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The Interaction of Polysaccharides Isolated from *Auricularia Polytricha* with Human Serum Albumin

Wei Wang* · Guoguang Zhang · Jinmei Zou

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Abstract Polysaccharides have attracted great attention for their wide range of applications in biological and medical fields. In this paper, the interaction of polysaccharides with human serum albumin (HSA) was systematically investigated by fluorescence (FL) spectroscopy and circular dichroism (CD) spectra under different conditions. The Stern-Volmer quenching constants (K_a) at different ionic strength and pH were calculated, and information of the structural features of HSA was discussed. FL and CD results indicate that both hydrophobic and electrostatic interactions play important roles during the binding process. The quenching of the fluorescence resulting the binding of polysaccharides and HSA is static.

Keywords *Auricularia polytricha* · fluorescence · human serum albumin · interaction · polysaccharides

Introduction

Edible mushrooms are known as highly nutritious foodstuffs, and exhibit tonic and medicinal attributes in folk medicine. Recently, more attention has been paid to their therapeutic values, and more researchers are becoming interested in finding new functional compounds in mushrooms. The medicinal characteristics of some mushroom species have been proved, including their antitumor, immunomodulating, antiviral, hypocholesterolemia, and hepato-protective activities (Niemeyer, 2001; Nathanie and Mirkin, 2005; Whitesides, 2005). Many polysaccharides have been isolated from mushrooms, fungi, yeast, algae, lichens, and plants in recent years, and screened for biological research. It has been confirmed that most polysaccharides derived from natural plants are relatively non toxic and cause no significant side effects. These polysaccharides might be effective natural anticancer pharmaceuticals for their few side effects.

Auricularia polytricha, which belongs to the Auriculariaceae family, is widespread in many districts of China. Modern pharmacology research indicates that *Auricularia polytricha* has antioxidant, antitumor, and immunomodulatory activities (Sahin and Burgess, 2003; Byon and Choi, 2006). Polysaccharides are the major pharmacologically active components in *Auricularia polytricha*. *Auricularia polytricha* have showed many interesting biological activities, including anti-tumor and anti-inflammatory effects. Much effect could be attributed to polysaccharides in *Auricularia polytricha*. However, the transportation and delivery of Polysaccharide in biological system has not yet been thoroughly studied.

Human serum albumin (HSA) was selected as the carrier to transport the polysaccharides isolated from *Auricularia polytricha*. As the major soluble protein of the circulatory system, HSA has been used as a model protein for many different biophysical, biochemical and physicochemical studies (Yamada et al., 2003; Kalodimos et al., 2004; Ramachandran et al., 2004). Serum albumins play important roles in the transport of many exogenous and endogenous ligands, binding covalently or reversibly to these ligands and increasing the tumor selectivity of the ligands by enhanced permeation and retention effect (Hansen, 1981; Carter and Ho, 1994; Bordbar et al., 2002; Tanaka et al., 2004). Therefore, the binding of ligands to serum albumin is an important determinant of their distribution and fate in the body (Bhattacharya et al., 2000). Due to its unusual binding properties, HSA is widely applied in many fields.

Based on the research achievement in the past, major interactions involved in protein adsorption are classified as follows: electrostatic, hydrophobic and hydrogen-bonding. In this paper, we studied the interaction between HSA and polysaccharides isolated from *Auricularia polytricha*. The effects of the ionic strength and pH were studied on the interaction of polysaccharides with HSA by changing the concentration of NaCl and pH to make clear the binding mechanism of polysaccharides and HSA. Both fluorescence

W. Wang · G. Zhang · J. Zou
School of Biological Sciences and Biotechnology, Minnan Normal University, Zhangzhou 363000, China

*Corresponding author (W. Wang: wangwei0596@163.com)

spectra and circular dichroism (CD) spectra were used to determine the Stern-Volmer quenching constant and the conformation change of HSA.

Materials and Methods

Materials. Sodium hydroxide (NaOH), Tris (hydroxymethyl) aminomethane, and other routine chemicals were purchased from Shenshi Chem. Ltd. (China) HSA was purchased from Sigma. All the chemicals were of analytical grade and double distilled deionized water was used in all experiments.

Polysaccharides from a crude extract of *Auricularia polytricha* were separated by high-speed countercurrent chromatography (HSCCC) (Xiao et al., 2010). The sample solution contained 2.0 g of the crude P, and 50 mL of each phase of the two-phase solvent system. The separation was performed at 700 rpm at a flow rate of 2.5 mL/min, using the lower phase as the mobile phase. The solvent front of mobile phase emerged in the 46th tube, which indicates the retention of the stationary phase was 42.5%. Fractions 22–50, 60–84, and 92–110 corresponded to the first (P-1), second (P-2), and third (P-3) polysaccharide components, respectively. The structure of polysaccharide (P-2) was shown in scheme 1.

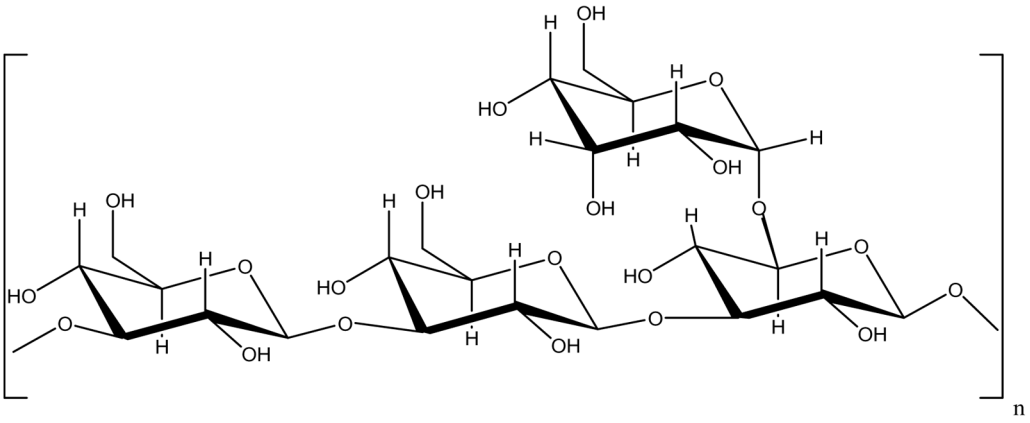
Instrumental. A Hitachi F-4500 spectrofluorophotometer (Hitachi, Japan) was used to record the fluorescence spectra and measure the intensities of fluorescence. The scan underwent in the wavelength range of 285-450 nm upon excitation at 400 nm with slit widths for excitation and emission at 5 nm when HSA samples were titrated with polysaccharides. In each titration, the fluorescence spectrum was collected under the concentration of HSA at 1.0×10^{-6} mol L^{-1}. HSA was dissolved in Tris-HCl buffer solution (0.05 mol L^{-1} Tris, 0.10 mol L^{-1} NaCl, and pH 7.4±0.1). 10 μL 5×10^{-5} mol L^{-1} polysaccharides was added to 2.5 mL HSA and mixed. Different amount (5, 15, and 40 μL) of HCl was added to change pH at room temperature and different amount (5, 10, and 15 μL) of NaCl (0.10 mol L^{-1}) was added to change the ionic strength of the mixture, respectively.

The CD spectra were recorded on Jasco (J-810-150S) automatic recording spectropolarimeter, using a cylindrical cuvette with 0.1 cm path length. Scan speed was set at 500 nm/min and response time was 0.5 s. In order to maintain accuracy, each spectrum was obtained from the average of three successive scans and corrected by adding Tris-HCl buffer solution. Far-CD spectra were recorded from 200 to 260 nm in 0.05 mol L^{-1} Tris-HCl (containing 0.10 mol L^{-1} NaCl, pH 7.4±0.1), at 25°C. Appropriate baseline corrections were made in the spectra and the concentration of HSA was kept at 1.0×10^{5} mol L^{-1}. The solution of polysaccharides (0.4 mL, 5×10^{-5} mol L^{-1}) was added into 2 mL HSA and mixed. Then different amount (5, 15, and 40 μL) of HCl was added to change pH and different amount (5, 10, and 15 μL) of NaCl (0.10 mol L^{-1}) was added to change the ionic strength of the mixture.

Results and Discussions

Fluorescence quenching studies. P-2 was chosen as the model polysaccharide molecule in this study. P-2 showed a single and symmetrically sharp peak, indicating its homogeneity (data not shown). According to the retention time, its molecular weight was estimated to be 2.59×10^{5} Da.

The influences of polysaccharides in the conformational changes of HSA were evaluated by fluorescence methods. The information about fluorophore functional groups was provided by fluorescence measurements. Fluorescence spectra were obtained by simultaneous scanning of excitation and emission monochromators. According to the fluorescence data, we analyzed the binding of polysaccharides to HSA quantitatively and the result was shown in Fig. 1. The analysis of the binding of polysaccharides to HSA in the presence of different amount (pH≈4, pH≈5, pH≈6) of 0.1 mol L^{-1} HCl was carried out by using the fluorescence quenching as shown in Fig. 2. The analysis under different amount (5, 10, and 15 μL) of 0.1 mol L^{-1} NaCl was carried out as shown in Fig. 3. The concentration of HSA was fixed at 1.0×10^{-6} mol L^{-1}. The aqueous solution of HSA (1×10^{-6} mol L^{1-}) was titrated with increasing concentration of polysaccharides (0-2.0×10^{5} mol L^{1}).



Scheme 1 The structure of polysaccharide.

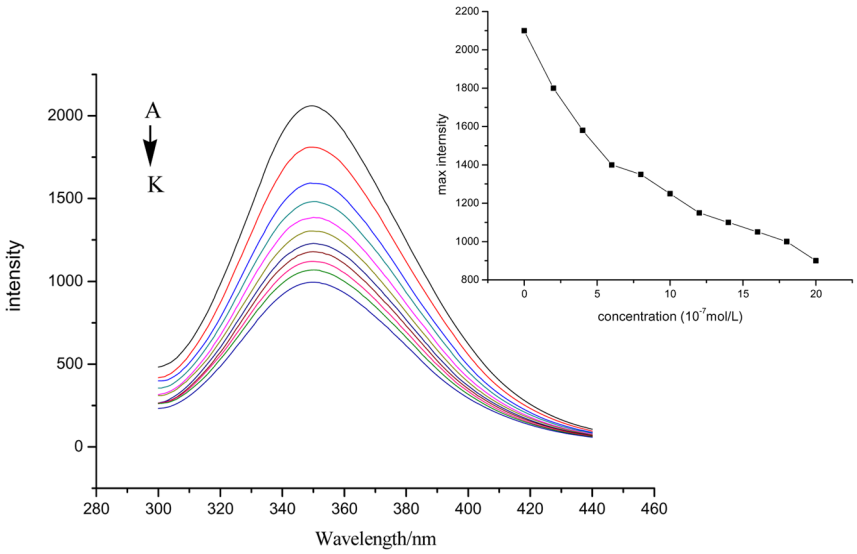


Fig. 1 Fluorescence emission spectra of Polysaccharides-HSA complex in the presence of different concentrations of polysaccharides. The insert graph shows the plot of the max intensity vs concentration. The concentration of HSA was 1.0×10^{-6} mol L^{-1}. Concentrations of Polysaccharides were (from A to K), 0, 2.0×10^{-7} mol L^{-1}, 4.0×10^{-7} mol L^{-1}, 6.0×10^{-7} mol L^{-1}, 8.0×10^{-7} mol L^{-1}, 1.0×10^{-6} mol L^{-1}, 1.2×10^{-6} mol L^{-1}, 1.4×10^{-6} mol L^{-1}, 1.6×10^{-6} mol L^{-1}, 1.8×10^{-6} mol L^{-1}, and 2.0×10^{-5} mol L^{-1}.

In Fig. 1, the fluorescence intensity of HSA was quenched obviously with the concentration of polysaccharides increasing and at the same time, it can be observed that there is a slight blue shift of the maximum emission wavelength (Table 1). In the present of HCl and NaCl, the fluorescence intensity decreased more apparently and regularly in Fig. 2 and Fig. 3 and also accompanied by a slight blue shift (Table 1). The decreasing effect of fluorescence intensity was caused by ground-state complex formation between the fluorophores (HSA) and the quencher (polysaccharide). Without polysaccharides, the fluorescence intensity would not be quenched with addition of HCl or NaCl, although the conformational change of HSA may be occurred.

The blue shift of the maximum emission wavelength indicates the tertiary change of HSA, which results a more hydrophobic environment of the tryptophan residue. The essential reason of the tertiary change probably is that the HSA tends to be a more flexible conformation in favor of the approaching of tryptophan residues to the bulk surface of polysaccharides.

Table 1 The max wavelength in Fig. 1-3. There is a slight blue shift of the maximum emission wavelength

Concentration ($\times10^{-6}$ mol/L)	Max wavelength (nm)	Max wavelength (nm)	Max wavelength (nm)
0	352	352	352
2	351	351	351
4	351	351	351
6	351	350	350
8	350	349	350
10	350	349	349
12	350	348	349
14	350	347	348
16	349	347	347
18	349	346	347
20	348	345	356

Fluorescence quenching is described by the well-known Stern-Volmer equation (Gao et al., 1998):

$$\frac{F_0}{F}=1+k_q\tau_q[Q]=1+K_{SV}[Q] \quad (1)$$

Where F_0 and F are the fluorescence intensities in the absence and presence of quencher, k_q is the bimolecular quenching constant, τ_q is the lifetime of the fluorescence in the absence of quencher, $[Q]$ is the concentration of the quencher, and K_{SV} is the Stern-Volmer quenching constant. Hence the above equation could be used to determining K_{SV} by linear regression of a plot of F_0/F against $[Q]$.

The nature of such fluorescence quenching can be either "static" or "dynamic". According to Lakowicz (2006), for dynamic quenching, the maximum scatter collision quenching constant of various quenchers with the biopolymer is 2.0×10^{-10} L mol^{-1} s^{-1}. Considering that the rate constants of the HSA quenching procedure initiated by polysaccharides were much greater than 2.0×10^{-10} L $mol^{-1}\cdot s^{-1}$ in our experiment, it can be concluded that the nature of quenching was probably static rather than dynamic. The static mechanism resulting in ground-state complex formation between the fluorophores and the quencher while the dynamic quenching was a diffusive encounters collision between the fluorophores and the quencher in the excited-state. In conclusion, the quenching mechanism of the binding reaction between polysaccharides and HSA might be initiated by compound formation but not dynamic collision.

The calculation of K_{SV} from Stern-Volmer equation (Fig. 4A)

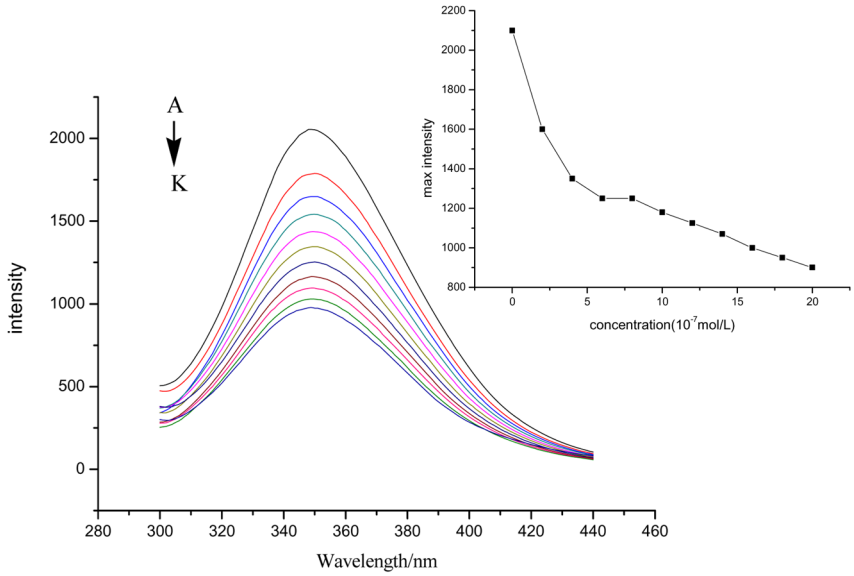


Fig. 2 Fluorescence emission spectra of Polysaccharides-HSA complex when joined 5 μL hydrochloric acid solution (concentrations of HCl was 1.0 mol L^{-1}) in the presence of different concentrations of polysaccharides. The insert graph shows the plot of the max intensity vs concentration. The concentration of HSA was 1.0×10^{-6} mol L^{-1}. Concentrations of polysaccharides were (from A to K), 0, 2.0×10^{-7} mol L^{-1}, 4.0×10^{-7} mol L^{-1}, 6.0×10^{-7} mol L^{-1}, 8.0×10^{-7} mol L^{-1}, 1.0×10^{-6} mol L^{-1}, 1.2×10^{-6} mol L^{-1}, 1.4×10^{-6} mol L^{1}, 1.6×10^{-6} mol L^{1}, 1.8×10^{-6} mol L^{-1}, and 2.0×10^{-5} mol L^{-1}.

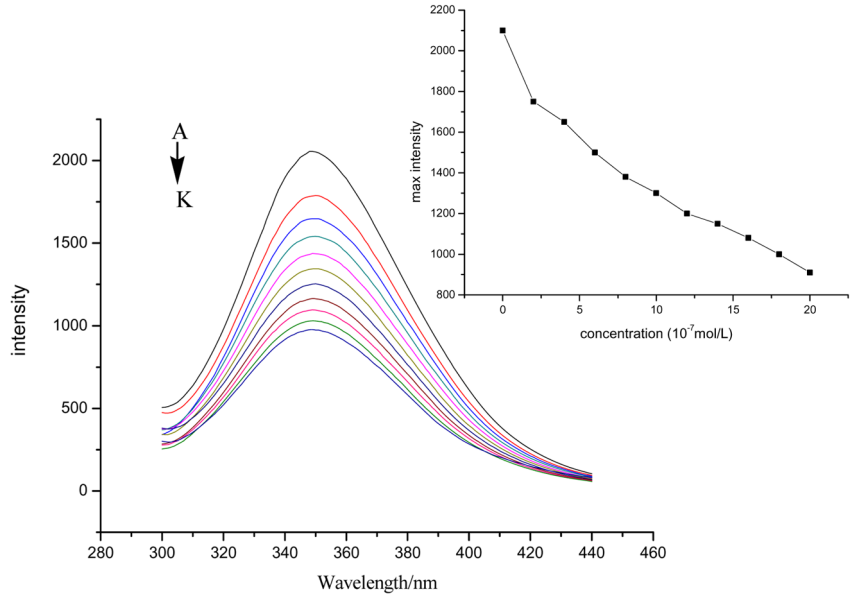


Fig. 3 Fluorescence emission spectra of Polysaccharides-HSA complex when joined 5 μL sodium chloride solution (concentrations of NaCl was 1.0 mol L^{-1}) in the presence of different concentrations of Polysaccharides. The insert graph shows the plot of the max intensity vs. concentration. The concentration of HSA was 1.0×10^{-6} mol L^{-1}. Concentrations of Polysaccharides were (from A to K), 0, 2.0×10^{-7} mol L^{-1}, 4.0×10^{-7} mol L^{-1}, 6.0×10^{-7} mol L^{-1}, 8.0×10^{-7} mol L^{-1}, 1.0×10^{-6} mol L^{-1}, 1.2×10^{-6} mol L^{1}, 1.4×10^{-6} mol L^{1}, 1.6×10^{-6} mol L^{-1}, 1.8×10^{-6} mol L^{-1}, and 2.0×10^{-6} mol L^{-1}.

revealed that HCl took a moderate role in the process of fluorescence quenching. This also proved that the probable quenching mechanism of the Polysaccharides-HSA binding reaction was initiated by compound formation but not dynamic collision.

Therefore, the quenching data were analyzed according to the modified Stern–Volmer equation (Li et al., 2007):

$$\frac{F_0}{\Delta F}=\frac{1}{f_a K_a[Q]}+\frac{1}{f_a} \quad (2)$$

ΔF is the difference in fluorescence in the absence and presence of the quencher at concentration [Q], f_a is the mole fraction of solvent-accessible fluorophores, and K_a is the effective quenching

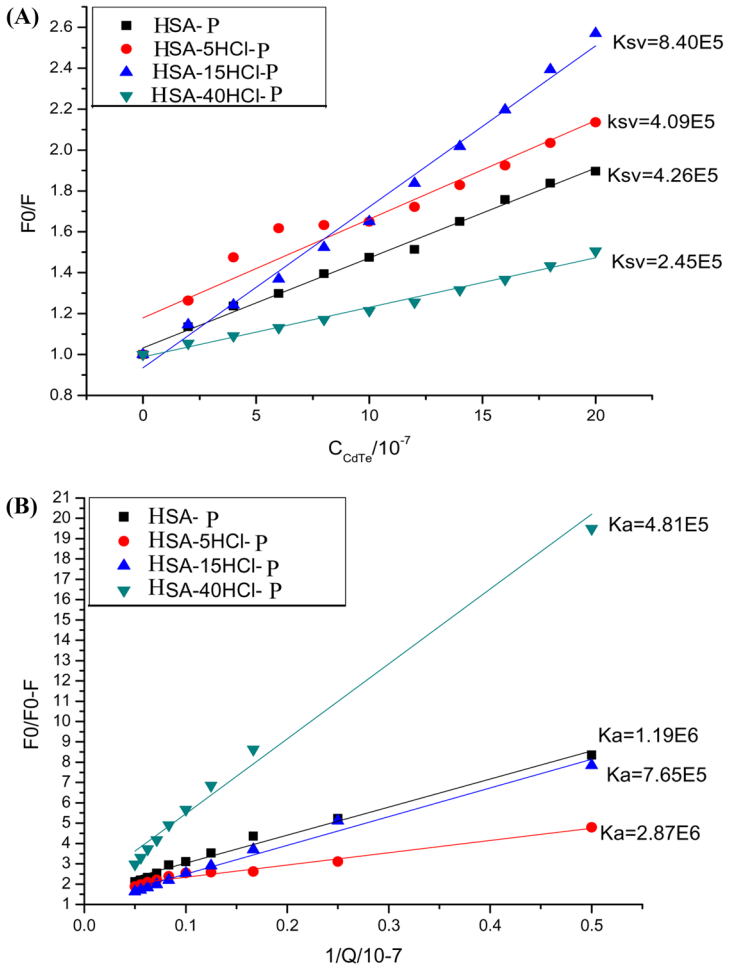


Fig. 4 Stern-Volmer plots (A) and modified Stern-Volmer plots (B) for the quenching of HSA by polysaccharides in the presence of different amount (5, 15, and 40 μL) of HCl. The inset shows the relationship of A) the Stern-Volmer quenching constants K_{SV}; B) modified Stern-Volmer quenching constants K_a.

constant for the accessible fluorophores (Xiao et al., 2008). The dependence of $F_0/\Delta F$ on the reciprocal value of the quencher concentration 1/[*Q*] is linear with the slope equalling the value of $1/(f_a \cdot K_a)$. The value $1/f_a$ is fixed on the ordinate. The constant K_a is the quotient of the ordinate $1/f_a$ and the slope $1/(f_a \cdot K_a)$.

The modified Stern-Volmer plots were shown in Fig. 4B and the corresponding quenching constants in the presence of different amount of HCl was presented. The corresponding quenching constants in the presence of different amount of NaCl were shown in Fig. 5B. Within the scope, the variation of K_a was in accordance with K_{SV}. The values of K_a indicated that electrostatic interaction played a dominate role in the binding reaction. The complex of the polysaccharides and HSA was formed by electrostatic interaction due to negative charge on the polysaccharides and positive charge carried by amino acid (Trp) residues of HSA. We can also draw a conclusion that with the increasing of 1/[*Q*], the fluorescence intensity of HSA reduced correspondingly. The Stern-Volmer quenching constant changed with the addition of HCl or NaCl, indicating that HCl and NaCl changed the binding force of Polysaccharides-HSA complex. HCl and NaCl also changed the structure of polysaccharides. Under low concentration, a slight change of HSA was facilitating to formation of Polysaccharides-HSA complex. Under high concentration, the presence of H^+ or Na^+ weakened the binding force of Polysaccharides-HSA. So the slopes were not proportioned to the concentration of HCl or NaCl.

Circular dichroism studies. To study the possible factor of polysaccharides binding on the secondary structure of HSA, we have performed far-UV CD spectroscopy without polysaccharides and with 1.0×10^{-5} mol L^{-1} polysaccharides under different conditions. Consistent with the literature, the CD spectra of HSA exhibited two negative bands in the UV region at 208 and 222 nm, which was the characteristic peak of á-helical structure of protein. The reasonable explanation may be that the negative peaks near 208 nm and 222 nm both contribute to the n→π* transfer for the bond of the α-helix. The CD results were expressed in terms of mean residue ellipticity (MRE) in mdeg cm^2 d mol^{-1}. According to the following equation:

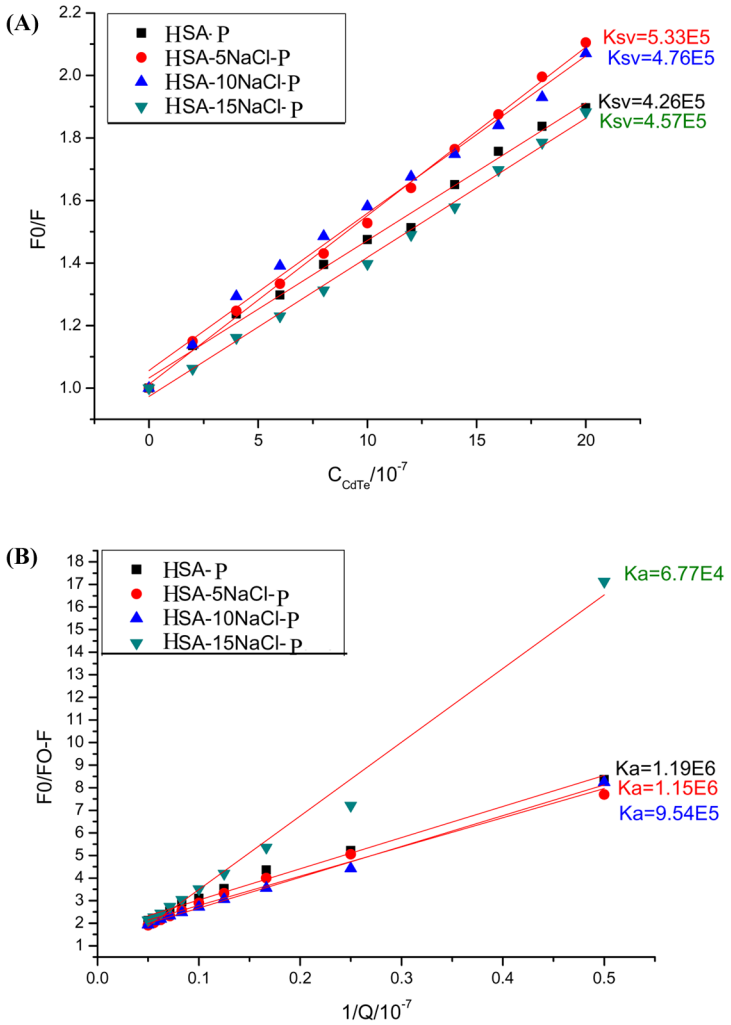


Fig. 5 Stern-Volmer plots (A) and modified Stern-Volmer plots (B) for the quenching of HSA by Polysaccharides in the presence of different amount (5, 10, and 15 μL) of NaCl. The inset shows the relationship of A) the Stern-Volmer quenching constants K_{SV}; B) modified Stern-Volmer quenching constants K_a.

$$\text{MRE} = \frac{\text{Observed CD (mdeg)}}{C_p n l \times 10} \quad (3)$$

where C_p is the molar concentration of the protein, n the number of amino acid residues and l is the path length (0.1 cm). The α-helical contents of free and combined HSA were calculated from MRE values at 208 nm using the equation:

$$\alpha\text{-helix } (\%) = \frac{-\text{MRE}_{208} - 4000}{33000 - 4000} \quad (4)$$

Where MRE_{208} is the observed MRE value at 208 nm, 4000 is the MRE of the β-form and random coil conformation cross at 208 nm and 33000 is the MRE value of a pure α-helix at 208 nm.

Figure 6 presented a CD spectrum for the Polysaccharides-HSA system, and the inset showed the helicity of HSA versus polysaccharides at 208 nm. As can be seen, the helicity of HSA decreased significantly in the presence of polysaccharides, suggesting a stronger structural change that related to a low degree of surface coverage (Wang et al., 2012). The conformation changes here suggest that the conformation state of HSA will be more incompact on the surface of polysaccharides, thus result the exposure of the hydrophobic cavities.

Figure 7 presented a set of representative CD spectra for the Polysaccharides-HSA system in the presence of different amount of HCl. The helicity of HSA increased in the presence of 5 μL HCl, but the helicity of HSA decreased in the presence of 15 μL and 40 μL HCl. Figure 8 presented a set of representative CD spectra for the Polysaccharides-HSA system in the presence of different amount of NaCl. We could see that with the amount of NaCl increased, the helicity of HSA decreased. It is known that the secondary structure contents have an enormous influence on the biological activity of the protein. Therefore, a decrease in α-helical led to a loss of the biological activity of HSA upon the interaction with the polysaccharides.

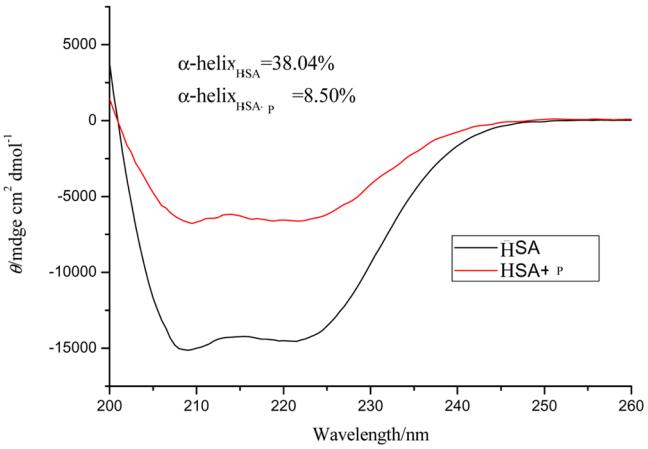


Fig. 6 The far-UV CD spectra of the HSA-Polysaccharides system obtained in 0.05 mol L^{-1} Tris-HCl buffer of pH 7.4±0.1 at room temperature. HSA concentration was kept xed at 1.0×10^{-5} mol L^{-1}, of which 2 mL HSA in the presence alone, and in the presence of 0.4 mL polysaccharides.

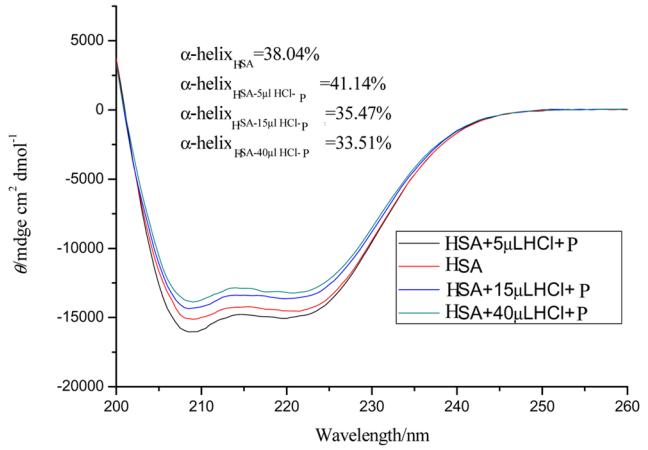


Fig. 7 The far-UV CD spectra of the HSA-Polysaccharides system obtained in 0.05 mol Lv^{1} Tris-HCl buffer at pH 7.4±0.1 at room temperature. HSA concentration was kept xed at 1.0×10^{-5} mol L^{-1}, of which 2 mL HSA in the presence alone, and in the presence of 0.4 mL polysaccharides and different amount (5, 15, and 40 μL) of HCl.

Figs. 7 and 8 suggested that both NaCl and HCl could change the structure of HSA, and the effect of HCl was more significant. We can get a conclusion that the binding of polysaccharides to HSA is the combined effects of electrostatic interactions and H bond. In the experiment, a tendency of decrease in α-helical was confirmed. As to Polysaccharides-HSA complex, HCl and NaCl not only change the structure of HSA, but also polysaccharides. In the process of interaction, polysaccharide would compete for H bond and charge in an instant due to polar group. So the change in α-helical was not proportioned to the concentration of HCl and NaCl in Figs. 7 and 8.

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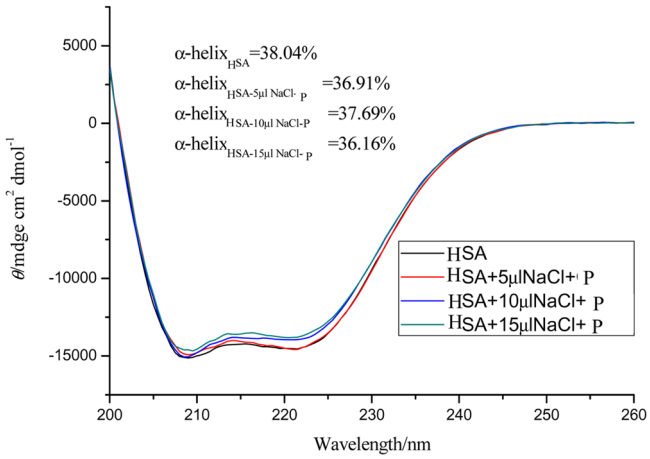

Fig. 8 The far-UV CD spectra of the HSA-Polysaccharides system obtained in 0.05 mol L^{-1} Tris-HCl buffer of pH 7.4±0.1 at room temperature. HSA concentration was xed at 1.0×10^{-5} mol L^{-1}, of which 2 mL HSA in the presence alone, and in the presence of 0.4 mL polysaccharides and different amount (5, 10, and 15 μL) of NaCl.

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