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Influence of pH on viscoelastic properties of heat-induced gels obtained with a β-Lactoglobulin fraction isolated from bovine milk whey hydrolysates

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ABSTRACT

A β-Lactoglobulin fraction (r-βLg) was isolated from whey hydrolysates produced with cardosins from *Cynara cardunculus*. The impact of the hydrolysis process on the r-βLg structure and the rheological properties of heat-induced gels obtained thereafter were studied at different pH values. Differences were observed between r-βLg and commercial β-Lg used as control. Higher values for the fluorescence emission intensity and red shifts of the emission wavelength of r-βLg suggested changes in its tertiary structure and more solvent-exposed tryptophan residues. Circular dichroism spectra also supported these evidences indicating that hydrolysis yielded an intermediate (non-native) β-Lg state.

The thermal history of r-βLg through the new adopted conformation improved the microstructure of the gels at acidic pH. So, a new microstructure with better rheological characteristics (higher conformational flexibility and lower rigidity) and greater water holding ability was founded for r-βLg gel. These results were reflected in the microstructural analysis by scanning electron microscopy.

1. Introduction

Whey proteins have a wide variety of functional applications in the food industry. Modification of whey proteins structure allows modifying its functionality and thus finding novel functional attributes for ingredients developed from them. This has been done applying physical (Nicorescu et al., 2011), chemical (Martinez-Alvarenga et al., 2014) or enzymatic (Otte, Lomholt, Ipsen, & Qvist, 2000) treatments. Among all, enzymatic hydrolysis has been recurrently used to design functionality for specific requirements such as to decrease allergenicity (Babij et al., 2015), produce bioactive peptides (Le Maux, Nongonierma, Barre, & Fitzgerald, 2016) or nanotubes (Tarhan & Harsa, 2014), and to improve functional properties including solubility (Severin & Xia, 2006), gelation (Doucet, Gauthier, & Foegeding, 2001) and foaming characteristics (Davis, Doucet, & Foegeding, 2005).

Since enzymes are usually very specific in their mode of action, a carefully selected enzyme formulation can be used to produce whey hydrolysates for different food applications. Plant origin enzymes, such as cardosins obtained from dried flowers of *Cynara cardunculus*, have been much less explored than microbial or gastric proteases. Because of its natural origin, low cost, and easy extraction, cardosins are interesting enzymes for the production of functional foods. In addition, cardosins extracts are characterised by the presence of a mixture of specific and unspecific proteases, which represents another advantage for varied industrial applications.

In our group, a process to obtain ACE-inhibitory peptides by hydrolysis of whey proteins with cardosins was developed. Together with bioactive peptides, a non-hydrolysed β-Lactoglobulin (β-Lg) fraction (r-βLg) was also obtained. Other authors have confirmed the inability of cardosins to hydrolyse β-Lg to a significant extent after 24 h of hydrolysis (Barros & Malcata, 2006). Therefore, in addition to the production of highly bioactive peptide mixtures, there is a potential for this process to allow purifying β-Lg, a valuable protein.

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In a previous paper (Estévez et al., 2016), the impact of the proteolysis with cardosins on the thermal behaviour and gelation capability of the surplus β-Lg was studied. Other authors have shown that the enzymatic modification of native β-Lg, a very stable and compact globular protein, represents a potential opportunity for enhancing its functional properties. For example, Nieuwenhuizen, Dekker, Gröneveld, De Koster, and De Jong (2004) reported a method that allowed the modification of native β-Lg with a transglutaminase yielding a protein derivative with increased affinity for the water–air interface and stronger surface tension lowering capacities. Pescuma et al. (2011) demonstrated that a *Lactobacillus* proteinase was able to degrade pure β-Lg and its antigenic epitopes *in vitro*, reducing the allergenic response of human sera towards this protein. Hydrolysis with different proteases has also being used to improve the rheological properties of β-Lg gels (Doucet, Otter, Gauthier, & Foegeding, 2003; Otte et al., 2000). Thus, Chen, Swaisgood, and Foegeding (1994) showed that the partially hydrolysed β-Lg formed weaker gels at a high gelling temperature (80 °C), while stronger gels were formed at a low temperature (60 °C) as compared to those of obtained with the native protein.

In our case, limited proteolysis with cardosins did not lead to a decrease in the r-βLg molecular weight, i.e. r-βLg was resistant to the proteolysis. However, the process induced changes in the β-Lg native conformation causing exposure of hydrophobic groups, lower thermal stability and also, shorter thermal treatments were needed to obtain non-native and aggregates species. Such species have been described repeatedly in the literature for β-Lg as a result of heat treatments (Croguennec, Mollé, Mehra, & Bouhallab, 2004; Delahaije, Gruppen, Van Eijk-Van Boxtel, Cornacchia, & Wierenga, 2016). In addition, at acidic pH (3.2) the r-βLg protein showed the same two-staged gelation mechanism as commercial intact β-Lg, with the advantage that r-βLg was able to form stable gels at lower temperature (Estévez et al., 2016).

In this work, we focus on the study of the effect of pH because this is a factor that highly determines the production of β-Lg gel matrices with enhanced appearance, microstructure and rheological properties (Langton & Hermansson, 1992). Therefore, the objective of the present work was to complete a systemic study of the thermal gelation of r-βLg protein under different pH conditions with an emphasis on the viscoelastic, mechanical and water retention properties of the gels because these are the most important gel characteristics for food applications.

2. Materials and methods

2.1. Materials

The bovine whey protein used in this study was obtained from Queizuar S.L. (A Coruña, Spain). The enzymatic extract used consisted of cardosins A and B purified in our laboratory (milk-clotting activity: 0.46 U/mg protein, determined using skim milk as substrate; protein concentration: 2.2 mg/mL) from dried flowers of *C. cardunculus* provided by Suministros García Gallardo S.L. (Badajoz, Spain). Commercial bovine β-Lg (>85% purity), acetonitrile (HPLC grade, ACN) and trifluoroacetic acid (HPLC grade, TFA) were all purchased from Sigma-Aldrich (St Louis, MO, USA). All other chemicals were of analytical grade. All solutions were prepared with ultrapure water (Milli-Q Advantage A10, Millipore, CA, USA).

2.2. Production and isolation of the residual β-Lg protein

Whey protein was submitted to proteolysis with purified extracts of *C. cardunculus* at an enzyme/substrate ratio of 1/150 (w/w), incubated for 24 h at 60 °C and pH 5.3. Aliquots samples were taken at the beginning of the reaction and after 24 h of hydrolysis, being immediately frozen at −80 °C until further analysis by reversed-phase high-performance liquid chromatography (RP-HPLC). The final hydrolysate was kept overnight at 5 °C and the next day was subjected to ultrafiltration through a Prep/Scale-TFF cartridge (0.56 m^2 spiral polyethersulfone membrane) with 10 kDa molecular weight (MW) cut-off (Millipore, CA, USA), operated in full recirculation mode, under 2-bar pressure and room temperature. The retentate obtained after the ultrafiltration (mainly β-Lg) was lyophilised (LyoQuest −85 °C, Telstar, Spain) and stored at room temperature until analysis.

2.3. RP-HPLC

The whey hydrolysates, at 0 and 24 h, and the 10 kDa retentate (before lyophilisation) were analysed by RP-HPLC with an Agilent 1200 HPLC system (Agilent Technologies, Waldbronn, Germany). The solvent A was 0.1% (v/v) TFA in Milli-Q water, and solvent B was 0.1% (v/v) TFA in 100% (v/v) ACN. Elution was performed by applying 100% A for 5 min, then a linear gradient of 0–50% B over the following 50 min, from 50 to 100% B over the next 2.5 min, and then maintained at 100% B for 2.5 min. Finally, solvent A was increased to 100% in 5 min and the column was re-equilibrated for 5 min more. The eluate absorbance was monitored at 220 nm. All samples were filtered through 0.45 μm filters (Sartorius Stedim, Germany). The operating conditions were: ACE 5 C18 column (250 × 4.6 mm, 5 μm, 300 Å, Advanced Chromatography Technologies), flow rate 0.7 mL/min, injection volume 20 μL.

The concentration of β-Lg in the 10 kDa retentate (r-βLg) was determined using a calibration curve of commercial β-Lg, with concentration ranging between 0.5 and 14 mg/mL.

2.4. Characterisation of the residual β-Lg

2.4.1. Sample preparation

A known amount of r-βLg or commercial pure β-Lg (p-βLg) powder was dispersed in water or buffer (see below) and magnetically stirred overnight at room temperature. The dispersions were centrifuged at 10,000×*g* for 15 min (Beckman Coulter, Model Avanti J-26 XP), and the protein concentration of supernatant was determined by the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA) following the manufacturer's instructions and using bovine serum albumin (BSA) as standard. After adjusting to an adequate pH (range 2–9) using HCl or NaOH, the stock solutions of both proteins were diluted to the desired protein concentration (0.03–7 mg/mL), and used for the following experiments.

2.4.2. Zeta potential (Zp)

Zp was measured by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) at 25 °C. The measurement was determined considering the refractive index of β-Lg material as 1.45 and that of the dispersant medium as 1.33. Protein samples were prepared at 0.03 mg/mL in water at pH range 2–7.2. The measurements were repeated at least 3 times for each sample. Before each measurement, the samples were filtered through a 0.45 μm filter (Sartorius Stedim, Germany).

2.4.3. Intrinsic fluorescence spectroscopy

The intrinsic fluorescence due to tryptophan (Trp) and tyrosine (Tyr) residues was measured using a Jasco FP-750 fluorescence spectrophotometer. The spectra were recorded from 300 to 400 nm at an excitation wavelength of 280 nm. The β-Lg samples were prepared at 0.03 mg/mL of protein concentration in water in the pH range 2–9 and were filtered through a 0.45 μm filter

(Sartorius Stedim, Germany). The fluorescence intensities of each sample were normalised dividing the measurements by their absorbance at 280 nm. Measurements were made with a minimum of 3 replicates at room temperature, using a 10 mm quartz cell.

2.4.4. Circular dichroism (CD) spectra

CD scans were carried out using a Jasco spectropolarimeter (Model J-815). The ellipticity values [θ] were obtained in millidegrees (mdeg) directly from the instrument. A 0.1 cm cell pathlength was used for the spectral range 200–270 nm. For this, each protein sample was prepared at 0.5 mg/mL in 4 mM sodium phosphate buffer at pH 3.2 and 7.2. All the solutions were filtered through a 0.45 μm filter. In all cases, the spectra were a mean of five scans and a blank without protein was subtracted.

2.5. Preparation of β-Lg gels

Prior to each experiment, 5, 7.5 or 10% (w/v) r-βLg solutions were made in water at pH 3.2, 5.2 and 7.2, as described above. Then, each solution was poured into a 12 mL polypropylene tube with a screw cap and heated in a thermostatic water bath at 80 °C for 3 h. Subsequently, the gels were cooled down in an ice bath for 30 min, and stored overnight at 5 °C. On the following day, gels were equilibrated to room temperature for rheological and water holding analysis.

Control gels with p-βLg at 7.5% (w/v) and pH 3.2, 5.2 and 7.2 were prepared following the same procedure to compare their structures with those of gels made from r-βLg.

2.6. Water holding capacity (WHC)

The WHC of protein gels was determined using the microcentrifuge-based method described by Otte et al. (2000) with some modifications. Gels were made in 1.5 mL Eppendorf tubes using 1 mL final volume of protein dispersions. Gel samples of 0.26 g were placed in tubes coupled to a 0.1 μm Ultrafree-MC hydrophilic PVDF membrane (Millipore, CA, USA) and the unit was centrifuged (Model 5415 R, Eppendorf AG, Hamburg, Germany) at 590 × g for 20 min. The volume of supernatant released was determined by weighing and the tube was centrifuged at 12,000×g for another 20 min. Finally, the expelled fluid was measured by weighing again. Three replicates were tested for each gel, and the WHC was quantified after each centrifugation as follows:

$$\text{WHC } (\%) = (m_f/m_i) \times 100$$

where m_i and m_f are the weights of the gel samples before and after centrifugation, respectively. After the last centrifugation, the amount of soluble protein remaining in the total supernatant was determined by the BCA method.

2.7. Dynamic rheometry measurements of β-Lg gels

Small amplitude oscillatory shear (SAOS) tests were performed using a Bohlin CVO controlled stress rheometer (Bohlin Instruments, Inc. Cranbury, NJ). The temperature was maintained at 25 ± 0.1 °C by a Peltier element in the lower plate. The measurements were carried out using parallel-plate geometry (20 mm diameter and 1 mm gap). Before measurement, the gels were removed from the tubes and carefully cut into disk-shaped slices by using a scalpel. Disk samples were put on the lower plate of the rheometer and the excess sample protruding beyond the upper plate was carefully removed. A solvent trap was used to avoid evaporation during the measurements. Samples were allowed to rest for 15 min before analyses to ensure both thermal and mechanical equilibrium at the time of measurement. At least 6 replicates were made.

2.7.1. Stress sweep tests

To determine the linear viscoelastic (LVE) region, stress sweeps were run at 6.28 rad/s at 25 °C with the shear stress (σ) of the input signal varying from 0.24 to 1000 Pa. 300 points on the continuous mode were used in all instances. Changes in the storage modulus (G'), loss modulus (G''), complex modulus (G^*), and loss tangent ($\tan\delta$) were recorded. The critical (maximum) values of shear strain (γ_{max}) and shear stress (σ_{max}) on the limit of LVE range were derived by the method previously described in Campo-Deaño and Tovar (2009).

2.7.2. Frequency sweep tests

Samples were subjected to stress that varied sinusoidally with time at variable frequencies from 10 to 0.01 Hz. The strain amplitude was set at γ = 1% within the LVE range.

2.8. Scanning Electron Microscopy (SEM)

The microstructure of protein gels was analysed with a Scanning Electron Microscope JSM 6700F (CACTI, University of Vigo, Vigo, Spain), using the method described by MacIerzanka et al. (2012).

3. Results and discussion

3.1. Hydrolysis

The hydrolysis of the major whey proteins, α-Lactalbumin (α-La) and β-Lg, by cardosins from *C. cardunculus* was followed by RP-HPLC, and the degree of hydrolysis (DH) at 24 h was determined according to the following equation:

$$DH_{24h}\ (\%) = 100 - [(A_{24h} \times 100)/A_{0h}]$$

where, A_{0h} and A_{24h} are the peak area of α-La or β-Lg before and after of 24 h of hydrolysis, respectively.

The protein profiles obtained from the hydrolysate at 0 and 24 h and the respective retentate of 10 kDa are shown in Fig. 1. The results after a 24 h-reaction showed that the DH for α-La (close to 100%) was much higher than for β-Lg (21.47%). These

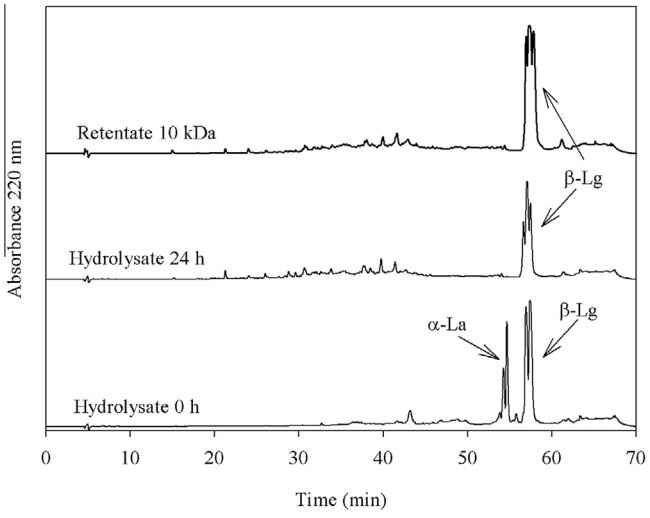

Fig. 1. RP-HPLC profiles of the hydrolysates obtained at the beginning and after 24 h of the whey hydrolysis using cardosins from *C. cardunculus* and the 10 kDa retentate fraction from the hydrolysate at 24 h. Major whey proteins (α-La and β-Lg) are identified.

results are consistent with those described by other authors, and being attributed to a higher affinity of cardosins for α-La than for β-Lg (Barros & Malcata, 2006). Since, 21.47% of native β-Lg was degraded to small peptides with antihypertensive activity (unpublished results), the 24 h-hydrolysate was subjected to ultrafiltration using a 10-kDa membrane and the obtained retentate was essentially free of peptides as shown by the very low absorbance signal on the chromatogram, containing only β-Lg (final β-Lg concentration was 16.36 mg/mL).

3.2. Characterisation of the residual β-Lg

Previous results reported by our group (Estévez et al., 2016) showed that even though r-βLg is resistant to hydrolysis with cardosins (no decrease in the molecular weight detected by high-performance gel permeation chromatography (HP-GPC) or electrophoresis), the process induced changes in its native structure. In essence, hydrophobic groups otherwise hidden inside the β-Lg molecule became exposed resulting in different intrinsic fluorescence spectra, particularly in higher values of maximum wavelength (λ_{max}) for the r-βLg compared to those of p-βLg used as control.

Assuming that these changes would influence the heat-induced gelation of this protein at different pH values, here besides the intrinsic fluorescence, we use additional techniques like dynamic light scattering and circular dichroism to throw more light into the r-βLg conformational state.

3.2.1. Surface charge/zeta potential

Zp measurements obtained by analysing the shear surface electric potential of colloid particles in solution provide an estimate of the effective surface charge. We studied the effect of pH on the Zp of p-βLg and β-Lg obtained after hydrolysis of whey with *C. cardunculus* proteases (r-βLg). In Fig. 2A, the surface charge of both proteins varied similarly with pH; both proteins displayed a maximum Zp around + 25.75 ± 0.78 mV at pH 2.5 and decreased progressively towards negative values as the pH increased. Along the pH scale, zeta potential crosses at zero when close to the isoelectric point, which was around pH 4.0 for either p-βLg or r-βLg. This value is slightly lower than the isoelectric point reported in the bibliography for β-Lg, which varies within the pH interval 4.7–5.2 (Sawyer & Kontopidis, 2000).

Minor differences were observed above pH 4 from which, for the same pH condition, Zp was lower for r-βLg than for p-βLg. This change might be consistent with either the burial or neutralization of basic surface side chains or to the increased exposure of acidic moieties induced on the β-Lg molecule by the proteolysis of whey that allowed recovering the r-βLg.

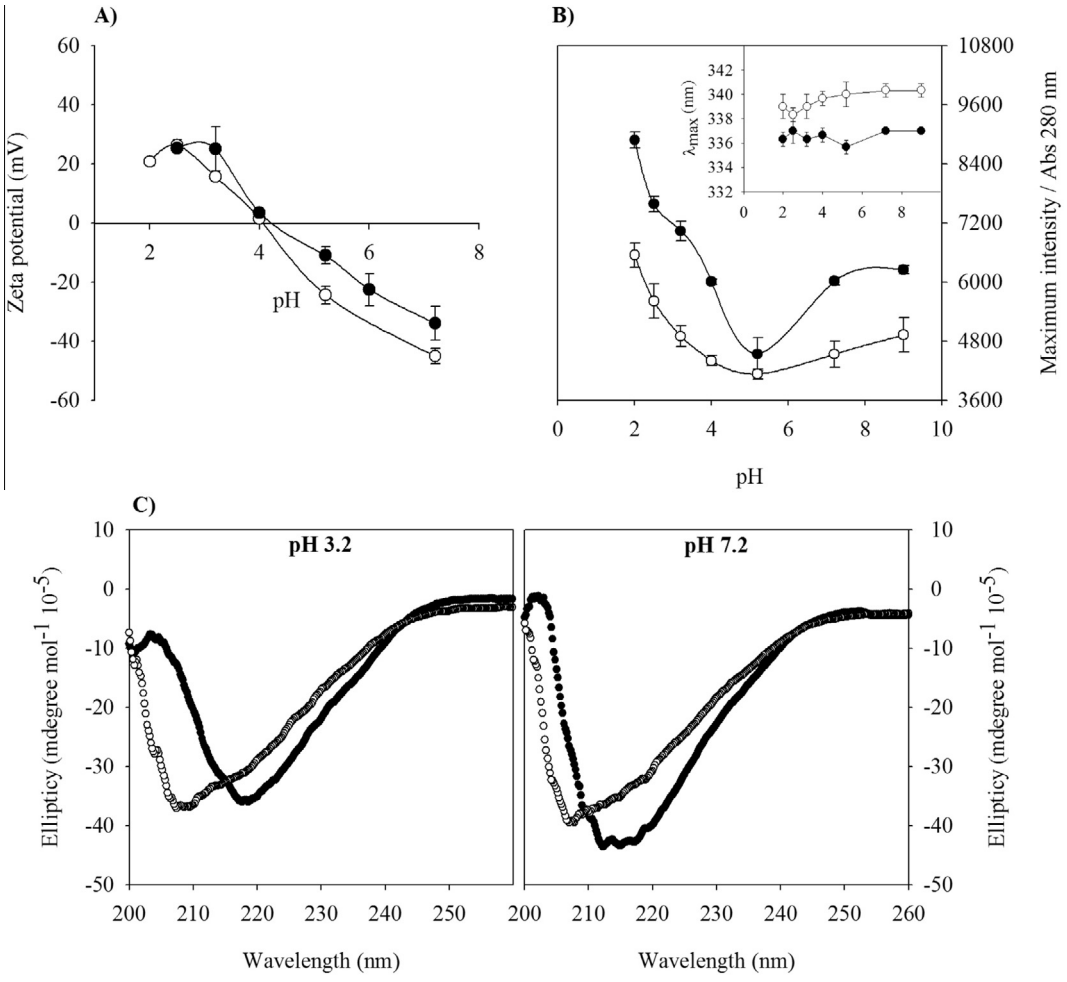

Fig. 2. A) Variation of the zeta potential of p-βLg (●) and r-βLg (○) as a function of the pH. Error bars represent the standard deviation. B) Effect of pH on the maximum fluorescence intensity of p-βLg (●) and r-βLg (○). Excitation wavelength of 280 nm. Error bars represent the standard deviation. Inset is λ_{max} observed for each protein. C) Circular dichroism spectra at pH 3.2 and 7.2 for p-βLg (●) and r-βLg (○). Conditions: 4 mM sodium phosphate buffer, temperature 25 °C, 0.5 mg/mL protein concentration.

3.2.2. Intrinsic fluorescence

In an attempt to have more information about the conformational state of r-βLg protein, both the intrinsic Trp fluorescence emission intensity (I_E) and the λ_{max} were monitored as a function of pH.

Fig. 2B shows sharp I_E changes with the pH for p-βLg, I_E values increased below and above pH 5 (closed to the bibliographic pI). The higher I_E at the extreme pH values (9 and 2), compared to the I_E around the isoelectric point, may reflect the difference between the fluorescence yields of monomers and dimers, as has been reported (Renard, Lefebvre, Griffin, & Griffin, 1998). β-Lg exists as a homodimer at neutral pH, but it dissociates into monomers at acidic pH (<3) and as pH approaches 9. According to Renard et al. (1998), the fluorescence increase above pH 7.0 may reflect, in addition to the dissociation effect, the formation of a β-Lg structure described by Tanford, Bunville, and Nozaki (1959), which is characterized by the ionization of a carboxylic acid group and the exposure of Tyr and Trp residues.

The intrinsic fluorescence has been extensively used to study and characterize the conformational changes of proteins. β-Lg contains two Trp (Trp^{19} and Trp^{61}) and four Tyr (Tyr^{20}, Tyr^{42}, Tyr^{99} and Tyr^{102}) residues per monomer. When excited at a wavelength of 295 nm, only Trp produces a fluorescent emission, while Trp and Tyr residues both emit when excited at 280 nm. Trp^{19} is located in a non-polar environment and contributes to nearly 80% of the total native protein fluorescence. Trp^{61} is relatively exposed to the solvent but only contributes to 20% to the total Trp fluorescence as it is almost quenched by its proximity to the Cys^{66}-Cys^{160} disulphide bond and the guanidine group of Arg^{124}, which can quench its emission, and/or to the self-quenching of Trp^{61} in the other monomer of the β-Lg dimeric form (Stanciuc, Aprodu, Râpeanu, & Bahrim, 2012). The residues Tyr^{42} and Tyr^{102} are buried, while Tyr^{20} and Tyr^{99} are exposed (Brownlow et al., 1997). Exposure of the buried Trp and Tyr residues leads to a shift towards longer emission wavelengths (red shift).

Although almost identical I_E was observed for r-βLg and p-βLg near the pI (pH 5.2), changes on I_E were much smoother throughout the entire pH interval for r-βLg. This might indicate that the conformation of the monomers of these proteins, predominant forms at the extreme pH values, might not be fully equivalent as was also seen in the analysis of their λ_{max} (inset in Fig. 2B).

p-βLg did not undergo significant conformational changes affecting the Trp and Tyr residues, as the λ_{max} value, 336 nm at pH 2.0, was not modified by pH (inset in Fig. 2B). This λ_{max} value corresponds to the emission of a tryptophan residue in a non-polar environment (Sharma & Kalonia, 2003). On the contrary, λ_{max} was higher for r-βLg (339 nm) indicating a more solvent exposed location of the buried Trp and Tyr residues. In addition, from pH 2.0 onwards the λ_{max} for r-βLg experienced red shifts over a wavelength span of 1.33 nm, suggesting that some structural changes were still taking place as pH increased.

3.2.3. CD spectra

The CD spectra were registered at pH 3.2 and 7.2 for both β-Lg proteins (Fig. 2C). At pH 7.2 p-βLg exhibited a wide minimum in its CD spectrum around 218 nm, which is characteristic of β-rich proteins (Brownlow et al., 1997), being the relative amounts of α-helix and β-sheet of 19 and 39%, respectively. At the secondary structure level, β-Lg has been shown to contain high levels of β-sheet and a small proportion of α-helix (Sawyer & Kontopidis, 2000). At the acidic pH a shift to 220 nm (Fig. 2C) was observed in agreement with an increase of the alpha-helix content. At both pH values, r-βLg showed a minimum at 208 nm, which is an evidence of a lost of alpha-helix content.

Proteins converting to the molten globule state usually show little change in the secondary structure and, hence conserve native-like far-UV spectra. However, β-Lg is an exception and shows a strong change in the secondary structure upon conversion to the molten globule state (Aouzelleg, 2014).

From our results it cannot be clearly stated that the r-βLg protein is in the molten globule state. Other authors have observed the formation of this particular state after heat treatments above 65 °C and at neutral pH (Carrotta, Bauer, Waninge, & Rischel, 2001; Croguennec et al., 2004; Qi et al., 1997) or at 80 °C and pH 2.59 (Tavel, Moreau, Bouhallab, Li-Chan, & Guichard, 2010), which are far from those existing during the hydrolysis of the milk whey substrate with cardosins.

However, our results at both molecular and structural levels clearly indicate that this technological process induced conformational changes yielding an intermediate β-Lg structure that is distinct from the native and fully unfolded structures. Different authors have demonstrated that during the protein folding/unfolding a multiplicity of intermediate stages (non-native states) can be obtained (Croguennec et al., 2004; Delahaije et al., 2016).

3.3. Gelation

3.3.1. Water holding capacity

The ability of gels from p-βLg (7.5%) and r-βLg (5–10%) to retain water was checked at two centrifugal forces as described in Otte et al. (2000). Results strongly depended on the gel and pH considered (Fig. 3A).

The WHC of p-βLg and r-βLg was compared at the intermediate protein concentration of 7.5% (v/w) and at three pH values. At pH 3.2 and 7.2, far from the isoelectric point of β-Lg (5.2), the percentage of water retained was higher than 70% for either gel, regardless the centrifugal force applied. Nonetheless, the WHC was lower for gels prepared with the pure protein sample. The difference was more noticeable at the lower centrifugal force; at pH 3.2 the WHC of r-βLg protein (91.9 ± 0.6%) was larger than of p-βLg (81.4 ± 1.8%). These results are also related to the gel characteristics described in the next sections (rheological data).

However, at the pI, centrifugation caused an important compression of p-βLg gel. Thus, the WHC was 42.8 ± 8.9% after centrifugation at low centrifugal force, and after subsequent centrifugation decreased to 18.1 ± 3.6% (Fig. 3A). The r-βLg gels underwent lower water losses but, as happened with p-βLg gels, the WHC was higher at low (74.8 ± 1.1%) than at high centrifugal forces (38.6 ± 5.1%).

The effect of protein concentration (10, 7.5 and 5%) was further analysed for r-βLg. At 7.5% and 5%, essentially the same trend on WHC was observed, no matter the pH studied, although differences were more pronounced at the pI -pH 5.2-. At this pH, a strong decrease on WHC was observed after the low (64.7 ± 1.1%) and the high centrifugal force (15.3 ± 1.5%) applied. At the highest protein concentration (10%), the most noticeable feature was the lower WHC (50%) at pH 7.2 after applying the higher centrifugal force.

These data agree with those reported by Otte et al. (2000), who found that water is largely expelled from gels upon centrifugation and the coarser the gel structure (as at the pI) the more water is expelled. Water retained in a protein matrix includes bound water, capillary water and water physically entrapped in the gel network (Damodaran, 1997). Close to the pI of β-Lg the electrostatic intermolecular repulsion is low, which leads to the formation of a turbid and less elastic gels, composed of much coarser particulate aggregates (can be as large as micrometres) from which water is easily lost even under low compression forces (Langton & Hermansson, 1992).

In all cases studied, protein retained in the gel network after centrifugation at any of the two forces was higher than 67% (see Fig. 3A). For r-βLg gels, protein loss followed the same trend to that

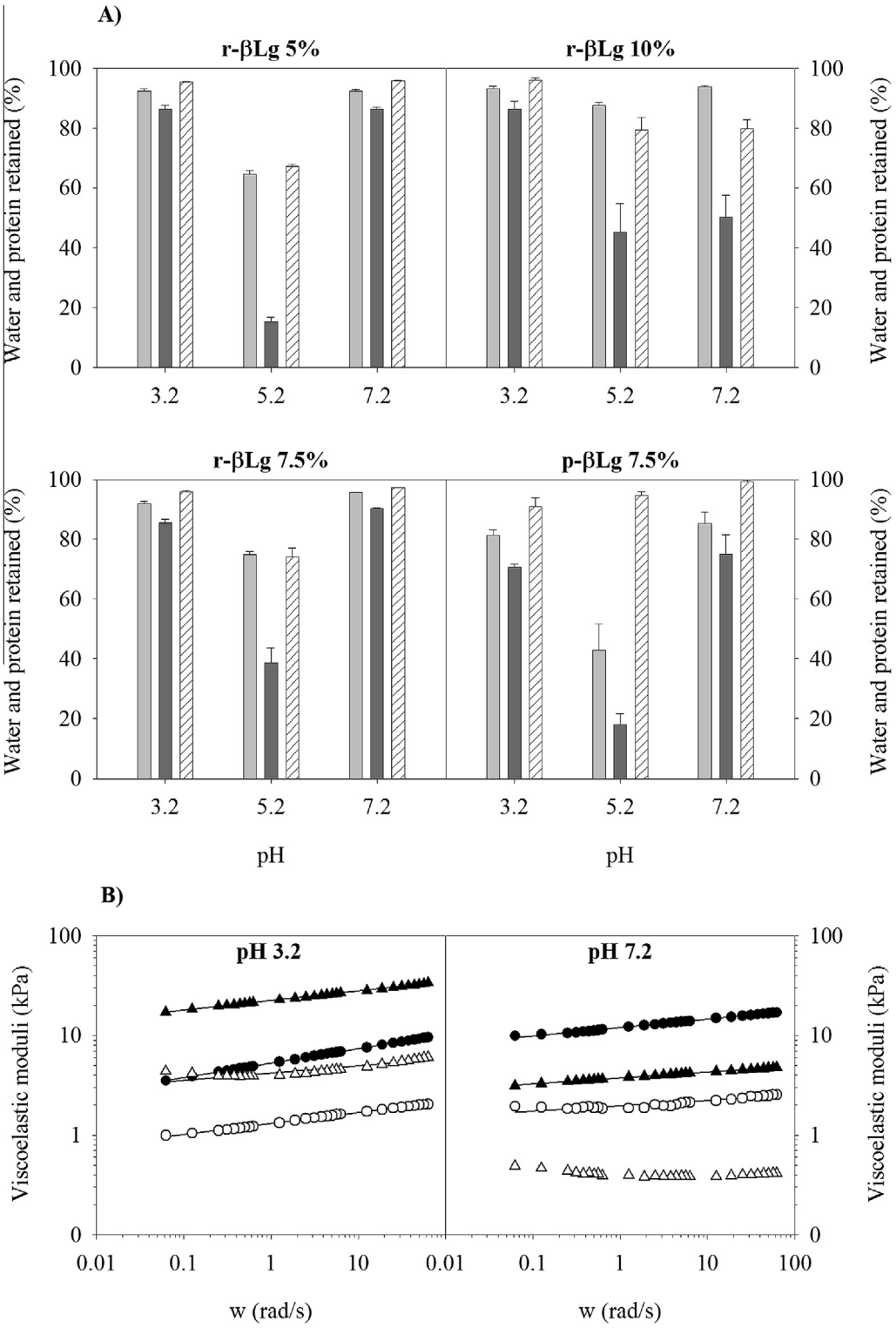

Fig. 3. A) Effect of pH on the protein retained and the water holding capacity of 5–10% (w/v) r-βLg and 7.5% (w/v) p-βLg gels. Water retained after centrifugation at 590 × g (light grey). Water (dark grey) and protein (line-filled) retained after centrifugation at 12,000×g. Error bars represent the standard deviation. *B*) Effect of pH on the mechanical spectra recorded at 25 °C for β-Lg gels at 7.5% (w/v). Symbols: experimental values of *G′* for p-βLg (▲) and r-βLg (●) and *G″* for p-βLg (△) and r-βLg (○). Lines: power law fit.

obtained before with water, i.e. more protein retained at high WHC.

3.3.2. Effect of pH on the LVE parameters of β-Lg gels

At acidic pH, p-βLg gels exhibited significantly higher σ_{max} and lower γ_{max} than r-βLg gels (7.5% protein, Table 1) indicating a greater structural stability of the protein network (high σ_{max}), corresponding to a progressive development of a physical structure being able to form a strongly cross-linked system (low γ_{max}) (Lapasin & Pricl, 1999). It is assumed that at pH∼3, β-Lg contains a subdomain with a highly ordered resistant β-sheet core in a fine-stranded network with shorter and stiffer linear strands

Table 1
Comparative values of the characteristic parameters of the LVE range for p-βLg and r-βLg gels at 7.5% (w/v) and pH 3.2 and 7.2. Effect of r-βLg concentration on the LVE parameters of gels at pH 3.2 or 7.2. Measures were made at 25 °C and 1 Hz frequency.

pH	Sample	% Protein	σ_{max} (kPa)	γ_{max} (%)	G^* (kPa)	tanδ
3.2	p-βLg	7.5	0.359 ± 0.036^{aA}	1.45 ± 0.25^{aA}	25.4 ± 4.8^{aA}	0.176 ± 0.004^{aA}
	r-βLg	5	0.0028 ± 0.0003^{1}	11.75 ± 0.56^{1}	0.026 ± 0.001^{1}	0.213 ± 0.015^{1}
		7.5	0.200 ± 0.020^{cB2}	4.0 ± 0.96^{cB2}	5.79 ± 1.1^{cB2}	0.230 ± 0.004^{cB1}
		10	0.913 ± 0.091^{3}	4.39 ± 0.75^{2}	21.0 ± 3.7^{3}	0.260 ± 0.005^{2}
7.2	p-βLg	7.5	0.401 ± 0.040^{aC}	10.40 ± 0.45^{bC}	3.90 ± 0.17^{bC}	0.100 ± 0.004^{bC}
	r-βLg	5	0.00105 ± 0.0001^{1}	5.51 ± 0.90^{1}	0.021 ± 0.003^{1}	0.281 ± 0.039^{1}
		7.5	0.200 ± 0.020^{cD2}	1.72 ± 0.64^{dD2}	12.4 ± 4.1^{dD2}	0.156 ± 0.016^{dD2}
		10	0.674 ± 0.067^{3}	2.6 ± 2.4^{12}	22 ± 19^{2}	0.163 ± 0.028^{2}

Values are given as mean ± expanded uncertainty limit (EUL).
[a-d]Different small letters in the same column indicate significant differences ($p < 0.05$) among diverse pH for each kind of β-Lg gel at 7.5% protein concentration.
[A-D]Different capital letters in the same column indicate significant differences ($p < 0.05$) between gels at fixed pH.
[1–3]Different numbers in the same column indicate significant differences ($p < 0.05$) in r-βLg gels with the protein concentration.

(Langton & Hermansson, 1992), which could explain the low conformational flexibility (lowest γ_{max}) and large stiffness (high G^*) in p-βLg gels (Renkema, 2004). These results are consistent with lower WHC in p-βLg (Fig. 3A) because acidic conditions induce brittle (low γ_{max}) and stiffer strands reducing the ability to retain bound water in the protein matrix. However, the r-βLg network was less rigid with greater conformational flexibility (higher γ_{max}) (Table 1). In addition, r-βLg samples with more exposed hydrophobic groups could form a less packed superstructure reducing the network stability (low σ_{max}) and the elasticity degree (high tanδ), resulting in a more open and deformable (high γ_{max}) network with, as previously analysed, greater WHC (Fig. 3A).

At neutral pH, p-βLg was a transparent fine-stranded gel, with a more flexible and curled strands in the network (Langton & Hermansson, 1992). These characteristics were evidenced in the significantly higher γ_{max} and lower G^* values comparing with those in r-βLg gel (Table 1). Heating at pH 7.2 induced the formation of p-βLg aggregates stabilised by intermolecular S–S bonds due to SH/S-S interchange reactions. These aggregates were detected by size exclusion chromatography and also in the electrophoresis patterns of heated p-βLg solutions at pH 7.2 (Estévez et al., 2016). These covalent bonds make a more stable structure whose cross-links have greater energy content (Walstra, 2003) as evidenced in the lower tanδ values (Table 1).

However, the r-βLg gel had a stiffer (high G^*) and some brittle (low γ_{max}) network, and less solid-like character (high tanδ). So, it is possible that the r-βLg conformation, with greater exposed hydrophobic groups and lower thermal stability (Estévez et al., 2016) favours the initial formation of intermolecular aggregates that could induce a disordered protein aggregation during gelling (Mounsey & O'Kennedy, 2007). As a result, rigid fibrils with straighter strands could be formed, increasing the gel strength and reducing the conformational flexibility and the solid-like character (Renkema, 2004).

At isoelectric pH (5.2) a coarse network was obtained in p-βLg gel, whose viscoelastic moduli and stress amplitude were much larger than those of r-βLg gel (data not shown). These rheological data were in agreement with less WHC in p-βLg *vs* r-βLg gel (Fig. 3A). As both β-Lg gels showed poor rheological properties at pH 5.2 that do not fit the function of interest of WHC and encapsulation for bioactive peptides and taking into account the possible functional application, especially for r-βLg as matrix for the encapsulation of bioactive peptides, these gels were discarded for subsequent analyses.

Thereafter, considering the industrial interest of the r-βLg protein, the rheological study was extended to analyse the effect of the protein concentration (5, 7.5 and 10%) on the viscoelastic parameters of the LVE.

At acidic pH, σ_{max} regularly increased from 5 to 10%, however γ_{max} decreased between 5 and 7.5% remaining practically constant between 7.5 and 10% (Table 1). Moreover G^* and tanδ increased to greater concentration (10%), indicating a greater cross-linking density (higher G^*) (Sperling, 2001; Walstra, 2003) with lower energy stability in the intermolecular interactions (Walstra, 2003). Thus, the r-βL sample at pH 3.2 and 10% protein concentration had the highest gel strength in a less solid-like system.

At pH 7.2, when the r-βLg concentration increased from 5 to 7.5% (results obtained for gels at 10% should be taken with caution due the high error), σ_{max} also increased and simultaneously γ_{max} decreased, which naturally corresponds to a growth of a reticular system with greater network density (Sperling, 2001). At higher protein concentrations, strand connectivity increased, thus, G^* augmented, but tanδ decreased (Table 1), which is the principal difference with the corresponding r-βLg gels made at pH 3.2. This different trend of tanδ could be explained because at neutral pH r-βLg gel was structured by intermolecular cross-links with covalent (S–S) bonds, enhancing the energy stability and the reticular order (low tanδ) in the network (Shimada & Cheftel, 1989).

3.3.3. Effect of pH on the mechanical spectra parameters of β-Lg gels

The experimental frequency dependence of elastic (G') and viscous (G'') moduli gives the mechanical spectra of gels (Mezger, 2006). G' and G'' were fitted to angular frequency (ω), as power law functions of Eqs. (1) and (2).

$$G' = G'_0 \cdot \omega^{n'} \quad (1)$$

$$G'' = G''_0 \cdot \omega^{n''} \quad (2)$$

where G'_0 and G''_0 are the stored and dissipated energy respectively, at $\omega = 1$ rad/s. In addition, n' and n'' quantify the frequency-dependence of both viscoelastic parameters at short time scale (Steffe, 1996).

In general, p-βLg and r-βLg gels showed high elastic character, being G' significantly higher than G'' exhibiting both G' and G'' small frequency dependence in all samples (Fig. 3B).

At pH 3.2, p-βLg gels exhibited remarkably higher viscoelastic parameters G'_0 and G''_0, and significantly lower frequency dependence than in r-βLg gels (Table 2). So, at acidic pH the main polypeptide chains may form "amyloid fibrils", which have a core structure composed by β-sheets (strongly stabilised by hydrogen bonding) producing higher rigidity (Dobson, 2003) as be noted in the greater stiffness in p-βLg gels (Table 2). The lower values of both n' and n'' exponents in p-βLg gels indicate higher temporal stability of the protein network (Steffe, 1996).

Conversely, at pH 7.2 G'_0 and G''_0 in r-βLg gels were higher than in p-βLg. Specifically, the relationship G''_0/G'_0 (0.166 ± 0.004) for r-βLg was also higher than that for p-βLg (0.108 ± 0.003), indicating that the gel-network in r-βLg gels was less solid-like (more disordered) with greater dissipative capacity. Moreover, n' exponent was

Table 2
Power law parameters from Eq. (1) and (2) for p-βLg gels at 7.5% (w/v) and pH 3.2 or 7.2 and r-βLg gels at 5, 7.5 or 10% (w/v) and pH 3.2 or 7.2. The measurements were performed at 25 °C.

pH	Sample	% Protein	G'_0(kPa)	n'	r^2	G''_0(kPa)	n''	r^2
3.2	p-βLg	7.5	22.32 ± 0.01	0.096 ± 0.001	0.999	4.20 ± 0.03	0.066 ± 0.007	0.790
	r-βLg	5	0.0228 ± 0.0001	0.143 ± 0.006	0.963	0.0045 ± 0.0002	0.081 ± 0.006	0.889
		7.5	5.243 ± 0.001	0.145 ± 0.0003	0.999	1.301 ± 0.001	0.110 ± 0.001	0.997
		10	16.43 ± 0.01	0.1448 ± 0.0005	0.999	4.15 ± 0.01	0.122 ± 0.001	0.996
7.2	p-βLg	7.5	3.72 ± 0.002	0.059 ± 0.001	0.998	nf	nf	–
	r-βLg	5	0.0201 ± 0.0011	0.087 ± 0.009	0.842	nf	nf	–
		7.5	11.840 ± 0.004	0.0872 ± 0.0003	0.999	1.97 ± 0.01	0.048 ± 0.004	0.818
		10	20.92 ± 0.01	0.087 ± 0.001	0.999	3.49 ± 0.01	0.051 ± 0.004	0.852

Values are given as mean ± standard deviation of fitted parameters.
nf: no fit.

something greater in the r-βLg gel than in p-βLg (Table 2). These data indicate that at neutral pH the gel network of r-βLg gel was more irregular, stiffer and less time-stable than in p-βLg. This result could be related with the fact that r-βLg was initially more reactive, exhibiting some protein aggregates that could induce denser strands, resulting in a less cohesive network.

Analysing the effect of protein concentration on the mechanical-spectra parameters of r-βLg, at both pH values (3.2 and 7.2), G'_0 and G''_0 increased when the protein concentration augmented from 5 to 10% (Table 2). This is a natural consequence of the increase in the total cross-links per unit volume to form a denser gel network at 10% r-βLg concentration.

3.3.4. Microstructure

More detailed information about the structures of r-βLg and p-βLg gels at 7.5% protein concentration was obtained from SEM micrographs of the gels. Fig. 4A and B show that the gel structures obtained with p-βLg at pH 3.2 and 7.2 resulted in regular, dense and compact networks, where individual strands and pores between the clusters of protein were hard to distinguish at low (Fig. 4) or high magnification (100,000×; data not shown). The more ordered-compact and fine-stranded protein matrix observed for p-βLg is typical of fine-stranded β-Lg gel structures (Langton & Hermansson, 1992).

However, important differences between r-βLg and p-βLg gel microstructures were observed at the pH values assayed. At pH 3.2 the r-βLg gel structure was composed of a more open and less homogenous network with locally denser clusters and larger pores showing a less packed network (Fig. 4C) compared to the p-βLg gel (Fig. 4A). So, the r-βLg microstructure reflects its lower gel stiffness (low G^*), greater tanδ and time frequency-dependence of viscoelastic moduli (higher n' and n'').

At pH 7.2, the p-βLg gel structure was composed of a regular, continuous and cohesive network (Fig. 4B) compared to the r-βLg gel (Fig. 4D), which is consistent with the higher bound flexibility (high γ_{max}), more temporal (low n') and energy stability (low tanδ).

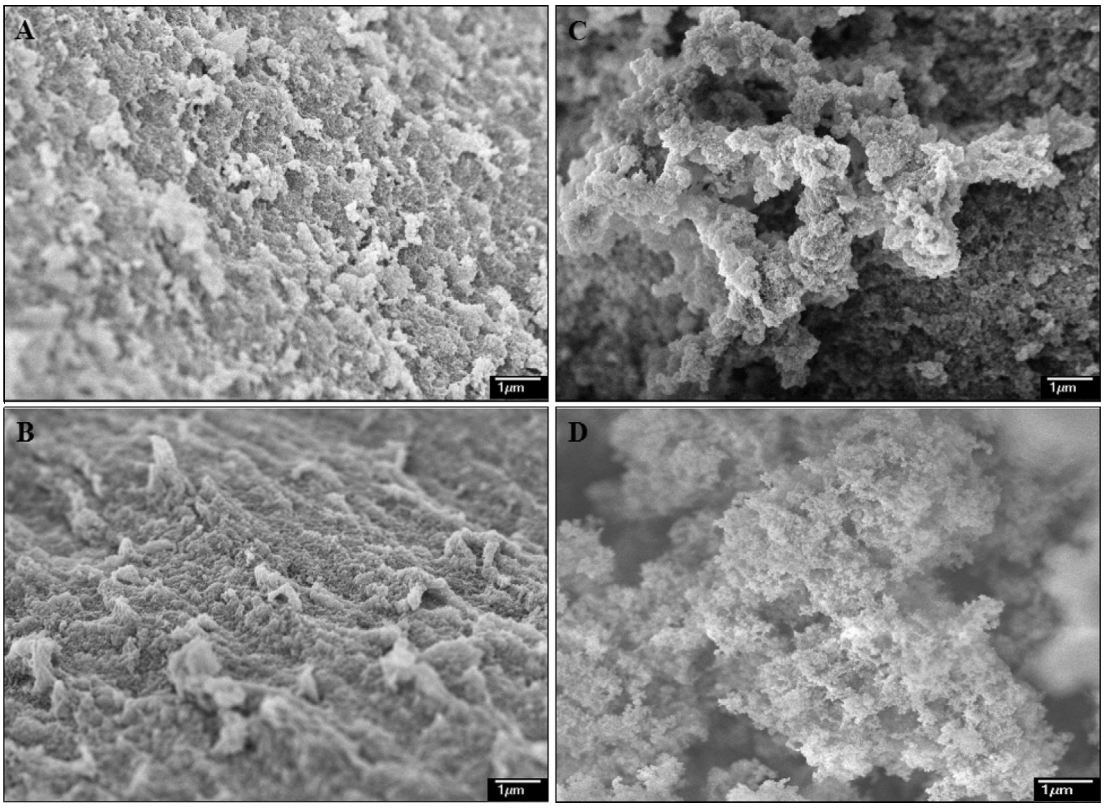

Fig. 4. SEM images of β-Lg gels at 7.5% (w/v). p-βLg at pH 3.2 (A) and (B) 7.2 and r-βLg at pH 3.2 (C) and (D) 7.2.

Some differences in r-βLg gels microstructures were found between pH 3.2 and 7.2. In gels at pH 7.2 (Fig. 4D), the particles formed defined strings of beads that in some cases were more like threads with filament-like appearance. At pH 3.2 (Fig. 4C) the r-βLg gels also exhibited linear strands of particles, but they were grouped in larger clusters and the microstructure was more discontinuous than at neutral pH. This structural discontinuity is consistent with the greater viscous component and frequency dependence of viscoelastic moduli n' and n'' (Table 2).

4. Conclusions

The results indicate that the β-Lg protein remaining from the whey hydrolysis with cardosins had the same pI as the p-βLg used as control, and both proteins underwent the characteristic monomer–dimer transition as observed studying the effect of pH on tryptophan fluorescence spectra. Nonetheless, differences in their tertiary structure were pictured from the analysis of zeta potential and intrinsic fluorescence spectra recorded. The differences in the 3D-structure were confirmed by the circular dichroism analysis, which indicated that the proteolysis conditions likely led to an intermediate β-Lg conformation that is between the totally denatured and the native protein.

At neutral pH, the p-βLg gel had a fine-stranded network with more flexible strands producing a more homogeneous, stable and cohesive gel network compared to r-βLg gel. Under acidic conditions (pH = 3.2), r-βLg gel exhibited less stiffness and more conformational flexibility, associated with a greater ability to retain water than p-βLg gel, rheological characteristics that may be useful in the food manufacture of gel-like products. Further studies are needed to determine whether this new r-βLg gel structure can be used as carrier to design an encapsulation system for bioactive peptides.

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References

Aouzelleg, A. (2014). High pressure control of protein structure and functionality. *Nutrition and Food Science, 44*(1), 41–46.

Babij, K., Bajzert, J., Dabrowska, A., Szoltysik, M., Zambrowicz, A., Lubec, G., et al. (2015). Hydrolysis with *Cucurbita ficifolia* serine protease reduces antigenic response to bovine whey protein concentrate and as-casein. *Amino Acids, 47*(11), 2335–2343.

Barros, R. M., & Malcata, F. X. (2006). Molecular characterization of peptides released from ß-lactoglobulin and α-lactalbumin via cardosins A and B. *Journal of Dairy Science, 89*(2), 483–494.

Brownlow, S., Morais Cabral, J. H., Cooper, R., Flower, D. R., Yewdall, S. J., Polikarpov, I., et al. (1997). Bovine ß-lactoglobulin at 1.8 Å resolution – still an enigmatic lipocalin. *Structure, 5*(4), 481–495.

Campo-Deaño, L., & Tovar, C. (2009). The effect of egg albumen on the viscoelasticity of crab sticks made from Alaska pollock and Pacific whiting surimi. *Food Hydrocolloids, 23*(7), 1641–1646.

Carrotta, R., Bauer, R., Waninge, R., & Rischel, C. (2001). Conformational characterization of oligomeric intermediates and aggregates in ß-lactoglobulin heat aggregation. *Protein Science, 10*(7), 1312–1318.

Chen, S. X., Swaisgood, H. E., & Foegeding, E. A. (1994). Gelation of ß-lactoglobulin treated with limited proteolysis by immobilized trypsin. *Journal of Agricultural and Food Chemistry, 42*(2), 234–239.

Croguennec, T., Mollé, D., Mehra, R., & Bouhallab, S. (2004). Spectroscopic characterization of heat-induced nonnative ß-lactoglobulin monomers. *Protein Science, 13*(5), 1340–1346.

Damodaran, S. (1997). Food proteins: An overview. In S. Damodaran & A. Paraf (Eds.), *Food proteins and their applications* (pp. 1–24). New York: Marcel Dekker.

Davis, J. P., Doucet, D., & Foegeding, E. A. (2005). Foaming and interfacial properties of hydrolyzed ß-lactoglobulin. *Journal of Colloid and Interface Science, 288*(2), 412–422.

Delahaije, R. J. B. M., Gruppen, H., Van Eijk-Van Boxtel, E. L., Cornacchia, L., & Wierenga, P. A. (2016). Controlling the ratio between native-like, non-native-like, and aggregated ß-lactoglobulin after heat treatment. *Journal of Agricultural and Food Chemistry, 64*(21), 4362–4370.

Dobson, C. M. (2003). Protein folding and misfolding. *Nature, 426*(6968), 884–890.

Doucet, D., Gauthier, S. F., & Foegeding, E. A. (2001). Rheological characterization of a gel formed during extensive enzymatic hydrolysis. *Journal of Food Science, 66*(5), 711–715.

Doucet, D., Otter, D. E., Gauthier, S. F., & Foegeding, E. A. (2003). Enzyme-induced gelation of extensively hydrolyzed whey proteins by alcalase: Peptide identification and determination of enzyme specificity. *Journal of Agricultural and Food Chemistry, 51*(21), 6300–6308.

Estévez, N., Fuciños, P., Bargiela, V., Pastrana, L., Tovar, C. A., & Rúa, M. L. (2016). Structural and thermo-rheological analysis of solutions and gels of a ß-lactoglobulin fraction isolated from bovine whey. *Food Chemistry, 198*, 45–53.

Langton, M., & Hermansson, A. (1992). Fine-stranded and particulate gels of β-lactoglobulin and whey protein at varying pH. *Food Hydrocolloids, 5*(6), 523–539.

Lapasin, R., & Pricl, S. (1999). *Rheology of industrial polysaccharides: Theory and applications*. Gaithersburg: Aspen Publishers.

Le Maux, S., Nongonierma, A. B., Barre, C., & Fitzgerald, R. J. (2016). Enzymatic generation of whey protein hydrolysates under pH-controlled and non pH-controlled conditions: Impact on physicochemical and bioactive properties. *Food Chemistry, 199*, 246–251.

Maclerzanka, A., Böttger, F., Lansonneur, L., Groizard, R., Jean, A.-S., Rigby, N. M., et al. (2012). The effect of gel structure on the kinetics of simulated gastrointestinal digestion of bovine ß-lactoglobulin. *Food Chemistry, 134*(4), 2156–2163.

Martinez-Alvarenga, M. S., Martinez-Rodriguez, E. Y., Garcia-Amezquita, L. E., Olivas, G. I., Zamudio-Flores, P. B., Acosta-Muniz, C. H., et al. (2014). Effect of maillard reaction conditions on the degree of glycation and functional properties of whey protein isolate – Maltodextrin conjugates. *Food Hydrocolloids, 38*, 110–118.

Mezger, T. G. (2006). *The rheology handbook: for users of rotational and oscillatory rheometers* (2nd ed.). Hannover, Germany: Vincentz Network.

Mounsey, J. S., & O'Kennedy, B. T. (2007). Conditions limiting the influence of thiol-disulphide interchange reactions on the heat-induced aggregation kinetics of ß-lactoglobulin. *International Dairy Journal, 17*(9), 1034–1042.

Nicorescu, I., Vial, C., Talansier, E., Lechevalier, V., Loisel, C., Della Valle, D., et al. (2011). Comparative effect of thermal treatment on the physicochemical properties of whey and egg white protein foams. *Food Hydrocolloids, 25*(4), 797–808.

Nieuwenhuizen, W. F., Dekker, H. L., Gröneveld, T., De Koster, C. G., & De Jong, G. A. H. (2004). Transglutaminase-mediated modification of glutamine and lysine residues in native bovine ß-lactoglobulin. *Biotechnology and Bioengineering, 85*(3), 248–258.

Otte, J., Lomholt, S. B., Ipsen, R., & Qvist, K. B. (2000). Effect of partial hydrolysis with an immobilized proteinase on thermal gelation properties of ß-lactoglobulin B. *Journal of Dairy Research, 67*(4), 597–608.

Pescuma, M., Hébert, E. M., Rabesona, H., Drouet, M., Choiset, Y., Haertlé, T., et al. (2011). Proteolytic action of *Lactobacillus delbrueckii* subsp. *bulgaricus* CRL 656 reduces antigenic response to bovine β-lactoglobulin. *Food Chemistry, 127*(2), 487–492.

Qi, X. L., Holt, C., Mcnulty, D., Clarke, D. T., Brownlow, S., & Jones, G. R. (1997). Effect of temperature on the secondary structure of ß-lactoglobulin at pH 6.7, as determined by CD and IR spectroscopy: A test of the molten globule hypothesis. *Biochemical Journal, 324*(1), 341–346.

Renard, D., Lefebvre, J., Griffin, M. C. A., & Griffin, W. G. (1998). Effects of pH and salt environment on the association of ß-lactoglobulin revealed by intrinsic fluorescence studies. *International Journal of Biological Macromolecules, 22*(1), 41–49.

Renkema, J. M. S. (2004). Relations between rheological properties and network structure of soy protein gels. *Food Hydrocolloids, 18*(1), 39–47.

Sawyer, L., & Kontopidis, G. (2000). The core lipocalin, bovine ß-lactoglobulin. *Biochimica Et Biophysica Acta – Protein Structure and Molecular Enzymology, 1482*(1–2), 136–148.

Severin, S., & Xia, W. S. (2006). Enzymatic hydrolysis of whey proteins by two different proteases and their effect on the functional properties of resulting protein hydrolysates. *Journal of Food Biochemistry, 30*(1), 77–97.

Sharma, V. K., & Kalonia, D. S. (2003). Steady-state tryptophan fluorescence spectroscopy study to probe tertiary structure of proteins in solid powders. *Journal of Pharmaceutical Sciences, 92*(4), 890–899.

Shimada, K., & Cheftel, J. C. (1989). Sulfhydryl group/disulfide bond interchange reactions during heat-induced gelation of whey protein isolate. *Journal of Agricultural and Food Chemistry, 37*(1), 161–168.

Sperling, L. H. (2001). *Introduction to physical polymer science* (3rd ed.). New York: Wiley-Interscience.

Stanciuc, N., Aprodu, I., Râpeanu, G., & Bahrim, G. (2012). Fluorescence spectroscopy and molecular modeling investigations on the thermally induced structural changes of bovine ß-lactoglobulin. *Innovative Food Science and Emerging Technologies, 15*, 50–56.

Steffe, J. F. (1996). *Rheological methods in food process engineering* (2nd ed.). East Lansing, USA: Freeman Press.

Tanford, C., Bunville, L. G., & Nozaki, Y. (1959). The reversible transformation of ß-lactoglobulin at pH 7.5. *Journal of the American Chemical Society, 81*(15), 4032–4036.

Tarhan, O., & Harsa, S. (2014). Nanotubular structures developed from whey-based α-lactalbumin fractions for food applications. *Biotechnology Progress, 30*(6), 1301–1310.

Tavel, L., Moreau, C., Bouhallab, S., Li-Chan, E. C. Y., & Guichard, E. (2010). Interactions between aroma compounds and β-lactoglobulin in the heat-induced molten globule state. *Food Chemistry, 119*(4), 1550–1556.

Walstra, P. (2003). *Physical chemistry of foods*. New York: Marcel Dekker Inc.