β-Lactoglobulin heat-induced aggregates as carriers of polyunsaturated fatty acids

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ABSTRACT

The aim of this work was to obtain heat-induced β-lactoglobulin (BLG) aggregates in order to test them as carriers of a model polyunsaturated fatty acid (PUFA), linoleic acid (LA). BLG aggregates were obtained at 85 °C by varying the heating time (0–60 min) and pH of protein dispersion (6.5–7.5). Aggregates were characterised by intrinsic and extrinsic fluorescence and surface hydrophobicity (S_0). Binding experiments were conducted by fluorescence spectroscopy. Results showed increased BLG aggregate S_0 values which could strongly depend on the pH of aggregate formation. Aggregates obtained at pH 6.5 showed the greatest S_0 values, so they could find application as LA carriers. Nevertheless, conjugation of LA to BLG aggregates showed complex behaviour depending on the aggregate producing conditions (pH, heating time and/or combination). The LA binding properties of BLG aggregates were not linked to their hydrophobic characteristics, suggesting that conjugation could require the structural preservation of the LA binding site.

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1. Introduction

β-Lactoglobulin (BLG) is one of the most studied proteins in the field of food chemistry. Its molecular characteristics have been extensively researched in the last few decades (Bhattacharjee & Das, 2000; Bolder, Vasbinder, Sagis, & van der Linden, 2007). The acquired knowledge has allowed understanding of BLG functional properties, leading to development of new technologies and food products (Matalanis, Jones, & McClements, 2011; McClements & Li, 2010).

Currently, BLG binding properties have received considerable attention due to their potential uses in nutraceutical delivery strategies (Livney, 2010; Sponton, Perez, Carrara, & Santiago, 2014; Zimet & Livney, 2009). BLG has the ability to solubilise and transport lipophilic bioactive nutrients such as retinol, fatty acids and vitamin D, and it is believed that this property could be linked to a possible biological function (Qin, Creamer, Baker, & Jameson, 1998; Wang, Allen, & Swaisgood, 1999). BLG is classified into a super family of carriers of small hydrophobic molecules called lipocalins (Kontopidis, Holt, & Sawyer, 2004). These proteins share a tridimensional structure which consists of eight stranded antiparallel β-sheets forming a hydrophobic β-barrel (Frapin, Dufour, & Haertle, 1993; Sawyer & Kontopidis, 2000). Therefore, BLG binding properties could be utilised for the development of nano and microparticles for encapsulation of lipophilic bioactive compounds (polyunsaturated fatty acids, carotenoids, polyphenols, etc.) in order to protect them from deterioration factors such as oxygen, UV radiation, moisture, temperature, etc. (McClements & Li, 2010; Zimet & Livney, 2009).

On the other hand, it is well known that BLG functional properties are easily modified by different processes including: heating (Durand, Gimel, & Nicolai, 2002; Foegeding, 2006), high pressure (Lee, Lefèvre, Subirade, & Paquin, 2009), enzymatic hydrolysis (Perez, Carrera Sánchez, Rodríguez Patino, Rubiolo, & Santiago, 2012a; Perez, Carrera Sánchez, Rodríguez Patino, Rubiolo, & Santiago, 2012b), etc. In a recent paper, Fioramonti, Perez, Aríngoli, Rubiolo, and Santiago (2014) have shown that a controlled heat treatment could promote conformational changes in the tertiary structure of whey proteins, affecting their functional properties and interactions with other molecules. Structural modifications were attributed to exposure of reactive and hydrophobic amino acid residues buried in the protein (Cairoli, Iametti, & Bonomi, 1994; Iametti, De Gregori, Vecchio, & Bonomi, 1996). Therefore, the induction of a suitable structural modification could be used in the development of delivery systems with improved encapsulation efficiency, changes in binding affinity and/or have repercussions on the release of lipophilic bioactive molecules (Fioramonti

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et al., 2014; Santipanichwong, Suphantharika, Weiss, & McClements, 2008; Sponton et al., 2014).

According to literature, formation and characteristics of BLG aggregates strongly depend on heating temperature and on bulk conditions such as pH, ionic strength, salt type (Durand et al., 2002; Hoffmann & van Mil, 1997; Nicolai, Britten, & Schmitt, 2011; Verheul & Roefs, 1998) and cosolute presence, such as sorbitol and glycerol (Chanasattru, Jones, Decker, & McClements, 2009). Around neutrality, the formation of protein filamentous aggregates was reported, whereas at pH values close to pI (4.8–5.2), spherical or particulate aggregates were obtained (Hoffmann & van Mil, 1997; Jung, Savin, Pouzot, Schmitt, & Mazzenga, 2008; Schmitt et al., 2009; Verheul & Roefs, 1998). Thus, aggregate morphology could be strongly linked to the bulk environment conditions. Although BLG aggregates have been produced and characterised in different conditions, there is not enough information about their potential uses as carriers of lipophilic bioactive molecules. Moreover, there are not enough data about the hydrophobicity of BLG aggregates and its relationship with fatty acid binding properties.

In this context, the aim of this work was to obtain heat-induced BLG aggregates in order to test them as carriers of a model polyunsaturated fatty acid (PUFAs), linoleic acid (LA). Our hypothesis was to modify the BLG binding properties by heat treatment in order to observe if BLG aggregates had an improved ability for binding LA. Modifications in heat-induced aggregate hydrophobicity could promote important changes in encapsulation properties. Therefore, this information could be interesting for the development of new PUFAs encapsulation strategies and thus novel nutraceutical products.

2. Materials and methods

2.1. Materials

β-Lactoglobulin (BLG) sample was provided by Davisco Food International (USA) and its chemical composition was (wt.%): 90.82% protein, 0.20% fat, 1.90% ash, 4.80% moisture and 2.28% others. In conjugation experiments, linoleic acid (cis, cis-9, 12-octadecadienoic acid) was used as a model of poly-unsaturated fatty acid (PUFA). Linoleic acid (LA) was purchased from Sigma (USA).

2.2. Production of heat-induced aggregates

BLG stock dispersion at 2.0 wt.% was prepared in ultrapure Milli-Q water. In order to complete the protein hydration, the stock dispersion was stirred for 2 h at room temperature (25 °C). Next, the BLG stock dispersion was filtered using a glass microfibre pre-filter and cellulose ester filters of 0.22 and 0.45 μm pore size (Millipore, USA). From the filtered stock dispersion, 1.0 wt.% BLG dispersions were prepared at pH 6.5, 7.0 and 7.5 using 0.1 M HCl or 0.1 M NaOH (Schmitt et al., 2009). BLG dispersions at different pH values were shaken for 30 min and stored at 4 °C overnight. Afterwards, 15 ml of each sample was dispensed into a glass tube (diameter 2 cm and height 15 cm) and subsequently subjected to heat treatment in a water bath (Dalvo instruments, BTMP model) at 85 °C and for different lengths of time: 15, 30, 45 and 60 min. The heating temperature was selected according to Fioramonti et al. (2014) who have shown that at 85 °C whey protein aggregate formation with improved hydrophobic properties can be produced. Finally, the heat-induced aggregate dispersions were allowed to cool in a water bath at room temperature. It is noteworthy that after heat treatment the pH of the aggregate dispersions was monitored and no pH variations were registered. Subsequently, these samples were stored at 4 °C overnight.

2.3. Characterisation of heat-induced aggregates

2.3.1. Determination of denatured/aggregated protein content

Denatured/aggregated protein content (DP) was determined according to the method described by Meza, Verdini, and Rubiolo (2009). Treated BLG dispersions were equilibrated at room temperature and the pH was adjusted to 4.6 using 0.1 M HCl or 0.1 M NaOH (de Wit, Hontelez Back, & Adamse, 1988; Verheul & Roefs, 1998). Afterwards, dispersions were centrifuged at 20,000g for 30 min at 10 °C (Heal force/Neofuge 18R, China). Supernatant aliquots were taken and soluble protein concentration (SP) was measured at 280 nm using a UV–Vis spectrophotometer (Lambda 20, Perkin Elmer). DP values were calculated according to the following expression:

$$DP = \frac{TP - SP}{TP} \times 100\% \tag{1}$$

where TP is the total protein concentration (1.0 wt.%). All determinations were performed at least in triplicate.

2.3.2. Spectroscopic characteristics of heat-induced aggregates

In order to evaluate some molecular characteristics of heat-induced BLG aggregates, fluorescence (intrinsic and extrinsic) spectroscopy was applied (Fioramonti et al., 2014). Protein aggregate dispersions were prepared by re-dispersion of the pellets obtained in the previous section. Pellet re-dispersion was performed in potassium phosphate buffer pH 7, 0.05 M and the protein aggregate concentration was calculated as the difference between TP (1.0 wt.%) and SP (as described in the previous section). For determination of spectroscopic characteristics, protein aggregate concentration was adjusted to 0.01 wt.% by dilution using the same buffer. Fluorescence measurements were performed at room temperature (25 °C) using a spectrofluorimeter (Hitachi 2000, Japan) equipped with a 1-cm pathlength quartz cell. Intrinsic fluorescence determination (due to Trp emission) was performed at 295 nm excitation wavelength and the emission spectra were recorded at 300–450 nm. Maximum fluorescence intensity (FI^M_{Trp}) and its corresponding wavelength (λ^M_{Trp}) were identified from the spectra.

Extrinsic fluorescence measurements were performed using the hydrophobic probe 1-anilino-8-naphtalenesulphonic acid (ANS, Fluka Chemie), which binds onto the protein surface mainly via hydrophobic interactions. For this, 100 μl 8.0 mM ANS were added to 2 ml of BLG aggregate dispersion. The excitation wavelength was 350 nm and the emission spectra were recorded between 400 and 600 nm. The maximum fluorescence intensity (FI^M_{ANS}) and the wavelength corresponding to this maximum (λ^M_{ANS}) were identified from the spectra (Sponton et al., 2014). All measurements were performed in triplicate.

Surface hydrophobicity (S_0) measurements were made according to the method of Kato and Nakai (1980). Serial dilutions from BLG aggregate dispersions were performed with potassium phosphate (pH 7.0, 0.05 M) and 10 μl of 8.0 mM ANS were added to each dilution. ANS fluorescence intensity (FI) was determined at 350 nm excitation wavelength and at 470 nm emission wavelength (Perez, Carrara, Carrera Sánchez, Rodríguez Patino, & Santiago, 2009). The slope of an FI vs. protein concentration plot was considered to be a measure of S_0. Experiments were performed at least in triplicate.

2.4. Conjugation of LA to heat-induced aggregates

In order to know the suitable conjugation conditions, assays with native BLG were conducted. Native BLG dispersion was prepared at 0.6 wt.% in potassium phosphate buffer pH 7.0, 0.05 M. This dispersion was stirred for 1 h at room temperature and subsequently filtered as described above. Dilutions from native

BLG dispersion were prepared in the concentration range of 0.04–0.08 wt.%. Also, a stock ethanolic solution of 4.0 M LA was prepared. A conjugation process was carried out by titration of 2 ml BLG dispersion with increasing aliquots of 4.0 M LA stock solution. The range of LA concentration was 0–50 µM. It is important to highlight that in no cases did final ethanol concentration exceed 2%, therefore, no protein structural modification could take place (Cogan, Kopelman, & Shinitzky, 1976). After LA addition, tubes were vigorously stirred for 2 min. It was assumed that ethanol dissipates in aqueous medium and that LA molecules conjugate to BLG (Sponton et al., 2014). Conjugation was monitored by means of fluorimetry at excitation and emission wavelength, 295 and 332 nm, respectively. Titration curves were obtained by plotting the relative fluorescence intensity (RFI) as a function of LA concentration (Frapin et al., 1993). RFI values were calculated as: RFI = F/F_0, where F is the fluorescence intensity of LA-BLG conjugates and F_0 corresponds to fluorescence intensity of pure BLG. Blank solutions were made by titration of 2 ml of buffer solution with increasing aliquots of LA stock solution. Binding parameters, number of binding sites (n) and apparent dissociation constant (K'_d), were calculated from titration curves according to the method developed by Cogan et al. (1976). Parameters were obtained through lineal regression from the ($P_0 \cdot \alpha$) vs. $R_0 \cdot [\alpha/(1 - \alpha)]$ plot which corresponded to the following equation:

$$P_0 \cdot \alpha = \frac{1}{n} \cdot \frac{R_0 \cdot \alpha}{1 - \alpha} - \frac{K'_d}{n} \qquad (2)$$

where P_0 is the total protein concentration (µM), R_0 is the total LA concentration, α is the fraction of ligand binding sites in free protein molecules and is given by:

$$\alpha = \frac{\text{RFI}_{\text{Trp Max}} - \text{RFI}_{\text{Trp}}}{\text{RFI}_{\text{Trp Max}} - \text{RFI}_{\text{Trp0}}} \qquad (3)$$

where RFI_{Trp0}, RFI and RFI_{max} are relative fluorescence intensity at zero LA concentration, at a given concentration and at the LA saturation, respectively.

Conjugation of LA to heat-induced BLG aggregates was monitored by fluorimetry as mentioned above. In these experiments, emission at wavelength 338 nm was detected due to redshift behaviour. All determinations were performed at least in triplicate.

2.5. Statistical analysis

Statistical differences were determined through one way analysis of variance (ANOVA) using StatGraphics Plus 3.0 software. For this, LSD testing, a 95% confidence level was applied.

3. Results and discussions

3.1. BLG aggregation

The experimental conditions (pH and heating time) for heat-induced BLG aggregate production were evaluated. Fig. 1 shows the effect of heating time (0–60 min) and aqueous medium pH (6.5–7.5) on denatured/aggregated protein content (DP). In general, it was observed that DP values gradually increased with heating time. At a constant temperature of 85 °C, the increase in heating time could promote the aggregate formation due to gradual exposure of hydrophobic and sulfhydryl residues buried in the BLG (Baussay, Nicolai, & Durand, 2006; Hoffmann & van Mil, 1997). Different mechanisms for BLG aggregate formation have been described. However, the kinetic model proposed by de Jong, Bouwman, and van der Linden (1992) could be most suitable for describing the heat-induced BLG aggregation process. This model consists of two stages: (i) denaturation and (ii) aggregation. The
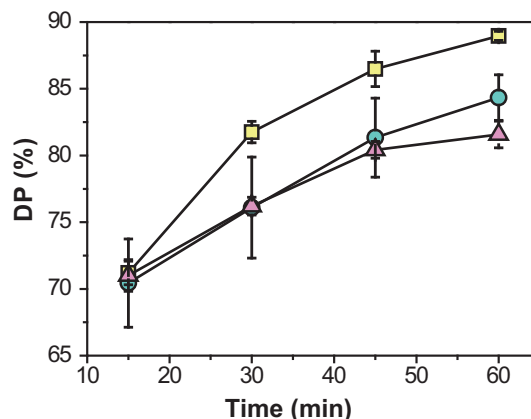

Fig. 1. Effect of heating time and aqueous medium pH on denatured/aggregated BLG content (DP). Symbols pH: 6.5 (■), 7.0 (●) and 7.5 (▲). Heating temperature (85 °C). WPI concentration 1.0 wt.%.

denaturation stage is considered as an equilibrium first order reaction between native and partially unfolded protein. In this stage, BLG dimers dissociate into monomers and the free sulfhydryl group is exposed on the protein surface (Hoffmann & col., 1997). The second step consists of several aggregation reactions. In contrast to the first stage, these ones are bimolecular second order reactions, driving the irreversible formation of protein aggregates via thiol-disulphide interchange reactions and disulphide bounds (Verheul & col., 1998). In addition to covalent bonds, other non covalent interactions (ionic, hydrophobic and van der Waals) could be involved in heat-induced BLG aggregation processes (Hoffmann & van Mil, 1997).

It was observed that at a given heating time, BLG dispersions heated at pH 6.5 showed the highest DP values, whereas no significant differences ($p < 0.05$) were observed for DP values of BLG dispersions heated at pH 7.0 and 7.5 (except at 60 min). It is possible that greater BLG aggregation at pH 6.5 is promoted, considering that proteins are in proximity (low electrostatic repulsion) when pH is relatively close to pI (Verheul & Roefs, 1998). At pH far enough from pI, low protein aggregate formation could be produced as consequence of an increased protein electrostatic repulsion (Majhi et al., 2006; O'Kennedy et al., 2006).

3.2. Some molecular characteristics of heat-induced BLG aggregates

As mentioned previously, our hypothesis was that heat treatment could promote the formation of BLG aggregates with increased hydrophobic properties and so the ability to bind PUFAs might be improved. In this way, some molecular characteristics of heat-induced BLG aggregates were evaluated by means of fluorescence (intrinsic and extrinsic) spectroscopy.

3.2.1. Intrinsic fluorescence

Intrinsic fluorescence strongly depends on the different conformations that a protein adopts in response to bulk solution conditions, such as pH, ionic strength, temperature, etc. Therefore, it could very be sensitive to protein structural changes upon heat treatment. BLG has two Trp residues: Trp_{19} and Trp_{61} (Bhattacharjee & Das, 2000). Trp_{19} is located at the base of the central hydrophobic *calyx*, while Trp_{61} is found close to the protein surface (Wang, Allen, & Swaisgood, 1998). BLG intrinsic fluorescence is mainly due to Trp_{19} (~70% of the fluorescence) while Trp_{61} is a minor contributor due to the adjacent disulphide link Cys_{160}–Cys_{166}, which decreases its fluorescence (Fessas, Lametti, Schiraldi, & Bonomi, 2001). Fig. 2 shows the effect of heating time (0–60 min)
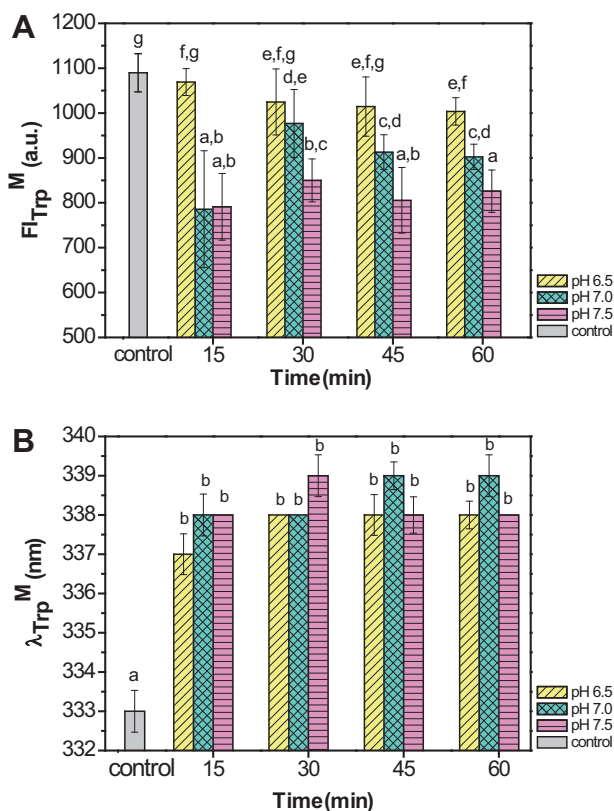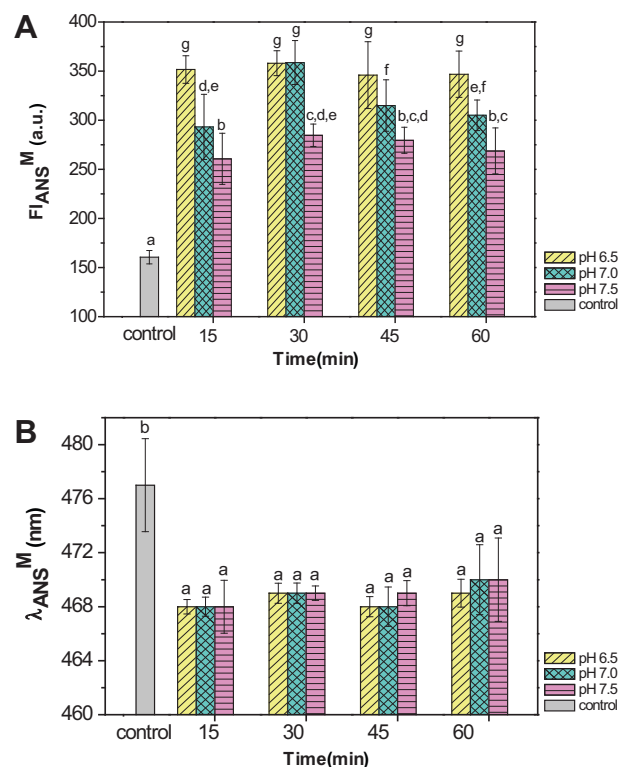
Fig. 2. Effect of heating time and aqueous medium pH on the maximum emission fluorescence intensity, FI_{Trp}^{M} (A) and its corresponding wavelength, λ_{Trp}^{M} (B). In legend pH refers to pH of BLG aggregate obtention and control refers to BLG without treatment (native). Measurement conditions: pH 7.0, 25 °C and protein or aggregate concentration 0.01 wt.%. Different letters indicate significant differences ($p < 0.05$).

Fig. 3. Effect of heating time and aqueous medium pH on the ANS maximum emission fluorescence intensity, FI_{ANS}^{M} (A) and its corresponding wavelength, λ_{Trp}^{M} (B). In legend pH refers to pH of BLG aggregate obtention and control refers to BLG without treatment (native). Measurement conditions: pH 7.0, 25 °C and protein or aggregate concentration 0.01 wt.%. Different letters indicate significant differences ($p < 0.05$).

at 85 °C and pH (6.5–7.5) on the maximum intrinsic fluorescence intensity, FI_{Trp}^{M} (Fig. 2A) of BLG dispersion (1.0 wt.%) and its corresponding wavelength, λ_{Trp}^{M} (Fig. 2B). FI_{Trp}^{M} and λ_{Trp}^{M} values for BLG dispersion without heat treatment were added as controls. With respect to the BLG control, it was observed that BLG aggregates obtained at pH 6.5 and 15, 30 and 45 min heating time showed no significant differences ($p < 0.05$) for FI_{Trp}^{M} values (Fig. 2A). These results could indicate that heat treatment caused minor conformational changes in protein tertiary structure (Bhattacharjee & Das, 2000). However, BLG aggregates obtained at pH 6.5 and 60 min, and at pH 7.0 and 7.5 for all evaluated heating times showed a significant decrease in FI_{Trp}^{M} values ($p < 0.05$), suggesting the existence of important changes on protein tertiary structure as consequences of heat treatment (Cairoli et al., 1994). Furthermore, as can be observed in Fig. 2B, all BLG aggregates showed increased λ_{Trp}^{M} values. This result corresponds to a redshift behaviour related to a Trp_{19} exposure in a more polar microenvironment, probably due to some enhanced penetration of water molecules around the Trp_{19} in the aggregates. (Bhattacharjee & Das, 2000; Vivian & Callis, 2001). However, this Trp_{19} exposure was observed to be independent of pH magnitude and heat treatment time.

3.2.2. Extrinsic fluorescence

Extrinsic fluorescence can be determined from the molecular interaction between a fluorescence probe and a protein. Changes in some probe spectroscopic characteristics can give information about conformational transitions at protein surfaces. Fig. 3 shows the effect of heating time (0–60 min) at 85 °C and pH (6.5–7.5) on the maximum ANS fluorescence intensity, FI_{ANS}^{M} (Fig. 3A) of BLG dispersion (1.0 wt.%) and its corresponding wavelength, λ_{ANS}^{M}

(Fig. 3B). FI_{ANS}^{M} and λ_{ANS}^{M} values for BLG dispersion without heat treatment were added as controls. In general, BLG aggregates showed greater FI_{ANS}^{M} values in comparison to control BLG. Native BLG has hydrophobic patches or clefts on its surface, which could interact with ANS molecules. However, these domains are not totally accessible to ANS. Afterwards, heat-induced BLG aggregates exhibited a greater ANS fluorescence intensity, suggesting an increased exposed hydrophobic area (Bhattacharjee & Das, 2000). Moreover, it was observed that at a given pH value, heating time had no influence on aggregate FI_{ANS}^{M} values. However, at a given heating time, aggregate FI_{ANS}^{M} values gradually decreased with increased pH. These results suggest that the pH for BLG aggregate production could strongly affect the extrinsic fluorescence, i.e. the exposure of hydrophobic domains on the BLG aggregate surfaces.

On the other hand, BLG aggregates showed a significant decrease in λ_{ANS}^{M} values ($p < 0.05$) with respect to control BLG. This result suggests blueshift behaviour linked to an exposure of ANS binding sites in a more hydrophobic microenvironment (Bhattacharjee & Das, 2000; Vivian & Callis, 2001). Additionally, it was observed that heating time and aqueous medium pH had no effect on the BLG aggregate λ_{ANS}^{M} values. In summary, these results suggest that exposure of hydrophobic domains on aggregates strongly depends on the aqueous medium pH at which they were formed.

3.2.3. Surface hydrophobicity

Surface hydrophobicity is an important molecular characteristic that could explain in part some protein functional properties (Perez et al., 2009, 2012a, 2012b). Therefore, in order to link the

heat-induced BLG aggregate hydrophobicity with its binding properties, aggregate surface hydrophobicity (S_0) was analysed. Fig. 4 shows the effect of heating time (0–60 min) at 85 °C and pH (6.5–7.5) on the S_0 value of the BLG dispersion (1.0 wt.%). The S_0 value for the BLG dispersion without heat treatment was added as a control. With respect to control BLG, a significant increase in BLG aggregate S_0 values was observed ($p < 0.05$). BLG aggregates more hydrophobic than native BLG (due to heat treatment) could promote a greater exposure of buried hydrophobic domains (Bhattacharjee & Das, 2000; Iametti et al., 1996). These changes in protein tertiary structure could be irreversible (Cairoli et al., 1994). Moreover, it was observed that BLG aggregate S_0 values depended on the aqueous medium pH and heating time in a complex way. It can be seen that the most hydrophobic aggregates were obtained at pH 6.5 and 7.0 (especially for 30 and 45 min). These results could be compared with those obtained by extrinsic fluorescence discussed above (Fig. 3A).

3.3. LA binding properties of heat-induced BLG aggregates

The study of protein binding properties involves the analysis of the parameters which govern such interaction. In this work, the interaction between BLG (native and aggregated) and LA was monitored through the changes in fluorescence intensity (Sponton et al., 2014). Firstly, the effect of native BLG concentration (0.04–0.08 wt.%) on LA binding properties was evaluated. Effect of BLG concentration on the relative fluorescence intensity (RFI) as a function of LA concentration (0–50 μM) is shown in Fig. 5A. Data were plotted as averages and standard deviations. In general, it was observed that the increase in LA concentration produced an increase in RFI values. This behaviour would correspond to an increase in LA-BLG conjugate amount in solution. It is well known that BLG has two lipophilic ligand binding sites: a central hydrophobic β-barrel (or *calyx*) and a superficial *pocket* (Wang et al., 1998, 1999). The superficial *pocket* is located close to the dimer contact region, in a groove between the α-helix (sited laterally to the *calyx*) and the *calyx* surface (Frapin et al., 1993; Wang et al., 1998, 1999). According to some authors, fatty acids bind at the superficial *pocket*, while other molecules (such as retinol) bind to the *calyx*. Thus, BLG could simultaneously link two lipophilic molecules (Frapin et al., 1993; Wang et al., 1999). In these studies it was reported that the BLG fluorescence intensity increased when fatty acids bound to the superficial *pocket* (Cogan et al., 1976; Frapin et al., 1993; Wang et al., 1999). Therefore, this behaviour could explain the results shown in Fig. 5A. Moreover, it was observed that RFI values of conjugate system at 0.08 wt.% BLG adequately adjusted to the model
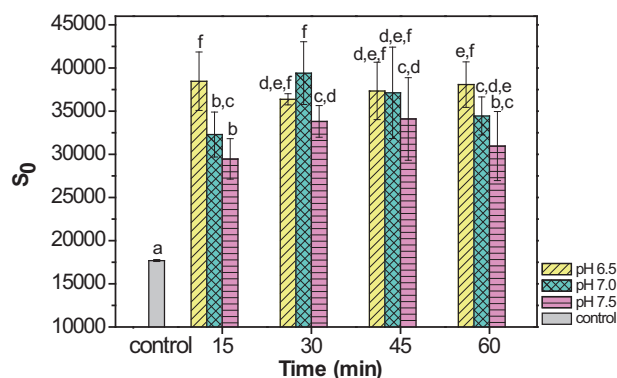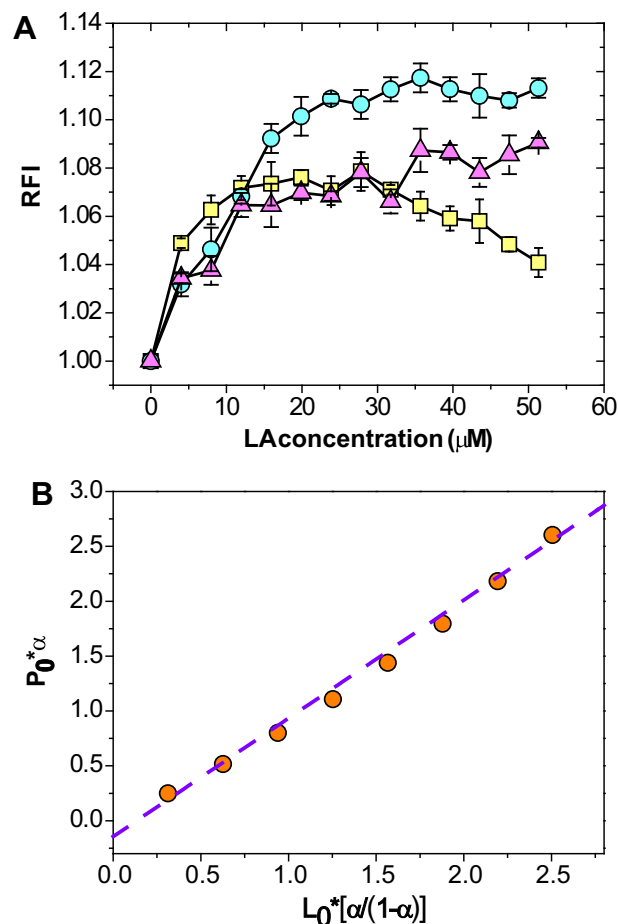

Fig. 5. Effect of BLG concentration on the LA binding properties (A). Symbols: 0.04 (■), 0.06 (●) and 0.08 wt.% (▲). Application of Cogan binding model to the titration of BLGn with LA. Measurement conditions: 0.08 wt.%, pH 7.0 and 25 °C.

described by Cogan et al. (1976). At this concentration the best correlation coefficient was obtained ($R = 0.989$). According to these results, interaction might occur at an optimal concentration both of BLG and LA (Wang et al., 1998). The application of the Cogan model to the titration curve for 0.08 wt.% BLG is shown in Fig. 5B. The adjustment of experimental data yielded the following binding parameters: binding site, $n = 0.93 \pm 0.05$ and apparent dissociation constant $K'_d = 1.3 \times 10^{-7}$ M. These results were consistent with Frapin et al. (1993) who reported $n = 0.83 \pm 0.08$ and $K'_d = 1.90 \times 10^{-7}$ M. However, discrepancies in K'_d values could be attributed to different origins and protein pre-treatments (Sponton et al., 2014).

Additionally, heat-induced BLG aggregates were assayed for their LA binding properties. Fig. 6 shows the LA binding properties of BLA aggregates which were produced at different pH (6.5–7.5) and heating time (0–60 min). In general, complex conjugation phenomena depending on the experimental conditions used for producing BLG aggregates (pH, heating time and/or combination of both) was observed. It is important to highlight that, in spite of the small differences between the RFI values, significant differences were observed mainly at higher LA concentrations. Conjugation phenomena were observed for aggregates obtained at pH 6.5 and at 30 and 60 min heating time (Fig. 6A) as well as at pH 7 and at 60 min heating time (Fig. 6B) mainly at low LA concentration, where RFI values decreased, probably due to an excess of ligand amount (Laligant, Dumay, Casa, Cuq, & Cheftel, 1991). However, binding parameters for aggregates were not calculated because their molecular weights were required (not determined

Fig. 4. Effect of heating time and aqueous medium pH on the BLG S_0. In legend pH refers to pH of BLG aggregate obtention and control refers to native BLG without treatment (native). Measurement conditions: pH 7.0, 25 °C and protein or aggregate concentration 0.01 wt.%. Different letters indicate significant differences ($p < 0.05$).

in this work). At pH 7.5 and for all heating times, an increase in RFI values was not observed, indicating little interaction between LA and these BLG aggregates (Fig. 6C). From these results, a relationship between S_0 and conjugation properties of heat-induced aggregates was not observed, i.e., it was not observed that the greater S_0, the greater LA binding ability. However, for some conditions, aggregate conjugation properties were consistent with intrinsic fluorescence behaviour (as discussed above Fig. 2A), mainly for
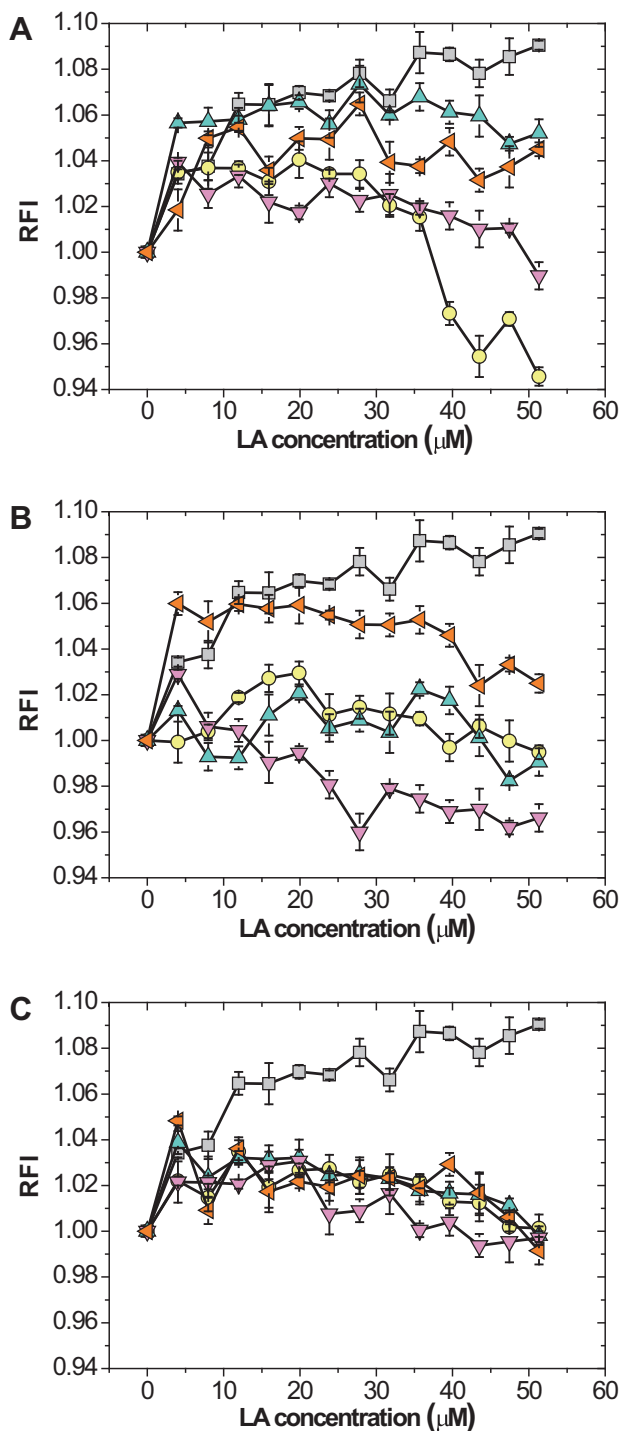
those aggregates obtained at pH 6.5 suggesting that minor modifications in tertiary structure could not considerably modify LA binding properties. Finally, the decreased LA conjugation ability of BLG aggregates obtained at pH 7.5 could be related to greater conformational changes on the protein tertiary structure, which could alter the LA binding site. In general, these results support the idea that LA binding properties of proteins and/or aggregates could involve the conservation of the binding site structure, i.e. a BLG superficial *pocket* which could no longer be available in certain aggregation conditions.

4. Conclusions

In this work, the hypothesis that heat-induced BLG aggregates with improved hydrophobic properties could have a greater ability for binding LA onto their hydrophobic surfaces with respect to native BLG was tested. It was concluded that the pH, in the range of 6.5 and 7.5, had a greater effect on the heat-induced BLG aggregate formation than other conditions. BLG aggregates formed at pH 6.5 were the most hydrophobic and these could find application as carriers of bioactive, lipophilic molecules. However, conjugation of LA onto the hydrophobic surfaces of BLG aggregates was characterised by a complex behaviour which could depend on aggregate formation conditions (pH, heating time and/or combination of both). The obtained heat-induced aggregates did not show that the greater surface hydrophobicity, the greater the ability for binding LA. This finding could suggest that conjugation requires preservation of the LA binding site. Finally, information derived from this study called attention to the impact of heat treatment (temperature and time) and pH on the development of PUFAs encapsulation strategies using BLG as a carrier.

Fig. 6. Effect of heating time and aqueous medium pH on the LA binding properties of heat-induced BLG aggregates. Symbols: 15 min (●), 30 min (▲), 45 min (▼) y 60 min (◄). (A) pH 6.5; (B) 7.0 y (C) 7.5. Control: BLG dispersion without heat-induced treatment (■). Protein concentration 0.08 wt.%. Measurement condition: pH 7.0–25 °C.

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