the affinity for bHB. The affinity of polydatin for bHB was 1.86 times higher than that of resveratrol.

Nature of bHB−Polyphenol Interaction. The lipophilicity of the compounds under study was assessed by their partition coefficient values ($XLogP_3$) according to PubChem Public Chemical Database.[33] There is a positive relationship between the $XlogP_3$ values and lg K_a values for polyphenols (Figure 5). The linear regression equation using Origin 7.5 software was

$XlogP_3 = -2.752 + 1.072 \lg K_a$ ($R = 0.5309$). The affinities of flavonoids increased with increasing partition coefficient. From this point, the binding interaction between polyphenols and bHB was mainly caused by hydrophobic forces. bHB consists of large numbers of NH, OH, and COOH groups, which indicates that bHB is a highly polar macromolecule.

To further investigate whether or not the hydrogen bond force plays an important role in binding polyphenols to bHB, the relationships of the hydrogen bond acceptor/donor numbers (N, data from ref 33) of polyphenols with affinities for bHB are shown in Figure 6. The affinities for bHB obviously decreased with increasing hydrogen bond donor and acceptor numbers of polyphenols. These results support the above-mentioned conclusion that the hydrophobic interaction is the main force to bind polyphenols to bHB.

Relationship of Topological Polar Surface Area (TPSA) and Affinity for HB. The TPSA is defined as the sum of surfaces of polar atoms in a molecule. TPSA has been shown to be a very good descriptor characterizing drug absorption, including intestinal absorption, bioavailability, Caco-2 permeability, and blood−brain barrier penetration.[33] The compounds with high TPSA are transported, whereas those with low TPSA are not. A strong correlation between TPSA and transport properties (K_m) was also found. In our present study, the relationship between TPSA and the binding affinity for bHB was studied. The TPSA values were obtained from PubChem Public Chemical Database.[33] There is a negative relationship between the TPSA values and lg K_a values for polyphenols. The lg K_a values were found to decrease with increasing TPSA for polyphenols (Figure 7). The linear regression equation using Origin 7.5 software was $TPSA = 404.210 - 67.308 \lg K_a$ ($R = 0.6493$). The polyphenols with low TPSA are bound tightly, whereas those with high TPSA are not.

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