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The dynamics of heat gelation of casein glycomacropeptide – β-lactoglobulin mixtures as affected by interactions in the aqueous phase

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ABSTRACT

The dynamics of heat gelation of casein glycomacropeptide (CMP) and β-lactoglobulin (β-lg) mixtures as affected by their interactions in the aqueous phase were studied at pH 3.5 and 7.0. At pH 7.0, whereas CMP did not gel, all the mixed systems gelled, but strong synergism was observed for 25:75 CMP:β-lg ratio. In comparison, at pH 3.5 where both components gelled on their own, a strong antagonism was observed, mainly at 75:25 CMP:β-lg ratio. The behaviour of mixed gels is ascribed to the formation of electrostatically driven assembled CMP:β-lg structures, modulated by pH, which were observed by dynamic light scattering and DSC measurements.

1. Introduction

Whey protein concentrates (WPC) and whey protein isolates (WPI) are widely used as food ingredients because of their gelling properties, which allow control of the texture and stability of food products. β-Lactoglobulin (β-lg), α-lactalbumin (α-la) and bovine serum albumin (BSA) are present at 50, 20 and 7% respectively in whey proteins (De Wit, 1998; Oakenfull, Pearce, & Burley, 1997). The ability of whey proteins to gel has been generally ascribed to β-lg, which is the major protein in bovine whey. Nevertheless, whey comprises other proteins that can gel and hence interact with β-lg. Consequently, several studies exist on the heat gelation of mixtures of β-lg and α-la or BSA (Hines & Foegeding, 1993; Kavanagh, Clark, Gosal, & Ross-Murphy, 2000; Kehoe, Morris, & Brodkorb, 2007). Next to β-lg and α-la, casein glycomacropeptide (CMP) is the most abundant polypeptide in cheese whey with typical concentrations between 15 and 25% of total proteins (El-Salam, El-Shibiny, & Buchheim, 1996; Thomä-Worringer, Sørensen, & López Fandiño, 2006). However, its presence and influence on functional properties of whey protein products has generally been neglected. Studies on the functional properties of CMP are scarce and sometimes contradictory (Thomä-Worringer et al., 2006). Few studies may be found in the literature that report about CMP gelation. Burton and Skudder (1987) reported the gelation of a 9.3% (w/w) CMP solution at pH 4.5 and 20 °C. However, Wang (2007) reported that CMP was also able to form a gel only at pH < 4.0. In a recent paper, we described the pH-driven cold gelation of CMP and proposed a model to explain it (Farías, Martinez, & Pilosof, 2010). As a result of the spontaneous pH-driven self-assembly of CMP at room temperature, the solutions at pH below 4.5 gelled with time. The gels formed were opaque. CMP concentration for gelation was pH-dependent. At pH 3.0 or 3.5 CMP gelled even at concentrations as low as 3% (w/w).

Few studies exist on the influence of CMP on the functional performance of whey proteins. In a recent work, Thomä-Worringer et al. (2006) studied the impact of interactions between CMP and WPI on foaming properties, suggesting the convenience of using CMP and WPI in combination. Martinez, Carrera Sanchez, Rodríguez Patino, and Pilosof (2009) showed that CMP presented greater surface activity than β-lg but in mixed systems with β-lg, the last dominated the static and dynamic surface pressure and the rheological properties of interfacial films. This behaviour was attributed to binding of CMP to β-lg in the aqueous phase that prevents CMP adsorption on its own. Regarding the impact of CMP on heat-induced gelation of whey proteins, Veith and Reynolds (2004) reported that at pH 7.0 the presence of CMP in the WPC was detrimental to gel strength and water-holding capacity.

CMP comprises the 64 amino acids of the hydrophilic C-terminal portion of κ-casein released after its specific cleavage by chymosin or pepsin. CMP contains all the posttranslational modifications (glycosylation and phosphorylation) present in κ-casein that contribute to its marked heterogeneity (Mikkelsen et al., 2005). Glycosylated forms of CMP (gCMP) represent about 50% of the total CMP (Mollé & Leonil, 2005) and it contains all carbohydrates originally present in κ-casein. The A and B forms of non-glycosylated CMP (aCMP) have molecular masses of 6787 and 6755 Da,

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respectively, and the highest molecular mass up to 9631 corresponds to highly glycosylated CMP (Mollé & Leonil, 2005). The most predominant carbohydrate is N-acetylneuraminic acid (sialic acid) (Coolbear, Elgar, & Ayers, 1996).

Sialic acid was found to be particularly important for biological and pharmacological activity. It is an acidic sugar with pKa value of 2.2. A higher concentration of sialic acid in CMP gives lower pI of this glycopeptides. The pI of aCMP is close to 4.1, which is related to the high amount of acidic amino acid side chains (Kreuß, Strixner, & Kulozik, 2009). The positive charge of aCMP at pH levels lower than the pI originates from the three Lys residues as well as from the positively charged N-terminus, while all Glu and Asp residues are protonated. The pI of gCMP, in contrast, is at 3.15, as the negative charge of the sialic acid residues reduces the net charge of the amino acid backbone (Kreuß et al., 2009). CMP has been the subject of growing interest due to its beneficial biological and physiological properties, as reviewed by El-Salam et al. (1996) and Thomä-Worringer et al. (2006).

The aim of the present work was to study the dynamics of heat-induced gelation of CMP and β-lg mixtures as affected by their interactions in the aqueous phase at pH 3.5 and 7.0.

2. Materials and methods

2.1. Materials

BioPURE-GMP® casein glycomacropeptide (CMP) and BioPURE® β-lactoglobulin (β-lg) was provided by DAVISCO Foods International, Inc. (Le Sueur, MN, USA). Composition of CMP was: protein (dry basis) 83.0% (w/w) ($N \times 6.47$) being CMP 90.0% (w/w) ($N \times 7.07$) of total proteins, 0.6% (w/w) fat, 6.3% (w/w) ash and 6.0% (w/w) moisture. Composition of β-lg was: protein (dry basis) 97.8% (w/w) ($N \times 6.38$) being β-lactoglobulin 93.6% (w/w) of total proteins, 0.3% (w/w) fat, 1.8% (w/w) ash and 5.0% (w/w) moisture.

Powder samples of β-lg and CMP were dissolved separately in Milli-Q ultrapure water at room temperature under agitation. The CMP:β-lg mixed systems were prepared by mixing the appropriate volume of each protein solution up to achieve a total concentration of 4% (w/w) for dynamic light scattering (DLS) measurements and 15% (w/w) for differential scanning calorimetry (DSC) and dynamics rheological measurements. The CMP:β-lg ratio in mixed systems was 0:100 (pure β-lg), 25:75, 50:50, 75:25 and 100:0 (pure CMP). The solutions were prepared freshly and kept 24 h at 4 °C. After that, the pH was adjusted to 3.5 or 7.0, immediately before the measurement, by using 1 M HCl or NaOH. To prevent bacterial growth 0.02% (w/w) NaN_3 was added.

2.2. Particle size determination

Dynamic light scattering (DLS) was carried out in a Zetasizer Nano-Zs (Malvern Instruments, Malvern, UK), provided with a He-Ne laser (633 nm) and a digital correlator, Model ZEN3600. Measurements were carried out at a fixed scattering angle of 173°. Samples were contained in a disposable polystyrene cell.

To obtain size information, Contin's algorithm was used as described elsewhere (Martinez et al., 2009).

The samples for DLS were filtered through a 0.45, 0.22 and 0.02 μm microfilter Whatman International Ltd. (Maidstone, England) before use. The assay was performed on three individual samples.

2.3. Differential scanning calorimetry

Differential scanning calorimetry (DSC) was used to study the thermal transition involved in β-lg, CMP and mixed systems. A DSC 822 Mettler Toledo Calorimeter (Schwerzenbach, Switzerland) was used. The instrument was calibrated with indium (156.6 °C), lead (327.5 °C) and zinc (419.6 °C). Thermograms were evaluated using Mettler Stare program. The onset temperature (T_o), peak temperature (T_p) were determined by heating 60 μL of each sample into 160 μL capacity pans at 10 °C min^{-1} from 5 to 100 °C. An empty pan was used as reference. The reported values are the average of two individual samples.

2.4. Sol–gel transition

The sol–gel transition was determined by a modified tilting test (Relkin, Meylheuc, Launay, & Raynal, 1998b). CMP solutions at 10 and 12% (w/w; 2 g) were heated in closed tubes at different temperatures (25–70 °C) and observed over time. The gelation time (t_{til}) was assumed to be reached when there was no deformation of the meniscus upon tilting. The average value of three individual samples is reported, with an experimental error < 10%.

2.5. Dynamic oscillation measurements

Dynamic oscillation measurements were performed using a Paar Physica controlled stress Rheometer (MCR 300) (Graz, Austria). The CMP:β-lg mixed systems 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'') and the loss tangent (tan δ) were measured. The loss tangent (G''/G') indicates the relative viscoelasticity of the sample.

The temperature at which the storage and loss modulus crossed over (at tan $\delta = 1$) was taken as the gel point, and the time (t_{gel}) and temperature (T_{gel}) at this point were evaluated. The data reported are means of at least three individual samples with an experimental error lower than 10%.

3. Results

3.1. Size distribution of particles at pH 7.0

The intensity-size distribution of β-lg solution (4%, pH 7.0, deionized water) presented two populations, one with a maximum value at 3 nm, which is close to the value reported for the monomeric form of this protein (3.6 nm) (Mehalebi, Nicolai, & Durand, 2008), and another population at 20 nm (Fig. 1A). At neutral pH, β-lg exists in a dynamic equilibrium between its dimeric and monomeric form (Aymard, Durand, & Nicolai, 1996; Mc Kenzie, 1971; Verheul, Pedersen, Roefs, & de Kruif, 1999). When the ionic strength is low as in the present work, the equilibrium is shifted towards the monomeric form. This behaviour agrees with the results presented by Mehalebi et al. (2008) who found a dependence of the hydrodynamic diameter (d_H) with the concentration, with lower values at lower concentrations. At pH 7.0 and concentrations lower than 5% (w/w) the interactions between monomers could be negligible because the molecular mass and hydrodynamic diameter obtained corresponded to the monomeric form of β-lg (18.4 kDa and 3.6 nm, respectively). The population with size

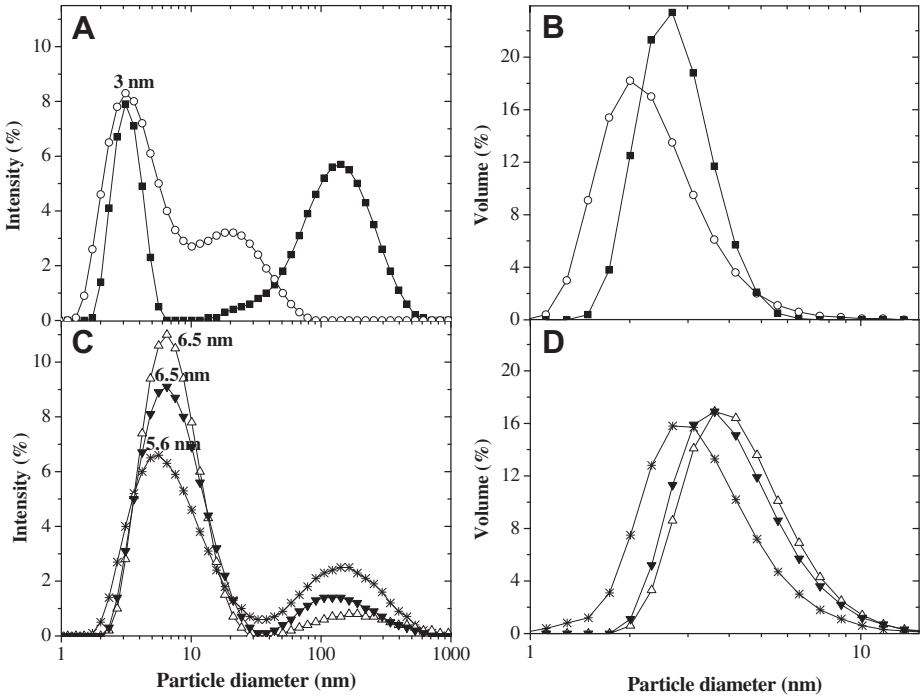

Fig. 1. Intensity (A and C) and volume (B and D) particle size distribution at pH 7.0 and 25 °C of 4% (w/w) protein solutions containing CMP (■), β-lg (○) and mixtures of CMP and β-lg at a ratio of 25:75 (△), 50:50 (▼), and 75:25 (✳).

20 nm could be due to the presence of aggregated forms. In fact, Verheul et al. (1999) found, by small-angle neutron scattering, structures higher than dimers at pH 6.9 and concentrations > 3% (w/w). However, in number this population was negligible as it is observed in the volume-size distribution (Fig. 1B).

The intensity-size distribution of CMP (4%, pH 7.0, deionized water) presented a predominant lower size peak with d_H between 2 and 6 nm with a maximum at 3 nm (Fig. 1A). A rough estimate of size of CMP made with the software of the Zetasizer Nano-Zs instrument indicated that the monomeric form of CMP (Mw between 7 and 11 kDa) should have a size of approximately 3 nm. Thus, in CMP solution at neutral pH predominated the monomeric form together with more aggregated forms like dimers, trimers and tetramers which would have d_H within 3.5 and 5 nm. Aggregates of higher size, between 20 and 500 nm were also apparent in the CMP intensity-size distribution. However their number was negligible as can be deduced from the volume-size distribution plots (Fig. 1B).

Upon mixing CMP and β-lg at different ratios a similar and bimodal distribution could be observed (Fig. 1C), decreasing the intensity of the lower size peak with the increase of CMP concentration in the mixed system. Additionally, the peak corresponding to CMP aggregates, with maximum values around 100 nm, increased with increasing CMP concentration in the mixed system, but was negligible in number (Fig. 1D). It is important to note that the position of the predominant lower size peak in the mixed system shifted to higher sizes (5.6–6.5 nm) as compared with solutions of single CMP or β-lg suggesting the formation of assembled CMP:β-lg structures. Moreover, monomers were not observed in the mixtures.

In a previous work (Martinez et al., 2009), a similar behaviour was found in trizma buffer (>99.5%) pH 7.0. Haque, Casay, Wilson, Antila, and Antila (1993) reported that β-lg A associated markedly in the presence of large and small peptides hydrolysates from casein at pH 7.0. They attributed this tendency to the following: (i) a subtle alteration in the structure as a result of the change in free energy due to protein–peptide binding; (ii) the formation of mixed micellar structures that may be better able to decrease hydrocarbon–aqueous interface, a thermodynamically favourable phenomenon. Recently, the complexation of β-lg with chitosan was evidenced by the shift of the particle size distribution to much higher sizes and by the disappearance of the native β-lg (Mounsey, O'Kennedy, Fenelon, & Brodkorb, 2008).

3.2. Size distribution of particles at pH 3.5

Solutions of β-lg (4%, pH 3.5, deionized water) showed a bimodal distribution with a predominant lower size peak at 5 nm (Fig. 2A). The shift of this peak to higher sizes as compared with pH 7.0, indicates that the equilibrium monomer–dimer is shifted towards the dimeric form at this pH. Between pH 2.0 and 3.7 β-lg has been reported to form dimers (Pessen, Purcell, & Farrell, 1985). Haque and Sharma (2002) also found by DLS that at pH 3.5 β-lg showed a tendency to association.

CMP (4%, pH 3.5, deionized water) presented a higher degree of association at pH 3.5 as compared with neutral pH, with maximum values of the predominant lower size peak at 6 nm (Fig. 2A). According with the Mw estimation by the software of Zetasizer Nano-Zs instrument, the increased peak sizes could correspond to CMP self-assembled forms like dimers, tetramers, hexamers and other oligomers, depending on the degree of glycosylation of CMP.

In both β-lg and CMP size-intensity distributions, other peaks with higher size were also observed, but they can be considered negligible in number from the volume-size distribution (Fig. 2B).

Due to effect on the net surface charge, the pH has a major influence on CMP interactions (Kreuβ et al., 2009) and hence on the intensity particle size distribution of CMP immediately after pH adjustment at pH 3.5 (Fig. 2A). At room temperature, CMP undergoes a pH-dependent self-assembly at pH < 4.5 forming different self-assembled structures that depending on CMP concentration can form gels. Self-assembled structures are almost

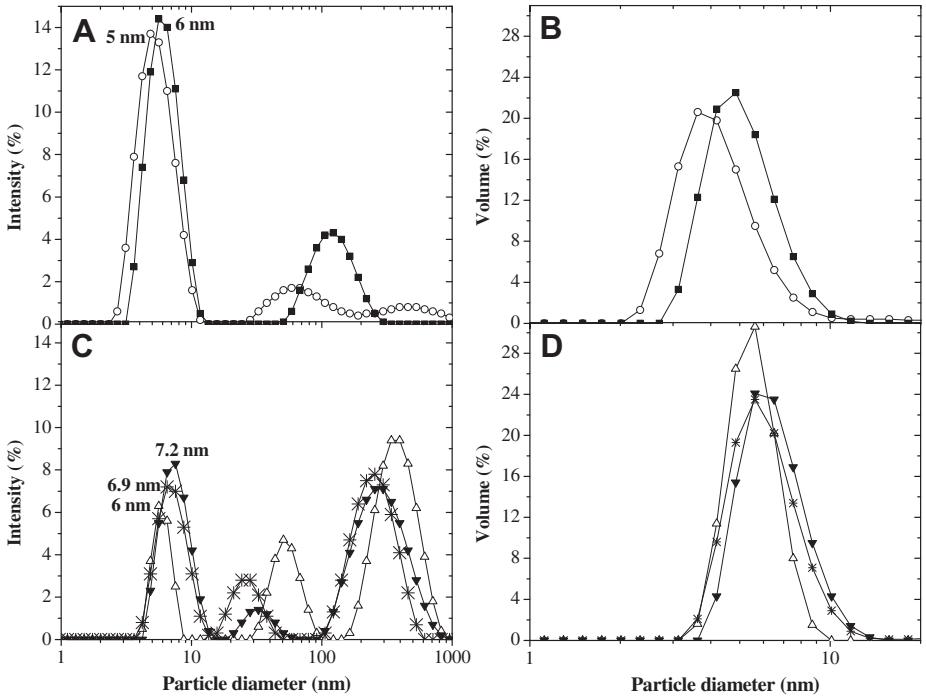

Fig. 2. Intensity (A and C) and volume (B and D) particle size distribution at pH 3.5 and 25 °C of 4% (w/w) protein solutions containing CMP (■), β-lg (○) and mixtures of CMP and β-lg at a ratio of 25:75 (△), 50:50 (▼), and 75:25 (✳).

pH-reversible, but dimers appear to be resistant to pH changes once formed (Farías et al., 2010). CMP self-assembly would include a first stage of hydrophobic self-assembly to form dimers which further interact trough electrostatic bonds to form gels with time.

Mixed systems presented multimodal intensity distributions at all the CMP:β-lg ratios studied (25:75, 50:50 and 75:25) (Fig. 2C). The predominant lower size peaks in the intensity-size distribution showed maximum values at 6, 7.2 and 6.9 nm, respectively. These values were higher than those for single components, indicating again the presence of CMP:β-lg assembled structures. The mixed systems presented also more aggregated forms even with sizes between 100 and 1000 nm, which points out the formation of big aggregates at this pH; however, the only peak to be considered important in number was the lower size peak as can be deduced from Fig. 2D. In the same figure it can be observed that most of the particles in the mixed systems had d_H higher than 4 nm, whilst in the single systems a significant number of particles were below 4 nm.

In a previous study, the existence of associative interactions between CMP and β-lg in the aqueous phase at neutral pH has been demonstrated by dynamic light scattering. DSC studies of these mixtures indicated the formation of CMP:β-lg complexes driven by electrostatic interactions and/or by hydrogen bonding (Martinez et al., 2009). However, complementary measurements to support complexes formation at pH 3.5, is necessary. To this end, DSC thermal transitions are reported in Table 1. For β-lg the apparent enthalpy changes correspond to dimer dissociation and denaturation processes (endothermic), a necessary step in the heat denaturation process (Cairoli, Iametti, & Bonomi, 1994; Iametti, De Gregori, Vecchio, & Bonomi, 1996) as was explained in a previous work (Martinez et al., 2009). This endothermic process is superimposed to an aggregation process (exothermic) during the time scale of DSC measurements (Baeza & Pilosof, 2002). For β-lg T_o and T_p were much higher at pH 3.5 than at pH 7.0 in agreement with previous studies (Relkin, Launay, & Liu, 1998a). It is well known that at pH 3.0–4.0, β-lg has its highest denaturation temperature (Burova, Grinberg, Visschers, Grinberg, & de Kruif, 2002; Mulvihill & Donovan, 1987; Relkin, 1996; Verheul, Roefs, & de Kruif, 1998). The maximum stability of β-lg at pH below its isoelectric point is probably related to the neutralization of carboxyl groups leading to extra internal hydrogen bonding and loss of localized unfavourable electrostatic interactions (Kella & Kinsella, 1988; Verheul et al., 1998).

CMP did not show any thermal transition at pH 3.5 and 7.0, because of its random coil structure and the absence of disulphide bonds. Ono, Yada, Yutani, and Nakai (1987) studied the conformation of CMP by circular dichroism and suggested that it has little organized structure. Wang (2007) observed an exothermic peak corresponding to aggregation of CMP 5% (w/w) at pH 3.5 using a microcalorimeter (heating about 0.3 °C min^{-1}), but in the equipment used in this work it was not observed. Thus thermograms of mixed CMP:β-lg systems, at both pH 3.5 and 7.0, mainly revealed β-lg transitions. The transition temperatures of β-lg (Table 1) were decreased by the presence of CMP in the mixed systems. The decrease in denaturation temperature on mixtures of proteins has been ascribed to complex formation by electrostatic

Table 1
Thermal parameters for 15% w/w solutions of CMP, β-lg and CMP:β-lg mixed systems at pH 3.5 and 7.

CMP:β-lg ratio	pH	T_{onset} (°C)[a]	T_{peak} (°C)[a]
0:100	3.5	79.37 ± 0.2	84.10 ± 0.1
	7[b]	66.46 ± 0.1	74.21 ± 0.2
25:75	3.5	78.31 ± 0.1	83.69 ± 0.1
	7[b]	60.53 ± 0.1	71.78 ± 0.1
50:50	3.5	77.11 ± 0.1	82.33 ± 0.2
	7[b]	63.58 ± 0.2	71.53 ± 0.1
75:25	3.5	74.94 ± 0.2	82.43 ± 0.1
	7[b]	62.58 ± 0.1	71.48 ± 0.1

[a] Mean ± SD of at least two replicates.
[b] From Martinez et al. (2009).

interactions (Ivinova, Izumrudov, Muronetz, Galaev, & Mattiasson, 2002; Martinez et al., 2009).

Electrostatic interactions have been reported to perturb the structure of myoglobulin and bovine serum albumin causing decreased thermal stability (Imeson, Ledward, & Mitchell, 1977). Van de Weert, Benedix Anderson, and Frokjaer (2004) found that lysozyme stability was reduced by 16 °C due to complexation with heparin (determined using DSC). Barbeau, Gauthier, and Pouliot (1996) studied the thermal stability of β-lg alone and in the presence of non-fractionated whey peptides, by chromatographic techniques, and they found that thermal denaturation of β-lg was greatly influenced by pH and by the addition of non-fractionated hydrolysate, decreasing the denaturation temperature in the pH range studied (between pH 4.6 and 8.0) through a mechanism similar to that in urea (Griko & Privalov, 1992). This solute disrupts the hydrogen-bonded structure of water and facilitates protein unfolding by weakening hydrophobic interactions (Pearce & Kinsella, 1978). The maximum decrease of the onset of β-lg denaturation at pH 7.0. (6 °C) was observed at CMP:β-lg ratio 25:75, but at pH 3.5 (4.5 °C) it was observed at CMP:β-lg ratio 75:25.

3.3. CMP gelation

In a recent work (Farías et al., 2010) we showed that CMP at pH below 4.5 undergoes a time-dependent self-assembly at room temperature which leads with time to the formation of opaque gels. The minimum CMP concentration for this cold-gelation depended on pH. Below pH 4.0, CMP gelled even at low concentrations (3% w/w) but the times needed for gelation were as long as 6–15 days. When CMP concentration was higher than 7–8% (w/w), the gelation time was shorter (<50 h) than for lower concentrations and kept almost constant. At pH higher than 4.0, gelation was not observed.

Fig. 3 shows the effect of temperature on the time needed to form a gel at pH 3.5 and high CMP concentrations, 10–12% (w/w), determined by a tilting test. The increase of temperature from 25 to 50 °C dramatically decreased the time for CMP gelation from 150–180 min to 10–30 min. A further increase of temperature slightly reduced the time for gelation. According to the model proposed to explain CMP self-assembly and the formation of a network-like structure (gel) at room temperature (Farías et al., 2010), it would involve a first step of hydrophobic self-assembly to form dimers which further interact trough electrostatic bonds to form gels with time. Temperature increases the potential for hydrophobic interactions (Bryant & McClements, 1998) increasing the rate of the first step involving hydrophobic bonds between CMP monomers, thus increasing the overall gelation rate.

Fig. 4 describes the dynamics of CMP gelation (dynamic oscillation measurements) at pH 3.5 upon heating up to 90 °C and holding the sample for 15 min, then cooling down to 25 °C. The crossover of G' and G'' can be taken as the gel point, from which the time to gel and the gelation temperature may be estimated as 9.4 min and 70 °C, respectively. The magnitude of time to gel agrees with times determined by the tilting test. After that time the G' continued to develop up to 90 °C where it levelled off. Interestingly, G' did not further increase upon cooling, as typically occurs for other protein gels, whose structures are reinforced by enhanced hydrogen bonding, promoted by lowering temperature (Bryant & McClements, 1998). The relative viscoelasticity of CMP gel was very high as tan δ was 0.044. For CMP at pH 7.0, no crossing between G' and G'' was observed even at concentrations as high as 40% (w/w), corroborating the absence of gelation previously observed by the tilting test.

3.4. Gelation of β-lactoglobulin

The heat-induced gelation of β-lg has been extensively studied and it presents high pH dependence (Clark, Kavanagh, & Ross-Murphy, 2001; Ould Eleya & Turgeon, 2000a; Sittikijyothin, Sampaio, & Gonçalves, 2007; Stading & Hermansson, 1990). At neutral pH, a minimal concentration of 12% (w/w) was needed for β-lg to form a gel, while at pH 3.5 it was possible to form a gel at lower concentrations as 8% (w/w) (data not shown). Fig. 5 shows the G' and G'' evolution for β-lg solutions, 15% (w/w), at pH 7.0 and 3.5, upon heating. It is possible to observe that it gelled at both pH, as shown by the crossover of G' and G''. A great increment in G' and G'' occurred with increasing temperature up to 90 °C and also during the holding time at 90 °C. On subsequent cooling from 90 to 25 °C both moduli further increased with decreasing temperature and finally during the holding time at 25 °C they reached almost constant values.

The increase of both moduli with decreasing temperature has been previously observed for β-lg and other whey and soy proteins (Ould Eleya & Turgeon, 2000b) and it was attributed to a reduction in entropy, which consolidated the attractive forces (hydrogen bonding, van der Waals forces) between the protein particles in gel. Similar maximum values of G' and G'' were attained, although slightly higher for the gel at pH 3.5. The gelation temperature of β-lg at pH 3.5 (90 °C) was higher than at pH 7.0 (88.3 °C) in agreement

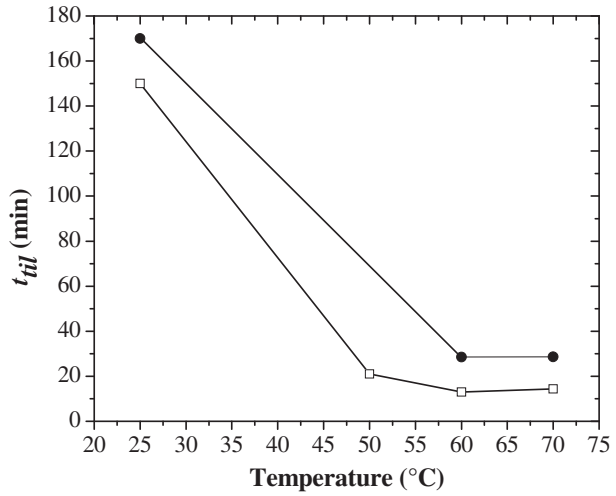

Fig. 3. Gelation time (t_{til}) as a function of temperature of CMP solutions at pH 3.5 at concentration at: (●) 10 and (□) 12% w/w.

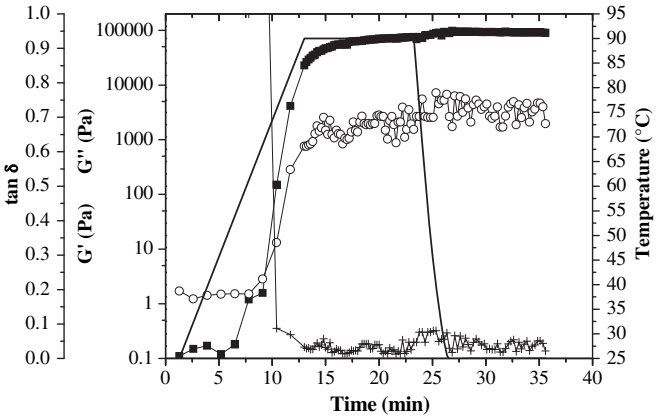

Fig. 4. G' (■), G'' (○) and tan δ (+) evolution during the heat-induced gelation of 15% w/w CMP solution at pH 3.5. The (–) temperature profile is also depicted.

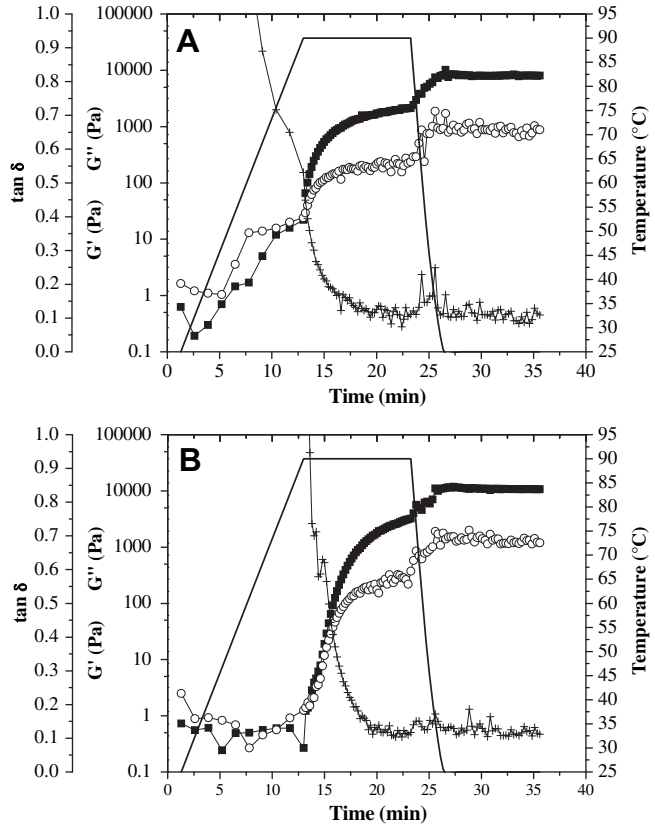

Fig. 5. G' (■), G'' (○) and tan δ (+) evolution during the heat-induced gelation of 15% w/w β-lg solutions at pH (A) 7.0 and (B) 3.5. The (–) temperature profile is also depicted.

with the higher denaturation temperatures reported at pH 3.5 (Table 1). Nevertheless the differences in denaturation temperature were much higher. Both gels were transparent as previously described by Langton and Hermansson (1992) composed of fine strands with a diameter less than 5 nm. At pH far away from isoelectric point, β-lg is highly charged, experiencing strong repulsion under conditions of low ionic strength resulting in fine stranded networks (Mudgal, Daubert, & Foegeding, 2009).

3.5. Gelation of CMP:β-lg mixed systems

All the mixed systems at both pH showed a crossover of G' and G'', indicating that gelation upon heating occurred and also exhibited the increment of G' and G'' during cooling from 90 to 25 °C, as was seen for β-lg solutions pointing out that β-lg dominated the gelation process in mixed systems.

Fig. 6 shows the evolution of G' for CMP:β-lg systems at pH 3.5 upon heating up to 90 °C and cooling down to 25 °C, in comparison with single components. At pH 3.5, all the mixed systems gelled, even when β-lg was present at concentrations lower than 8% (w/w) which is the critical concentration for gelation at pH 3.5. Nevertheless this behaviour does not call the attention because CMP gelled at pH 3.5 even at very low concentrations (Farías et al., 2010).

The G' evolution of systems with CMP:β-lg 25:75 and 50:50 at pH 3.5 was similar to single β-lg but with a more rapid G' development during the holding period at 90 °C, which reveals the presence of CMP. G' for system CMP:β-lg 75:25 increased more slowly during the heating period than each single component and reached lower values upon cooling.

The first conclusion is that CMP gelation is prevented by the presence of β-lg. In fact, as CMP content increased in the mixture the evolution of G', that is the evolution of the solid character of gels, lowered. This antagonic behaviour is better seen in the inner plot in Fig. 6 where G' and tan δ after cooling at 25 °C are plotted. It can be seen that CMP gel had a G' almost 10 times higher than that of β-lg gel. At the ratio CMP:β-lg 25:75, the G' had a value proportional to the content of each protein, but at 50:50 and 75:25 ratio the values were much lower than the expected ones if no interaction takes place. The gel 75:25 had a very low G' and was very soft. The relative viscoelasticity of CMP gel was very high (tan $\delta = 0.044$) in comparison with β-lg gel (tan $\delta = 0.109$), but all the mixed gels had values higher than single components, pointing out again their antagonistic behaviour in mixture at this pH.

Fig. 7 shows the evolution of G' for CMP:β-lg systems at pH 7.0 upon heating up to 90 °C and cooling down to 25 °C, in comparison with β-lg alone; as shown above, CMP did not gel at pH 7.0. Therefore the curves mainly reflect the gelation of β-lg. Interestingly, at pH 7.0 the three mixed systems gelled despite (i) CMP did not gel even at high concentrations and (ii) β-lg concentration in mixtures 50:50 and 75:25 was below the critical concentration for gelling, 12% (w/w). In fact β-lg concentration in those mixtures was 7.5 and 3.75% (w/w), respectively. It may be concluded that a synergistic interaction between CMP and β-lg takes place at pH 7.0. The β-lg gel was transparent and hard and those containing CMP were white with increasing softness as the content of CMP increased. The system CMP:β-lg 25:75 exhibited particular features because showed an unexpected high G' value (57 kPa) as compared with β-lg alone (8 kPa). The β-lg concentration in this system (11.75% w/w) allows the protein to gel but the presence of CMP (3.75% w/w) strongly enhanced its elastic character. Thus a strong synergism is clear at this CMP:β-lg ratio.

Veith and Reynolds (2004) reported that at pH 7.0 the presence of CMP in the WPC was detrimental to gel strength and water-holding capacity. This result agrees with the behaviour of mixed gels at 50:50 and 75:25 CMP:β-lg ratio which showed much lower G' than single β-lg gels. Nevertheless β-lg still gelled even though at pH 7.0, gels were not expected to form for pure β-lg at concentrations less than 12%, being zero the CMP contribution (inner plot in Fig. 7).

Fig. 8 shows the gelation temperature (T_{gel}) for the mixed systems as well as for single components. At pH 7.0, T_{gel} decreased from 88 °C to 74 °C with increasing CMP concentration from 0 to 11.25% (w/w), but the major decrease occurred for CMP:β-lg 25:75 system. This result matches the DSC results as the major decrease in the onset and peak temperatures was observed in this mixture. This indicates that denaturation of β-lg would be the rate determining step in the gelation at pH 7.0 of this protein or in admixture with CMP, which decreases β-lg thermostability by forming assembled structures in solution (Table 1 and Fig. 1).

At pH 3.5 T_{gel} of mixed systems was constant and equal to T_{gel} of β-lg (90 °C), even if T_{gel} for single CMP was very low (70 °C). At this pH, it looks that β-lg denaturation did not control the gelation as the decrease in the onset and peak temperatures in the presence of CMP (Table 1) was not revealed in T_{gel}. Therefore, the aggregation step would control the gelation at pH 3.5.

4. Discussion

Dynamic light scattering and DSC studies at molecular level, supports the existence of non-covalent interactions between CMP and β-lg in solutions of very low ionic strength (no added salts) at room temperature, at both pH 3.5 and 7.0, modulated by CMP:β-lg ratio. The assembly between CMP and β-lg at pH 3.5 would

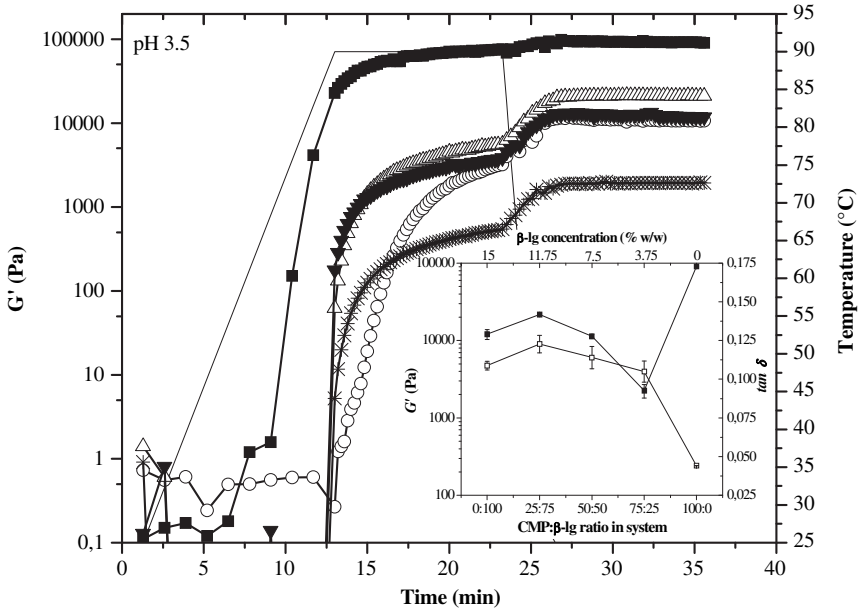

Fig. 6. *G′* evolution during the heat-induced gelation of CMP:β-lg systems mixed at different ratio: 0:100 (○), 25:75 (△), 50:50 (▼), 75:25 (✳) and 100:0 (■) at pH 3.5. The (–) temperature profile is also depicted. Total protein concentration: 15% w/w. Inner plot: *G′* (full symbol) and tan *δ* (empty symbol) for CMP:β-lg systems mixed at the end of the cooling at pH 3.5.

probably involve stronger interactions than at pH 7.0 due to the electric charges of each protein.

Thus different assembled structures driven mainly by electrostatic interactions, formed prior to heat treatment, would prevent the gelation of each single component by its own, leading to synergic or antagonic gel properties.

The antagonistic effect observed on *G'* at pH 3.5 would arise from formation of strong electrostatic complexes between CMP and β-lg. In fact, β-lg at pH 3.5 is far below its isoelectric point (pI) which is between 4.6 and 5.2 (Bromley, Krebs, & Donald, 2005; Das & Kinsella, 1989; Harnsilawat, Pongsawatmanit, & McClements, 2006), being its ξ-potential being approximately +20 mV (Harnsilawat et al., 2006) which represents a strong positive net charge.

CMP is considered to be affected by changing pH as it contains two Asp, seven or eight Glu (depending on CMP variant B or A, respectively), one phospho-Ser and three Lys residues (Dziuba & Minkiewicz, 1996). Below the isoelectric point (pI), the CMP molecules loose the negative charge of the Glu and Asp residues as well as the carboxyl group at the C-terminus and the sialic acid residues in gCMP. At pH 3.5, the aglyco-form of CMP (aCMP) is below its pI (4.15) with ξ-potential about +5 mV and the glyco-form

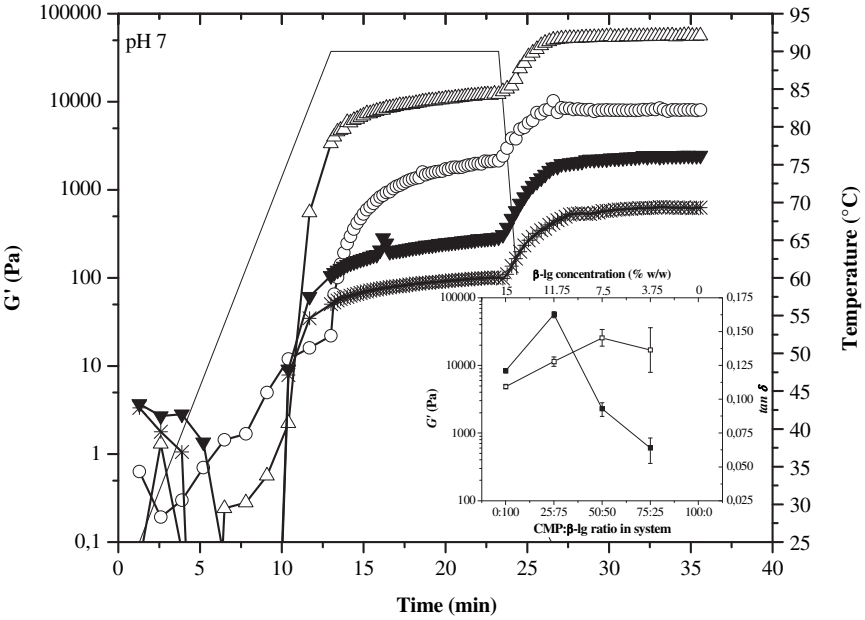

Fig. 7. *G′* evolution during the heat-induced gelation of CMP:β-lg systems mixed at different ratio: 0:100 (○), 25:75 (△), 50:50 (▼) and 75:25 (✳) at pH 7.0. The (–) temperature profile is also depicted. Total protein concentration: 15% w/w. Inner plot: *G′* (full symbol) and tan *δ* (empty symbol) for CMP:β-lg systems mixed at the end of the cooling at pH 7.0.

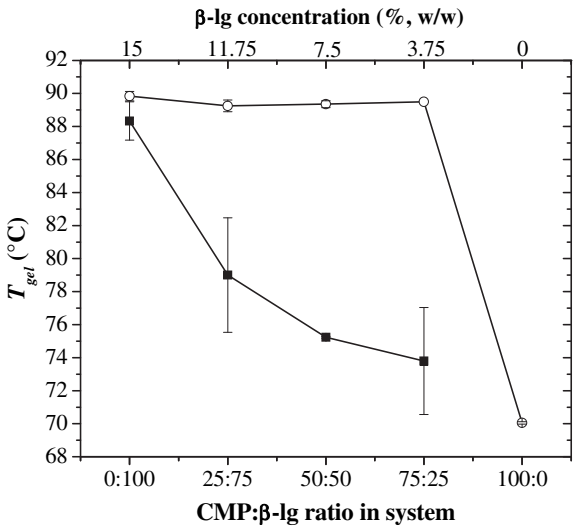

Fig. 8. Gelation temperature (T_{gel}) for CMP:β-lg systems at different ratio at pH 7.0 (■) and pH 3.5 (○).

(gCMP) is above its pI (3.15) with ξ-potential about −5 mV, as the negative charge of the sialic acid residues (pK of sialic acid: 2.2) reduces the net charge of the amino acid backbone (Kreuβ et al., 2009).

So, at pH 3.5 gCMP, which presents a negative net charge, can electrostatically interact with β-lg. Moreover, because of the low pK of sialic acid residues (2.2) local negative charges would be located on the glycan up to pH 2.2, allowing it to interact with β-lg too. The assembly between CMP and β-lg could be reinforced by hydrophobic interactions.

β-Lg belongs to the lipocalin family of proteins, which is folded as an eight-stranded antiparallel β-barrel that forms around a central cavity, the calyx with a hydrophobic pocket. Like most lipocalins, β-lg can bind small hydrophobic molecules within this central cavity (Papiz et al., 1986). CMP could interact throughout the hydrophobic domain N-terminal of CMP (amino acid 1–5) which is not covered by the negative charge, followed by the hydrophobic domains located in the centre of the peptide chain.

The mechanism of CMP cold gelation, proposed in a recent work (Farías et al., 2010), includes a first stage of hydrophobic self-assembly to form dimers which starts at pH below 6.5, followed by their interaction trough electrostatic bonds at pH below 4.5, to form gels with time. CMP self-assembly at pH 3.5 would be hindered by the presence of positively charged β-lg that, by complexing aCMP and gCMP, would prevent its assembly and further gelation, causing a strong decrease in *G'* of mixed gels at 50:50 or 75:25 CMP:β-lg (inner plot of Fig. 6).

Maximum interaction according to DSC results (Table 1) and *G'* behaviour (Fig. 6) occurs at 75:25 CMP:β-lg, which corresponds to a molar CMP:β-lg ratio of 6:1. The resulting assembled structure would have a Mw of about 70 kDa which roughly agrees with the Mw estimated from the hydrodynamic diameter of the lower size peak in Fig. 2. The gelation of β-lg, when assembled with CMP, would be hindered sterically or because hydrophobic domains interacting with CMP are not available to interact each other upon heating. As a result of these complex interactions the mixed gels exhibited an antagonistic behaviour, except at 25:75 CMP:β-lg, where *G'* value of the composite gel could be related to pure component behaviour by a simple blending law. For this mixture the DLS and DSC results showed the lowest degree of interaction between CMP and β-lg in solution.

At pH 7.0 the electric state of CMP and β-lg is similar being the ξ-potential of β-lg −20 mV (Harnsilawat et al., 2006) and that of aCMP and gCMP −18 and −24 mV, respectively (Kreuβ et al., 2009). Therefore in this condition only weak electrostatic interactions can take place between CMP and β-lg throughout the positive patches of β-lg and negative patches of CMP or sialic acid. Contrarily to pH 3.5, in this case the assembly was beneficial; the CMP contribution was always zero because it did not gel at pH 7.0, and given the critical concentration to gel for single β-lg, 12% (w/w), modulus values would not be expected for the mixed systems 50:50 and 75:25 that have 7.5 and 3.75% β-lg, respectively. In spite of this, gels were formed (inner plot of Fig. 7) but they were white, very soft and had a low *G'* (2291 and 601 Pa). The mixture CMP:β-lg 25:75 showed an outstanding performance as *G'* was greater than that of pure β-lg at 15% (w/w). The molar CMP:β-lg ratio for this mixture was 1:2 which gives a molecular weight of 45 kDa that agrees with the Mw estimated from the hydrodynamic diameter of the corresponding peak (6.5 nm) in the intensity-size distribution (Fig. 1). This kind of assembly facilitates not only denaturation of β-lg (Table 1) but also its aggregation.

5. Conclusions

It has been proved that CMP and β-lg strongly interact in solutions at pH 3.5 and 7.0 forming assembled structures driven by electrostatic interactions. However, the assembly impacted in a different way on gel formation: at pH 7.0 a strong synergism was observed because pure CMP did not gel but allowed β-lg to gel even at concentrations far below the critical concentration. Contrarily, at pH 3.5 where both CMP and β-lg gelled on its own the assembly was strongly detrimental, mainly because hindered gelation of CMP.

The present results and the previous ones point out that the assembly of CMP and β-lg in aqueous solutions is more a rule than an exception and have a strong impact on gelation. Probably because β-lg dominates the behaviour of mixed CMP:β-lg systems the importance of studying the influence of CMP in whey products has not been highlighted.

Finally, the results show that the synergism between CMP and β-lg in some conditions can be exploited for the design of foods with desired texture containing CMP as a bioactive ingredient.

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