INVESTIGATION OF THE EFFECTS OF TEMPERATURE AND IONS ON THE INTERACTION BETWEEN ECG AND BSA BY THE FLUORESCENCE QUENCHING METHOD

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Abstract - The effects of temperature and common ions on binding (-)-epicatechin gallate (ECG) to bovine serum albumin (BSA) are investigated. The binding constants (K_a) between ECG and BSA are 1.20×10^6 (17 °C), 1.38×10^6 (27 °C), and 5.69×10^6 L mol^{-1} (37 °C), and the number of binding sites (n) were 1.14, 1.15, and 1.26, respectively. These results showed that the increasing temperature improves the stability of the ECG-BSA system, which results in a higher binding constant and the number of binding sites of the ECG-BSA system. The presence of Co^{2+} and Zn^{2+} ions decreased the binding constants (K_a) and the number of binding sites (n) of ECG-BSA complex. However, the presence of Cu^{2+} and Ni^{2+} increased the affinity of ECG for BSA largely. The positive ΔH and positive ΔS indicated that hydrophobic forces might play a major role in the binding between ECG and BSA.

Key words: Bovine serum albumin, interaction, ECG, temperature, ion

INTRODUCTION

The interaction between biomacromolecules and drugs has attracted great interest among researchers for several decades (Xiao et al., 2008; Sirk et al., 2008). Among biomacromolecules, the serum albumins are the major soluble protein constituent of the circulatory system; they have many physiological functions (Xiao et al., 2007, 2008). Bovine serum albumin (BSA) has been one of the most extensively studied proteins, particularly because of its structural homology with human serum albumin (HSA) (Wang et al., 2007). The interaction between protein and drug molecules results in the formation of a stable protein-drug complex, which may be considered as a model for gaining general fundamental insights into drug-protein binding Xiao et al., 2008 a, b).

Flavan-3-ols, more commonly known as catechins, are a group of polyphenols. These compounds are ubiquitously found in green tea, which mainly contains (-)-epicatechin (EC), (-)-epicatechin gal-late (ECG, Fig. 1), (-)-epigallocatechin (EGC), and (-)-epigallocatechin gallate (EGCG) (Hu et al., 2009; Soares et al., 2007). ECG is a polyphenol similar to EGCG that is abundant in green tea. Numerous workers have proposed that EGCG has antioxidant (Hu et al., 2009) and anticancer (Cho et al., 2007) properties and that it can protect epidermal cells against UVB-induced damage (Huang et al., 2007). Serum albumin facilitates the disposition and transport of a variety of ligands. Thus, it is important to study the interaction of the drugs with albumins, especially its effect on drug pharmacokinetics. To gain some insights into the interaction of ECG and BSA, the effects of temperature and ions were investigated.

MATERIALS AND METHODS

Apparatus

Fluorescence spectra were obtained using a *Hitachi* F-4500 spectrofluorometer. A quartz cell of 1.00 cm was used for the measurements. The pH measure-
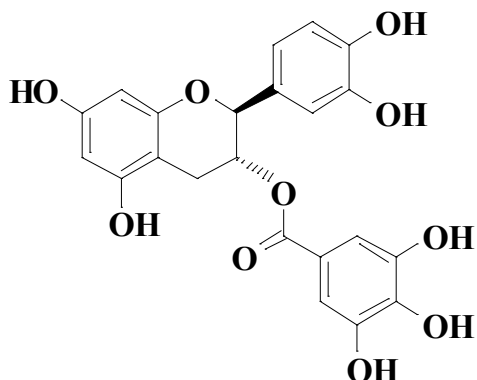
Fig. 1. Structure of ECG.

ments were carried out on a PHS-3C Exact Digital pH meter (Leici, Shanghai), which was calibrated with standard pH buffer solutions.

Reagents

BSA (fraction V) and Tris-base were purchased from Beijing Ding Guo Biotechnology Limited Liability Company (Beijing, China). ECG (98%) was obtained commercially from the BIOSZUNE Co. (Beijing, China). The stock solution of ECG (1.0×10^{-4} mol L^{-1}) was prepared by dissolving flavone with double-distilled water and it was stored in a refrigerator prior to use (it is stable for one month.). *Tris*-HCl buffer (0.05 mol L^{-1}, pH 7.4) containing 0.10 mol L^{-1} NaCl was selected to maintain the pH value and ionic strength of the solution. The working solution of BSA (1.0×10^{-5} M) was prepared by dissolving the BSA in *Tris*-HCl buffer, and was stored in a refrigerator prior to use. All other reagents and solvents were of analytical reagent grade and used without further purification. All aqueous solutions were prepared using freshly double-distilled water.

Fluorescence spectra

Appropriate quantities of 1.0×10^{-4} mol L^{-1} ECG solution were transferred to a 10 mL volumetric flask. One mL of BSA solution was added and diluted to 10 mL with *Tris*-HCl buffer. The resultant mixture was subsequently incubated at 17°C, 27°C, and 37°C for 30 min. The solution was scanned in a fluoropho-

tometer with the range of 290-500 nm. The widths of both the excitation slit and emission slit were set at 5.0 nm. The fluorescence intensity at 340 nm was determined under the excitation at the wavelength of 280 nm.

Fluorescence quenching was described by the Stern-Volmer equation (Lakowicz):

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

where F_0 and F represent the fluorescence intensities of fluorophore in the absence and in the presence of flavone; K_q is the quenching rate constant of the bimolecular; K_{SV} is the dynamic quenching constant, τ_0 is the average lifetime of the fluorophore without ECG, and $[Q]$ is the concentration of ECG.

The relationship between the intensity of fluorescence quenching and the concentration of ECG can be described by the binding constant formula (Lakowicz, 1999)

$$\lg(F_0 - F)/F = \lg K_a + n \lg[Q] \qquad (2)$$

where F_0 and F represent the fluorescence intensities in the absence and in the presence of flavonoid, and $[Q]$ is the concentration of ECG. K_a is the binding constant, and n is the number of binding sites per BSA. After the fluorescence quenching intensities on BSA at 340 nm were measured, the double-logarithm algorithm was assessed by Eq. (2).

RESULTS AND DISCUSSION

Characteristics of the fluorescence spectra

The interaction of ECG with BSA was evaluated by monitoring the intrinsic fluorescence intensity changes of the BSA upon the addition of ECG. The fluorescence quenching spectra of the BSA in the presence of various concentrations of ECG at different temperatures are shown in Fig. 2. Under the experimental conditions, the fluorescence spectrum of ECG was absent. As illustrated in Fig. 2, the addition of ECG led to a concentration-dependent quench-
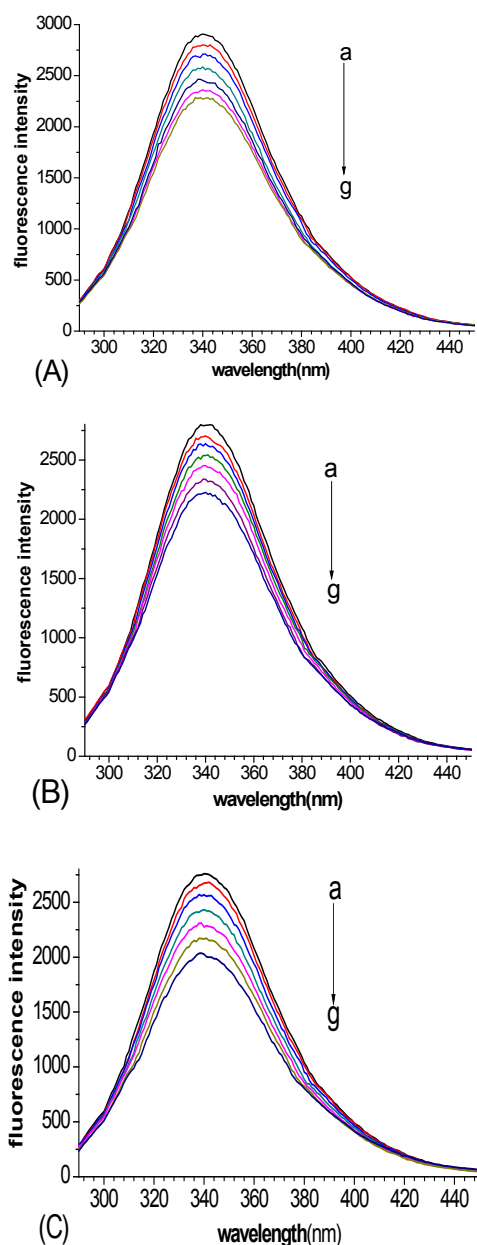
Fig. 2. The quenching effect of ECG on BSA fluorescence at 17°C (A), 27°C (B), and 37°C (C). λ_{ex} =280 nm, BSA, 1.00×10^{-6} mol L^{-1}; 0.00, 0.25, 0.50, 0.75, 1.00, 1.25, 1.50×10^{-6} mol L^{-1} of ECG (a-g).

ing of the BSA intrinsic fluorescence intensity. These results suggested that there was a change in the immediate environment of the tryptophan and tyrosine residues of the BSA (Xiao et al., 2010; Cao and Liu,

2009), and that ECG was situated at close proximity to the tryptophan and tyrosine residues for the quenching to occur. The fluorescence of BSA is primarily from two tryptophan and eighteen tyrosine residues. When the excitation wavelength is 280 nm, the fluorescence emission shows the characteristic of tyrosine and tryptophan residues. In the present study, the information about other amino acid residues was not understood. This means that the molecular conformation of the protein was affected, which is in agreement to recent studies that have shown that the tertiary structure of proteins changes upon binding to polyphenols (Xiao et al., 2010; Wei et al., 2010). The fluorescence of ECG with the excitation wavelength of 280 nm did not affect the fluorescence spectra of the BSA (data not shown).

Quenching constants

To clarify the fluorescence quenching mechanism of BSA by ECG, it was first assumed that the interaction occurred in a dynamic way. The temperature-dependent fluorescence quenching of BSA by ECG was then carried out. Fig. 3 shows the Stern-Volmer plots for the BSA fluorescence quenching by ECG. According to Eq. (1), the corresponding dynamic quenching constants (K_{SV}) for the interaction between ECG and BSA were 2.0×10^5 (17°C, R = 0.9986), 1.75×10^5 (27°C, R = 0.9992), and 1.63×10^5 L mol^{-1} (37°C, R = 0.9974), respectively. Because the fluorescence life time of the biopolymer is about 10^{-8} s (Förster, 1965), the quenching constants K_q at 17°C, 27°C and 37°C were calculated to be 2.00×10^{13}, 1.75×10^{13} and 1.63×10^{13} L mol^{-1} s^{-1}, respectively. According to the literature (Xiao et al., 2008 a, b), 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}, and the K_{SV} increases with increasing temperature. Considering that in our experiment the rate constant of the BSA quenching procedure initiated by ECG was much greater than 2.0×10^{10} L mol^{-1} s^{-1} and that the K_{SV} decreased with increasing temperature, it can be concluded that the quenching was not initiated by dynamic quenching, but probably by static quenching resulting in the formation of a ECG-BSA complex.
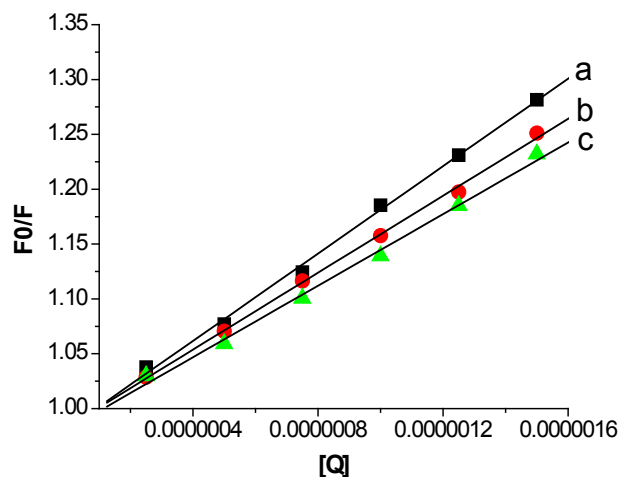
Fig. 3. The Stern-Volmer curves of fluorescence quenching of BSA by ECG at different temperatures (a, 17°C; b, 27°C; c, 37°C).

Fig. 4. Double-log plots of ECG quenching BSA fluorescence at different temperatures (a, 17°C; b, 27°C; c, 37°C).

Binding constant and number of binding sites

Fig. 4 shows the double-logarithm curves of ECG quenching BSA and HSA fluorescence at different temperatures, and Table 1 gives the corresponding calculated results. The apparent binding constants (K_a) between ECG and BSA were 1.20×10^6 (17°C, R=0.9978), 1.38×10^6 (27°C, R=0.9995), and 5.69×10^6 L mol^{-1} (37°C, R=0.9988), and the binding sites values (n) were 1.14, 1.15, and 1.26, respectively. The correlation coefficients are larger than 0.990, indicating that the interaction between ECG and BSA agrees well with the site-binding model underlying Eq. (2). The data clearly shows that there was one binding site on the BSA for ECG independent of temperatures from 17°C to 37°C. The binding constant increased with the increasing temperature in the range of 17-37°C. The temperature may affect the diffusion coefficient and stability of the ECG-BSA system. The increasing temperature may result in the increasing diffusion coefficient, and also it leads to the lower stability of the ECG-BSA system. The competition of the diffusion coefficient and stability of the ECG-BSA system with increased temperature may induce the above results.
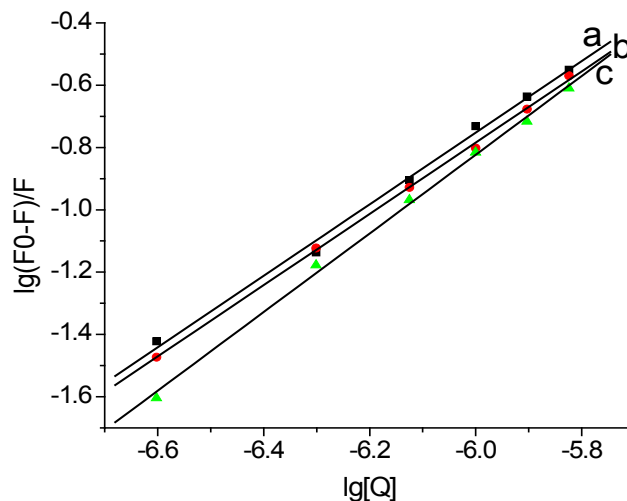
Table 1. The binding parameters of ECG-BSA system

| Temp./°C	K_a/L mol^{-1}	n	R
17 °C	1.20×10^6	1.14	0.9978
27 °C	1.38×10^6	1.15	0.9995
37 °C	5.69×10^6	1.26	0.9988

*Effects of common ions on the affinity
of ECG for BSA*

Metal ions, especially bivalent, are vital to the human body and play essential structural roles in many proteins as a result of coordinate bonding. The presence of metal ions in the plasma can affect the interaction of drugs with plasma proteins. The effects of common bivalent metal ions (e.g. Co^{2+}, Zn^{2+}, Cu^{2+}, and Ni^{2+}) on the affinity of ECG for BSA were investigated at 37°C. The four metal ions (1.0×10^{-4} M) can decrease the fluorescence intensity of the ECG-BSA systems. This possibly resulted in the formation of metal ion-BSA complexes, which also quench BSA fluorescence. The fluorescence quenching spectra of the ECG-BSA system in the presence of metal ions are shown in Fig. 5. Fig. 6 shows the double-logarithm curves and the Stem-Volmer curves in the

presence of Co^{2+}, Zn^{2+}, Cu^{2+}, and Ni^{2+}. The values of the binding constant (K_a) and the number of binding sites (n) acquired in the present of metal ions are listed in Table 2.
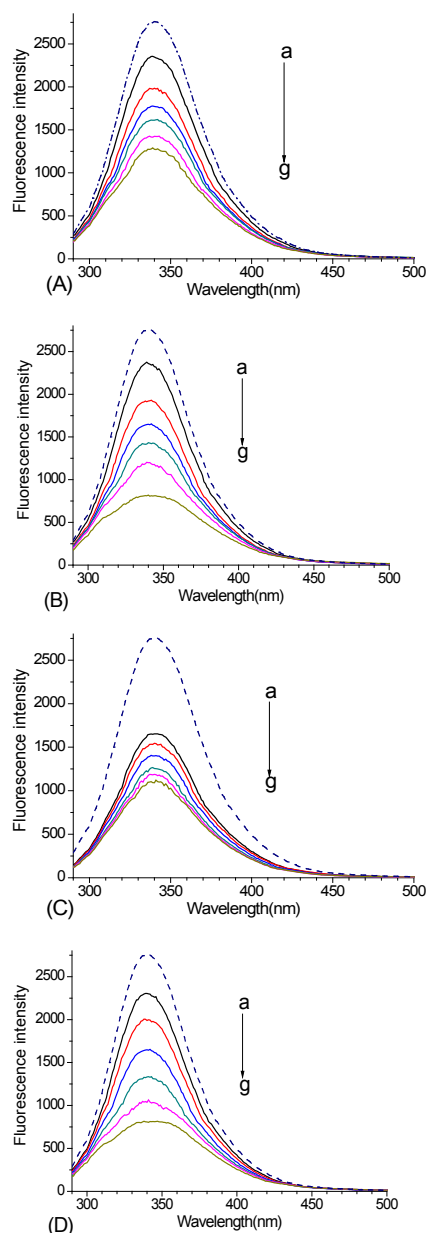
Fig. 5. The fluorescence spectra of ECG-BSA-ion systems. λ_{ex} =280 nm, (a) BSA,1.00×10^{-6} mol L^{-1}, (b-g) BSA,1.00×10^{-6} mol L^{-1}, and Co^{2+}(A), Zn^{2+}(B), Cu^{2+}(C), and Ni^{2+}(D) 1.0×10^{-4} mol L^{-1} with 0.00, 0.25, 0.50, 0.75, 1.00, 1.25, 1.50×10^{-6} mol L^{-1} of ECG (37°C).
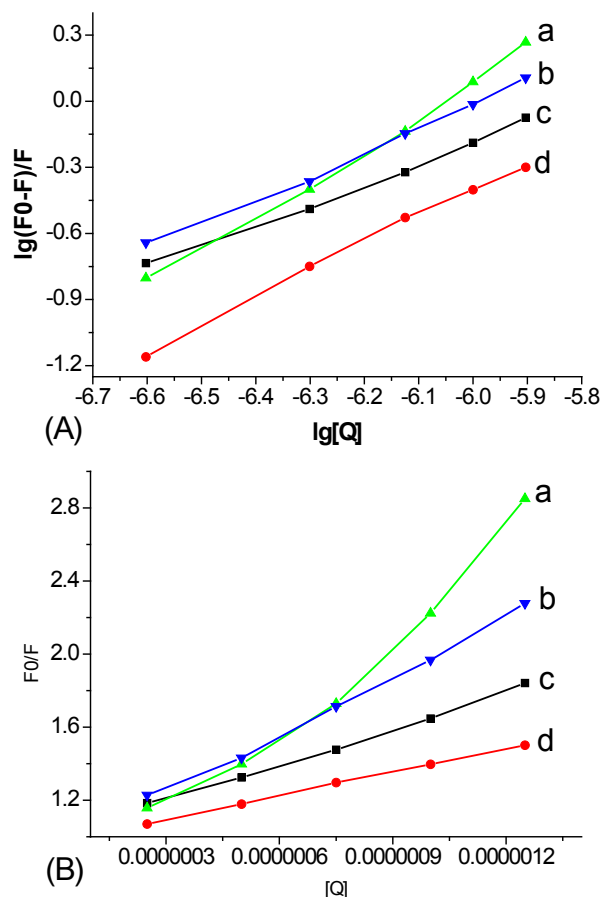
Fig. 6. Double-log plots (A) and Stem-Volmer curves (B) of the ECG quenching effect on BSA in presence of Ni^{2+} (a), Zn^{2+} (b), Co^{2+} (c), and Cu^{2+} (d) (37°C).

It can be seen from Table 2 that the presence of Cu^{2+} and Ni^{2+} increased the affinities of ECG for BSA, whereas the affinities of ECG for BSA decreased in the presence of Co^{2+} and Zn^{2+}. The higher binding constant possibly results from the formation of metal ion-ECG complexes via a metal ion bridge (Liu et al., 2005). This may prolong the storage period of ECG in the blood plasma and enhance its maximum effects. On the contrary, the decrease in the binding constant may be due to the formation of metal ion-BSA complexes. The formation of metal ion-albumin complexes is likely to affect the conformation of protein, which may influence ECG binding kinetics and even inhibit ECG-BSA binding.

Table 2. The binding parameters of ECG-BSA complex in the presence of metal ions at 37°C.

	Ka (mol L^{-1})	n	R
ECG-BSA	5.69×10^6	1.2630	0.9988
ECG-BSA (Cu^{2+})	1.02×10^7	1.3345	0.99803
ECG-BSA (Co^{2+})	2.75×10^5	0.9478	0.99745
ECG-BSA (Zn^{2+})	2.71×10^6	1.0781	0.9981
ECG-BSA (Ni^{2+})	1.60×10^9	1.5296	0.99732

Thermodynamic parameters and nature of the binding forces

The interaction between a drug and a biomolecule may involve hydrophobic forces, electrostatic interactions, van der Waals interactions, hydrogen bonds, etc. Thermodynamic parameters, free energy changes (ΔG), enthalpy changes (ΔH) and entropy changes (ΔS) of interactions are essential to interpret the binding mode. In order to elucidate the interaction of ECG with BSA, we calculated the thermodynamic parameters from Eqs. (3) - (5). If the temperature does not vary significantly, the enthalpy change (ΔH) can be regarded as a constant. The free energy change (ΔG) can be estimated from the following equation, based on the binding constants at different temperatures:

$$\Delta G = -RT\ln K_a \qquad (3)$$

where R is the gas constant, T is the experimental temperature and K_a is the binding constant at corresponding T. Then the enthalpy change (ΔH) and entropy change (ΔS) can be calculated from the following equations:

$$\ln \frac{K_2}{K_1} = \left[\frac{1}{T_1} - \frac{1}{T_2}\right]\frac{\Delta H}{R} \qquad (4)$$

where K_1 and K_2 are the binding constants at the experiment temperatures T_1 and T_2, respectively.

$$\Delta G = \Delta H - T\Delta S \qquad (5)$$

The thermodynamic parameters for the interaction of ECG with BSA are shown in Table 3. The negative sign for ΔG means that the interaction process is spontaneous. The positive ΔH and positive ΔS indicate that hydrophobic forces may play a major role in the binding between ECG and BSA (Ross and Subramanian, 1981).

Table 3. The thermodynamic parameters of the ECG-BSA binding procedure.

T (K)	ΔH (kJ mol^{-1})	ΔG (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})
290	10.13	-33.75	151.31
300	-	-35.26	151.30
310	-	-40.09	162.00

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