

Whey protein interactions in acidic cold-set gels at different pH values

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Abstract – Cold-set whey protein isolate gels (7% w/w) were produced by the addition of different amounts of glucono- δ -lactone to thermally denatured protein solutions. After 48 h of incubation at 10 °C, different final pH values were obtained (5.2 to 3.9). The gels were analysed by uniaxial compression measurements, water-holding capacity and protein solubility. The water-holding capacity of the gels decreased at pH values near the pI, this being associated with the lower protein solubility at this pH value. Protein solubility in the presence or absence of denaturing and reducing agents indicated that electrostatic interactions were responsible for the maintenance of the acidified gel structure at pH values from 5.2 to 4.6, but at pH 4.2, more hydrophobic interactions were present in relation to other final gel pHs, in spite of β -lactoglobulin (β -Lg) and BSA being far from their pI values. Complementary PAGE assays showed that disulphide bonds were associated with internal stabilisation of the protein aggregates formed during heat treatment. The mechanical properties of the gels were influenced by the final system pH value, showing that the strongest network was observed at pH 5.2. The results allowed the conclusion that although the interactions amongst the aggregates in the network of cold-set whey protein gels were essentially the same at the pH values evaluated, β -Lg played an important role in gel stabilisation due to its high concentration in WPI, strengthening the structure at its pI and, at the same time, providing good water-retention capacity.

whey proteins / glucono-delta-lactone / cold gelation / protein interactions

摘要 – 酸冷凝固性乳清蛋白凝胶中蛋白质间的相互作用。在热变性的乳清分离蛋白溶液加入不同量的葡萄糖酸- δ -内酯后制成冷凝固性的乳清分离蛋白凝胶(7%, w/w)。在10 °C下保温48 h后,可以获得不同pH(5.2–3.9)的凝胶。采用单轴压缩测定法分析了凝胶的力学性质、凝胶的持水能力和蛋白质的溶解性。在接近等电点时凝胶的持水能力随着pH的降低而下降,在等电点pH下蛋白质的溶解度较低。在变性剂和还原剂存在和不存在的条件下,根据蛋白质的溶解性,可以确定在pH 5.2~4.6范围内酸性凝胶结构的保持是由于静电相互作用的结果,然而在pH 4.2时,尽管其远离 β -乳球蛋白(β -Lg)和BSA的等电点,蛋白质分子之间疏水作用仍占主导作用。根据PAGE分析进一步证明了二硫键是热处理过程中形成蛋白质凝聚物内在稳定的根本原因。凝胶的力学性质受反应体系最终pH的影响。在pH 5.2时可以形成最强的凝胶网络。由此可以说明:虽然在本研究选定的pH下,冷凝固性乳清蛋白凝胶中蛋白质之间相互作用基本上是相同的,但是 β -Lg在凝胶的稳定性方面起着重要

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要作用,因为 β -Lg在乳清分离蛋白中浓度高,在等电点时可以加固凝胶结构,同时还可以赋予了凝胶较好的保水能力。

乳清蛋白 / 葡萄糖酸- δ -内酯 / 冷凝胶 / 蛋白质间的相互作用

Résumé – Interactions entre protéines de lactosérum dans des gels acides obtenus à froid à différents pH. Des gels d'isolats de protéines de lactosérum (7 % m/m) à froid ont été produits par ajout de différentes quantités de glucono- δ -lactone à des solutions de protéines dénaturées thermiquement. Après 48 h d'incubation à 10 °C, différentes valeurs finales de pH ont été obtenues (5,2 à 3,9). Les gels ont été analysés par mesures de compression uniaxiale, capacité de rétention d'eau et solubilité protéique. La capacité de rétention d'eau des gels diminuait aux valeurs de pH proches du pI, ce qui est associé à la solubilité protéique inférieure à cette valeur de pH. La solubilité protéique en présence ou en absence d'agents dénaturants ou réducteurs indiquait que les interactions électrostatiques étaient responsables du maintien de la structure du gel acidifié aux valeurs de pH de 5,2 à 4,6, mais à pH 4,2 les interactions hydrophobes étaient plus nombreuses par rapport aux autres pH finaux des gels, alors que la β -Lg et la BSA étaient loin de leur valeur de pI. Des essais complémentaires PAGE ont montré que les ponts disulfures étaient associés à la stabilisation interne des agrégats protéiques formés durant le traitement thermique. Les propriétés mécaniques des gels étaient influencées par la valeur de pH du système final, montrant que le réseau le plus ferme était observé à pH 5,2. Les résultats ont permis de conclure que, bien que les interactions entre les agrégats du réseau des gels de protéines de lactosérum obtenus à froid étaient essentiellement les mêmes aux valeurs de pH évaluées, la β -Lg jouait un rôle important dans la stabilisation du gel en raison de sa concentration élevée dans l'isolat de protéines de lactosérum, raffermissant sa structure à son pI et, en même temps, procurant une bonne capacité de rétention d'eau.

protéine de lactosérum / glucono- δ -lactone / gélification à froid / interaction protéique

1. INTRODUCTION

Whey proteins (WP) are widely used as an ingredient in the food industry because of their excellent functional and nutritional properties. They are globular proteins with molar mass ranging from 14 to 1000 kg·mol⁻¹ and are composed of 60% β -lactoglobulin (β -Lg), 22% α -lactoalbumin, (α -La), 5.5% bovine serum albumin (BSA) and 9% immunoglobulins (Ig). The iso-electric points of these proteins are 5.2 for β -Lg, from 4.2 to 4.5 for α -La, from 4.7 to 4.9 for BSA and between 5.5 and 6.8 for Ig [8, 26].

Heat-induced aggregation and gelation of whey proteins has been extensively studied and reported in the literature [21]. The combined control of thermal WP denaturation and solvent quality can lead to a gelling process called “cold gelation” that can be used in a variety of products, such as surimi, mayonnaise and gelatine-like desserts [8]. This method of gelation consists of two steps. First, a solution of native globular proteins is heated at neutral

pH (well above the iso-electric point), at low ionic strength and a protein concentration lower than the gelation concentration. In this first step, unfolding of the native proteins is followed by aggregation into disulphide cross-linked aggregates. Under the conditions described, the proteins have a net surface charge and repulsive forces will prevent random aggregation, resulting in the formation of soluble aggregates. After cooling to room temperature, a stable dispersion of aggregates is obtained, which does not gel for a period of hours, depending on the denaturation conditions [2, 19]. In the second step, a change in the quality of the solvent induces gelation. Turbid, particulate gels are formed after the addition of relatively large amounts of salt or after acidification in the direction of the iso-electric point. Usually, acid-induced cold-set gels are stronger than salt-induced cold-set gels, for the same protein concentration [19].

Among the functional properties of proteins, solubility is of primary importance due to its significant influence on the other

functional properties of proteins. High solubility provides good emulsions, foam, gelation and whipping properties. Since most conventional food products contain more than 50% water, good water-holding capacity is essential, because consumers tend to avoid products that show free water in the package [4]. Protein solubility is considered to be the result of surface-active properties such as protein-protein and protein-solvent interactions [10]. The balance of these interactions between adjacent polypeptide chains will also interfere in gel formation [8]. The solubility of whey protein gels in different buffer media has been used to understand interactions between whey protein molecules [25]. Solubility in different buffer media was also used in an attempt to associate solubility properties with the network molecular interactions, which could be responsible for the rheological and water-holding properties of the systems [22–25, 29]. The knowledge of system rheological or mechanical properties is of great importance, since texture attributes can be significantly correlated with these properties.

The objective of this study was to investigate the interactions between whey proteins under cold-set gelation conditions produced by heat treatment and by pH modifications in the direction of the WP pI. Acidification conditions were used in order to obtain gels with final pH values near the pI of the different fractions of WP, after 48 h of acidification at 10 °C. Interactions between the WP fractions in the gel network were studied by protein solubility experiments followed by polyacrylamide gel electrophoresis. The protein network structure formed was also evaluated by measurement of the water-holding capacity and uniaxial compression.

2. MATERIAL AND METHODS

2.1. Material and chemicals

The whey protein isolate (WPI), Lactoprodan, was kindly donated by Arla Foods

Ingredients (Viby J, Denmark). According to SDS-PAGE experiments [20], this WPI is comprised mainly of β -Lg (56%), α -La (31%) and BSA (7%), plus some other minor fractions and denatured protein, as determined by densitometry (see Sect. 3.1). The protein concentration in the powder was 92.4% and the lactose content 0.6%, as determined by the Kjeldahl procedure [3] and phenol sulphuric method [16], respectively. The total solids content was 94.3% (5.7% moisture, wet basis). The ion content of the WPI was determined by atomic absorption spectroscopy, showing the following composition (w/w %): 0.05 Ca^{2+} , 0.63 Na^+ and 0.65 K^+ . The ester GDL was purchased from Sigma Aldrich Corporation (St Louis, USA).

2.2. Gel preparation

Seven % (w/w) WPI solutions were prepared by dissolution of the powder in deionised distilled water, with magnetic stirring for 90 min at room temperature. During this period the pH was kept at 6.7. These solutions were then subjected to heat treatment at 80 °C for 30 min in a stainless steel jacketed vessel. WPI solutions (0.3 L) were poured into a 0.75-L internal volume vessel and the temperature kept constant at 80 °C by rapid water circulation in the inner part of the jacket, using a controlled-temperature water bath (QUIMIS, São Paulo, Brazil). During heat treatment the WPI solutions were subjected to gentle mechanical agitation. Temperature measurements made in preliminary experiments with WPI solutions showed values of around 80 °C within 5 min after pouring the solutions into the vessel. PAGE experiments [20] were performed on samples removed after this heating step to evaluate the extent of thermal WP denaturation.

After the heat treatment, the solutions were rapidly cooled to 10 °C in an ice bath.

Different concentrations of GDL powder were then added to the cooled solutions and gently stirred for 1 min. All the systems were then stored at 10 °C for 48 h and different final pH values were achieved.

Immediately after adding the GDL to the thermally denatured protein solutions, part of each system was gently poured into small plastic tubes with internal diameters of 20 mm and 25 mm in height, and sealed in order to avoid evaporation during storage. Just before each analysis, the gels were gently removed from the plastic tubes and subjected to uniaxial compression measurements. Some gels were also cut with a sharp blade and a small piece from the centre subjected to the WHC experiment, or blended with water or buffers in the protein solubility experiment. The GDL concentration used to prepare the systems depended on the protein concentration and final pH desired, which was expressed as the GDL/WPI (% w/w) ratio. WHC and protein solubility experiments were performed using GDL/WPI ratios of 0.084, 0.115, 0.135, 0.186, 0.220 and 0.350, which resulted in final pH values of 5.2, 4.9, 4.7, 4.6, 4.2 and 3.9 after 48 h of storage at 10 °C, respectively. Some conditions were chosen due to their closeness to the iso-electric point of the main whey proteins: 5.2 for β-Lg, and between 4.2 and 4.8 for α-La and BSA [26], but the pH value of 3.9 corresponded to a system condition of over-acidification. The uniaxial compression tests were performed using GDL/WPI ratios between 0.084 and 0.220. At pH 3.9, the mechanical properties were not determined because the gels were not self-supporting (were not able to maintain their form sufficiently for the compression experiments). In all the studies, the fall in pH caused by GDL hydrolysis was measured using a Sentron 2001 pH meter (Sentron Inc., Gig Harbor/Washington, USA) equipped with an electrode calibrated at the reaction temperature, over the pH range from 7.0 to 4.0.

2.3. Mechanical properties

Uniaxial compression experiments were carried out using a TA-XTIII Texture Analyser (Stable Microsystems Ltd., Godalming, UK) equipped with a lubricated acrylic cylindrical plate (40 mm diameter). The gels were compressed to 80% of their original height using a crosshead speed of 1 mm·s⁻¹. All measurements were done with five replications at 10 ± 1 °C. The force and height data were transformed into Hencky stress (σ_H)-Hencky strain (ε_H) curves [30]. The rupture properties (stress and strain) were obtained from the maximum point of the stress-strain curve, while Young's modulus was the slope of the initial linear region of this curve.

2.4. Water-holding capacity (WHC)

The WHC of the gels was determined by weighing the gel pieces before and immediately after centrifugation. The gels were wrapped in Whatman 1 filter papers (Maidstone, UK) and gently placed in centrifuge tubes. The tubes were centrifuged at 120×*g* for 5 min [23] at 10 °C using an Allegra 25 R BECKMAN centrifuge (Fullerton-USA). The WHC was expressed according to equation 1:

$$\text{WHC (\%)} = 100 * \left[\left(\frac{\text{water}_{\text{remaining}} (\text{g})}{\text{water}_{\text{gel}} (\text{g})} \right) \right] \quad (1)$$

where $\text{water}_{\text{gel}}$ and $\text{water}_{\text{remaining}}$ represent the amounts of water in the gel before and after centrifugation, respectively.

2.5. Determination of protein solubility

The protein solubility of the WPI gels was determined in deionised distilled water (DW) and in four different buffer systems:

a standard pH 8.0 buffer ($0.086\text{ mol}\cdot\text{L}^{-1}$ Tris, $0.09\text{ mol}\cdot\text{L}^{-1}$ glycine, $4\text{ mmol}\cdot\text{L}^{-1}$ Na_2EDTA) (B), and the same standard buffer containing 0.1% β -mercaptoethanol (BM), $6\text{ mol}\cdot\text{L}^{-1}$ urea (BU) or 0.1% β -mercaptoethanol plus $6\text{ mol}\cdot\text{L}^{-1}$ urea (BUM). During the dissolution experiments the water and buffers were kept at refrigerated temperatures, and after dissolution the supernatants obtained were immediately subjected to a refrigerated environment. After dissolution in water, no modifications in pH values of the systems of more than 0.2 pH units were observed. Gel dispersions were prepared by adjusting the protein concentration of the dispersions to 0.1% (w/w). The solutions were homogenised at room temperature using an Ultra-Turrax IKA model T18 basic (Staufen, Germany) at $10\,000$ rpm for 2 min. Homogenised solutions were then centrifuged at $20\,000\times g$ for 15 min in an Allegra 64 R Beckman centrifuge (Fullerton, USA) and the dissolved protein determined in the supernatant fraction. The protein concentration in the supernatant was determined immediately after centrifugation (less than 30 min) at 280 nm in a Beckman Du 70 Spectrophotometer (Fullerton, USA) using an apparent extinction coefficient ($E_{1\text{ cm}}^{0.1\%}$) of 9.1 . The extinction coefficient was obtained by measuring the absorbance at 280 nm of a 0.1% whey protein solution, the concentration of which was determined by the Kjeldhal method. These soluble fractions were also immediately subjected to PAGE analysis (less than 1 h after centrifugation) for the characterisation of whey protein fractions in the supernatant.

2.6. Polyacrylamide gel electrophoresis (PAGE)

The soluble fractions from the WP gels extracted in water and in Tris buffer (B or BM in the solubility experiments)

were analysed by polyacrylamide gel electrophoresis (PAGE) using a vertical slab Mini-Protean electrophoresis system (Bio-Rad Laboratories, Hercules, CA, USA). Native PAGE and sodium dodecyl sulphate SDS-PAGE (reducing and non-reducing conditions) analyses [20] were performed. The resolving and stacking gels contained 5% and 15% of acrylamide, respectively, giving an effective separation range between 12 and $200\text{ kg}\cdot\text{mol}^{-1}$ [11]. After protein quantification for each protein solubility extraction, $10\text{ }\mu\text{g}$ of protein (except when specified in the results and discussion section) were diluted in equal volumes of 3 different loading buffers with the same base of $0.05\text{ mol}\cdot\text{L}^{-1}$ Tris-HCl (pH 6.8), 10% glycerol and 0.1% bromophenol blue. This buffer was used for the Native PAGE runs (non-dissociating conditions). For the SDS-PAGE analyses, the samples were diluted in the same buffer, but with the addition of 2% SDS (dissociating non-reducing conditions) or with the addition of 2% SDS plus $0.1\text{ mol}\cdot\text{L}^{-1}$ β -mercaptoethanol (β -ME) (dissociating and reducing conditions). Under dissociating conditions, the proteins were completely dissociated by immersing in boiling water for 2 min. These samples were then loaded into polyacrylamide wells in the stacking gel. The gels were run at 200 V and then stained with Coomassie Brilliant Blue in methanol:acetic acid:water (45:10:45 v/v) and diffusion-destained by repeated washing in a methanol:acetic acid:water solution (10:5:85 v/v). A pre-stained InvitrogenTM Bench Marker protein leader (Bioagency International Corp-Jacksonville, USA) and pure fractions of β -lactoglobulin, α -lactalbumin, BSA and α , β , and κ -caseins (Sigma Aldrich Corporation, St Louis, USA) were used as markers.

Pictures of the gels were taken using an Eagle Eye video system (Stratagene, La Jolla, USA), which also allowed the densitometric analysis of the bands

using the software Stratagene Eagle Sight (Stratagene, La Jolla, USA). The band intensities were used to determine the protein composition of each WP fraction ($C_{WP_{sup}}$) in the water-supernatant of the water solubility experiments. The data were analysed in relation to the total initial protein content (Eq. (2)) or initial WP protein fraction content in the gel (Eq. (3)):

$$C_{WP_{sup}} (\%) = \frac{m_{WP}S}{m_{total} GP} * 100 \quad (2)$$

$$C_{WP_{sup}} (\%) = \frac{m_{WP}S}{m_{WP} gel} * 100 \quad (3)$$

where $m_{WP}S$ is the mass composition of each WP water-soluble fraction, $m_{total} GP$ is the initial total amount of protein and $m_{WP} gel$ is the initial WP protein fraction content in the gel. The precipitated fraction of WP (X_{WP}) was calculated by difference.

2.7. Protein electro-elution from the polyacrylamide gel

Protein aggregates observed in the Native PAGE of the water-soluble proteins were extracted from the polyacrylamide gels using a Bio-Rad Protein Electro-Eluter model 422 Module (Bio-Rad Laboratories, Hercules, CA, USA). The stained bands were separated and cut off from at least 8 Native PAGE gels and minced into small pieces. The gel slices were then loaded into an electro-eluter and the equipment filled with the running buffer. The running buffer used in the elution was the same running buffer used in the Native PAGE and consisted of $25 \text{ mmol} \cdot \text{L}^{-1}$ Tris plus $192 \text{ mmol} \cdot \text{L}^{-1}$ glycine diluted in deionised distilled water. Elution was done at a constant current of 10 mA for 6 h against membrane caps with a $3.5 \text{ kg} \cdot \text{mol}^{-1}$ cut-off. At the end of elution a volume of about 0.6 mL was collected from the membrane caps, and a second elution carried out for a further 6 h

using the same sample, under the same elution conditions. The eluted samples were then dialysed for 24 h against deionised distilled water with a $3.5 \text{ kg} \cdot \text{mol}^{-1}$ cut-off membrane. The water used for dialysis was changed twice with fresh distilled water. The sample was then collected and the volume reduced in an Eppendorf Vacufuge concentrator model 5301 (Westbury, USA) at room temperature. After concentration, the samples were analysed by polyacrylamide gel electrophoresis (PAGE) under reducing conditions, as described above.

2.8. Statistical analysis

Significant differences ($P < 0.05$) between the treatments were determined by the Tukey procedure. Statistical analyses were performed using the software Statistica 5.5 (Statsoft Inc., Tulsa, USA).

3. RESULTS AND DISCUSSION

Turbid gel formation was observed during acid gelation, with a gradual pH reduction. The formation of cold-set whey protein gels takes place at pH values above and around the $\beta\text{-Lg}$ pI [1] since the formation of disulphide bonds occurs predominantly under these pH conditions. However, in the present research we studied the gels produced at different pH values, especially at $\beta\text{-Lg}$ pI, because we were interested in evaluating the mechanical properties and consequently the molecular interactions of cold-set whey proteins formed at the pI of BSA and $\alpha\text{-La}$. Previous results indicated that at slower acidification rates, the protein gels were stronger [5, 9], which was attributed to molecular rearrangements after achieving the final pH values [9]. According to this study, the whey protein gels showed a great intensity of pH change up to 500 min (8 h) and then steadily decreased. Gels with a final pH of 5.2 were

achieved in 29 h whilst gels with a pH of 4.2 were obtained in about 17 h. Since the systems showed no changes in pH after 48 h of acidification, the water-holding capacity, protein solubility and mechanical properties of the gels were evaluated at this point.

3.1. Soluble whey protein thermal aggregates

The protein composition and formation of the soluble protein aggregates due to heat treatment of the initial whey protein solution were evaluated by polyacrylamide gel electrophoresis (Fig. 1). From the Native PAGE gels (Fig. 1A) the initial characteristics of the WPI and the effects of heat treatment on the whey protein were observed by comparing the unheated solutions (lane 4) with the heat-denatured ones (lane 5). The pure milk fractions were loaded in order to identify the main WP in the sample (lanes 1, 2 and 3). The initial whey protein solution (not heated) showed a smeared band of aggregates in the top stacking gel and accordingly, the densitometry measurements of such aggregates corresponded to almost 5% of the initial whey protein composition. Such aggregates showed a molar mass above $200 \text{ kg} \cdot \text{mol}^{-1}$, since they were unable to enter the 5% polyacrylamide stacking gel. The bands of the main whey protein fractions (β -Lg A and B, and α -La and BSA) can be visualised in the unheated solution, but were almost absent in the heated solution. However, an aggregate can be visualised at the top of the gel (disulphide-bonded material), corresponding to a high molar mass protein aggregate formed during heating. In the denatured WPI solution such aggregates corresponded to approximately 80% of the proteins loaded in the Native PAGE (Fig. 1, lane 5). Densitometry measurements also allowed the observation that almost 100%

of the BSA and α -La were associated with large aggregates formed during heating, but a fraction of the initial amount of β -Lg (corresponding to approximately 30%) remained in solution and was not linked to the aggregates. Such results suggest that the majority of the whey protein fractions was involved in thermal aggregate formation under these heating conditions. Similar results of whey protein denaturation were observed in Native PAGE for whey protein concentrate [15] and WPI solutions with different protein concentrations [31] under similar conditions of heating to those applied in the present study.

An increase in intensity of bands corresponding to β -Lg and α -La can be observed in the heated WP solutions under non-reducing conditions, as compared with Native PAGE (Figs. 1A and 1B, respectively). Such results indicate that the SDS disrupted the non-covalent interactions amongst the WP aggregates, probably hydrophobic interactions, but disulphide-bonded material still remained in the top stacking gel [15, 31]. Moreover, the heated solution (lane 4) showed bands of β -Lg and α -La monomers with lower intensities than those observed in the unheated solution (lane 3) and faint BSA bands. In the $176.5 \text{ kg} \cdot \text{mol}^{-1}$ region, a small and intense band can be observed in lane 3 and also in lane 4, but with weaker intensity. These bands could correspond to the Ig fraction [26], since these proteins have high molar mass and are extremely heat-labile in the presence of other WP, possibly leading to interactions with the β -Lg and BSA via disulphide bond formation [26]. Other smearable bands can be visualised in the gel region between 26 and $37.4 \text{ kg} \cdot \text{mol}^{-1}$, which could correspond to β -Lg dimers, as reported by some authors [14, 15, 18, 28].

In the SDS-PAGE under reducing conditions (Fig. 1C), the high molar mass aggregates showed weaker intensities at the top of the stacking gel as compared with

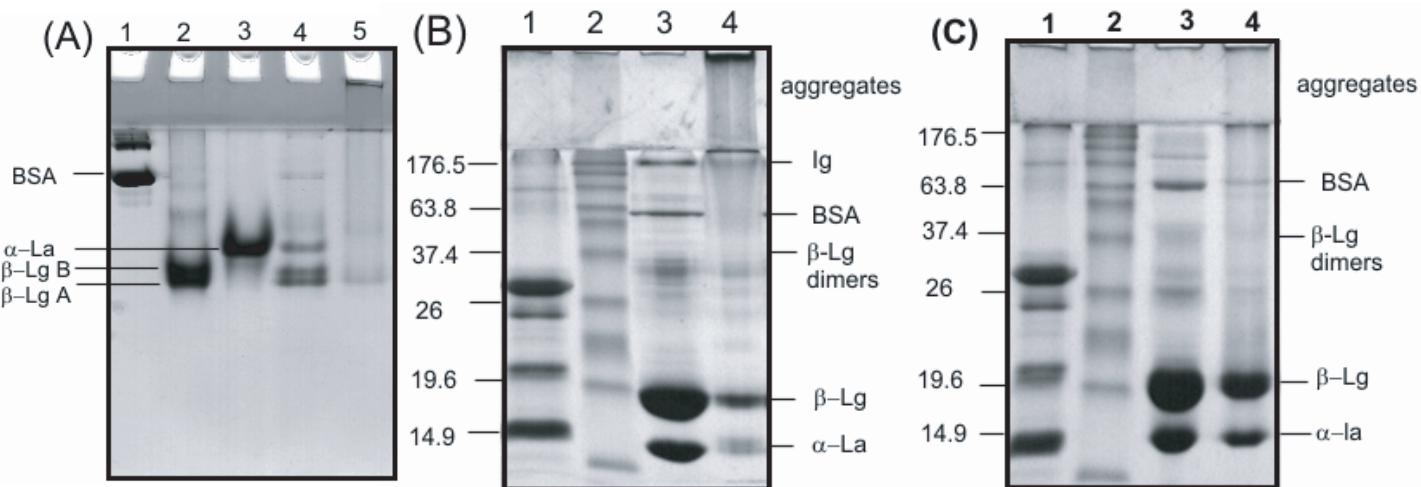


Figure 1. Heat-induced polymerisation of whey proteins: (A) Native PAGE, (B) SDS-PAGE under non-reducing conditions and (C) SDS-PAGE under reducing conditions. In (A) lanes 1, 2 and 3 are pure milk protein fractions. In (B) and (C) lane 1 = mixture of pure milk proteins, lane 2 = commercial molar mass marker. The unheated WP solution was loaded into lane 4 in (A) and into lane 3 in (B) and (C). The heat-denatured WP solution ($80^{\circ}\text{C}/30\text{ min}$) was loaded into lane 5 in (A) and into lane 4 in (B) and (C).

under non-reducing conditions (Fig. 1B). Consequently, the intensity of the WP bands (β -Lg, α -La and BSA) was greater under reducing conditions, showing similar patterns between unheated and heated solutions. These results indicate that intermolecular disulphide bonds between the WP fractions are also associated with WP aggregate stabilisation, despite the occurrence of non-covalent interactions (Fig. 1B).

Several models have been proposed in the literature to explain the possible mechanisms for the polymerisation of proteins during heating. In all these models the formation of aggregates are based on disulphide bonds between protein molecules during heating, with little or no emphasis on the importance of non-covalent bonding. However, these conclusions were based mainly on heating experiments with pure whey protein solutions, giving a clue of more complex mechanisms that may occur in protein isolate solutions, which are a mixture of whey proteins.

3.2. Protein solubility in water and in different buffer systems of cold-set gels

Soluble thermal aggregate solutions were acidified with different GDL concentrations in order to form gels. Figure 2 shows the results for the protein solubility of 6 different WP cold-set gels (pH values of 3.9, 4.2, 4.6, 4.7, 4.9 and 5.2) dispersed in water and in different pH 8.0 buffers. Comparing the values for protein solubility in DW one can observe that the WP gel with the highest protein solubility was obtained at pH 3.9 (far from the pI of the whey proteins). The lowest solubility values (5%) were obtained at pH values between 4.2 and 4.9, close to the iso-electric point of α -La and BSA, while solubility was slightly higher (9%) near the pI of β -Lg. It was observed that the WP gels were

more soluble in all the pH 8.0 buffers than in water (Fig. 2). The solubility values in the standard buffer (B) and in the same buffer with the addition of β -ME (BM) were between 75% and 90%, and the lowest solubility values (75–77%) were observed at pH 4.2. No significant differences in the solubility values of the gels dispersed in buffers B or BM for each gel final pH evaluated were observed. However, when gels were dispersed in buffers containing urea (BU and BUM), the protein solubility was nearly 100% for all the gels studied.

The patterns obtained for gel protein solubility in water (U-shaped curves with minimal values around the pI) can be attributed to the characteristics of the protein interactions occurring during gel formation. When the pH was lowered by GDL hydrolysis, the negative charge of the proteins was progressively neutralised, diminishing protein electrostatic repulsion and favouring protein-protein interaction. As a result, there was less water to interact with the protein molecules near the pI, favouring protein precipitation [32].

The higher solubility in pH 8.0 buffer (B) than in water (DW) suggests that the interactions amongst the acidified WP proteins were mainly electrostatic in nature for all the pH values studied [26]. Such behaviour could be explained by the fact that the solubility of the proteins increased at higher pH values, far from the pI values of the proteins (buffer pH 8.0). In such conditions the proteins show a predominance of negative charges, leading to an increase in electrostatic repulsion between protein molecules, causing protein dissolution and thereby increasing protein solubility [32].

No significant differences were found between the extractions made in (B) and (BM) buffers, despite the formation of S-S bonds between the WP during the heating step, as attested by the PAGE experiments (Sect. 3.1). This fact could be attributed to

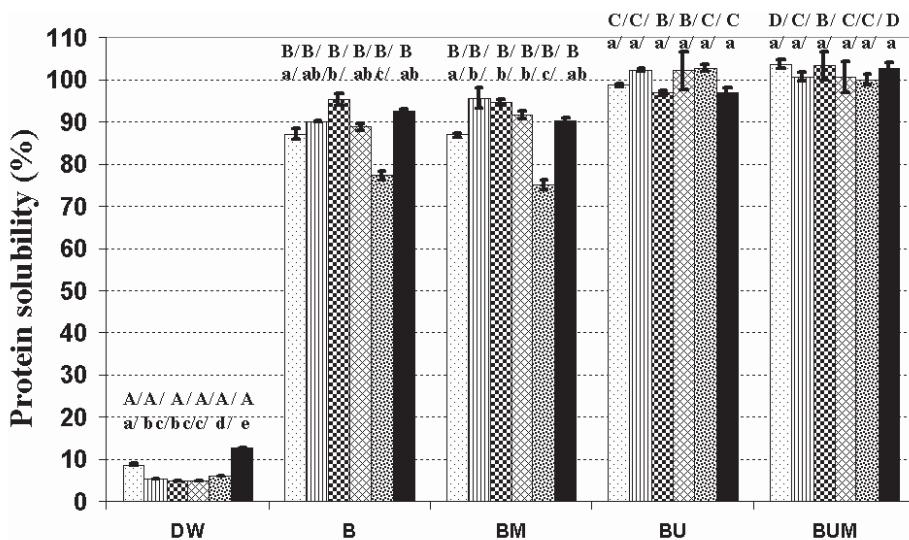


Figure 2. Solubility profile of 7% (w/w) WPI cold-set gels acidified with GDL in water (DW) and in different standard pH 8.0 Tris buffers (B) also containing β -mercaptoethanol (BM), urea (BU) and β -mercaptoethanol plus urea (BUM). System pH values after 48 h at 10 °C: □ 5.2, ▨ 4.9, ■ 4.7, ▨ 4.6, ▨ 4.2 and ■ 3.9. Different letters indicate significant differences ($P < 0.05$). Small letters: differences amongst the pH values in each buffer system. Capital letters: differences amongst the buffer systems at each pH.

the greater amount of electrostatic as compared with S-S bonds, making it difficult to identify and quantify these disulphide interactions.

The presence of urea (a denaturant agent) produced dissolution by disrupting non-covalent interactions such as hydrophobic and hydrogen bonding [27] among whey protein aggregates in the gel network. At pH 4.2 the differences in solubility between the buffers BM and BU or BUM was greater than observed at other pH values, suggesting that under these acidification conditions (final pH at the α -La pI) hydrophobic and hydrogen bonding were present in higher amounts in the aggregates forming the gel network, in spite of the β -Lg and BSA fractions being far from their isoelectric points at this pH.

Figure 3 shows the water-holding capacity properties of the systems studied.

The results show that cold-set WP gels produced at the lowest and highest GDL/WPI ratios (pH values of 5.2 and 3.9) retained more water after centrifugation than gels with intermediate pH values. Systems with a final pH value of 5.2 showed WHC of 74%, and with a final pH of 3.9 (over acidification conditions), the WHC was 84.80%. Gels with final pH values near the pI of BSA and α -La (4.9 to 4.2) exhibited WHC between 62 and 68%. WHC patterns were very similar to those observed for protein solubility in water (the same U-shaped curves with minimal values around the pI), suggesting that protein interactions around the pI (mainly electrostatic) also reduced water retention amongst protein segments in the gel.

The solubility patterns obtained in DW and in the different pH 8.0 buffers allowed 2 kinds of interaction to be distinguished

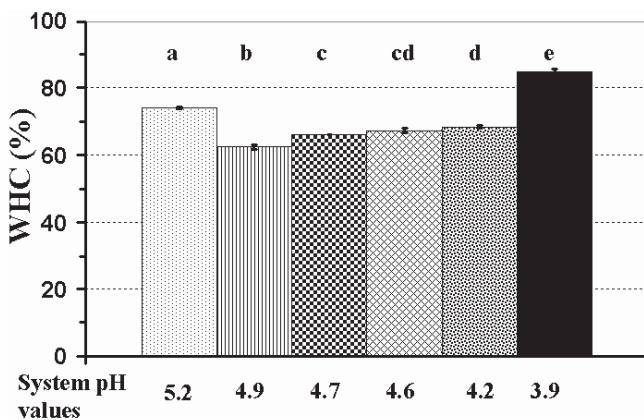


Figure 3. Water-holding capacity of 7% (w/w) WPI cold-set gels acidified with GDL. Gel pH values after 48 h at 10 °C: □ 5.2, ▨ 4.9, ■ 4.7, ▨ 4.6, ▨ 4.2 and ▲ 3.9. Small letters mean significant differences ($P < 0.05$) amongst the acidification conditions studied.

in the protein network of the acidified gels: (1) interactions between part of the proteins with water, by hydrogen bonding, and (2) interactions amongst the greater part of the proteins by electrostatic and hydrophobic interactions and even by hydrogen bonding, the latter two being more significant at pH 4.2. Disulphide bond interactions were probably responsible for keeping the protein aggregates formed during heat treatment, but they could not be identified in the solubility experiments. Electrophoretic experiments were performed in order to evaluate the aggregates or individual WP fractions that were soluble in DW or in the pH 8.0 buffers.

3.3. PAGE of water-soluble protein fractions

Figure 4 shows the electrophoretic mobility patterns of the soluble protein extracted in water (DW) in Native (A) and SDS-PAGE under non-reducing (B) and reducing conditions (C). Samples at pH values between 4.6 and 5.2 showed pronounced bands for β -Lg A and β -Lg B (particularly at pH 4.6), and weaker bands

corresponding to α -La (Fig. 4A). Samples at pH 3.9 and 4.2 showed bands of lesser intensity for β -Lg (A and B) and α -La. Protein aggregates (marked as aggregates 1 and 2) can be seen in the resolving gel between the α -La band and the stacking gels at all pH values, but almost no bands corresponding to BSA could be visualised.

The water-soluble protein observed in Figure 4A could be participating in the gel network through weak non-covalent interactions formed during the pH decrease or could have been entrapped in the network, and was then dissociated from the gel matrix during the dissolution procedure (Fig. 2). The total BSA and α -La present in the initial WPI were linked to the protein aggregates formed after the initial heat treatment step, while the β -Lg was only 70% linked to the aggregates (Sect. 3.1). Such results suggest that α -La, part of the β -Lg and a small fraction of BSA, as well as aggregates 1 and 2 (Fig. 4A), could be dissociated from the protein aggregates formed in the initial heating step, due to possible rearrangements in such aggregates, which were induced by the acidification conditions during the cold-set gelation

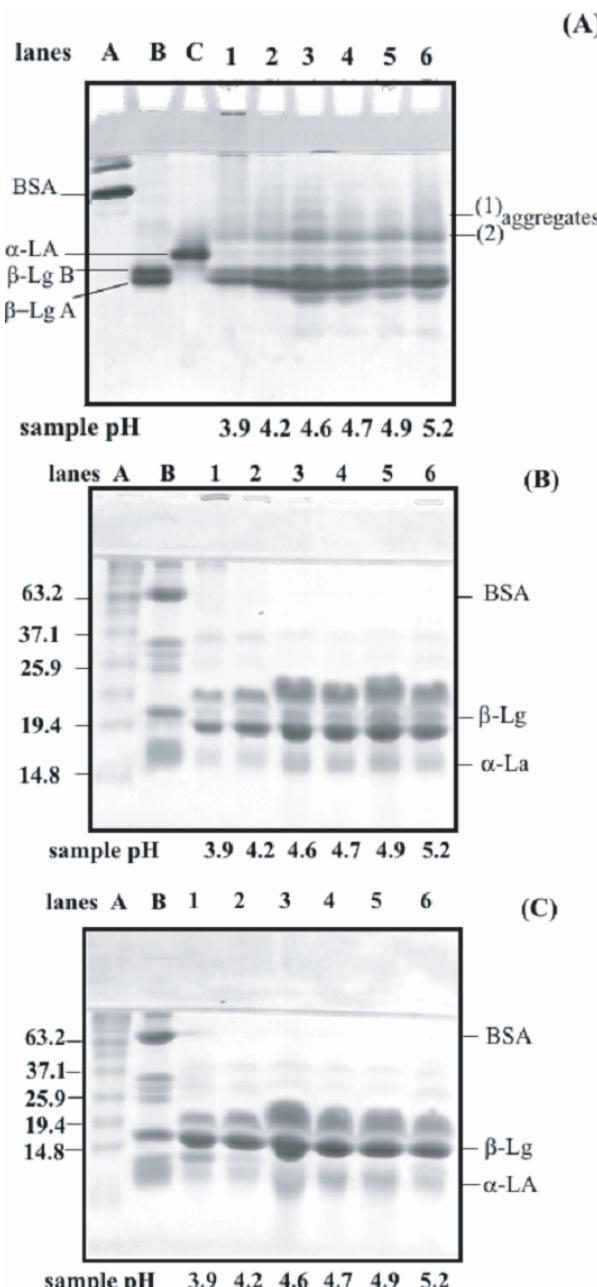


Figure 4. (A) Native PAGE, (B) SDS-PAGE under non-reducing conditions and (C) SDS-PAGE under reducing conditions, of water-soluble protein fractions of WPI cold-set gels. In (A) pure milk proteins were loaded as markers into the single lanes 1, 2 and 3. In (B) and (C) lanes 1 and 2 were the commercial molar mass marker and the mixture of pure milk proteins, respectively.

step. However, part of the observed β -Lg fractions soluble in water after acidification (Fig. 4A) probably came from the β -Lg not linked into soluble whey protein aggregates.

The PAGE patterns observed in Figures 4B and 4C were similar, showing intense bands for β -Lg and weaker bands for α -La in all samples, and BSA bands were almost not observed. Although the ideal sample load corresponds to 10 μg of protein, in these experiments the samples were overloaded (20 μg) in an attempt to visualise the α -La and BSA bands better. The lack of bands for aggregates 1 and 2 in Figure 4B could indicate that these aggregates were stabilised by non-covalent interactions, such as hydrophobic and electrostatic interactions. Moreover, the similar PAGE patterns found in Figures 4B and 4C could indicate that disulphide bonds were not involved in the structure of these water-soluble aggregates.

Electro-elution experiments were performed for a qualitative analysis of the protein constituents of aggregates 1 and 2 observed in Native PAGE (Fig. 4A). The bands extracted were then analysed by SDS-PAGE under reducing conditions and the results can be seen in Figure 5. An intense β -Lg band and very weak BSA bands can be observed in aggregate 1, which could be related to the formation of hydrophobically-bonded aggregates between β -Lg and BSA during the first heating step [12, 13]. However, aggregate 2 constituted β -Lg and α -La, as can be visualised in lane 4. Although BSA bands were almost not observed in Figures 4A, 4B and 4C, the electro-elution results suggested that BSA was present in aggregate 1, indicating that this protein was mostly linked to another protein in the water-soluble fractions.

The weak intensity of the α -La bands in the water-soluble fraction, as shown in Figure 4 (A, B and C), could be correlated with the following facts: (1) formation of

large disulphide-bonded polymers between this whey protein fraction due to the first heating step [13], (2) α -La aggregation when heated in the presence of β -Lg [12] or (3) precipitation of α -La at pH values near its iso-electric point or even lower pH values, due to conformational transition of the α -La structure, involving dissociation of the strongly bound calcium ion [6, 7]. The first and second facts could partly explain the minor intensity of α -La bands in the water-soluble fractions, but the lower solubility of the gels at pH 4.2 and 3.9 as compared with that at higher pH values could be related to the third fact.

The quantification of each WP fraction in the DW supernatants was determined from the PAGE band densities (Fig. 4), considering the amount of protein applied to the gel for each pH evaluated. Figures 6A and 6B show the WP composition in relation to the total protein in the gel (Eq. (2)), and Figure 6C shows the values calculated in relation to the initial amount of individual WP in the gel (Eq. (3)). The data shown in Figure 6A were obtained from the quantification of the Native PAGE (Fig. 4A), while Figures 6B and 6C show the composition after disruption of aggregates 1 and 2 by the addition of SDS and β -mercaptoethanol (PAGE loading buffer, Fig. 4C).

The composition of the WP fractions in water (DW) showed the U-shaped curve pattern as a function of pH like that of the total soluble protein (Fig. 2), and WHC (Fig. 3), with minimal values between pH 4.9 and 4.2. Greater values for solubility were observed for all fractions between pH 5.2 and 3.9, as also for WHC.

It was observed that β -Lg represented the greater part of the soluble protein (Fig. 6A) even before disruption of aggregates 1 and 2 (Fig. 6B). The α -La fraction was the second most soluble protein fraction, while BSA was the least soluble (Figs. 6A and 6B). It should be mentioned that when the composition was analysed

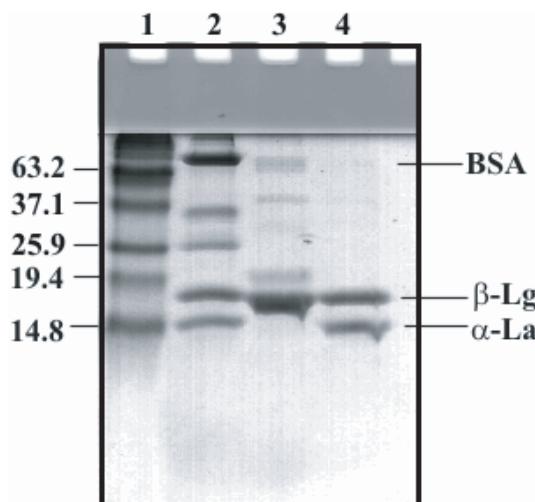


Figure 5. Reducing SDS-PAGE of the aggregates extracted from Native PAGE (Fig. 4A) by electroelution. Lanes 1 and 2 are the commercial and pure milk protein markers, respectively. Lane 3 corresponds to the aggregate 1 observed in Figure 4A, and lane 4 to aggregate 2.

with respect to the total protein of the gel (Fig. 6A), the sum of each WP fraction and amount of aggregate at each pH was always the total water-soluble protein at each pH (data in Fig. 2). The difference from 100% corresponds to the amount of water-insoluble protein. When the composition was calculated in relation to the individual WP, the values for the composition were relatively greater (Fig. 6C), but in this way it was possible to evaluate that β -Lg was the most soluble WP fraction.

Figure 7 shows the precipitated fractions of the main whey proteins. BSA was the most precipitated fraction, with maximum precipitation at pH values between 5.2 and 4.2. α -La was the second most precipitated fraction, followed by β -Lg. These proteins showed maximum precipitation at pH values between 4.9 and 4.2. The occurrence of minimal solubility near the iso-electric point is due primarily to the lack of electrostatic repulsion, which promotes aggregation and precipitation via hydrophobic interactions. In addition, more protein is dissociated at pH

values lower than 4.0, due to the predominance of repulsive electrostatic charge. Under both conditions, WP dissociated from the gel network would be available to interact with water by hydrogen bonding, leading to the higher values for WHC at pH 5.2 and below 4.0.

3.4. PAGE of buffer-soluble protein fractions

Figure 8 shows the PAGE patterns for soluble WP proteins extracted by the different pH 8.0 buffers, (B) and (BM). Figures 8A and 8C are SDS-PAGE under non-reducing conditions and Figures 8B and 8D are SDS-PAGE under reducing conditions. Considering the soluble components extracted by buffer (B) and analysed by SDS-PAGE under non-reducing conditions (Fig. 8A), aggregates with molar mass greater than $200 \text{ kg} \cdot \text{mol}^{-1}$ were visualised at the top of the resolving polyacrylamide gel. Under these conditions, weaker bands of β -Lg and α -La were also

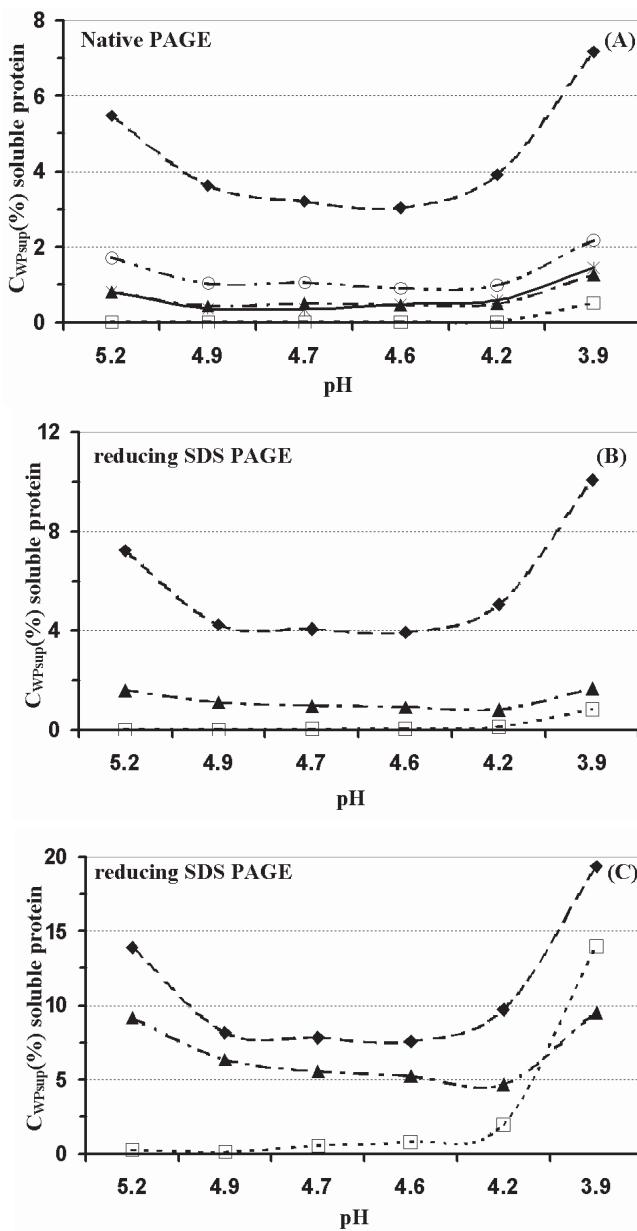


Figure 6. Whey protein composition of water supernatant in solubility experiments (C_{WPSup}). (A) and (B) are protein compositions in relation to the initial total protein in the gel. (C) is the protein composition in relation to the initial amount of each WP in the gel. Whey protein fractions: $\blacklozenge \beta\text{-Lg}$, $\blacktriangle \alpha\text{-La}$, \square BSA, \ast aggregate 1 and \odot aggregate 2 (the aggregates are those observed in Fig. 4A).

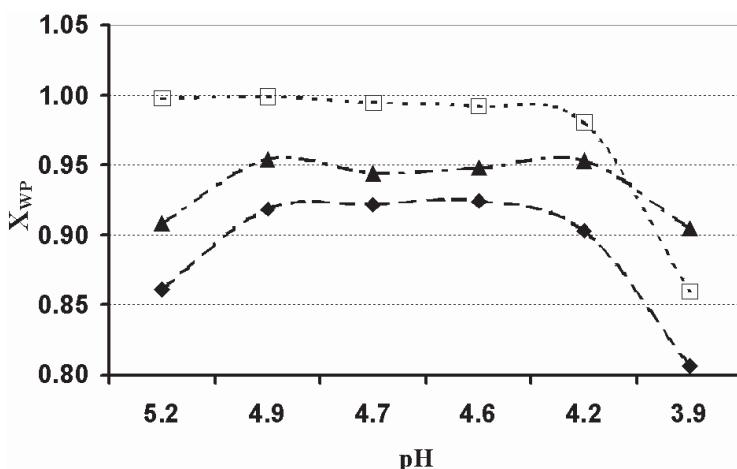


Figure 7. Precipitated fraction of each whey protein in water, calculated in relation to its initial amount. Whey protein fractions: \blacklozenge $\beta\text{-Lg}$, \blacktriangle $\alpha\text{-La}$, and \square BSA.

visualised, while BSA bands were not seen at any of the pH values studied. Thus it was assumed that in buffer (B) large aggregates were separated from the protein matrix in the gel, and were probably linked to each other mainly by electrostatic interactions, confirming the protein solubility results. However, under reducing conditions (Fig. 8B), the main WP bands were clearly marked, especially $\beta\text{-Lg}$ and $\alpha\text{-La}$. Therefore, the large aggregates observed in Figure 8A were probably maintained internally by S-S bonds formed between their WP constituents during the first heating step before gelation, or by a small fraction of $\beta\text{-Lg}$ denatured due to a disulphide bonds reshuffle occurring while the protein was maintained in the pH 8.0 buffer [17]. The PAGE results were more accurate at determining the S-S bonds than the solubility experiments, and this difference between the methods could be related to the amount of electrostatic interaction in relation to S-S bonding, showing that the combination of the two methods used was essential for an adequate analysis of the system interactions. This assumption was confirmed by the electrophoretic patterns

obtained for the supernatants of the extracts obtained with (BM) under reducing and non-reducing conditions. Figures 8C and 8D show that the main WP constituents were able to migrate in the resolving gel. Under both conditions, intense bands for $\beta\text{-Lg}$ and less intense bands for $\alpha\text{-La}$ were visualised, while weaker bands for BSA were observed. Such a result confirms the role of S-S bonds in the stabilisation of the WP aggregates, as observed in Figures 8A and 8B. The PAGE results for the (BUM) extracts were similar to those of the (BM) supernatants and are not shown.

3.5. Mechanical properties of the gels at equilibrium pH

The mechanical properties are shown in Figure 9. The values for stress at rupture (Fig. 9A) and the elasticity modulus (Fig. 9B) exhibited a similar trend. At pH 5.2 (pI of the $\beta\text{-Lg}$ fraction) the gels showed greater values for stress at rupture and for the elasticity modulus, while at pH 4.9 and 4.7 (pI range of the BSA fraction) no significant differences were observed.

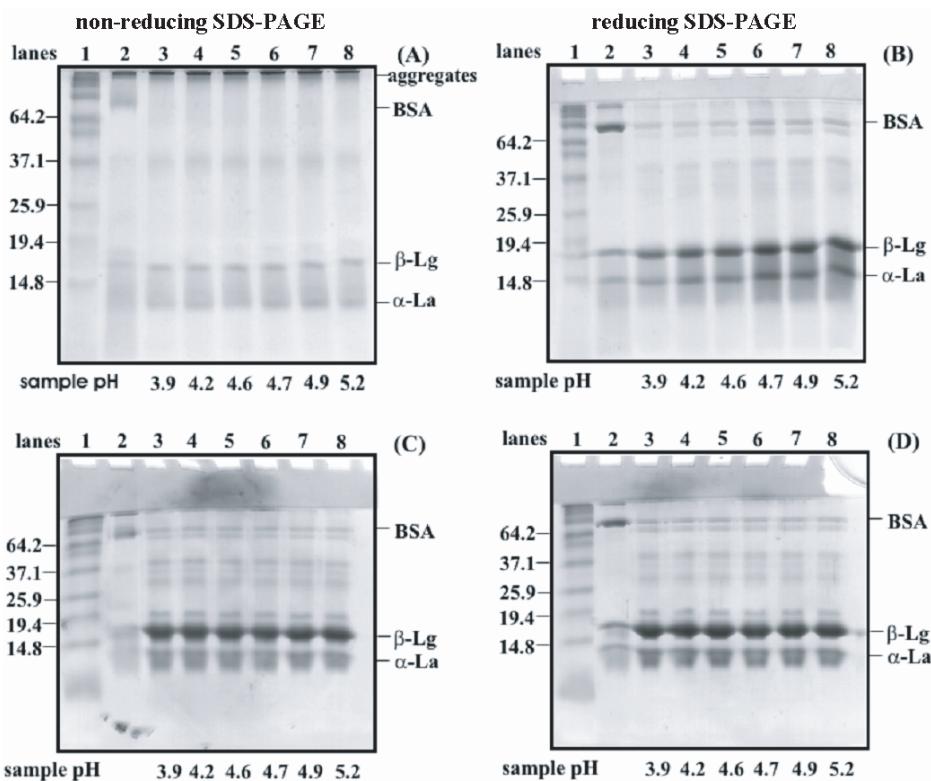


Figure 8. SDS-PAGE under non-reducing (A and C) and reducing (B and D) conditions for the soluble WPI protein aggregates extracted in different pH 8.0 buffers. Extraction solutions: (Figs. A and B) buffer pH 8.0 (B) and (Figs. C and D) buffer pH 8.0 plus β-ME (BM). The commercial molar mass marker was loaded into lane 1 and pure milk proteins into lane 2.

At pH 4.2 (pI of α -La) the lowest values for stress at rupture and elasticity modulus were found. The different acidification conditions and final pH values did not influence the strain at rupture properties of the systems studied (Fig. 9C). This fact suggests that despite the different network strengths of the systems studied, the networks were similar with respect to their deformability properties.

The decreasing profile of stress at rupture and elasticity modulus in the pH range between 5.2 and 4.2 was similar to that observed for water solubility and WHC in the same pH range (Figs. 2 and 3,

respectively). The former fact can be attributed to electrostatic repulsion of the β -Lg fraction, which weakened the structure, since at pH values below 5.2 the system departed from the β -Lg pI. This result suggests that the higher amounts of β -Lg in the WPI play an important role in gel network stabilisation, due to its ability to interact with proteins and aggregates close to its pI on account of its globular structure. However, at pH values below 5.2, a decrease in the solubility of the protein in water and in the WHC was also observed. The WHC properties were more closely linked to the protein disposable to

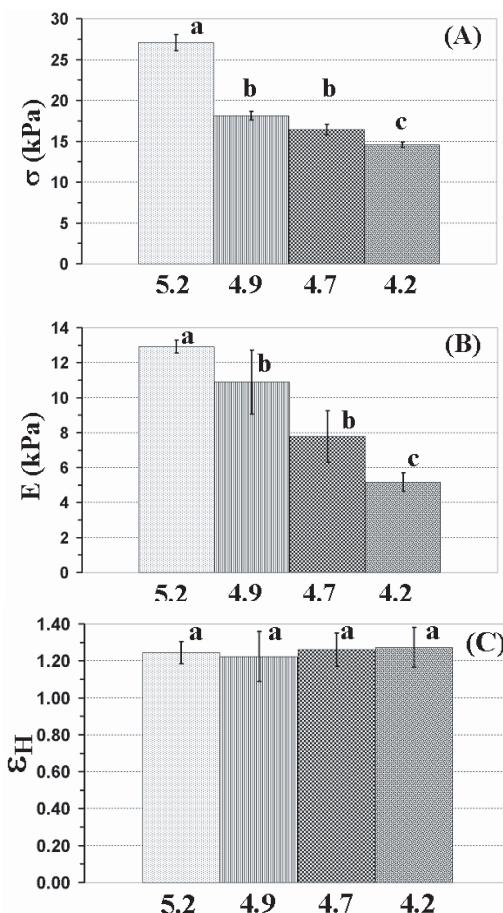


Figure 9. Mechanical properties of 7% (w/w) heat-denatured WPI solutions with different GDL/WPI ratios. (A) Stress at rupture, (B) elasticity modulus and (C) strain at rupture. System pH values after 48 h at 10 °C: □ 5.2, ▨ 4.9, ■ 4.7, ▨ 4.6 and ▨ 4.2. Small letters mean significant differences ($P < 0.05$) among the acidification conditions studied.

interact with water than the gel network structure, suggesting that the whey protein fractions, especially β -Lg, provided water-holding capacity to the system due to their hydrophilic characteristics, including their pI.

4. CONCLUSION

Gel solubility in different buffer systems, in the presence or absence of

denaturant agents, permitted the qualitative evaluation of the molecular interactions amongst the thermal WP aggregates in the gel network, when the final equilibrium pH values were achieved. Under the conditions studied, the interactions were mainly electrostatic, independent of the final pH, but hydrophobic and hydrogen bonds were present in minor intensity. However, it was observed that at pH 4.2 more hydrophobic interactions were present in relation to other final gel pH values, despite β -Lg and

BSA being far from their pI values. Complementary electrophoretic analyses of the soluble protein in each buffer system were important for further evaluation. It was verified that disulphide bonds were present on a minor scale, and were probably associated with the internal stabilisation of WP aggregates (formed in the first heating step), that interacted with each other by electrostatic and hydrophobic bonding and also by hydrogen bonds in the gel network. The mechanical properties of the gels were influenced by the final system pH, especially at pH 5.2 and 4.2. The results suggested that although the interactions amongst the aggregates in the gel network were essentially the same at the pH values evaluated, the β -Lg played an important role in gel stabilisation due to its higher amount of WPI, strengthening the structure at its pI and at the same time providing good water-holding capacity.

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REFERENCES

- [1] Alting A.C., de Jongh H.H.J., Visschers R.W., Simons J., Physical and chemical interactions in cold gelation of food proteins, *J. Agric. Food Chem.* 50 (2002) 4682–4689.
- [2] Alting A.C., Hamer R.J., de Kruif C.G., Paques M., Visschers R.W., Number of thiol groups rather than the size of the aggregates determines the hardness of cold set whey protein gels, *Food Hydrocoll.* 17 (2003) 469–479.
- [3] AOAC, Official Methods of Analysis of AOAC International, Association of Official Analytical Chemists, Patricia Cunniff, Gaithersburg, USA, 1997.
- [4] Barbut S., Determining water and fat holding, in: Hall G.M. (Ed.), *Methods of testing protein functionality*, Chapman & Hall, London, 1996, pp. 187–225.
- [5] Braga A.L.M., Menossi M., Cunha R.L., The effect of the glucono-delta-lactone/caseinate ratio on sodium caseinate gelation, *Int. Dairy J.* 16 (2006) 389–398.
- [6] Bramaud C., Aimar P., Daufin G., Thermal isoelectric precipitation of Alpha-Lactalbumin from a whey-protein concentrate - influence of protein-calcium complexation, *Biotechnol. Bioeng.* 47 (1995) 121–130.
- [7] Bramaud C., Aimar P., Daufin G., Whey protein fractionation: Isoelectric precipitation of alpha-lactalbumin under gentle heat treatment, *Biotechnol. Bioeng.* 56 (1997) 391–397.
- [8] Bryant C.M., McClements D.J., Molecular basis of protein functionality with special consideration of cold-set gels derived from heat-denatured whey, *Trends Food Sci. Technol.* 9 (1998) 143–151.
- [9] Cavallieri A.L.F., Cunha R.L., The effects of acidification rate, pH and ageing time on the acidic cold set gelation of whey proteins, *Food Hydrocoll.* 22 (2007) 439–448.
- [10] Cheftel J.C., Cuq J.L., Lorient D., Aminoacidos, peptidos y proteínas, in: Fennema O.R. (Ed.), *Química de los alimentos*, Editorial Acritia, Zaragoza, 1996, pp. 275–414.
- [11] Copeland R.A., Electrophoretic and chromatographic methods for assessing protein purity, in: Copeland R.A. (Ed.), *Methods for protein analysis: A practical guide to laboratory protocols*, Chapman & Hall, New York, USA, 1990, pp. 59–98.
- [12] Gezimati J., Singh H., Creamer L.K., Aggregation and gelation of bovine beta-lactoglobulin, alpha-lactalbumin, and serum albumin, in: Paris N., Kato A., Creamer L.K., Pearce J. (Eds.), *Macromolecular Interactions in Food Technology*, Amer. Chemical Soc., Washington, 1996, pp. 113–123.
- [13] Havea P., Singh H., Creamer L.K., Characterization of heat-induced aggregates of beta-lactoglobulin, alpha-lactalbumin and bovine serum albumin in a whey protein concentrate environment, *J. Dairy Res.* 68 (2001) 483–497.
- [14] Havea P., Singh H., Creamer L.K., Heat-induced aggregation of whey proteins: Comparison of cheese WPC with acid WPC and relevance of mineral composition, *J. Agric. Food Chem.* 50 (2002) 4674–4681.
- [15] Havea P., Carr A.J., Creamer L.K., The roles of disulphide and non-covalent bonding in the functional properties of heat-induced

- whey protein gels, *J. Dairy Res.* 71 (2004) 330–339.
- [16] Hodge J.R., Hofreiter B.T., Determination of reducing sugar and carbohydrates: Phenol sulfuric test, in: Whistler R.L., Wolfrom M.L. (Eds.), *Methods in Carbohydrate Chemistry*, Academic Press, New York, USA, 1962, pp. 380–394.
- [17] Hoffmann M.A.M., van Mil P.J.J.M., Heat-induced aggregation of β -Lactoglobulin: Role of the free thiol group and disulphide bonds, *J. Agric. Food Chem.* 45 (1997) 2942–2948.
- [18] Hong Y.H., Creamer L.K., Changed protein structures of bovine β -lactoglobulin B and α -lactalbumin as a consequence of heat treatment, *Int. Dairy J.* 12 (2002) 345–359.
- [19] Ju Z.Y., Kilara A., Effects of preheating on properties of aggregates and of cold-set gels of whey protein isolate, *J. Agric. Food Chem.* 46 (1998) 3604–3608.
- [20] Laemmli U.K., Cleavage of structural proteins during assembly of head of bacteriophage-T4, *Nature* 227 (1970) 680–685.
- [21] Livney Y.D., Corredig M., Dalgleish D.G., Influence of thermal processing on the properties of dairy colloids, *Curr. Opin. Colloid Interface Sci.* 8 (2003) 359–364.
- [22] Lupano C.E., Effect of heat-treatments in very acidic conditions on whey-protein isolate properties, *J. Dairy Sci.* 77 (1994) 2191–2198.
- [23] Lupano C.E., Gelation of mixed systems whey protein concentrate-gluten in acidic conditions, *Food Res. Int.* 33 (2000) 691–696.
- [24] Lupano C.E., Dumay E., Cheftel J.C., Gelling properties of whey-protein isolate - influence of calcium removal by dialysis or diafiltration at acid or neutral pH, *Int. J. Food Sci. Technol.* 27 (1992) 615–628.
- [25] Lupano C.E., Renzi L.A., Romera V., Gelation of whey protein concentrate in acidic conditions: Effect of pH, *J. Agric. Food Chem.* 44 (1996) 3010–3014.
- [26] Morr C.V., Ha E.Y.W., Whey protein concentrates and isolates: Processing and functional properties, *Crit. Rev. Food Sci. Nutr.* 33 (1993) 431–476.
- [27] Puppo M.C., Lupano C.E., Anon M.C., Gelation of soybean protein isolates in acidic conditions - effect of pH and protein-concentration, *J. Agric. Food Chem.* 43 (1995) 2356–2361.
- [28] Schokker E.P., Singh H., Creamer L.K., Heat-induced aggregation of β -lactoglobulin A and B with α -lactalbumin, *Int. Dairy J.* 10 (2000) 843–853.
- [29] Shimada K., Cheftel J.C., Texture characteristics, protein solubility, and sulphydryl-group disulfide bond contents of heat-induced gels of whey-protein isolate, *J. Agric. Food Chem.* 36 (1988) 1018–1025.
- [30] Steffe J.F., *Rheological methods in food process engineering*, Freeman Press, East Lansing, USA, 1996.
- [31] Vardhanabuti B., Foegeding A., Rheological properties and characterization of polymerized whey protein isolates, *J. Agric. Food Chem.* 47 (1999) 3649–3655.
- [32] Vojdani J., Solubility, in: Hall G.M. (Ed.), *Methods of testing protein functionality*, Chapman & Hall, London, UK, 1996, pp. 11–55.