

# Effects of heat-treated $\beta$ -lactoglobulin and its aggregates on foaming properties

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## ABSTRACT

The effects on foaming properties of the aggregates formed by heating concentrate beta-lactoglobulin solutions ( $55 \text{ mg mL}^{-1}$ , pH 6.8) at  $85^\circ\text{C}$  from 1 to 15 min were investigated. Structural characteristics (size and molecular conformation), hydrophobicity and protein aggregates proportion were also studied. All tested methods pointed at 3 min of heating as a critical time, in terms of conformational changes and aggregation processes. At this time, the most significant conformational changes took place: non-native monomers were present and the greatest amount of dimers and trimers was produced, which was proved with the results of gel densitometry of SDS-PAGE, fluorescence quenching and circular dichroism tests. Foamability and foam stability were both improved by pre-heating the protein. A constant proportion among beta-lactoglobulin species (monomer 51%, dimer 33% and trimer 16%), regardless the protein concentration, led to the same results on foaming properties, confirming the link with structural changes. Aggregates formed by heating beta-lactoglobulin up to 10 min produced more stabilized foams, slowing down disproportionation, because of the formation of stiffer films. The increase in surface hydrophobicity was considered a decisive factor in the improved foamability and hydrophobic interactions improved the foam stability through the rapid formation of a viscoelastic film.

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## 1. Introduction

In food industry, foams are mainly stabilized by proteins. A number of papers have been devoted to the characterization of the foaming behaviour of proteins under a variety of conditions (Foegeding, Luck, & Davis, 2006; Murray, 2007).

Proteins from aqueous solutions adsorbed spontaneously to air/water interface where their free energy is lower than in the non adsorbed state. During adsorption process, proteins unfold and establish intermolecular interactions with other proteins at the interface, or close to the interface, leading to the formation of an interfacial film of proteins. Compared to low molecular weight surfactants, proteins are less effective to reduce the air/water interfacial tension but they form an interfacial film exhibiting viscoelastic properties that are thought to improve the resistance of the foam under stress or aging conditions (Damodaran, 2005; Foegeding et al., 2006).

Whey proteins, milk proteins that remain soluble after rennet or acid precipitation of caseins, have been increasingly utilized as a source of protein as well as a functional ingredient in many formulated foods. Heating induces denaturation/aggregation of

whey proteins, modifying their functional properties. The majority of the studies on whey protein aggregation have been confined to model systems which use purified beta-lactoglobulin ( $\beta$ -LG), because it is the most abundant protein among whey proteins and readily purified (De la Fuente, Singh, & Hemar, 2002).

Under physiological conditions,  $\beta$ -LG associates as a non-covalent dimer. It is thought that  $\beta$ -LG native dimer is in rapid equilibrium with  $\beta$ -LG native monomer (McKenzie, 1971).  $\beta$ -LG monomer is a single polypeptide chain of 162 amino acids, with a molecular weight of 18.3 kDa and its structure involves eight strands of anti-parallel  $\beta$ -sheets and one  $\alpha$ -helix, as it was determined by X-ray crystallography (Papiz et al., 1986). Each monomer has two disulfide bonds (Cys66–Cys160 and Cys106–Cys119), which stabilize the protein tertiary structure and one free sulfhydryl group at position Cys121 buried within the protein structure at pH > 7.5.

Using pre-heated  $\beta$ -LG solutions ( $1.0 \text{ mg mL}^{-1}$ , pH 7.0) at  $80^\circ\text{C}$  during different periods of time (5–30 min), Kim, Cornec, and Narsimhan (2005) found that thermal treatment enhanced foam stability. These authors did not characterize the species in solution. However, taking into account the low protein concentration they used and the temperature and time of heating, it can be inferred from previous works (Croguennec, O'Kennedy, & Mehra, 2004; Manderson, Hardman, & Creamer, 1998; Prabakaran & Damodaran, 1997; Schokker, Singh, Pinder, Norris, & Creamer,

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1999) that the species of  $\beta$ -LG which are responsible for this particular behaviour were native monomers, non-native monomers and oligomers of low molecular weight (dimers and trimers).

On the other hand, Rullier, Novalles, and Axelos (2008) heated  $\beta$ -LG solutions of different concentrations (1, 2, 8 and 10 mg mL<sup>-1</sup>) at 80 °C for 24 h, in order to obtain aggregates of high molecular weight. Their results showed that these aggregates varied between 35 and 197 nm and were not able to improve the foaming properties.

However, there are some differences between the experimental conditions in which those cited works were carried out and the conditions used in several industrial processes, e.g.: the protein concentrations currently used in food industry are greater, i.e.  $\geq 50$  mg mL<sup>-1</sup> (McIntosh et al., 1998) and most of the industrial heat treatments applied to the samples are not as drastic as the long time of heating used by Rullier et al. (2008). In that sense, the aim of the present work was to investigate the effect on foam properties of the aggregates formed by heating concentrated  $\beta$ -LG solutions (55 mg mL<sup>-1</sup>, pH 6.8) at 85 °C for periods of time from 1 to 15 min, conditions which are similar to those in industrial processes. Structural characteristics (size and molecular conformation), hydrophobicity and protein aggregates proportion were also studied.

## 2. Materials and methods

### 2.1. Heat treatment of $\beta$ -LG

A stock 55 mg mL<sup>-1</sup>  $\beta$ -LG solution was prepared in 20 mM phosphate buffer at pH 6.8. Aliquots from this solution were placed in small glass tubes and heated in a water bath at 85 °C. The heating times varied from 1 to 15 min. The samples were cooled to room temperature and analyzed as it is described in the following sections. The concentration of  $\beta$ -LG solutions was verified through the measurement of absorbance at 280 nm and calculated with the use of Beer's law with an extinction coefficient of 0.966 mL mg<sup>-1</sup> cm<sup>-1</sup>.

### 2.2. Electrophoresis

SDS-PAGE of native and heated  $\beta$ -LG was performed as Laemmli (1970) described, using a stacking gel of 10% and a running gel of 15%. The gels were run under nonreducing conditions to avoid cleavage of intermolecular disulfide bonds formed during the heating treatment. After electrophoresis, gels were stained with Coomassie Brilliant Blue R250 and scanned using a Hewlett–Packard ScanJet 5p connected to a computer. To quantify the relative intensities of the stained protein bands, the pixel densities of digitized images were analyzed using software developed by our group (Palazolo, Rodriguez, Farruggia, Picó, & Delorenzi, 2000). The molecular weight of each protein band was matched to known standard proteins.

In order to identify the  $\beta$ -LG species which are responsible for the foaming behavior obtained by Kim et al. (2005), a  $\beta$ -LG solution 1.0 mg mL<sup>-1</sup> was heated at 80 °C, with heating times varying from 5 to 30 min, and also studied by SDS-PAGE.

### 2.3. Circular dichroic spectral measurements

Circular dichroism (CD) of  $\beta$ -LG solutions was measured using a Jasco J-810 automatic recording spectropolarimeter (Japan), with an integration time of 2 s and 2 nm bandwidth. Four scans at a rate of 50 nm min<sup>-1</sup> were carried out and their results were averaged for the wavelength range employed. In the experiments in far UV (190–250 nm), 25  $\mu$ M protein solutions were assayed in 0.1 cm

path length cell. In the near UV (250–320 nm), 164  $\mu$ M protein solutions were assayed in 1 cm path length cell. The CD data were reported as mean residue ellipticity ( $[\theta]_{MRW}$ ) in units of deg cm<sup>2</sup> dmol<sup>-1</sup>, using a mean residue weight of 113 g.

The CD spectra of the native and heat-treated protein solutions were compared to assess any conformational changes. The far UV spectra were analyzed to infer different proportions of  $\alpha$ -helix,  $\beta$ -sheet and random coil using CONTIN software that compared the spectrum with the spectra of 16 standard proteins of different conformations (Chang, Wu, & Yang, 1978). The near UV CD spectra were used to characterize the tertiary structure of proteins mainly because of the constrained asymmetries in the environment of the aromatic amino acids (Greenfield, 1996).

In order to compare these spectra with those of chemically denatured  $\beta$ -LG, solutions of this protein in 8 M urea, without any heating treatment, were also assayed.

### 2.4. Measurement of $\beta$ -LG surface hydrophobicity using acrylamide

Three milliliters of a sample, 10  $\mu$ M in  $\beta$ -LG, was placed in the cell of a Jasco FP-770 spectrofluorometer, and the fluorescence intensity ( $F_0$ ) was measured at 337 nm, using excitation at 295 nm. Aliquots of 7 M acrylamide, used as fluorescence quencher, were sequentially added in the cell content, and the new fluorescence intensities were measured ( $F$ ). Acrylamide concentrations ranged from 0 to 0.2 M. The  $F_0/F$  ratio was plotted versus the quencher concentration (Stern–Volmer plot). In the used range, this plot was linear and the Stern–Volmer equation can be expressed as

$$\frac{F_0}{F} = 1 + K_{app}[\text{acrylamide}] \quad (1)$$

$K_{app}$  is an apparent constant because  $\beta$ -LG has more than one tryptophanyl residue that can be quenched by acrylamide. The initial slope of Stern–Volmer plots,  $K_{app}$ , is an index of protein hydrophobicity.

Two other methods were tested to a further study of surface hydrophobicity: binding *cis*-parinaric acid (CPA) or 1-anilino-8-naphthalenesulfonate (ANS) to the protein. Their results completely agreed with the fluorescence quenching method and led to similar conclusions (Moro, Gatti, & Delorenzi, 2001). The fluorescence quenching of  $\beta$ -LG in urea 8 M without any heating treatment was also studied, in order to compare chemically and thermally denatured structures.

### 2.5. Foaming properties

Foams were formed using a bubbling apparatus (Hagolle, Relkin, Popineau, & Bertrand, 2000; Loisel, Guéguen, & Popineau, 1993). Native and heated  $\beta$ -LG was diluted to 0.1% (w/v) in 20 mM phosphate buffer, pH 6.8. Determinations were made in a transparent acrylic tube (3.5 cm  $\times$  20.0 cm) equipped with a pair of electrodes located at the base of the column and with a porous disk through which air, at a flow rate of 5 mL s<sup>-1</sup>, was passed and forced through the liquid ( $V_{init}$  = 10 mL), creating foam. Bubbling stopped when the foam reached a fixed volume of 115 mL ( $V_f$ ). During the test, the conductivity and the volume of foam were recorded by a computer and a digital camera Olympus DS-580 4.0 mega pixels.

Conductivity measurements at different times ( $C_t$ ) and with reference to the initial conductivity ( $C_{init}$ ) were used to calculate the volume of liquid in the foam ( $V_{LF}$ ) (Chevalier, Chobert, Popineau, Nicolas, & Haertle, 2001; Loisel et al., 1993):

$$V_{LF} = V_{init} \left[ 1 - \frac{C_t}{C_{init}} \right] \quad (2)$$

As reported previously, foams are compared on the basis of (i) maximum foam density (FD), as a measure of foamability; and (ii) half-life time of drainage ( $T_{1/2}$ ) and (iii) volume variation with time, as measures of foam stability (Croguennec, Renault, Bouhallab, & Pezennec, 2006; Fains, Bertrand, Baniel & Popineau, Y., 1997; Hagolle et al., 2000).

FD is defined as the ratio between the maximal liquid incorporated into the foam ( $V_{LFmax}$ ) and the foam volume reached at the end of the sparging period ( $V_{Fmax}$ ):

$$FD = \frac{V_{LFmax}}{V_{Fmax}} \quad (3)$$

It has been noted that  $V_{Fmax} = V_f - (V_{init} - V_{LFmax})$ .

$T_{1/2}$ , the half-life time of drainage, is equal to:

$$T_{1/2} = t_{1/2} - t_0 \quad (4)$$

where  $t_{1/2}$  is the time when half of the maximum volume of liquid in the foam came back to the solution ( $V_{LFmax}/2$ ) and  $t_0$  is the time at the end of bubbling.

On the other hand, in terms of volume variation with time, the longer it takes for the foam to collapse, the more stable the foam is (Wilde & Clark, 1996). This can be measured through the one quarter time of foam volume ( $T_{1/4}$ ), which is defined as:

$$T_{1/4} = t_{1/4} - t_0 \quad (5)$$

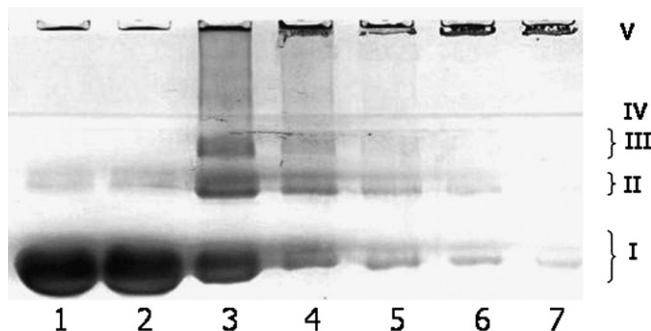
where  $t_{1/4}$  is the time required for  $V_{Fmax}$  to decay 25% and  $t_0$  is the time at the end of bubbling.

Both times,  $T_{1/2}$  and  $T_{1/4}$ , and FD are plotted versus heating time (min), as ratios in reference to initial values (without heating treatment).

### 3. Results and discussion

#### 3.1. Electrophoresis

$\beta$ -LG solutions ( $55 \text{ mg mL}^{-1}$ ) heated for 0, 1, 3, 5, 7, 10 and 15 min at  $85^\circ\text{C}$ , were analyzed by SDS-PAGE (non reduced) (Fig. 1). The unheated sample ran as a dense band (region I), containing monomeric  $\beta$ -LG and a faint band (region II) containing dimers. With increasing heating time, the amount of monomeric  $\beta$ -LG decreased and aggregates of various sizes were also formed: dimers (region II), trimers (region III) and oligomers and polymers (from region IV to region V), which did not enter the running gel. With heating, the region I and region II seemed to be split into two bands, indicating the presence of different species, produced by a diversity of intramolecular disulfide bonds for monomers and by inter and



**Fig. 1.** SDS-PAGE (non reduced) of  $\beta$ -LG ( $55 \text{ mg mL}^{-1}$ ) unheated (lane 1) and heated at  $85^\circ\text{C}$  for different periods of time (min): 1 (lane 2), 3 (lane 3), 5 (lane 4), 7 (lane 5), 10 (lane 6) and 15 min (lane 7). I: SDS-monomeric  $\beta$ -LG; II: dimers; III: trimers; IV: aggregates not entering the running gel; V: aggregates not entering the stacking gel.

intramolecular bonds for the aggregates. Moreover, the split of the monomeric band indicates the presence of irreversibly altered monomer (non-native  $\beta$ -LG) (Manderson et al., 1998; Schokker et al., 1999).

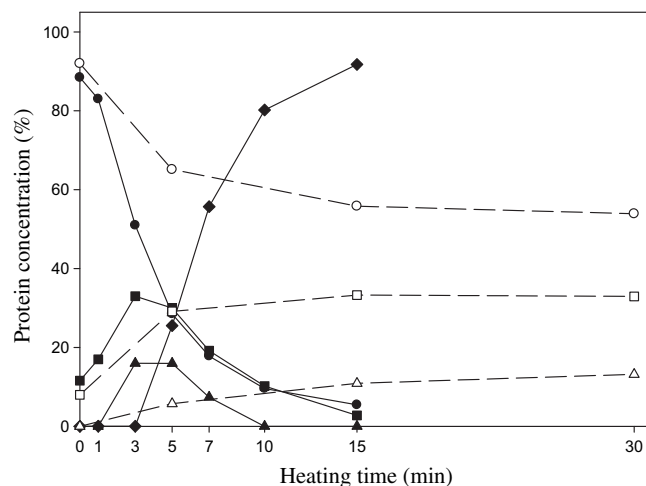
The loss of monomeric  $\beta$ -LG and the appearance of other species were quantified by gel densitometry (Fig. 2). With heating at  $85^\circ\text{C}$ , the quantities of dimers and trimers decreased, whereas the amount of aggregates larger than trimers increased abruptly. The largest amount of dimers and trimers was found at 3 min of heating and at 10 min of heating, oligomers and polymers represented more than 80% of the species in solution.

Gel densitometry for the heating conditions assayed by Kim et al. (2005) (gel not shown) proved that the quantity of  $\beta$ -LG monomers decreased slowly with heating time, whereas the quantities of dimers and trimers increased with time until 15 min (Fig. 2). Between 15 min and 30 min the protein concentration of the different species seemed to be approximately constant. On the other hand, the presence of aggregates larger than trimers was not detected in the range of the used heating times.

#### 3.2. Circular dichroism

The effects of heating  $\beta$ -LG solutions at  $85^\circ\text{C}$  on the far UV CD spectra are shown in Fig. 3. There is a shift to shorter wavelengths in the spectra, being particularly pronounced between 3 min and 5 min of heating, with smaller changes for subsequent heating times. The far UV CD spectra largely reflect the secondary structure of proteins. The different proportions of secondary structures inferred from far UV CD spectra of  $\beta$ -LG samples, heated during different periods of time, agreed with the results reported by Qi et al. (1997) and by Kim et al. (2005), despite different protein concentrations were used. The analysis of CD spectra showed a decrease in  $\alpha$ -helix and  $\beta$ -sheet and a corresponding increase in random coil conformation with heating time.

In previous works, it has been suggested that  $\beta$ -LG unfolding is independent of protein concentration, whereas the aggregation phenomenon is highly dependent on protein concentration (Iametti, Cairolì, De Gregori, & Bonomi, 1995). Confirming this, heating  $\beta$ -LG solutions of different protein concentration ( $1 \text{ mg mL}^{-1}$  and  $55 \text{ mg mL}^{-1}$ ) resulted in irreversible and concentration-independent



**Fig. 2.** Protein concentrations (%) of different species formed by heating  $\beta$ -LG solutions ( $55 \text{ mg mL}^{-1}$ ) at  $85^\circ\text{C}$  and for different periods of time: monomers (●), dimers (■), trimers (▲) and aggregates not entering the running gel (oligomers and polymers) (◆). Protein concentrations (%) of different species formed by heating  $\beta$ -LG solutions ( $1 \text{ mg mL}^{-1}$ ) at  $80^\circ\text{C}$  and for different periods of time: monomers (○), dimers (□) and trimers (△).

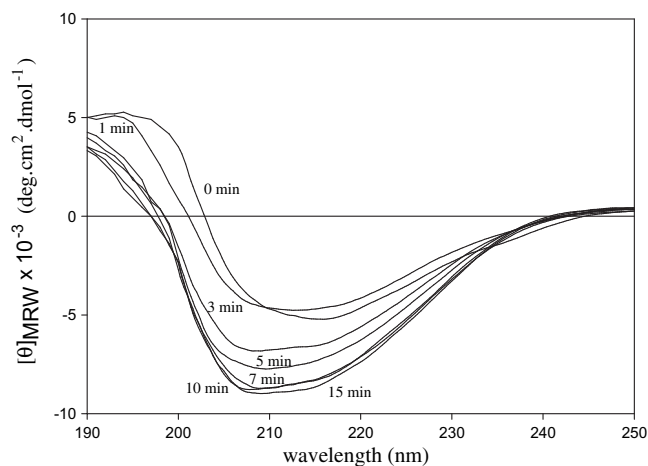


Fig. 3. CD spectra in the far ultraviolet of  $\beta$ -LG solutions subjected to heating at 85 °C for different periods of time.

modifications of the secondary structure of the protein, as shown by CD spectra analysis (Fig. 3). Contrarily, showing the dependence of aggregation on protein concentration, the proportion of monomeric and multimeric species after the heating treatment was quite different when concentration changed, as the analysis of SDS-PAGE confirmed.

The effects of heating  $\beta$ -LG solutions at 85 °C on the near UV CD spectra are shown in Fig. 4. The analysis of near UV CD spectra were used to characterize the tertiary structure of proteins mainly because of the constrained asymmetries in the environment of the aromatic amino acids (Greenfield, 1996). The two negative peaks of the ellipticity at  $\sim 285$  and  $\sim 292$  nm in the CD spectra of native  $\beta$ -LG are mainly due to Trp19 absorbance (Creamer, 1995; Gast, Siemer, Zirwer, & Damaschew, 2001). These two negative peaks diminished their intensities with the heating treatment at 85 °C (Fig. 4), reflecting structural changes within the calyx of the  $\beta$ -LG molecule, where the indole side chain of Trp19 is located (Manderson, Creamer, & Hardman, 1999), and being particularly pronounced between 3 min and 5 min of heating, with smaller changes for subsequent heating times. It should be noted that some ordered tertiary structures have remained in the protein molecules after heat treatment, since the chemical denaturation with 8.0 M

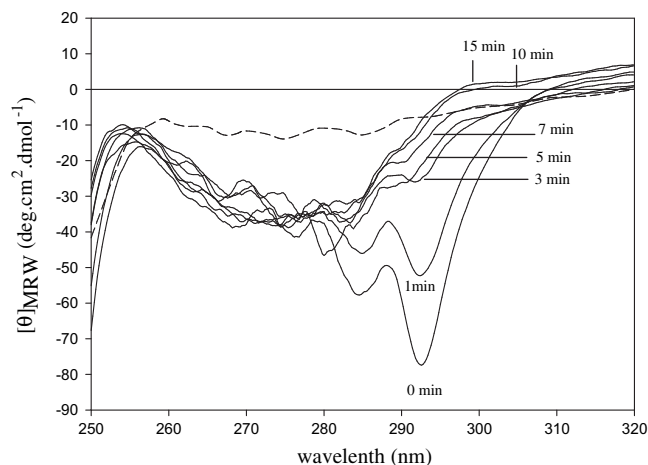


Fig. 4. CD spectra in the near ultraviolet of  $\beta$ -LG solutions subjected to heating at 85 °C for different periods of time. CD spectrum of  $\beta$ -LG in 8 M urea without any heat treatment (dashed line).

urea solution caused a further unfolding, which became evident in the CD spectra between 260 and 292 nm (Fig. 4).

It can be noted that  $\beta$ -LG solutions became turbid for heating times longer than 15 min, due to the presence of protein aggregates. In these conditions, spectrophotometric determinations were impossible.

### 3.3. Fluorescence quenching

Fluorescence quenching of proteins by acrylamide is an accepted method for determining surface hydrophobicity (Moro et al., 2001). Protein unfolding causes a red shift on the fluorescence emission spectrum due to the major exposition of Trp residues to the aqueous solvent, which promotes an increase in the fluorescence quenching of denatured proteins by acrylamide (Busti, Gatti, & Delorenzi, 2006; Moro et al., 2001; Palazolo et al., 2000). The more flexible the protein structure and/or the higher the unfolded species concentration, the more pronounced the slope in the Stern–Volmer plot and then, greater  $K_{app}$  is (Equation (1)). Besides, during the unfolding process, the protein surface becomes more and more hydrophobic due to the appearance of non-polar amino acids, which were previously inside the protein structure.

The results of the present work were analyzed from Fig. 5. The effectiveness of the quenching process, measured through the  $K_{app}$  values, increased with the length of heating treatment, verifying the minimum value for the unheated protein and the maximum, after 3 min of heating time, which corresponds to the conformational and surface hydrophobicity changes. The  $K_{app}$  increment can be ascribed to the presence of a percentage of non-native monomers and aggregates of low molecular weight of denatured molecules, irreversibly formed during heat treatment.

The effect of acrylamide on the fluorescence of heated  $\beta$ -LG was substantially less than for  $\beta$ -LG denatured by urea ( $K_{app} = 12.05 \text{ M}^{-1} \pm 0.10 \text{ M}^{-1}$ ). These results point at the presence of a residual structure in the denatured cross-linked species that hinders the full Trp accessibility to the acrylamide. This conclusion agrees with and strengthens what was inferred from the near CD spectra.

### 3.4. Conformational changes

In the conditions assayed in this work, all tested methods pointed at 3 min of heating as a critical time, in terms of conformational changes and aggregation processes. At this time, the more

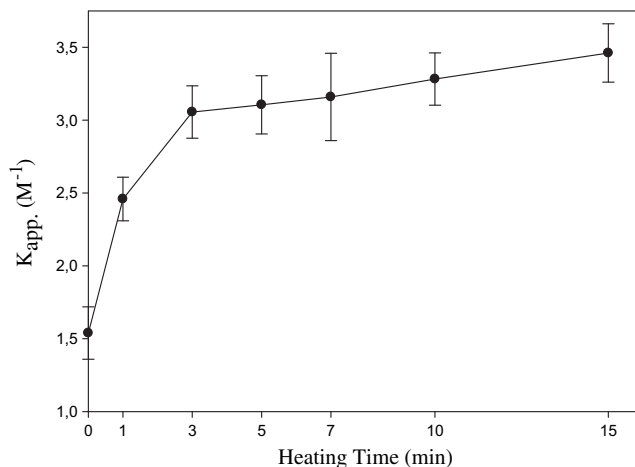


Fig. 5.  $K_{app}$  ( $\text{M}^{-1}$ ) from the Stern–Volmer graphs with the time of heating at 85 °C of  $\beta$ -LG solutions. Each point is the average of three replications.

significant conformational changes take place: non-native monomers are present and the greatest amount of dimers and trimers is produced, which was proved with the results of SDS-PAGE, surface hydrophobicity and circular dichroism tests. The proportion among the  $\beta$ -LG species was: monomer 51%, dimer 33% and trimer 16% (Figs. 1 and 2). Kim et al. (2005) reached a similar proportion among these species (monomer 54%, dimer 33% and trimer 13%) but only after 30 min of heating treatment (Fig. 2). This fact will be relevant at the time of discussing foam properties (Results and Discussion, 3.5).

In previous works, it was quoted that during heating treatments at temperatures higher than 80 °C, native  $\beta$ -LG molecules unfold and lead to the formation of non-native monomers with increasing exposure of inner hydrophobic amino acids to the solvent (Bauer, Carrotta, Rischel, & Ogendal, 2000; Croguennec, Bouhallab, Moullé, O'Kennedy, & Mehra, 2003; Croguennec et al., 2004; Manderson et al., 1998; Palazolo et al., 2000; Schokker et al., 1999; Surroca, Haverkamp, & Heck, 2002). When these non-native monomers were characterized, it was found that either molecules with Cys121 free sulfhydryl group (Mcys121) or molecules with Cys119 free sulfhydryl group (Mcys119) were exposed to the solvent. Mcys119 is formed by intramolecular sulfhydryl/disulfide bond exchange reaction between Cys121 and Cys106–Cys119. It was stated that Mcys121 and Mcys119 constitute the only monomeric species present in  $\beta$ -LG solutions after heating treatments. During the first stages of heating, Mcys119 accumulated and Mcys121 concentration decreased, confirming it as the most reactive species in the aggregation process. Thus, Mcys121 is probably involved as a major element in the mechanism of formation of oligomers, which then act as intermediates in the formation of larger aggregates. On the other hand, on cooling, Mcys121 reverse to native  $\beta$ -LG molecules, whereas Mcys119 remains as the non-native monomer (Croguennec et al., 2003, 2004). In view of these works, the  $\beta$ -LG species present in solution after heating treatment followed by cooling are native monomers, Mcys119 and protein aggregates.

However, SDS-PAGE of the samples, showed a split of the monomeric band (region I) for heating times longer than the critical time of 3 min (Fig. 1), which strengthens the idea of two different non-native species, not only MCys119, as previous works have stated. Some authors have suggested that other changes take place during the process, like  $\beta$ -elimination of the sulfhydryl group or deamidation of asparagines or glutamine groups (Morgan, Léonil, Mollé, & Bouhallab, 1997; Schokker et al., 1999). Further studies to confirm this would be necessary.

### 3.5. Foaming properties

There are two distinct phases to protein foaming: (i) the effectiveness of gas encapsulation (foamability) and (ii) the lifetime of the foam (foam stability) (Foegeding et al., 2006; Wilde & Clark, 1996). The effects of heating treatment on foaming properties of  $\beta$ -LG solutions in the assayed conditions are shown in Fig. 6 and Fig. 7. Foamability and foam stability were both modified by heating.

Foamability, estimated by foam density (FD), was improved (Fig. 7) with a maximum increment of ~40% up to 10 min of heating. It is known that adsorption of a protein in a sufficient amount, within the time scale of foam production is a condition for efficient foam formation, strongly correlated with the rate at which surface tension can be lowered. Hence, one of the most important factors for foam formation is the protein adsorption rate, which depends on protein concentration, molecular weight of the protein, protein structure, and solution conditions such as pH (Martin, Grolle, Bos, Cohen Stuart, & van Vliet, 2002). Therefore,

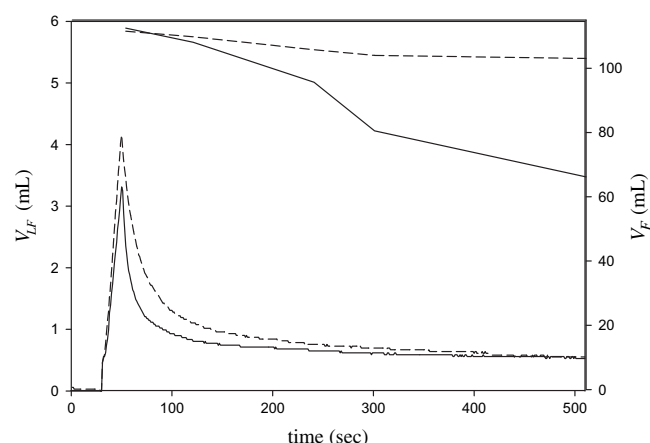


Fig. 6. Foam curves of  $\beta$ -LG without heat treatment (continuous lines) and pre-heated 3 min at 85 °C (dashed lines).

disordered, smaller and more flexible proteins are better surface agents than ordered, larger and rigid ones.

Foamability is enhanced by an increment in protein surface hydrophobicity (Foegeding et al., 2006; Moro et al., 2001; Kato & Nakai, 1980; Townsend & Nakai, 1983). The higher the extent of denaturation a protein suffers, the higher surface hydrophobicity it will present. This leads to a greater affinity of the protein for the interface, which allows it to overcome the barrier against adsorption, which is developed at the interface while proteins are closely packed (Wilde & Clark, 1996). This fact promotes a rapid decrease in surface tension and then, an increase in protein foamability.

As it has been mentioned, after 3 min of heating treatment, there was only non-native  $\beta$ -LG monomers and other denatured species of higher molecular weights (Fig. 2). The heating treatment produces simultaneously two consequences: (i) the production of  $\beta$ -LG aggregates of higher molecular weights and (ii) the thermal unfolding with the exposure of hydrophobic patches on non-native  $\beta$ -LG. While the presence of aggregates produces a reduction in the diffusion coefficient, an unfavorable factor for foam formation; the exposure of hydrophobic patches is a favorable factor for foamability. In view of the observed results, the increased surface hydrophobicity became a more important factor, promoting foam formation. Besides, in the assayed conditions, convection phenomenon, which enhances even more the importance of

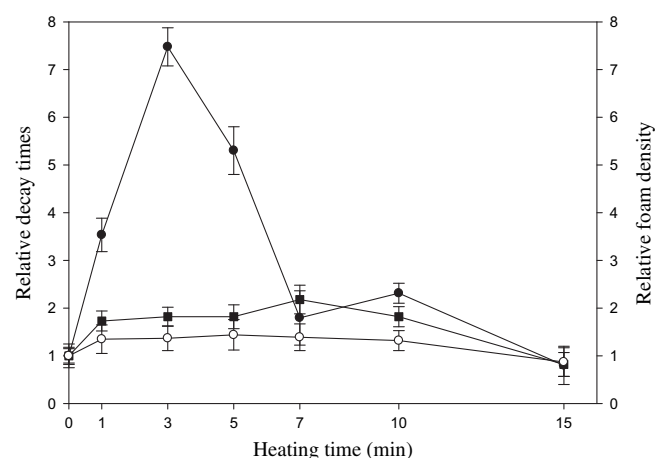


Fig. 7. Variation of relative foam density (O), relative decay of  $T_{1/2}$  (■) and relative decay of  $T_{1/4}$  (●) with heating time. Each point is the average of three replications.

surface hydrophobicity of the aggregates, cannot be disregarded. This is consistent with the conclusions for heating treatment of ovalbumin recently researched (Croguennec, Renault, Beaufils, Dubois, & Pezenec, 2007).

During foam formation, gas bubbles are surrounded by an interfacial film of proteins assuring the protection of the foam against destabilization. The processes involved in foam destabilization are: liquid drainage, a close approach of adjacent bubble surfaces which leads to film rupture (coalescence) and gas diffusion into the continuous phase (Ostwald ripening or disproportionation) resulting in bubble coarsening. All these mechanisms occur simultaneously after the air bubbling stops. While drainage and coalescence prevail at the beginning, when the bubbles are mainly spherical, disproportionation is more important at advanced stages, when the cells are polyedric. At the end, the foam collapses.

Foaming stability, estimated in this work either through  $T_{1/2}$  or  $T_{1/4}$  values, increased in different degrees, in comparison with the unheated sample, for each studied pre-heating time up to 10 min of heating (Figs. 6 and 7). Around 15 min, foam stability decreased. After 15 min of heating, the foam quickly collapses and then, stability cannot be measured.

$T_{1/2}$ , the half time of drainage, verified an increment of ~80% all over the range of heating time. Only the line representing results of 3 min of heating is shown in Fig. 6; nevertheless, the other pre-heating times produced similar results. The observed increase of  $T_{1/2}$  with heating is directly associated with an increase in protein solution viscosity due to the presence of aggregates of denatured  $\beta$ -LG with high hydrodynamic sizes, which slows the drainage rate (Foegeding et al., 2006).

On the other hand,  $T_{1/4}$ , the time required for a decay of 25% for  $V_{Fmax}$ , increased even more than  $T_{1/2}$  in the same range of time. Like this, after heating treatment up to 10 min a peak at 3 min appeared, where this increment was 800%, which is consistent with the most significant conformational changes on  $\beta$ -LG. Kim et al. (2005) reached similar results but only after heating  $\beta$ -LG solutions for a longer time (30 min). It has been mentioned that, at that time of heating, these authors found the same proportion of monomer, dimer and trimer as the one reported for 3 min of heating in this work (Section 3.4). This fact shows that whatever the time of heating was, the foam stability is closely linked with structural changes of the protein.

As it can be observed in Fig. 6, while the drainage phenomenon was almost finished, the foam volume was still considerable and, according to the stage, corresponded to a polyedric foam, which was confirmed through the recorded video. Besides, the foam broke easily in the case of native  $\beta$ -LG, whereas better foam stability was observed for heated samples of  $\beta$ -LG, showing the greatest stabilization when the protein was heated for 3 min. These results agree with those reached by Martin et al. (2002) in the way that interfacial rheology plays an important role against disproportionation. Thus, aggregates formed by heating  $\beta$ -LG up to 10 min led to more stabilized foams, slowing down disproportionation, because of the formation of stiffer films that resist compression and may reduce gas transport.

Croguennec et al. (2006) have suggested that the initial rheology (short-term) of the interfacial film is more important in the general mechanism of foam stabilization than the viscoelasticity that the interfacial film could reach on aging (long-term). Following the conclusions of these authors, it was assumed for this discussion that the higher flexibility of  $\beta$ -LG species (non-native monomers, dimers, trimers and oligomers of low molecular weight, produced in  $\beta$ -LG solutions heated up to 10 min) could facilitate their reorganization at the interface, leading to faster associations with neighbouring molecules and so, the rapid development (short-term) of a viscoelastic film. The main forces involved in the

formation of this closely packed layer of adsorbed proteins could be hydrogen bonds, hydrophobic associations and electrostatic interactions rather than disulfide bonds formed by sulfhydryl–disulfide exchange reactions. For  $\beta$ -LG solutions heated over 10 min, the presence of oligomers and polymers of higher molecular weight produced a steric impediment which did not lead to the formation of a closely packed layer but to an open interfacial film, decreasing foam stability.

However, in some cases, protein aggregates of high molecular weight improve foam stability by remaining in aqueous phase (Saint-Jalmes, Peugeot, Ferraz, & Langevin, 2005). These aggregates, when large enough, can act as a cork in the Plateau borders, leading to a decrease of the drainage rate. On the other hand, Rullier et al. (2008) reached a different conclusion, studying the behavior of large fractal aggregates of  $\beta$ -LG formed by heating. These large particles, from a few hundred proteins for the small aggregates to around 5000 proteins for the larger ones, imply a size range of 35 nm–197 nm, approximately. It was shown that these aggregates are not able to improve foaming stability, unless non-aggregated monomers were present, which might act as anchors for the aggregates in the interface. The same authors have also demonstrated that large protein aggregates, when the monomers are absent, cannot resist hydrodynamic flow inside the film and are expelled from it to the periphery, leaving a thin film vulnerable to rupture (Rullier et al., 2008; Rullier, Axelos, Langevin, & Novales, 2009).

According to the results of this work, after 10 min of heating, there were monomers and dimers (~10%) and oligomers and polymers (80%). In this case, although the aggregates were not large and monomers were present, the same effect of the large protein aggregates was verified, and foam stability strongly decayed.

#### 4. Conclusions

Gel densitometry of SDS-PAGE for native and treated  $\beta$ -LG showed that the largest amount of dimers and trimers appears at 3 min of heating and at 10 min of heating, oligomers and polymers represented more than 80% of the species in solution. All tested methods pointed at 3 min of heating as a critical time, in terms of conformational changes and aggregation processes. At this time, the most significant conformational changes occur: non-native monomers are present and the greatest amount of dimers and trimers is produced (monomer 51%, dimer 33% and trimer 16%), which was in agreement with the results of SDS-PAGE, fluorescence quenching and circular dichroism tests.

When foams are formed and stabilized by the presence of  $\beta$ -LG, the time of previous heating of the protein is a crucial variable, in terms of the different molecular species formed. Foamability and foam stability were both modified by heating.

Foamability, estimated through FD, was improved with a maximum increment of ~40% up to 10 min of heating. Foamability is enhanced by an increment in protein surface hydrophobicity.

Foam stability, estimated through  $T_{1/2}$  and  $T_{1/4}$  values and in comparison with foam stability of the unheated sample, was increased in different degrees up to 10 min of heating and around 15 min foam stability decreased. The greater stability could be due to an increase in protein solution viscosity because of the presence of aggregates, which slows the drainage rate, and mainly due to rheology factors such as the stiffening of the interfacial film which makes the bubbles more resistant to disproportionation and collapse. The best foam stabilization is achieved at 3 min of heating treatment, coincidentally with the occurrence of the most significant conformational changes. On the other hand, with heating treatment over 10 min, the presence of larger aggregates leads to

the opposite effect, the decay of stability, even in the presence of remaining unfolded monomers.

Foamability and foam stability are closely linked with structural changes of the protein. The increase in surface hydrophobicity is considered a decisive factor in the improved foamability in spite of the presence of aggregates of higher molecular weight. Besides, hydrophobic interactions improve foam stability through rapid formation of a viscoelastic film.

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