Chemistry

DENATURATION OF HEMOGLOBIN IN THE PRESENCE OF TANNIC ACID
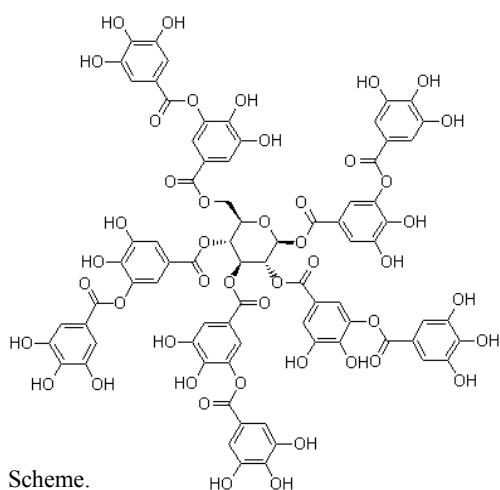
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Thermal denaturing of human hemoglobin (HHb) in the presence of tannic acid (TA) was studied by fluorescence and UV/vis spectroscopy in 30–66°C temperature range. To study more detailed structural changes in HHb molecule caused by temperature rise in the presence of TA, the excitation/emission fluorescence (3D spectra) matrix method has been used. It has been shown, that the increase of temperature causes more pronounced structural changes in the flexible areas of protein containing aromatic amino acids than in the rigid α, β polypeptide chains. It was revealed that the presence of TA causes structural changes in HHb.

Keywords: human hemoglobin, tannic acid, denaturation, fluorescence spectroscopy, 3D spectra, UV/vis spectroscopy.

Introduction. Human hemoglobin (HHb) and human serum albumin (HSA) are the two major blood protein components in human circulatory system and erythrocytes. These proteins can reversibly bound several endogenous and exogenous agents and, hence, responsible for distribution through the circulation [1]. HHb is a globular tetramer protein comprised of two α- and two β-subunits, bounded non-covalently. Each of α- and β-subunits is comprised of 141 and 146 amino acid residues respectively. This is denoted as $\alpha_2\beta_2$, in which α-Trp^{14}, β-Trp^{15} and β-Trp^{37} are located [2]. The subunits are structurally similar and of about the same size. Since the four chains are stabilized by weak non-covalent interactions, the biologically active tetramers can easily undergo dissociation. Exposure to temperatures around 50°C, therefore, leads to rapid irreversible denaturation followed by precipitation [3].

Scheme.

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Tannic acid (TA) is a natural gallotannin and is comprised of a pentagalloylglucose core esterified at all functional hydroxyl groups with gallic acid molecules (see Scheme) [4]. TA belongs to the class of hydrolysable tannins and is found in numerous plants and foods. It generally can be extracted from grape seeds. TA is known as an effective antioxidant, antimicrobial/antiviral agent, which binds to blood proteins [5–8]. The binding of a ligand to HSA and HHb can stabilize or destabilize the protein structure.

Fluorescence emission and UV/vis absorption spectroscopy are informative and sensitive methods for the studies dealing with structural changes caused by binding of ligands and external factors (temperature, pH, presence of additives).

In order to gain the effect of high temperatures on HHb structure in the presence of TA, we have studied thermal denaturing of the protein in 30–66°C temperature range using fluorescence and UV/vis spectroscopy.

Materials and Methods. HHb and TA were purchased from "Sigma Aldrich Ltd". All other materials and reagents were of analytical grade. HHb was desalted by Sephadex-G25 column. Double distilled water was used for the preparation of solutions. HHb and TA solutions were prepared in 0.2 *M* phosphate buffer (pH=7.2).

UV/vis Spectroscopy Measurements. The UV/vis studies were perfomed using a Specord 50 UV/vis spectrophotometer (Germany) equipped with a water circulating thermostat LAUDA. The UV/vis spectra were recorded with a 1.0 *cm* quarz cell in the range of 250–500 *nm*. Thermal denaturation of HHb in the presence of TA was studied in the 36.6–66.0°C temperature range. These experiments were carried out by keeping the concentration of HHb constant ($3.956 \cdot 10^{-6}$ *M*), while the concentration of TA was varied $4.08 \cdot 10^{-6} - 1.65 \cdot 10^{-5}$ *M*. The thermal denaturation curves of HHb in the presence of TA were normalized (from 0 to 1). Each sample of TA–HHb was measured after reaching the equilibrium state. Temperature scanning rate: 1°C /*min*.

Fluorescence Spectroscopy Measurements. The fluorescence spectroscopy measurements were performed using Cary Varian Eclipse fluorescence spectrophotometer (Australia), which is also equipped with a water circulating thermostat. The 3D spectra of HHb were recorded in the temperature range of 30–66°C. Temperature scanning rate was 1°C/*min*. The 3D spectra were recorded under the following conditions: λ_{em}=200–400 *nm*, λ_{ex}=200 *nm*, $\Delta\lambda_{incr}$=10 *nm*, number of scans 26; entrance and exit slit width 10 *nm*. Concentration of HHb was $3.95 \cdot 10^{-6}$ *M*.

Each experiment was performed triply and the average data were used for analysis. Origin 8.0 software was used to construct the graphs.

Results and Discussion.

Fluorescence Studies. Using the fluorescence approach, many authors have reported movements of aromatic Tyr and Trp residues in various proteins and focused on the dynamics of particular area within the protein [5–7]. Indeed, the various regions of proteins cannot have the same dynamic behavior and should be more or less sensitive to thermal energy. The present study introduces a new chance of using 3D fluorescence spectra to compare the temperature dependence of particular areas of proteins. We have used this method to study thermal denaturing of HSA in the presence of potassium chloride in the temperature interval 36–90°C [8, 9].
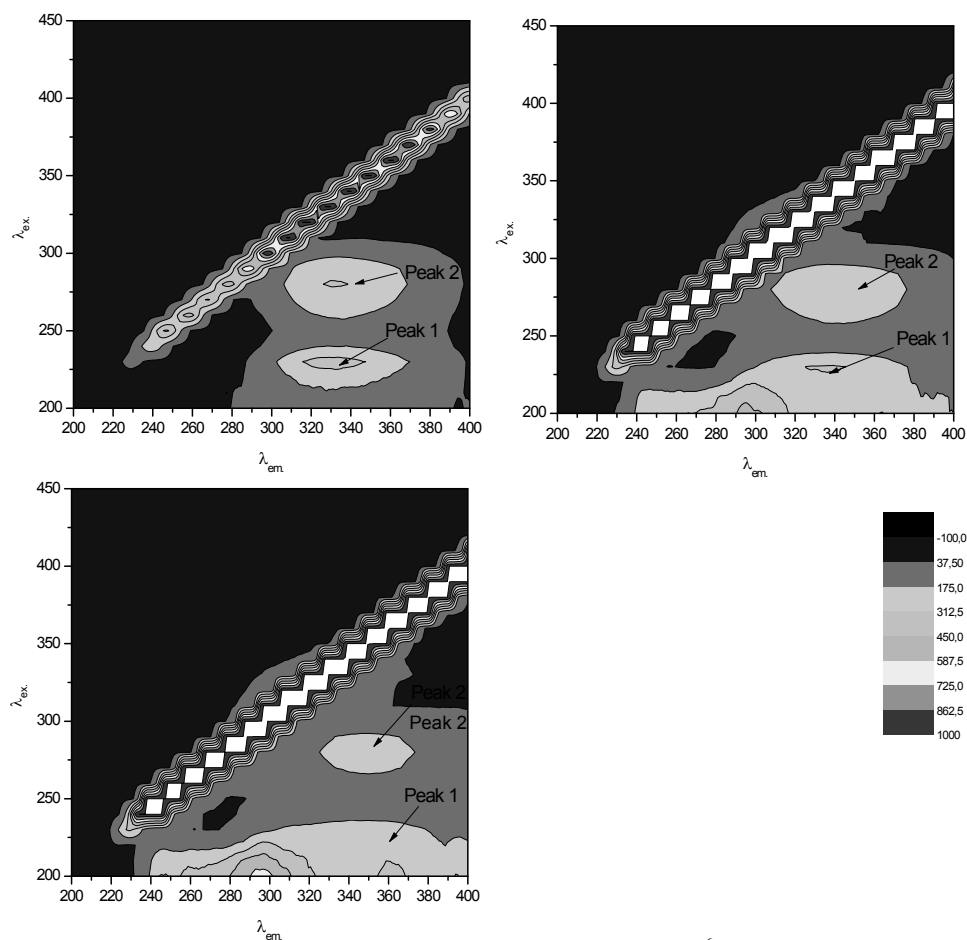
Fig. 1. 3D projections of the fluorescence spectra of HHb, C_{HHb}=3.95·10^{-6} M: a – 30; b – 50; c – 66 °C.

Spectral characteristics of fluorescence 3D spectra for thermal denaturating of HHb

t, °C	Peak 1			Peak 2		
	$\frac{\lambda_{ex}}{\lambda_{em}}$, nm	$\Delta\lambda$, nm	F	$\frac{\lambda_{ex}}{\lambda_{em}}$, nm	$\Delta\lambda$, nm	F
30	230/333	103	369.64	280/333	53	320.1
34	230/332	102	364.9	280/335	55	322.65
38	230/332	102	367.16	280/336	56	328.75
42	230/334	104	362.82	280/336	56	329.31
46	230/334	104	358.11	280/337	57	328.08
50	230/335	105	338.78	280/339	59	314.01
54	230/335	105	324.16	280/339	59	295.00
58	230/335	105	301.2	280/339	59	268.77
62	230/335	105	276.12	280/339	59	238.35
66	230/335	105	229.51	280/339	59	219.03
70	230/335	105	245.04	280/339	59	213.63

The fluorescence 3D spectra of HHb are described by two peaks: peak 1 at $\lambda_{ex.}/\lambda_{em}$=230/335 (*nm*) characterizes the fluorescence behavior of polypeptide chains of HHb and peak 2 at $\lambda_{ex}/\lambda_{em}$=280/339 (*nm)* characterizes the aromatic amino acid residues Trp, Tyr and Phe (Fig. 1). Decrease in the values of fluorescence intensities of these two peaks with temperature increase and a pronounced shift in 6 *nm* for peak 2 is mentioned. On the bases of 3D spectra we have constructed the fluorescent profiles of HHb thermal denaturation (Fig. 2), using intensities of peak 1 ($\lambda_{ex}/\lambda_{em}$=230/335 *nm*) and peak 2 ($\lambda_{ex.}/\lambda_{em}$=280/339 *nm*). The curve 1 ($\lambda_{ex}/\lambda_{em}$=230/335 *nm*) is shifted to higher temperature range compared with curve 2 ($\lambda_{ex}/\lambda_{em}$=280/339 *nm*). The analysis of these facts revealed that increase of temperature causes more pronounced structural changes in the flexible areas of protein containing aromatic amino acids than in the rigid α, β polypeptide chains.
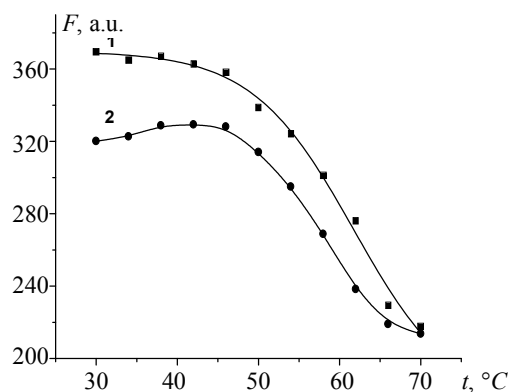

Fig. 2. Fluorescent profiles for thermal denaturation of HHb:
1 − $\lambda_{ex.}/\lambda_{em}$ = 230/335 *nm*;
2 − $\lambda_{ex.}/\lambda_{em}$ = 280/338 *nm*;
$C_{HHb} = 3.95 \cdot 10^{-6}$ *M.*

UV/vis Studies. UV/vis spectrum of HHb in the rage of 250–500 *nm* is described by two peaks at around λ=280 and λ=407 *nm*. The peak at 280 *nm* corresponds to Trp residues and the peak at 407 *nm* to heme group [3]. Denaturation of HHb causes changes in light absorption in UV/vis region. For HHb denaturation studies we have followed the optical density change at 280 *nm*. With the increase of temperature (36.6–66.0°C) intensity of this peak increases, from which denotes the protein denaturing. On the basis of obtained data the UV/vis melting profiles of HHb in the presence of TA have been constructed (Fig. 3). The curves 1–3 are characterized by

Fig. 3. The melting curves of HHb at the presence of TA. Concentration of TA:
1 − 0; 2 − 4.08·10⁻⁶ *M*; 3 − 8.17·10⁻⁶ *M.*

sigmoidal shape. T_m values of HHb thermal denaturation have been determined as a midpoint of curve to be 51.10 (±0.19), 50.02 (±0.14) and 47.40°C (±0.06) respectively.
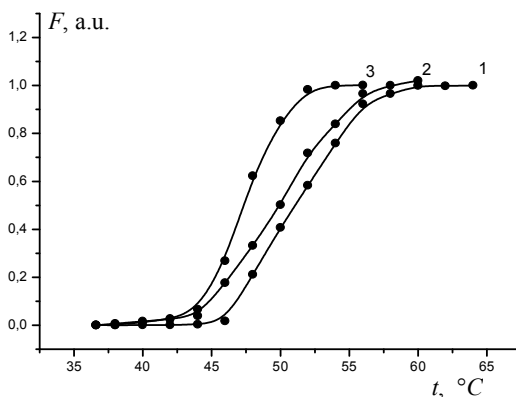
At higher concentrations of TA (>10⁻⁵ *M*) sigmoidal form of melting profiles is collapsed as a result of protein aggregation and precipitation. As it follows from

Fig. 3, in the presence of TA the melting curves shift to the region of low temperatures with decreasing T_m. It means that TA itself causes structural changes in HHb.

Conclusion. Fluorescence and electronic absorption spectroscopy in the UV region was used to study thermal denaturation (30–66°C) of HHb in the presence of TA. Temperature rise and the presence of TA cause denaturation of HHb. Excitation/emission fluorescence (3D spectra) matrix method can be used to determine structural changes in various areas of protein especially in parts containing aromatic amino acids and rigid α, β polypeptide chains.

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