American Journal of Biochemistry and Biotechnology

Original Research Paper

Binding Interaction between Bovine Serum Albumin and Chicoric Acid, a Food Functional Component

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Article history
Received: 26-06-2016
Revised: 18-07-2016
Accepted: 15-09-2016

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Abstract: Fuorescence, FTIR and UV-Vis absorption spectroscopy were used to explore the binding between chicoric acid and Bovine Serum Albumin (BSA). Binding characteristics at various levels of temperature have been calculated. The results indicated that chicoric acid statically quenched the intrinsic fluorescence of BSA. The binding constants (K_a) were 4.14×10^5 L mol^{-1} at 273K and 4.29×10^6 L mol^{-1} at 298 k. The numbers of binding sites between chicoric acid and BSA were both approximately equal to 1 at the two temperatures. Furthermore, the binding distance between chicoric acid and BSA was 2.69 nm which was calculated according to the Förster's resonance energy transfer. Thermodynamic parameters suggested that BSA bind chicoric acid spontaneously mainly *via* hydrophobic interaction. Results demonstrated that the conformation and microenvironment of BSA were changed after binding with chicoric acid. Moreover, chicoric acid showed stronger binding with tryptophan (Trp) residue than with tyrosine (Tyr) residue. Our results can provide scientific basis for studying availability and distribution of chicoric acid.

Keywords: Chicoric Acid Bovine Serum Albumin, Spectroscopy, Interaction, Conformation

Introduction

Phenolic acids widely occur in plant leaves, roots and especially fruits, are aromatic acid compounds and secondary plant metabolites (Herrmann and Nagel, 1989). Hydroxybenzoic and hydroxycinnamic acids are two groups of phenolic acids that widely distribute in plants (Ghasemzadeh and Ghasemzadeh, 2011). Much attention has been paid to these natural phenolic acids because of their functional activities in intervening diabetes, inflammatory and cancer as well as anti-oxidative and anti-microbial properties (Chao *et al*., 2009; Cueva *et al*., 2010; Hsu *et al*., 2000; Maurya *et al*., 2010; Nayaka *et al*., 2010). Moreover, previous reports revealed that the binding between some phenolic acids and biomolecules such as DNA and proteins played a certain role in their biological properties (Labieniec and Gabryelak, 2005).

Chicoric acid (Fig. 1) is a member of phenolic acids and found in many edible plants such as *Echinacea purpurea*, dandelion, basil, iceberg lettuce, chicory, cat's Whisker (Baur *et al*., 2004; Innocenti *et al*., 2005; Lee and Scagel, 2009; Liu *et al*., 2006; Olah *et al*., 2003; Schütz *et al*., 2005). Many literatures have reported that chicoric acid possesses anti-oxidative, antivirus and anti-diabetic activities (Dalby-Brown *et al*., 2005; Robinson *et al*., 1996; Tousch *et al*., 2008). Moreover, previous study also revealed that chicoric acid possessed a stimulatory effect on phagocytes (Bone, 1997). Recently, the binding study of chicoric acid with HIV-1 integrase has been carried out (Healy *et al*., 2009).

Binding studies of small molecules to proteins are very important in their disposition and efficacy because protein binding can influence the effective solubility, distribution and biological half-life of small molecules *in vivo* as well as interaction between small molecules and other endogenous or exogenous compounds. Therefore, it is of great necessity for explaining the pharmacodynamics and pharmacokinetics of small molecules to investigate the binding between them and proteins (Cui *et al*., 2008; Qin *et al*., 2007). Serum albumins, lipoproteins and al-glycoprotein are proteins commonly participated in protein binding (Abdi *et al*., 2012). Among them, the most abundant blood proteins are serum albumins which play an important role in balancing the oncotic pressure and pH of blood (Carter and Ho, 1994).

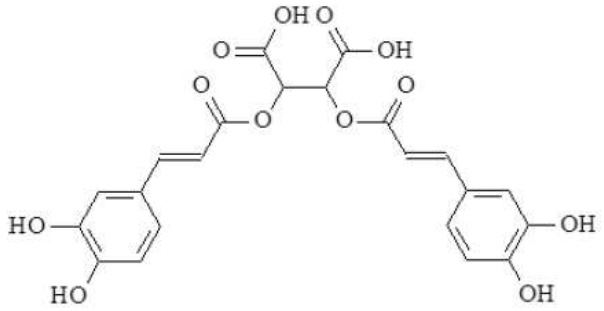

Fig. 1. Molecule structure of chivoric acid

The most prominent characteristic of serum albumins is that they can act as the depot proteins and transporters for numerous endogenous and exogenous small molecules (Huang *et al*., 2004). BSA was used frequently in previous studies because of its advantages such as highly stability, cheap and homology with Human Serum Albumin (HSA) in structure (Carter *et al*., 1994; Naik *et al*., 2010). BSA contains two tryptophan (Trp) residues including Trp-134 and Trp-212 which possess intrinsic fluorescence. In the first domain Trp-134 is located on the surface of the molecule and in the second domain Trp-212 is located within a hydrophobic binding pocket of the protein (He and Carter, 1992; Hamdanim *et al*., 2009). The conformation of BSA would be changed upon interacting with small molecules.

UV-Vis absorption and fluorescence spectroscopy were used to investigate the interactions between serum albumins and small molecules for their outstanding sensitivity, selectivity, reproducibility, convenience and theoretical foundation (Zhang *et al*., 2012). FTIR spectroscopy is reliable method to illustrate the conformational changes of proteins after binding with small molecules (Darwish *et al*., 2010). Recently, several researches on the binding between phenolic acid and serum albumins have been undertaken using spectroscopic technology (Kang *et al*., 2004; Labieniec and Gabryelak, 2006; Meng *et al*., 2012; Rawel *et al*., 2005; Soares *et al*., 2007). However, no report of chicoric acid-serum albumins interaction has been found so far. Therefore, this research was carried out to explore the interaction between chicoric acid and BSA under simulated physiological conditions using fuorescence, FTIR and UV-Vis absorption spectroscopy. The mechanism of interaction between chicoric acid and BSA including quenching mechanism, binding parameters, binding distance, thermodynamic parameters and conformational change were explored.

Materials and Methods

Chemicals and Reagents

Chicoric Acid and BSA was obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents used in this study were of analytical purity. Water used throughout the experiments was ultrapure.

Instrumentations

Hitachi F-4500s pectrofluorimeter (Tokyo, Japan) with a 1.0 cm quartz cell and a 150 W xenon lamp was employed in this study to record fluorescence spectra. Excitation wavelength was set at 285 nm. The widths of excitation slit and emission slit were both 10 nm. The UV-Vis absorption spectra were measured by Shimadzu UV-2550 s pectrophotometer (Kyoto, Japan) in the wavelength range 250-350 nm. FTIR spectra were measured using Thermo-Nicolet Avatar330 FTIR spectrometer (Rochester, NY, USA) using KBr pellets. The weight of samples was measured by Sartorius BP211D analytical balance with a precision of 0.1 mg (Göttingen, Germany). PHS-3Cdigital pHmeter (Shanghai, China) was used to detect pH values.

Preparation of Stock Solutions

To keep the ionic strength of solution NaCl (0.10 M) was used in Tris-HCl buffer (0.10M, pH 7.4). All BSA solutions were prepared in Tris-HCl buffer solution and kept at 0-4°C before used. The stock solution of chicoric acid was prepared in methanol.

Fluorescence Studies

Equal volumes of chicoric acid solutions with various concentrations were added to protein solutions, respectively. All solutions were mixed thoroughly. The final concentrations of chicoric acid were 0, 2, 3, 4, 5, 6, 7, 8, 9 and 10 μM. Then the mixtures of chicoric acid and BSA were equilibrated at 273 or 298 K for 20 min. The fluorescence emissions spectra were recorded in the range of 300-450 nm and the binding constants of chicoric acid-BSA systems were calculated in the base of fluorescence data. The synchronous fluorescence spectra of BSA with or without chicoric acid were recorded with the excitation and emission wavelength intervals ($\Delta\lambda$) at 15 and 60 nm, respectively. All the experiments were carried out in triplicate and the measurement error was less than 1%.

Absorption Studies

The UV-Vis spectra were collected by Shimadzu UV-2550 spectrophotometer in the region of 200-450 nm at 298K. The final concentrations of chicoric acid were 0, 2, 3, 4 and 5μM, respectively. While that of BSA was 1 μM.

FTIR Spectroscopic Measurements

The FTIR spectra of Tris-HCl buffer, BSA in the absence and presence of chicoric acid were collected in the spectral region 1000-2000 cm^{-1}, respectively. Then the FTIR spectra of the sample solution were obtained by subtracting that of Tris-HCl buffer which taken as blank.

Results and Discussion

Binding Characteristics

Molecular interaction is one of the causes to decrease the fluorescence intensity of a fluorophore (Vijayabharathi *et al*., 2012). Therefore, the interactions between small molecules and proteins were revealed through detecting fluorescence quenching. In this study, the fluorescence spectra of BSA (λ_{ex} = 285 nm) mixed with chicoric acid were obtained at 273 and 298 K. Figure 2 shown that chicoric acid decreased the fluorescence intensity of BSA and there was a positive correction between concentrations of chicoric acid and fluorescence intensity of BSA. These results indicated that the interaction between chicoric acid and BSA occured and the non-fluorescent complex chicoric acid-BSA formed. Moreover, the emission maximum (λem) of BSA slight red-shifted in the present of chicoric acid, indicating that Trp chromophore in BSA was located in a more hydrophilic environment because of the interaction of chicoric acid with BSA. This result was further confirmed by synchronous fluorescence spectra described below.

Dynamic and static quenching are two main mechanisms of fluorescence quenching and different in dependence on temperature and viscosity. Stern-Volmer equation (Lakowicz and Weber, 1973) (Equation 1) was usually used to analyze the quenching mechanism in the previous studies:

$$F_0 / F = 1 + K_{sV}[Q] = 1 + K_q \tau_0 [Q] \quad (1)$$

Where:

F_0 and F = The fluorescence emission intensities with and without quencher, respectively
K_{sv} = The Stern-Volmerquenching constant
K_q = The quenching rate constant
$[Q]$ = The concentration of quencher
τ_0 = The average lifetime of the molecules without quencher and its value is about 10^{-8}s

Figure 3 showed the Stern-Volmer plots for BSA fluorescence quenched bychicoric acid. Satisfactory linearity of the Stern-Volmer equations was obtained in the investigated concentrations of chicoric acid. Table 1 listed the values of K_{sv} and K_q. The results suggested that with temperatures rising the values of K_{sv} decreased, indicating that static quenching was the probable machenism of fluorescence quenching between chicoric acid and BSA. Moreover, the quenching rate constants (K_q) of BSA were determined to be 9.835×1012 and 7.454×1012L $mol^{-1}s^{-1}$, respectively, which were far greater than the maximum diffusion collision quenching rate constant (2.0×10^{10} mol^{-1} Ls^{-1}), further demonstrating that the dominant mechanism was static quenching in the fluorescence quenching process of BSA by chicoric acid.

Binding Constants and Binding Sites

The double-logarithm equation (Bandyopadhyay *et al*., 2012) (Equation 2) was used to caculate the binding constant (K_a) and the number of binding sites (n) in static quenching interaction:

$$\lg\left[\frac{F_0 - F}{F}\right] = \lg K_a + n\lg[Q] \quad (2)$$

Figure 4 demonstrated plots of $\lg(F_0\text{-}F)/F$ versus $\lg[Q]$ for chicoric acid-BSA. The values of K_a and n can be obtained from the intercept and the slope, respectively. The calculated K_a and n at different levels of temperature were summarized in Table 2. The values of n at 273 and 298 K were both equal to 1, suggesting single class of binding site in BSA for chicoric acid.

Thermodynamic Parameters and Binding Force

Generally, the non-covalent interaction of small molecules and proteins cover hydrogen-bonding forces, vander Waals forces, hydrophobic interactions and electrostatic interactions. The major evidences for determining the binding mode of small molecule-protein are thermodynamic parameters such as free energy change (ΔG), enthalpy change(ΔH) and entropy change (ΔS). The parameters above can be estimated from Equation 3 and 4:

$$In\frac{K_{a2}}{K_{a1}} = \frac{1}{R}\left[\frac{1}{T_1} - \frac{1}{T_2}\right]\Delta H \quad (3)$$

$$\Delta G = \Delta H - T\Delta S = -RT\ln K_a \quad (4)$$

Where:

K_{a1} and K_{a2} = Binding constants at temperature T_1 and T_2, respectively
R = The gas constant

The interaction researches were implymented at 273 and 298 K. Table 3 listed the thermodynamic parameters for the binding of chicoric acid and BSA. The value of ΔG was negative indicating the binding process of chicoric acid and BSA was spontaneous. The values of ΔH and ΔS were positive implied that the interaction between chicoric acid and BSA was mainly an endothermic and entropy-driven reaction. Meanwhile, the main force between chicoric acid and BSA was hydrophobic force (Zhang *et al*., 2012).

Binding Distance

The spectral researches revealed that a complex was formed between chicoric acid and BSA. Additionally, Fig. 5 showed the fluorescence emission spectrum of BSA and the absorption spectrum of chicoric acid.

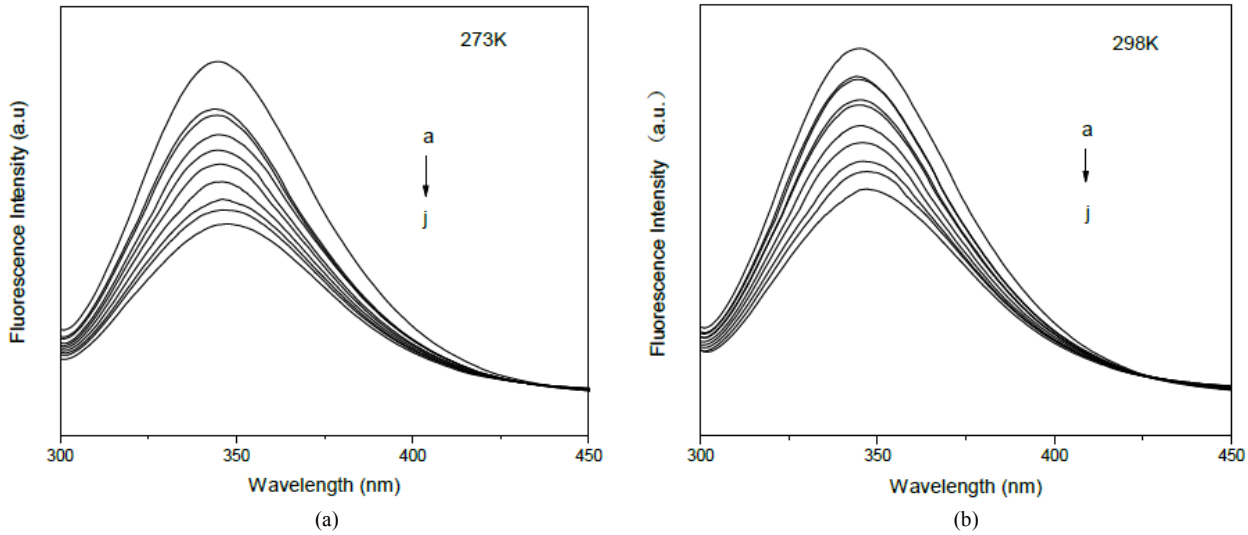

Fig. 2. The fluorescence spectra of BSA quenched by chicoric acid at 273 K (A) and 298 K (B). λ_{ex} = 285 nm; BSA, 1 μM; chicoric acid (a-j): 0, 2, 3, 4, 5, 6, 7, 8, 9 and 10 μM, respectively

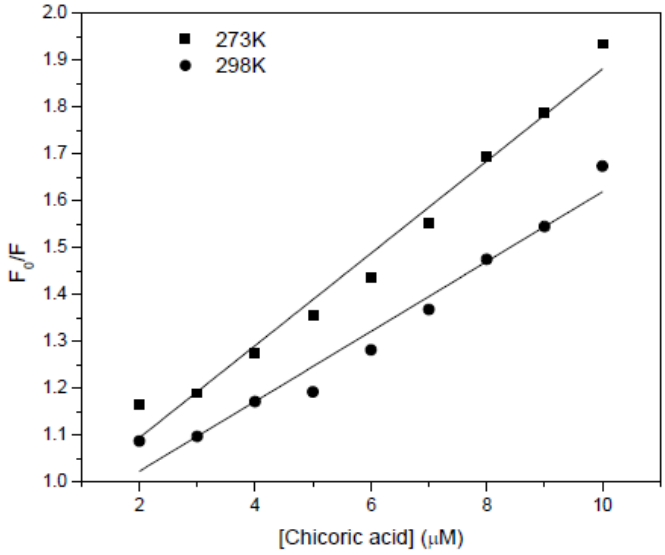

Fig. 3. Stern-Volmer plots for fluorescence quenching of chicoric acid-BSA at 273K (■) and 298K (●)

An overlap of the two spectra could be found. Therefore, the binding distance of chicoric acid and BSA can be obtained upon fluorescence resonance energy transfer. Equation 5 (Pang *et al*., 2012) could be used to determine the efficiency of energy transfer between the donor and acceptor (E):

$$E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + r^6} \quad (5)$$

Where:

F and F_0 = The fluorescence intensities of BSA with and without chicoric acid, respectively

r = The binding distance of BSA and chicoric acid 50% of the excitation energy is shifted to the acceptor at the critical distance (R_0) which can be determined by the Equation 6 (Pang *et al*., 2012):

$$R_0^6 = 8.8 \times 10^{-25} K^2 n^{-4} \varnothing J \quad (6)$$

Where:

K_2 = The spatial orientation factor of the dipole

n = The refractive index of the medium

φ = fluorescence quantum yield of the donor

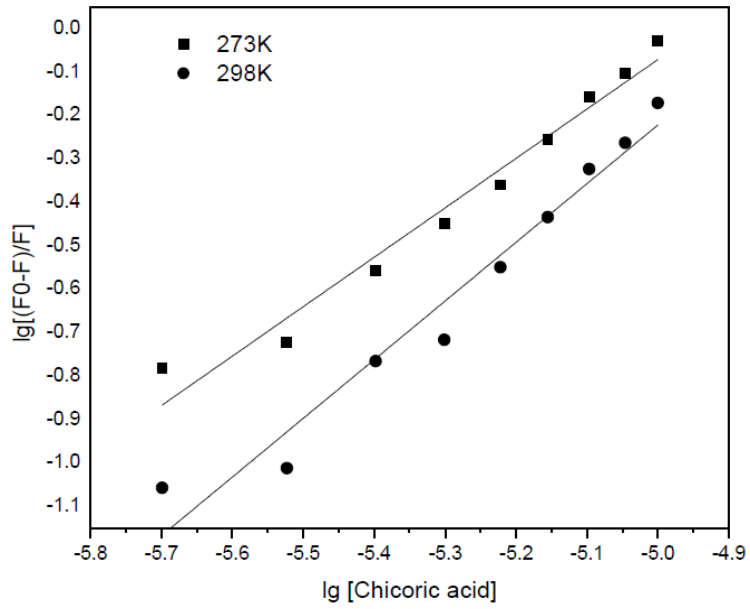

Fig. 4. Double log plots of BSA with chicoric acid at 273K (■) and 298K (●)

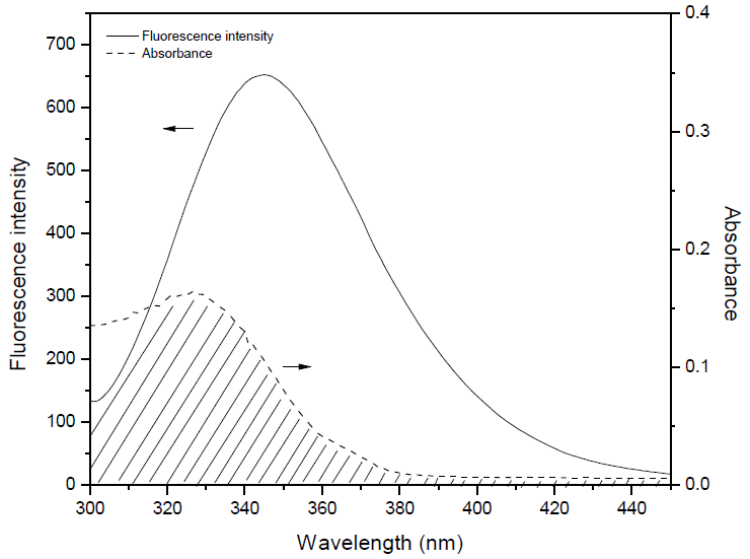

Fig. 5. The overlap of the fluorescence spectrum of BSA and the absorption spectrum of chicoric acid. BSA, 1 μM; chicoric acid, 10 μM; T = 298K

Table 1. Stern-Volmer quenching constants for the interaction of chicoric acid with BSA

Temperature (K)	K_{sv} ($\times 10^4$ Lmol^{-1})	K_q ($\times 10^{12}$ L mol^{-1}s^{-1})	r
273K	9.835	9.835	0.9749
298K	7.454	7.454	0.9589

Table 2. Binding constants and binding sites for the interaction of chicoric acid with BSA

Temperature (K)	K_q (L mol^{-1})	n	r
273K	9.835	9.835	0.9749
298K	7.454	7.454	0.9589

Table 3. Thermodynamic parameters for the binding of chicoric acid and BSA

Temperature (K)	ΔG (kJ·mol^{-1})	ΔS (J·mol-1·K^{-1})	ΔH (kJ·mol^{-1})
273K	-12.75	147.33	27.47
298K	-16.43		

The values of K^2, φ and n have been reported for BSA are 2/3, 0.14 and 1.36, respectively (Zhuang *et al*., 2012). *J*, the overlap integral of the fluorescence emission spectrum of donor and absorption spectrum of the acceptor, can be obtained using Equation 7 (Pang *et al*., 2012):

$$J = \frac{\Sigma F(\lambda)\varepsilon(\lambda)\lambda^4\Delta\lambda}{\Sigma F(\lambda)\Delta\lambda} \quad (7)$$

Where:

$F(\lambda)$ = The fluorescence intensity of fluorescent donor at wavelength λ

$\varepsilon(\lambda)$ = The molar absorption coefficient of the acceptor at wavelength λ

Basing on Equation 5, 6 and 7, it could be obtained that $J = 1.07\times10^{-14}$ $cm^3\cdot L\cdot mol^{-1}$, $R_0 = 2.52$ nm, $E = 0.40$, $r = 2.69$ nm. The value of r is less than 8 nm, suggesting the shift of non-radiative energy between BSA and chicoric acid. Moreover, the value of r was larger than that of R_0 also suggested that the quenching mechanism of chicoric acid to BSA was static (Zhuang *et al*., 2012). Base on the above, the static quenching combined with non-radiative energy transfer was the quenching mechanism for chicoric acid to BSA.

Conformation Investigation

Synchronous Fuorescence Spectra

Synchronous fluorescence spectroscopy can be used to investigate the changes in structure and conformation of proteins. The shift in maximum emission wavelength corresponds to changes in polarity around the chromosphere molecules (Jayabharathi *et al*., 2012). Synchronous fluorescence spectra offer information about the characteristics of Tyrresidue and Trpresidue when Δλ between excitation wavelength and emission wavelength is fixed at 15 and 60 nm, respectively (Shi *et al*., 2012). Figure 6 showed that with the increasing concentration of chicoric acid the fluorescence intensities at Δλ = 15 and Δλ = 60 nm were both decreased gradually. The emission maximum of Tyr residue kept unchanged at 288 nm upon addition of chicoric acid, suggesting that chicoric acid had no obvious change on the microenvironment of the Tyr residue. Whereas, it was observed that the emission maximum of Trp residue had a weak red shift by about 1 nm from 283 nm to 284 nm, indicating that Trp residue was close to chicoric acid, the hydrophobicity around the Trp residue decreased, however, the polarity increased (Zhuang *et al*., 2012). Additionally, we calculated fluorescence quenching ratios (R_{SFQ}) basing on the equation $R_{SFQ} = 1\text{-}F/F_0$. In this equation, F and F_0 are the synchronous fluorescence intensities of BSA with and without chicoric acid, respectively (Meng *et al*., 2012). As shown in Fig. 7, the R_{SFQ} for Δλ = 15 nm were smaller than the R_{SFQ} for Δλ = 60 nm, suggesting that chicoric acid was more accessible to Trp residue than to Tyr residue (Wang *et al*., 2012).

FTIR Measurements

Different amide bands in infrared spectra of proteins indicate different vibrations of the peptide moiety. Two amide bands related with the secondary structure of protein were the protein amide I band at 1600-1700 cm^{-1} (mainly C=O stretching) and II band at 1500-1600 cm^{-1}(C–N stretching coupled with N–H bending mode). In this study, the binding between chicoric acid and BSA was demonstrated using infrared spectroscopy to obtain more information on mechanism of this interaction and conformational changes of BSA. Figure 8 showed the FTIR spectra of BSA and chicoric acid-BSA complex. The peak position of amide I and II bands shifted from 1654 to 1649 and 1542 to 1552 cm^{-1}, respectively. The changes of these peak positions indicated that in the protein structural subunits C=O and C–N groups were bound with chicoric acid and then rearranged the polypeptide carbonyl hydrogen bonding pattern, finally changed the secondary structure of BSA (Tantipolphan *et al*., 2007).

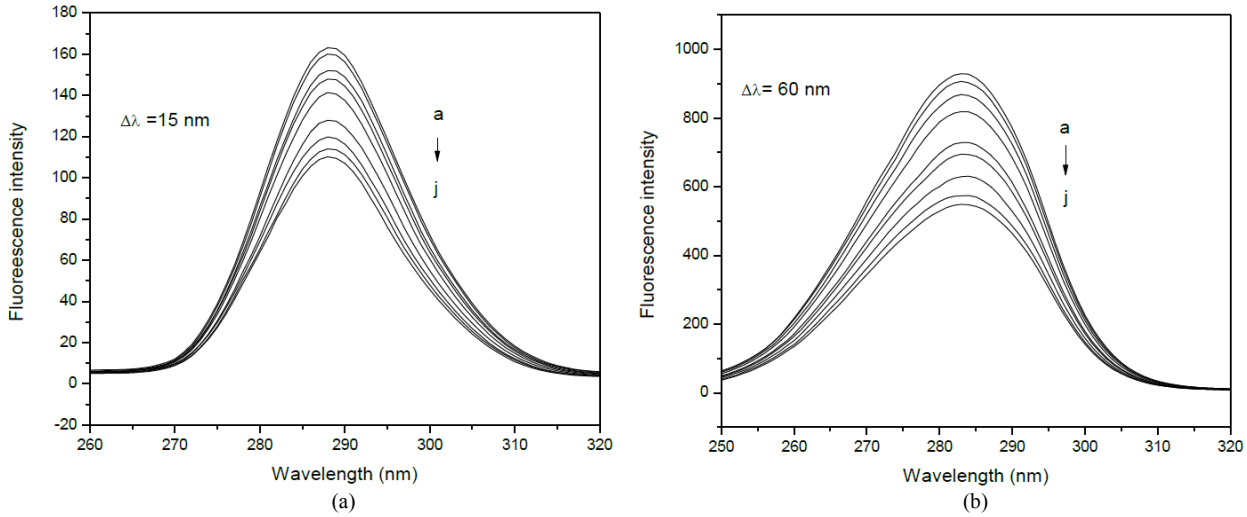

Fig. 6. The synchronous fluorescence spectra of BSA. (A) Δλ = 15 nm, (B) Δλ = 60 nm; BSA, 1 μM; chicoric acid (a–j): 0, 2, 3, 4, 5, 6, 7, 8, 9 and 10 μM, respectively; T = 298K

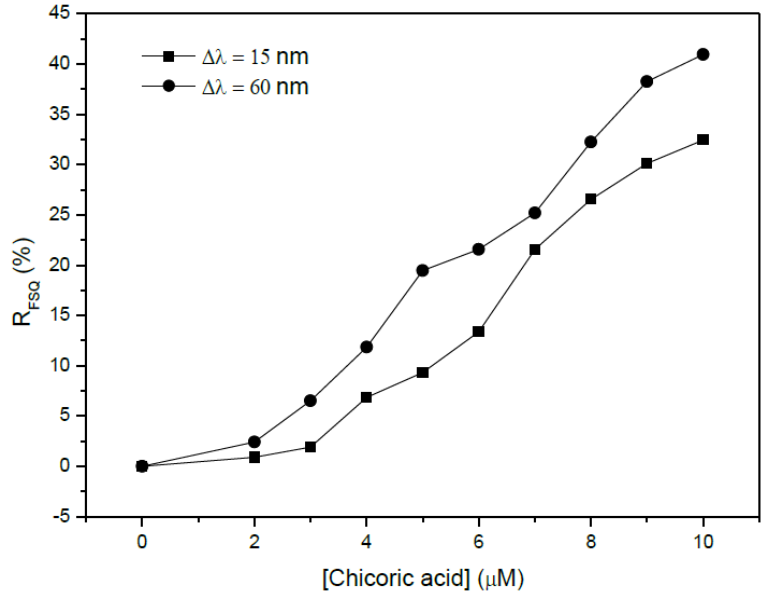

Fig. 7. Ratios of Synchronous Fluorescence Quenching (RSFQ) of BSA in the presence chicoric acid. BSA, 1 μM; chicoric acid: 0, 2, 3, 4, 5, 6, 7, 8, 9 and 10 μM, respectively; T = 298 K. Δλ = 15 nm (■), Δλ = 60 nm (●)

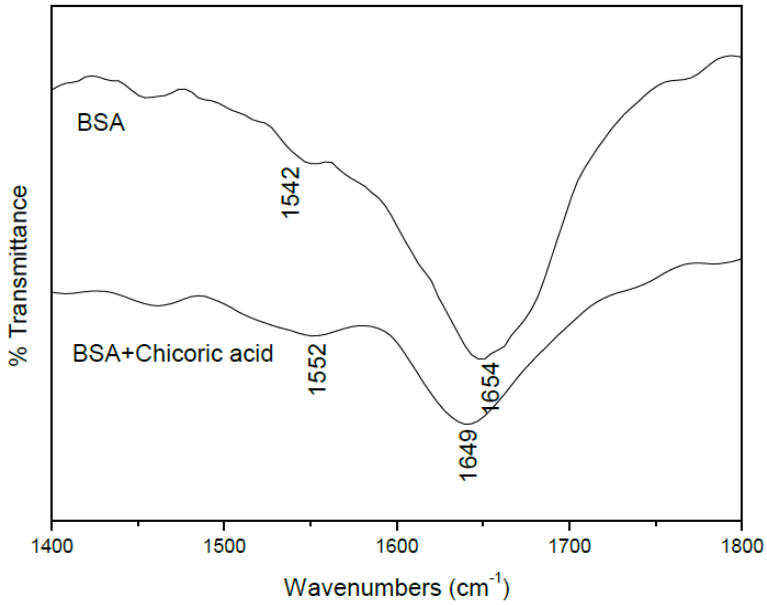

Fig. 8. FTIR spectrum of BSA and difference spectrum of BSA-chicoric acid complex. The FTIR spectrum of BSA was obtained by subtracting the spectrum of buffer solution from the spectrum of the protein solution; the FTIR difference spectrum of BSA-chicoric acid complex was obtained by subtracting the spectrum of chicoric acid-free form from that of chicoric acid-BSA complex form; BSA, 1 μM; chicoric acid, 10 μM; T = 298K

UV-Vis Absorption Studies

For reconfirming the conformational change of BSA by the addition of chicoric acid, UV-Vis absorption spectra of BSA with varying concentrations of chicoric acid were obtained. In the present of chicoric acid, the absorption peak intensity of BSA increased as well as the peak red shifted from 278 to 286 nm (Fig. 9). It was reported that dynamic quenching did not change the absorption spectrum, but the formation of non-fluorescence ground-state complex can change it (Du *et al*., 2012). Thus, these results indicated that the interaction between chicoric acid and BSA caused the formation of ground sate complex and reconfirmed the static quenching mechanism of this interaction (Liu *et al*., 2010).

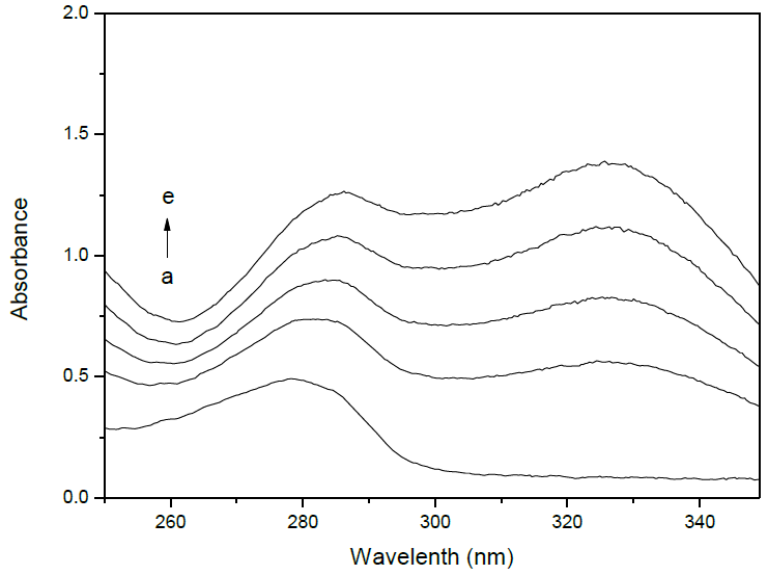

Fig. 9. UV-Vis absorption spectra of BSA and chicoric acid-BSA complex. BSA, 1 μM; chicoric acid, (a–e): 0, 2, 3, 4, 5 μM; T = 298K

Conclusion

In this study, the interaction of chicoric acid and BSA was studied using spectroscopic analysis. The results demonstrated that static quenching process was probable the quenching mechanism of BSA by chicoric acid. It was calculated that one binding site in BSA was accessible to chicoric acid. Thermodynamic parameters revealed that the binding reaction was spontaneous and hydrophobic force played a major role during the interaction. The distance between chicoric acid and BSA was less than 8 nm base on the Förster's resonance energy transfer. Additionally, BSA undergone conformational and microenvironment changes upon binding to chicoric acid and the binding site is mainly at Trp residue.

Acknowledgement

This work was supported by Shandong Provincial Natural Science Foundation, China (No. ZR2014CQ002). Therefore, we are grateful for this funding and support of this research.

Funding Information

This work was supported by Shandong Provincial Natural Science Foundation, China (No. ZR2014CQ002).

Author's Contributions

Haifang Xiao: Took part in all experiment process as well as data analysis and manuscript preparation.

Quancai Sun: Involved in study design and data analysis.

Xuebo Liu: Coordinated the study.

Yuanda Song: Integrated all data and improved the manuscript.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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