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Human serum albumin interaction with formononetin studied using fluorescence anisotropy, FT-IR spectroscopy, and molecular modeling methods

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Abstract—Interaction of formononetin with a model transport protein, human serum albumin (HSA), has been studied using fluorescence anisotropy, FT-IR spectroscopy, and molecular modeling methods. Upon binding with HSA, the fluorescence spectrum of formononetin exhibits appreciable hypsochromic shift along with an enhancement in the fluorescence intensity. Gradual addition of HSA led to a marked increase in fluorescence anisotropy (r). From the value of fluorescence anisotropy, it is argued that the drug is located in a restricted environment of protein. The binding constant ($K \approx 1.6 \times 10^5\ M^{-1}$) and the standard free energy change ($\Delta G^0 \approx -29.9$ kJ/mol) of formononetin–HSA interaction have been calculated according to the relevant fluorescence data. Fourier transform infrared measurements have shown that the secondary structures of the protein have been changed by the interaction of formononetin with HSA. Computational mapping of the possible binding sites of formononetin revealed the molecule to be bound in the large hydrophobic cavity of subdomain IIA.

1. Introduction

Serum albumins are the most widely studied abundant proteins in plasma. In order to understand how serum albumins affect the functionality of foods including proteins, many researchers have studied the structures and properties of serum albumins as well as their interaction with other proteins. Human serum albumin is the most abundant protein which is composed of blood plasma. Its three-dimensional structure has been determined through X-ray crystallographic measurements.[1] It is a single-chain 66 kDa protein, which is largely α-helical. It consists of three structurally homologous domains that assemble to form a heart-shaped molecule. Each domain contains two subdomains, which are predominantly helical and extensively cross-linked through several disulfide bridges. Its amino acid sequence contains a total of 17 disulfide bridges, one free thiol (Cys-34) and a single tryptophan (Try-214). Albumin is known as binding and transporting many ligands, including fatty acids, amino acids, steroids, metal ions, and a variety of pharmaceuticals.[2] It is suggested that the principal regions of ligand binding to HSA are located in hydrophobic cavities in subdomains IIA and IIIA, which are consistent with sites I and II, respectively, and one tryptophan residue of HSA is in subdomain IIA.[1,3] HSA can interact with many endogenous and exogenous substances including many drugs.[4,5] The binding form of the drug and the serum albumin is the storage form for the drug. It is important to study the interaction of the drug with the protein because protein–drug binding plays an important role in pharmacology and pharmacodynamics. The information on the interaction of serum albumin and drug can help us better understand the absorption and distribution of the drug. Extensive investigations into the interaction between the serum albumin and internal compound or pharmaceutical molecule have been made, but the interaction of protein with the main component of herbal medicine has seldom been reported.[6,7] Traditional medicine prescriptions have been used for over 2000 years, and in recent years concentrated dosage form has been widely adopted for clinical use. Isoflavones, in general, are known as biologically active compounds, possessing many properties such as

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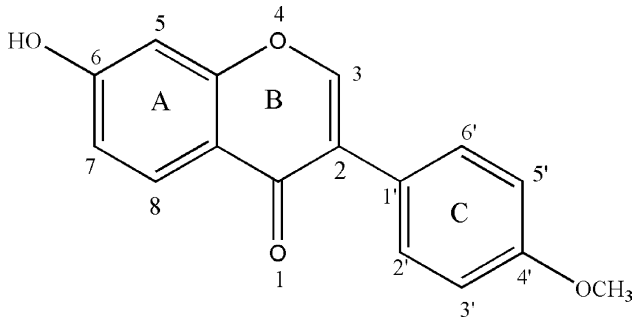

Figure 1. The chemical structure of formononetin.

antioxidant and reducing cardiovascular diseases. Formononetin (Fig. 1), one of the major bioactive components, is a natural estrogenic compound present in large amounts in seeds, fruits, and vegetables. The work is worthy to be done and can benefit understanding the transports and metabolic process for isoflavones in herbal medicine. The knowledge of the structural features that determine the binding capacity of the compound and HSA may open up new avenues for the design of the most suitable isoflavone derivatives with structural variants.

A series of study methods concerning the interaction between drugs and protein are often monitored using optical techniques as these methods are sensitive and relatively easy to use.[8,9] Among these, fluorescence anisotropy is found to be intrinsically high[10] and sensitive[11,12] to relevant environmental changes. The present work was undertaken in an attempt to assess, for the first time, the usefulness of formononetin as an extrinsic optical probe for protein, exploiting its fluorescence anisotropy as a useful parameter. FT-IR, a powerful technique for the study of hydrogen bonding, has recently become very popular for detecting structural characterization of proteins. The most important advantage of FT-IR spectroscopy for biological studies is that spectra of almost any biological system can be obtained in a wide variety of environments.

In this paper, the fluorescence anisotropy (r) parameter dramatically reveals the binding characteristics of formononetin with the protein, and based on fluorescence anisotropy measurements on formononetin, the binding constant for the formononetin–HSA complex has been calculated as $1.6 \times 10^5\ M^{-1}$. We have also estimated the standard free energy change ($\Delta G^0 \approx -29.9$ kJ/mol) for the association reaction. Furthermore, FT-IR difference spectrum technology was used for illustrating the changes of protein secondary structure. The changes of secondary structure were quantitatively analyzed by the curve-fitting method.

2. Results and discussion

2.1. Interaction of formononetin with HSA and binding constant

Fluorescence measurements were carried out to investigate whether formononetin interacts with HSA. Figure 2 shows the fluorescence emission spectra of formononetin in the absence and presence of HSA. Formononetin has a strong fluorescence emission peak at 466 nm on excitation at 336 nm. Gradual addition of HSA to the buffer solution leads to a hypsochromic shift of the emission maximum associated with an enhancement of fluorescence intensity. The observations reflect that the microenvironment around the fluorophore in the protein solution is quite different from that of pure aqueous phase. The changes in the fluorometric behavior of the fluorophore with the addition of HSA in buffer solution can be rationalized in terms of binding of the drug with the protein leading to a less polar microenvironment around the fluorophore. A small increase in the fluorescence yield of formononetin in HSA is also ascribed to a lowering in the polarity in the near vicinity of formononetin. A blueshift in the fluorescence maximum also suggests a reduction in the polarity of the microenvironment.

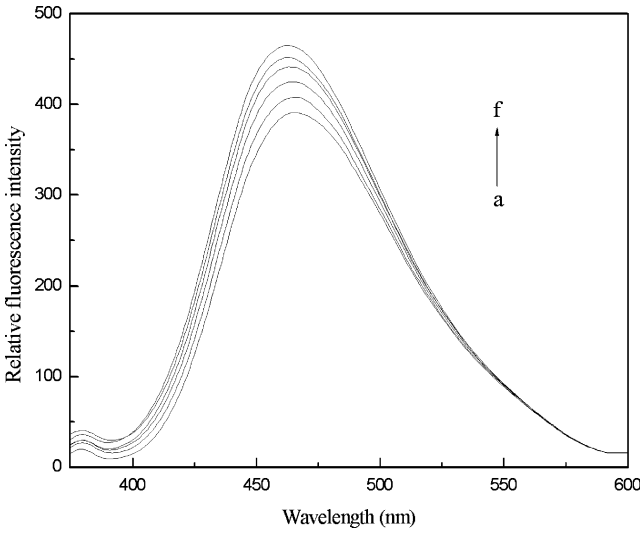

Figure 2. The fluorescence emission spectra of formononetin–HSA system: (a) 5 μM formononetin; (b)–(f) 5 μM formononetin in the presence of 1–5 μM HSA. (λ_{ex} = 336 nm, λ_{em} = 466 nm; pH 7.4; T = 301 K).

To ascertain the binding of formononetin with HSA, we measured the fluorescence anisotropy of formononetin in the presence of HSA. Figure 3 presents the variation of the fluorescence anisotropy value for formononetin with increase in HSA concentration. The plot shows that the anisotropy of formononetin increases from 0.019 ([HSA] = 0 μM) to 0.14 ([HSA] ≈ 15 μM) and gradually levels off after that. The gradual increase in anisotropy of formononetin admission suggests that more and more formononetin molecules bind with HSA, until [HSA] ≈ 15 μM. After the HSA concentration reaches 15 μM, no significant increase in binding of formononetin with HSA takes place with further increase in HSA concentration.

In order to understand the interaction between formononetin and the protein, the binding constant was determined from the fluorescence intensity considering the following equation as developed by Bhattacharya et al.[13,14]

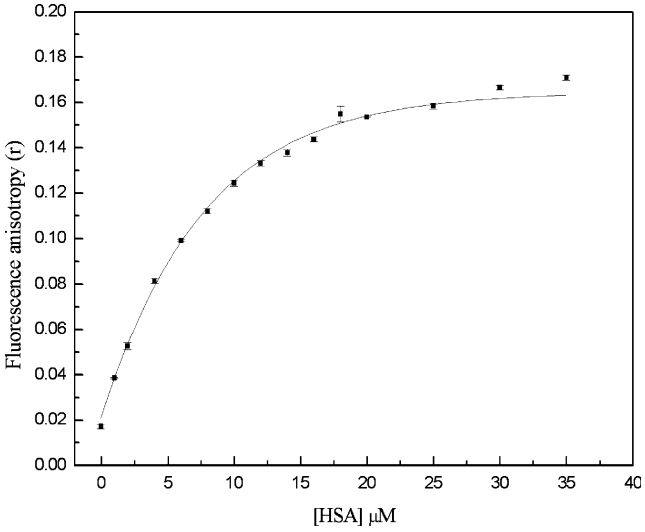

Figure 3. Variation of fluorescence anisotropy (r) of formononetin with HSA concentration. ([Formononetin] = 5 μM; λ_{ex} = 336 nm, λ_{em} = 466 nm; pH 7.4; T = 301 K). Each data point indicates the average of three determinations where each bar indicates the standard deviation.

$$1/\Delta F = 1/\Delta F_{max} + (1/K[L])(1/\Delta F_{max}),$$

where $\Delta F = F_X - F_0$, and $\Delta F_{max} = F_\infty - F_0$ where F_0, F_X and F_∞ are the fluorescence intensities of formononetin in the absence of HSA, at an intermediate concentration of HSA, and at the saturation of interaction, respectively, K being the binding constant and $[L]$ the protein concentration. Rearranging the above equation we have the following form:

$$(F_\infty - F_0)/(F_X - F_0) = 1 + \left(K[L]\right)^{-1}.$$

The linearity in the plot of $(F_\infty - F_0)/(F_X - F_0)$ against $[L]^{-1}$ suggests the existence of only one class of binding sites with a unique binding constant (Fig. 4). The slope of the plot led to the determination of the binding constant between the two and the value obtained was $1.6 \times 10^5\ M^{-1}$. The standard free energy change ($\Delta G^0 = -RT\ln K$) comes out to be approximately −29.9 kJ/mol at 301 K, which indicates the spontaneity of binding of formononetin and HSA.

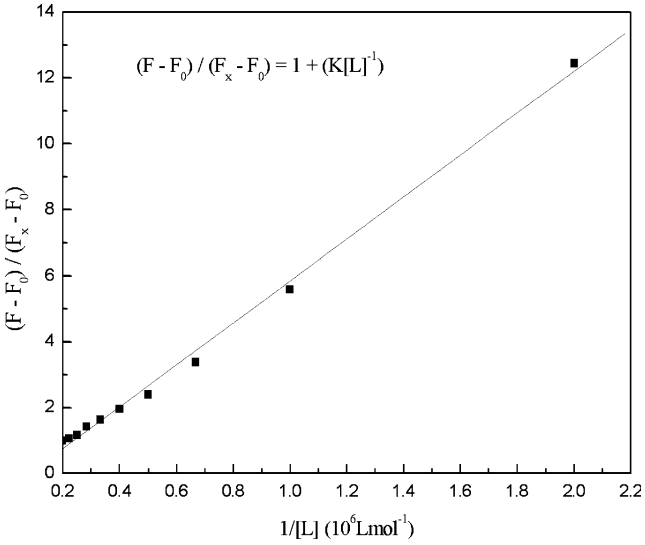

Figure 4. Plot of $(F_\infty - F_0)/(F_X - F_0)$ against $[L]^{-1}$. ([Formononetin] = 5 μM; λ_{ex} = 336 nm, λ_{em} = 466 nm; pH 7.4; T = 301 K).

2.2. FT-IR spectra of formononetin–HSA complexes

Infrared spectroscopy has long been used as a powerful method for investigating the secondary structures of proteins and their dynamics. In the IR region, the frequencies of bands due to the amide I–III vibrations are sensitive to the secondary structure of proteins. Particularly, the amide I band is useful in studying the secondary structure. The amide I peak position occurs in the region 1600–1700 cm^{-1} and the amide II band ≈1548 cm^{-1}. Amide I band is more sensitive to the change of protein secondary structure than amide II.[15,16] Figure 5 shows the FT-IR spectrum of free HSA in Tris–HCl buffer and the difference spectra after binding with formononetin. In general, the spectra range in 1650–1660 cm^{-1} in amide I bands can be attributed to α-helix. As shown in Figure 5, the peak position of amide I moved from 1645 to1639 cm^{-1} in HSA indicating that the secondary structure of HSA has been changed because of the interaction of formononetin with HSA. As the formononetin concentration was increased, the intensities of the amide I band and the amide II decreased further in the spectra of all the formononetin–HSA complexes. The reduction in the intensity of the amide I and II bands is related to the formononetin–HSA interaction.

To compare more meaningfully the structure of the protein in the absence and presence of formononetin, the curve-fitted IR spectra of HSA and its components, assignments and compositions with the increase of drug concentration are illustrated in Figure 6. The secondary structure of a protein can be characterized via IR spectrum and the use of amides I and II frequencies as indicators. Using the amide I band and a sensitive indicator of conformation and structure,[17,18] the present study demonstrated that the native HSA in pH 7.4 buffer solution consisted of 54.4% α-helix, 38.7% β-sheet, and 6.9% β-turn (Table 1). The structural composition of HSA incubation with 30 μM formononetin consisted of 19.5% α-helix, 56.0% β-sheet, and 24.5% β-turn. Once 60 μM formononetin was incubated with HSA, the composition of HSA changed to 39.3% α-helix, 42.3% β-sheet and 18.4% β-turn. The observed spectral changes are consistent with the major perturbations to HSA secondary structure upon formononetin interaction. These data indicate that interaction of formononetin can affect HSA protein α-helical domains.

2.3. Computational modeling of the formononetin–HSA complex

The crystal structure of HSA taken from the Protein Data Bank (entry PDB code 1h9z) was used to find the binding site of formononetin. The best energy ranked result is shown in Figure 7. It can be seen that formononetin is situated within subdomain IIA in Sud-

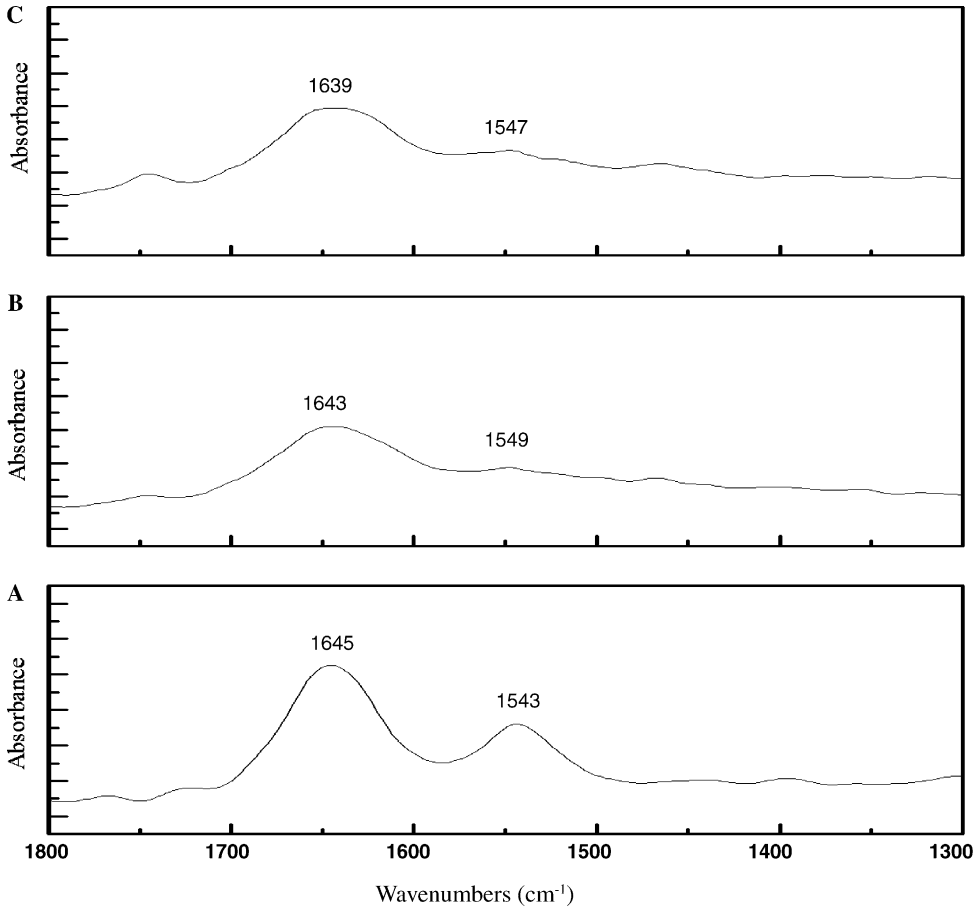

Figure 5. FT-IR spectra and difference spectra of HSA in aqueous solution. (A) FT-IR spectrum of free HSA; (B and C) FT-IR difference spectrum [(HSA solution + formononetin solution) − (formononetin solution)] in buffer solution in the region of 1800–1300 cm^{-1}. Drug to HSA (30 μM) ratios: A 0:1, B 1:1, C 2:1; pH 7.4.

low's site I formed by helices. The C ring locates within the binding pocket but the B ring protrudes from it and points toward the interface of IIA and IIIA subdomains. The A and B rings are practically co-planar, while the C ring is rotated by 60° around the C-bond. The interaction between formononetin and HSA is not exclusively hydrophobic in nature since there are several ionic and polar residues in the proximity of the bound ligand playing important roles in stabilizing the negatively charged formononetin molecule via H-bonds and electrostatic interactions. It is important that the only tryptophan residue of HSA (Try214) is in close proximity to the 4′-CH_3 of formononetin, suggesting the existence of hydrophobic interaction between them. As shown in Figure 7, the atoms of Lie 290, His242, and Arg222 are in suitable positions to form an intermolecular H-bond interaction with the 6-OH, 1-O, and 4-O, respectively, and site I is large enough to accommodate the formononetin molecule.

3. Conclusions

Interaction between the isoflavone formononetin and HSA was investigated by fluorescence anisotropy in combination with Fourier transform infrared spectroscopy and molecular modeling methods. Albumin is the most abundant plasma protein and isoflavone interacts with this macromolecule that crucially determines their bioavailability and toxicology. As a result of specific binding of formononetin to HSA, its fluorescence spectrum is blueshifted. The binding constant is calculated from the fluorescence data. The formononetin binding properties of HSA were also mapped by molecular modeling. Docking calculations found formononetin to be located in site I of HSA within subdomain IIA. Additionally, FT-IR spectroscopy shows that the secondary structure of HSA changed after formononetin bound to HSA. Formononetin induced protein conformational changes with a reduction of α-helices from 54.4% for free HSA to 19.5–39.3% and β-sheet from 38.7% for free HSA to 42.3–56.0%.

4. Experimental

4.1. Materials

Human serum albumin (fatty acid free), purchased from Sino-American Biotechnology Company (China), was used without further purification and its molecular weight was assumed to be 66,500. All HSA solution

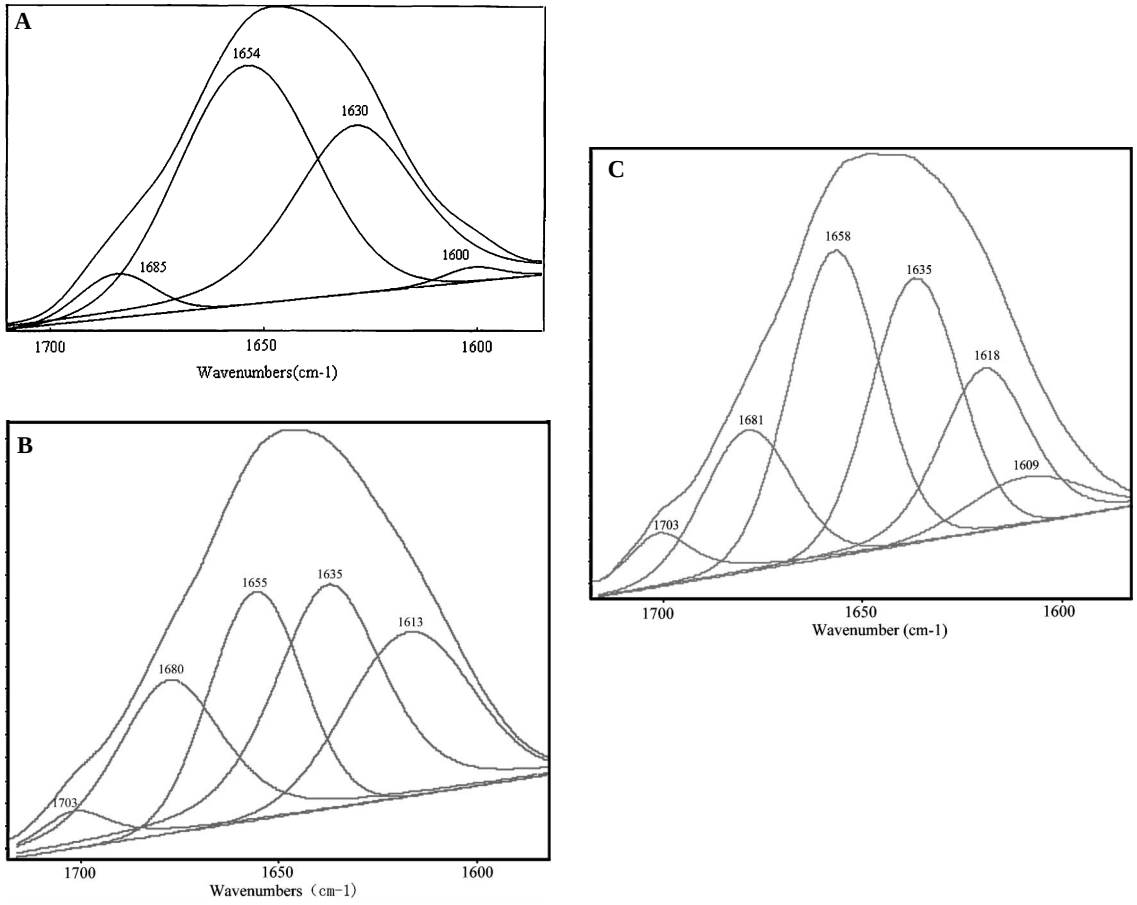

Figure 6. The curve-fit amide I region with secondary structure determination of the formononetin–HSA complexes at different drug to HSA ratios in buffer solution in the region of 1700–1600 cm^{-1}. Drug to HSA (30 μM) ratios: A 0:1, B 1:1, C 2:1; pH 7.4.

Table 1. The effect of formononetin on the components and assignments of the curve-fitted amide I of HSA before and after treatment with different concentrations of formononetin

Structure components	Free HSA, % (cm^{-1})	Formononetin (30 μM), % (cm^{-1})	Formononetin (60 μM), % (cm^{-1})
α-Helix	54.4 (1656)	19.5 (1655)	39.3 (1658)
β-Sheet	38.7 (1616)	56.0 (1635, 1613)	42.3 (1618, 1609)
β-Turn	6.9 (1672, 1694)	24.5 (1680)	18.4 (1681)

was prepared in the pH 7.4 buffer solution and HSA (30 μM) stock solution was kept in the dark at 277 K. Formononetin was of analytical grade and was purchased from the National Institute for Control of Pharmaceutical and Bioproducts (China), and the stock solution was prepared in absolute ethanol (1 mM). Buffer (pH 7.40) consisted of Tris (0.2 M) and HCl (0.1 M), and the pH was adjusted to 7.40. The pH was checked with a suitably standardized pH meter.

4.2. Apparatus and methods

The absorption and steady state fluorescence anisotropy measurements were performed using RF-5301PC spectrofluorophotometer (Shimadzu), the excitation and emission band widths both being 5 nm. Steady-state anisotropy (r) is defined by

$$r = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH}),$$

where I_{VV} and I_{VH} are the intensities obtained with the excitation polarizer oriented vertically and the emission polarizer oriented in vertical and horizontal directions, respectively. The G factor is defined as:

$$G = I_{HV}/I_{HH},$$

where I refers to the similar parameters as mentioned above for the horizontal position of the excitation polarizer.

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 under the same condition. Then, the absorbance of buffer

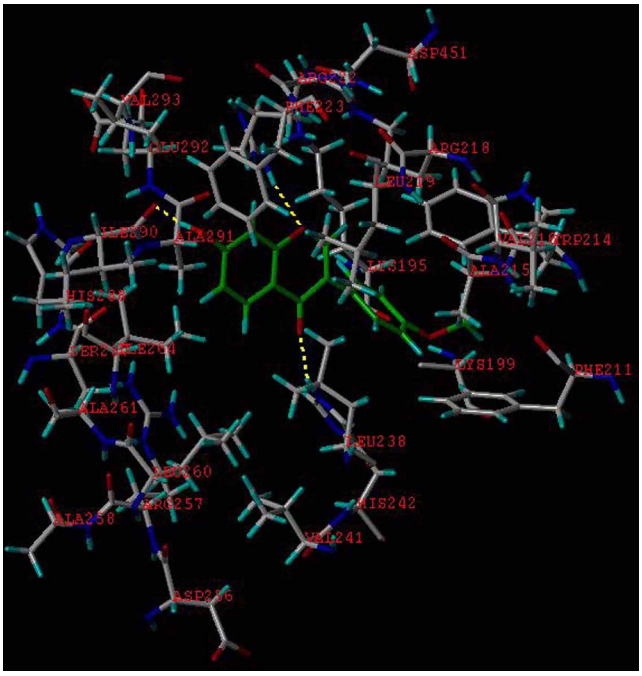

Figure 7. The interaction mode between formononetin and HSA. The residues of HSA are represented using lines and the ligand structure is represented using a ball and stick model. The hydrogen bond between formononetin and HSA is represented using a dashed line.

solution is subtracted from the spectra of sample solution to get the FT-IR spectra of proteins. The subtraction criterion was that the original spectrum of protein solution between 2200 and 1800 cm^{-1} was featureless.[19] Fourier self-deconvolution and secondary derivatives were applied to this range, respectively, to estimate the number, position, and width of component bands. Based on these parameters a curve-fitting process was carried out by Galactic Peak to get the best Gaussian-shaped curves that fit the original protein spectrum. After identification the individual bands with its representative secondary structure of HSA is calculated by the area of their respective component bands.

The crystal structure of HSA in complex with *R*-warfarin was taken from the Brookhaven Protein Data Bank (entry codes 1h9z).[20] The potential of the 3-D structures of HSA was assigned according to the Amber 4.0 force field with Kollman-all-atom charges. The initial structures of all the molecules were generated by molecular modeling software Sybyl 6.9.[21] The geometries of these compounds were subsequently optimized using the Tripos force field with Gasteiger–Marsili charges. FlexX program was used to calculate the interaction modes between the ligands and HSA.

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