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Fluorescence Spectral Study on the Interaction Between Brilliant Blue and Trypsin

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The interaction between brilliant blue and trypsin was studied by using fluorescent technique. The fluorescence quenching data were analyzed according to Stern-Volmer equation and Line weaver-Burk equation. The binding constants, thermodynamic parameters and binding sites were obtained. According to the thermodynamic parameters, the main binding force between brilliant blue and trypsin was ascertained as electrostatic interaction. The binding distance between brilliant blue and trypsin was also given based on the Förster energy transfer theory.

Keywords: Brilliant blue, Trypsin, Fluorescence spectrum, Thermodynamic parameters.

INTRODUCTION

Food color comprises both natural and synthetic colorants. Synthetic colorants are a very important class of food additives. They are widely used to compensate for the loss of natural color of food and to provide the desired colored appearance[1]. However, some of these colorants pose a potential risk to human health, especially if they are consumed in excess. Thus, each synthetic food colorant has been evaluated by the Food and Agricultural Organization (FAO) and World Health Organization (WHO)[2].

Trypsin is a serine protease found in the digestive system, where it breaks down proteins. Trypsin predominantly cleaves peptide chains at the carboxyl side of the amino acids lysine and arginine[3]. As a blue synthetic colorant, brilliant blue (BB) is often used in ice cream, dairy products, sweets and drinks. It is also used in soaps, shampoos and other hygiene and cosmetics applications.

The molecular structure was shown in Fig. 1. Since synthetic colorants were consumed by human beings, the study on the interaction between synthetic colorants and trypsin would be of biological significance in pharmacology and clinical medicine.

In this work, we reported our studies on the interaction of brilliant blue with trypsin using UV-visible spectrophotometry and fluorescence spectrometry. The binding parameters had been evaluated by fluorescence quenching methods. The quenching mechanism, binding constants, binding sites and binding mode were also investigated.

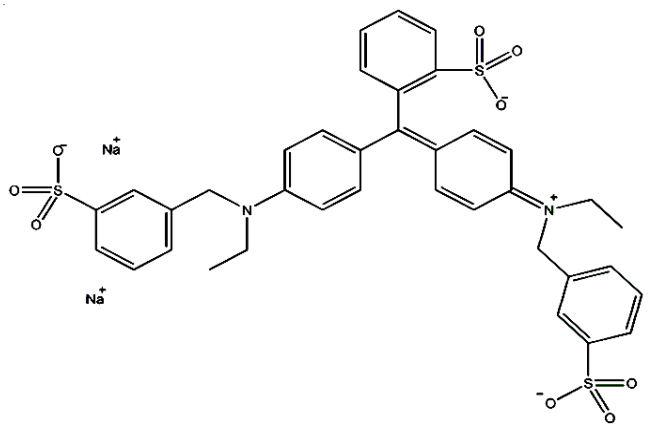

Fig. 1. Molecular structure of brilliant blue

EXPERIMENTAL

Trypsin (Shanghai Wenming Biochemical Technology Co., Ltd.) was dissolved in deionized water to prepare stock solution of 2.0×10^{-4} mol/L, which was stored at 0-4 °C. Brilliant blue (Sinopharm Group Chemical Reagents Co., Ltd.) was dissolved in water to prepare stock solution of 1.0×10^{-3} mol/L. Britton-Robinson (B-R) buffer solutions were prepared by mixing the mixed acid (composed of 0.04 mol L^{-1} H_3PO_4, CH_3COOH and H_3BO_3) with 0.2 mol L^{-1} NaOH in proportion. The buffers were used to control the acidity of the interacting system. All other reagents were of analytical-reagent grade and were used without further purification.

Apparatus and method: 1 mL of 2.0×10^{-4} mol/L trypsin, a known volume of brilliant blue solution and 1 mL of B-R buffer were added to a 10 mL calibrated tubes. Then the solution was diluted to 10 mL with deionized water and mixed well. After reaction for 7 min, the solutions were taken into the optical cell. The absorption spectra were recorded on a TU1901 UV-visible Spectrophotometer (P-General, Beijing, China). The absorbances were measured at 300-450 nm. An F-4500 fluorescence spectrometer (Hitachi, Japan) was applied to record the fluorescencespectra at 300-400 nm. The pH values were measured with a PHS-23 meter (Shanghai Secondly Analytical Instruments, China).

RESULTS AND DISCUSSION

Fluorescence quenching: The fluorescence intensity of a compound could be decreased by a variety of molecular interactions, including excited-state reactions, molecular rearrangements, energy transfer, ground state complex formation and collisional quenching[4]. Such decrease in fluorescence intensity was called quenching. In order to investigate the binding of brilliant blue to trypsin, the fluorescence emission spectra were recorded in the range of 300-400 nm with excitation wavelength of 278 nm. Fig. 2 showed the fluorescence emission spectra of trypsin in the presence of various concentrations of brilliant blue. Under the same condition, no fluorescence (curve 9) of brilliant blue itself was observed. When different amounts of brilliant blue were added into a fixed concentration of trypsin, the fluorescence intensity of trypsin decreased regularly (curves 1-8) with no shift of the emission wavelength, which suggesting that brilliant blue could interact with trypsin and quench its intrinsic fluorescence[5].

In order to clarify the fluorescence quenching mechanism induced by trypsin, the Stern-Volmer equation (eqn. 1)[6] was utilized to process the data.

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

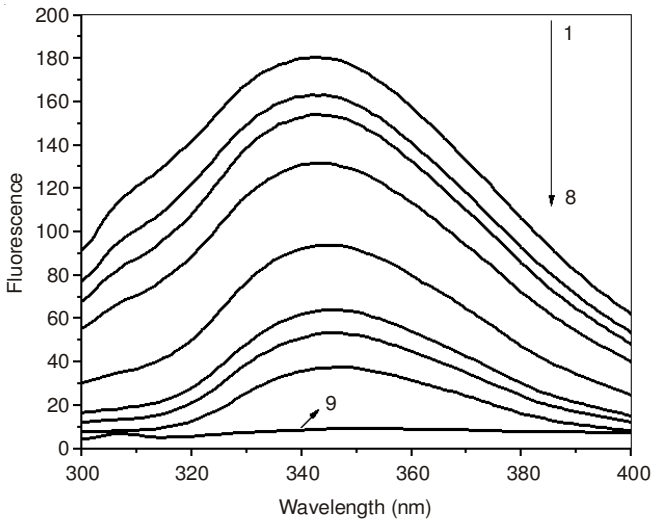

Fig. 2. Fluorescence emission spectra of the trypsin-brilliant blue system. (1-8) 4.0×10^{-5} mol L^{-1} trypsin in the presence of 0.0, 1.0, 2.0, 3.0, 6.0, 9.0, 12.0, 15.0×10^{-5} mol L^{-1} brilliant blue, (9) 6.0×10^{-5} mol L^{-1} brilliant blue, λ_{ex} = 278 nm, T = 298 K, pH = 7.24

where F_0 and F represented the fluorescence intensities of trypsin in the absence and presence of the quencher (brilliant blue). [Q] was the concentration of the quencher and K_{SV} was the Stern-Volmer quenching constant, which was equal to $K_q\tau_0$. K_q was the quenching rate constant of the biomolecule macromolecule and $k_q = K_{SV}/\tau_0$. τ_0 was the average lifetime of the biomolecule without any quencher and the fluorescence lifetime of the biopolymer is10^{-8} s[7]. For dynamic quenching, the maximum scattering collision quenching constant of various quenchers[8] was 2×10^{10} L mol^{-1} s^{-1}. Fig. 3 displayed the Stern-Volmer plots of the fluorescence quenching of trypsin by brilliant blue. The plot showed that within the investigated concentrations, the results agreed with the Stern-Volmer eqn. 1. Table-1 showed the calculated K_{SV} and k_q at each temperature. The results showed that the values of Stern-Volmer quenching constants K_{SV} decreased with increasing temperature and the values of k_q were much greater than 2×10^{10} L mol^{-1} s^{-1}, which indicated that the probable quenching mechanism was not initiated by dynamic collision but compound formation[8]. In other words, the fluorescence quenching of trypsin resulted from complex formation was predominant, while from dynamic collision could be negligible.

Binding constant: In a static quenching process, small molecules would be independently bound to a set of equivalent sites on a macromolecule. Thus, an equilibrium between free and bound molecules was given by reference[9]. When small molecules were bound independently to a set of equivalent sites on a macromolecule, the equilibrium between free and bound molecules was given by eqn. 2[10]:

$$\log \frac{(F_0 - F)}{F} = \log K_b + n \log[Q] \quad (2)$$

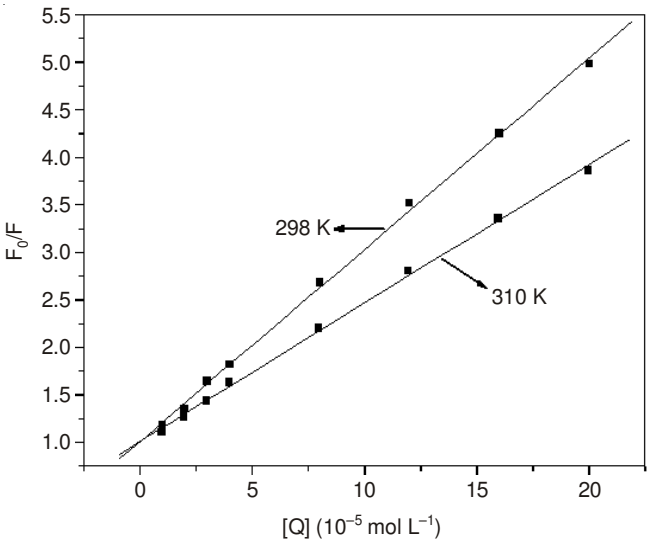

Fig. 3. Stern-Volmer plots for the quenching of trypsin by brilliant blue at different temperatures. c (trypsin) = 4.0×10^{-5} mol L^{-1}, pH = 7.24

TABLE-1
STERN-VOLMER QUENCHING CONSTANTS FOR THE INTERACTION OF BRILLIANT BLUE WITH TRYPSIN

T (K)	K_{SV} ($\times 10^4$ L mol^{-1})	K_q ($\times 10^{12}$ L mol^{-1} s^{-1})	R
298	2.02	2.02	0.9991
310	1.46	1.46	0.9990

where K_b was the binding constant and n was the number of binding sites. For the brilliant blue-trypsin system in the lower concentration range, the values for K_b and n at different temperatures could be derived from the intercept and slope of plots of log $(F_0\text{-}F)/F$ *versus* log [Q] based on Eqn. 2, which presented in Table-2. K_b indicated a strong interaction between brilliant blue and trypsin and formation of a complex. Furthermore, it could be inferred from the values of n, there was an independent class of binding sites on trypsin for brilliant blue. As can be seen from Table-2, K_b decreased with the increasing temperature, which indicated the forming of an unstable compound in the association reaction. The unstable compound would be partly decomposed with the rising temperature. Thus, the values of K_b decreased. The values of n at the experimental temperatures were approximately equal to 1, which indicated that there was one class of binding sites in trypsin for brilliant blue.

TABLE-2
BINDING CONSTANTS K_b AND BINDING SITES n AT DIFFERENT TEMPERATURES

T (K)	K_b ($\times 10^4$ L mol^{-1})	n	R
298	2.97	1.05	0.9993
310	1.39	0.98	0.9989

Binding mode: Generally, the acting force between a small molecule and macromolecule mainly included hydrogen bond, van der Waals force, electrostatic and hydrophobic interactions . The thermodynamic parameters, enthalpy (ΔH^0) and entropy (ΔS^0) of a reaction were important for confirming the acting force. For this reason, the temperature dependence of the binding constant was studied. The temperatures were chosen from 298 to 310 K so that trypsin did not undergo any structural degradation. The thermodynamic parameters could be determined from the van't Hoff equation:

$$\ln K = -\frac{\Delta H^0}{RT} + \frac{\Delta S^0}{R} \quad (3)$$

Consequently, a free energy change (ΔG^0) for a binding interaction at different temperatures could be evaluated:

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

where K was the binding constant and R was the gas constant. The value of ΔH^0 and ΔS^0 were obtained from linear van't Hoff plot (Fig. 4) and were presented in Table-3. As shown in Table-3, the signs for ΔH^0 and ΔS^0 of the binding reaction between trypsin and brilliant blue were found to be negative and positive, respectively. Thus, the formation of the complex was an exothermic reaction accompanied by positive ΔS^0 value. Ross and Subramanian[11] had characterized the sign and magnitude of the thermodynamic parameter associated with various individual kinds of interaction that may take place in protein association processes. Small ΔH^0 value and a positive ΔS^0 value was frequently taken as typical evidence for electrostatic

TABLE-3
THERMODYNAMIC PARAMETERS OF THE BRILLIANT BLUE-TRYPSIN SYSTEM

T (K)	ΔG^0 (kJ mol^{-1})	ΔH^0 (kJ mol^{-1})	ΔS^0 (J mol^{-1} K^{-1})
298	-25.75	-0.24	85.63
310	-26.79	-0.24	85.63

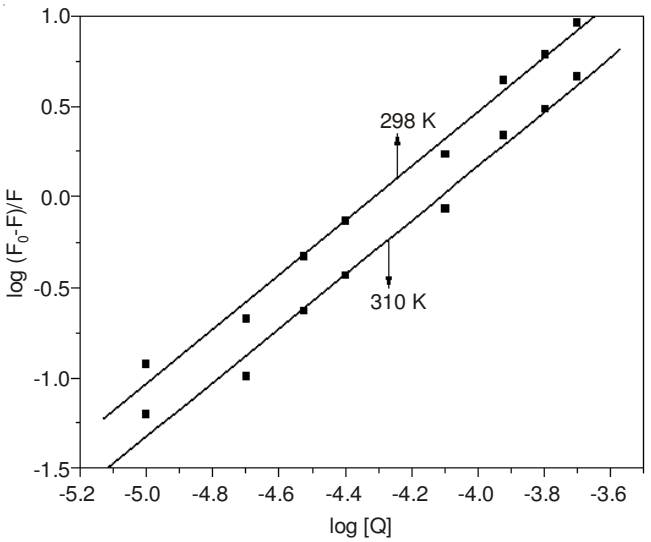

Fig. 4. Plots of log $(F_0\text{-}F)/F$ *versus* log[Q]. c (trypsin) = 4.0×10^{-5} mol L^{-1}, pH = 7.24

interaction. So, in the binding of brilliant blue to trypsin process, electrostatic forces most likely played a major role.

Energy transform between brilliant blue and trypsin: In order to determine the precise location of brilliant blue in trypsin, the efficiency of energy transfer was studied according to the Förster resonance energy transfer theory[12]. The fluorescence quenching of trypsin after binding with brilliant blue indicated that the energy transfer between brilliant blue and trypsin had occurred. The efficient ligand-protein interaction gave rise to energy, from which the distance between the two interacting molecules can be easily evaluated. The efficiency of energy transfer (E) was described by the following equation[13].

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

F_0 and F were the fluorescence intensity of donor in the absence and presence of acceptor respectively. R was the distance between acceptor and donor. R_0 was the critical distance when the transfer efficiency reaches 50 % and the value of R_0 was calculated by following equation:

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

where K_2 was the spatial orientation factor of the dipole, N was the refractive index of the medium, F was the fluorescence quantum yield of the donor, J was the overlap integral of the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor. J was given by

$$J = \frac{\int_0^\infty F(\lambda)\varepsilon(\lambda)\lambda^4 d\lambda}{\int_0^\infty F(\lambda)d\lambda} \quad (7)$$

In this equation, $F(\lambda)$ was the fluorescence intensity of the fluorescent donor of wavelength, $\varepsilon(\lambda)$ was the molar absorption coefficient of the acceptor at wavelength λ = 100 nm. It was reported earlier that K^2 = 2/3, N = 1.336 and F = 0.118[14]. Fig. 5 showed the overlap of UV absorbance spectrum of brilliant blue with fluorescence spectrum of trypsin. From

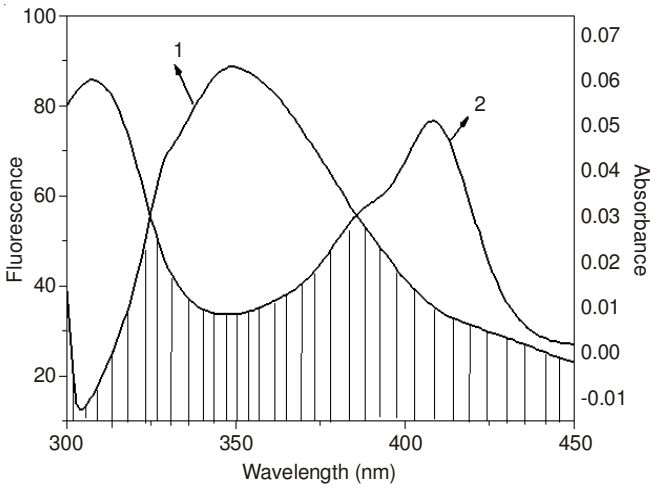

Fig. 5. Overlapping between the fluorescence emission spectrum of trypsin (1) and UV/visible absorption spectrum of brilliant blue (2). c (trypsin) = 2.0×10^{-5} mol L^{-1}, c (brilliant blue) = 3.0×10^{-5} mol L^{-1}; pH = 7.24, T = 298 K

the above relationships, the values for E and R were evaluated as follows: J = 1.037×10^{-15} cm^3 L mol^{-1}, R_0 = 1.68 nm, E = 0.33 and R = 3.36 nm. The distance R < 8 nm between donor and acceptor indicated that the energy transfer from trypsin to brilliant blue occurred with high possibility[15]. This agreed with the conditions of Forster energy transfer theory and indicated again a static quenching between brilliant blue and trypsin.

Conclusion

The interaction between brilliant blue and trypsin has been investigated under physiological condition (pH 7.24) by fluorescence emission spectra in combination with UV/visible. It was shown that the fluorescence of trypsin has been quenched for reacting with brilliant blue. The quenching belongs to static fluorescence quenching type, with non-radiative energy transfer happening within single molecule. The thermodynamic parameter elucidated that electrostatic interaction was the dominant intermolecular force in stabilizing the complex. The results obtained herein would be of biological significance in pharmacology and clinical medicine.

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