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Original article

Mineral modulation of thermal aggregation and gelation of whey proteins: from β-lactoglobulin model system to whey protein isolate

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Abstract – Solutions of 100 g·kg^{-1} of β-lactoglobulin (β-Lg), β-Lg + α-lactalbumin (α-La), β-Lg + α-La + bovine serum albumin (BSA) or whey protein isolate (WPI) were heated at 75 °C, pH 6.8 in water and in the presence of either 100 mmol·L^{-1} NaCl or 10 mmol·L^{-1} $CaCl_2$. The subsequent polymerisation-aggregation processes in solution before gelation and the physical properties of the formed gels were determined. The disappearance of native-like proteins, formation of β-Lg covalent dimer and the nature of the interactions involved in the formed aggregates were addressed. Whatever the protein system, both NaCl and $CaCl_2$ increased gel strength and decreased gelation time. At gelling time, relatively small aggregates, formed by the contribution of the total initial amount of proteins, were observed in samples without added salts. In contrast, with NaCl or $CaCl_2$, only part of the initial amount of proteins was aggregated before gel time. Very large aggregates were formed in the presence of calcium. Under these two mineral conditions, as well as in samples without added salt, covalent disulphide bonds were seen to be the major forces involved in the aggregation process at gel time.

Whey protein / aggregation / gelation / calcium / sodium

Résumé – **Modulation de l'agrégation et de la gélification des protéines de lactosérum par les minéraux : de la β-lactoglobuline pure à l'isolat de protéines sériques.** Des gels de différentes solutions de protéines (β-lactoglobuline (β-Lg), β-Lg + α-lactalbumine (α-La), β-Lg + α-La + sérum albumine bovine (BSA), isolat de protéines sériques) à 100 g·kg^{-1} étaient formés à pH 6.8 dans l'eau ou dans 100 mmol·L^{-1} NaCl ou 10 mmol·L^{-1} $CaCl_2$. Les propriétés physiques des gels ainsi que les réactions d'agrégation des protéines (disparition des protéines natives, formation de dimères covalents de β-Lg et nature des liaisons entre protéines agrégées) ont été étudiées. Le NaCl et le $CaCl_2$ augmentent la force des gels et diminuent les temps de gel (tg). Au tg, sans ajout de sels, les agrégats formés, de petites tailles, impliquent la totalité des protéines présentes en solution. En revanche, en présence de NaCl ou de $CaCl_2$, seule une partie des protéines est impliquée dans la formation des agrégats au tg. La taille des agrégats formés est plus élevée en présence de $CaCl_2$. Dans toutes les conditions, les ponts disulfures interprotéiques semblent être les liaisons majoritairement impliquées dans la formation des agrégats au tg.

Protéine de lactosérum / agrégation / gélification / calcium / sodium

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1. INTRODUCTION

Whey protein isolates (WPI) and concentrates (WPC) are highly functional food proteins that are extensively used as basic ingredients. The gel formation following heating of these proteins is among the most important functionality, great explored by the food industry. The gelation process involves the physical and chemical transformations of one type of structure into another.

The overall process that occurs during thermally-induced protein gelation can be divided into reactions that occur in liquid and solid phases [7, 21]. In the liquid phase, the main reactions involve denaturation, interaction and aggregation of proteins. The formed aggregates transit to the solid phase with subsequent macro-molecular reorganisations to form structured gel.

There is a relative abundance of information in the literature on factors affecting the denaturation steps and gel properties of whey proteins [2, 3, 7, 13]. From these studies, it can be clearly deduced that the structures and the properties of formed gels can be modulated by various factors including pH, protein concentration, ionic strength, ion type, time and temperature of heating. In this area, the major part of the published work can be classified into two major areas: (1) the fist one concerns all studies which focus on determining the effect of various factors on the structure of formed gels i.e. "solid state studies"; and (2) the second group concerns studies which aimed to determine the effect of these factors on the denaturation, polymerisation and/or aggregation of whey protein systems i.e. "liquid state studies" [10, 15, 23–26].

A better knowledge of the link between these two scientific areas and how these factors affect the denaturation-polymerisation processes of the proteins before their gelation under industrially relevant conditions will offer strong possibilities for controlled transformation of one type of structure to another. The objective of this study was to examine how optimum mineral concentration affects the formation of aggregates in concentrated whey protein systems that lead to well-described properties of the resulting gels. For this purpose, we compared the characteristics of aggregates and gels formed by β-Lg as a single component or in more complex systems under fixed total protein concentration, pH and heating temperature. The three ionic strengths used in the present work were chosen in order to produce gels with different microstructures [7]. In fact, thermally-induced gels of globular proteins are generally classified as fine-stranded, particulate or mixed gels. A fine-stranded matrix is favoured in protein solution with low mineral content. Mixed or particulate gel matrixes were formed, respectively, in the presence of about 100 $mmol \cdot L^{-1}$ of monovalent cations or about 10 $mmol \cdot L^{-1}$ divalent cations.

2. MATERIALS AND METHODS

2.1. Materials

WPI was obtained from an industrial acid whey by combined membrane processes as described by Fauquant et al. [5]. The overall composition is shown in Table I. The denaturation level of individual proteins was determined by size exclusion chromatography analysis of samples after acid precipitation at pH 4.6. The obtained WPI was used for the purification of α-lactalbumin (α-La) and β-lactoglobulin (β-Lg) (see below). Defatted bovine serum albumin (BSA, Product No. A-7030, purity 98%) as well as standard proteins were from Sigma Chemical Co. (St Louis, MO, USA). All other reagents were of analytical grade. For all experiments, ultrapure water from a Milli-Q water purification was used.

2.2. Purification of α-lactalbumin and β-lactoglobulin

α-La and β-Lg were purified from WPI using a Pharmacia BioPilot system fitted with a preparative column (Q-Sepharose fast

Table I. Composition of initial whey and prepared whey protein isolate.

	Whey		Whey protein isolate	
	Total ($g \cdot L^{-1}$)	Denatured (%)	Total ($g \cdot kg^{-1}$)	Denatured (%)
Calcium + magnesium	0.81		2.1	
Sodium + potassium	1.2		7	
Nitrogen × 6.38	5.7		964	
α-Lactalbumin	0.7	1	118	1
β-Lactoglobulin	2.4	0	580	0
Serum albumin	0.4	17	95	34
IgG	0.2	7	61	42

flow 100 × 500 mm, Amersham Pharmacia biotech, Uppsala, Sweden) equilibrated with 0.02 $mol \cdot L^{-1}$ Tris/HCl buffer, pH 7. Elution was performed by stepwise gradient with the same buffer containing 1 $mol \cdot L^{-1}$ NaCl. The collected fractions were concentrated by ultrafiltration on a 3 $kg \cdot mol^{-1}$ membrane and extensively diafiltered with Milli-Q water before lyophilisation. The purity of the obtained protein powders was higher than 98% based on reverse-phase HPLC analysis, and contained 0.37 mmol sodium and 1.6 μmol calcium per g of β-Lg and 0.26 mmol sodium and 60 μmol calcium per g of α-La.

2.3. Heat-induced gelation

All the heat-induced gels were formed in 150 mm × 16 mm I.D. glass tubes. WPI, β-Lg and protein mixtures (100 $g \cdot L^{-1}$) were dissolved at room temperature in either Milli-Q water (control) or in mineral-enriched solutions (100 $mmol \cdot L^{-1}$ NaCl or 10 $mmol \cdot L^{-1}$ $CaCl_2$) and the solutions were stirred for 1 h at room temperature and then held for 1 h under vacuum. The pH was then adjusted to 6.8. The binary mixture combined 8 g β-Lg and 2 g α-La in 100 g solution. The ternary mixture was performed by dissolving 6.45 g β-Lg, 2 g α-La and 1.55 g BSA, and made up to 100 g. The solutions were heated for 30 min at 75 °C. In all experiments, the formed gels were immediately cooled at 4 °C and stored at this temperature for 20 h. The gels were equilibrated at room temperature before subsequent measurements. For each sample, the gelation experiments were performed two times.

2.4. Gel properties

2.4.1. Firmness measurement

The gels formed were penetrated with a stainless steel rod (8 mm diameter) connected to a 10 N load cell of a 4501 Instron apparatus (Guyancourt, France). Force-time curves were obtained at a crosshead speed of 10 $mm \cdot min^{-1}$ for a 10 mm displacement. Firmness results were expressed as the slope of strength versus penetration between 2 and 4 mm.

2.4.2. Dynamic viscoelastic measurements

In thermal scanning experiments, the following gradient steps were applied: 40 to 66 °C at 20 $°C \cdot min^{-1}$; 66 to 73 °C at 5.4 $°C \cdot min^{-1}$; 73 to 75 °C at 0.92 $°C \cdot min^{-1}$; and held at 75 °C for 25 min and then the solutions were cooled to 15 °C at –12.5 $°C \cdot min^{-1}$ and to 4 °C at –1.1 $°C \cdot min^{-1}$. Storage (G') and loss moduli (G'') were determined at a frequency of 1 Hz. Tan σ, the ratio between G'' and G' was small (0.14) for all gels. The samples (4 mL) were covered with a layer of paraffin to avoid evaporation during heating. For each sample, the measurements were performed in triplicate. The G' value was also used to define the gel time of various samples during

heating at 75 °C. Gel time (tg) is defined as the time after which the G' value was higher than 1 Pa.

2.4.3. Colour of gels

The lightness (L) of the prepared gels was measured on samples placed on a black surface with a minolta CR-300 colorimeter (Chromameter, Osaka, Japan).

2.5. Size exclusion chromatography (SEC)

Samples were analysed by SEC on a Superdex 75 HR 10/30 column (Amersham Pharmacia Biotech) equilibrated with 0.1 mol·L^{-1} Tris/HCl buffer + 0.15 mol·L^{-1} NaCl, pH 8 (buffer A). Samples were diluted in cold buffer (dilution 1/50, 4 °C) to stop the aggregation step, filtered through 0.45 µm pore size, diluted once again four-fold and 50 µL were injected onto the column. The elution was performed at a flow rate of 0.5 mL·min^{-1}. The apparatus used was the Pharmacia Fast Protein Liquid Chromatography system equipped with a LCC-500 system controller, two P500 pumps and a UV detector. The absorbance was monitored at 214 nm. The standard proteins used for calibration were aldolase (158 kg·mol^{-1}), human serum albumin (66 kg·mol^{-1}), ovalbumin (43 kg·mol^{-1}),β-lactoglobulin (36.6 kg·mol^{-1}), α-chymotrypsinogen (25 kg·mol^{-1}) and ribonuclease A (13.6 kg·mol^{-1}).

2.6. Reverse-phase high performance liquid chromatography (RP-HPLC)

The formation of heat-induced β-Lg covalent dimer was monitored by RP-HPLC in the conditions adapted from Elgar et al. [4]. Briefly, the column (a 3 mL Resource RPC column, Amersham Pharmacia Biotech) was operated at room temperature and at a flow rate of 2 mL·min^{-1}. The column was equilibrated in 70% solvent A (0.1%, v/v, TFA in Milli-Q water) and after sample injection, a 1-min isocratic period was applied, followed by a series of linear gradients to 100% solvent B (0.09%, v/v, TFA; 90% (v/v) acetonitrile in Milli-Q water) as follows: 1–6 min: 30–40% B; 6–16 min: 40–45% B; 16–19 min: 45–50% B; 19–22 min: 50% B; 22–25 min: 50–70% B; 25–26 min: 70–100% B; 26–31 min: 100% B; 31–33 min: 100–30% B; 33–35 min. Samples were diluted four-fold with water, filtered through 0.45 µm pore size, and 50 µL were injected onto the column.

2.7. Protein aggregation at gelling time

The aggregation state of the protein solutions and the disappearance of native-like proteins before gelation point were determined by SEC analysis as described above. To determine the role of covalent and noncovalent interactions in the aggregates formed at gelling time, all samples were incubated in buffer A containing either 6 mol·L^{-1} urea or 6 mol·L^{-1} urea + 10 mmol·L^{-1} DTT. For samples heated in the presence of $CaCl_2$, treatment with 2 mmol·L^{-1} EDTA, 6 mol·L^{-1} urea ± 10 mmol·L^{-1} DTT was also performed. Samples were incubated at room temperature for 1 or for 24 h before SEC analysis with buffer A containing 6 mol·L^{-1} urea. The formation of β-Lg covalent dimer was monitored by RP-HPLC as described above.

The experiments to determine the protein state after heating (native-like, dimers and aggregates) were replicated two times for all samples.

3. RESULTS AND DISCUSSION

3.1. Effect of mineral and protein composition on gel properties

It is known that the physical properties of whey protein gels that contain salt are strongly related to the concentration and the nature of the salt [16, 19]. In this study, we chose 100 mmol·L^{-1} and 10 mmol·L^{-1} of added sodium and calcium, respectively, because these concentrations were found, as expected, to be optimum in forming the

Table II. Effect of sodium, calcium and protein composition on the lightness and firmness of heat-induced (75 °C, 30 min; pH 6.8) whey protein gels (100 g·kg^{-1}).

	Lightness score	Resistance to penetration (N·mm^{-1})
WPI	41	< 0.01
WPI + NaCl	87	0.62
WPI + $CaCl_2$	89	0.65
Ternary mixture	39	< 0.01
+ NaCl	59	1.47
+ $CaCl_2$	74	0.89
Binary mixture	39	< 0.01
+ NaCl	63	1.66
+ $CaCl_2$	72	0.37
β-Lactoglobulin	40	< 0.01
+ NaCl	56	0.82
+ $CaCl_2$	61	0.27

strongest gels (results not shown). Table II gives the effect of these optimum salt concentrations on the properties of gels formed with various protein systems.

Without added salt, the "L" value as well as the hardness of the gel were relatively low for all protein mixtures studied, indicating the formation of transparent and less rigid gels. As expected, the formation of heat-induced gels was promoted at higher ionic strength. Addition of NaCl or $CaCl_2$ induced a significant increase in the "L" value as more light was reflected back due to the formation of more opaque gels. Also, the firmness of the gel increased significantly, underlining that the thicker protein strands formed a more rigid matrix, as previously reported for whey protein isolates [1]. However, the effect of these two salts depended on the nature of the proteins in the mixture. With NaCl, similar firmness behaviour was obtained for gel made with β-Lg alone (0.82) as well as with mixtures of the three major proteins (0.89). In contrast, the presence of BSA seemed to be required to induce the strongest gel in the presence of calcium. Relatively soft gels were obtained for samples lacking this protein. Its addition markedly increased the gel firmness which became two-to three-fold higher than in samples without BSA. From their transmission electron microscopy data performed on gels that had been stored for 24 h at 4 °C, Boye et al. [2] have suggested that the presence of BSA appeared to inhibit the development of large β-Lg and α-La aggregates. They attributed this effect to a steric hindrance due to the earlier gelation of BSA, which has a lower denaturation temperature.

The gel time (tg), here defined as the time after which $G' > 1$ Pa, was higher for all protein samples heated under low ionic strength (Tab. III). Samples with added calcium gelled more rapidly and had the lowest tg. Samples heated in the presence of NaCl exhibited intermediary tg. These results are in agreement with those previously reported by other authors who studied whey proteins or purified β-Lg [6, 19]. For samples without added minerals, β-Lg alone seemed to gel faster than the protein mixtures, but this phenomenon was not observed after addition of NaCl or $CaCl_2$.

The gel stiffness, expressed here by the elastic modulus value, G', increased as a function of heating time in all studied samples. The maximum value of G', obtained after heating and cooling cycles for various samples is given in Table III. Overall, the results showed that G' values increased in the following order: without added mineral

Table III. Effect of sodium, calcium and protein composition on the G′ and gelling time of whey protein mixtures.

	Initial ionic strength		+ 100 mmol·L^{-1} NaCl		+ 10 mmol·L^{-1} $CaCl_2$	
	Gelling time (s)	G′ (Pa)	Gelling time (s)	G′ (Pa)	Gelling time (s)	G′ (Pa)
β-Lg	1919 ± 251	15.6	384.0 ± 24.6	20400 ± 600	195.0 ± 8.4	14000
β-Lg + α-La + BSA	3481 ± 529	3.61 ± 0.34	362.0 ± 7.9	26337 ± 405	197.0 ± 11.9	14153 ± 850
WPI	2205 ± 28.7	10.72 ± 1.12	363 ± 14.0	15743 ± 875	177.5 ± 10.5	11127 ± 935

< + 10 mmol·L^{-1} $CaCl_2$ < + 100 mmol·L^{-1} NaCl. The initial mineral environment was not sufficient to form strong gels. G′ obtained in the presence of NaCl were higher than in the presence of $CaCl_2$. This is consistent with results reported by other authors [9]. In a given mineral environment, significant differences were observed between different mixtures, without any clear correlation between final G′ and protein nature. These differences could be attributed to the state of ions, in particular, calcium in solution. It has already been reported that the relative distribution of calcium between free, colloidal and protein-bound forms may have a significant influence over gelling properties [16]. This relative distribution of calcium is greatly governed by its affinity towards various proteins when used in mixtures.

The above-mentioned effect of BSA on the gel hardness was not recovered when the gel times and G′ of various samples were compared. Consequently, it is likely that the specific event linked to BSA occurred during gel equilibrium phase following storage at 4 °C.

3.2. Events that occurred before sol-gel transition

3.2.1. Heat-induced disappearance of native-like β-Lg and α-La

The disappearance of native-like proteins as a function of heating time is a common criterion used to monitor the first steps of protein aggregations. It allows comparison of the denaturation kinetics under different experimental conditions.

In our experimental conditions, reliable data were only obtained for the major proteins, i.e. β-Lg and α-La. As expected, the proportion of native-like proteins decreased as a function of heating time whatever the experimental conditions. The results obtained after 3 min of heating (the lowest gelation time in our study) are summarised in Table IV. For a given mineral environment, the concentration of both proteins had decreased slightly with the same rate for all protein mixtures. However, in the pure β-Lg system, a higher proportion of native-like species had disappeared, indicating the contribution of the other proteins to this initial heating phase. The decrease in native-like proteins was reported to be faster when 0.1 mol·L^{-1} NaCl was added to the β-Lg solution before heating [17, 27]. This was not observed in the present work, probably because the high protein concentration by itself promoted the denaturation-aggregation kinetics in samples without added salt. The effect of the mineral environment is best illustrated by the residual native-like proteins just before tg. As shown in Table V, the proportion of native proteins that was still present in solution at sol-gel transition depended on the mineral environment. The proportion of both proteins involved in the formed aggregates decreased from 100% in the

Table IV. Residual native-like β-Lg and α-La after 3 min of heating at 75 °C (total protein concentration 100 g·kg^{-1}, pH 6.8) as determined by size exclusion chromatography.

Experimental conditions	Samples	Residual native like β-Lg after 3 min heating (%)	Residual native like α-La after 3 min heating (%)
No minerals added	β-Lg	64	-
	Binary mixture	80	87
	Ternary mixture	83	84
	WPI	78	82
+ NaCl	β-Lg	66	-
	Binary mixture	63	73
	Ternary mixture	62	71
	WPI	79	80
+ $CaCl_2$	β-Lg	47	-
	Binary mixture	68	100
	Ternary mixture	59	68
	WPI	67	82

absence of added salt to 40–50% with NaCl and further to 35–40% only in the presence of $CaCl_2$. Other workers have reported that only part of the total amount of proteins present in solution were aggregated at tg in the presence of NaCl [3, 28]. Le Bon [17] showed that the proportion of non-aggregated β-Lg at gel point increased from 0.25 to 0.55 when the concentration of NaCl was increased from 16 to 150 mmol·L^{-1} (initial protein concentration = 60 g·L^{-1}). Our results confirmed and extended these observations showing that at high protein concentration and without added salt, all protein molecules were involved at tg.

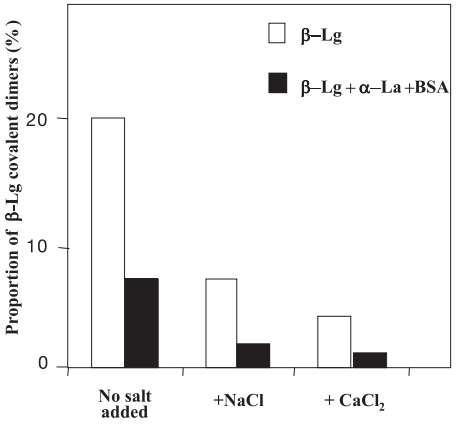

Figure 1. Effect of protein mixture and mineral environment on the proportion of β-Lg covalent dimers formed at tg in heated β-Lg and WPI solutions.

3.2.2. Formation of β-Lg covalent dimers

In earlier studies, the formation of heat-induced disulphide-bonded dimers as intermediates was reported to be an important step in the further aggregation of β-Lg [18, 22]. The presence of these intermediates at tg was determined under different protein and mineral environments. As shown in Figure 1, the proportion of denatured covalent dimers of β-Lg depended on both the salt environment and protein mixtures. Whatever the mineral environment, the proportion of this molecular species was 2.5 to 3 times higher in pure β-Lg solution than in the other protein solutions. This lower amount in the mixed systems can be attributed either to the lower concentration of β-Lg or more probably to the concomittant formation of covalent heterodimers, e.g. between β-Lg and α-La, as already shown by Havea et al. [11]. Figure 1 also shows that, for a

Table V. Residual native-like β-Lg and α-La at gel time of samples heated at 75 °C (total protein concentration 100 g·kg^{-1}, pH 6.8) as determined by size exclusion chromatography.

Experimental conditions	Samples	Residual native like β-Lg before tg (%)	Residual native like α-La before tg (%)
No minerals added	β-Lg	0	-
(tg = 33 min)	Binary mixture	0	0
	Ternary mixture	0	0
	WPI	0	0
+ NaCl	β-Lg	40	-
(tg = 6 min)	Binary mixture	57	67
	Ternary mixture	55	59
	WPI	57	60
+ $CaCl_2$	β-Lg	47	-
(tg = 3 min)	Binary mixture	68	90–100
	Ternary mixture	59	68
	WPI	67	82

given protein mixture, the proportion of this covalent β-Lg dimer at tg was lower in samples with added salts than in those without added salt. Such results may be attributed to the effect of salt on the size of aggregates formed just before sol-gel transition. Addition of salt favoured the formation of large aggregates throughout association of smaller ones [3].

3.2.3. Nature of stabilising forces involved in formed aggregates

Since all samples were filtered through a 0.45 μm filter before SEC analysis, the results in Figure 2 indicate that the size of aggregated particles was larger (> 0.45 μm) in the calcium-supplemented samples than in the other samples (free added salt and + NaCl). These results suggest that in the presence of 10 mmol·L^{-1} $CaCl_2$, heating induced the formation of large aggregates, which were removed by the filtration step. This is consistent with the results of Havea et al. [12] who reported that, after heating, the size of formed aggregates was larger in cheese WPC (calcium-rich WPC) than in acid WPC.

To elucidate the role of covalent and noncovalent interactions in the aggregates formed under different mineral and protein environments at tg, heated samples were incubated with link-dissociating agents: 6 mol·L^{-1} urea, 6 mol·L^{-1} urea + DTT, and 6 mol·L^{-1} urea + DTT + EDTA for samples containing samples calcium. The treated samples were then analysed by SEC in the presence of urea. Figure 3 shows the relative proportion of native-like β-Lg or its aggregates that involved either covalent or noncovalent bonds. It is immediately apparent that (1) after treatment with reducing agents, a considerable increase in native-like proteins occurred, indicating that intermolecular disulphide bonds predominated in all samples; (2) treatment with urea alone and urea + EDTA had little effect on the fraction of aggregated material – noncovalently associated aggregates represented less than 10% of the total species in all mixtures; (3) in the absence of added salt, the major β-Lg species (= 80%) involved covalent bonds, and this did not depend on the presence of other proteins during heating; and (4) the amount of residual native-like proteins at tg was higher after addition of NaCl or $CaCl_2$ and covalent interactions were still

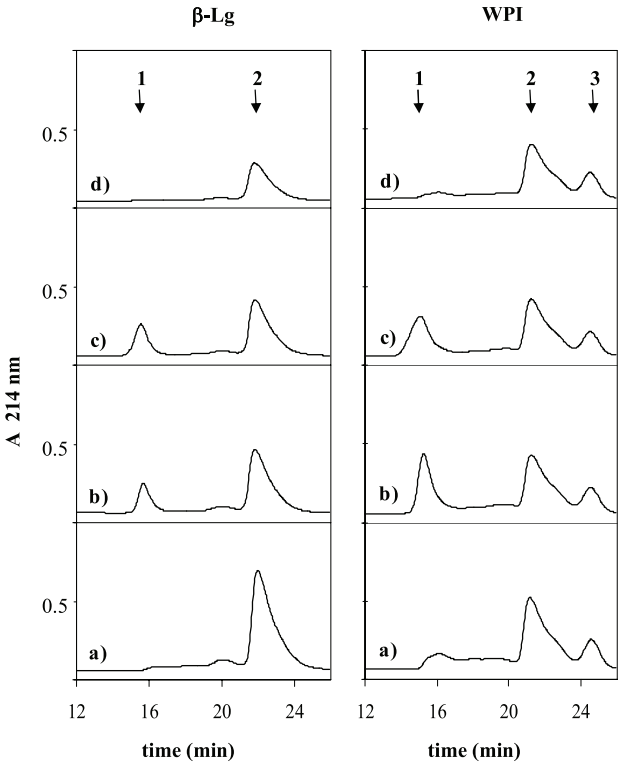

Figure 2. SEC profiles at gel time of heated β-Lg and WPI solutions. (a) Control unheated samples; (b) Heated samples without added salt; (c) Heated samples with 100 mmol·L^{-1} NaCl; (d) Heated samples with 10 mmol·L^{-1} $CaCl_2$. Protein solutions (100 g·kg^{-1}) were heated during 3 min at 75 °C, pH 6.8. 1; aggregates; 2: β-Lg; 3: α-La.

dominant in formed aggregates. Hence, at a total protein concentration of 100 g·L^{-1}, covalent disulphide bonds were the major forces involved in the aggregation process of β-Lg alone as well as of more complex mixtures. It must be noted, however, that a possible urea-induced dissociation of high molecular weight aggregates into smaller ones cannot be excluded. It is possible, as previously suggested by Hoffman et al. [14], that some disulphide linked aggregates had formed larger ones via noncovalent interactions. On the other hand, our results suggest that even if noncovalently linked aggregates were formed at a high proportion, they were rapidly converted into disulphide bonded aggregates. The predominance of covalent interactions shown in this study is in agreement with the results on the aggregation of β-Lg in skim milk reported by Olfield et al. [20].

The contribution of the different types of bond to the aggregation process of whey proteins is not fully understood, probably because it depends on experimental conditions such as pH, ionic strength, temperature and protein concentration. Hoffmann et al. [14] showed that noncovalent interactions are of minor importance in the formation of β-Lg aggregates after heating at neutral pH and 65 °C. They postulated the prominent role of disulphide bonds. Other authors supported the view that β-Lg aggregates through a series of parallel and consecutive steps which involve both covalent (thiol/disulphide bond interchanges) and noncovalent (hydrophobically driven associations) reactions [18]. The relative proportion of these two types of interactions seems to be highly temperature-dependent. For instance, Galani and Apenten [8] showed that below 75 °C, the noncovalent

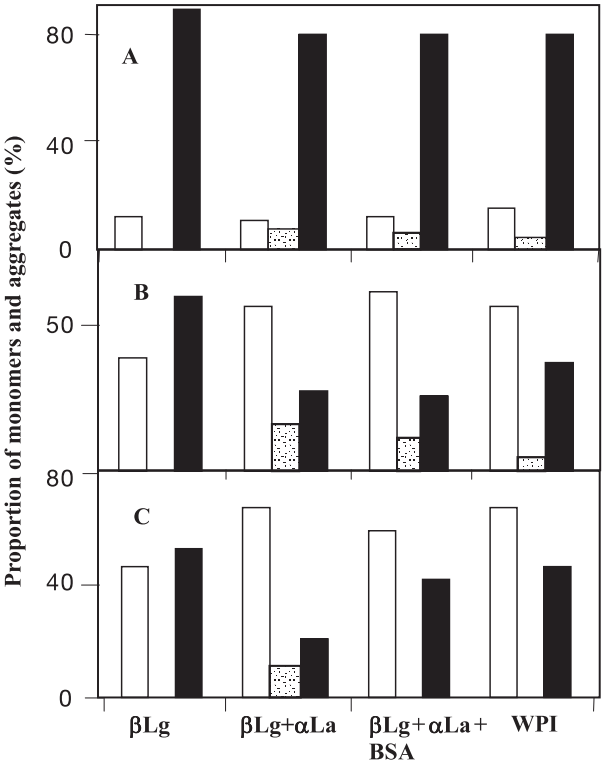

Figure 3. Effect of protein mixture on the proportion of β-Lg residual monomers and polymerised species at tg under different mineral environments. **A** = without added salt; **B**: + NaCl; **C**: + $CaCl_2$. □: native-like β-Lg; ▨: noncovalent interactions; ■: covalent interactions.

interactions contributed little to the overall aggregation process. They found that the contribution of noncovalent interactions to the aggregation becomes important at temperatures higher than 90 °C.

4. CONCLUSION

The gelation time of whey protein solutions (100 g·kg^{-1}) at pH 6.8 decreased six and ten-fold in the presence of NaCl and $CaCl_2$, respectively. Addition of NaCl or $CaCl_2$ salt considerably increased the aggregation kinetics of whey protein throughout intramolecular electrostatic shielding of negative charges on the proteins and/or by forming complexes throughout intermolecular cross-linking of adjacent anionic molecules in the case of calcium. Progressive substitution of β-Lg up to about 40% by the other proteins did not alter this gelation behaviour.

The properties of whey protein gels can be related to two aggregation pathways: the first one involved the formation of aggregates with limited size concomitantly to the decrease in native-like proteins. This aggregation pathway was observed under low ionic strength and in the presence of monovalent cations, whatever the protein mixture. The size of the formed aggregates was shown to be larger in the presence of NaCl as determined by light scattering measurements [3]. This is expressed here by the fact that the proportion of initial proteins aggregated at tg is greatly lowered after addition of NaCl. The second aggregation-gelation pathway, that occurred in the presence of calcium ions in the four protein

mixtures, involved the formation of large aggregates not detected during SEC analyses after filtration on a 0.45 μm filter. The resulting gel constitutes approximately an equal amount of large aggregates and native-like proteins. Hence, the occurrence of intermolecular cross-linking of proteins and the formation of very large aggregates are among the main differences between the two mineral environments, i.e. Na^+ and Ca^{++}. This probably explains why the gel stiffness (G' values) obtained after addition of $CaCl_2$ was lower than in the presence of added NaCl.

Finally, our results suggest that sol-gel transition is governed by the size of the aggregates rather than the number of aggregated molecules. Whatever their size, it seems that covalent interactions are the main forces involved between proteins in the formed aggregates, at pH 6.8.

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