Molecular structure-affinity relationship of dietary flavonoids for bovine serum albumin

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Abstract: The relationship between the molecular structure of dietary flavonoids and their affinities for bovine serum albumin (BSA) were fully investigated by fluorescence titration analysis. The binding process with BSA was significantly affected by the molecular structure of flavonoids under study. The methylation of hydroxyl group in flavonoids enhanced their binding affinities for BSA by 1 to 794 times. Hydroxylation on rings A, B and C also strongly influenced the affinity for BSA. The glycosylation weakened the affinities for BSA by 1-2 orders of magnitude depending on the conjugation site and the class of sugar moiety. The hydrogenation of the C2=C3 double bond slightly increased the binding affinity. The galloylated catechins and pyrogallol-type catechins exhibited higher binding affinities for BSA than non-galloylated and catechol-type catechins, respectively. The affinities for BSA increased with increasing partition coefficients and decreased with increasing hydrogen bond donor and acceptor numbers of flavonoids, which suggested that the binding interaction was mainly caused by hydrophobic forces.

Keywords: BSA, flavonoids, protein binding, structure-affinity

Introduction

Dietary flavonoids are most important polyphenols in plant foods, such as fruits, vegetables, nuts, and tea (Blade et al., 2010; Haghi & Hatami, 2010; Truchado et al., 2010). Flavonoids are of great interest for their bioactivities, which are basically related to their antioxidative properties (Cohen & Kennedy, 2010; Côté et al., 2010a; Cheung et al., 2010). The structural differences between the various classes concern the chemistry of the ring C, as well as the number and distribution of hydroxyl groups and their substitutions on the rings A and B (Côté et al., 2010b; Yang et al., 2011). The flavonol moiety, i.e. the 2,3-double bond in conjugation with a 4-oxo group and a 3-hydroxyl group, as well as the 5,7-dihydroxylation at the A-ring has been found to be important structural features for significant antioxidant activity (Morabito et al., 2010). In addition to -OH moieties in the structural arrangements of flavonoids, the resonance of electrons between the rings A and B was reported to be essential for the antioxidant and biological activities of the compounds (Natella et al., 2010).

Recently, the interactions between flavonoids and proteins have attracted great interest (Xiao et al., 2007a and 2007b; Xiao et al., 2008; Xiao et al., 2010a and 2010b; Xiao et al., 2011). Most of the reports mainly focused on the binding process and parameters. Few reports, however, have focused on their molecular structure-affinity relationship. Our group has studied the effects of the hydroxylation on ring A of isoflavone (Zhao & Ren, 2009) and ring B of flavonol (Xiao et al., 2008a), as well as the glycosylation of flavonoids on binding to bovine serum albumin (BSA) (Cao et al., 2009; Xiao et al., 2009). Furthermore, the molecular property-affinity relationship of flavonoids for human serum albumin (HSA) in vitro was described recently (Xiao et al., 2010; Xiao et al., 2011a). However, the comprehensive relationship between the molecular structure of dietary flavonoids and their affinities for BSA was still not understood. In previous reports, appropriate quantities of flavonoids solution were mixed with BSA and subsequently incubated at 300.15 K for 1 h. Then, the solution was scanned on the fluorometer. Here, the fluorescence titration method was carried out by using trace syringes. The binding affinities obtained by these two methods are certain different. The present work fully investigated the relationship between the molecular structure of dietary flavonoids and their affinities for BSA. Thirty-one flavonoids (Table 1) were studied.

Materials and Methods

Apparatus and reagents

The fluorescence spectra were recorded on a JASCO FP-6500 fluorometer (Tokyo, Japan). The
Table 1. Chemical structures of the various flavonoids and their affinities for BSA in vitro.

<table>
<thead>
<tr>
<th>Subclass</th>
<th>Name</th>
<th>Substitutions</th>
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<td>7-β-D-glucuronide</td>
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<td>7-glucoside</td>
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<td>6.24</td>
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<tr>
<td></td>
<td>EGC (2,3-cis)</td>
<td>3,5,7,3',4',5'</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>C (2,3-trans)</td>
<td>3,5,7,4',5'</td>
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</table>

pH measurements were carried out on a Cole-Parmer PHS-3C Exact Digital pH meter (IL, USA). BSA (~99%, lyophilized powder) and 7-hydroxyflavone (99.5%) were purchased from Sigma Co. (MO, USA). 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, genistin, (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin gallate (EGCG), gallocatechin gallate (GCG), and tectorigenin (>98.0%) were obtained commercially from Tongtian Co. (Shanghai, China). The working solutions of the flavonoids (1.0 × 10⁻³ mol/L) were prepared by
dissolving each flavonoid with methanol. Tris-HCl buffer (0.20 M, 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 tris-HCl buffer and stored in 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

Fluorescence analysis was performed on a Varian Cray/E spectrofluorophotometer in the ratio mode with temperature maintained by circulating bath. The spectra were recorded in the wavelength range of 310-450 nm upon excitation at 295 nm when BSA samples were titrated with QDs or flavonoid. Slit widths (5 nm), scan speed (240 nm/min), and excitation voltage (400 V) were kept constant within each data set and each spectrum was the average of three scans. Quartz cells (1 cm path length) were used for all measurements. Titrations were performed manually by using trace syringes. In each titration, the fluorescence spectrum was collected with the concentrations of BSA at 1.0 ×10⁻⁵ mol/L. The experiments were repeated and found to be reproducible within experimental errors.

Results and Discussion

The binding constants (Kₐ) and the number of binding sites (n)

As representative examples, the fluorescence spectra of BSA after addition of kaempferide are shown in Figure 1 (the fluorescence spectra of BSA quenched by other flavonoids are not given here). In these and all other cases, the fluorescence intensities of BSA decreased remarkably with increasing concentration of flavonoids.

The binding constants were calculated according to the double-logarithm equation (Zhao & Ren, 2009; Xiao et al., 2008b; Cao et al., 2009; Xiao et al., 2009; Xiao et al., 2010c; Xiao et al., 2011b):

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

where \( F_0 \) and \( F \) represent the fluorescence intensities of BSA in the absence and in the presence of flavonoids, \( K_a \) is the binding constant, \( n \) is the number of binding sites per BSA, and \([Q]\) is the concentration of flavonoids. Table 1 summarizes the results correspondingly calculated results according to equation (1). The values of \( \lg K_a \) are proportional to the number of binding sites (n) (Figure 2), which indicates that the equation (1) used here is suitable to study the interaction between flavonoids and BSA (Berezhkovskiy, 2007; Xiao et al., 2008c).

Effects of methylation of hydroxyl group in dietary flavonoids on the affinities for BSA

As shown in Figure 3, the methylation of hydroxyl group in flavonoids enhanced the binding affinities for BSA. In general, the methylation of hydroxyl group in flavonoids enhanced their binding affinities for BSA by 1 to 794 times. Extremely, the affinity of daidzein for BSA was found to be 794-time higher than that of formononetin (Figure 3). On the other side, the affinity of nobiletin for BSA was only 1-time higher than that of tangeretin (Figure 3). This result supports that the methylation of flavonoids enhanced the transporting ability, which leads to facilitated absorption and greatly enhanced bioavailability. The methylation of hydroxyl groups in the flavonoids significantly improved their intestinal absorption and metabolic stability (Wen & Walle, 2006; Walle 2009). Walle found that the methylation seems to be an effective method to enhance the metabolic resistance and transport of flavonoids (Walle 2009). Courts and Williamson concluded that the C-glycoside aspalathin...
was methylated and glucuronidated \textit{in vivo} (Courts & Williamson 2009). The potential therapeutic utility of the methylated flavones has also been advanced by finding that the methylation of flavonoids reduces the possibility of toxic side-effects (Walle 2009). The oligomethoxyflavones occurring in plants are less utilized for human consumption compared to the polymethoxyflavones and polyhydroxyflavones.

### Effects of hydroxylation of flavonoids on the affinities for BSA

Table 2 gave the effects of hydroxylation of flavonoids on the affinities for BSA \textit{in vitro}. As seen from these data, the hydroxylation on the rings A, B and C of flavonoids dramatically influenced the binding affinities for BSA.

#### Hydroxylation on ring A of flavones

As illustrated in our recent report (Xiao et al., 2010c), it appears that the optimal number of hydroxyl groups introduced to the ring A of flavones is one for HSA, as the highest binding was observed with 7-hydroxyflavone (containing only one hydroxyl group) (Xiao et al., 2010c). Here, the similar conclusion was found for BSA. 7-hydroxyflavone showed strongest affinity with BSA. However, when more hydroxyl groups are introduced to C-5, C-6, and/or C-7 of flavones, the affinities for BSA slightly decreased.

#### Hydroxylation on ring B of flavones

As shown in Table 2, the binding constants ($K_b$) between flavones and BSA decreased with the increasing numbers of hydroxyl groups on the B-ring. The hydroxylation on position 4' of flavone significantly improves the binding affinity for BSA. The affinities of apigenin (5,7,3') for BSA were about 7.41-time higher than that of chrysin (5,7).

#### Hydroxylation on ring C of flavones

As shown in Table 2, the affinities of galangin (3,5,7) for BSA were about 3.72-time higher than that of chrysin (5,7). On the other side, the affinity of apigenin (5,7,3') and luteolin (5,7,3',4') for BSA are about 3.02 and 1.82-time higher than those of kaempferol (3,5,7,3') and quercetin (3,5,7,3',4') for BSA.

#### Hydroxylation of rings A and B of flavonols

In this study, it was found that the hydroxylation of rings A and B of flavonols slightly affected their binding affinities for BSA. The affinity of quercetin (3', 4') and myricetin (3', 4', 5') for BSA was 1.51-time higher than that of kaempferol (4'). On the other side, the affinity of galangin (no hydroxyl groups on ring B) for BSA was 1.51-fold higher than that of kaempferol (4'). Flavonols are the most prominent flavonoids in plants. The most prominent flavonols such as quercetin and kaempferol in foods exist with 5,7-dihydroxy groups on ring A. Here, it was found that the hydroxylation on position 5 of flavonol slightly enhances the binding affinity for BSA by 1.78 times.

#### Hydroxylation on ring A of isoflavones

As shown in Table 2, the affinity of genistein for BSA was about 8-time higher than that of daidzein. The affinity of formononetin for BSA was about 13.49-time higher than that of biochanin A.

#### Comparing the affinities of flavonoid isomers with BSA

Previously, we have compared the affinities of flavonoid isomers (apigenin, baicalein, genistein and luteolin, kaempferol) with BSA (Cao et al., 2008). Here, the binding constants ($K_b$) were determined as: apigenin > baicalein > genistein and luteolin > kaempferol. In this study, these results are in good agreement with above data.

#### Effects of glycosylation of dietary flavonoids on the affinities for BSA

The dietary flavonoids in nature occur mostly as β-glycosides. The flavonols are found mainly as the 3- and 7-O-glycoside, although the 4' position may also be glycosylated in some plants (Table 1). Most recently, we have reported the glycosylation of flavonoids lowered the affinity for BSA by 1 to 3 orders of magnitude depending on the conjugation site and the class of sugar moiety (Xiao et al., 2009).

Herein, the effect of glycosylation of dietary
flavonoids on the affinities for BSA was investigated. In our present study (Figure 4), the glycosylation of flavonoids lowered the affinity for BSA by 1 to 2 orders of magnitude. The affinity of naringenin for BSA was about 29.51-fold higher than that of naringin, but the affinity of kaempferol for BSA is only 1.32-time higher than that of kaempferitrin. The glucopyranosylation (genistin) of genistein lowered the affinity for BSA by 7.41 times. All above, the sugar moieties are in 3 or 7-positions of flavonoids. The decreasing affinity for BSA after glycosylation may be caused by the non-planar structure. After the hydroxyl group is substituted by a glycoside, steric hindrance may take place, which weakens the affinity for BSA (Xiao et al., 2009). On the other side, compared with the affinity of daidzein for BSA, the affinity of puerarin (daidzein-8-C-glucose) for BSA increased 3.16 times.

Figure 4. Glycosylation decreases the affinity of the flavonoids for BSA.

Effects of hydrogenation of the C2=C3 double bond of dietary flavonoids on the affinities for BSA

The C2=C3 double bond in conjugation with a 4-oxo group plays a very important role for the affinity for BSA. It was found that hydrogenation of the C2=C3 double bond of flavonoids increased the binding affinities for BSA. As shown in Table 1, the affinities of apigenin and myricetin for BSA were about 4.68-time and 5.25-time higher than those of naringenin and dihydromyricetin, respectively. Hydrogenation of the C2=C3 double bond for many flavonoids increased the binding affinity for BSA by 2-4 orders of magnitude. Planarity of the C ring in flavonoids maybe 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 p-conjugation of the bond linking the B and C rings, which favors near-planarity of the two rings (Edenharder et al., 1993). Molecules with near-planar structure easier enter the hydrophobic pockets in proteins.

Catechins

Catechins are the major polyphenols in green tea leaves. The major catechins of green tea extract are (-)-catechin (C), (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin gallate (EGCG), and galloallocatechin gallate (GCG). Recent studies have suggested that the catechins form complexes with BSA for transport in blood, and their binding affinity for albumin is believed to modulate their bioavailability. Here, we determined the affinities between catechins and BSA by fluorescence quenching method with double logarithm regression curve. The binding constants (log10Ka) between ECG, EGCG and GCG for BSA were 6.81, 6.24 and 4.36, respectively. However, EC, EGC, and C hardly quenched the fluorescence of BSA. It illustrates that the galloylated catechins have higher binding affinities with BSA than non-galloylated catechins and the catechol-type catechins had higher affinities than pyrogallol-type catechins. The presence of the galloyl moiety is the most decisive factor; the increasing hydroxyl groups on ring B decreases the affinity for BSA. In our present study, the affinity of the catechin with 2,3-trans structure (GCG) for BSA was lower than that of the catechin with 2,3-cis structure (EGCG).

Relationship of partition coefficient and the affinity for BSA

The lipophilicity of the compounds under study was assessed by their partition coefficient values (XLogP3) according to PubChem Public Chemical Database [30]. There is a relationship between the XlogP3 values and lgK a values for flavonoids (Figure was not shown here). The linear regression equation using the Origin 7.5 software was XlogP3 = 0.30487+0.28577lgK a (R=0.21792). From this equation, the binding interaction between flavonoids and BSA was slightly caused by hydrophobic forces. BSA consists of large number of NH, OH and COOH groups, which indicates that BSA is a highly polar macromolecule.

To further investigate whether or not the hydrogen bond force plays an important role in binding flavonoids to BSA, the relationships of the hydrogen bond acceptor/donor numbers (N, Data were from reference [30] of flavonoids with the affinities for BSA were shown in Figure 5. The affinities for BSA obviously decreased with increasing hydrogen bond donor and acceptor numbers of flavonoids. These results support the above-mentioned conclusion that the hydrophobic interaction is the main force to bind flavonoids to BSA.
Relationship of topological polar surface area and the affinity for BSA

The topological polar surface area (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. Fernandes and Gattass used TPSA to analyze drug permeability and blood-brain barrier penetration. Including intestinal absorption, bioavailability, Caco-2 permeability, number of binding sites of the protein. TPSA values were found to decrease with the increasing lgK_a for flavonoids (Figure was not shown here). The linear regression equation using the Origin 7.5 software was TPSA=167.94371-9.35675 lgK_a (R=-0.1383). The flavonoids with low TPSA are bound tightly while those with high TPSA are not.

Acknowledgments

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