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Formation and colloidal stability of ovalbumin-retinol nanocomplexes

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ABSTRACT

This work is aimed to obtain and characterize nanocomplexes formed by non-covalent attractive interactions between ovalbumin (OVA) and retinol (RET). In order to gain some ideas about the effect of OVA aggregation on RET complexation, OVA nanoparticles (OVAn) obtained by controlled heat treatment (85 °C, 5 min) were also evaluated. Nanocomplex formation was monitored by intrinsic and extrinsic fluorescence spectroscopy. Results suggest that OVA was more effective for binding RET than OVAn, possibly because aggregate formation could employ some hydrophobic patches needed for RET binding. Furthermore, nanocomplex colloidal stability was studied by means of absorbance (at 400 nm), particle size distribution (PSD) and ζ potential determinations. At pH values of common foods (4.0 and 7.0), nanocomplex colloidal stability mainly depended on aqueous phase behaviour of the proteins (OVA and OVAn). Finally, long-term photochemical stability against light and oxygen of RET nanocomplexes depended on solution pH and protein aggregation.

1. Introduction

Retinol (RET) or vitamin A is a fat-soluble compound critical for the correct performance of many biological processes including vision, fetal growth, immune response, cell differentiation and proliferation, and it plays an important role in different pathologies (Stephensen, 2001; Sun & Kawaguchi, 2011). It is the immediate precursor to two important active metabolites or retinoids: retinal, which plays a key role in vision; and retinoic acid, which serves as an intracellular messenger in gene transcription (Noy, 2000). Humans cannot synthesize it, so its intake must be performed through consumption of vegetables and fruits (e.g. sweet potatoes, carrots, dark leafy greens, winter squashes, lettuce, dried apricots, cantaloupe, bell peppers, fish, liver, and tropical fruits) rich in provitamin A carotenoids (Castenmiller & West, 1998).

RET deficiency cases are usually reported in developing countries, so that food fortification strategies should be implemented (Sommer & Davidson, 2002). However, RET incorporation into foods could present two disadvantages: (i) their poor solubility in water, which turn it generally incompatible with aqueous matrices, and (ii) their high sensitivity to light and oxygen, which leads to their photochemical decomposition, and consequently, to the deterioration of their biological properties (Allwood & Plein, 1986; Failloux, Bonnet, Perrier, & Baron, 2004; Vilanova & Solans, 2014). In order to solve these problems, several encapsulation technologies could be implemented, e.g. emulsions (Carlotti, Rossatto, & Gallarate, 2002), micelles (Blayo, Marchal, Lange, & Dumay, 2014), lipid solid nanoparticles (Jenning & Gohla, 2001), liposomes (Lee et al., 2002), polymer systems (Hwang, Oh, & Oh, 2005), etc. Nevertheless, these technologies have not been widely applied in food industry (Loveday & Singh, 2008).

The ability for binding lipophilic ligands that shows some globular proteins could be used as a strategy for introducing RET into food matrices. In this sense, the main milk whey protein, β-lactoglobulin (BLG), is the most recognized protein showing the ability for binding RET (Muresan, van der Bent, & de Wolf, 2001; Puyol, Perez, Ena, & Calvo, 1991). This ability is driven by non-covalent attractive forces, involving particular protein domains. BLG has two hydrophobic pockets that are potentially capable for binding lipophilic ligands: one in *calyx* formed by β-barrel, and the other one between α-helix and β-barrel surface. Although binding sites for ligands remain controversial, most evidence indicates that RET binds at the *calyx* (Kontopidis, Holt, & Sawyer, 2002; Wang, Allen, & Swaisgood, 1999). Finally, it was demonstrated that the BLG-RET complexes formation promoted a considerable RET protection against heat, oxidation and UV radiation (Hattori, Watabe, &

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Takahashi, 1995; Shimoyamada, Yoshimura, Tomida & Watanabe, 1996).

In order to extend the application of RET vehiculization strategies based on protein-ligand complexes, the current research should be focused to the evaluation of other food globular proteins. In this sense, the main protein of egg white protein (EWP), ovalbumin (OVA), could be used because of its great availability in the agro-food sector. OVA is a monomeric protein of 43 KDa, and it has 4 sulfhydryl groups (-SH) and one disulfide bond (S-S) per monomer (Weijers & Visschers, 2002). It is constituted of 385 amino-acids, from which a half is hydrophobic and mainly buried into the protein structure and a third are charged aminoacids (Nisbet, Saundry, Moir, Fothergill, & Fothergill, 1981). The OVA properties to bind hydrophobic ligand have been poorly studied; furthermore, OVA ability to bind RET is not known at the present. According to this, the aim of the present paper was to study the obtention of nanocomplexes formed by non-covalent attractive interactions between RET and OVA. Moreover, because of heat-induced protein aggregates are often produced in food processing (Croguennec, Renault, Beaufils, Dubois, & Pezennec, 2007; Le Maux, Bouhallab, Giblin, Brodkorb, & Croguennec, 2013; Perez, Andermatten, Rubiolo, & Santiago, 2014; Sponton, Perez, Carrara, & Santiago, 2015a; Sponton, Perez, Carrara, & Santiago, 2015b), this research was extended to the evaluation of nanosized heat-induced OVA aggregate (OVAn) previously characterized in our lab (Sponton et al. 2015b). In recent papers, the ability of OVAn for binding a model hydrophobic ligand, linoleic acid (LA), was highlighted (Sponton et al., 2015a, 2015b, Sponton, Perez, Carrara, & Santiago, 2016). Hence, OVAn could be assayed for knowing the effect of controlled heat treatment on OVA ability to bind RET. For predicting the nanocomplex colloidal stability at pH values of common foods, particle size and ζ potential measurements were performed. Finally, the photochemical stability of RET nanocomplexes was also examined.

2. Materials and methods

2.1. Materials

Native ovalbumin (OVA, product A5503, purity 98% according to agarose gel electrophoresis) was purchased from Sigma (USA). Ovalbumin heat-induced nanoparticle (OVAn) was produced according to Sponton et al. (2015b). Briefly, OVA dispersion was prepared at 10 g/L, 50 mM NaCl and pH was adjusted 7.5 by using 0.1 M NaOH. Then, 2 mL aliquots were dispensed in glass tubes and were heated in a water bath at 85 °C for 5 min. Subsequently, tubes were removed and immediately cooled in an ice bath. Tubes containing OVAn were kept at 4 °C until further analysis. Retinol (RET, product 17772, purity ≥95.0% according to HPLC) was also obtained from Sigma (USA). RET was kept in darkness under N_2 atmosphere at −20 °C according to manufacturer advice. Fluorescence probe 1-anilino-8-naphthalene sulfonic acid (ANS) was purchased from Fluka Chemie AG (Switzerland). Additional analytical reagents were supplied from Cicarelli (Argentina).

2.2. Protein-retinol complexes formation

2.2.1. Intrinsic fluorescence

Formation of OVA-RET and OVAn-RET complexes was monitored by intrinsic fluorescence spectroscopy. For this, OVA and OVAn were dispersed in phosphate buffer (pH 7, 50 mM) at 1 μM final protein concentration. On the other hand, a stock RET solution was prepared at 20 mM concentration in ethanol. Then, 3 mL of protein (OVA or OVAn) dispersion was titrated with increasing volumes (0–30 μl) of stock RET solution. It is important to remark that final ethanol concentration in mixed systems was lower than 1 vol%; therefore, it could be assumed that no protein structural modifications were induced (Cogan, Kopelman, & Shinitzky, 1976). Protein-RET complexes dispersions were stored in darkness for 2 h for reaching equilibrium. Intrinsic fluorescence experiments were performed in a Hitachi F-2000 spectrophotometer (Japan) exciting at 280 nm (Trp and Tyr excitation), and registering emission spectra between 300 and 425 nm. Fluorescence intensity (FI) data were corrected by inner filter effect due to RET absorption at 250–400 nm, according to van de Weert and Stella (2011). Corrected FI (FI_{corr}) data for OVA-RET and OVAn-RET complexes were obtained as following:

$$FI_{corr} = FI_{obs} \times 10^{\frac{A_{ex}d_{ex}}{2} + \frac{A_{em}d_{em}}{2}} \quad (1)$$

where FI_{obs} is the observed fluorescence intensity, A_{ex} and A_{em} are the measured change in absorbance value at the excitation and emission wavelength, respectively, caused by ligand addition, and d_{ex} and d_{em} are the cuvette length (cm) in direction of excitation and emission, respectively. Absorption spectra for OVA-RET and OVAn-RET complexes were collected from 250 to 450 nm by using a Perkin Elmer Lambda 20 UV/Vis spectrophotometer (USA). The FI_{corr} values at the maximum emission wavelength were also expressed in terms of relative fluorescence intensity (RFI), being $RFI = FI/FI_0$, where FI is the intrinsic fluorescence intensity of protein-RET complexes and FI_0 corresponds to intrinsic fluorescence intensity of pure protein (OVA or OVAn) in solution (Perez et al., 2014; Sponton, Perez, Carrara, & Santiago, 2014). Usually, ligand addition to protein dispersions can produce increasing or decreasing RFI values, which would correspond to the formation of protein-ligand complexes in solution (Frapin, Dufour, & Haertle, 1993; Le Maux, Bouhallab, Giblin, Brodkorb, & Croguennec, 2013; Perez, Sponton, Andermatten, Rubiolo, & Santiago, 2015; Sponton et al., 2015b).

Furthermore, FI_{corr} data were used to calculate the binding parameters: stoichiometry or number of RET molecules bounds (n) and association constant (K_a). For this, modified Scatchard model was applied (Le Maux et al., 2013):

$$P_{total}(1 - f_i) = \frac{[RET]}{n}\left(\frac{1}{f_i} - 1\right) - \frac{1}{nK_a};\ f_i = \frac{FI_i - FI_0}{FI_{máx} - FI_0} \quad (2)$$

where P_{total} is the total protein (OVA or OVAn) concentration, RET is the retinol concentration, n is the number of RET molecules bound at protein saturation, K_a is the association constant, f_i is the fraction of one site of the protein occupied by the RET and FI_0, FI_{max} and FI_i are the fluorescence intensity initial, at saturation and at the ligand/protein ratio, respectively. It is important to remark that OVAn molar concentration was expressed in terms of native OVA monomeric unit (Sponton et al 2016). Fluorescence and absorption measurements were performed at room temperature (20 °C) in triplicate.

2.2.2. Extrinsic fluorescence

The binding mode of RET to proteins (OVA and OVAn) was examined by extrinsic fluorescence spectroscopy. Extrinsic experiments were conducted by using ANS as a fluorescent probe. Usually, ANS is bound to proteins by non-covalent attractive forces, mainly by hydrophobic interactions. OVA and OVAn were dispersed in phosphate buffer (pH 7, 50 mM) at 1 μM final protein concentration. Then, protein-RET complex dispersions were prepared as it was described previously. Subsequently, 9 μl of 15 mM ANS solution were added to each system (Perez, Carrera-Sanchez, Rodriguez-Patino, Rubiolo & Santiago, 2012). Extrinsic fluorescence emission spectra were obtained from 420 to 600 nm at 390 nm excitation