MOLECULAR SPECTROSCOPY EVIDENCE OF EFFECTIVE CONSTITUENTS IN THE BINDING OF *Angelica sinensis* TO BOVINE SERUM ALBUMIN: COMPARATIVE BINDING AND THERMODYNAMIC PROFILE

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Some bio-effective constituents, such as organic acids and phthalides, from the root of Angelica sinensis can be observed in the blood after oral intake. In this work, two organic acids, ferulic acid and anisic acid, and two phthalides, senkyunolide I and butylidenephthalide, were selected to investigate the interaction with bovine serum albumin systematically, using a fluorescence spectroscopic method under physiological conditions. The Stern–Volmer quenching constant, binding constant, and the corresponding thermodynamic parameters and binding numbers were measured. Synchronous fluorescence and three-dimensional fluorescence spectroscopy were also used. The results provided molecular spectroscopy evidence that some constituents of Angelica sinensis are absorbed and transported into the blood.

Keywords: *Angelica sinensis, fluorescence, bovine serum albumin, ferulic acid, butylidenephthalide, senkyunolide I.*

МОЛЕКУЛЯРНО-СПЕКТРОСКОПИЧЕСКОЕ ИССЛЕДОВАНИЕ ВЗАИМОДЕЙСТВИЯ ЭФФЕКТИВНЫХ КОМПОНЕНТОВ *Angelica sinensis* С БЫЧЬИМ СЫВОРОТОЧНЫМ АЛЬБУМИНОМ: СРАВНЕНИЕ ХАРАКТЕРИСТИК СВЯЗЫВАНИЯ И ТЕРМОДИНАМИЧЕСКИХ ПАРАМЕТРОВ

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Методами синхронной и трехмерной флуоресцентной спектроскопии проведено систематическое исследование взаимодействия биоэффективных компонентов корня дудника китайского (Angelica sinensis) — органических кислот и фталидов — с бычьим сывороточным альбумином в физиологических условиях с использованием органических кислот (феруловой и анисовой) и двух фталидов (сенкюнолида I и бутилиденфталида). Измерены константы тушения Штерна–Фольмера и константы связывания, а также соответствующие термодинамические параметры и числа связывания. Полученные результаты свидетельствуют о том, что некоторые компоненты дудника китайского поглощаются и переносятся в кровь.

Ключевые слова: *Angelica sinensis (дудник китайский), флуоресценция, бычий сывороточный альбумин, феруловая кислота, бутилиденфталид, сенкюнолид I.*

Introduction. The root of *Angelica sinensis*, also called Danggui in China, is a highly valued medicinal herb known as female ginseng that has been widely used in China for over 1800 years. Over 70 constituents have been isolated and identified according to the comprehensive and thorough research on its chemical components carried out since the 1980s [1]. Recent research indicates that the many biological effects of Danggui are due to two kinds of chemical components, organic acids and phthalides [2–5]. Previous research reported that some constituents in *Angelica sinensis*, especially organic acids had strong interactions with cell membranes and could be transported into the plasma of experimental animals [6, 7]. From a biopharmaceutical point of view, the intensity, and duration of the efficacy and biotransformation of drugs are primarily dependent on the transporting capability of albumins. Although some basic information for understanding bioavailability and bio-efficacy of the *Angelica sinensis* extract is available, there is a dearth of data for plasma protein binding.

The study of drug binding affinities for serum albumin is helpful for understanding the availability of the drug for diffusion or transport into the drug's target organ. Bovine serum albumin (BSA) has been extensively used for drug-serum albumin interactions because of its intrinsic structural homology with human serum albumin [8]. BSA labeled with fluorescent probes is commonly used as a model system to investigate surface-induced conformational changes at protein interfaces. Numerous experiments have been carried out to characterize the binding capacity and sites of albumins [9–11].

The analysis monitors the change of a physicochemical property of the protein–probe system upon binding. Fluorometry, which is used extensively in this field, does not disturb the binding equilibrium upon separation [12]. The spectral changes observed on binding of the fluorophores with proteins are an important tool for investigating the topology of the binding sites, conformational changes, and characterization of the substrate to ligand binding [9].

In this work, four effective constituents of *Angelica sinensis*, namely, two organic acids, ferulic acid (FA) and anisic acid (AA), and two phthalides, senkyunolide I (SI) and butylidenephthalide (BP), were chosen to analyze the binding characteristics with BSA. The Stern–Volmer quenching constant, binding constant, and the corresponding thermodynamic parameters and binding numbers were acquired from the fluorescence spectroscopic data under physiological conditions. Analysis of synchronous fluorescence and three-dimensional fluorescence were used to evaluate the effect of the four effective constituents to the secondary structure of BSA.

Experimental. Ferulic acid and anisic acid were purchased from the Institute for the Control of Pharmaceutical and Biological Products of China (Beijing, China). Butylidenephthalide and senkyunolide I were obtained from the Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian, China. The purities of the four reference compounds were found to be >98% based on the percentage of total peak area by HPLC analysis. An Agilent 1200 series HPLC system with a diode array detector (Agilent, California, USA) was employed. The chromatographic separation was performed on a Sinochrom ODS-AT column (C18, 5μm, 4.6×250 mm, Dalian Elite Analytical Instruments, Dalian, China).

BSA (fraction V, purity ≥95%) was obtained from Roche, Shanghai, China. The molecular weight of the BSA was assumed to be 66000 to calculate the molar concentrations. All BSA solutions (1.0×10^{-5} mol/L) were dissolved in Tris-HCl buffer solution (0.05 mol/L, pH 7.4±0.1), containing NaCl (0.10 mol/L), which was selected to keep the pH value constant and to maintain the ionic strength of the solution.

All fluorescence spectra were recorded on a LS-55 spectrofluorometer (Perkin-Elmer, USA) equipped with quartz cells (1.0 cm) and a thermostat bath (HAAKE D3, Germany).

The fluorescence measurements were performed at the same temperature (298 K). The excitation wavelength was 280 nm, and the emission spectra were recorded from 220 to 500 nm. The slit widths for excitation and emission were set to 15.0 and 2.5 nm, respectively. The fluorescence intensity at 358 nm was determined under excitation at a wavelength of 280 nm. All data and each spectrum were the average of three scans. Titrations were performed manually by using trace syringes. In each titration, the fluorescence spectrum was collected with the concentration of BSA at 1.0×10^{-5} mol/L.

Binding of the four constituents to BSA was studied using fluorescence spectroscopy. To study the effect of temperature on the quencher-BAS interactions, titration experiments were carried out at four different temperatures: 293, 298, 303, and 308 K, by recording the fluorescence intensity after an equilibration time of 3 min at each temperature. Fluorescence quenching can be dynamic, resulting from collisional encounters between the fluorophore (protein) and the quencher, or static, resulting from the formation of a ground state between the fluorophore and the quencher. The fluorescence data were analyzed using the Stern–Volmer equation [13]:

$$F_0/F = 1 + k_q\tau_0[Q], \quad (1)$$

where F_0 and F are the fluorescence intensities in the absence and presence of the quencher, respectively, k_q is the bimolecular quenching constant, τ_0 is the lifetime of the fluorescence in the absence of the quencher, and $[Q]$ is the concentration of the quencher. The Stern–Volmer quenching constant, K_{SV}, is given by $k_q\tau_0$.

The binding constant (K) and binding sites (n) are calculated by the double-logarithm equation for static quenching [14]:

$$\lg[(F_0 - F)/F] = \lg K + n\lg[Q]. \quad (2)$$

Binding data at different temperatures were used to analyze the thermodynamic parameters using the van't Hoff equation:

$$\ln K = -\Delta H^0/RT + \Delta S^0/R, \quad (3)$$

where K is the association constant, T is the absolute temperature, and R is the gas constant (8.3145 $J \cdot mol^{-1} \cdot K^{-1}$). Values of the enthalpy change (ΔH^0) and the entropy change (ΔS^0) were obtained from the slope and intercept, respectively. Subsequently, the free energy change (ΔG^0) of the reaction at different temperatures was calculated by substituting the values of ΔH^0 and ΔS^0 thus obtained into the equation:

$$\Delta G^0 = \Delta H^0 - T\Delta S^0. \quad (4)$$

Synchronous fluorescence spectra of BSA in the absence and presence of increasing amounts of the four constituents were measured under the same conditions with steady-state fluorescence. Synchronous fluorescence spectra of the protein samples were obtained after scanning them at the wavelength range of 220–340 nm between excitation and emission wavelengths ($\Delta\lambda$) of 60 nm, and other scanning parameters were the same as those of the fluorescence spectra.

Three-dimensional fluorescence spectroscopy was performed under the following conditions: the emission wavelength was recorded from 220 to 550 nm, the initial excitation wavelength was set to 220 nm with an increment of 5 nm, the number of scanning curves was 27, and the slit widths for excitation and emission were set to 15.0 and 3.5 nm, respectively.

Results and discussion. The intrinsic fluorescence of proteins has been widely used to study the folding and association reactions of protein [15]. BSA contains two tryptophan residues, Trp212 (located within a hydrophobic pocket of the protein) and Trp134 (located on the surface of the protein molecule), which show intrinsic fluorescence [16, 17]. Fluorescence examinations took advantage of the intrinsic tryptophan fluorescence of BSA to probe the interaction with organic acids and phthalides. Figure 1 shows the fluorescence emission spectra (λ_{ex} = 280 nm) of BSA obtained in the absence and presence of increasing

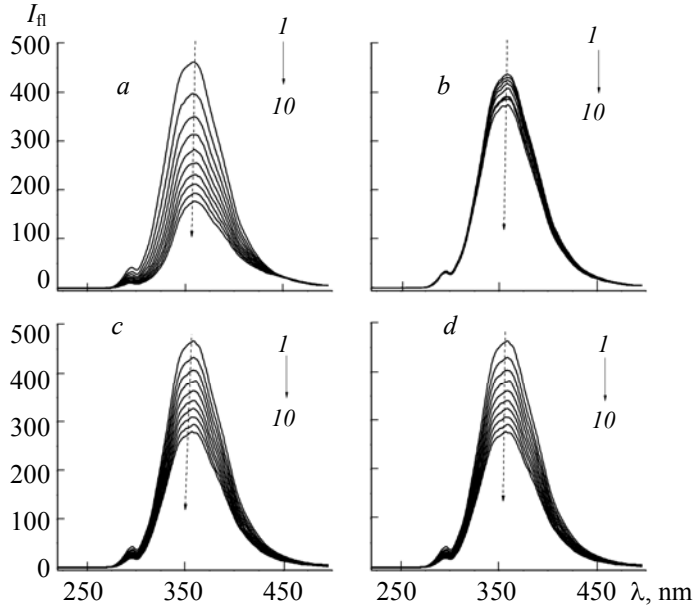

Fig. 1. Fluorescence emission spectra (1–10) of bovine serum albumin (10.0 μM) in Tris-HCl buffer solutions upon addition of quencher: 0–29.0 μM of ferulic acid (a), senkyunolide I (c), butylidenephthalide (d), and 0–74.0 μM of anisic acid (b). λ_{ex} = 280 nm, pH 7.4, T = 293 K.

amount of FA, AA, SI, and BP, respectively. All fluorescence emission spectra of the four compounds showed fluorescence quenching regularly during titration. This demonstrated that there were some suitable binding sites for these compounds on BSA. Furthermore, the order of emission fluorescence quenching ability at 358 nm in the presence of BSA was FA>BP>SI>AA, which reveals that this was the order of accessing the tryptophan residues. Small red shifts of maximum were also observed for all the tested constituents, suggesting an increase in the polarity of the tryptophan environment of BSA in the presence of the tested constituents. This was probably due to the loss of the compact structure of the hydrophobic subdomain IIA where tryptophan-214 is placed [18]. Therefore it can be inferred that the binding interaction between FA/AA/SI/BP and BSA resulted in an enhancement of the hydrophilicity around the tryptophan residues of BSA.

The mechanisms of the fluorescence quenching process for BSA binding with the organic acids and phthalides were investigated by fluorescence titration experiments at four different temperatures: 293, 298, 303, and 308 K. The data were further analyzed using the well-known Stern–Volmer equation (Fig. 2). It can be seen from the linearity of the Stern–Volmer plots that all four constituents bound to a single class of binding site on BSA. The quenching constants (K_{SV}) and quenching rate constant (k_q) were determined and are listed in Table 1. The K_{SV} values for quenching of BSA fluorescence with the addition of FA/AA/SI/BP were inversely correlated with temperature, which indicates that the probable quenching mechanism of BSA fluorescence by FA/AA/SI/BP is not initiated by dynamic collision, but by complex formation [13]. All the values of k_q was 10-fold higher than the maximum value possible for diffusion-limited quenching in water ($\sim 10^{10}$ $M^{-1} \cdot s^{-1}$), which suggests that the fluorescence quenching is not initiated by dynamic collision and that there is a specific interaction occurring [19] between BSA and the organic acids and phthalides studied here.

TABLE 1. Stern–Volmer Quenching Parameters of FA, AA, SI, and BP Binding with BSA at pH 7.4

T (K)	K_{SV}, 10^4 M^{-1}				k_q, 10^{11} $M^{-1} \cdot s^{-1}$			
	FA-BSA	AA-BSA	SI-BSA	BP-BSA	FA-BSA	AA-BSA	SI-BSA	BP-BSA
293	5.46	0.16	2.27	2.52	109.18	4.60	45.46	50.34
298	5.03	0.20	1.98	2.15	100.67	4.04	39.56	42.97
303	4.93	0.23	1.96	2.04	98.69	3.25	39.14	40.71
308	4.71	0.15	1.93	1.98	94.26	3.10	38.54	39.51

[a] The quenching rate constant (k_q) were calculated using the equation $k_q = K_{SV}/\tau_0$, $\tau_0 = 5\times10^{-9}$ s [10].

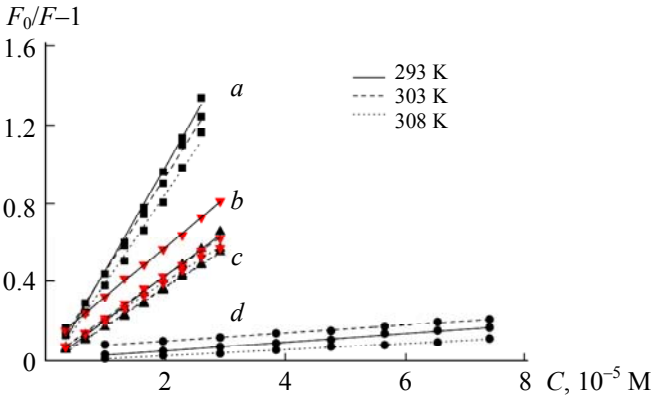

Fig. 2. Stern–Volmer plots for fluorescence quenching data of the four constituents with BSA at three different temperatures: 293 K, 303 K and 308 K. Ferulic acid-BSA (a), anisic acid-BSA (d), senkyunolide I-BSA (c), and butylidenephthalide-BSA (b). F_0 and F are the fluorescence intensities in the absence and presence of the quencher.

A more precise way of investigating the affinities of the organic acids and phthalides for BSA, the binding constants (K_a), and binding sites (n) is by using the double-logarithm Eq. (3). Table 2 lists the corresponding calculated results with all the regression coefficients over 0.995, which confirmed that the mathematical model used in the experiment was suitable to study the degree of binding to BSA.

TABLE 2. Binding Constants and the Number of Binding Sites of FA, AA, SI, and BP with a BSA Binding System at pH 7.4

T (K)	FA-BSA			AA-BSA		
	K, 10^4 M^{-1}	n	R	K, 10^4 M^{-1}	n	R
293	16.38	1.1109	0.9991	0.60	0.9519	0.9952
298	10.51	1.0843	0.9965	0.46	0.9705	0.9969
303	5.79	1.0194	0.9956	0.29	0.9609	0.9953
308	3.06	0.9592	0.9936	0.14	1.1442	0.9957
	SI-BSA			BP-BSA		
293	4.33	1.0764	0.9995	2.55	1.0149	0.9970
298	4.16	1.0714	0.9994	1.30	0.960	0.9995
303	3.99	1.0651	0.9976	0.83	0.9119	0.9993
308	2.40	1.0235	0.9980	0.25	0.7737	0.9994

On the basis of the data above, the values of the binding affinities can be ranked in the order FA>SI> > BP>AA. The values of n indicate that in each case all four constituents were located in one binding site.

To further elucidate which types of noncovalent interactions may play a crucial role in the binding process, the thermodynamic parameters, enthalpy change (ΔH^0), entropy change (ΔS^0), and free energy change (ΔG^0) of the binding reaction were obtained as follows. There are essentially four types of non-covalent interactions: hydrogen bond, van der Waals force, electrostatic force, and hydrophobic interaction force. According to the binding constants of the four compounds with the BSA system obtained at the four temperatures mentioned above, the value of ΔH^0 and ΔS^0 can be calculated by the van't Hoff Eq. (3), and the free energy change (ΔG^0) can be calculated by Eq. (4). The results of the thermodynamical parameters (ΔH^0, ΔS^0, and ΔG^0) are listed in Table 3. The negative values for ΔG^0 support the assertion that the interaction of the four compounds with the BSA complex was spontaneous. The negative signs for ΔG^0 and ΔH^0 indicate that FA/AA/SI/BP binding of BSA was an exothermic reaction, and the positive ΔS^0 value is frequently taken as evidence for hydrophobic interaction [20].

TABLE 3. Thermodynamic Parameters of FA, AA, SI, and BP with the BSA Binding System at pH 7.4

T, K	ΔH^0, kJ/mol	ΔS^0, J · mol^{-1} · K^{-1}	ΔG^0, kJ/mol	ΔH^0, kJ/mol	ΔS^0, J · mol^{-1} · K^{-1}	ΔG^0, kJ/mol
	FA-BSA			AA-BSA		
293			–139.37			–120.80
298	–84.38	187.65	–140.30	–71.13	169.52	–121.65
303			–141.24			–122.50
308			–142.18			–123.35
	SI-BSA			BP-BSA		
293			–27.48			–196.61
298	–26.86	2.12	–27.49	–110.77	292.96	–198.08
303			–27.50			–199.54
308			–27.51			–201.01

The interaction of BSA with FA, AA, SI, and BP as well as the information about the microenvironment in the vicinity of the fluorophore molecules were further investigated in detail through synchronous fluorescence spectra. As shown in Fig. 3, the fluorescence quenching of the tryptophan residues at the $\Delta\lambda$ = 60 nm was observed by the addition of FA, AA, SI, and BP. This suggests that FA, AA, SI, and BP quenched the BSA fluorescence spectrum mainly by quenching the tryptophan residue. With increasing FA, AA, SI, and BP concentration, the fluorescent emission at 280 nm was efficiently quenched with a slight red shift of the tryptophan fluorescence peak. The binding of FA, AA, SI, and BP to BSA appears to increase the polarity of the environment where the tryptophan residues are located.

To obtain more information on the precise configuration of BSA upon FA, AA, SI, and BP binding, three-dimensional (3-D) fluorescence spectroscopy was performed on BSA in the absence and presence of the quencher. The 3-D fluorescence spectra of free BSA and BSA in the presence of FA, SI, and BP with the molar ratios of 3:1, and AA at 8:1, are shown in Fig. 4. Peaks *a* and *b* represent the Rayleigh scattering peak

($\lambda_{ex} = \lambda_{em}$) and the second-order scattering peak ($2\lambda_{ex} = \lambda_{em}$), respectively. The strong peak 1 (λ_{ex} = 280 nm, λ_{em} = 358 nm), mainly revealed the spectral characteristics of tryptophan and tyrosine residues and reflected changes in the tertiary structure of BSA [21–23]. The intensity of peak 1 was quenched upon the separate addition of FA, AA, SI, and BP (70.64, 39.97, 53.62, and 55.34%, respectively), but with hardly any shift of λ_{em}. From the above results, we can deduce that the structural and size features of FA, AA, SI, and BP probably have some effect on the molecular conformation of BSA.

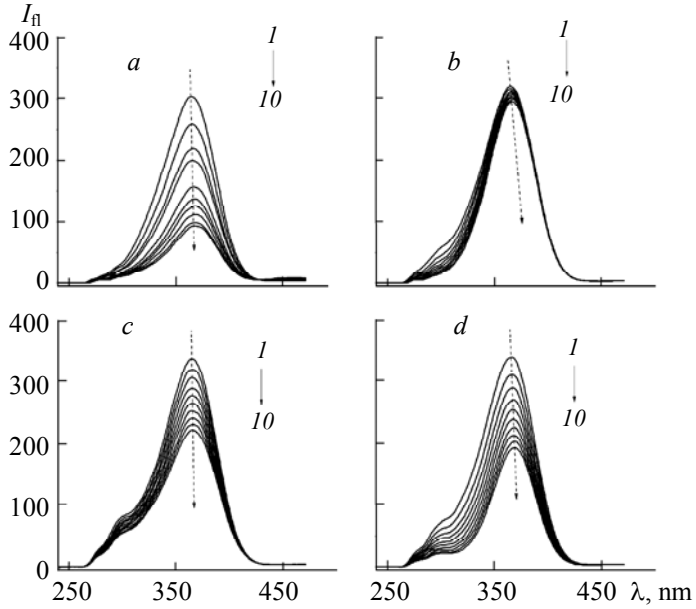

Fig. 3. Synchronous fluorescence spectra (1–10) of bovine serum albumin (10.0 μM) in Tris-HCl buffer solutions upon addition of quencher: 0–29.0 μM of ferulic acid (a), senkyunolide I (c), butylidenephthalide (d), and 0–74.0 μM of anisic acid (b). $\Delta\lambda$ = 60 nm, T = 298 K.

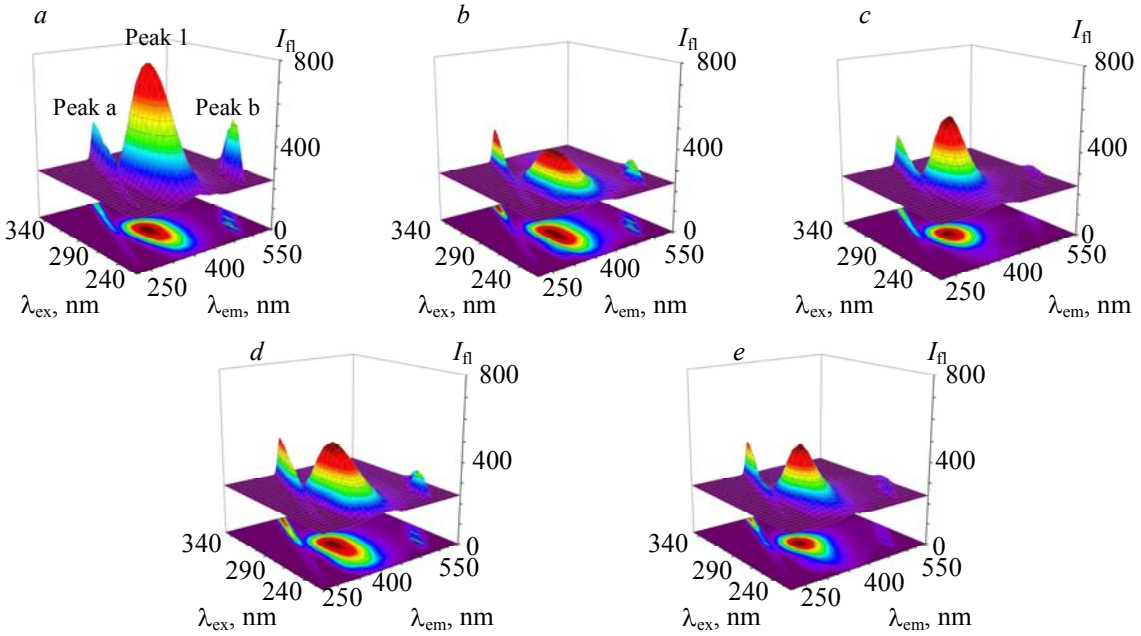

Fig. 4. Three-dimensional fluorescence spectra (fluorescence signal axis offset 30%) and corresponding contour maps of BSA (a) and quenchers FA, AA, SI, and BP binding with BSA systems: [FA]/[BSA] = 3:1 (b), [AA]/[BSA] = 8:1 (c), [SI/[BSA] = 3:1 (d), and [BP/[BSA] = 3:1 (e), at 298 K.

Conclusion. The present study reports the binding mechanism of several organic acids and phthalides in *Angelica sinensis* with BSA by fluorescence spectroscopy under simulated physiological conditions. The fluorescence of BSA mainly originates from the tryptophan residues, which can be quenched by all four constituents (FA, AA, SI, and BP) through a static quenching mechanism. The Stern–Volmer quenching constant, binding constant, and the corresponding thermodynamic parameters and binding numbers were also determined. The results showed that all four constituents (FA, AA, SI, and BP) could bind to BSA, mainly through van der Waals forces and hydrogen bonds. The interaction of AA with BSA was much weaker than the other three compounds. The microenvironment around the tryptophan residues exhibited obvious changes during the binding process. The results from the analysis of synchronous fluorescence and three-dimensional fluorescence showed that the binding of all four constituents (FA, AA, SI, and BP) to BSA induced changes in the secondary structure of the protein. The binding mechanism of SI and BP with BSA was investigated and compared in this work by fluorescence spectroscopy for this first time.

All the results mentioned above provide important information for understanding the transport process of effective compounds of *Angelica sinensis* in vivo and provides a useful foundation for further pharmaceutical mechanistic studies of *Angelica sinensis*.

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