THERMODYNAMIC STUDY OF BINDING BETWEEN BOVINE ALBUMIN SERUM (BSA) AND RED PONCEAU 4R

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RESUMO – A interação entre o vermelho Ponceau 4R (P4R) e albumina de soro bovino (BSA) foi estudada, in vitro, em pH 3,5 e 7,4, a diferentes temperaturas, na ausência e na presença de marcadores de sítios de interação. Os espectros de fluorescência mostraram que a intensidade de fluorescência da BSA diminuiu com o aumento da concentração do P4R. A extinção de fluorescência foi classificada como supressão estática devido à formação de complexos BSA/P4R em todas as condições estudadas, os quais foram conduzidos por redução da entalpia e aumento da entropia. Para todas as condições analisadas os valores de energia livre de Gibbs foram negativos, indicando que a formação do complexo foi mais favorável do que reagentes. No entanto verificou-se que o corante se liga em sítios diferentes na proteína dependendo do pH do meio. Este trabalho contribui para o conhecimento sobre a interação entre a BSA e corantes sintéticos em alimentos (pH 3,5) e em condições do sangue (pH 7,4).

ABSTRACT - The interaction between the red Ponceau 4R (P4R) and bovine serum albumin (BSA) was studied in vitro at pH 3.5 and 7.4, at different temperatures and in the absence or presence of BSA site markers. Fluorescence spectra showed that BSA fluorescence intensity decreased as P4R enhanced concentration. The fluorescence quenching was classified as static quenching due to complex formation for all conditions studied, which were driven by enthalpy reduction or increase of entropy. For all conditions analyzed, the standard free Gibbs energy change of complex formation (ΔG°) values were negative, indicating that complex formation was more favorable than the reactants. However it found that the dye binds to the protein in different places depending on the pH. This work contributes to the understanding of the interaction between BSA and synthetic dyes in foods (pH 3.5) and blood conditions (pH 7.4).

PALAVRAS-CHAVE: fluorescência; entalpia; marcadores; corante sintético
1. INTRODUCTION

Natural food color is partially lost during food processing and storage. To overcome this problem, industries add dyes in food formulations to improve product appearance and acceptability. The addition of synthetic dyes is widely used due to its higher stability and lower cost compared to natural dyes. The synthetic dyes most used in food industries contain at least one azo functional group (C=N) and two or three aromatic ring structures. Various studies have demonstrated adverse reactions caused by these dyes in humans, as headaches, allergies, as well as neurotoxicity, genotoxicity, carcinogenicity (Sasaki et al., 2002).

Bovine serum albumin (BSA) is the major protein in cow blood plasma and plays important physiological role in the transport of hydrophobic compounds. BSA has been extensively studied due to its high homology to human serum albumin (HSA). The connection between the food additives such as azo food dyes and serum albums can dramatically affect the structure and function of these proteins, besides change metabolism, distribution and elimination of these additives (Wang et al., 2014). Therefore, the investigation of the interaction between food dyes and BSA is important from the point of view of food chemistry, medicine and life sciences.

Umer Abdullah et al., (2008) studied the interaction between BSA and allura red (AR), another azo-dye, evaluating the digestibility of protein in vitro. They found that AR bound to BSA at low and high temperatures and a wide range of pH (4.0 to 7.5) however, this interaction did not affect the digestibility of protein by trypsin and fungal proteases. Basu et al. (2014) investigated the binding of carmosine, a monoazo-dye, with BSA and found that carmosine/BSA complex was formed with binding constant (K) of the order of 10\(^6\) mol.L\(^{-1}\) and stoichiometry (n) of 1:1. The complex formation occurred with reduction of standard enthalpy change and increasing in the entropy system.

There are some studies elucidating the interaction between food azo-dyes and proteins. However, there is lack of information about the binding between BSA and ponceau 4R (E124), which is a water-soluble monoazo dye, responsible for giving red color for different foods, such as ice cream and soda. It has been related to adverse reactions as allergy, toxicity and hyperactivity in children being not recommended for consumption by children in Europe (Pourreza et al., 2011). In this context, we report a thermodynamic approach of P4R x BSA binding, in different conditions, using fluorescence spectroscopy.

2. MATERIAL AND METHODS

2.1. Materials
BSA (> 99% wt.), citric acid, sodium citrate, sodium phosphate (reagent grade), ibuprofen (>98% wt.), digitoxin (>92% wt.) and warfarin (analytical standard) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Ponceau 4R was kindly provided by Gemacom (Juiz de Fora, Brazil).

2.2. Fluorescence experiment

BSA-dye interaction: Fluorescence spectra were obtained with a CaryEclipse Fluorescence (Agilent) spectrophotometer equipped with a thermostat bath, according to Wang et al., 2014 with some modifications. For fluorescence measurement, 3.0 mL of BSA solution (3.01 x 10^{-5} mol.L^{-1}) containing different concentrations of P4R (3.0 x 10^{-6} to 5 x 10^{-5} mol.L^{-1}), at pH 3.5 and 7.4, was added to a 1.0 cm quartz cell. The fluorescence emission spectra were then measured at three different temperatures (288, 298, 308, 318 and 328 K) in the range of 281–450 nm (excitation wavelength 280 nm).

Competitive binding studies: The competitive binding studies were carried out using site probes for sites I, II and III of BSA (warfarin, ibuprofen and digitoxin, respectively). For this, BSA and site probes were at fixed concentration (3.01 x 10^{-5} mol.L^{-1}) and the fluorescence quenching titration (with P4R) was performed as described before at pH 3.5 and 7.4. Thus, binding parameters for AR-BSA interaction were determine in the presence of site markers.

2.5. Statistical analysis

All assays were performed in 3 repetitions. The mean values and standard deviations were evaluated using analysis of variance (ANOVA); all statistical data were processed using Statistical Analysis System (SAS) version 9.1.

3. RESULTS AND DISCUSSION

3.1. Fluorescence experiments

Fluorescence spectroscopy is an important tool to investigate protein interaction with small ligands. When BSA interacts with food dyes, its intrinsic fluorescence, which is mainly attributed to tryptophan, often changes as additive concentration increases. BSA has two tryptophan residues, Trp 134 and Trp 212, which are located at protein hydrophilic surface and hydrophobic core, respectively (Wu et al., 2015). If ground-state complexes are formed in Trp surroundings, the fluorescence intensity of protein is quenched, enabling to follow intermolecular interactions by determining the fluorescence dependence on the food dye concentration.

The fluorescence emission spectra of BSA showed a strong fluorescence emission peak at 342 nm, which reduced as increasing P4R concentrations were added (Figure 1).

Figure 1 – Effect of P4R on BSA fluorescence at 298 K and pH 7.4 (λ_{ex}= 280 nm and λ_{em}= 342 nm). Arrow indicates an increase in the P4R concentration (from 0 to 5.0 x 10^{-5} mol.L^{-1}).
Fluorescence quenching could be due to collision between BSA and P4R molecules or formation of BSA/P4R complex. In order to distinguish between both mechanisms, we analyzed the temperature-dependence of fluorescence behavior and applied the Stern-Volmer model.

For both pHs studied the Stern-Volmer constant ($K_{SV}$) values decreased with increasing temperature, indicating that the BSA fluorescence quenching was induced by the formation of BSA-P4R complex. Since a complex was formed we determined the binding constant (K) and stoichiometry (n) for this complex by plotting the double-logarithm regression curve of fluorescence data using the modified Stern-Volmer equation (Wu et al., 2015).

At pH 7.4, both BSA and P4R are negatively charged. At this pH the K values ranged 2.24 x 10^6 L.mol⁻¹ to 1.09 x 10^6 L.mol⁻¹, for temperatures varying between 298 and 328 K, with “n” values about 1.20. The thermodynamic parameters (standard enthalpy change ($\Delta H°$), standard entropy change ($\Delta S°$) and standard Gibbs free energy change ($\Delta G°$)) for the BSA/P4R complex formation were calculated following the van't Hoff approach and the fundamental Gibbs relations (Lu et al., 2011).

The negative values of $\Delta G°$ indicated that P4R + BSA ↔ BSA/P4R equilibrium favored the formation of the complex in all temperatures (Table 1). Despite the electrostatic repulsion due to the negative charges on both chemical species at physiologic pH, the formation of the complex was governed by enthalpy decreasing with a slight entropic gain.

<table>
<thead>
<tr>
<th>T (K)</th>
<th>$\Delta H°$ (kJ.mol⁻¹)</th>
<th>$\Delta S°$ (kJ.mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>288</td>
<td>-33.78</td>
<td>6.72</td>
</tr>
<tr>
<td>298</td>
<td>-34.50</td>
<td>7.43</td>
</tr>
<tr>
<td>308</td>
<td>-27.06</td>
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<tr>
<td>318</td>
<td>-37.85</td>
<td>10.78</td>
</tr>
<tr>
<td>328</td>
<td>-38.16</td>
<td>11.09</td>
</tr>
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</table>

BSA is made up three homologous sites linearly arranged, structurally distinct, and evolutionarily related domain (I-III), which are involved in transport of different molecules such as drugs. Thus, the knowledge about specific binding domain on BSA of molecules present in food is important. Moreover, it has been described that binding sites occurring in human probably remain the same in bovine albumin (Jattinagoudar et al., 2016).
In order to identify the P4R binding site on BSA, we performed competitive binding experiments with three specific site probes. It is known that warfarin binding specifically on site I of BSA, while ibuprofen and digitoxin are markers for BSA sites II and III, respectively (Li and Hagerman, 2014).

At pH 7.4 the K values remarkable decreased in the presence of warfarin ($7.08 \pm 0.53 \times 10^5$ L.mol$^{-1}$), while decreased less in the presence of ibuprofen ($1.96 \pm 0.87 \times 10^6$ L.mol$^{-1}$) and increased the presence of digitoxin ($1.23 \pm 0.98 \times 10^7$ L.mol$^{-1}$). These results indicate that P4R competed with warfarin and the BSA/P4R interaction occurs preferably subdomain IIA, named Sudlow’s site I. P4R shows a heterocyclic chemical structure, with four benzene rings that confer hydrophobicity to the molecule, thus justifying its binding on Sudlow’s site I of BSA, since this site comprises a large hydrophobic cavity (Fasano et al., 2005). Considering that many relevant molecules interact with BSA in this same domain with lower K values P4R could be a competitive molecule to interact with BSA and compromising vehicle function of BSA.

At pH 3.5, below the isoelectric point (pI) of BSA, for all temperatures, BSA/P4R complexes showed K values ranging from $3.72 \times 10^6$ L.mol$^{-1}$ to $3.80 \times 10^7$ L.mol$^{-1}$ and “n” around 1.4, suggesting that the existence of one binding site for P4R on BSA.

Thermodynamic parameters for formation of BSA/P4R complex at pH 3.5 are presented at Table 2.

<table>
<thead>
<tr>
<th>T (K)</th>
<th>ΔH° (kJ.mol$^{-1}$)</th>
<th>r$^2$</th>
<th>ΔG° (kJ.mol$^{-1}$)</th>
<th>TΔS° (kJ.mol$^{-1}$)</th>
</tr>
</thead>
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<td>298</td>
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<tr>
<td>308</td>
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<td>0.98</td>
<td>24.93</td>
<td></td>
</tr>
<tr>
<td>318</td>
<td>- 41.51</td>
<td></td>
<td>25.00</td>
<td></td>
</tr>
<tr>
<td>328</td>
<td>- 41.30</td>
<td></td>
<td>24.79</td>
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</table>

The negative values of ΔG° indicated that formation of BSA/P4R complex was favored in all temperatures, being the complex formation driven by both enthalpy and entropy. Negative values of ΔH° resulted mainly of attractive electrostatic interaction between sulfide groups of the dye and amino acid residues; while the entropic contribution came from the increasing in degree of translational freedom of water molecules released from solvation layer of both P4R and BSA during complex formation.

On the contrary to that occurred at physiological pH, at acid condition and at 298 K, the K value for P4R-BSA binding showed a slight reduction in the presence warfarin ($1.99 \times 10^7$ L.mol$^{-1}$) compared to P4R-BSA without any marker ($3.80 \times 10^7$ L.mol$^{-1}$), pointing to another binding site at pH 3.5. However, at this pH BSA is denatured, and therefore, the site I lost its conformation, changing the binding behavior of P4R on BSA. It seems that on denatured BSA, P4R competed with digitoxin for BSA binding, since a considerable reduction was noted in K value at digitoxin presence ($3.56 \times 10^6$ L.mol$^{-1}$).

The values of ΔH° in pH 3.5 were less negative than observed at pH 7.4 where both molecules were negatively charged. These less negative enthalpy value may be attributed to a higher cost of energy to change the conformation of BSA at pH 3.5.
4. CONCLUSION

Interaction between P4R and BSA was investigated at different pH conditions. The results showed P4R and BSA formed a complex driving the enthalpic and entropic contribution independent of pH. However, the conformational change of protein plays an important role in the interaction between the BSA and P4R. This study provided important information about the interaction between the P4R, a synthetic dye, and BSA in a food (pH 3.5) and a blood (pH 7.4) conditions.

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5. References


