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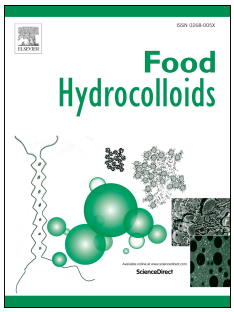

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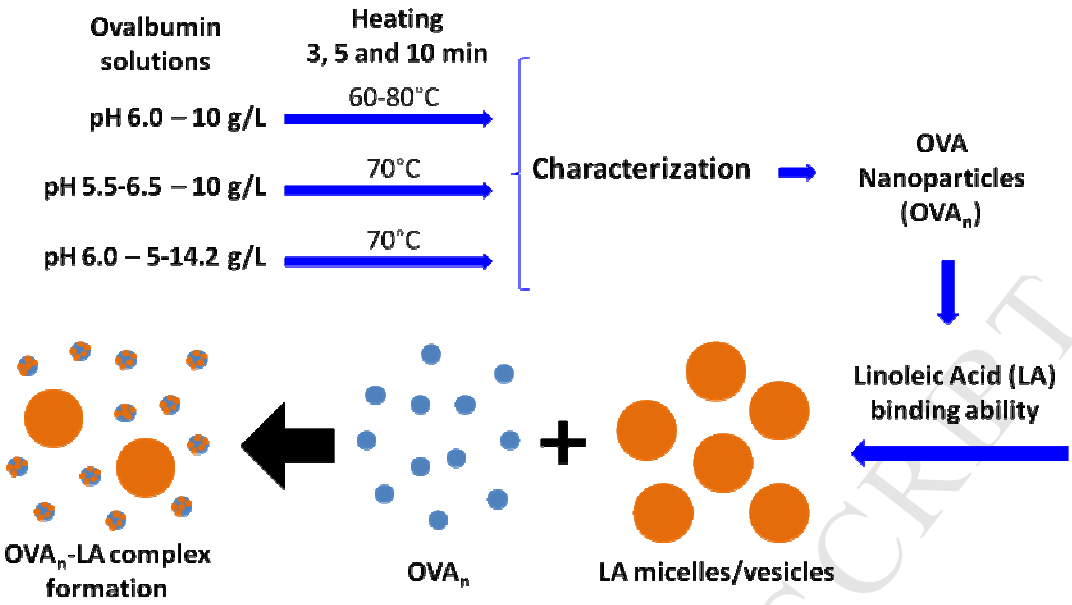

Impact of environment conditions on physicochemical characteristics of ovalbumin heat-Induced nanoparticles and on their ability to bind PUFAs

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Abstract

In this work, OVA heat-induced aggregates were obtained by controlled heat treatment varying temperature (60, 70 and 80ºC), heating time (3, 5 and10 min), aqueous medium pH (5.5, 6.0 and 6.5) and protein concentration (5, 10 and 14.2 g/L). Particle size distribution and surface characteristics derived from fluorescence spectroscopy (both intrinsic and extrinsic) were determined. Evaluation of these physicochemical properties allowed knowing experimental conditions under which nanometric OVA aggregates (OVA_n), with suitable surface hydrophobicity, could be produced. OVA_n ability to bind polyunsaturated fatty acid (PUFA) was carried out by turbidity measurement. In these experiments, linoleic acid (LA) was used as a PUFA model. In general, OVA aggregate sizes increased with temperature, time and protein concentration, furthermore at higher pH value, OVA aggregate sizes were lower. OVA aggregates surface hydrophobicity increased with rising heating temperature and the pH value indicating, on one hand, larger protein unfolding and on the other hand lower aggregation via hydrophobic interaction at higher pH value, respectively. However, surface hydrophobicity decreased with concentration suggesting greater aggregation. OVA_n obtained at 70°C, 10 g/L, pH 6-6.5 and 70°C, 5 g/L, pH 6 were assayed by LA binding ability. This property was 1.4-2.0 folds greater than for native OVA. Information derived from this work could be of practical interest for the development of innovative PUFAs carrier systems.

Keywords: Ovalbumin, Heat-induced aggregation, Nanoparticles, Linoleic acid, Polyunsaturated fatty acids, Fluorescence.

1. Introduction

Proteins are natural biopolymers with a lot of physicochemical and functional properties. Ovalbumin (OVA), which is the main protein of egg white proteins (EWP), has been extensively studied,

because of its good functionality, particularly due to OVA is the main responsible for EWP foaming, and gelling properties (Nyemb, Guérin-Dubiard, Dupont, Jardin, Rutherfurd & Nau, 2014; Weijers, Sagis, Veerman, Sperber & van der Linden, 2002). OVA is a globular protein of 43 kDa molecular weight and it is composed by 385 aminoacids, of which a half is hydrophobic and mainly buried into the protein structure. Otherwise, charged aminoacids (a third part of total aminoacids) are mainly located on protein surface in contact with aqueous medium (Croguennec, Renault, Beaufils, Dubois & Pezennec, 2007; Giosafatto, Rigby, Wellner, Ridout, Husband, & Mackie, 2012). OVA has one disulphide bond and four free sulfhydryl groups. Protein globular structure can be modified through different treatments such as heating, high pressure, enzymatic reaction, shaking, etc. (Galazka, Smith, Ledward, & Dickinson, 1999; Weijers M., Barneveld P. A., Cohen Stuart M. A. & Visschers R. W., 2003; Wolfe, 1966). The effect of heat treatment on the OVA molecular structure has been extensively studied (Galazka et al., 1999; Hayakawa & Nakai, 1985; Matsudomi, Takahashi, & Miyata, 2001; Nakamura, Sugiyam & Sato, 1978; Nyemb et al., 2014; Weijers et al., 2003). Heating promotes protein unfolding, phenomenon known as denaturation, in which hydrophobic aminoacids are exposed conferring an increase in protein surface hydrophobicity (Croguennec et al., 2007; Weijers et al., 2003). Environment conditions under which denaturation take place can lead to OVA aggregation. This phenomenon is normally driven by molecular interactions between exposed hydrophobic patches and disulphide bonds formation (Sánchez-Gimeno & López-Buesa, 2006). Moreover, under determinated ionic strength and pH conditions, heated OVA dispersions can produce protein aggregates with different sizes and morphologies (Nyemb et al., 2014).

Otherwise, one of the functional properties that have recently received great interest is the ability of some globular proteins to bind bioactive lipophilic compounds. In general, these compounds are very sensitive to environment factors that promote bioactivity deterioration, such as light, oxygen, metals, enzymes, etc. Polyunsaturated fatty acids (PUFAs) are examples of very sensitive bioactive molecules, especially to oxidation (Zimet, & Livney, 2009). PUFAs are essential in human nutrition but they have very low water solubility which limits their incorporation in aqueous products. The development of protein nanostructures with suitable surface hydrophobicity able to bind PUFAs might be important for several reasons: i) to increase their stability (Zimet, & Livney, 2009); ii) to increase their solubility and so allow their incorporation in aqueous systems (Ilyasoglu & El, 2014; Zimet, & Livney, 2009); iii) to have potential antitumor activity (Fontana, Spolaore, & Polverino de Laureto, 2013).

Binding ability of proteins is linked with the presence of hydrophobic regions on the protein surface (Perez, Andermatten, Rubiolo, & Santiago, 2014). Considering that heat treatment promotes a great exposition of hydrophobic residues on globular protein surfaces, this treatment could be taken into account for increase binding ability of proteins (Fioramonti, Perez, Aríngoli, Rubiolo & Santiago, 2014). In a recent paper, it was demonstrated that β-Lactoglobulin aggregates could increase their PUFAs binding

ability depending on the environment conditions under which aggregates were produced (Perez et al., 2014). Therefore, the systematic study of heating and environment variables is very important in order to control OVA aggregates physicochemical properties required to the enhancement of PUFAs binding ability.

In this framework, the aim of the present paper was to determine the influence of environment conditions such as pH, protein concentration and thermal treatment (temperature and time) on physicochemical properties of OVA aggregates (particle size and surface characteristics derived from fluorescence measurements). One set of OVA aggregates (called OVA_n) was selected according to their nanometric particle size and surface hydrophobicity to carry out binding assays using linoleic acid (LA) as PUFA model. The original feature of this work was to obtain knowledge about formation of OVA_n at heating temperature below OVA denaturation (80.1 ºC) (Matsudomi et al., 2001) and to evaluate the impact of OVA_n physicochemical characteristics on the PUFA binding ability. This last aspect has not been evaluated yet. Information derived from this study could provide some useful data for the design of innovative OVA_n with increased PUFAs binding ability.

2. Materials and methods

2.1. Materials

Ovalbumin (product A5503, purity 98% according to agarose gel electrophoresis) and linoleic acid (LA) sample were obtained from Sigma (USA). 1-anilino-8-naphthalene sulfonic acid (ANS) was purchased from Fluka Chemie AG (Switzerland). The other reagents were purchased in Cicarelli (Argentina).

2.2. Heat treatment

OVA dispersions were prepared in 50 mM NaCl and were kept overnight. Then, pH was adjusted at the corresponding values (5.5, 6.0 and 6.5) using NaOH or HCl solutions. OVA dispersions were filtered with 0.22 µm cellulose ester membrane (Millipore) in order to eliminate possible protein aggregates. OVA extinction coefficient of 0.712 L g^{-1} cm^{-1} was used in order to determinate protein concentration from the absorbance of the dispersion at 280 nm (Croguennec et al., 2007). Protein concentrations evaluated were 5, 10 and 14.2 g/L. For heat treatment, 2 ml of OVA dispersion were heated in a water bath at 60, 70 and 80°C into stoppered glass tubes. Tubes were removed at different times (3, 5, 10 min) and immediately cooled in an ice bath. They were kept at 4°C until further analysis.

2.3. Particle size distribution

Particle size distribution (PSD) was obtained using a Nano Zetasizer (Nano ZS90, Malvern Instruments Ltd., UK), which allow determining the particle size by means of dynamic light scattering

(DLS). The instrument has a He-Ne laser as light source which operates at 632.8 nm wavelength. DLS measurements were performed at a set angle of 90°C. The refractive indexes used for solvent and OVA aggregates were 1.33 and for 1.50, respectively (Croguennec et al., 2007). The mean hydrodynamic diameter (nm) was obtained from the peak of the intensity particle size distribution (PSD) (Carvalho, Carvalho, Alves & Tabak, 2013). However, volume transformation PSD (PSDv) was obtained in order to report the mass composition of the evaluated systems (Niidome, 2014). PSDv is derived from scattered light intensity distribution applying Mie theory (Arzenšek, Podgornik, & Kuzman, 2010). The percentage values given after each mean diameter correspond to the area under the curve of PSDv of the corresponding mean diameter. Measurements were performed at 25°C.

2.4. Fluorescence spectroscopy

Fluorescence spectroscopic measurements were performed using a fluorescence spectrophotometer (Hitachi 2000, Japan). Intrinsic fluorescence determination (due to Trp emission) was done at 280 nm excitation wavelength and the emission spectra were recorded at 310-360 nm. Fluorescence intensity data were informed as Trp Relative Fluorescence Intensity (RFI-Trp), which was defined as the ratio between the maximum fluorescence intensity (FI-Trp) for heat-treated OVA to FI-Trp for native OVA. Wavelength shift (Δλ-Trp) was also informed, and it was calculated as the difference between maximum fluorescence wavelength for treated OVA and native OVA.

On the other hand, extrinsic fluorescence was determined using ANS as a fluorescence probe. Ten microliters of 8 mM ANS solution were added to 2 ml of 0.05 g/L protein dispersion (Sponton, Perez; Carrara & Santiago, 2014). Then, the extrinsic fluorescence spectra were obtained at 390 nm excitation wavelength between 450-550 nm emission wavelengths (Croguennec et al., 2007). Fluorescence intensity data were informed as ANS Relative Fluorescence Intensity (RFI-ANS), which was defined as the ratio between the maximum fluorescence intensity (FI-ANS) for heat-treated OVA to FI-ANS for native OVA. RFI-ANS value was considered as a measure of protein surface hydrophobicity. Wavelength shift (Δλ-ANS) was also obtained and it was calculated as the difference between maximum ANS fluorescence wavelength for treated OVA and native OVA.

Both intrinsic and extrinsic fluorescence experiments were performed in triplicate at room temperature (25°C).

2.5. Turbidity measurement

Turbidity measurements were performed using a Jenway 7305 spectrophotometer (UK). Absorbance at 400 nm was taken as an index of turbidity (Fontana et al., 2013). Firstly, protein dispersions were diluted at 0.5 g/L in 50 mM pH 7 potassium phosphate buffer. Then, 8 µl of 100 mM LA in ethanolic solution were added to 2 ml of protein solution. A blank solution (protein without LA) for

each sample was prepared and its turbidity value was subtracted from the corresponding sample. From turbidity measurements, a percentage of LA binding ability was calculated for some systems as follow: Binding ability (%) = $(T_{LA}-T_{LA+OVA})/T_{LA} \times 100$, where T_{LA} and T_{LA+OVA} correspond to the turbidity values of LA solution and LA+OVA (native/aggregated), respectively.

2.6. Statistical analysis

All assays were measured at least in duplicate. Averages and standard deviations were calculated from these measurements. Differences between means were determined with LSD test at $p<0.05$ significance level (StatGraphics Centurion XV).

3. Results and discussion

In order to evaluate OVA aggregates formation, the following experimental conditions: temperature (60, 70 and 80 ºC), heating time (3, 5 and 10 min), pH (5.5, 6.0 and 6.5) and protein concentration (5, 10 and 14.2 g/L) were systematically studied. The process conditions that promote OVA aggregates with nanometric size (OVA_n) and improved fluorescence properties were selected for LA binding assays.

3.1. Particle size distribution

Results of particle size distribution will be discussed in terms of the effect of heat treatment (temperature and heating time) and environment variables (protein concentration and pH) as following.

3.1.1. Effect of heating temperature and time

Heating temperature is a critical variable in protein aggregates formation. It is well known that OVA denaturation temperature is around 80.1 ºC (Matsudomi et al., 2001). In this work, we explore the effect of heating temperature, below the denaturation one, at 10 g/L OVA and pH 6.0, on OVA aggregates sizes. Figure 1 shows the effect of temperature (60, 70 80ºC) and heating time (3, 5 and 10 min) on OVA PSDv. The y axis shows the volume distribution while the x axis shows the particle hydrodynamic diameter (nm) which is defined as the equivalent sphere diameter that diffuses at the same mean rate that the particle under examination (Niidome, 2014). From Figure 1, it can be deduced that:

(i) Heat treatment at 60 ºC, for all times produced a bimodal PSDv (Fig. 1a). A main peak at 6 nm corresponding to native OVA size (Croguennec et al., 2007) and a secondary peak, around 100 nm, it can be observed. In all cases, the last one represented < 2% of the total volume, indicating that these conditions practically did not produced changes in native OVA size.

(ii) Heat treatment at 70 ºC for both, 3 and 10 min produced monomodal PSDv with peaks at 225 and 1900 nm, respectively (Fig. 1b). Nevertheless, heating at 70 ºC, 5 min produced a bimodal PSDv with a main peak at 550 nm (81 %) and a secondary peak at 120 nm (20 %). In the food sector, the definition of nanometric materials is not clear. Particles with both, size strictly < 100 nm and with

few hundred nm could be named "nanoparticles" (Gutiérrez, Albillos, Casas-Sanz, Cruz, García-Estrada, García-Guerra, et al., 2013). In our work, particles with few hundred nm were considered as "nanoparticles". According to this definition, OVA nanoparticles (OVA_n) could be those obtained at 70 ºC for 3 min (225 nm) and 5 min (120 nm).

(iii) At 80 ºC and 3 min (Fig. 1c), two peaks were obtained; a main peak at 3280 nm (90 %) and a secondary one at 575 nm (10 %). These aggregates (similar to the ones obtained at 70ºC for 10 min) have a micrometric size. Treatments at 80ºC for 5 and 10 min produced bigger aggregates which precipitated impeding the particle size determination.

As it can be noted, size of OVA aggregates increased with the rise in heating time and temperature, which also increases strongly the aggregation rate. Hence, at a given heating time, aggregates were larger at higher temperature (Mehalebi, Nicolai, & Durand, 2008). Moreover, a higher temperature would allow a larger protein unfolding, and consequently, greater exposition of hydrophobic residues, which might be involved in protein aggregation via hydrophobic interactions (Kato, Nagase, Matsudomi & Kobayashi, 1983).

3.1.2. Effect of aqueous medium pH and heating time

The aqueous medium pH during heating is considered as a key factor in particle size and morphology of protein aggregates, because modulate the magnitude of intermolecular interactions between protein macromolecules (Nyemb et al., 2014). In this sense, aggregate sizes could be controlled modifying pH.

Particle sizes of OVA aggregates obtained at 70ºC (3, 5 and 10 min), 10 g/L, and pH 5.5, 6.0 and 6.5 were determined. Figures 2 shows the effect of heating time and pH, 5.5 (Fig. 2a) and 6.5 (Fig. 2b), on OVA aggregates PSDv. It can be noted that:

(i) At pH 5.5, 70 ºC and 3 min, OVA aggregates showed a bimodal PSDv distribution, and a main peak at 2107 nm (98 %) and a secondary peak at 476 nm (2 %) were registered (Fig. 2a), while at 5 min a bimodal PSDv distribution with a main peak at 2711 nm (99 %) and a minor peak at 587 nm (1 %) was registered. Finally, at 10 min, a monomodal PSDv distribution with a peak at 4077 nm molecular size was obtained.

(ii) Results of the effect of heat treatment at 70 ºC, pH 6 on particle size of OVA aggregates were discussed in the previous section (3.1.1) (Fig. 1b).

(iii) At pH 6.5, 70 ºC and 3 min a bimodal PSDv with peaks at 10 nm (53 %) and at 76 nm (47 %) (Fig. 2b) was obtained, whereas at 5 min, a bimodal PSDv with a main peak at 96 nm (97 %) and a secondary peak at 4491 nm (3 %) was observed. Finally, at 10 min heating a bimodal PSDv with a main peak at 192 nm (98 %) and a secondary peak at 4550 nm (2 %) was registered.

In general, the results from all pH tested suggest that the increase in heating time caused an increase in OVA aggregates sizes. Differences in particle sizes could be attributed to the effect of pH on protein

surface charge, which could promote the approach (lower electrostatic repulsion) and/or distancing (higher electrostatic repulsion) between OVA molecules (Croguennec et al., 2007). Thus, at pH 5.5 OVA macromolecules are in the proximity of isoelectric pH (pI ~ 4.5), with a low net charge and, consequently, a reduced electrostatic repulsion between protein macromolecules. This condition could induce protein molecules to approach and consequently, promote OVA aggregates with micrometric molecular sizes (2107-4077 nm). On the other hand, pH 6.5 is quite distant from protein pI, so electrostatic repulsion between OVA macromolecules should be higher. This condition would induce OVA aggregates with nanometric molecular sizes (OVA_n) (Nyemb et al., 2014). Finally, an intermediate behaviour could be deduced at pH 6.0.

3.1.3. Effect of protein concentration and heating time

Another important process variable in protein aggregate formation is protein concentration. (Nakamura et al., 1978). According to this, OVA aggregate formation at 70 ºC, pH 6.0 and 5 g/L (Fig. 2c), 10 g/L (Fig. 1b) and 14.2 g/L (Fig. 2d) was examined. It can be observed that:

(i) At 5 g/L, 70 ºC and 3 min a monomodal PSDv with a mean diameter of 122 nm was obtained (Fig. 2c) nevertheless, for 5 min heating a bimodal PSDv with a main peak at 207 nm (97 %) and a secondary peak at 4946 nm (3 %) was registered. Finally, for 10 min a bimodal PSDv with a major peak at 665 nm (97 %) and minor peak at 4909 nm (3 %) was observed. In general, results obtained suggest that heat treatment at 70 ºC and at pH 6 produced OVA aggregates mainly with particle sizes in nanometric and/or submicron scale (122-665 nm).

(ii) Results of the effect of heating time on the OVA aggregates PSDv obtained at 10 g/L and pH 6 was previously discussed in section 3.1.1 (Fig. 1b).

(iii) At 14.2 g/L, 70 ºC and 3 min a bimodal PSDv with a main peak at 317 nm (96 %) and a secondary peak at 65 nm (4 %) was registered while at 5 min heating time a bimodal PSDv with peaks at 377 nm (78 %) and at 2103 nm (22 %) was observed. Finally, at 10 min a bimodal PSDv with a main peak at 2666 nm (98% volume) and a minor peak at 261 nm (3 %) was obtained. It can be noted that in this condition, OVA aggregates sizes were in nanometric and/or submicron scale.

The size of aggregate formed during heating increases with protein concentration until a critical value where a gel is formed (Mehalebi et al., 2008). This critical value depends of temperature, pH and ionic strength (Weijers, Visschers & Nicolai, 2004). In the present work, gel formation was not registered. In general, it was observed that an increase in heating time and protein concentration caused larger aggregates. This result could be explained taking into account that protein aggregate size would be influenced by the initial number of protein molecules during heat treatment. Thus, larger aggregates would be formed at higher concentration in the range evaluated.

3.2. Intrinsic fluorescence

Protein fluorescence behaviour due to the Trp residues emission is considered an important tool in the characterization of protein tertiary structure changes upon heat treatment (Perez et al., 2014, Shpigelman, Israeli, & Livney, 2010). OVA has three Trp residues: Trp 148, Trp 267 and Trp 184 (Wang, Yin, Li, Wang, Pu,Wang et al, 2013). Therefore, OVA intrinsic fluorescence emission properties could be considered as the average of the fluorescence contribution of their three Trp residues. Upon heat treatment, variations in protein emission fluorescence intensity (FI-Trp) are usually accomplished with shifts in the wavelength that corresponds to the maximum FI-Trp (λ_M). In Table 1, these shifts were expressed as a $\Delta\lambda$–Trp and their positive or negative signs represent red (increase in λ_M) and blue (decrease in λ_M) shift behaviours, respectively. Results of intrinsic fluorescence spectroscopy will be discussed in terms of heat treatment (temperature and time) and environment variables (pH and protein concentration) as follow.

3.2.1. Effect of heating temperature and time

Figure 3a shows the effect of temperature (60, 70 and 80 ºC) and heating time (3, 5 and 10 min) on OVA intrinsic fluorescence at pH 6.0 and 10 g/L. OVA intrinsic fluorescence behaviour was expressed as relative fluorescence intensity (RFI-Trp) taking into account a protein control without heat treatment (native OVA). It was observed that:

(i) At 60 ºC, the increase in heating time did not produce changes in RFI-Trp until 10 min. At 10 min, RFI-Trp decreased slightly indicating that some protein irreversible structural changes could take place (Cairoli, Iametti, & Bonomi, 1994). Otherwise, $\Delta\lambda$–Trp values did not change with the increase in heating time ($p>0.05$) indicating that molecular environment of Trp residues did not alter upon heat treatment. These results would suggest low aggregation and minimal changes in tertiary structure.

(ii) At 70 ºC, 3 and 5 min, RFI-Trp was not significantly different of RFI-Trp of native OVA ($p>0.05$). However, heating for 10 min caused a decrease in RFI value ($p<0.05$).

(iii) At 80 ºC, the RFI-Trp values strongly decrease at 3 min heating and subsequently, there was an increase in RFI-Trp values with heating time reaching a maximum at 10 min ($p<0.05$).

Results suggest that OVA tertiary structure was modified depending on temperature and heating time. As it can be noted in Table 1, heating at 70 and 80 ºC caused slight red shift behaviour. This trend would be consistent with slight Trp residues exposition in a more polar environment (Cairoli et al., 1994). For one hand, the decrease in fluorescence for OVA heated at 70°C, 10 min and at 80°C, 3 and 5 min could be explained taking into account that heating promoted protein aggregates with Trp occluded inside them, probably participating in hydrophobic interactions (Cairoli et al., 1994). These Trp residues would

not contribute to the fluorescence emission. Hence, decrease in RFI could be explained in terms of those Trp residues that not participated in hydrophobic interactions which could be in a minor proportion but in a more polar environment (slight red shift behaviour) than native OVA. On the other hand, at 80°C and 3 min, the decrease in fluorescence may be explained considering initial stages of protein aggregation by mean of hydrophobic interactions (Sánchez-Gimeno & López-Buesa, 2006) and Trp residues could be occluded into the formed structures. The further increase in fluorescence (at 5 and 10 min) could be due to great protein unfolding and exposure of Trp buried into native OVA.

3.2.2. Effect of aqueous medium pH and heating time

Figure 3b shows the effect of pH (5.5, 6.0 and 6.5) and heating time (3, 5 and 10 min) on OVA intrinsic fluorescence behaviour at 70 ºC and 10 g/L protein concentration. Protein control without heat treatment was also included. It can be deduced that:

(i) At pH 5.5 and pH 6.0, the increase in heating time caused a decrease in RFI-Trp ~~and an increase~~ in $\Delta\lambda$–Trp values (Table 1), especially at pH 6 ($p<0.05$). These results could be linked with changes in OVA tertiary structure and, consequently, with aggregate formation via hydrophobic interaction due to less electrostatic repulsion as it was discussed in the previous section.

(ii) At pH 6.5, RFI-Trp value was maximum for 3 min heating ($p<0.05$) then gradually decrease until reach the control RFI-Trp value ($p>0.05$). Moreover, it was observed an increase in $\Delta\lambda$–Trp values with heating (Table 1). These results are consistent with the idea that the increase in RFI-Trp and the slight red shift behaviour could be due to minor Trp residues involved in hydrophobic interaction as a consequence of higher intermolecular electrostatic repulsion and so lower aggregation. Consequently, OVA aggregates would be smaller (as it can be observed in Fig. 2b).

3.2.3. Effect of protein concentration and heating time

Figure 3c shows the effect of protein concentration (5, 10 and 14.2 g/L) and heating time (3, 5 and 10 min) at 70 ºC and pH 6.0, on OVA intrinsic fluorescence behaviour. Protein control, without heat treatment, was also included. In the range of OVA concentration studied it was observed that the increase of heating time caused a decrease in RFI-Trp values suggesting modifications in protein tertiary structure and aggregate formation via hydrophobic interactions. This hypothesis was especially confirmed for aggregates obtained at 10 g/L which presented increased $\Delta\lambda$ with heating time increment (Table 1), indicating that Trp (Trp residues probably not involved in hydrophobic interactions) would be exposed in a more polar environment (Table 1). Conversely, at 5 g/L there was not a wavelength shift, suggesting that Trp environment would be similar to that in native OVA. On the other hand, at 14.2 g/L there was a slight blue shift wavelength which suggests Trp residues are in a more hydrophobic environment (Keerati-u-rai, Miriani, Iametti, Bonomi, & Corredig, 2012).

3.3. Extrinsic fluorescence

Protein extrinsic fluorescence is an important tool in examination of protein surface hydrophobicity (Perez et al., 2014). It is well established that ANS binds on protein exposed hydrophobic regions which increase its fluorescence. Moreover, blue shift behaviour is normally registered suggesting that ANS is in a more hydrophobic environment (Albani, 2004). Table 2 shows the effect of temperature (60, 70 and 80 ºC), pH (5.5, 6.0 and 6.5), protein concentration (5, 10 and 14.2 g/L) and heating time (3, 5 and 10 min) on blue shift behaviour (Δλ-ANS) experimented by ANS when it is bound on OVA aggregates surface. Results of extrinsic fluorescence spectroscopy will be discussed as follow.

3.3.1. Effect of heating temperature and time

Fig. 4a presents effect of temperature (60, 70 and 80 ºC) and heating time (3, 5 and 10 min) on the extrinsic fluorescence intensity expressed as RFI-ANS, at pH 6.0 and 10 g/L. A protein control without heat treatment was also included. It can be observed that:

(i) At 60 ºC, RFI-ANS values increased slightly respect to the control, and no significant differences among heating times were registered ($p>0.05$). However, the increase in heating time caused a decrease in Δλ-ANS values indicating that ANS binding sites became gradually more apolar (Table 2).

(ii) At 70 ºC, RFI-ANS increased (around 4 folds with respect to the control) and a strong decrease in Δλ-ANS was detected with the increase in heating time ($p<0.05$) (Table 2).

(iii) At 80 ºC, RFI-ANS values strongly increased (around 6-8 folds respect to control) and Δλ-ANS values decreased with heating time ($p<0.05$) reaching the lowest values (Table 2).

It is important to remark that for all examined heating times RFI-ANS increased with the increase in temperature, suggesting an increase in OVA surface hydrophobicity (Croguennec et al., 2007). Moreover, the decrease in Δλ-ANS with the increase in temperature was indicative that ANS was bound in more hydrophobic sites. Hence, higher temperature and time promoted more hydrophobic sites became exposed.

3.3.2. Effect of aqueous medium pH and heating time

Fig. 4b shows the effect of pH (5.5, 6.0 and 6.5) and heating time (3, 5 and 10 min) on the extrinsic fluorescence intensity expressed as RFI-ANS at 70 ºC and 10 g/L. In this figure a protein control without heat treatment was also included. It was observed that for all examined heating times RFI-ANS increased with the increase in pH. Specifically, it can be noted that:

(i) At pH 5.5, the increase in heating time produced an increase in RFI-ANS (2-3 folds respect to the control) and a decrease in Δλ-ANS values ($p<0.05$). In this case, the blue shift behaviour observed was the maximum in comparison with the others obtained at another pH values (Table 2).

(ii) At pH 6.0, RFI-ANS values increased with the increase in heating time (around 4 folds with respect to control) finding a maximum value for 5 min heating. Moreover, it was observed a decrease in Δλ-ANS values with heating time ($p<0.05$) (Table 2).

(iii) At pH 6.5, the increase in heating time caused an increase in RFI-ANS values (5 folds respect to the control) and a decrease in Δλ-ANS values ($p<0.05$). This behaviour could be explained considering that at this pH condition, electrostatic repulsion could be higher than a lower pH, so aggregation via hydrophobic interaction could be less promoted. In this sense, the hydrophobic sites would be more exposed and consequently more accessible to ANS.

3.3.3. Effect of protein concentration and heating time

Fig. 4c shows the effect of protein concentration (5, 10 and 14.2 g/L) and heating time (3, 5 and 10 min) at 70°C and pH 6.0 on the extrinsic fluorescence behaviour expressed as RFI-ANS. For all examined heating times, RFI-ANS and Δλ-ANS values decreased with the increase in protein concentration ($p<0.05$). Moreover, at a given concentration, RFI-ANS increased with heating time. The increase in RFI-ANS indicates higher surface hydrophobicity according to the results obtained by Perez et al. (2014). Hence, at lower concentration, surface hydrophobicity was higher, probably due to minor aggregation via hydrophobic interactions. This result is consequent with the lower size of OVA aggregates obtained at the minor concentrations. The increase in protein concentration promoted protein aggregation phenomenon possibly, due to the increased protein macromolecules number and so the probability that they get together (Nakamura et al., 1978).

3.4. PUFA binding ability

PUFA molecules are formed by hydrophilic heads composed by carboxylic groups and hydrophobic tails which correspond to aliphatic chains (Fennema, 1996). Hydrophobic tails confers to PUFAs low solubility in aqueous solutions (Fontana et al., 2013). When PUFA concentration in aqueous medium exceeds the critical micelle concentration (CMC), around 60 μM at pH 7.6, fatty acid molecules assembles forming micelles and vesicles (Fontana et al., 2013; Zimet, & Livney, 2009). These supramolecular assemblies are mainly formed by hydrophobic interaction between aliphatic chains. In the resulting configuration, polar heads are located in the outside of supramolecular structure in contact with water molecules (Fennema, 1996). Formation of micelles and vesicle confers appreciable turbidity to the PUFA aqueous solution. On the other hand, some proteins have the ability to bind hydrophobic compound such as PUFAs. Hence, turbid PUFAs aqueous solution becomes clear when these protein solutions are added. At this respect, it was demonstrated that micelles and vesicles formed by oleic acid (OA) were disrupted in presence of α-lactalbumin (α-La) at neutral pH (Fontana et al., 2013). In these last conditions, system turbidity decreased suggesting the formation of hybrid α-La-OA nanocomplexes. In the same way,

turbidity technique was applied in the present study in order to evaluate the ability of OVA nanoparticles (OVA_n) to bind LA. According to the results discussed in previous sections, one set of OVA_n was selected in terms of nanometric particle size and surface hydrophobicity. The chosen OVA_n were those obtained at the following conditions: (i) 70°C pH 6.0, 5 g/L and 10 g/L, 3 and 5 min heating time and (ii) 70°C pH 6.5, 10 g/L, 3, 5 and 10 min.

Figure 5 shows turbidity values for LA solution, LA+native OVA and LA+OVA_n set. In order to facilitate the understanding of the results, main diameter (nm), surface hydrophobicity (RFI-ANS) and LA binding ability (%) of LA+native OVA and LA+OVA_n set systems are shown in Table 3 as a summary. Figure 5 shows that LA addition to native OVA caused a decrease in LA turbidity value ($p<0.05$) indicating LA supramolecular assemblies disruption and hybrid nanocomplexes formation, results in accordance with Fontana et al. (2013). As it can be observed in Table 3, LA binding ability of native OVA was around 34 %.

Comparing the binding ability of the native OVA with OVA nanoparticles, it can be observed that LA addition to the OVA_n set produced lower turbidity values than with native OVA ($p<0.05$) (Figure 5), in addition LA binding ability calculated for OVA_n set was in the range of 49-69 %, i.e. 1.4-2.0 folds greater than binding ability of native OVA (Table 3). Lower LA binding ability of native OVA could be explained in terms of the occluded hydrophobic groups less available for binding LA. In general, these results were consistent with a greater surface hydrophobicity of OVA_n as it was discussed previously.

On the other hand, considering OVA_n, the lowest binding ability and the highest turbidity were obtained for those produced at pH 6.0 and 10 g/L (Table 3, Figure 5). It is important to remark that OVA_n obtained at pH 6.5, 10 g/L and pH 6.0, 5 g/L presented high LA binding ability. We hypothesized that these results could have a close relationship with the specific surface area of the OVA_n, that is, a larger size would generate minor specific surface area for LA binding and conversely, lower sizes would produce higher specific surface and higher LA binding. However, binding ability not only would depend on specific surface area and hydrophobicity but also on the spatial conformation of hydrophobic surface as was found by Perez et al., (2014), so more studies should be done about these features. Nevertheless, it was demonstrated that manipulating heat treatment and environmental conditions, OVA_n with different sizes, surface hydrophobicity and binding ability were obtained.

4. Conclusions

In this paper we provide information about the particle size and fluorescence (both intrinsic and extrinsic) characteristics of OVA heat-induced aggregates produced in a range of temperature below to the OVA denaturation temperature (80.1 ºC). The systematic study of heat treatment temperature (60, 70 and 80 ºC) and time (3, 5 and 10 min), and environment variables, pH (5.5, 6.0 and 6.5) and protein

concentration (5, 10 and 14.2 g/L), allowed to choose those experimental conditions for which OVA heat-induced aggregates had nanometric particle size (OVA_n) and a suitable surface hydrophobicity. In all cases, this OVA_n set had greater LA binding ability than native OVA due the greater surface hydrophobicity. Differences in LA binding ability OVA_n could be attributed not only to differences in surface hydrophobicity but also to differences in their size and so their specific surface area. In general, the highest binding ability was for OVA_n with the lowest size. The fact that LA was bound to OVA_n in a more effective way than native OVA may suggest that they could be applied as vehicles for PUFAs favoring its incorporation in aqueous systems. Information derived from this study provides some practical data about requirements of particle size and surfaces properties of OVA nanoparticles with improved binding ability.

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6. References

Albani, J. R. (2004). Structure a dynamics of macromolecules: Absorption and fluorescence studies, Paris: Elsevier, (Chapters 2 & 3).

Arzenšek, D., Podgornik, R., & Kuzman, D. (2010). Dynamic light scattering and application to proteins in solutions. University of Ljubljana, Slovenia.

Bryant, C. M., & McClements, D. J. (1998). Molecular basis of protein functionality with special consideration of cold-set gels derived from heat-denatured whey. *Trends in Food Science & Technology, 9*, 143-151.

Cairoli, S., Iametti, S, & Bonomi, F. (1994). Reversible and Irreversible Modifications of β-Lactoglobulin Upon Exposure to Heat. *Journal of Protein Chemistry, 13*, (3), 347-354.

Carvalho, F. A. O, Carvalho, J. W. P., Alves, F.R, Tabak, M. (2013). pH effect upon HbGp oligomeric stability: characterization of the dissociated species by AUC and DLS studies. *International Journal of Biological Macromolecules, 59*, 333– 341.

Croguennec, T., Renault, A., Beaufils, S., Dubois, J., & Pezennec S. (2007). Interfacial properties of heat-treated ovalbumin. *Journal of Colloid and Interface Science, 315*, 627–636

Fennema, O. R. (1996). Food chemistry (3rd ed.). New York: Marcel Dekker Inc. (Chapter 3).

426 Fioramonti, S. A., Perez, A. A., Aríngoli, E. E., Rubiolo, A. C., Santiago, L. G. (2014). Design and
427 characterization of soluble biopolymer complexes produced by electrostatic self-assembly of a
428 whey protein isolate and sodium alginate. *Food Hydrocolloids*, *35*, 129-136.
429 Fontana, A., Spolaore, B., & Polverino de Laureto, P. (2013). The biological activities of protein/oleic
430 acid complexes reside in the fatty acid. *Biochimica et Biophysica Acta*, *1834*, 1125–1143.
431 Galazka, V. B., Smith, D., Ledward, D. A., & Dickinson, E. (1999). Interactions of ovalbumin with
432 sulphated polysaccharides: effects of pH, ionic strength, heat and high pressure treatment. *Food*
433 *Hydrocolloids*, *13*, 81–88.
434 Giosafatto, C.V.L., Rigby, N.M., Wellner, N., Ridout, M., Husband, F., & Mackie, A.R. (2012).
435 Microbial transglutaminase-mediated modification of ovalbumin. *Food Hydrocolloids*, *26*, 261-267.
436 Gutiérrez, F.J., Albillos, S. M., Casas-Sanz, E., Cruz Z., García-Estrada, C., García-Guerra, A. et al.
437 (2013). Methods for the nanoencapsulation of β-carotene in the food sector. *Trends in Food Science*
438 *& Technology*, 32, 73-83.
439 Hayakawa, S., & Nakai, S. (1985). Contribution of Hydrophobicity, Net Charge and Sulfhydryl Groups to
440 Thermal Properties of Ovalbumin. *Can. Insf. Food Sci. Technol. J.*, *18*, (4), 290-295.
441 Ilyasoglu, H., & El, S.H. (2014). Nanoencapsulation of EPA/DHA with sodium caseinate-gum arabic
442 complex and its usage in the enrichment of fruit juice. *Food Science and Technology*, *56*, 461-468.
443 Kato, A., Nagase, Y., Matsudomi, N., & Kobayashi, K. (1983). Determination of Molecular Weight of
444 Soluble Ovalbumin Aggregates during Heat Denaturation Using Low Laser Light Scattering
445 Technique. *Agric. Biol. Chem.*, *47* (8), 1829-1834.
446 Keerati-u-rai, M., Miriani, M., Iametti, S., Bonomi, F., & Corredig, M. (2012). Structural changes of soy
447 proteins at the oil–water interface studied by fluorescence spectroscopy. *Colloids and Surfaces B:*
448 *Biointerfaces*, *93*, 41– 48.
449 Matsudomi, N., Takahashi, H., & Miyata, T. (2001). Some structural properties of ovalbumin heated at
450 80°C in the dry state. *Food Research International*, *34*, (2–3), 229-235.
451 Mehalebi, S., Nicolai, T., & Durand, D. (2008). Light scattering study of heat-denatured globular protein
452 aggregates. *International Journal of Biological Macromolecules*, *43*, 129–135.
453 Nakamura, R., Sugiyam, H., & Sato, Y. (1978). Factors Contributing to the Heat-induced Aggregation of
454 Ovalbumin. *Agric. Biol. Chem.*, *42* (4), 819-824.
455 Niidome (2014). Application note: The Use of Light Scattering to Study the Structure and charge of
456 Dendritic Poly(L-Lysine). Malvern Instruments Ltd.
457 Nyemb, K., Guérin-Dubiard, C., Dupont, D., Jardin, J., Rutherfurd, S. M., & Nau, F. (2014). The extent of
458 ovalbumin in vitro digestion and the nature of generated peptides are modulated by the morphology
459 of protein aggregates. *Food Chemistry*, *157*, 429–438

460 Perez, A. A., Andermatten, R. B., Rubiolo, A. C., & Santiago, L. G. (2014). β-Lactoglobulin heat-induced
461 aggregates as carriers of polyunsaturated fatty acids. *Food Chemistry*, *158*, 66-72.
462 Sánchez-Gimeno, A. C., & López-Buesa, A. V. P. (2006). Studies of ovalbumin gelation in the presence
463 of carrageenans and after manothermosonication treatments Innovative. *Food Science and*
464 *Emerging Technologies*, *7*, 270–274
465 Shpigelman, A., Israeli, G. & Livney, Y. (2010). Thermally-induced protein-polyphenol co-assemblies:
466 beta lactoglobulin-based nanocomplexes as protective nanovehicles for EGCG. *Food*
467 *Hydrocolloids*, *24*, 735-743.
468 Sponton, O. E., Perez, A. A., Carrara, C. R., Santiago, L. G. (2014). Effect of limited enzymatic
469 hydrolysis on linoleic acid binding properties of β-lactoglobulin. *Food Chemistry*, *146*, 577–582.
470 Wang, R., Yin, Y., Li, H., Wang, Y., Pu, J., Wang, R. et al. (2013). Comparative study of the interactions
471 between ovalbumin and three alkaloids by spectrofluorimetry. *Mol Biol Rep*, 40, 3409–3418
472 Weijers, M., Sagis, L.M.C., Veerman, C., Sperber, B. & van der Linden, E. (2002).
473 Rheology and structure of ovalbumin gels at low pH and low ionic strength. *Food*
474 *Hydrocolloids*, *16*, (3), 269-276.
475 Weijers, M., Barneveld, P. A., Cohen Stuart, M. A., & Visschers, R. W. (2003). Heat-induced
476 denaturation and aggregation of ovalbumin at neutral pH described by irreversible first-order
477 kinetics. *Protein Science*, 12, 2693–2703.
478 Weijers, M., Barneveld, P. A., Cohen Stuart, M. A., & Visschers R. W. (2003). Heat-induced denaturation
479 and aggregation of ovalbumin at neutral pH described by irreversible first-order kinetics. *Protein*
480 *Science*, *12*, 2693–2703.
481 Weijers, M., Visschers, R. W., & Nicolai, T. (2004). Influence of the Ionic Strength on the Structure of
482 Heat-Set Globular Protein Gels at pH 7. Ovalbumin. *Macromolecules*, *37*, 8709-8714
483 Wolfe, F. H. (1966). A quantitative study on the denaturation of ovalbumin by shaking. A Dissertation In
484 Chemistry. Faculty of Texas Technological College (US).
485 Zimet, P., & Livney, Y. D. (2009). Beta-lactoglobulin and its nanocomplexes with pectin as vehicles for
486 ω-3 polyunsaturated fatty acids. *Food Hydrocolloids*, *23*, 1120–1126.
487

Captions

Tables

Table 1. Wavelength shift for intrinsic fluorescence (Δλ-Trp). Different letters indicate a significant difference ($p < 0.05$).

Table 2. Wavelength shift for extrinsic fluorescence (Δλ-ANS). Different letters indicate a significant difference ($p < 0.05$).

Table 3. Summary of data for selected samples for LA binding experiments. Heating temperature: 70°C. Different letters indicate a significant difference ($p < 0.05$).

Table 1

Heating time	10 g/L, pH 6			10 g/L, 70°C			pH 6, 70°C		
	60°C	70°C	80°C	pH 5.5	pH 6	pH 6.5	5 g/L	10 g/L	14.2 g/L
0 min	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a,b}	0.0±0.0^{a,b}	0.0±0.0^{a,b}	0.0±0.0^{b}	0.0±0.0^{b}	0.0±0.0^{b}
3 min	0.3±0.4^{a}	1.0±0.0^{b}	0.8±0.4^{b}	-0.3±0.4^{a}	1.0±0.0^{e}	0.6±0.4^{d}	0.0±0.0^{a,b}	1.0±0.3^{c}	-0.3±0.5^{a}
5 min	0.3±0.5^{a}	1.0±0.0^{b}	0.8±0.4^{b}	0.2±0.5^{b,c}	1.0±0.0^{e}	0.4±0.5^{c,d}	0.0±0.0^{a,b}	1.0±0.0^{c}	0.0±0.0^{a,b}
10 min	0.4±0.5^{a}	1.0±0.0^{b}	1.0±0.0^{b}	0.2±0.5^{b,c}	1.0±0.0^{e}	0.8±0.0^{d,e}	0.2±0.4^{b}	1.0±0.3^{c}	-0.3±0.5^{a}

Table 2

Heating time	10 g/L, pH 6			10 g/L, 70°C			pH 6, 70°C		
	60°C	70°C	80°C	pH 5.5	pH 6	pH 6.5	5 g/L	10 g/L	14.2 g/L
0 min	0.0±0.0^{g}	0.0±0.0^{g}	0.0±0.0^{g}	0.0±0.0^{e}	0.0±0.0^{e}	0.0±0.0^{e}	0.0±0.0^{e}	0.0±0.0^{e}	0.0±0.0^{e}
3 min	-2.5±0.5^{f}	-12.0±0.0^{d}	-29.9±1.5^{b}	-15.0±4.9^{c}	-12.0±0.0^{d}	-12.0±0.0^{d}	-8.6±0.5^{c,d}	-12±0.0^{b}	-20.7±1.7^{a}
5 min	-3.0±0.9^{f}	-12.8±0.5^{d}	-33.7±2.5^{a}	-21.3±1.0^{a,b}	-12.8±0.5^{c,d}	-12.6±0.5^{d}	-7.8±0.5^{d}	-12.8±0.5^{b}	-20.7±1.7^{a}
10 min	-5.3±0.6^{e}	-19.5±2.1^{c}	-30.5±0.4^{b}	-22.8±1.3^{a}	-19.5±2.1^{b}	-13.5±0.5^{c,d}	-9.3±0.0^{c}	-19.5±2.1^{a}	-19.0±2.5^{a}

Table 3

Sample	Heating conditions			Main Diameter* (nm) (% volume)	ANS Fluorescence	Binding ability (%)
	pH	Protein concentration (g/L)	Heating time (min)			
Native OVA	-	-	-	6	1.0 ± 0.0^{a}	34 ± 9^{a}
OVA_n	6	5	3	122	4.2 ± 0.1^{c}	69 ± 1^{e}
OVA_n	6	5	5	207	4.7 ± 0.1^{e}	$60 \pm 3^{d,e}$
OVA_n	6	10	3	225	3.9 ± 0.2^{b}	$50 \pm 2^{b,c}$
OVA_n	6	10	5	120 (20%) 550 (81%)	4.4 ± 0.1^{d}	49 ± 2^{b}
OVA_n	6.5	10	3	10 (53%) 76 (47%)	$4.2 \pm 0.1^{c.d}$	$60 \pm 3^{c,d,e}$
OVA_n	6.5	10	5	96	4.7 ± 0.1^{e}	$55 \pm 7^{b,c,d}$
OVA_n	6.5	10	10	192	5.8 ± 0.1^{f}	$63 \pm 2^{d,e}$

* Peaks with area higher than 5% in PSDv are shown.

Figures

Captions

Figure 1. Volume PSD of heat-treated OVA solutions. Heating conditions: 10 g/L, pH 6.0, **(a)** 60°C, **(b)** 70°C, **(c)** 80°C.

Figure 2. Volume PSD of heat-treated OVA solutions. Heating conditions: 70°C **(a)** pH 5.5, 10 g/L; **(b)** pH 6.5, 10 g/L; **(c)** pH 6.0, 5 g/L; **(d)** pH 6.0, 14.2 g/L.

Figure 3. Trp Relative Fluorescence Intensity (RFI-Trp) of heat-treated OVA solutions. **(a)** Effect of temperature (heating conditions: pH 6.0, 10 g/L) **(b)** Effect of aqueous medium pH (heating conditions: 70°C, 10 g/L) **(c)** Effect of concentration (heating conditions: 70°C, pH 6.0).

Figure 4. ANS Relative Fluorescence Intensity (RFI-ANS) of heat-treated OVA solutions. **(a)** Effect of temperature (heating conditions: pH 6.0, 10 g/L) **(b)** Effect of aqueous medium pH (heating conditions: 70°C, 10 g/L) **(c)** Effect of concentration (heating conditions: 70°C, pH 6.0).

Figure 5. Absorbance at 400 nm of LA solutions in presence of different OVA solutions.

Figure 1

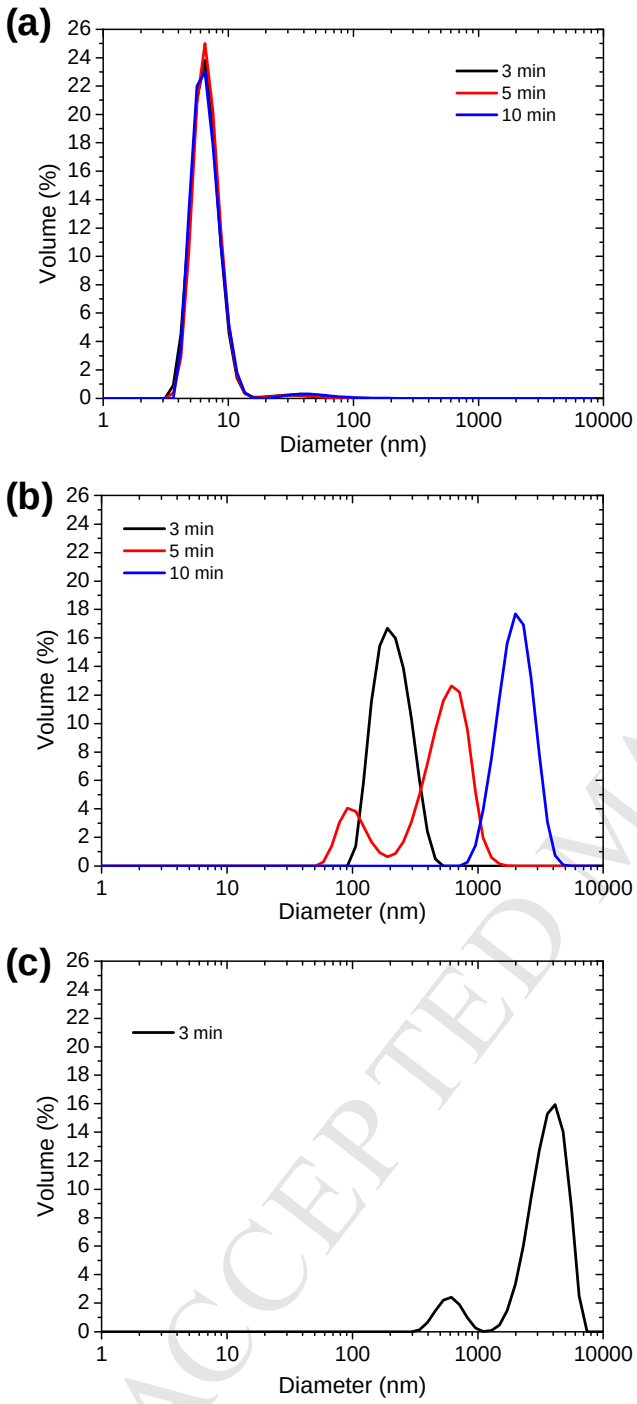


Figure 2

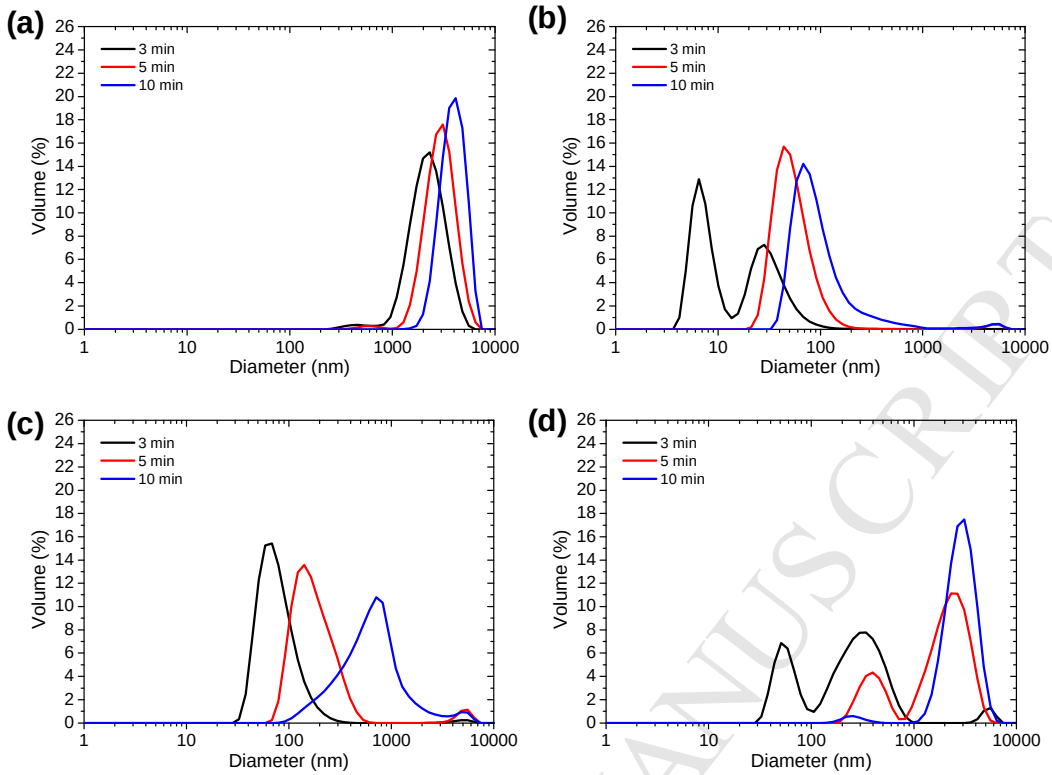


Figure 3

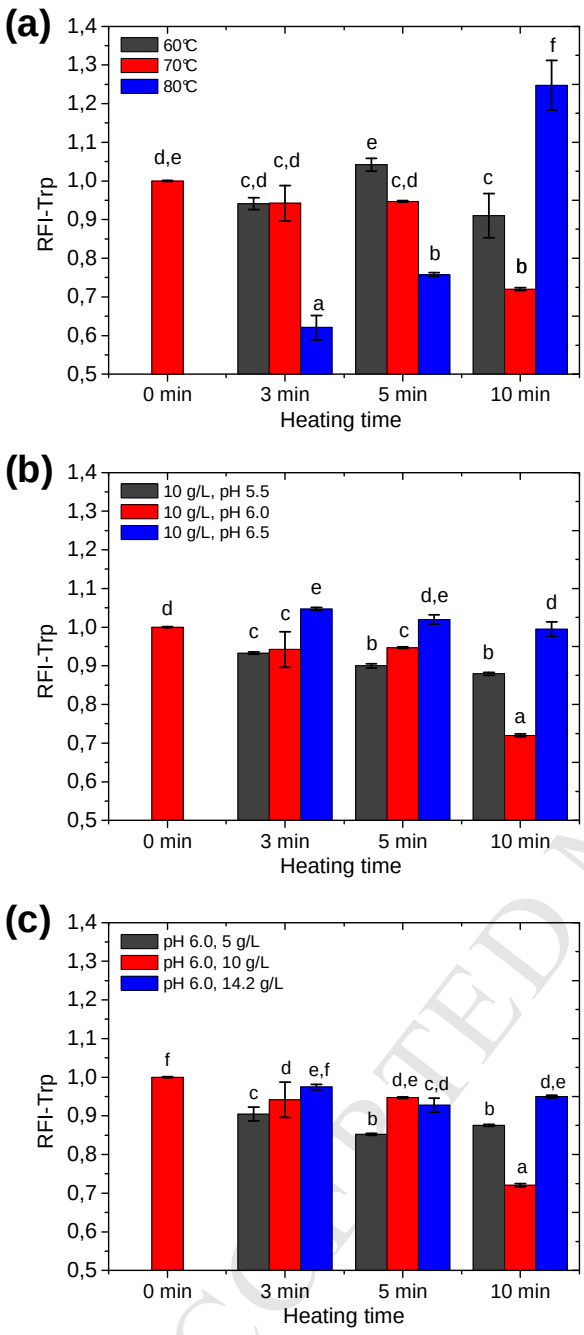


Figure 4

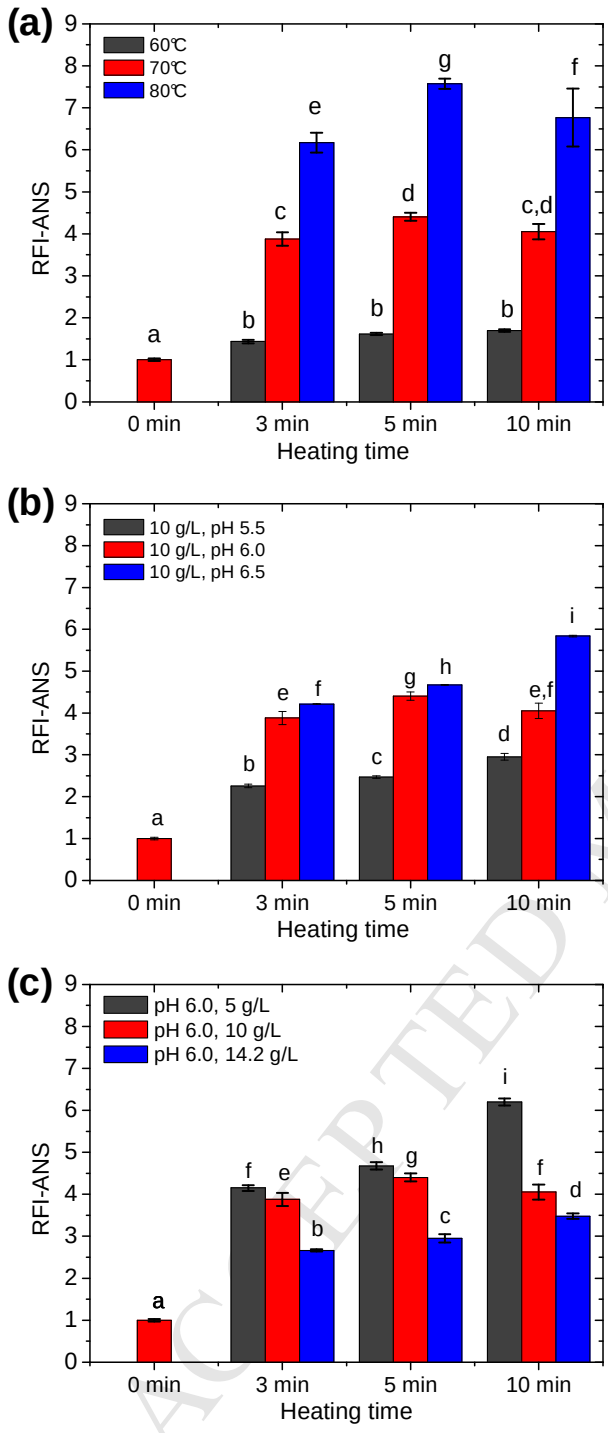
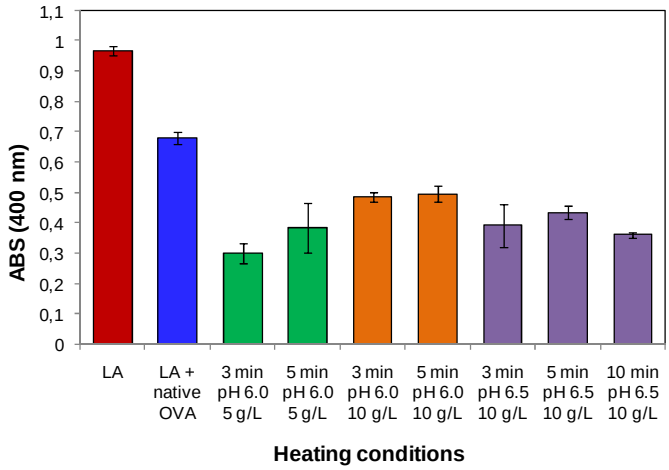


Figure 5



Ovalbumin aggregates were obtained at different heating and environment conditions.

Ovalbumin aggregate sizes increased with temperature, time and protein concentration.

At higher pH value, ovalbumin (OVA) aggregate sizes were lower.

OVA nanoparticles showed 1.4-2.0 fold greater LA binding ability than native OVA.