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On the relationship between pH-dependent β-lactoglobulin self assembly and gelation dynamics

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Abstract

There are a lot of works in literature about the size particle of β-lactoglobulin (β-lg) at different conditions and also about its rheological properties; however, there are not works which connect both results. The aim of this work was precisely to relate the state of association of β-lg in solution with the heat-induced aggregation and the dynamics of gelation upon heating in a wide range of pH. The state of association and the heat-induced aggregation of β-lg were evaluated by the determination of its size particle at room temperature and upon heating by dynamic light scattering, while the dynamics of gelation was studied by rheological measurements in a controlled stress rheometer. The state of association of β-lg was highly dependent on pH at room temperature increasing near to its isoelectric point. The rate of heat aggregation, the size of aggregates and the dynamic of gelation of β-lg were also highly dependent on pH. Finally, a mechanism involved in β-lg gelation at different pH values is proposed. The dynamic light scattering technique proved to be a useful tool to characterize the state of association and the onset of β-lg aggregation upon heating for understanding the behaviour of these proteins, for example, under the effect of heating on the gelling properties.

Introduction

Dynamic light scattering (DLS) has been used for studying the state of association and the aggregation of β-lactoglobulin (β-lg) over a wide range of conditions. Depending on pH, temperature and salt concentration, β-lg can exist as a monomer, dimer or larger oligomer. At room temperature and neutral pH, β-lg exists mainly as a non-covalently linked dimer, although many authors described a mixture of monomers and dimers (Verheul *et al*., 1999; Gottschalk *et al*., 2003; Mehalebi *et al*., 2008). At pH 2 – 3, where the protein has a strong net-positive charge, β-lg is essentially monomeric under salt-free conditions (Uhrínová *et al*., 2000) because it seems that intra and intermolecular disulfide bridge formation is suppressed at this acidic condition because of the low reactivity of the sulfhydryl residue (Wada *et al*., 2006). In the pH range 3.7 to 5.2, β-lg reversibly forms larger oligomers (Townend and Timasheff, 1960; McKenzie and Sawyer, 1967; Verheul *et al*., 1999; Gottschalk *et al*., 2003; Mehalebi *et al*., 2008). This self-association process has a maximum around pH 4.6, just below the isoelectric point of β-lg (Harnsilawat *et al*., 2006; Haug *et al*., 2009). The heat-induced aggregation of β-lg is also dependent on pH and the kinetics of aggregation and the size of β-lg aggregates is different at pH above and below pI (Roefs and de Kruif, 1994; Hoffmann *et al*., 1996; Verheul *et al*., 1998; Hoffmann and van Mil, 1999; Surroca *et al*., 2002; Mehalebi *et al*., 2008).

The behaviour of β-lg during the heat induced gelation has also been extensively studied in different conditions and the properties of thermally induced β-lg gels are influenced by several factors, including concentration, extent of denaturation, heating rate and temperature, pH, concentration (Stading and Hermansson, 1990; Langton and Hermansson, 1992; Relkin *et al*., 1998; Ould Eleya and Turgeon, 2000a; Sittikijyothin *et al*., 2007). The formation of a gel involves different types of interactions, such as hydrogen bonds, van der Waals, hydrophobic and electrostatic interactions and also covalent bonds (e.g. disulfide) (Ziegler and Foegeding, 1990; Aguilera, 1995). Depending on the pH values, β-lg forms opaque (pH 4 – 6) or transparent (pH values above or below pH range 4 – 6) gels with different mechanical and structural properties and they are called aggregate/particulate gels and fine-stranded gels, respectively (Stading and Hermansson, 1990).

Despite the numerous works in this topic, there is no work which relates the results obtained by dynamic light scattering and rheometry in order to understand

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the effect of pH on the self assembly and heat-gelation of β–lg. So, the aim of this work was to evaluate the potential of dynamic light scattering analysis to assess the state of association of β-lg in solution and to relate it with the heat-induced aggregation and the dynamic of gelation upon heating in a wide range of pH in order to understand and characterize the effect of pH on the association, self assembly, aggregation and gelation of β-lg.

Materials and Methods

Sample preparation

BioPURE β-lactoglobulin (β-lg) was supplied by DAVISCO Foods International, Inc. (Le Sueur, MN, USA). Its composition was: protein (dry basis) 97.8% being β-lactoglobulin 93.6% of total proteins, fat 0.3%, ash 1.8% and moisture 5.0% (data provided by the supplier). The β-lg powder was dissolved at room temperature under agitation in distilled water at 3% w/w and 15% w/w, for DLS and rheological measurements, respectively, and stored overnight for complete hydration. To prevent bacterial growth, 0.02% w/w sodium azide was added to each sample. The pH was adjusted from 2.5 to 7.0 immediately before the measurements by using 0.1 or 1 M of hydrochloric acid or sodium hydroxide.

Particle size determination

DLS experiments were carried out in a Zetasizer Nano-Zs, Malvern Instruments (Worcestershire, United Kingdom) provided with a He-Ne laser (633 nm) and a digital correlator, Model ZEN3600. Measurements were carried out at a fixed scattering angle of 173º. Samples were contained in a disposable polystyrene cuvette. In DLS, the sample is illuminated with a laser beam and the intensity of the resulting scattered light produced by the particles fluctuates at a rate that is dependent upon the size of the particles. Analysis of these intensity fluctuations yields the diffusion coefficient of the particle and hence the particle size using de Stokes-Einstein equation (1):

$$d(H) = \frac{kT}{3\pi\eta D} \qquad (1)$$

where, d(H): hydrodynamic diameter; D: translational diffusion coefficient; k: Boltzmann's constant; T: absolute temperature; η: viscosity.

Size particle was determined at 25°C and also upon heating inside the DLS equipment from 25 to 85°C in steps of 5°C and holding them at each temperature for 5 min. The autocorrelation function at each temperature was obtained in this equipment and then the size of particles. Two approaches were utilized to obtain size information: (i) cumulative method was used to find the mean average (Z-average) or the size of a particle that corresponded to the mean of the intensity distribution and also to know the polydispersity index as an indicator of the degree of aggregation; (ii) contin method was used to analyze the data for percentile distribution of particle/aggregate sizes. The size distribution obtained is a plot of the relative intensity of light scattered by particles in various size classes and it is therefore known as an intensity size distribution. Through Mie theory, it is possible to convert the intensity distribution to volume and number distribution.

The selected concentration for these measurements was 3% w/w in order to evaluate the effect of pH on the state of protein association, but minimizing the effect of concentration. The samples were filtered through a 0.45, 0.22 and 0.02 μm microfilter (Whatman International Ltd., Maidstone, England) before analysis. The assay was performed in triplicate.

Dynamic oscillation measurements

Dynamic oscillation measurements were performed using a Paar Physica controlled stress Rheometer (MCR 300) (Graz, Austria). The samples initially at 25°C were poured onto the bottom plate of a parallel plate measuring system (PP30S), with a gap setting of 1 mm. The temperature of the bottom plate was controlled with a Peltier system (Viscotherm VT2, Paar Physica), and liquid paraffin was applied to the exposed surfaces of the sample to prevent evaporation and the adhesion of the sample to the plate. During gelling experiments, the frequency was held constant at 1 Hz and the strain was kept at 0.01%. The samples were heated from 25°C to 90°C at a rate of 5°C min^{-1}, then held at 90°C for 10 min, which was sufficient time to allow storage modulus (G') equilibration; after that the samples were cooled to 25°C at a rate of 25°C min^{-1} and held at 25°C for 10 min. During the measurements, the evolution of storage (G') and loss modulus (G'') were measured. The temperature at which the storage and loss modulus crossed over was taken as the gel temperature (T_{gel}).

The concentration selected (15% w/w) was above the critical concentration required for β–lg gelation at pH 7.0 (Stading and Hermansson, 1990). The data reported are means of at least three individual samples with an experimental error lower than 10%.

Results and Discussion

Effect of pH on β-lg self-assembly.

Figure 1 shows the intensity size distributions of β-lg at different pH values. The measurements were obtained immediately after pH adjustment. The intensity size distribution of β-lg at pH 7.0 (Figure 1A), as explained previously (Martinez *et al*., 2010), presented two populations, one with a maximum value at 3.3 nm, which is close to the value reported for the monomeric form of this protein (3.6 nm) (Mehalebi *et al.*, 2008), and another population at 20 nm. At neutral pH β-lg exists in a dynamic equilibrium between its dimeric and monomeric form (Verheul *et al*., 1999). In many conditions the equilibrium is shifted towards the monomeric form, like at low protein concentrations or low ionic strength as in this work. Mehalebi *et al.* (2008) found a dependence of the hydrodynamic diameter (dH) with the concentration, with lower values at lower concentrations. In fact, we studied β-lg solutions at higher concentrations (up to 20% w/w) and observed a predominant peak with maximum values of dH about 5 – 6 nm (data not shown), according with previous reports (McKenzie and Sawyer, 1967; Hoffmann and van Mil, 1999; Harnsilawat *et al*., 2006) which correspond to the dimeric form of β-lg (36.8 kDa). At pH 7.0 and concentrations lower than 5% w/w the interactions between monomers could be negligible because the molecular weight and hydrodynamic diameter obtained corresponded to the monomeric form of β-lg (18.4 kDa and 3.6 nm, respectively). The population with size 20 nm was negligible in mass as it is observed in the volume size distribution (Figure 2A) and could correspond to more associated forms.

At pH 6.5 only one peak was observed in the intensity (Figure 1B) and volume (Figure 2B) size distributions with a maximum value about 6 nm which corresponds to the dimeric form of β-lg. The lower size peak moved to higher sizes with decreasing pH, up to the isoelectric pH that has been reported at 4.8 (Bromley *et al*., 2005; Harnsilawat *et al.*, 2006; Haug *et al.*, 2009). At pH below or around the pI a strong decrease of the intensity of the lower size peak and the occurrence of a peak with size higher than 1000 nm in the intensity and volume size distributions (Figure 1E and F and Figure 2E and F) was observed. The driving force for protein association around the isoelectric point is a combination of hydrophobic, van der Waals and some electrostatic interactions (Harnsilawat *et al.*, 2006).

At pH values lower than the isoelectric range (from 4.0 to 2.5) a main peak at lower sizes and a decrease of the maximum value with decreasing pH

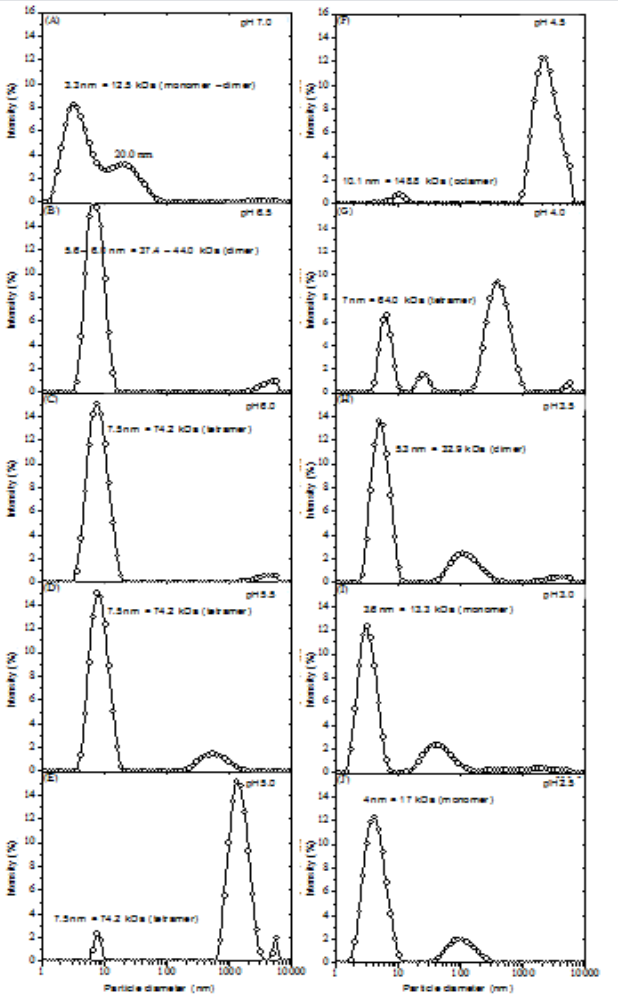

Figure 1. Intensity size distribution of β–lg solutions at 3 % w/w and different pH: 7.0 (A), 6.5 (B), 6.0 (C), 5.5 (D), 5.0 (E), 4.5 (F), 4.0 (G), 3.5 (H), 3.0 (I) and 2.5 (J). Temperature 25 °C.

up to values corresponding to the monomeric form of β-lg was observed. In the intensity size distributions of β-lg at pH lower than the pI another peak in size about 50 – 100 nm or higher was also observed (Figure 1G – J); however it represented a negligible population as can be deduced form the volume size distributions (Figure 2G – J).

From the dH corresponding to the maximum values of the lower size peaks in the intensity size distributions it was estimated the molecular weight (MW) of the protein by a tool in the software of the equipment for globular proteins (indicated in Figure 1). Additionally, it was calculated the predominant association state (PAS) of β-lg at each pH as a relation between the MW corresponding to the diameter of the maximum value of the lower size peak at each pH and the MW of β–lg monomer (18.4 kDa). PAS for each pH conditions is given in brackets in each size intensity distribution.

The behaviour observed at different pH values reveals the pH dependent self-assembly of β-lg and

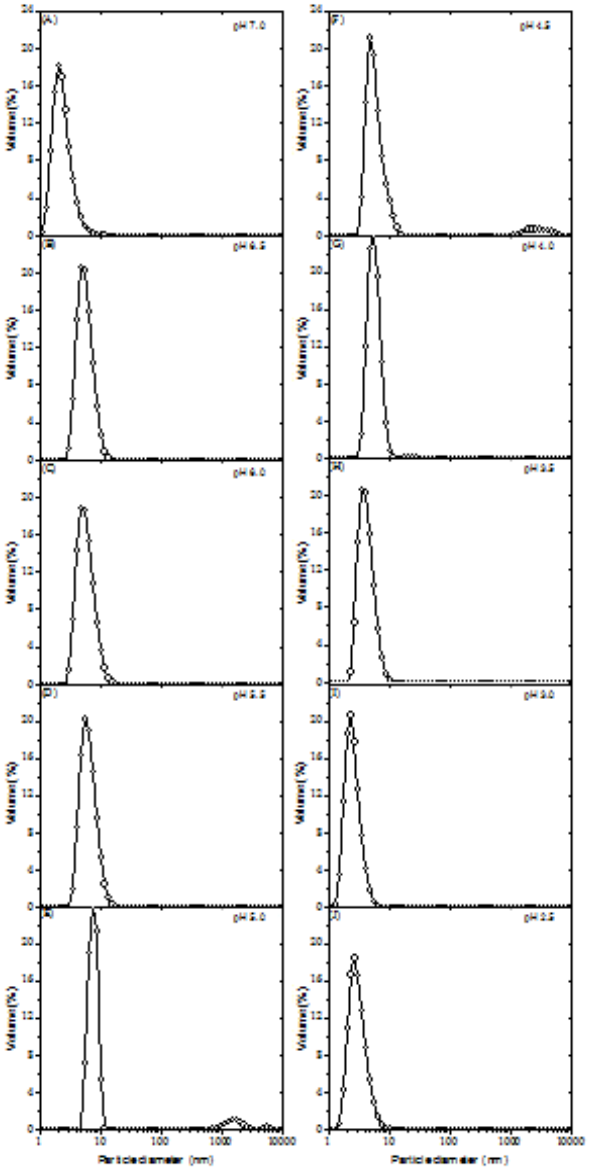

Figure 2. Volume size distribution of β–lg solutions at 3 % w/w and different pH: 7.0 (A), 6.5 (B), 6.0 (C), 5.5 (D), 5.0 (E), 4.5 (F), 4.0 (G), 3.5 (H), 3.0 (I) and 2.5 (J). Temperature 25 °C.

agrees with previously reports from several authors (Relkin, 1996; Relkin, 1998; Verheul *et al*., 1999; Gottschalk *et al*., 2003; Sakurai and Goto, 2007; Mehalebi *et al*., 2008). Mehalebi *et al.* (2008) determined the dH of β-lg at pH between 5.2 and 8.0 in a wide range of concentrations by DLS technique and they found higher differences in dH at higher concentrations of β-lg. At a concentration of 3 % w/w the results were similar to those reported in the present work.

At pH 4.6, β-lg is found as an octamer (Verheul *et al.*, 1999; Gottschalk *et al.*, 2003). Townend *et al.* (1960) reported that at pH between 3.7 and 5.2 occurs a specific association to tetramers and octamers with a MW of 144 kDa. This association presents a maximum at pH between 4.4 and 4.65, below the pI (Gottschalk

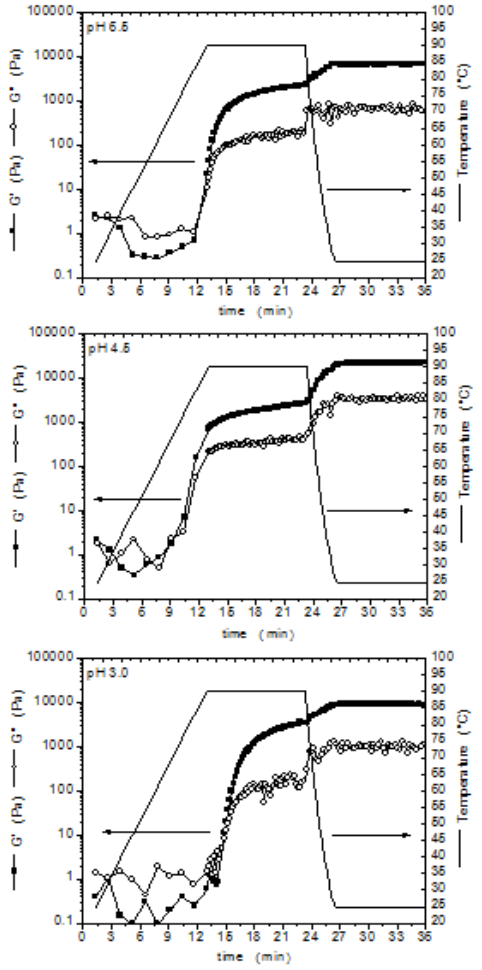

Figure 3. G' and G'' evolution during the heat induced gelation of β–lg solutions at 15 % w/w at pH 6.5, 4.5 and 3.0. The temperature profile is also depicted.

et al., 2003) which agrees with the present results. Sakurai *et al.* (2007) studied the tridimensional structure of β-lg by a principal component analysis (PCA) by simulation and reported changes in protein structure at pH 5.0; however, from their analysis of the fluorescence data, they found no evidence of a substantial conformational change at pH 5.0. So, they attributed the differences in the structure, found by PCA, to changes probably caused by the octamer's formation near to pI, which did not involve changes in the secondary structure of the protein. Uhrínová *et al.* (2000) found by spectroscopy of nuclear magnetic resonance at pH 2.6 more monomeric form than dimeric form of β-lg. At acidic pH (2.5 – 3.5), far away from pI, β-lg shows a tendency to dissociation due to the increased electrostatic repulsion (Verheul *et al*., 1999) which also agrees with the results obtained in the present work.

Dynamics of gelation

The G' and G'' evolution for β-lg solutions at 15% w/w in a wide range of pH (2.5 – 7.0) was determined and the crossover point between the elastic (G ')

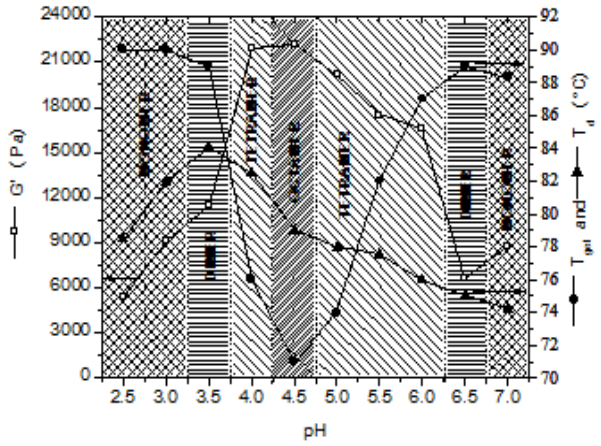

Figure 4. Elastic modulus (G') (□), gelation temperature (T_{gel}) (●) and denaturation temperature (T_d) (▲) as a function of pH of β–lg solutions at 15 % w/w. It is also depicted the state of association of β–lg solutions at 3 % w/w at each pH (from Figure 1).

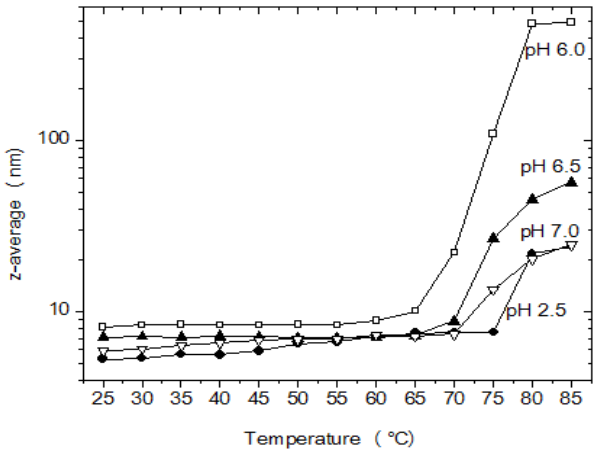

Figure 5. Change of z-average during the heating of β–lg solutions at pH: (●) 2.5, (□) 6.0, (▲) 6.5 and (▽) 7.0.

and viscous (G'') moduli was taken as the gel point to evaluate the gelation temperature (T_{gel}). It was possible to observe that β-lg gelled at all the studied pH values, as shown by the crossover of both moduli. Figure 3 shows the plots obtained for pH 3.0, 4.5 and 6.5 as an example. After the crossover G' and G'' values increased with increasing temperature up to 90°C and also during the holding time at 90°C. During the cooling from 90 to 25°C both moduli increased with decreasing temperature and then reached almost constant values. The increment of G' and G'' during the cooling is due to a reduction in entropy, which strengthens the attractive forces (hydrogen bonds, van der Waals forces) between the protein particles in the gel (Ould Eleya and Turgeon, 2000b). The values of G' at the end of cooling and the T_{gel} values at each pH are shown in Figure 4. The gelation temperature (and, in the same way, the sol-gel transition time, data not shown) strongly depended on pH, ranging from 71°C to 90°C. G' values also varied with pH within 5000 and 22000 Pa. T_{gel} showed the lowest value (71°C) at pH 4.5, close to β-lg pI, then as the pH moved away from the pI the T_{gel} increased up to near 90°C. The opposite behaviour was observed for G' values which were maximum at the pI and decreased at pH values far away of the pI.

In this figure are also shown the predominant forms of β-lg (PAS) estimated previously by DLS in order to relate the rheological behaviour with the self-assembly of the protein in solution previous to the heating. It can be observed that the lowest T_{gel} and the higher G' values were obtained when β-lg was present as the most associated state in solution (octamer).

The G' can be related to the size of self-assembled β-lg particles in solutions previous to heat-treatment that in turn determines the size of particles (or aggregates) formed upon heating. Thus G' increases as the size of β-lg particles forming the gel network increases. In the pI range (4.5 – 5.0) estimated by ζ–potential measurements (Harnsilawat *et al*., 2006; Haug *et al*., 2009) it was observed by DLS that the octameric form of β-lg was predominant immediately after the pH adjustment at 25°C (Figure 1E and F and Figure 2E and F); nevertheless more associated structures were formed with time, so that the size of particles rapidly exceeded the range of measurement of the DLS equipment (6 μm) (data not shown). The absence of charges can promote the association of β-lg to form large aggregates by physical interactions. Upon heating, these big particles rapidly grow and interact to form an initial gel matrix at the lowest T_{gel} (71°C). Donovan and Mulvihill (1987) also reported that in the pH range 4.5 – 5.5 occurred the highest degree of β-lg association.

Slightly increasing or decreasing the pH away from the pI (pH 5.5 or 4.0) where the tetrameric form of β-lg predominated once adjusted the pH at 25°C (Figure 1D and G and Figure 2D and G), it was observed that with time the tetrameric form disappeared to form more associated forms of z-average about 300 nm (data not shown). Thus G' for this pH values resulted lower than in the pI range where larger initial particles were formed.

When the pH was below 4.0 or above 5.5 no changes in the association state (dimers or monomers) of β-lg were observed with time at 25°C. For these pH conditions the size of β-lg aggregates only increased upon heating as shown in Figure 5 where the evolution of the mean diameter of particles (z-average) is plotted. Figure 5 indicates that above the pI higher aggregation rates and highest z-average values were obtained with decreasing pH from 7.0 to 6.0. These results agree with previous reports about the size of aggregates by different techniques

(Hoffmann *et al.*, 1996; Hoffmann *et al*., 1997; Baeza *et al*., 2001). At pH far away of pI (2.5 or 7.0), when β-lg is mostly present in the monomeric form, the highest T_{gel} and the lowest G' values were obtained (Figure 4), which are related to small size aggregates forming the gel network as shown in Figure 5. At pH 2.5, the z-average at the end of the heating was similar to that obtained at pH 7.0 (Figure 5); however, the aggregation of β-lg (observed like an increment on the z-average) occurred above 75°C for pH 2.5 and 70°C for pH 7.0, which could be related with the higher T_d of β-lg at the acidic pH as seen below. Verheul et al. (1998) studied the rate of aggregation of β-lg at 0.9% w/w and different heating temperatures and they also found that at temperatures higher than 75°C the rate of aggregation at pH 7.0 was higher than at pH 2.5.

In Figure 4 are included the denaturation temperatures (T_d) of β-lg at different pH values from data reported in the literature (Ruegg *et al*., 1977; Hegg, 1980; Bernal and Jelen, 1985; Relkin, 1996; Haug *et al*., 2009). As the pH decreased from 7.0 to 3.5, the T_d increased from 74 to 84°C. A further decrease of pH to 2.5 caused a decrease of T_d to 78.5°C. At first sight, the PAS of β-lg is not related to T_d. At neutral pH, β-lg has a strong negative charge resulting in repulsive interactions between protein molecules and in good interactions with the solvent facilitating its denaturation. On the other hand, T_d shows maximum values in the pH range 3.0 – 4.0. The energy needed to denature β-lg should reflect both the energy needed to dissociate the quaternary structures into simpler entities as well as to denature the protein. Therefore, the energy needed for denaturing β-lg should be higher when the protein is more stabilized and organized on the quaternary level (Haug *et al*., 2009). The differences in T_d would be related to pH-dependent conformational changes which allow the protein to conform a "closed" or "open" structure (Sakurai *et al*., 2009).

Thus, in Figure 4 is important to highlight that at pH higher than 5.5 and lower than 3.5, T_d is lower than T_{gel}, suggesting that in this pH range the denaturation is a prior step to gelation. The globular structure partially unfolds and the protein forms colloidal aggregates whose structures and properties are dependent of pH and ionic strength of the solution (Sagis *et al*., 2002). Later the aggregates interact to form a gel structure. On the other hand, in the pH range 3.5 – 5.5 T_d is higher than T_{gel}. In this pH region around the isoelectric range the decrease of the electrostatic repulsions promotes the self-assembly of β-lg to form tetramers or octamers and more associated forms at room temperature by non covalent interactions which further associate to form an initial gel network at T_{gel} before the denaturation occurs.

The nature of bonds involved in the formation of β-lg aggregates and gels is related with the electrostatic charge and the activity of thiol groups (Donovan and Mulvihill, 1987). During the conformational transition of β-lg at pH 6.0 – 9.0 the activity of thiol groups increases due to the pK value of thiol group is ~ 8.0 (Dunnill and Green, 1966); therefore the reactions of thiol-disulfide interchange are more probably at this pH range. In β-lg solutions at acidic pH the formation of intra and intermolecular disulfide bonds would be reduced due to the very low reactivity of thiol groups at this pH (Harwalkar, 1980; Liu *et al.*, 1994; Wada *et al*., 2006). So, the mechanism of aggregation upon heating at acidic pH is different from that at neutral pH, showing rod-like structures at pH 2.0 and aggregates of globules at pH 7.0 (Kavanagh *et al*., 2000).

Conclusion

From the results obtained in the present work and those reported in the literature we can summarize the mechanism involved in β-lg gelation at different pH values in three cases: i) at pH higher than 6.0 the native β-lg denatures upon heating at T_d and then the denatured molecule aggregates by means of disulfide bonds. Finally, if the protein concentration is high enough the aggregates interact to form a gel structure at T_{gel}. As it was previously observed the T_d of β-lg in this region is much lower than the T_{gel}; ii) around the isoelectric range ($3.5 < pH < 6.0$) the native β-lg self-assemble in solution and grow upon heating by means of non covalent interactions due to the existence of minimum electrostatic repulsions. Then the aggregates of native β-lg denature at higher temperatures. In this pH range T_d (78 – 82.5°C) of β-lg is higher than T_{gel} (71 – 76°C); iii) finally, at pH values lower than 3.5, the native protein denatures upon heating at T_d and then the molecules aggregate by means of weak interactions like van der Waals forces and hydrogen bond. In fact, as it was previously explained, in this pH range the denaturation of β-lg molecules did not exhibit the thiol residues and the reactivity of the thiol groups is low, so the reactivity of β-lg molecule to the thiol-disulfide interchange decreases forming more weak gels. Results obtained in this study allow concluding that dynamic light scattering is a useful technique to characterize β-lg behaviour at the molecular level upon heating, thus providing a better understanding of pH-dependent gelation of β-lg.

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