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Binding thermodynamics of synthetic dye Allura Red with bovine serum albumin

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ABSTRACT

The interaction between Allura Red and bovine serum albumin (BSA) was studied *in vitro* at pH 7.4. The fluorescence quenching was classified as static quenching due to the formation of AR–BSA complex, with binding constant (K) ranging from 3.26 ± 0.09 to 8.08 ± 0.06 10^4 $L.mol^{-1}$, at the warfarin binding site of BSA. This complex formation was driven by increasing entropy. Isothermal titration calorimetric measurements also showed an enthalpic contribution. The Allura Red diffusion coefficient determined by the Taylor-Aris technique corroborated these results because it reduced with increasing BSA concentration. Interfacial tension measurements showed that the AR–BSA complex presented surface activity, since interfacial tension of the water-air interface decreased as the colorant concentration increased. This technique also provided a complexation stoichiometry similar to those obtained by fluorimetric experiments. This work contributes to the knowledge of interactions between BSA and azo colorants under physiological conditions.

1. Introduction

Despite safety concerns, synthetic food dyes have been used widely in food production to improve appearance and make products more attractive to consumers (de Andrade et al., 2014; Saeed, Abdullah, Sayeed, & Ali, 2010). The most commonly used synthetic dyes contain at least one azo (N=N) functional group and two or three aromatic ring structures, and can be harmful to human health if consumed excessively (de Andrade et al., 2014; Vieira, Esquerdo, Nobre, Dotto, & Pinto, 2014).

Allura Red (E129, Fig. 1) is a water-soluble monoazo colorant, approved by Food and Drug Administration (FDA), responsible for giving a fascinating red color to different kinds of foods, and is also used in the pharmaceutical and cosmetic industries. However, if excessively, consumed Allura Red has been related to allergies, toxic effects, and hyperactivity in children (Pourreza, Rastegarzadeh, & Larki, 2011). Thus its Acceptable Daily Intake (ADI) is, at maximum, 7 mg/kg body weight (bw) per day (European Food Safety Authority, 2015; Food & Applied Nutrition. Certified Color Additives in Food, 2011).

Bovine serum albumin (BSA) is the major soluble protein in cow blood plasma and plays an important physiological role, mainly in transporting various compounds (Shahabadi, Maghsudi, & Rouhani, 2012). BSA has been extensively studied, particularly due to its homology with human serum albumin (HSA) (Bolel, Mahapatra, Datta, & Halder, 2013). The binding of food additives such as food dyes and serum albumins may dramatically affect the structure and transport functions of these proteins, as well as changing the metabolism, distribution, and elimination of food dyes (Shahabadi et al., 2012; Wang, Zhang, & Wang, 2014). Therefore, investigating the interactions between food colorants and BSA is important for the fields of chemistry, life science, and clinical medicine (Bolel, Mahapatra, & Halder, 2012).

Abdullah, Badaruddin, Asad Sayeed, Ali, and Riaz (2008) studied the interaction between BSA and Allura Red by evaluating protein digestibility *in vitro*. They found that Allura Red bound with BSA at low and high temperatures and over a wide pH range (4.0–7.5); however, their interaction did not affect protein digestibility by trypsin and fungal protease. Other work investigated the Allura Red-HSA interaction using UV–vis, fluorescence, and circular

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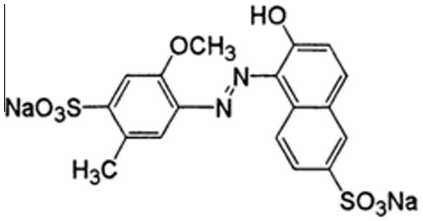

Fig. 1. Chemical structure of Allura Red.

dichroism spectroscopy to determine the Allura Red-HSA binding parameters, and the van't Hoff approach to obtain the thermodynamic parameters of the interaction. They found that fluorescence quenching resulted from the formation of Allura Red-HSA complex (named static mechanism) and not from collision between both molecules (named collisional mechanism). The ΔH° and ΔS° values suggesting that complex formation at pH 7.4 was driven enthalpically and entropically by the occurrence of hydrogen bonds and hydrophobic interactions (Wang et al., 2014).

Despite efforts in the field of food dye-protein interactions, to the best of our knowledge, the thermodynamic parameters of the Allura Red–BSA interaction have not been fully and directly determined. In this work, we report a thermodynamic approach to the Allura Red–BSA interaction using fluorescence spectroscopy, isothermal titration microcalorimetry (ITC), interfacial tension measurements, and the Taylor-Aris diffusion technique.

2. Material and methods

2.1. Materials

BSA (>99% wt.), warfarin (reagent grade), ibuprofen (>98% wt.), digitoxin (>92% wt.), citric acid ($C_6H_8O_7$), tribasic sodium citrate ($Na_3C_6H_5O_7.2H_2O$), bibasic sodium phosphate ($NaHPO_4$) and monohydrated sodium phosphate ($NaH_2PO_4.H_2O$) (all reagent grade) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Allura Red (>85% wt.) was kindly provided by Gemacom (Juiz de Fora, Brazil).

To prepare buffer pH 7.4, we used bibasic sodium phosphate (0.077 mol.L^{-1}) and monohydrated sodium phosphate (0.023 mol.L^{-1}); and for buffer pH 3.5, tribasic sodium citrate (0.020 mol.L^{-1}) and citric acid (0.080 mol.L^{-1}) we used. Deionized water was used in the preparation of both buffers, without addition of any acid or alkali to achieve the target pH.

2.2. Fluorescence experiments

2.2.1. BSA-colorant interaction

Fluorescence spectra were obtained with a CaryEclipse Fluorescence spectrofluorimeter (Agilent, Santa Clara, CA, USA) equipped with a thermostat bath, according to the procedure of Wang et al. (2014) with some modifications. For fluorescence measurements, 3.0 mL of BSA solution (3.01×10^{-5} mol.L^{-1}) containing different concentrations of Allura Red (3.0×10^{-6} to 5×10^{-5} mol.L^{-1}) at pH 7.4 was added to a 1.0 cm quartz cell. Fluorescence emission spectra were then measured at five different temperatures (288, 298, 308, 318 and 328 K) in the range 296–450 nm (excitation wavelength, 295 nm).

2.2.2. Competitive binding studies

Competitive binding studies were carried out using site probes for sites I, II, and III of BSA (warfarin, ibuprofen, and digitoxin, respectively). BSA and site probes were used at a fixed concentration (3.01×10^{-5} mol.L^{-1}), and fluorescence quenching titration (with Allura Red) was performed as described previously at pH 3.5 and 7.4. Thus, the binding parameters for the Allura Red–BSA interaction were determine in the presence of site markers.

2.3. Calorimetric experiments

2.3.1. Isothermal titration calorimetry (ITC)

ITC experiments were conducted at 298 K using a CSC 4200 microcalorimeter controlled by ITCRun software (TA Instruments, New Castle, DE, USA). Aqueous BSA solution at pH 7.4 was prepared at 3.01×10^{-4} mol.L^{-1}. The Allura Red solution (titrant) was prepared at 3.01×10^{-3} mol.L^{-1}, and both solutions were degassed before titration. The sample cell was loaded with 1.8 mL of protein solution and Allura Red solution was loaded into the injection syringe. After baseline stability was achieved, Allura Red solution (10 μL each) was injected into the sample cell 25 times. A dilution experiment was carried out, replacing BSA solution with a pH 7.4 buffer. Samples were stirred constantly at 300 rpm to ensure thorough mixing.

Raw data obtained from a plot of heat flow vs. injection number were transformed using the instrument software to construct a plot of enthalpy change vs. molar ratio. The values of enthalpy change of dilution were subtracted from those acquired in the presence of BSA. The resulting data were fitted in order to obtain the binding constant (k), the binding stoichiometry, and the calorimetric standard enthalpy change (ΔH^{o}_{cal}) for BSA-Allura Red complex formation.

2.3.2. Differential scanning calorimetry (DSC)

DSC analysis was carried out using a nanoDSC model 6300-A (TA Instruments, New Castle, DE, USA). BSA solution (3.01×10^{-5} mol.L^{-1}), at pH 7.4 or 3.5, was added into the cell (0.5 mL). The temperature ranged between 293 and 373 K, with a scan rate of 1 K.min^{-1}. The experiment was repeated using only buffer to achieve a stable and reproducible baseline. A plot of heat capacity vs. temperature was obtained and correlated with the change in BSA conformation.

2.4. Measurement of excess Gibbs free surface energy

The interfacial tension at the water-air interface was measured by a pendant drop method using a goniometer Easy Drop (Kruss GmbH, Hamburg, Germany). Samples of BSA solution (1 mL, 7.57×10^{-6} mol.L^{-1}) prepared in buffer (pH 7.4) containing different concentrations of Allura Red (ranging from 0 to 1.2×10^{-5} mol.L^{-1}) were used. Measurements were carried out at 298 K once a second for 15 min to establish the equilibrium time required to obtain a constant interfacial tension (Amine, Dreher, Helgason, & Tadros, 2014).

2.5. Determination of diffusion coefficient

Allura Red (1.6×10^{-3} mol.L^{-1}) and BSA solutions (concentration range, 0–6.8×10^{-5} mol.L^{-1}) were prepared at pH 7.4. Diffusion coefficient were determined at 298 K using the Taylor-Aris technique, according to Ye et al. (2012), with some modifications. The BSA solutions were used to fill the capillary (length, 30 m; diameter, 0.601×10^{-3} m) and Allura Red solution (20 μL) was injected into the capillary in a laminar flow. At the end of capillary, a UV detector recorded at 502 nm. Each sample was analyzed in triplicate and diffusion coefficient was obtained using Eq. (1).

$$D = \frac{r^2 t_r}{24\sigma^2} \quad (1)$$

where D is diffusion coefficient, r is the capillary radius, t_r is the retention time and σ is the peak width at average height.