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Interaction of wogonin with bovine serum albumin

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Abstract—The binding of wogonin with bovine serum albumin (BSA) was investigated at different temperatures by fluorescence, circular dichroism (CD) and Fourier transform infrared spectroscopy (FT-IR) at pH 7.40. The association constants K were determined by Stern–Volmer equation based on the quenching of the fluorescence of BSA in the presence of wogonin, which were in agreement with the constants calculated by Scatchard plots. The thermodynamic parameters were calculated according to the Van't Hoff equation and the result indicated that ΔH^0 and ΔS^0 had a negative value (−12.02 kJ/mol) and a positive value (58.72 J/mol K), respectively. On the basis of the displacement experimental and the thermodynamic results, it is considered that wogonin binds to site I (subdomain IIA) of BSA mainly by hydrophobic interaction. The studied results by FT-IR and CD experiment indicated that the secondary structures of protein have been perturbed by the interaction of wogonin with BSA.

1. Introduction

Serum albumins are the most abundant proteins in the circulatory system of a wide variety of organisms, being the major macromolecules contributing to the osmotic blood pressure.[1] Their functional and physiological properties have been extensively studied over several decades.[2] Moreover, these proteins have long been used as model proteins in both industrial and academic research areas.[3]

The most important physiological role of albumins is to bring numerous ligands, such as fatty acids, amino acids, steroids and metal ions, in the bloodstream to their target organs.[4]

The high-resolution crystal structures revealed the major ligand binding sites on serum albumin,[5] the exact location of many ligands being still obscure. Since the overall distribution, metabolism and efficacy of many drugs in the body are correlated with their affinities toward serum albumins, the investigation of pharmaceuticals with respect to albumin–drug binding is imperative and of fundamental importance. In the current work, bovine serum albumin is selected as our protein model because of its lone-standing interest in the protein community.[6] Like other serum albumin, BSA has a wide range of physiological functions involving the binding, transport, and delivery of fatty acids, porphyrins, bilirubin, tryptophan, thyroxine, and steroids and it is home to specific binding aggregation, and conformational dynamics in solution have been studied by NMR,[7] CD,[8] Raman,[9] attenuated total reflectance-Fourier transform infrared (ATR-FTIR),[10] UV–vis absorbance,[11] and fluorescence spectroscopy.[12] However, the binding of the components of natural plant medicine to proteins has seldom been investigated.[13–15]

The flavonoids are a large group of polyphenolic natural products that are widely distributed in higher plants.[16] Such compounds are increasingly being recognized as possessing a broad spectrum of biological activities and important therapeutic applications,[17] including novel features, for example, as anti-cancer, anti-tumor, anti-inflammatory, and anti-coagulant drugs. Interestingly, many biologically active flavonoids appear to have effects on various proteins, including enzymes. The nutritional and toxicological significance of consuming such protein–phenol derivatives is largely unknown and the microenvironments of the binding sites

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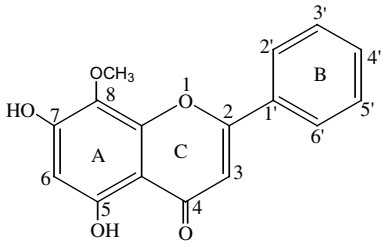

Scheme 1.

of such flavonoid molecules with proteins are expected to be complex in nature and essentially an unexplored area until now. On the other hand, to optimize the biological utilization of proteins, a better understanding is needed of the various interrelated parameters that influence their nutritional value. Many authors have studied the interaction of flavonoids with proteins including quercetin,[18] 4-hydroxy-5-azaphenanthrene (HAP)[19] and 3-hydroxyflavonol,[20] but the binding of wogonin to BSA has not been reported until now.

In this paper, the interaction of wogonin (Scheme 1, 4H-1-Benzopyran-4-one) with BSA was studied at pH 7.40 by spectroscopic methods including circular dichroism (CD), fluorescence spectroscopy, and Fourier transform infrared (FT-IR) spectroscopy. The binding site of flavonoids was designated site I, the PB site on the BSA molecule, as indicated by the displacement experimental study of the interaction between BSA and wogonin. The hydrophobic interaction was found to play a main role in the binding of this drug to BSA according to the thermodynamic parameters.

2. Materials and methods

2.1. Materials

Bovine serum albumin (essentially fatty acid free) was obtained from Sino-American Biotechnology Company and used without further purification. Wogonin (analytical grade) was obtained from the National Institute for Control of Pharmaceutical and Products, China. NaCl solution (0.5 mol/L) was used to keep the ion strength at 0.1. Tris–HCl buffer was selected to keep pH of the solution at 7.40. BSA solution of 1.5×10^{-5} mol/L was prepared in pH 7.40 Tris–HCl buffer solution. Wogonin (7.0×10^{-4} mol/L) solution was obtained by dissolving it in 50 mL ethanol. Phenylbutazone (PB), flufenamic acid (FA), and digitoxin (Dig) solutions were obtained by dissolving it in ethanol, respectively. All other chemicals were of analytical reagent grade.

2.2. Methods

Fluorescence spectra were recorded using a RF-5301 PC spectrofluorophotometer (Shimadzu) with a 150 W Xenon lamp and a 1 cm quartz cell. The excitation and emission bandwidths were both 5 nm. The temperature of the sample was maintained by recycling water throughout the experiment.

To confirm that wogonin–BSA interactions lead to unfolding of the protein, CD and FT-IR methods were used to analyze the interactions. Circular diachronic (CD) measurements were made on a Jasco-20c automatic recording spectropolarimeter (Japan), using a 2 mm cell at 296 K. The induced ellipticity was defined as the ellipticity of the drug–BSA mixture minus the ellipticity of drug alone at the same wavelength. The content of α-helix was calculated from the following equation:[21]

$$\alpha\% = \{(-[\theta]_{208} - 4000)/(33,000 - 4000)\} \times 100 \quad (1)$$

FT-IR measurements were carried out at room temperature on a Nicolet Nexus 670 FT-IR spectrometer (America) equipped with a Germanium attenuated total reflection (ATR) accessory, a DTGS KBr detector and a KBr beam splitter. All spectra were taken via the Attenuated Total Reflection (ATR) method with a resolution of 4 cm^{-1} and 60 scans. Spectra processing procedures: spectra of buffer solution were collected at the same condition. Then, the absorbance of the buffer solution was subtracted from the spectra of the sample solution to get the FT-IR spectra of proteins. The subtraction criterion was that the original spectrum of the protein solution between 2200 and 1800 cm^{-1} was featureless.[22]

2.2.1. Binding parameters. A quantitative analysis of potential interaction between wogonin and BSA was performed by fluorimetric titration. Three milliliters of solution containing 1.5×10^{-6} mol/L BSA was titrated by successive additions of wogonin solution (to give a final concentration of 7.0×10^{-6} mol/L) and the fluorescence intensity was measured (excitation at 285 nm and emission at 345 nm). All experiments were measured at different temperatures (296, 303, and 310 K).

The binding parameters have been calculated using the Scatchard's procedure.[23] This method is based on the general equation:

$$r/D_f = nK - rK \quad (2)$$

where r is in moles of drug bound per mole of protein, D_f is the molar concentration of free drug, n is binding site multiplicity per class of binding sites and K is the association binding constant.

Quenching data were also analyzed according to the Stern–Volmer equation:[24]

$$\frac{RF_0}{\Delta RF} = \frac{1}{[Q]}\frac{1}{fK} + \frac{1}{f} \quad (3)$$

where RF_0 and ΔRF are the relative fluorescence intensities of protein in the absence and the presence of quencher, respectively, f is the fractional maximum fluorescence intensity of protein summed up and K is a constant.

The dependence of $RF_0/\Delta RF$ on the reciprocal value of the quencher concentration $1/[Q]$ is linear with a slope equal to the value of $(fK)^{-1}$. Association constant K is a quotient of an ordinate $1/f$ and slope $(fK)^{-1}$.

2.2.2. Thermodynamic parameters. If the enthalpy changes (ΔH^0) do not vary significantly over the temperature range studied, then its value and that of ΔS^0 can be determined from the Van't Hoff equation:

$$\ln K = -\Delta H^0/RT + \Delta S^0/R \quad (4)$$

In Eq. 3, K is the binding constant at corresponding temperature and R is the gas constant. In this section, the association constants obtained by the Scatchard plots were used to calculate the hydrodynamic parameters. The free energy change is estimated from the following relationship:

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 \quad (5)$$

2.2.3. The displacement experiment. The displacement experiments were performed using the site probes keeping the HSA and the probes concentrations at 1.5 μM and 1.67 μM, respectively. The fluorescence titration was used as before to determine the binding constants of wogonin with HSA in presence of the site probes. Phenylbutazone (PB), flufenamic acid (FA) and digitoxin (Dig) are used as site probes of site I, II, and III, respectively, according to Sudlow et al.[5]

3. Results and discussion

3.1. Fluorescence spectra

The conformational changes of BSA were evaluated by the measurement of intrinsic fluorescence intensity of protein before and after addition of drug. Fluorescence measurements give information about the molecular environment in a vicinity of the chromophore molecules. The effect of drug on BSA fluorescence intensity was shown in Figure 1. When different concentration of wogonin solution was titrated into a fixed concentration of BSA, a remarkable fluorescence decrease of BSA was observed, which indicated that wogonin can interact with BSA. Furthermore, from Figure 1, the maximum wavelength of BSA shifted from 345 to 342 nm after the addition of wogonin, so a slight blue shift of the maximum emission wavelength was observed and it could be deduced that the chromophore of protein was placed in a more hydrophobic environment after the addition of wogonin.[25] The quantitative analysis of the binding of wogonin to BSA was carried out using the fluorescence quenching at 345 nm at various temperatures as shown in Figure 2, with the increase of the concentration of wogonin the fluorescence intensity of system gradually decreased, and with the further addition of wogonin, the fluorescence intensity of system decreased tardily in each titration curve which indicates the beginning of saturation of the BSA binding site.

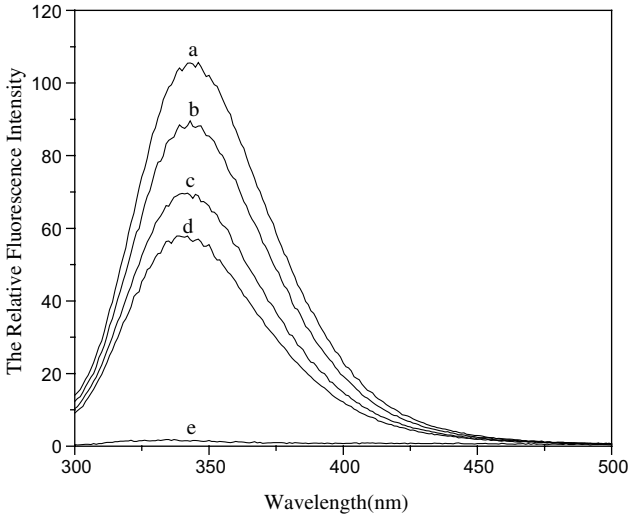

Figure 1. The fluorescence spectra of wogonin–BSA system: The concentration of BSA was 1.5×10^{-6} mol/L while the wogonin concentration corresponding to 0, 1.2, 1.8, 2.4×10^{-5} mol/L from (a) to (d); (e) [wogonin] = 1.2×10^{-5} mol/L. T = 296 K; pH 7.40; λ_{ex} = 285 nm; λ_{em} = 345 nm.

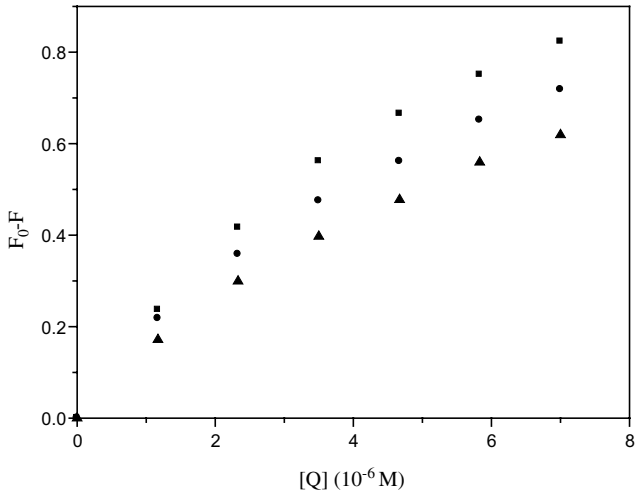

Figure 2. Relative fluorescence intensity for the wogonin–BSA interaction obtained by the titration with wogonin: BSA concentration was 1.5×10^{-6} mol/L; pH 7.40; (■) 296 K; (●) 303 K, (▲) 310 K; λ_{ex} = 285 nm; λ_{em} = 345 nm.

3.2. CD spectra and FT-IR spectra

To obtain an insight into the structure of the BSA, CD spectra were studied for the wogonin–BSA system. In the far ultraviolet region, such spectra relate to the polypeptide backbone structures. The comparison of the spectra of wogonin–BSA with BSA is shown in Figure 3 at pH 7.40. The CD spectra of BSA exhibited two negative minima at 208 and 217 nm, which is typical of the α-helix structure of class proteins.[26] The interaction between wogonin and BSA caused only a decrease in band intensity at all wavelengths of the far-UV CD without any significant shift of the peaks, indicating that this drug induces a slight decrease in the helix structure content of the protein. From Eq. 1, the α-helix content of BSA was decreased from 43.4% to 37%. From CD and fluorescence spectra results, we can conclude that the interaction of wogonin with BSA induced the slight unfolding of the constitutive polypeptides of protein, which resulted in a conformational change of the

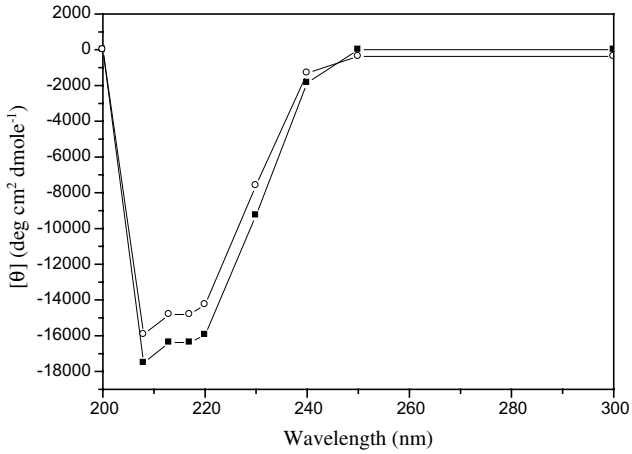

Figure 3. The CD spectra of wogonin-HSA. wogonin/HSA: (■) 0:1, (○) 1:1; [HSA] = 1.5×10^{-6} mol/L; pH 7.40; 296 K.

protein that increased the exposure of some hydrophobic regions which were previously buried.

Hydrogen bonding and the coupling between transition dipoles are amongst the most important factors governing conformational sensitivity of amide bands. The protein amide I band ≈1653 cm^{-1} (mainly C=O stretch) and amide II band ≈1548 cm^{-1} (C–N stretch coupled with N–H bending mode) both have a relationship with the secondary structure of the protein. Figure 4 showed the FT-IR spectrum of free BSA in Tris–HCl buffer and the difference spectra after binding with wogonin. The peak position of amide I has not remarkably changed but amide II moved from 1558.2 to 1546.7 cm^{-1} in the BSA infrared spectrum after interaction with wogonin, which indicate that wogonin can interact with BSA and the secondary structure of BSA has been changed because of the interaction of wogonin with BSA.

3.3. Binding parameters

The intrinsic fluorescence of BSA was obtained at 345 nm when excited at 285 nm. Using the fluorescence decrease the association constants K for the complex of wogonin with BSA at different temperatures were calculated.

Figures 5 and 6 were the Stern–Volmer plots and Scatchard plots for the wogonin–BSA system at different temperatures, respectively. The binding constants K calculated from the Stern–Volmer equation and Scatchard equation are shown in Table 1. It can be seen that the association constants from the Stern–Volmer plots were similar to the constants from the Scatchard plots and the interaction between wogonin and BSA is very strong and the binding constants decreased with increasing temperature (Figs. 5, 6 and Table 1). The linearity of the Scatchard plots indicated that BSA has one binding site for wogonin, which was in agreement with the binding site number from the Scatchard plots. We have studied the interaction of scutellarin, analogous compound of wogonin, with HSA,[28] and the interaction of wogonin with HSA.[29] The results indicated that the interaction of

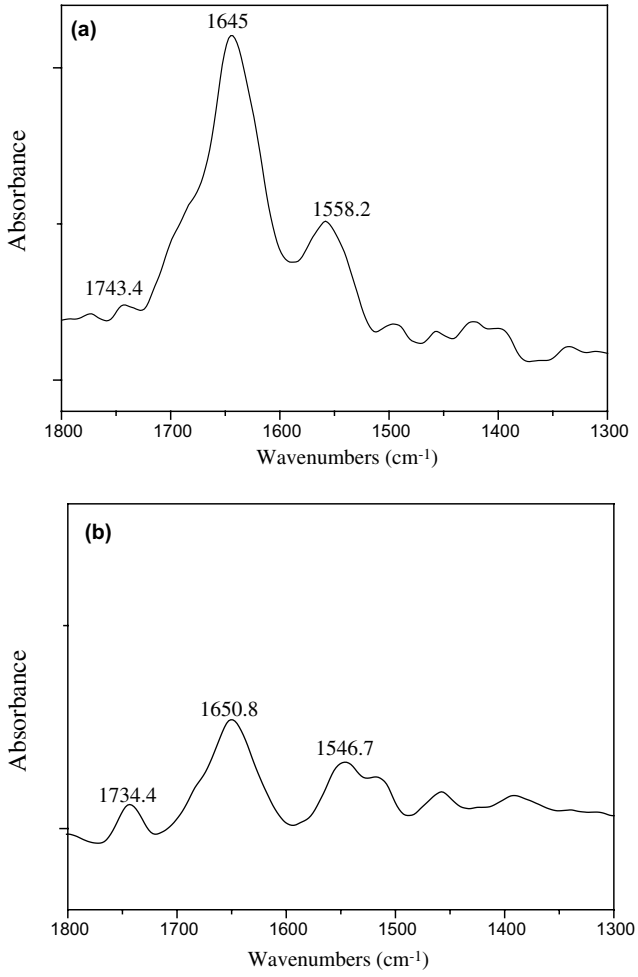

Figure 4. FT-IR spectra and difference spectra [(BSA solution + wogonin solution) − (BSA solution)] of free BSA: (a) and its wogonin complexes and (b) in buffer solution in the region of 1800–1300 cm^{-1} ([wogonin] = 6.0×10^{-5} mol/L, [BSA] = 3.0×10^{-5} mol/L).

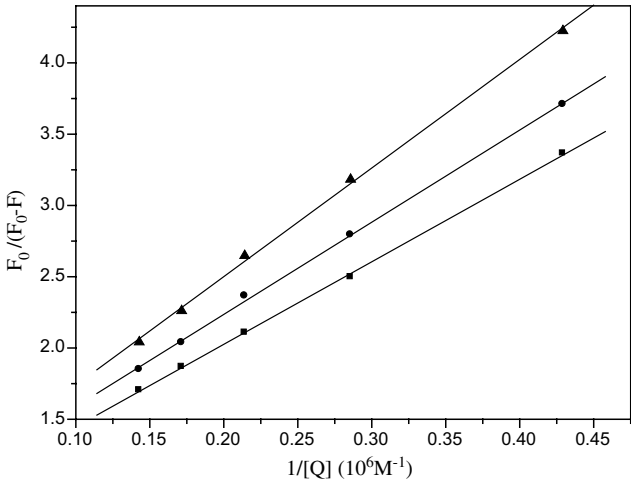

Figure 5. The Stern–Volmer plot for the wogonin–BSA at pH 7.40: BSA concentration was 1.5×10^{-6} mol/L; pH 7.40; (■) 296 K, (●) 303 K, (▲) 310 K; λ_{ex} = 285 nm; λ_{em} = 345 nm.

scutellarin with HSA is weaker than wogonin, which may be the result of spatial structure. But the binding

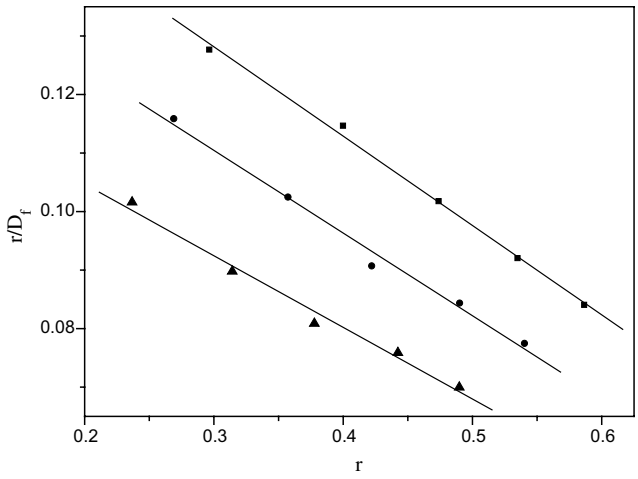

Figure 6. The scatchard plot for the wogonin–BSA at pH 7.40. BSA concentration was 1.5×10^{-6} mol/L; pH 7.40; (■) 296 K, (●) 303 K, (▲) 310 K; λ_{ex} = 285 nm; λ_{em} = 345 nm.

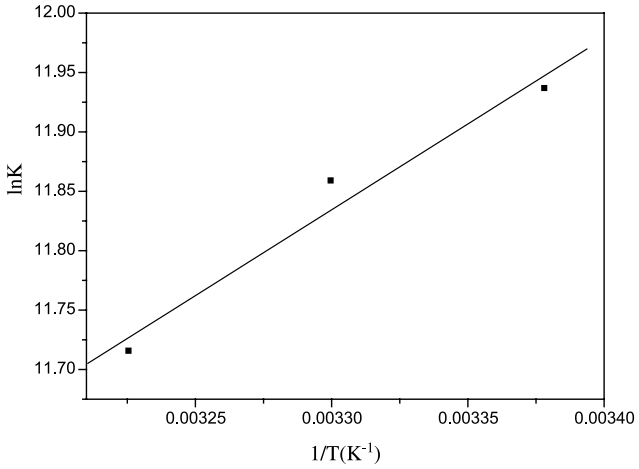

Figure 7. The Van't Hoff plot, pH 7.40, [BSA] = 1.5×10^{-6} mol/L.

Table 1. Binding parameters and thermodynamic functions for the binding of wogonin to BSA at pH 7.40 measured by fluorimetric titrations

Temperature (K)	Stern–Volmer method	Scatchard method	
	K (10^5 M^{-1})	K (10^5 M^{-1})	n
296	1.50	1.53	1.14
303	1.46	1.41	1.08
310	1.28	1.22	1.05

Table 2. Thermodynamic parameters of wogonin–BSA interaction at pH 7.4

Temperature (K)	ΔG^0 (kJ/mol)	ΔH^0 (kJ/mol)	ΔS^0 (J/mol K)
296	−29.40		
303	−29.81	−12.02	58.72
310	−30.22		

Table 3. The comparison of binding constant of wogonin to BSA before and after the addition of the site probe, 296 K

K (without the site probe) (10^5 M^{-1})	K (with PB) (10^5 M^{-1})	K (with FA) (10^5 M^{-1})	K (with Dig) (10^5 M^{-1})
1.53	1.26	1.62	1.42

constants of wogonin to HSA are similar to the constants of wogonin to BSA and the interaction of 3-hydroxyflavone with BSA.[30]

3.4. Binding mode and binding site

In order to elucidate the interaction of wogonin with BSA, the thermodynamic parameters were calculated from the Van't Hoff plots. The temperatures used were 296, 303, and 310 K. The enthalpy change (ΔH^0) is calculated from the slope of the van't Hoff relationship.

Figure 7 is the van't Hoff plot of the wogonin-BSA system. Table 2 shows the values of ΔH^0 and ΔS^0 obtained for the binding site from the slopes and the ordinates at the origin of the fitted lines. From Table 2 it can be seen that ΔH^0 and ΔS^0 has a small negative value (−12.02 kJ/mol) and a positive value (58.72 J/mol K), respectively. The negative sign for ΔG^0 means that the binding process is spontaneous. For drug–protein interaction, positive entropy is frequently taken as evidence for hydrophobic interaction, but it has been pointed out that positive entropy may also be a manifestation of electrostatic interaction.[27] Furthermore, the main source of ΔG^0 value is derived from a large contribution of ΔS^0 term with a little contribution from the ΔH^0 factor, so the main interaction is hydrophobic contact, but the electrostatic interaction cannot be excluded.

Sudlow et al.[6] have suggested two distinct binding sites on BSA, site I and site II, site I of BSA showed affinity for warfarin, phenylbutazone, etc. and site II for ibuprofen, flufenamic acid etc. Digitoxin binding is independent of sites I and II.[26] To determine the specificity of the drug binding, competition experiments were performed with phenylbutazone, flufenamic acid, and digitoxin in connection with Sudlow's classification of the binding sites. Table 3 shows the changes in fluorescence of wogonin bound to BSA on the addition of other drugs. Wogonin was not significantly displaced by flufenamic acid or by digitoxin (site III). However, phenylbutazone (site I) gave a significant displacement of wogonin suggesting that wogonin binding site on BSA is site I, so the site I is the main binding site for wogonin binding to BSA through hydrophobic force.

4. Conclusion

In this paper, we studied the interaction of wogonin with BSA by many spectroscopic methods. The experimental results indicate that wogonin can interact with BSA strongly in site I mainly through hydrophobic interaction, which induces the changes of secondary structures of BSA.

References and notes

1. Peters, T. J. *All About Albumin, Biochemistry, Genetics and Medical Applications*; Academic: San Diego, 1996.
2. He, X. M.; Carter, D. C. *Nature* **1992**, *358*, 209.
3. Carter, D. C.; Ho, J. X. *Adv. Protein Chem.* **1994**, *45*, 153.
4. Sudlow, G.; Birkett, D. J.; Wade, D. N. *Mol. Pharmacol.* **1975**, *11*, 824.
5. Curry, S.; Brick, P.; Franks, N. P. *Biochem. Biophys. Acta* **1999**, *1441*, 131.
6. Peters, T. *All About Albumin; Biochemistry, Genetics and medical applications*; Academic: San diego, CA, 1995.
7. Baroni, S.; Mattu, M.; Vanini, A.; Cipollone, R.; Aime, S.; Ascenzi, P.; Fasano, M. *Eur. J. Biochem.* **2001**, *268*, 6214.
8. Bhakuni, V.; Gupta, C. M. *FEBS Lett.* **1986**, *205*, 347.
9. Fleurya, F.; Kudelinab, I.; Nabieva, I. *FEBS Lett.* **1997**, *406*, 151.
10. Huai, Q.; He, Y. L.; Sheng, F. L.; Tao, Z. Y. *Spectrochim. Acta A* **1996**, *52*, 1795.
11. Baroni, S.; Mattu, M.; Vannini, A.; Cipollone, R.; Aime, S.; Ascenzi, P.; Mauro, F. *Eur. J. Biochem.* **2001**, *268*, 6214.
12. Rahman, M. H.; Maruyama, T.; Okada, T.; Yamasaki, K.; Otagiri, M. *Biochem. Pharm.* **1993**, *46*, 1721.
13. Jian Niao, T.; Jia Qin, L.; Ji You, Zh.; Zhi De, H.; Xing Guo, Ch. *Chem. Pharm. Bull.* **2003**, *51*, 579.
14. Jia Qin, L.; Jian Niao, T.; Ji You, Zh.; Zhi De, H.; Xing Guo, Ch. *Anal. Bioanal. Chem.* **2003**, *376*, 864.
15. Jian Niao, T.; Jia Qin, L.; Xuan, T.; Zhi De, H.; Xing Guo, Ch. *J. Mole. Struct.* **2004**, *691*, 197.
16. Cody, V.; Middleton, E.; Harborne, J. B. *Plant Flavonoids in Biology and Medicine*; Alan.R. Liss: New York, 1986.
17. Chen, S.; Hwang, J.; Deng, P. S. K. *Arch. Biochem. Biophys.* **1993**, *302*, 72.
18. Zsila, F.; Bikadi, Z.; Simonyi, M. *Biochem. Pharm.* **2003**, *65*, 447.
19. Sytnik, A.; Kasha, M. *Porc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 8627.
20. Sytnik, A.; Litvinyuk, I. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 12959.
21. Lu, Z. X.; Cui, T.; Shi, Q. L. *Applications of Circular Dichroism (CD) and Optical Rotatory Dispersion (ORD) in Molecular Biology*, 1st ed.; Science: Beijing, 1987.
22. Dong, A. C.; Huang, P.; Caughey, W. S. *Biochemistry* **1990**, *29*, 3303.
23. Scatchard, G. *Ann. NY Acad. Sci.* **1949**, *51*, 660.
24. Eftink, M. R.; Ghiron, C. A. *Anal. Biochem.* **1981**, *114*, 199.
25. Yuan, T.; Weljie, A. M.; Vogel, H. J. *Biochemistry* **1998**, *37*, 3187.
26. Gerbanowski, A.; Malabat, C.; Rabiller, C.; Gueguen, J. *J. Agri. Food Chem.* **1999**, *47*, 5218.
27. Maruyama, A.; Lin, C. C.; Yamasaki, K.; Miyoshi, T.; Imal, T.; Yamasaki, M.; Otagiri, M. *Biochem. Pharm.* **1993**, *45*, 1017.
28. Jian Niao, T.; Jia Qin, L.; Wen Ying, H.; Zhi De, H.; Xiao Jun, Y.; Xing Guo, C. *Biomacromolecules* **2004**, *5*, 1956–1961.
29. Jian Niao, T.; Jia Qin, L.; Jian Ping, X.; Xiao Jun, Y.; Zhi De, H.; Xing Guo, C. *J. Photochem. Photobiol. B Biology* **2004**, *74*, 39–45.
30. Guharay, J.; Sengupta, B.; Sengupta, P. K. *Proteins: Structure Function Genetics* **2001**, *43*, 75–81.