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Complexes between ovalbumin nanoparticles and linoleic acid: Stoichiometric, kinetic and thermodynamic aspects

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ABSTRACT

Stoichiometric, kinetic and thermodynamic aspects of complex formation between heat-induced aggregates of ovalbumin (ovalbumin nanoparticles, OVAn) and linoleic acid (LA) were evaluated. Extrinsic fluorescence data were fitted to modified Scatchard model yielding the following results: n: 49 ± 2 LA molecules bound per OVA monomer unit and K_a: $9.80 \pm 2.53 \times 10^5$ M. Kinetic and thermodynamic properties were analyzed by turbidity measurements at different LA/OVA monomer molar ratios (21.5–172) and temperatures (20–40 °C). An adsorption approach was used and a pseudo-second-order kinetics was found for LA-OVAn complex formation. This adsorption process took place within 1 h. Thermodynamic parameters indicated that LA adsorption on OVAn was a spontaneous, endothermic and entropically-driven process, highlighting the hydrophobic nature of the LA and OVAn interaction. Finally, Atomic Force Microscopy imaging revealed that both OVAn and LA-OVAn complexes have a roughly rounded form with size lower than 100 nm.

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1. Introduction

In the last years, development of nutraceutical vehiculization systems has gained considerable interest in food industry (Perez, Sponton, Andermatten, Rubiolo, & Santiago, 2015; Zimet & Livney, 2009). In this sense, several strategies to incorporate these compounds in food aqueous matrices have been evaluated (Gutiérrez et al., 2013; McClements & Li, 2010). One of them includes the formation of ligand-protein complexes, being the ligand a nutraceutical compound. Moreover, biopolymer nanoparticles can be produced by polysaccharide electrostatic deposition onto the surface of preformed ligand-protein complexes in order to improve colloidal stability and nutraceutical protection against environmental conditions such as oxygen, UV radiation, etc. (Perez et al., 2015; Zimet & Livney, 2009).

Overall, ligand-protein interactions are characterized by means of the binding parameters, n and K_a, which represent the binding stoichiometry and association constant, respectively (Joye, Davidov-Pardo, Ludescher, & McClements, 2015; Le Maux, Bouhallab, Giblin, Brodkorb, & Croguennec, 2013; Wang et al., 2013). In some cases, thermodynamic parameters such as enthalpy, entropy and free energy changes associated to ligand-protein complexes formation are also informed (Moyon, Islam, Phukan, & Mitra, 2013; Wang et al., 2013). Ligand-protein interactions can be analyzed by using several techniques such as intrinsic (Sponton, Perez, Carrara, & Santiago, 2015a) and extrinsic fluorescence spectroscopy (Lange, Kothari, Patel, & Patel, 1998), equilibrium dialysis (Muresan, van der Bent, & de Wolf, 2001), and currently, by isothermal titration calorimetry (Le Maux et al., 2013). However, at the present, fluorescence technique is the most widely used method.

In literature, there are a lot of works which characterize ligand-protein interactions in order to determine the intrinsic ability of native proteins to bind hydrophobic and hydrophilic ligands (Joye et al., 2015; Rhodes et al., 2014; Wang et al., 2013). About this, β-lactoglobulin (BLG) is one of the most studied proteins (Le Maux et al., 2013; Perez, Andermatten, Rubiolo, & Santiago, 2014; Perez et al., 2015; Sponton, Perez, Carrara, & Santiago, 2014; Zimet & Livney, 2009). However, studies of interaction between ligands and heat-induced protein aggregates have gained relevance. Moreover, it is well known that protein denaturation/aggregation processes lead to an increase in surface hydrophobicity under certain heat treatment conditions. Thus, higher surface hydrophobicity of protein aggregates can promote greater ligand binding capacity than native proteins (Croguennec, Renault, Beaufils, Dubois, & Pezennec, 2007; Le Maux et al., 2013; Perez

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et al., 2014; Sponton, Perez, Carrara, & Santiago, 2015b; Sponton et al., 2015a).

In a previous work, it was demonstrated that controlled heat treatment of ovalbumin (OVA) dispersion produced ovalbumin aggregates with a mean hydrodynamic diameter lower than 100 nm (ovalbumin nanoparticles, OVAn). These nanoparticles presented higher surface hydrophobicity and higher LA binding ability than native OVA. Moreover, from fluorescence and ζ-potential analysis, it was found that LA binds to OVAn by means of hydrophobic interaction (Sponton et al., 2015a); however, quantitative parameters were not determined. Hence, in order to quantify parameters associated to LA-OVAn interaction, additional studies should be performed taking into account stoichiometric, kinetic and thermodynamic aspects. The knowledge of these parameters would allow reaching a better understanding of the LA-OVAn complexes formation.

Linoleic acid is a polyunsaturated fatty acid (PUFA) which is essential to human diet because is not synthesized endogenously by humans. PUFAs play an important role in cardiovascular and inflammatory disease, mental health and brain development and function (Ruxton, Reed, Simpson, & Millington, 2004). In general, fatty acids have very low water solubility, which limit their incorporation in aqueous systems. On the other hand, they can form supramolecular structures (micelles, vesicles, etc.) in some buffer solutions, resulting in stable colloidal dispersion with appreciable turbidity (Fontana, Spolaore, & Polverino de Laureto, 2013; Sponton et al., 2015a). Moreover, PUFAs are susceptible to oxidative deterioration (Fioramonti, Arzeni, Pilosof, Rubiolo, & Santiago, 2015), so that complexation with protein could be a strategy for protect them.

In this context, the aim of the present work was to evaluate the stoichiometric, kinetic and thermodynamic aspects of LA-OVAn complex formation. This information would allow defining the most appropriate conditions to produce innovative nutraceutical vehiculization systems. For this, a set of complementary techniques and models were employed, including an adsorption mechanism to describe LA-OVAn complexes formation. Finally, AFM images were also included in order to describe some morphological and structural aspects.

2. Materials and methods

2.1. Materials

Ovalbumin (product A5503, purity 98% according to agarose gel electrophoresis) and linoleic acid (LA) samples were obtained from Sigma (USA). Extrinsic fluorescence probe, 1-anilino-8-naphthalene sulfonic acid (ANS), was purchased from Fluka Chemie AG (Switzerland).

2.2. Ovalbumin nanoparticles obtention

Ovalbumin nanoparticles (OVAn) were obtained by heat treatment (85 °C, 5 min) of 10 g/L OVA solution at pH 7.5 and 50 mM NaCl according to a previous work (Sponton et al., 2015a). The obtained OVA nanoparticles showed a monomodal particle size distribution (determinated by dynamic light scattering, DLS) with peak: 79 nm and polydispersity index (PdI): 0.208 as described in Sponton et al. (2015a).

2.3. Binding stoichiometry

In order to study LA-OVAn binding stoichiometry, fluorescence and turbidity (absorption at 400 nm) measurements were done. It is important to remark that binding parameters were expressed on the basis of OVA monomeric unit (i.e. considering individual OVA macromolecules), due to the ovalbumin aggregates are formed by the association of OVA molecules (Le Maux et al., 2013).

2.3.1. Fluorescence measurements

In order to determine the binding stoichiometry, i.e. number of LA molecules bound per OVA monomeric unit (n) and the association constant (K_a), an extrinsic fluorescence method was applied. For this, samples were prepared adding increasing volumes of 15 mM LA ethanolic solution to 2 ml of 0.043 g/L OVAn solution in 50 mM pH 7 potassium phosphate buffer. Then, 9 μL of 15 mM ANS solution was added to each sample. This experiment was performed under the assumption that ANS would not displace LA molecules from the LA-OVAn complexes (Lange et al., 1998; Takikawa & Kaplowitz, 1986). Emission spectra were registered between 410 and 600 nm, at 390 nm excitation wavelength by using a Hitachi F-2000 fluorescence spectrophotometer (Japan) (Croguennec et al., 2007; Sponton et al., 2015a). Experiments were performed in triplicate at room temperature (around 20 °C). ANS maximum emission fluorescence intensity (F) for each spectrum was registered, and relative fluorescence (RFI-ANS) was calculated as the ratio between F at a given LA concentration, and F at zero LA concentration (F_0). Titration curves were plotted as RFI-ANS *versus* LA concentration, and data were fitted to the modified Scatchard model:

$$[P] \cdot (1 - f_i) = \frac{[L]}{n} \cdot \left(\frac{1}{f_i}\right) - \frac{1}{K_a \cdot n} \quad (1)$$

where [P] is the total molar protein concentration (based on OVA monomer); [L] is the total molar ligand concentration; n is the number of ligand molecules bound to protein at the saturation and f_i is defined as:

$$f_i = \frac{F - F_0}{F_{max} - F_0} \quad (2)$$

where F_{max} is the fluorescence intensity at saturation. It is important to highlight that when F_{max} is not reached experimentally, its value can be determined by data fitting to an exponential decay model (Le Maux et al., 2013).

2.3.2. Turbidity measurements

In previous works, it was reported that LA dispersed in phosphate buffer showed appreciable turbidity as a consequence of supramolecular self-assemblies formation. However, when OVAn is added to LA dispersion, turbidity decreased as a consequence of the disassembly of micelles in favor of interaction with protein (LA-OVAn complexes formation) forming a stable colloidal system (Sponton et al., 2015a, 2015b). In the present paper, we have extended such studies for deepening the knowledge about binding stoichiometry of the LA-OVAn complex formation. Thus, turbidity of LA dispersions was determined as the absorbance (ABS) at 400 nm using a Jenway 7305 Spectrophotometer (UK). Systems were prepared adding increasing volumes of 100 mM LA ethanolic solution to 2 ml of 0.5 g/L OVAn in 50 mM pH 7 potassium phosphate buffer solution, up to 1.2 mM maximum total LA concentration. Measurements were performed at room temperature (~20 °C) in triplicate.

2.4. Kinetics of complex formation

Kinetics of LA-OVAn complexes formation was analyzed by means of a turbidity approach. Turbidity measurements were carried out in a Perkin Elmer Lambda 20 UV/Vis Spectrometer (USA) equipped with a water thermo-circulator. Determinations were performed in a temperature range of 20–40 °C. In first place,

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