Granulomorphometry: A suitable tool for identifying hydrophobic and disulfide bonds in β -lactoglobulin aggregates. Application to the study of β -lactoglobulin aggregation mechanism between 70 and 95°C

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ABSTRACT

This work deals with the investigation of β -lactoglobulin (β -LG) aggregation by granulomorphometry. In the first part of this study, we showed that the binding interactions involved in aggregate structure could be identified by their appearance in granulomorphometric pictures. The reliability of this analytical approach was demonstrated by comparing the appearance of β -LG aggregates in the presence and absence of a thiol-blocking agent (*N*-ethylmaleimide). The translucency of the aggregates was associated with hydrophobic interactions and their opacity was associated with disulfide bonds. We state, based on the morphology of the aggregates, along with the color of protein aggregates and insoluble materials, that hydrophobic interactions had a better water-holding capacity than disulfide bonds. Additionally, our results suggest that disulfide and hydrophobic bonds compete for β -LG aggregate shaping. In the second part of this work, interesting features of granulomorphometry useful for identifying aggregate binding interactions were highlighted to clarify the effect of temperature on the aggregation mechanisms occurring in a β -LG concentrate with a moderate calcium content (6.6 mmol·L⁻¹). Heat treatment experiments were performed between 70 and 95°C, and granulomorphometric measurements (aggregate size, aggregate number, and gray level of the picture) were conducted at different sampling times up to 4 h. Results, which were interpreted in light of calculated β -LG denaturation levels, revealed that the aggregation mechanism could be split into 2 steps. Initially, β -LG denatured quickly, leading to fast β -LG aggregation by disulfide bonds. The denaturation rate then declined, which drastically slowed the disulfide aggregation mechanism. From that point on, a second aggregation path became preponderant. It consisted of the agglomeration of small aggregates by hydrophobic

interactions and resulted in the formation of large aggregates containing both interaction types. This second aggregation mechanism was clearly favored at high temperatures because it was not detected in our experiments at temperatures below 85°C.

Key words: granulomorphometry, β -lactoglobulin, disulfide bond, hydrophobic interaction

INTRODUCTION

Globular proteins (especially β -LG) are commonly used as functional ingredients in many kinds of dairy food products. The industrial thermomechanical treatments influence β -LG denaturation, along with the aggregation and fouling mechanisms of β -LG. Thus, heat treatments, which are carried out to ensure sanitary requirements, are responsible for the size and nature of aggregates as well as the associated functionalities of dairy products, such as their gelation, emulsification, water retention, and stabilizing effect. The means by which the aggregation process occurs also influences the fouling behavior of heated dairy solutions, in particular because β -LG aggregates and deposits have the same precursory species (molten globule β -LG; Blanpain-Avet et al., 2012). Fouling is defined as protein deposition on the hot surfaces of heat exchangers, and it results in a decrease in heat exchange (René and Lalande, 1988; Benning et al., 2003), a greater pressure drop (Grijpspeerdt et al., 2004; Fryer et al., 2006), and bacterial growth (Fryer et al., 2006; Jun and Puri, 2007). Deposit formation in industrial heat exchangers makes frequent cleanings necessary, resulting in the excessive use of water and harsh chemicals (René and Lalande, 1988; Georgiadis et al., 1998). To limit the phenomenon of fouling, the lead-up and concomitant mechanisms, respectively, β -LG denaturation and aggregation, need to be clearly understood. Thus, the main information on β -LG denaturation and aggregation reported in the literature were gathered.

Bovine β -LG, one of the main whey proteins (along with α -LA and BSA), accounts for approximately 50%

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of the whey proteins (Mulvihill and Donovan, 1987; Havea et al., 2001), which corresponds to approximately $3.5 \text{ g} \cdot \text{L}^{-1}$ in milk (Lalande et al., 1989). Its isoelectric point is approximately 5.3 (René and Lalande, 1988) and its denaturation temperature is estimated at approximately 77°C (Nielsen et al., 1995; Relkin, 1996; Havea et al., 2001; Linmark-Mansson et al., 2005), depending greatly on the chemical environment. The tertiary structure of β -LG is consolidated by 2 disulfide bonds. A free thiol group $-\text{SH}$ is hidden inside its hydrophobic core in the native state (Relkin, 1996; Labouré et al., 2004; Perez and Pilosof, 2004). Under heat or chemical denaturation, β -LG loses its native tertiary structure to form reactive species, often called “molten globules,” which are able to form disulfide bonds with another whey protein via thiol group oxidation or disulfide interchange, which induces an alteration of the β -LG secondary structure (Mulvihill and Donovan, 1987; Labouré et al., 2004). This constitutes the aggregation phenomenon (Delplace et al., 1997; Santos et al., 2006).

The literature shows that 2 kinds of interactions take part in β -LG aggregate formation: covalent (disulfide) and noncovalent (hydrogen bonds, van der Waals interaction, etc.) interactions. The competition between these 2 interaction types leads to the formation of 2 main types of aggregates, depending on the environmental conditions. On one hand, at a low temperature ($<85^\circ\text{C}$; Hongsprabhas and Barbut, 1997; Spiegel, 1999), a pH close to the β -LG isoelectric point (Stading and Hermansson, 1991; Gault and Fauquant, 1992; Ikeda and Li-Chan, 2004), high ionic strength (Barbut, 1995a; Ikeda, 2003; Ikeda and Li-Chan, 2004; Nicolai et al., 2011), or high calcium concentration (Lupano et al., 1992; Barbut, 1995b; Hongsprabhas and Barbut, 1997; Nicolai et al., 2011), β -LG aggregates are mainly formed by hydrophobic interactions: they are rather spherical and rigid (Havea et al., 2004). Their size is important, and they show a good aptitude for water release (Ikeda, 2003), generally associated with a low water-holding capacity (Lupano et al., 1992; Barbut, 1995a; Nicolai et al., 2011). Moreover, hydrophobic aggregates appear opaque on transmission electron micrographs (Barbut, 1995a), which has been explained by the random location of noncovalent interactions.

On the other hand, disulfide interactions predominate at high temperatures (≥ 85 to 95°C ; Hongsprabhas and Barbut, 1997; Spiegel, 1999; Nicolai et al., 2011), neutral or acidic pH (Stading and Hermansson, 1991; Ikeda, 2003; Ikeda and Li-Chan, 2004; Nicolai et al., 2011), low ionic strength (Barbut, 1995a; Ikeda, 2003), or low calcium content (Gault and Fauquant, 1992; Lupano et al., 1992; Barbut, 1995b; Hongsprabhas and Barbut, 1997). The aggregates formed are smaller, soft,

and easily deformable (Havea et al., 2004); they have a translucent aspect in microscopic analysis (transmission electron microscopy, scanning electron microscopy), which is subsequent to their finely stranded structure and small size (Stading and Hermansson, 1991; Barbut, 1995a; Ikeda, 2003; Havea et al., 2004). Several authors (Lupano et al., 1992; Barbut, 1995a; Spiegel, 1999) have ascribed a good water-holding capacity to disulfide aggregates, based on the observation that water molecules bound to these aggregates are difficult to remove by centrifugation or gel squeezing. It is important to note that no unequivocal definition exists for the concept of water-holding capacity in the scientific community. In fact, water-holding capacity sometimes refers to the overall water retained by aggregates, equal to the sum of bound and removable water fractions. With this definition, hydrophobic gels are said to have a better water-holding capacity than disulfide ones (Clare et al., 2007), indicating that hydrophobic interactions are more prone to associate with water than are disulfide bonds.

This brief literature survey characterizing β -LG aggregates emphasizes the obvious need to develop an analytical technique that is able to relate the appearance of aggregates in solution to the hydration level of their binding interactions (i.e., the total amount of retained water in these aggregates).

As regards the temperature influence on aggregate shaping by hydrophobic and disulfide bonds, Briggs and Hull proposed for the first time in 1945 an aggregation mechanism involving both interaction types (Briggs and Hull, 1945), which was later confirmed by Sawyer (1968) and has been generally agreed on in the dairy community from that time on (Hoffmann and Van Mill, 1997; De La Fuente et al., 2002; Sava et al., 2005). According to Sawyer (1968), the formation of disulfide and noncovalent linked aggregates at near-neutral pH can be divided in 2 steps. During a primary reaction, small β -LG aggregates (presumably non-native β -LG dimers or tetramers) are formed by disulfide bonds. This reaction, occurring at temperatures above 65°C , is favored at high temperatures, especially around 97.5°C . Once this primary reaction is initiated, a secondary reaction takes place, which consists of the formation of large aggregates by noncovalent bonds.

In a recent review dealing with advances in β -LG thermal behavior, De Wit (2009) confirmed this global aggregation mechanism and provided more detail about the temperature ranges associated with disulfide and hydrophobic interactions in β -LG solutions at pH >6.8 and low ionic strength. It is well known that β -LG keeps its native structure up to 60°C . Above this temperature, β -LG unfolds and changes into a molten globule state prone to aggregation. Small aggregates

(essentially non-native β -LG dimers) begin to be produced from 65°C on, enabling further aggregation (Hoffmann and Van Mill, 1997). De Wit (2009) suggested that hydrophobic and disulfide interactions are concomitant up to 85°C and lead to the formation of large-sized aggregates. Above 85°C, the mechanism of β -LG aggregation is dominated by disulfide bond formation, and these short-range interactions are expected to contract the particle radius and strengthen the aggregate structure. Therefore, the aggregate size declines from 85 to 105°C, where the noncovalent interactions become absolutely negligible. These mechanisms may be altered in high-ionic-strength environments or in the presence of a significant calcium concentration, such as in the conditions of our study.

In sum, this literature review revealed that the nature of binding interactions governs β -LG aggregate morphological features (size, shape, and color) and these interactions are thus expected to have a strong effect on the functionalities of heat-treated β -LG solutions, such as their mouthfeel, color, aptitude for gelation, and water-holding capacity. According to the state of the art, however, it seems difficult to relate the appearance of aggregates to the nature of their binding interactions. Moreover, although many authors have been interested in shedding light on the dependence of aggregation mechanisms on process parameters, such as temperature, it is still impossible to propose guidelines for selecting the aggregate binding interactions and their water-holding capacities from an adequate choice of process parameters. For all these reasons, our study aimed to show interesting features of granulomorphometry as they relate to identifying binding interactions and elucidating aggregation mechanisms.

First, the suitability of granulomorphometry for identifying aggregate binding interactions was demonstrated by performing heat-denaturation experiments on a β -LG concentrate in the presence and absence of a thiol-blocking agent. Comparing the granulomorphometric pictures of β -LG aggregates and unsolvated dispersed phases (insoluble materials and air bubbles) permitted us to connect the water-holding capacity of aggregates to their appearance in pictures. The means by which binding interactions influence the aggregate water-holding capacity is also discussed from these experiments.

The second part of this study consisted of applying this granulomorphometric approach to clarify the role played by temperature in β -LG aggregation mechanisms. Heat treatment experiments were carried out between 70 and 95°C, and the temporal evolution of aggregate morphological characteristics (shape, color, and size) was recorded by granulomorphometry. The

acquired results, interpreted in relation to the β -LG denaturation level, permitted a better understanding of how binding interactions compete and confer their functionalities on β -LG aggregates in concentrated solutions.

MATERIALS AND METHODS

Chemicals and Reagents

For each experiment, a 53 g·L⁻¹ [i.e., 2.9 mmol·L⁻¹, by considering a 18.3-kDa molar mass (Verheul et al., 1998) in the calculation] β -LG solution was prepared by scattering 6 g of an industrial β -LG powder (composition: β -LG 88.85%, α -LA <0.01%, calcium 0.03%, phosphate 0.07%, sodium 0.76%, lactose 0.4%) in 94 g of deionized water (Millipore, Bedford, MA). Therefore, 660 μ L of a molar calcium chloride (96%, Prolabo; VWR International, West Chester, PA) solution was added to obtain a stock solution containing 5.3% β -LG and 6.6 mmol·L⁻¹ of calcium chloride. The composition of this stock solution was chosen so as to simulate an industrial β -LG concentrate. This solution was maintained at 40°C for 2 h for complete dissolution of the β -LG powder. The pH of this solution was 6.8.

To inactivate the β -LG thiol group, half of the samples were treated with *N*-ethylmaleimide (NEM, Sigma-Aldrich, St. Louis, MO) according to the following protocol adapted from literature (Hoffmann and Van Mill, 1997): 2.9 mmol·L⁻¹ of NEM was added to the β -LG stock solution, which was allowed to react for 1 h at ambient temperature. The NEM concentration was chosen to form an equimolar mixture of NEM and β -LG, which was sufficient to significantly inhibit β -LG thiol reactivity (Mounsey and O'Kennedy, 2007).

For granulomorphometric analyses, the heat-treated β -LG samples were diluted 100-fold in deionized water. This allowed accurate granulomorphometric results to be acquired while limiting larger particles to hide the others.

The opacity of unsolvated objects on the granulomorphometric pictures, such as air bubbles and insoluble minerals, was obtained by analyzing a simulated skim milk [composition: 5% (wt/wt) ultrafiltrate powder (Lactepi 452; Epi Ingredients, Ancenis, France) + 0.7% (wt/wt) whey protein powder (Promilk 852 FB1; Idi SAS, Arras, France) + 2.9% (wt/wt) phosphocaseinate powder (Promilk 852 B; Idi SAS)], a 10 g·L⁻¹ calcium carbonate suspension (Normapur, Prolabo; VWR International), and a 2 g·L⁻¹ phosphocaseinate (Promilk 852 B; Idi SAS) suspension in deionized water and in ethanol (99%, Thermo Fisher Scientific, Waltham, MA).

Heat Treatment

All thermal denaturation experiments were conducted on 1.4-mL samples in polypropylene vials (Eppendorf, Hamburg, Germany). Samples treated with NEM and those free of NEM were jointly heat treated so as to allow comparison of the experimental data. We chose to preheat the samples to 60°C [below the beginning of β -LG aggregation: 77°C (Nielsen et al., 1995; Relkin, 1996; Havea et al., 2001; Linmark-Mansson et al., 2005)] to limit β -LG denaturation before sample acquisition. This was achieved by first placing samples in a water bath at 62°C for 30 min. During the entire heat treatment procedure, the temperature was monitored by using a temperature probe inserted in a reference vial filled with the stock solution.

The rapid temperature increase from 60°C to the desired holding temperature was performed by placing the samples in a second water bath, whose temperature was fixed (as a rule) at 10°C higher than the holding temperature to shorten the temperature increase time and restrict β -LG denaturation before sample acquisition.

Once the holding temperature was reached, the samples were placed in a third water bath, whose temperature was fixed at 2°C higher than the holding temperature (e.g., 97°C for a holding temperature of 95°C). Six holding temperatures were investigated: 70, 75, 80, 85, 90, and 95°C. Sample immersion in this last water bath corresponded to time zero (i.e., the holding time began with sample immersion in the third water bath). At different holding times (10 min, 30 min, 1 h, 2 h, 3 h, and 4 h), the samples were removed and cooled immediately in a beaker half filled with melting ice to stop β -LG denaturation and aggregation immediately.

It was noted that during these heat treatments, NEM-treated samples had a strong tendency to form gels. Indeed, at 90 and 95°C, gelation occurred in less than 10 min, whereas at 85°C, the β -LG concentrate gelled from the 30-min heat treatment. This explains why no granulomorphometric analysis was performed for these samples. No such propensity for gelation was detected for samples free of NEM.

Granulomorphometry

In the heat-treated samples, the aggregate sizes were analyzed using a Flow-Cell 200 S-M instrument (Ochio, Angleur, Belgium) granulomorphometer. Granulomorphometry is a recently developed microscopy technique designed to acquire high-quality pictures of particles within a liquid solution. The granulomorphometric analysis consisted of pumping a liquid sample through a 300- μ m-wide flow cell, backlit at a 440-nm

wavelength (blue), and obtaining digital pictures from a high-resolution camera with a 0.38- μ m spatial resolution (9 $\times$ zoom factor). Sample pictures were acquired when the flow was completely stopped so as to ensure good sharpness of the digital pictures obtained.

The granulomorphometer was coupled with image analysis software (Callisto) designed for particle detection by using the gray level difference between the particle and background. It allowed the aggregate number to be counted, the size distribution to be measured, and the analyzed particles to be visualized. In addition, aggregate morphological characteristics, such as area, perimeter, or circularity, could be deduced. In our study, the mean aggregate diameter in volume, abbreviated D_{50} , was calculated by the image analysis software as the average area diameter of all analyzed particles, weighted by their estimated volume. The area diameter was defined as the equivalent diameter of a sphere having the same area in the granulomorphometric pictures as the analyzed particle. This size estimator was very similar to the Sauter mean diameter.

For each run, the mean aggregate size was determined by processing 16 pictures, which corresponded to a few thousand particles, depending on the heat treatment time and temperature. Each sample was analyzed 5 times so as to improve the accuracy of the granulomorphometric parameters determined. Light calibration was performed on deionized water between each sample, with an associated gray level of 200. The most accurate measurements were obtained with a threshold of 180 for particle detection. The same analytical method was used for acquiring pictures of bubbles, calcium carbonate, and phosphocaseinate particles.

Granulomorphometry has another advantage for studying the microstructure of aggregates. Indeed, the contrast level of the analyzed particles may reveal the ability of the solvent to interact with the dispersed phase. As the background calibration was carried out on the solvent, the solvent color was very light gray (gray level of 200) in the granulomorphometric pictures, whereas opaque particles corresponded to black patches (with a gray level far below 180). We show in the Results section that a contrast decrease inside a particle was indicative of greater solvent (here, water) retention.

Denaturation Level Calculation

The evolution of the β -LG denaturation level with time and temperature was calculated by means of the finite difference method implemented with Scilab software (Digiteo, Saclay, France), as presented in a previous work (Petit et al., 2011). These calculations were based on the denaturation mechanism, in which both

the unfolding and aggregation reactions are described by a kinetic differential equation (Petit et al., 2011), and on the temporal discretization of the experimentally acquired heat profile. We chose to fix the time step to 1 s, which provided sufficient precision for our calculations.

The kinetic parameters of β -LG denaturation related to the β -LG concentrate used in the current work (composed of 53 g·L⁻¹ of β -LG and 6.6 mmol·L⁻¹ of calcium) were determined in our laboratory in a previous study (Petit et al., 2011). At temperatures below 80°C, β -LG denaturation is limited by the unfolding reaction, $\ln k^o = 98.4$, and $E_A = 304.6$ kJ·mol⁻¹, whereas at temperatures above 80°C, β -LG denaturation is predominated by the aggregation mechanism, $\ln k^o = 32.1$, and $E_A = 110.9$ kJ·mol⁻¹, where $\ln k^o$ designates the logarithm of the denaturation frequency factor and E_A is the associated activation energy.

The heat treatment profiles used in our calculations mimicked those recorded by temperature monitoring during the heat treatment procedures. The temperature evolution in the samples was almost linear in 2 successive time ranges: after a fast, linear temperature increase from the initial temperature (60°C) to the desired one, the sample temperature remained constant.

The times needed to heat the β -LG samples from 60°C to the holding temperatures were as follows: 15

s for 70°C, 30 s for 75°C, 45 s for 80°C, 50 s for 85°C, 60 s for 90°C, and 90 s for 95°C. Heating rates for the selected holding temperatures ranged from 0.33 to 0.67°C·s⁻¹.

RESULTS

The first part of this study presents the granulomorphometric method developed to identify β -LG aggregate binding interactions. This method was applied to determine the structure of β -LG aggregates formed by heat treatments between 70 and 95°C.

The second part of this work investigates the role of temperature in the mechanism of β -LG aggregation by following the evolution of the gray level in the granulomorphometric picture as well as the aggregate number and size in the different heat treatment experiments. These observations were related to calculations of the β -LG denaturation level to suggest an aggregation mechanism adapted to β -LG concentrates with a moderate calcium content.

Use of Granulomorphometry for the Identification of Hydrophobic and Disulfide Bonds in β -LG Aggregates

This section shows that the appearance of β -LG aggregates in granulomorphometric pictures could be linked to the binding interactions constituting their structure. An attempt was made to relate this structural information to the water-holding capacity of the aggregate.

Figure 1 displays granulomorphometric pictures obtained at 80°C, illustrating the appearance of β -LG aggregates in the presence of NEM. Aggregates formed at temperatures less than or equal to 75°C were too small to be presented in this paper, whereas temperatures above 85°C were responsible for gelation of the β -LG solution, making granulomorphometric analysis irrelevant.

Aggregates formed in the presence of NEM are known to be almost exclusively composed of hydrophobic interactions (Xiong et al., 1993; Hoffmann and Van Mill, 1997) because NEM blocks the β -LG thiol function, preventing the formation of disulfide bonds between β -LG units by thiol oxidation or disulfide interchange. In Figure 1, the NEM-treated aggregates appear translucent (light gray) in the granulomorphometric pictures, which permitted us to state that hydrophobic aggregates had a translucent aspect in granulomorphometric analysis. It can be added that these hydrophobic aggregates had gray levels comparable with those of the picture background. Because the background calibration was performed on deionized

Figure 1. Appearance of aggregates in the presence of *N*-ethylmaleimide (NEM; Sigma-Aldrich, St. Louis, MO) for various sampling times at 80°C. The size scale is identical in all panels.

water, this may indicate that a large amount of water was entrapped in the hydrophobic aggregates. In other words, the similar refractive indexes of water and the hydrophobic aggregates suggest that water has a high affinity for hydrophobic interactions (i.e., hydrophobic bonds have a high water-holding capacity).

This hypothesis was corroborated by performing additional granulomorphometric analyses on insoluble materials suspended in water. On one hand, particles that did not contain water appeared in black in the granulomorphometric pictures because they were opaque to incident light, owing to their refractive index being very different from that of water. Figure 2 illustrates this point with pictures of air bubbles and calcium chloride (which is known to be highly insoluble) suspended in deionized water.

In Figure 2, the granulomorphometric picture on the left shows that bubbles were accidentally pumped in together with the skim milk being analyzed, causing the presence of black disks. The circularity of these black particles was extremely high (very close to 1), undoubtedly corresponding to air bubbles. One should note that some bubbles had a white spot in the center, owing to the straight path of the incident light through the bubble axis of symmetry. The black appearance followed from the strong refraction of the incident light

on the bubble surface: light rays out of the optical axis did not reach the acquisition camera.

In the picture on the right of Figure 2, calcium carbonate was also detected by granulomorphometry as black particles. This was related to its high insolubility. Indeed, almost no water molecules could enter the powder structure; thus, the calcium carbonate particles were expected to have a refractive index very different from that of the solvent. As in the case of air bubbles, light rays underwent a strong refraction at the solvent-particle interface, so they did not reach the detector.

On the other hand, solids containing a certain water fraction had a refractive index closer to that of water: some areas of these particles also appeared in light gray in the granulomorphometric pictures, as illustrated in Figure 3, which presents pictures acquired in the laboratory in a side study dealing with the rehydration mechanisms of a phosphocaseinate powder. Particle dissolution was followed by evaluating the solvent entering the powder particles by granulomorphometry. The pictures in Figure 3 allowed us to compare the particle appearance in ethanol (left picture), in which phosphocaseinates are totally insoluble, and in water (right picture), in which dissolution takes place. For each solvent, the granulomorphometric pictures were acquired 5 min after powder addition.

Figure 2. Left: Bubbles observed during the granulomorphometric analysis of a simulated skim milk. Right: Analysis of a $10 \text{ g}\cdot\text{L}^{-1}$ calcium carbonate suspension, carried out immediately after powder addition. Both panels are $1,280 \text{ }\mu\text{m}$ wide.

Figure 3. Left: Suspension of a phosphocaseinate powder in ethanol. Right: Suspension of the same phosphocaseinate powder in deionized water. Both panels are 1,280 μm wide. In both analyses, light calibration was performed on solvent [i.e., ethanol (left) or water (right)].

Powder particles appeared totally black in ethanol and ranged from light gray to black in water, which confirmed the difference in solubility between these solvents. From Figure 3, it is clear that soluble powder particles incorporate water during the rehydration process, which results in the appearance of light gray areas inside the particles. This did not occur in ethanol, in which phosphocaseinates are insoluble and which is thus impermeable to the entrance of the solvent. It can be deduced from these observations that the granulomorphometric gray level of a particle is linked to its solvent affinity.

Hence, the gray level of aggregates constituted an indicator of water-holding capacity: the lighter the β -LG aggregates appeared in the granulomorphometric pictures, the higher was their water fraction. It could be deduced that the translucency of hydrophobic aggregates in the granulomorphometric pictures was indicative that they had a good water-holding capacity.

After having characterized the hydrophobic aggregates by granulomorphometry, we showed the ability of this analytical method to discriminate between hydrophobic and disulfide bonds in β -LG aggregates formed without a thiol-blocking agent. Figure 4 displays the appearance of the aggregate at temperatures ranging from 80 to 95°C after different heating times in solutions free of NEM.

Figure 4 shows that the aggregates formed in the absence of NEM had a different appearance than did purely hydrophobic aggregates (observed in NEM-treated β -LG solutions, as in Figure 1). In fact, aggregates had both dark and translucent areas across the entire temperature range. The translucent areas corresponded to hydrophobic interactions, such as in the case of the NEM-treated β -LG solutions, whereas dark areas indicated the presence of another kind of binding interaction: disulfide bonds, which are known to take part in β -LG aggregation mechanisms.

A comparison of Figures 1 and 4 showed that the aggregates formed in NEM-treated solutions had a more homogeneous aspect and a more spherical structure than those produced in the absence of NEM. This could be explained by the competition between hydrophobic and disulfide bonds during the β -LG heat aggregation mechanism in the absence of NEM. Indeed, at temperatures below 85°C, the competition between these 2 interaction binding types led to the formation of either fully hydrophobic or fully covalent aggregates, corresponding, respectively, to the translucent and dark particles in the granulomorphometric pictures in Figure 4.

At temperatures above 90°C (Figure 4), the aggregation mechanism appeared to change: the majority of aggregates contained both opaque and translucent

Figure 4. Appearance of aggregates in β -LG concentrates at 80 (top left), 85 (top right), 90 (bottom left), and 95°C (bottom right). The size scale is the same in all panels.

areas, which was indicative of a more complex, hybrid structure. Hydrophobic and disulfide interactions were consistently segregated in different locations of the β -LG aggregate, validating the hypothesis of competition between hydrophobic and disulfide bonds across the entire temperature range.

Finally, the shape of the aggregates appeared to follow from this binding interaction competition. As shown in Figure 1, aggregates found in NEM-treated solutions after heat treatment had a rather spherical shape, owing to the random location of hydrophobic bonds. In contrast, Figure 4 displays aggregates formed in the absence of NEM, which seemed to be composed of small stick-shaped dark or translucent aggregates placed side by side.

Furthermore, the fact that aggregates at temperatures above 85°C were larger and contained more opaque areas was a sign that the aggregation mechanism changed around 85°C. In brief, at temperatures below 85°C, hydrophobic and disulfide aggregates did not associate, whereas at temperatures above 90°C, small aggregates of each type adhered to form large aggregates.

It can be noted that the picture background became more granular as the holding temperature increased, which denotes an increase in the number of β -LG aggregates with an increase in temperature. The light gray spots observed in the picture background most certainly correspond to small aggregates located near the walls of the analytical flow cell, which explains the fuzziness of the granulomorphometric picture background.

Role of Temperature in β -LG Aggregation Mechanisms

The evolution of granulometric parameters (size and number) of the β -LG aggregates during the different heat treatments was monitored to understand how hydrophobic and disulfide bonds contributed to the formation and shaping of aggregates as a function of temperature. We were able to calculate the evolution of the β -LG denaturation level during the heat treatment procedures to help interpret the granulomorphometric measurements. This approach led us to propose an aggregation mechanism for β -LG concentrates with a moderate calcium content in the temperature range of 70 to 95°C, explaining the formation of the β -LG aggregate structures observed by granulomorphometry.

Calculation of the Denaturation Level as a Function of the Heat Profile. In this subsection, we present the calculations performed to assess the evolution of β -LG denaturation during the heat treatment procedures. This is useful for interpreting the granulomorphometric analyses.

Figure 5 shows the results obtained for holding times of less than 30 min at the 6 experimental temperatures in the β -LG concentrate without a thiol-blocking agent. The denaturation level represents the proportion of β -LG proteins that were irreversibly denatured (i.e., converted to aggregates).

It is obvious in Figure 5 that the denaturation level increased strongly with temperature and moderately with time. Indeed, the time needed to denature 95% of the β -LG proteins was less than 5 min at the highest

Figure 5. Calculation of β -LG denaturation during the heat treatments performed.

Figure 6. Temperature influence on the gray level of granulomorphometric pictures acquired with and without *N*-ethylmaleimide (NEM; Sigma-Aldrich, St. Louis, MO).

temperatures (above 85°C), whereas at 70 or 75°C, more than 20 min was required (data not shown in Figure 5), and at 80°C, this time was reduced to approximately 10 min. Regarding the time effect, β -LG denaturation was faster in the first minutes of the heating experiment: at temperatures greater than 75°C, a 50% denaturation level was reached in less than 5 min of heat treatment. This indicates that most of the β -LG molecules were denatured in the samples free of NEM, except for heat treatment times of less than 30 min at 70°C and 10 min at 75°C.

Regarding the evolution of denaturation in NEM-treated samples, the associated denaturation rates were not determined, principally because of the tendency of NEM-treated β -LG solutions to gelate even at low temperatures and short heating times. Nevertheless, it was expected, based on the literature, that β -LG denaturation rates would be drastically reduced by NEM addition because NEM blocks the β -LG thiol group (Xiong et al., 1993; Hoffmann and Van Mill, 1997), precluding its irreversible denaturation via thiol group oxidation or disulfide interchange. This point permits us to state that the NEM-treated β -LG solutions had lower denaturation levels than did the raw β -LG solutions for all tested temperatures.

Influence of Temperature on β -LG Aggregate Size and Number. The gray level in the picture background was calculated by means of Image J freeware for the first granulomorphometric analysis of each sample (16 pictures). The results are reported in Figure 6.

Note that small gray level discrepancies were apparent between samples at different temperatures and holding times: all gray levels were between 195 and 198. Before each analysis, light calibration was performed by adjusting the gray level of deionized water to 200. So, the contribution of the aggregate to the overall gray level was significant enough to allow aggregation monitoring.

The gray level of β -LG solutions without NEM decreased from 70 to 80°C, which corresponded to a darkening of the picture background, and it remained constant around 195.5 at temperatures above 85°C. The similar results acquired at 1- and 4-h sampling times proved that no further aggregation occurred after a 1-h heat treatment. This is easily understandable, given that the denaturation level, which was already close to 100%, did not vary significantly after a 60-min heat treatment (data not shown in Figure 5).

The gray level of the picture background decreased at temperatures below 80°C, and its stable value at

temperatures above 85°C could be interpreted as a consequence of the increase in aggregation level with temperature, resulting in an increase in the growth and number of aggregates. On one hand, the presence of aggregates reduced the light transmitted through the sample. As the denaturation and aggregation processes were strongly enhanced by the temperature increase, the picture background darkened from 70 to 80°C, which is expressed in Figure 6 by a decrease in the gray level. On the other hand, most of the aggregates appeared lighter than the background in the granulomorphometric pictures (see Figure 4); this phenomenon tended to increase the overall gray level in the picture. As aggregates began to be apparent around 85°C, the background darkening and the appearance of small, light aggregates compensated in such a way that the gray level stabilized at around 195.5.

In the presence of a thiol-blocking agent, the β -LG solutions adopted a similar behavior, but the temperature range was slightly different: the darkening of the picture background attributable to aggregate formation reduced the gray level at temperatures between 70 and 75°C, whereas the appearance of light aggregates, which were noticeable from 80°C, tended to bring the gray level back to its initial value of 197.5 at 85°C. This confirms that hydrophobic aggregation was highly favored in the presence of a thiol-blocking agent such as NEM, in comparison with the raw β -LG solution.

The number of aggregates was equally monitored in these heat treatment experiments, and its temporal evolution at different temperatures is presented in Figure 7. The number of aggregates displayed in this figure corresponds to the total number of aggregates detected by the image analysis software on the 16 pictures of a single granulomorphometric analysis. The results presented were averaged on the basis of 5 granulomorphometric analyses.

The results presented in Figure 7 suggest that the β -LG aggregation mechanism changed between 85 and 90°C. The aggregation mechanism below 85°C was characterized by a small number of aggregates after a 4-h heat treatment, that is, fewer than 1,000 aggregates at 70°C and between 1,000 and 1,500 particles for other temperatures below 85°C. In this temperature range, the great majority of aggregates were formed after only a 10-min heat treatment (approximately 600 aggregates at 70°C, and between 1,000 and 1,500 particles for the other temperatures), owing to the maximal value of the β -LG denaturation rate in the first moments of the heat treatment (see Figure 5), which resulted in a high availability of molten globule β -LG for disulfide aggregation. After a few minutes (from 10 min to 1 h, depending on the temperature), β -LG denaturation was almost complete and the denaturation rate declined because of the

lack of native β -LG. From that time on, the availability of molten globule β -LG was strongly reduced and most of the newly formed aggregates were too small to be detected by granulomorphometry (their diameter was expected to be considerably less than the pixel scale of 0.38 μ m). This may explain why the measured number of aggregates increased slightly until the end of the heat treatment.

In addition, the large aggregates obtained after long heat treatment times (see Figure 4) were expected to proceed from the hydrophobic agglomeration of small aggregates rather than from aggregate growth by reaction with denatured β -LG (Hongsprabhas and Barbut, 1997; Ikeda, 2003) because to take place, the latter mechanism requires a sufficient amount of denatured β -LG and thus cannot occur in solutions in which the β -LG denaturation level is already close to 100%. Thus, we deduced that the agglomeration rate of small aggregates by hydrophobic interactions was very low at temperatures below 85°C in static conditions.

At temperatures above 90°C, β -LG denaturation was so enhanced that all the native β -LG were denatured in approximately 1 min (see Figure 5). Aggregation rates were drastically increased as well, as evidenced by the very high particle numbers recorded at 90 and 95°C after 4 h of heat treatment: 3,500 and 12,000 aggregates, respectively. The 1,500 particles measured in the 10-min sample were comparable with the value found at temperatures below 85°C, suggesting that the same mechanism acted in the first moments of the heat treatment, whatever the temperature; it dealt with aggregate growth by the addition of denatured β -LG. The large increase in aggregate numbers, which was observed only at temperatures above 90°C, permitted us to add that the formation of large aggregates by the agglomeration of small aggregates was favored at temperatures above 90°C.

Regarding the results obtained in the presence of NEM (data not shown), we found that the aggregate number at temperatures below 75°C was less in NEM-treated solutions than in raw β -LG solutions. This suggests that the β -LG denaturation rate was decreased by the addition of a thiol-blocking agent, and it confirms that aggregation in the first moments of the heat treatment was driven by disulfide interactions, provided that the β -LG thiol function was available.

In the presence of NEM, the aggregate numbers became very important at 80°C (approximately equal to 100,000 particles at the end of the heat treatment), indicating a marked increase in the rate of hydrophobic aggregation. It equally indicated the high gelation potential of NEM-treated solutions, which was perceptible in our experiments from the 10-min heat treatment at 85°C. It could be deduced from these

Figure 7. Temperature influence on the temporal evolution of the mean number of aggregates.

observations that the gelation temperature of the NEM-treated β -LG solution must have been between 80 and 85°C.

Last, in Figure 8 we report the mean aggregate sizes obtained in β -LG concentrates not treated with NEM. As indicated previously, the mean aggregate size considered here refers to the average area diameter in 5 granulomorphometric analyses.

The granulomorphometric analyses performed indicate that the aggregate size was quite affected by temperature: measured particle diameters were higher between 70 and 85°C than for temperatures greater than 90°C. First, this can be explained by the lower aggregate number obtained at temperatures below 85°C. In fact, a significant fraction of aggregates formed at a low temperature were expected to be too small to be detected in granulomorphometry (less than the spatial resolution of the granulomorphometer: 0.38 μ m), which tends to increase the measured aggregate mean diameter at temperatures less than 85°C.

The temporal variation in the aggregate size reinforces this interpretation. At all temperatures, the D_{50} globally decreased with increasing sampling time, corroborating the previously stated aggregation mechanism. Indeed, in the first moments, denaturation was fast and led to the formation of numerous large aggregates, which corresponded to the high aggregate diameters (from 4 to 8 μ m, depending on the temperature) obtained with the 10-min heat treatment. The denaturation was then slowed by the rarefaction of native β -LG, and the aggregation rate was also reduced. The newly formed aggregates had small diameters, owing to the few denatured β -LG available for aggregation, which decreased the measured aggregate size. Finally, the last step in the aggregation mechanism consisted of the agglomeration of already formed aggregates because 100% β -LG denaturation was already achieved. In this step, the aggregate size was expected to increase again, which was observed from the 3-h sampling times, particularly at the highest temperatures.

Figure 8. Temperature influence on the temporal evolution of the mean diameter of aggregates.

DISCUSSION

Finally, our aim was to gather the main findings of this work and compare them with literature studies to show how granulomorphometry, as a complementary technique to microscopy, might provide additional information on the properties of aggregates and improve the current understanding of β -LG aggregation mechanisms.

The first part of this work dealt with identifying by granulomorphometry the binding interactions responsible for β -LG aggregation. The use of a thiol-blocking agent (NEM) allowed us to relate hydrophobic interactions to aggregate translucency in the granulomorphometric pictures. As a consequence, we stated that opaque aggregates were mainly constituted from disulfide interactions. These conclusions disagree with microscopic or direct observations of β -LG aggregates in gels (Barbut, 1995a) and prevent the direct comparison of granulomorphometric and microscopic results. Furthermore, aggregate translucency in

granulomorphometry was correlated with a high level of retained water, and we deduced that hydrophobic interactions induced a better water-holding capacity than did disulfide bonds. This contradicts literature studies dealing with free water (water released by gel squeezing or centrifugation), in which it has been established that finely stranded gels (formed mainly by disulfide interactions) are more prone to water release than are hydrophobic gels (Lupano et al., 1992; Spiegel, 1999). Nevertheless, some authors (Hongsprabhas and Barbut, 1997; Clare et al., 2007) have shown that calcium-enriched or low-temperature gels (known to be composed mainly of hydrophobic bonds) are able to retain a higher amount of water in the form of bound water (water that cannot be removed by squeezing or centrifugation). The following approach is in accordance with our observations. Thus, granulomorphometry allowed us to evaluate the presence of water in β -LG aggregates qualitatively, but this technique seemed unable to differentiate between free or bound water fractions.

The second part of this paper was devoted to applying granulomorphometry to the investigation of the aggregation mechanisms occurring in the temperature range from 70 to 95°C. As reported in numerous papers (Barbut, 1995a; Hongsprabhas and Barbut, 1997; De Wit, 2009; Nicolai et al., 2011), we found that hydrophobic aggregates were more spherical and compact than were disulfide ones (compare Figures 1 and 4). Furthermore, the presence of disulfide bonds within β -LG aggregates is known to reduce their size. In our study, more small aggregates were observed at higher temperatures (see Figure 8), where covalent interactions are expected to be favored (De Wit, 2009). Regarding aggregate shapes, Nicolai et al. (2011) reported in their recent review that the shape of β -LG aggregates is extremely dependent on the pH value: they form long, rigid strands at pH 2.0, spheres at pH 5.8, and small curved strands at pH 7.0. This is consistent with our observations performed in a β -LG concentrate at pH 6.8: the β -LG aggregates adopted shapes ranging from spheres to curved sticks, depending on the temperature, as also shown in the work of Simmons et al. (2007). High-temperature aggregates (termed hybrid aggregates in our study) seemed to be formed by the association of small stick-shaped (see the upper right quadrant at 85°C in Figure 4) or spherical (compare the left upper quadrant at 90°C and the lower left quadrant at 95°C in Figure 4) aggregates, which coincides with the mechanisms reported by Barbut (1995b) and Simmons et al. (2007). Finally, the aggregation mechanism described in our study joins the model of Sawyer (1968): in a first step, primary aggregates are formed by disulfide bonds as a consequence of β -LG denaturation, and in a second step, these primary aggregates adhere under the influence of hydrophobic interactions, which lead to large aggregates with a hybrid structure. Our study permits us to emphasize the role of ionic strength and calcium in β -LG aggregation. In fact, the model of De Wit (2009), which is applicable for low ionic strengths, does not apply for our β -LG concentrate with a moderate calcium content because both interaction types were found in the temperature range from 70 to 95°C.

CONCLUSIONS

This study demonstrates that granulomorphometry has a good ability to depict aggregation phenomena occurring in β -LG solutions. This analytical technique was applied to study the β -LG aggregation mechanisms and their dependence on temperature between 70 and 90°C. Six heat-denaturation procedures were performed on β -LG concentrates at holding temperatures of 70 to 95°C, and aggregate formation was monitored by granulomorphometric measurements at holding times

ranging from 10 min to 4 h. Two categories of results were deduced from this experimental set: the binding interactions involved in aggregate formation were identified, and the influence of temperature on the aggregation mechanisms was clarified. Below 85°C, aggregates were either fully hydrophobic or fully covalent, whereas above 90°C, aggregates contained both kinds of interactions segregated in different locations. We suggested that disulfide and hydrophobic binding compete for β -LG aggregation and that the aggregation mechanism changes between 85 and 90°C. Across the entire temperature range, β -LG denaturation was initially fast, which resulted in a large availability of molten globule β -LG for aggregation by disulfide bonds. The β -LG denaturation was then completed between 10 min and 1 h, depending on the temperature, and the denaturation rate declined because of the lack of native β -LG. A second aggregation mechanism became preponderant after this denaturation step, namely, the agglomeration of small aggregates by hydrophobic interactions, which resulted in the formation of large aggregates containing both hydrophobic and disulfide bonds.

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