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FLUORESCENCE CHARACTERIZATION OF HUMAN HEMOGLOBIN BINDING WITH TANNIC ACID

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The nature of the interactions between human hemoglobin (HHb) and tannic acid (TA) has been studied using fluorescence spectroscopy. It has been shown that the quenching mechanism (dynamic or static) of HHb fluorescence in the presence of TA depends on structural changes of protein. Physicochemical characteristics of HHb-TA interactions (Stern-Volmer constant, the binding constant, thermodynamic parameters, Hill coefficient and distance between TA and HHb) have been determined. Thermodynamic analysis suggests that van der Waals forces and hydrogen bonds are the predominant forces in the binding of TA to HHb when the protein molecule has loosen structure, and hydrophobic association plays a major role when the protein molecule is in a nonseparated state.

Fig. 3, tables 2, ref. 14.

Introduction

Tannins are naturally occurring plant polyphenols. They are found in many fruits (*e.g.* grapes, persimmon, blueberry), in tea, in chocolate, in legume forages (trefoil) and also in grasses (*e.g.* sorghum, corn.). Tannins are responsible for the astringent taste of wine or unripe fruits [1]. Considerable amounts of tannins can be extracted from grape seed powder [2]. Tannins are water-soluble polyphenols and can be classified into two categories: hydrolysable and non-hydrolysable (condensed) tannins. Tannic acid (TA) is an important gallotannin belonging to the hydrolysable class of tannins [3]. Tannins, like many other polyphenols have received considerable attention because of their potential health benefits, especially because of their antioxidant properties. Interestingly, these antioxidant properties on plants are joined by the fact that polyphenols inhibit the growth of certain harmful micro-organisms. Here, tannins have

been reported to be bacteriostatic or bactericidal against *Staphylococcus aureus* [4]. Tannins can reversibly be bound by blood proteins, such as human hemoglobin (HHb), human serum albumin (HSA). Recently, the interactions of flavonoids with HHb have attracted considerable interest, as the distribution, metabolism and efficacy of many drugs *in vivo* are related to their respective affinities towards HHb [5, 6]. The study presented herein examines the details of TA-HHb interactions using fluorescence spectroscopy.

Experimental Materials

HHb and TA were purchased from Sigma Aldrich (Steinheim, Germany). All materials and reagents were of analytical grade. Solutions were prepared in 0.2 *M* phosphate buffer (pH = 7.2). Double distilled water was used for the preparation of solutions. Two samples of HHb were used: 1. hemoglobin without desalting and 2.desalted (group separated) hemoglobin. Group separation was performed with a Sephadex G-25 desalting column.

Methods

Fluorescence spectroscopy measurements

A Varian fluorescence spectrophotometer (Australia) equipped with a circulating water bath (Lauda 100) was used to record the fluorescence spectra at different temperatures (298.15, 303.15 and 309.75 *K*). The fluorescence spectra were scanned under the following conditions: entrance slit and exit slit widths at 10 *nm*, the excitation wavelength was adjusted at 280 *nm* and the emission spectra were recorded in the range 290-400 *nm*. The quenching experiments were carried out by keeping the concentration of HHb constant ($3.956 \cdot 10^{-6}$ *M*) while varying the concentration of TA ($4.088 \cdot 10^{-6}$-$1.635 \cdot 10^{-5}$ *M*). Measurements were performed after achieving equilibrium state (≈5 *min*). Each experiment was performed in triplicate and the average data were used for the analysis. Origin 8.0 software was used to construct the graphs.

Results and Discussion

Fluorescence studies

Fluorescence quenching mechanism of HHb by TA

HHb is an iron-containing protein, the main function of which is the reversible binding and transfer of dioxygen to different parts of an organism. HHb has tetramer structure which is assembled from two symmetrical αβ dimers. HHb contains three tryptophan (Trp) in each αβ dimer, for a total six in the tetramer: two α–14 Trp, two β–15 Trp and two β–37 Trp. The intrinsic fluorescence of HHb primarily originates from β – 37 Trp at the $\alpha_1\beta_2$ interface, though it may contain some contribution by the surface Trp residues, α–14 and β –15 Trp [7, 8]. Figure 1 shows the fluorescence spectra of HHb in the presence of TA at different concentrations. HHb has an emission peak at 334 nm with the excitation wavelength at 280 *nm*. TA causes concentration dependent quenching of the intrinsic fluorescence of HHb, simultaneously shifting the emission peak by 23 nm

to longer wavelengths. These results indicate that there are interactions between HHb and TA. To determine the mechanism of binding, the Stern-Volmer equation was used:

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

where F_0 and F are the fluorescence intensities of HHb in the absence and presence of quencher, respectively, $[Q]$ is the concentration of quencher and K_{SV} is the Stern-Volmer quenching constant. The values of K_{SV} at different temperatures are given in Table 1. The linearity of the F_0/F versus [Q] plots is shown in Fig. 2. As shown in Table 1, the quenching constant K_{SV} decreases with increasing temperature which indicates that the quenching mechanism of HHb is static [9].

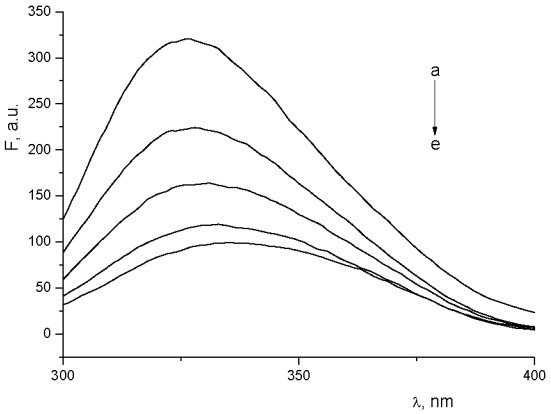

Fig. 1. Fluorescence spectra of HHb in the presence of TA at 298.15 K. Concentration of TA: (a) 0; (b) $4.088 \cdot 10^{-6}$; (c) $8.176 \cdot 10^{-6}$; (d) $12.264 \cdot 10^{-6}$; (e) $16.352 \cdot 10^{-6}$ *M*.

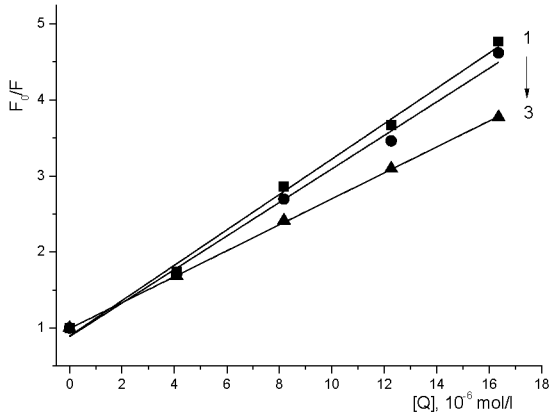

Fig. 2. Stern-Volmer plots for the quenching of HHb fluorescence by TA at different temperatures: (1) 298.15; (2) 303.15 and (3) 309.75 *K*.

Table 1

Stern-Volmer quenching constants for HHb-TA interactions

T, *K*	$K_{SV}, 10^5$ ($l \cdot mol^{-1}$)	k_q, 10^{13} ($l \cdot mol^{-1} \cdot s^{-1}$)	R
298.15	2.34	2.34	0.97733
303.15	2.28	2.28	0.97984
309.75	1.70	1.70	0.99957

R is the correlation coefficient

Binding constants and number of binding sites

For the static quenching process the binding constant (K_b) and the number of binding sites (n) can be calculated using the following equation [10].

$$\lg[(F_0 - F)/F] = \lg K_b + n\lg[Q], \quad (2)$$

where K_b and n are the binding constant and the number of binding sites, respectively. Thus, a plot of $\lg(F_0 - F)/F$ versus lg[Q] has been used to determine K_b and n. The values of K_b and n at 298.15, 303.15 and 309.75K are given in Table 2. The fact that K_b decreases with increasing temperature indicates that the stability of the complex becomes weaker with increasing temperature.

In the case, when the HHb molecule is in the nonseparated state, with the increase of temperature the K_{SV} increases, denoting that collisional mechanism (dynamic mechanism) prevails in HHb-TA complex formation. Increase of temperature brings to group separation in protein structure and K_b increases. Thermodynamic analysis suggests that in this case hydrophobic association plays a major role in the binding of TA to HHb [11].

Thermodynamic parameters of the HHb-TA interactions

The attractive forces between small molecules and macromolecules often include hydrogen bonds, van der Waals forces, hydrophobic and electrostatic interactions [12]. The thermodynamic parameters, enthalpy change (ΔH), entropy change (ΔS) and free energy change (ΔG), are the main parameters to determine the binding mode. If the temperature does not vary significantly, the enthalpy change can be regarded as a constant and the value of enthalpy change and entropy change can be estimated from the van't Hoff equation:

$$\ln K = -\Delta H/RT + \Delta S/R, \quad (3)$$

where the associative binding constant K is analogous to the effective quenching constant K_a at the corresponding temperature. The free energy change (ΔG) can be estimated from the following relationship:

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

The thermodynamic parameters were calculated from the relevant van't Hoff plot. The negative sign for ΔG indicates the spontaneity of the TA binding process to HHb. Based on the characteristic signs of the thermodynamic parameters at the various interactions, both negative ΔH and ΔS values (Table 2) generally represent van der Waals forces and hydrogen bonds are responsible for the binding between TA and HHb [12].

Table 2

Binding constant and thermodynamic parameters for HHb - TA interactions

T, K	K_b, 10^6 ($l \cdot mol^{-1}$)	ΔH, ($kJ \cdot mol^{-1}$)	ΔS, ($J \cdot mol^{-1} \cdot K^{-1}$)	ΔG, ($kJ \cdot mol^{-1}$)	n, (*Hill coeff.*)
298.15	4.57	-197.33	-532.20	-38.66	1.298
303.15	1.76			-36.00	1.20
309.75	0.21			-32.48	1.03

Energy transfer from HHb to TA

According to the Foster's non-radiation fluorescence energy transfer theory the distance between the donor and the acceptor can be calculated [13].The efficiency of energy transfer, E, is determined by using the equation:

$$E = 1 - F / F_0 = R_0^6 / R_0^6 + r^6, \quad (5)$$

where E is the energy transfer efficiency, F_0 and F are the fluorescence intensities of HHb in the absence and presence of TA, respectively, r is the distance between the donor and acceptor, and R_0 is the critical distance when energy transfer efficiency is 50% and which can be calculated by the following equation:

$$R_0^6 = 8.79 \times 10^{-25} K^2 n^{-4} \Phi J, \quad (6)$$

where K^2 is the space factor of orientation, n is the refractive index of the medium, Φ is the fluorescence quantum yield of the donor and J is the overlap integral of the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor. Therefore,

$$J = \sum F(\lambda)\ \varepsilon(\lambda)\ \lambda^4 \Delta\lambda) / \sum F(\lambda)\Delta\lambda, \quad (7)$$

where $F(\lambda)$ is the fluorescence intensity of the donor at the wavelength λ and $\varepsilon(\lambda)$ is the molar absorption coefficient of the acceptor at the wavelength λ. The overlap of the UV absorption spectrum of TA and fluorescence emission spectrum of HHb is shown in Fig. 3.

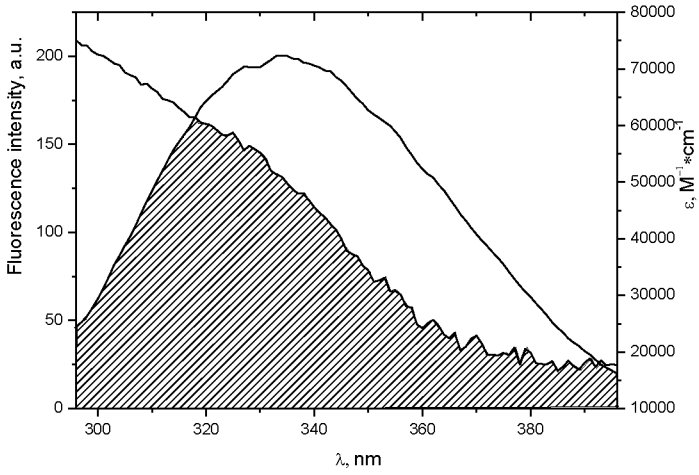

Fig. 3. Overlapping of fluorescence emission spectrum of HHb with UV/vis absorption spectrum of TA. c(TA) = $4.088 \cdot 10^{-6}$ M; c(HHb) = $4.088 \cdot 10^{-6}$ M; pH = 7.2, T = 298.15 K.

In the present case, K^2 = 2/3, n = 1.336, and Φ = 0.062 [14]. According to eq. 7, it was found J = $3.627 \cdot 10^{-14}$ $cm^3 \cdot l \cdot mol^{-1}$, R_0 = 2.69 *nm*, E = 0.456, r = 2.76 *nm*. The donor acceptor distance r was less than 8 *nm* and 0.5 R_0 < r <1.5 R_0, which indicates that in this

case, the energy transfer from HHb to TA is possible and a static quenching mechanism takes place [14].

Conclusions

The nature of the interactions between HHb and TA has been studied using fluorescence spectroscopy. This detailed study reveals that the quenching mechanism (dynamic or static) of HHb fluorescence in the presence of TA depends on structural changes of protein. The experimental results indicate that van der Waals forces, hydrogen bonds and hydrophobic association are responsible for the stability of formed complexes.

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ФЛУОРЕСЦЕНТНОЕ ИССЛЕДОВАНИЕ СВЯЗЫВАНИЯ ГЕМОГЛОБИНА ЧЕЛОВЕКА С ДУБИЛЬНОЙ КИСЛОТОЙ

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Природа взаимодействий между гемоглобином человека (ГЧ) и дубильной кислотой (ДК) при разных температурах (в температурном интервале 298.15-309.75K) изучена методом флуоресцентной спектроскопии. Показано, что механизм тушения флуоресценции ГЧ (динамическое или статическое) в присутствии ДК зависит от структурных изменений белка. Определены физико-химические характеристики связывания ГЧ с ДК (константа Штерна-Фольмера, константа связывания, термодинамические параметры, коэффициент Хилла, расстояние между ГЧ и ДК). Термодинамический анализ показывает, что Ван-дер-Ваальсовые силы и водородные связи являются превалирующими в связывании ГЧ-ДК, когда молекула белка имеет разрыхленную структуру, и гидрофобные взаимодействия, когда молекула белка находится в закрученной форме.

ՄԱՐԴՈՒ ՀԵՄՈԳԼՈԲԻՆԻ ԴԱԲԱՂԱԹԹՎԻ ՀԵՏ ԿԱՊՄԱՆ ՖԼՈՒՈՐԵՍՑԵՆՏԱՅԻՆ ՀԵՏԱԶՈՏՈՒԹՅՈՒՆԸ

Լ. Ս. ՍԱՐԳՍՅԱՆ, Կ. Ռ. ԳՐԻԳՈՐՅԱՆ, Շ. Ա. ՄԱՐԳԱՐՅԱՆ, Տ. ԲՈՒՐԿՀՈԼՑ և Կ. ՅԱԿՈԲ

Մարդու հեմոգլոբինի (ՄՀբ) և դարաղաթթվի (ԴԹ) միջև փոխազդեցությունների բնույթը ուսումնասիրվել է տարբեր ջերմաստիճաններում (298.15-309.75K տիրույթում)՝ օգտագործելով ֆլուորեսցենտային սպեկտրոսկոպիայի մեթոդը: Ցույց է տրվել, որ ՄՀբ-ի ֆլուորեսցենցիայի մարման մեխանիզմը ԴԹ-ի ներկայությամբ (դինամիկ կամ

ստատիկ) կախված է սպիտակուցի կառուցվածքային փոփոխություններից: Որոշվել են ՄՀբ-ի ԴԹ-ի հետ կապման ֆիզիկաքիմիական բնութագրերը (Շտերնի-Վոլմերի հաստատունը, կապման հաստատունը, հիլի գործակիցը, կապման թերմոդինամիկական պարամետրերը և ՄՀբ-ի ու ԴԹ-ի միջև հեռավորությունը): Թերմոդինամիկական վերլուծությունը ցույց է տվել, որ ՄՀբ–ԴԹ կապման համար էական նշանակություն ունի Վան-դեր-Վաալսի ուժերը և ջրածնական կապերը, երբ սպիտակուցի մոլեկուլը զտնվում է փխրուն վիճակում, և հիդրոֆոբ փոխազդեցությունները, երբ այն սեղմված վիճակում է:

REFERENCES

[1] *Haslam E.* Plant polyphenols. Cambridge University Press, Cambridge, UK, 1989.

[2] *Sun B., Belchior G., Ricardo-da-Silva J., Spranger M.* // J. Chromatogr. A, 1999, v. 841, p. 116.

[3] *Chung K., Wong T., Wei C., Huang, Y., Lin Y.* // Critical Reviews in Food Science and Nutrition, 1998, v. 38, p. 464.

[4] *Akiyama H, Fujii K., Yamasaki O., Oono T., Iwatsuki K.* // J. Antimicrob. Chemother., 2001, v. 48, №4, p. 487.

[5] *Jiahong Z., Xiaohong W., Xiaotian G., Lin Z., Kaixi S., Shaohua W., Yuying F., Jian S.* // Spectrochim. Acta, Part A, 2009, v.72, p. 151.

[6] *Paulami M., Tapan G.* // J. Phys. Chem. B, 2009, v. 113, p. 14904.

[7] *Swati D., Agnishwar G.* // J. Colloid Interface Sci., 2006, v. 296, p. 324.

[8] *Alpert B., Jameson D.M., Weber G.* // Photochem. Photobiol., 1980, v.31, p.1.

[9] *Lakowicz J.* Principles of Fluorescence Spectroscopy, Springer, New York, 2006, 960 p.

[10] *Wang Y., Zhang H., Zhang G., Liu S., Zhou Q., Fei Z.* // Int. J. Biol. Macromol., 2007, v. 41, p. 243.

[11] http://www.armchemfront.com/2013/abstracts/ACF2013_PS178_Grigoryan.pdf.

[12] *Ross P., Subramanian S.* // Biochem., 1981, v. 20, p. 3096.

[13] *Förster T., Sinanoglu O.* Modern Quantum Chemistry, Academic Press, New York, 1996, p. 93.

[14] *Chen T., Zhu Sh., Shang Y., Ge C., Jiang G.* // Spectrochim. Acta, 2012, Part A 93, p. 125.