CORA Cork Open Research Archive
Cartlann Taighde Oscailte Chorcaí

Title	Influence of chaperone-like activity of caseinomacropeptide on the gelation behaviour of whey proteins at pH 6.4 and 7.2
Authors	Gaspard, Sophie J.;Sharma, Prateek;Fitzgerald, Ciarán;Tobin, John T.;O'Mahony, James A.;Kelly, Alan L.;Brodkorb, André
Publication date	2021
Original Citation	Gaspard, S. J., Sharma, P., Fitzgerald, C., Tobin, J. T., O'Mahony, J. A., Kelly, Alan L. and Brodkorb, A. (2021) 'Influence of chaperone-like activity of caseinomacropeptide on the gelation behaviour of whey proteins at pH 6.4 and 7.2', Food Hydrocolloids, 112, 106249 (9pp). doi: 10.1016/j.foodhyd.2020.106249
Type of publication	Article (peer-reviewed)
Link to publisher's version	10.1016/j.foodhyd.2020.106249
Rights	© 2020, Elsevier Ltd. All rights reserved. - https://creativecommons.org/licenses/by/4.0/
Download date	2026-11-06 09:00:44
Item downloaded from	https://hdl.handle.net/10468/13484



UCC
University College Cork, Ireland
Coláiste na hOllscoile Corcaigh

Food Hydrocolloids 112 (2021) 106249

Contents lists available at ScienceDirect

Food Hydrocolloids

journal homepage: http://www.elsevier.com/locate/foodhyd

Influence of chaperone-like activity of caseinomacropeptide on the gelation behaviour of whey proteins at pH 6.4 and 7.2.

Sophie J. Gaspard [a,b], Prateek Sharma [a,c], Ciarán Fitzgerald [a], John T. Tobin [a], James A. O'Mahony [b], Alan L. Kelly [b], André Brodkorb [a,*]

[a] Teagasc Food Research Centre, Moorepark, Fermoy, Co, Cork, P61 C996, Ireland
[b] School of Food and Nutritional Sciences, University College Cork, Cork, Ireland
[c] Department of Nutrition, Dietetics, and Food Sciences, Utah State University, Logan, UT, USA

ARTICLE INFO

Keywords:
Caseinomacropeptide
Whey protein
Heat stability
Chaperone-like activity
Gelation

ABSTRACT

The effect of caseinomacropeptide (CMP) on the heat-induced denaturation and gelation of whey proteins (2.5–10%, w/v) at pH 6.4 and 7.2, at a whey protein:CMP ratio of 1:0.9 (w/w), was investigated using differential scanning calorimetry (DSC), oscillatory rheology (90 °C for 20 min) and confocal microscopy. Greater frequency-dependence in the presence of CMP suggested that the repulsive interactions between CMP and the whey proteins affected the network generated by the non-heated whey protein samples. At pH 6.4 or 7.2, CMP increased the temperature of denaturation of β-lactoglobulin by up to 3 °C and increased the gelation temperature by up to 7 °C. The inclusion of CMP strongly affected the structure of the heat-induced whey protein gels, resulting in a finer stranded structure at pH 6.4 and 7.2. The presence of CMP combined with a lower heating rate (2 °C/min) prevented the formation of a solid gel of whey proteins after heating for 20 min at 90 °C and at pH 7.2. These results show the potential of CMP for control of whey protein denaturation and gelation.

1. Introduction

Uncontrolled denaturation and aggregation of whey proteins can lead to undesirable precipitation during heat processing. In dairy science and technology, chaperone-like activity is defined as the ability of a molecule to protect another biomolecule against denaturation, aggregation and/or precipitation. κ-Casein has been shown to form heat-induced nanoparticles with whey proteins *via* hydrophobic interactions and disulphide bonds, and to exert a chaperone-like activity by limiting the size of the whey protein aggregates formed (Guyomarc'h, Nono, Nicolai, & Durand, 2009; Liyanaarachchi, Ramchandran, & Vasiljevic, 2015). As a result, the heat stability of dairy proteins can be enhanced as compared to non-heated whey protein systems (Gaspard, Auty, Kelly, O'Mahony, & Brodkorb, 2017)). However, κ-casein represents only 9–13% of total milk protein (Swaisgood, 2003), and isolating κ-casein at a reasonable cost and in sufficient quantity to observe a chaperone-like activity remains a challenge.

During cheese manufacture, chymosin cleaves κ-casein and releases a 64-amino acid glycopeptide, caseinomacropeptide (CMP), of size 7–9 kDa (Mikkelsen et al., 2005), representing up to 20–25% (w/w) of total whey protein, depending on the source of whey proteins and the method of fractionation employed (Thomä-Worringer, Sørensen, & López-Fandiño, 2006). CMP is highly hydrophilic and heat-stable, even at acidic pH, because of its disordered, random coiled structure, and the negative charges carried by the glutamate, aspartic acid, carboxyl groups, phosphorylation sites and the carbohydrate chain at neutral pH (Smith, Edwards, Palmano, & Creamer, 2002). The glycosidic residues, galactose, *N*-acetyl galactosamine and *N*-acetylneuraminic acid, originate from the C-terminal part of κ-casein and are attached to the peptide by O-glycosylation linkages. These residues are organised in mono-to tetrasaccharides, with *N*-acetylneuraminic acid generally located at the end of the carbohydrate chain (Saito & Itoh, 1992). The low pKa (2.6) of *N*-acetylneuraminic acid is due to its carboxylic functional group, making the carbohydrate chain highly negatively-charged at neutral pH.

Despite the cleavage of the *N*-terminus tail of κ-casein, which contains most of the hydrophobic amino acids and all the cysteine residues, by chymosin, several studies showed that CMP can still interact with whey proteins *via* non-covalent interactions and affect their heat-induced gelation. For example, spherical nanoparticles of CMP and

* Corresponding author.
E-mail address: andre.brodkorb@teagasc.ie (A. Brodkorb).

https://doi.org/10.1016/j.foodhyd.2020.106249
Received 30 May 2020; Received in revised form 31 July 2020; Accepted 8 August 2020
Available online 15 August 2020
0268-005X/© 2020 Elsevier Ltd. All rights reserved.

lactoferrin, an iron-binding glycoprotein, can be formed during heating, based on the electrostatic attraction of these two proteins at pH 5.0 (Bourbon et al., 2015); heating CMP or lactoferrin separately produced more branched particles rather than spherical particles. Other authors have shown that a complexation between CMP and the whey proteins occurs during heating, affecting the gelation behaviour and the strength of whey protein gels formed (Martinez, Farías, & Pilosof, 2010; Svanborg, Johansen, Abrahamsen, Schüller, & Skeie, 2016). This interaction depended on the pH of heating and the ratio of CMP to whey proteins. When β-lactoglobulin (β-lg) was heated with CMP at pH 6.7, the rate of denaturation of β-lg was accelerated by the presence of CMP (Croguennec et al., 2014); however, increasing the proportion of CMP reduced the size of the β-lg aggregates and the turbidity of the mixture. This interaction was pH-dependent and the authors postulated that the negative charges carried by the glycopeptide prevented extensive aggregation of β-lg. On the basis of these studies, more investigations are needed to establish and elucidate the mechanism of the potential chaperone-like activity of CMP on whey protein aggregation.

As a bioactive peptide, CMP is already incorporated in commercial products such as sports nutrition products (Pasin & Miller, 2000), diet supplements for infants (Lacprodan® CGMP-10, Arla Foods Ingredients, Viby J, Denmark), medical nutrition products (Ney et al., 2009) and products for treatment of dental caries (Neeser, 1991; Zhang & Gaffar, 2001). Modulation of the immune system response, improvement of learning abilities, promotion of the growth of the gut microbiota and prevention of bacterial and viral adhesion to cells are some of the most cited positive effects of CMP (Brody, 2000; Thomä-Worringer et al., 2006).

The present study aimed to assess the chaperone-like activity of CMP on whey protein aggregation and to determine the nature of the interactions formed before, during and after heating using integrated analytical approaches (i.e., rheology, microscopy and calorimetry). The range of protein contents used for nutritional beverages was tested (2.5–10, w/w, % protein) at pH 6.4 and 7.2, in the presence of a relatively high calcium content (9–18 mM) and at two heating rates (2 and 25 °C/min).

2. Materials and methods

2.1. Materials

The caseinomacropeptide (CMP) used in this study was supplied by Moorepark Technology Ltd. (Teagasc, Moorepark, Fermoy, Ireland). The CMP powder contained 87.4% CMP (w/w), 5.4% (w/w) moisture and 4.8% (w/w) ash, of which 0.72% (w/w) was calcium. Glycosylation affects the nitrogen-to-protein conversion factor for CMP; therefore the protein content of the CMP powder in this study was measured by deducting the moisture, fat, lactose and ash content from the dry matter content. This resulted in a conversion factor of 7.29, which was close to the values of 7.34 and 7.37 for the genetic variants of caseinomacropeptide A and B, respectively, calculated by Karman and Van Boekel (1986) on the basis of the amino acid sequence and taking into account the carbohydrate content.

The ratio of glycosylated to non-glycosylated CMP was analysed using anion-exchange chromatography (see section 2.3), with detection at 220 nm, following a modification of the method of Kreuß, Krause, and Kulozik (2008) and the chromatograms showed that the CMP was mostly glycosylated at pH 4.0 (Fig. 1). It is noteworthy that the degree of glycosylation of CMP is affected by the conditions of manufacture, in particular pH and intensity of pre-heat treatment (Siegert, Tolkach, & Kulozik, 2012). Whey protein isolate (WPI) was purchased from Davisco Bipro® (Davisco Food International, U.S.A.) and contained 91.9% (w/w) protein (Kjeldahl analysis, nitrogen to protein conversation factor of 6.38). The calcium content of the WPI powder was 0.11% (w/w). The denaturation level of the WPI powder was 8.5% (w/w). The mineral compositions of all dairy powders were measured by inductively coupled plasma mass spectrometry method (Reid et al., 2015). All reagents were purchased from Sigma Aldrich (St. Louis, Missouri, United States) unless stated otherwise.

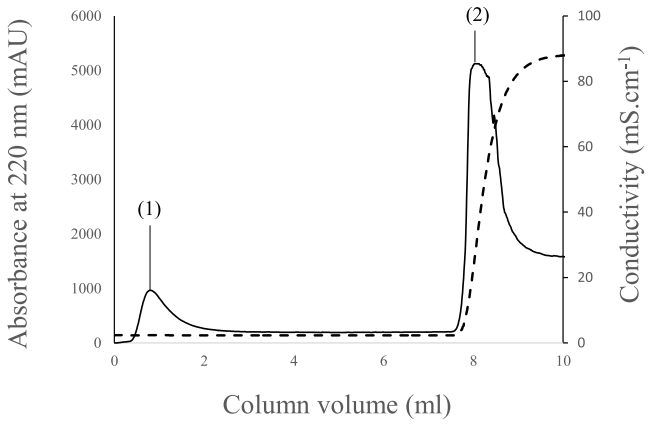


Fig. 1. Anion-exchange chromatography on a fast protein liquid chromatography system of (—) a solution of 0.125% (w/v) caseinomacropeptide (CMP). The peak labelled (1) corresponds to the non-glycosylated CMP and the peak labelled (2) corresponds to the glycosylated CMP. The equilibration buffer was 20 mM sodium acetate at pH 4.0, the elution buffer was 1 M NaCl and (– –) the change in conductivity is also shown on the chromatogram.

2.2. Protein rehydration

Solutions of 2.5 and 5% (w/v) whey protein, abbreviated as $[WP]_{2.5}$ and $[WP]_5$, and a mixture of whey proteins and CMP (abbreviated $[WP/CMP]_5$) at a whey protein:CMP ratio of 1:0.9 (w/w) and containing a total protein content of 5% (w/v), were reconstituted in MilliQ® Water at 40 °C for 2 h and, held overnight at 4 °C with 0.05% (w/v) of sodium azide to prevent microbial growth. Calcium chloride ($CaCl_2.2H_2O$) was added to achieve 9 mM of total calcium in the samples. In the same way, solutions of 5 and 10% (w/v) whey protein, abbreviated $[WP]_5$ and $[WP]_{10}$, and a mixture of whey protein and CMP (abbreviated $[WP/CMP]_{10}$) at a whey protein:CMP ratio of 1:0.9 (w/w) and containing a total protein content of 10% (w/v), were reconstituted in MilliQ® Water. Calcium chloride ($CaCl_2.2H_2O$) was added to achieve 18 mM total calcium in these samples.

The pH was adjusted for all samples to 6.4 or 7.2 using a large range of sodium hydroxide and hydrochloric acid concentrations (0.1 M–8 M) to limit the dilution of proteins. The pH of the samples was readjusted after 1 h stirring at room temperature. Fig. 2 summarizes the steps of rehydration, heating and analysis carried out on the solutions of whey proteins and CMP.

2.3. Anion-exchange chromatography (AEC)

A solution of 1 ml of 0.125% (w/v) CMP was analysed by anion-exchange chromatography, using a 5 ml-sepharose Hi Trap Q FF column (GE Healthcare, Chicago, IL, USA). The equilibration buffer A was 20 mM sodium acetate at pH 4.0 and the elution buffer B was 1 M NaCl. The elution gradient was 0% buffer B for 6 column volume (30 ml) then 100% buffer B for 4 column volume (20 ml), at a flow rate of 5 ml/min. The absorbance was monitored at 220 nm by an AKTA Purifier 10 system (GE Healthcare) connected to a computer with the software Unicorn 4.10 (GE Healthcare).

2.4. Oscillation rheology

2.4.1. Onset of gelation

The onset of gelation was measured on samples containing 2.5–5% (w/v) protein and 9 mM calcium at pH 6.4 or 7.2. An aluminium plate of

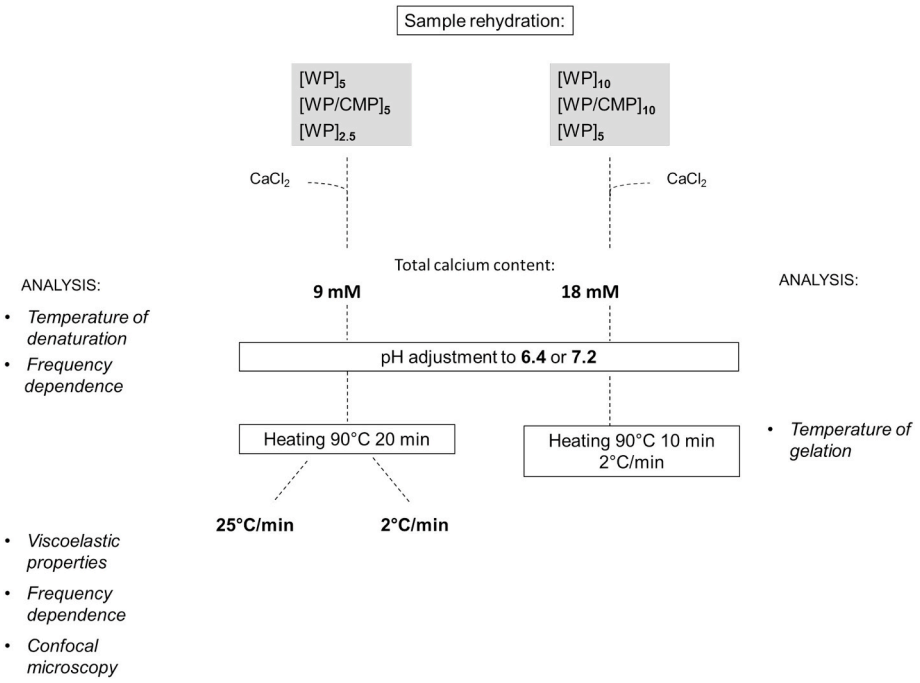


Fig. 2. Flowchart for the preparation and analysis of whey protein and caseinomacropeptide (CMP) mixtures. Solutions of 2.5, 5 or 10% (w/v) whey protein (abbreviated $[WP]_{2.5}$, $[WP]_5$ or $[WP]_{10}$, respectively) and mixtures of whey proteins and CMP containing 5 or 10% (w/v) total protein (abbreviated $[WP/CMP]_5$ or $[WP/CMP]_{10}$, respectively), were reconstituted in MilliQ® water. The mixtures contained CMP and whey proteins at a whey protein:CMP ratio of 1:0.9 (w/w).

diameter 60 mm was used together with a Peltier plate to measure the storage modulus, G′, the loss modulus, G″, and the loss tangent, δ, of the samples during heating and cooling. The parallel plate was covered with a solvent trap in order to prevent the evaporation during heating. The rheometer was an AR 2000ex from TA Instrument (New Castle, Delaware, USA) and the results were analysed with TA Instrument Data Analysis software (New Castle, Delaware, USA). The samples were maintained at 22 °C for 1 min of equilibration. Measurements were taken at 22 °C for 2 min, then the samples were heated to 90 °C at 2 °C/min or 25 °C/min and held at 90 °C for 20 min. Finally, all samples were cooled down to 22 °C at a rate of 10 °C/min and maintained at 22 °C for 1 min. The strain and frequency used were 0.02 and 1 Hz, respectively. The onset of gelation was arbitrarily determined at 0.4 Pa, where G′ increases steeply above the background noise, during heating for all samples.

2.4.2. Temperature of gelation

A multiple frequency temperature sweep was performed during the heating-up step and the holding step at 90 °C, on samples containing 5–10% (w/v) protein and 18 mM calcium, using the same equipment as above. The samples were subjected to the same heating process as that for the onset of gelation measurement, but the holding time at 90 °C was 10 min. In addition, the equipment collected the values of loss tangent δ point by point at each frequency. Therefore, the heating rate to 90 °C was decreased to 2 °C/min to allow a higher accuracy of the temperature recording. The frequency varied from 0.1 to 10.0 Hz and the strain was maintained at 0.02. The critical transition point from liquid to solid state was established by applying Winter-Chambon criteria of a gel transition point as indicated by the loss tangent becoming independent of frequency as a function of temperature (Winter & Chambon, 1986).

2.4.3. Frequency sweep

Before heating and after measuring the onset of gelation and the temperature of gelation of the samples, a frequency sweep was performed from 0.1 to 4 Hz on non-heated samples, 0.1–50 Hz on heated samples containing 2.5–5% (w/v) protein and 9 mM calcium, and 0.1–63 Hz on heated samples containing 5–10% (w/v) and 18 mM calcium. The strain amplitude (0.02) and the temperature (22 °C) were kept constant during the measurement. All experiments were conducted within the linear viscoelastic range. The storage modulus and the loss modulus were plotted (log-log plot) against the frequency and the value of the slope (n) of the storage modulus was reported as indices of the strength and nature of the molecular bonds forming the gel (Tunick, 2010).

2.5. Differential scanning calorimetry

For calorimetric measurements, 20–30 mg of liquid sample containing 2.5–10% (w/v) protein were placed into an aluminium pan and heated in parallel with an empty reference pan to 100 °C at 5 °C/min. Despite the starting concentration of the samples (2.5%) being relatively low, the denaturation peak for β-lg could still be identified. The peak of denaturation of α-lactalbumin (α-la) could not be identified in this study. The differential scanning calorimetry (DSC) equipment used for this experiment was a DSC Q2000 (TA Instrument, Newcastle, Delaware, USA) equipped with a refrigerator and was computer-interfaced. The thermograms were analysed by the software TA Universal Analysis (TA Instrument).

2.6. Confocal microscopy

After heating the samples at 90 °C for 20 min, at pH 6.4 or 7.2 and at a heating rate of 25 °C/min using an AR 2000ex rheometer (TA instrument), the gels were dyed with a 0.1% (w/v) Fast Green FCF solution, designed to specifically stain proteins, after which the samples were incubated in the dark for 20 min to allow for the penetration of the dye into the gels. The samples were analysed at room temperature on a confocal microscope Leica DM6000 B (Wetzlar, Germany) with a 63 × oil immersion objective (numerical aperture 1.40) at an excitation wavelength of 633 nm, provided by the He/Ne633 laser. Images were captured in 1024 × 1024 pixels.

2.7. Statistical analysis

All the experiments were carried out using the same batch of powder and the measurements were carried out on at least three independent replicates. The DSC and anion-exchange chromatography measurements were carried out on at least two independent replicates. Several microscopy images of one representative sample were recorded for each condition tested. One way ANOVA, post hoc Tukey tests were used and the results are presented as the mean ± SD. The superscripts indicate the statistical significance with $p < 0.05$.

3. Results and discussion

3.1. Interactions between CMP and whey proteins before heating

Fig. 3 presents the frequency dependence of mixtures of whey proteins and CMP (5%, w/v protein), and the frequency dependence of the control samples containing whey proteins only (2.5–5%, w/v) before heating. The storage (G′) and the loss modulus (G″) describe the elastic and viscous behaviour of a material in shear, respectively, and define the ability of a material to reverse its deformation. By keeping the amplitude constant and varying the frequency of oscillation during the measurement of G′ and G″, it is possible to vary the rate of internal deformation and estimate the rigidity of the network formed by the cross-linking of the proteins, *i.e.*, the strength of their bonds. For example, in a solution of polymers with a low degree of crosslinking, the molecules glide along each other at the lower frequencies and get entangled at the higher frequencies. Therefore, G′ will increase with frequency until reaching a maximum in rigidity. In contrast, the G′ of a strongly cross-linked gel will be relatively constant for the whole frequency range because the interactions between the molecules make it impossible for them to glide along each other without destruction of the network (Mezger, 2006; Tunick, 2010). A power law can apply to the log-log plot of frequency *vs.* G′, whereby the slope (n) is used to describe the network of proteins, with a value close to 0 describing a very cross-linked gel, and a slope value closer to 1 being characteristic of a weak physical gel (Sharma, Munro, Dessev, & Wiles, 2016; Tunick, 2010).

For all non-heated samples, G′ was higher than G″ (results not shown) over most of the frequency range, which is representative of a dominant elastic behaviour and could be due to the relatively high protein content of the samples (5%, w/v) and the presence of calcium (9 mM). Similar phenomena have been observed before in the same range of frequencies and whey protein concentration in whey protein concentrate solutions (Meza, Verdini, & Rubiolo, 2009). Because of the dominance of the elastic behaviour in all samples, the frequency-dependence of G′ only was presented in Fig. 3. At pH 6.4 or 7.2, the G′ values of the mixtures of CMP and whey protein were more frequency-dependent than those of the control samples containing whey protein only (Fig. 3), with n values of 0.4–0.5 and 0.2–0.3, respectively. In addition, the G′ value of the mixture at pH 7.2 was lower than that of the control samples containing whey protein only across the entire frequency range (Fig. 3). This suggests that whey proteins formed a network at room temperature that was disrupted by the presence of CMP. As the whey proteins and CMP are negatively charged at pH 6.4 and 7.2, additional electrostatic repulsion provided by CMP could explain this result.

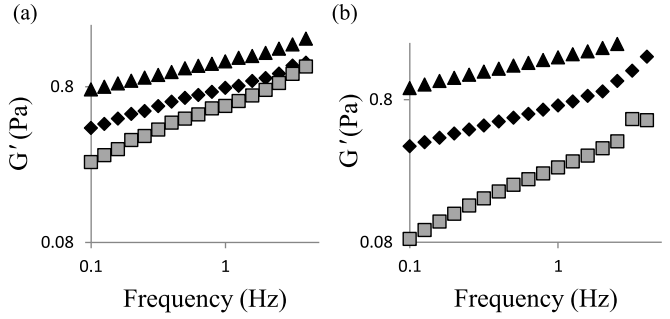


Fig. 3. Storage modulus (G′) as a function of the frequency at (a) pH 7.2 or (b) 6.4 for non-heated samples containing (◆) 5% (w/v) whey protein, (▲) 2.5% (w/v) whey protein or (■) a mixture of whey proteins and caseinomacropeptide (CMP) at a total protein level of 5% (w/v). The whey protein:CMP ratio in the mixtures was 1:0.9 (w/w). The experiment was performed in three independent replicates.

Interactions between β-lg and CMP before heating were proposed previously, through the formation of aggregates of β-lg and CMP, as measured by dynamic light scattering at pH 7.0 (Martinez et al., 2010); the latter authors suggested that the whey proteins and CMP interacted *via* electrostatic interactions. It is possible that these interactions affected the formation and the final structure of the heat-induced whey protein aggregates through changes in the conformation of whey proteins. However, Croguennec et al. (2014) did not find any major change in the fluorescence of β-lg in the presence of CMP or evidence of interactions, as measured by isothermal titration calorimetry (ITC), at pH 6.7; the authors concluded that CMP contributes mainly to the denaturation of whey protein when β-lg is already unfolded (Croguennec et al., 2014).

3.2. Effect of CMP on the gelation of whey proteins

Fig. 4 shows a typical profile of the heat-induced changes in G′, G″ and the loss tangent, δ, in a 5% (w/v) whey protein sample and in a mixture of whey proteins and CMP. G′ and G″ values increased on

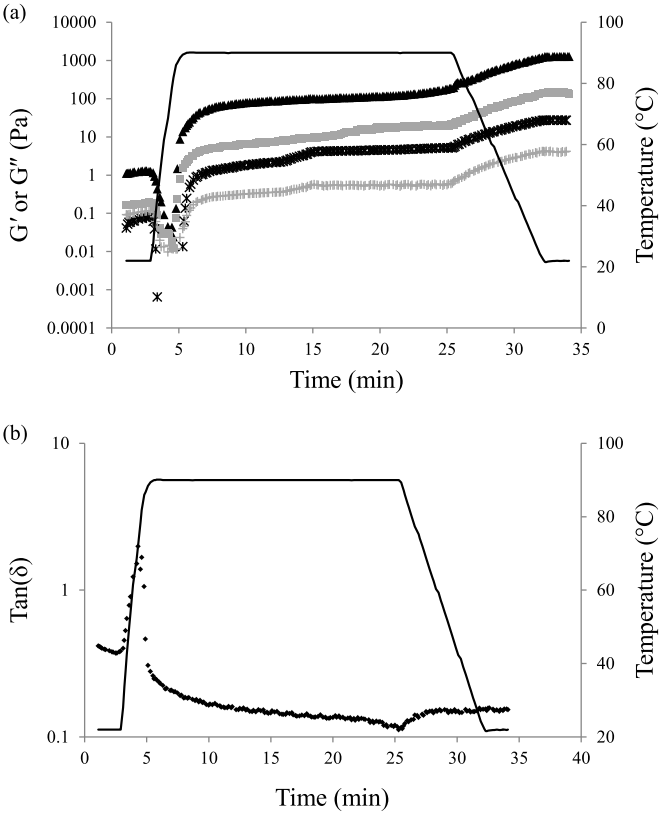


Fig. 4. Typical profile of (a) (▲) storage modulus (G′) and (■) loss modulus (G″) of a 5% (w/v) whey protein solution and (✱) G′ and (+) G″values of a mixture of whey proteins and caseinomacropeptide (CMP) at a total protein content of 5% (w/v) during heat treatment. All samples were heated at 90 °C for 20 min at pH 7.2. The whey protein:CMP ratio in the mixture was 1:0.9 (w/w). (b) Typical profile of the loss tangent δ in all samples tested. The temperature was represented by a continuous line. The heating rate was 25 °C/min.

Table 1
Viscoelastic properties of solutions containing 2.5–5% (w/v) whey protein (abbreviated $[WP]_5$ and $[WP]_{2.5}$) and a mixture of caseinomacropeptide (CMP) and whey proteins ($[WP/CMP]_5$) at a total protein content of 5% (w/v) and a whey protein:CMP ratio of 1:0.9 (w/w). All samples contained 9 mM calcium and were heated at 90 °C for 20 min at a heating rate of 25 °C/min. The experimental data were the average of at least three independent replicates. The superscripts indicate significant differences with $p < 0.05$.

	pH	G′ after holding at 90 °C for 20 min (Pa)	G′ after cooling to 22 °C (Pa)[a]	Gelation onset (min)[b]	n value[c]
$[WP]_5$	7.2	175 ± 44 [a]	1117 ± 137 [a]	4.8 ± 0.1 [a]	0.09 ± 0.01 [a]
$[WP/CMP]_5$	7.2	7 ± 3 [b]	38 ± 9 [b]	5.7 ± 0.1 [b]	0.15 ± 0.04 [b]
$[WP]_{2.5}$	7.2	66 ± 33 [c]	253 ± 91 [c]	4.9 ± 0.3 [a]	0.09 ± 0.01 [ab]
$[WP]_5$	6.4	339 ± 113 [d]	NA	4.5 ± 0.2 [a]	0.10 ± 0.00 [ab]
$[WP/CMP]_5$	6.4	124 ± 4 [e]	NA	4.9 ± 0.1 [a]	0.09 ± 0.01 [a]
$[WP]_{2.5}$	6.4	22 ± 8 [bc]	NA	4.9 ± 0.4 [a]	0.11 ± 0.01 [ab]

[a] G′ is the storage modulus of the sample. The samples heated at pH 6.4 could not be analysed after cooling, as the geometry became embedded in the gels (NA).
[b] The gelation onset was determined at the point at which the storage modulus (G′) increased sharply above the background noise and reached 0.4 Pa.
[c] The multiple frequency measurement was taken after heating at 90 °C for 20 min and cooling to 22 °C, with frequency varying from 0.1 to 50 Hz. A power law can apply to the log-log plot of frequency against G′ and the n value corresponds to the slope of each curve.

heating from 22 to 90 °C and reached a plateau when the temperature was maintained at 90 °C for 20 min. The moduli increased further, but to a minor extent, during cooling (from around 200 to 1000 Pa in 5% (w/v) whey protein sample). G′ values were higher than G″ before, during and after heating. The peak of the loss tangent (δ) indicated that the gelation was heat-induced, with a reinforcement of the elastic component during cooling. This phenomenon has been observed previously and has been attributed to the strengthening of hydrogen and van der Waals interactions during cooling (Lefèvre & Subirade, 2000; Martinez et al., 2010).

Table 1 presents the viscoelastic properties of the mixtures of whey proteins and CMP (5%, w/v protein) and those of the control samples containing whey protein only (2.5–5%, w/v). The samples were all heated at 90 °C for 20 min, at pH 6.4 or 7.2, and at a heating rate of 25 °C/min. At pH 7.2, the G′ values of the mixture of whey protein and CMP were considerably lower than those of the controls, before and after cooling (Table 1 and Fig. 4). This could be due to additional electrostatic repulsion during heating provided by CMP, which could have limited the extent of whey protein aggregation. Previous authors reported that the presence of CMP affected the viscoelastic properties of gels of whey proteins, with a slower increase in the storage modulus of whey protein concentrate gels during heating at pH 7.0, and a significant reduction in gel strength (Svanborg et al., 2016; Xianghe, Pan, Peilong, Ismail, & Voorts, 2012).

The onset of gelation of whey proteins was determined at 0.4 Pa, corresponding to a distinct steep increase in G′. At pH 7.2, the onset of gelation of the mixtures of whey proteins and CMP was delayed by 1 min, compared to that of the controls containing whey protein only (Table 1 and Fig. 4). This effect of CMP on the onset of gelation was not observed at pH 6.4, possibly due to a larger difference in the surface charges between the whey proteins and CMP, leading to greater attractive interactions. This may also be responsible for the higher G′ and the lower frequency-dependence of the mixture at pH 6.4 than that at pH 7.2 (Table 1). In agreement with our findings, Croguennec et al. (2014) reported that the denaturation kinetics and gelation of whey proteins in the presence of CMP depended partly on electrostatic interactions, which were modulated by the pH of heating.

To determine accurately the exact temperature of gelation of the proteins, and also due to the limited sensitivity of the equipment used in this study, the protein content of the samples and the total calcium content were increased up to 10% (w/v) and 18 mM, respectively. This was performed in a proportional manner to maintain the same calcium: protein ratio, which has been reported to be more important in influencing the heat-induced aggregation rate than the concentration of protein or calcium *per se* (Sherwin & Foegeding, 1997). During heating, G′ value was recorded as a function of the frequency from 0.1 to 10 Hz, with the frequencies from 5.1 to 10 Hz being the most adequate for the measurement of the temperature of gelation. The temperature at which the loss tangent (δ) was independent of the frequency, i.e., the temperature at which the proteins formed a strong network, was defined as the temperature of gelation (Fig. 5).

The viscoelastic properties of these samples are reported in Table 2. Whether the controls contained 5 or 10% (w/v) whey protein, the temperatures of gelation were between 68.5 and 72.1 °C; thus, these temperatures may be characteristic of the whey proteins at the pH studied. At pH 6.4 or 7.2, the presence of CMP increased the temperature required for gelation of whey proteins by up to 7 °C, confirming that CMP had a chaperone-like activity on the aggregation of whey proteins (Table 2). Previously, the temperature of gelation of a mixture of CMP and β-lg in the same ratio (1:1) was reported to be around 75 °C, at pH 7.0 (Martinez et al., 2010). This value is close to that obtained in the present study (75.7 °C). However, the gelation temperature of the control sample containing β-lg only (88 °C) was much higher than that measured in the present study for whey protein samples; therefore, the authors concluded that β-lg was less heat stable in the presence of CMP during heating. This can be explained by differences in heating conditions, in particular in terms of concentration and composition of proteins and minerals between the studies (Mahmoudi, Mehalebi, Nicolai, Durand, & Riaublanc, 2007). It is noteworthy that the lower value of G′ of the mixtures at pH 7.2 was not observed at 10% total protein (Table 2), which could be due to a greater extent of aggregation of whey proteins at higher protein concentration (Mehalebi, Nicolai, & Durand, 2008). The higher temperature of gelation of the mixtures, compared to those of the samples containing whey protein only, could be due to additional electrostatic repulsions provided by the negatively-charged CMP. Greater repulsions between proteins could hinder the intermolecular interactions necessary for the heat-induced formation of a solid network.

The strength and nature of the bonds between proteins after heating was determined by plotting G′ as a function of frequency. The n values are reported in Tables 1 and 2; when the samples were heated at 25 °C/

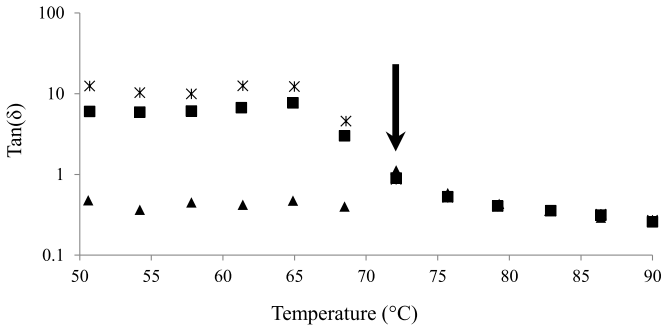


Fig. 5. Typical profile of the loss tangent (δ) of a solution of 10% (w/v) whey protein as a function of the temperature, measured at (▲) 5.1, (■) 7.5 and (✳) 10.0 Hz. The arrow indicates the gel point of the sample, i.e., the collapse of the loss tangent values at the temperature of gelation of the sample.

Table 2
Temperature of gelation and viscoelastic properties of heated solutions containing 5–10% (w/v) whey protein (abbreviated $[WP]_5$ and $[WP]_{10}$) or a mixture of caseinomacropeptide (CMP) and whey proteins (abbreviated $[WP/CMP]_{10}$) at 10% (w/v) total protein content. All samples contained 18 mM calcium and were heated at 90 °C for 10 min at pH 6.4 or 7.2. The whey protein:CMP ratio in the mixtures was 1:0.9 (w/w). The heating rate was 2 °C/min. The experimental data were the average of at least three independent replicates. The superscripts indicate significant differences with $p < 0.05$.

	pH	Temperature of gelation (°C)	G′ after holding at 90 °C for 10 min (Pa) [a]	Frequency slope of G′ [b]
$[WP]_{10}$	7.2	71.2 ± 1.8^{ab}	1637 ± 358^{a}	0.10 ± 0.00^{a}
$[WP/CMP]_{10}$	7.2	75.7 ± 0.0^{c}	80 ± 23^{b}	0.10 ± 0.01^{a}
$[WP]_5$	7.2	68.5 ± 0.0^{a}	125 ± 11^{b}	0.08 ± 0.00^{a}
$[WP]_{10}$	6.4	69.7 ± 2.1^{ab}	1511 ± 165^{a}	0.08 ± 0.01^{a}
$[WP/CMP]_{10}$	6.4	75.7 ± 0.0^{c}	69 ± 21^{b}	0.09 ± 0.01^{a}
$[WP]_5$	6.4	72.1 ± 0.0^{b}	44 ± 12^{b}	0.11 ± 0.03^{a}

[a] G′ is the storage modulus of the samples and was measured at 2.575 Hz.
[b] The multiple frequency measurement was taken after heating at 90 °C for 10 min and cooling to 22 °C, with frequency varying from 0.1 to 63 Hz.

min, the presence of CMP did not affect the frequency dependence of the gels. The low values of n (around 0.1) indicated that all gels, with or without CMP, were highly cross-linked.

The structure of the gels formed on heating 2.5–5% (w/v) protein at a rate of 25 °C/min and holding at 90 °C for 20 min was also analysed using confocal microscopy, with the proteins being selectively stained using Fast Green (Fig. 6). The microscopy images revealed a fine-stranded gel structure in the samples that contained whey protein only (Fig. 6 a,c, d and f), and an even finer gel structure for the samples containing CMP (Fig. 6 b and e). A fine-stranded structure is expected at pH values greater than 6.0 for heat-induced gels of whey proteins. However, the differences in the gel networks between the samples containing whey protein only and the mixtures of CMP and whey proteins were not reflected in the frequency sweep measurement (Table 1). At pH 7.2, a finer structure of the strands could explain the lower G′ of the mixtures after heating (Table 1), whereas at pH 6.4, the storage modulus of the mixture was not lower than that of the control containing 2.5% (w/v) whey protein only (Table 1), despite a clear difference in gel structure (Fig. 6 b); admittedly, the details of the fine-strands could not be captured by the confocal microscope, due to its limited resolution. For example, the structure of the strands and the interactions between the strands could be affected by the reduction in negative charges on the whey proteins when lowering the pH from 7.2 to 6.4 in the presence of CMP, and could explain a higher G′ at pH 6.4 than that at pH 7.2. Nevertheless, the presence of CMP modified the temperature of gelation of the whey proteins and altered the network of the whey protein gels.

Morand, Guyomarc'h, and Famelart (2011) reported a smaller fractal dimension (Df) of around 1.1 for the whey protein and κ-casein

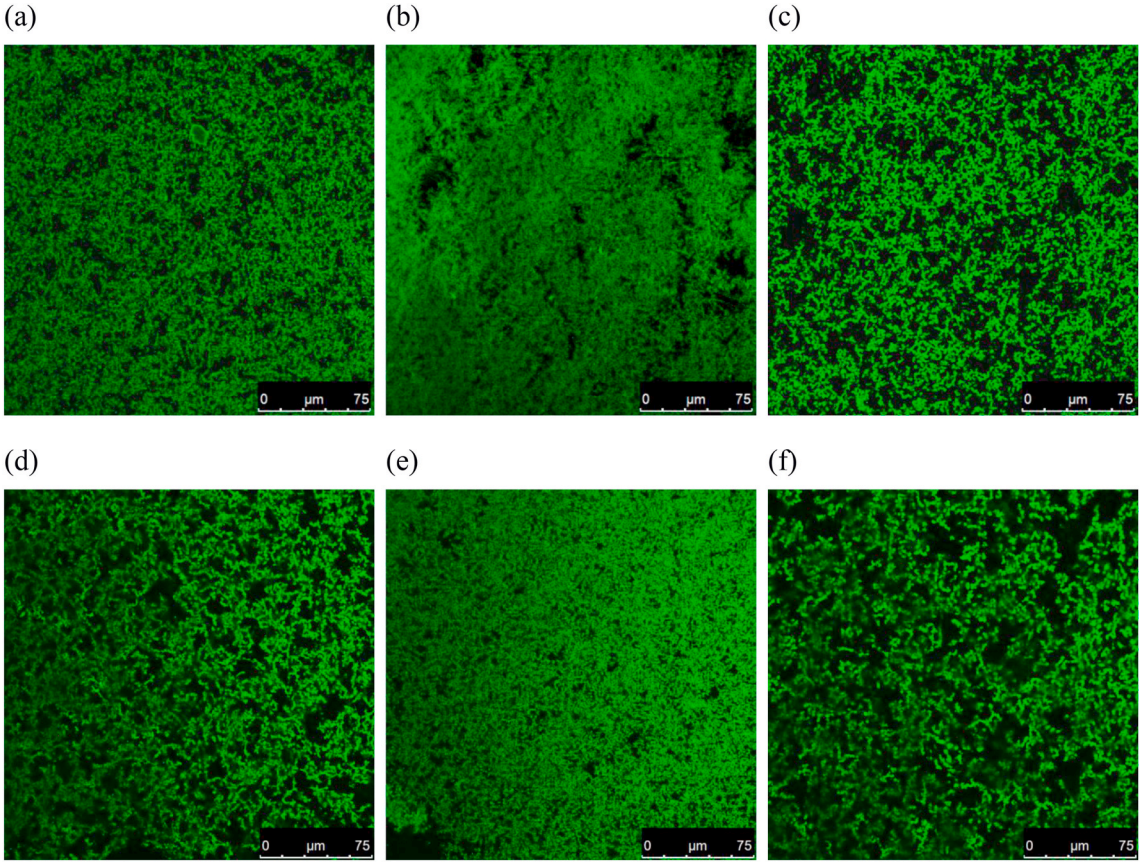


Fig. 6. Confocal microscopy images of gels formed after heating (a,d) 5% whey protein solution, (b,e) a mixture of whey proteins and caseinomacropeptide (CMP) at 5% (w/v) total protein (w/v) and (c,f) 2.5% (w/v) whey protein solution at 90 °C for 20 min, and at (a,b,c) pH 7.2 or (d,e,f) pH 6.4. The heating rate was 25 °C/min and the proteins were selectively stained green using 0.1% (w/v) of Fast Green FCF. The whey protein:CMP ratio in the mixtures was 1:0.9 (w/w). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

aggregates formed during aggressive heating (80 °C for 24 h in 0.1 M NaCl), while the Df of those made of whey proteins only was around 2, i.e., the aggregates containing κ-casein were more thread-like. κ-Casein carries a pole of highly-negative charge in its C-terminus region, due to negatively charged amino acids and post-translational modification. This pole of negative charges could be responsible for the preferential strand-like orientation of the whey protein and κ-casein aggregates. Hence, those structural properties, also shared by CMP, could impact the final structure of the whey protein and CMP-based aggregates. In addition, Xianghe et al. (2012) found that CMP decreased the number of disulphide bonds formed by whey proteins during heating. Finally, the glycosylation of CMP may modify the water-holding capacity of the protein network during heating and affect the final structure of the gel (Guyomarc'h et al., 2009).

3.3. Effect of CMP on the denaturation of whey proteins

The denaturation of the whey proteins at pH 6.4 or 7.2, with or without CMP, was analysed by DSC (Figs. 7 and 8). One endothermic peak was observed, with an onset of denaturation around 60 °C and a maximum at 71–79 °C (Fig. 7), which corresponds to the denaturation temperature of β-lg and may partly overlap with the peak of denaturation of α-la, which is reported to have a maximum around 65 °C (Patel, Kilara, Huffman, Hewitt, & Houlihan, 1990).

The temperature of denaturation for all samples decreased with increasing pH at heating (Fig. 8). This can be explained by increased intramolecular repulsions when the whey proteins were heated at a pH further away from their isoelectric point, and higher thiol reactivity promoting the formation of irreversible disulphide bonds above pH 6.0 (Hoffmann & van Mil, 1997; Verheul, Roefs, & de Kruif, 1998). All mixtures containing CMP exhibited a higher temperature of denaturation than those of the controls, except for the mixtures containing the lowest protein content (5%, w/v) and heated at pH 7.2 (Fig. 8). This is in agreement with the report of Svanborg et al. (2016) of a higher denaturation peak at pH 7.0 for the whey proteins in the presence of CMP (Svanborg et al., 2016). However, previous authors found that the temperature of denaturation of β-lg decreased in the presence of CMP (Martinez, Sanchez, Patino, & Pilosof, 2009). In addition, the kinetics of denaturation of β-lg are accelerated in the presence of CMP at pH 6.7 (Croguennec et al., 2014). The differences from the results reported by these previous studies could be due to differences in proteins present and mineral profile between samples used in different studies. A higher temperature of denaturation could contribute to the delay in gelation observed in the mixtures of whey proteins and CMP.

As noted above, the increase in temperature of denaturation in the presence of CMP was not observed at pH 7.2 for the mixtures containing a lower protein content (5%, w/v), suggesting that the pH of heating and the protein content are the major factors influencing whey protein denaturation in this study (Fig. 8 a).

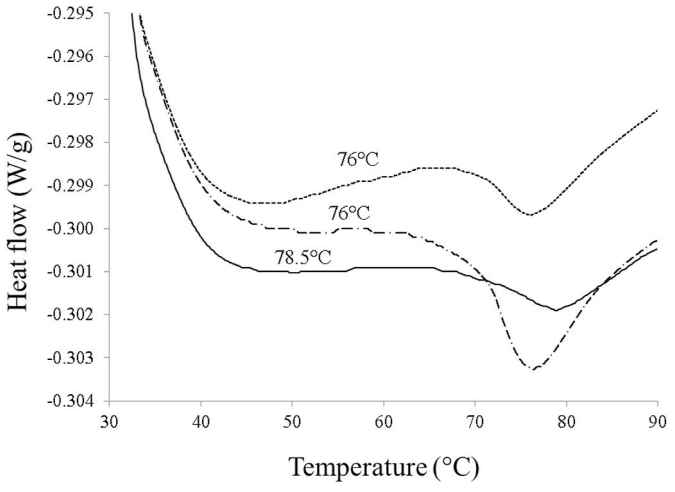


Fig. 7. Typical thermograms of (-·-·-) 5% (w/v) whey protein, (——) a mixture of whey proteins and caseinomacropeptide (CMP) at 5% (w/v) total protein and (·····) 2.5% (w/v) whey protein containing 9 mM calcium at pH 6.4. The whey protein:CMP ratio in the mixtures was 1:0.9 (w/w). Above the lines, the temperature of denaturation is indicated for each endothermic peak.

3.4. Effect of heating rate on the interactions between CMP and whey proteins

Two different heating rates (2 and 25 °C/min) were applied to the samples containing 2.5–5% protein and 9 mM calcium. The samples were heated at 90 °C for 20 min and cooled to 22 °C, then a frequency sweep was performed (Fig. 9).

As reported in the previous section, all samples showed frequency-independent behaviour (n = 0.09–0.15) after heating at 25 °C/min, indicative of the formation of highly cross-linked protein gel with permanent covalent bonds (Table 1). However, the mixtures of whey proteins and CMP at pH 7.2 exhibited high frequency dependency (n close to 1) after heating at a slower rate (2 °C/min), whereas the control samples containing only whey proteins remained frequency-independent (n = 0.1–0.2). The samples containing CMP were liquid in appearance, while the samples containing whey proteins formed a soft white gel.

Previous authors have reported that decreasing the heating rate can affect the gelation of proteins. Stading and Hermansson (1990) found that the temperature of gelation of β-lg was lower when decreasing the heating rate from 1 to 0.01 °C/min, at pH 2.5, 6.5 or 7.5 and assumed that a slower heating rate gives the time necessary for the protein network to develop. Relkin, Eynard, and Launay (1992) reported that the denaturation of β-lg at acidic pH values (3.5) was partially reversible at heating rates above 10 °C/min. The authors suggested that only a slower heating rate gives enough time to the proteins to complete the formation of intermolecular disulphide bonds, leading to the irreversibility of their denaturation. Stading, Langton, and Hermansson (1992) reported that β-lg gels formed at pH 7.5 on heating at a rate of less than 5 °C/min had a lower storage modulus than those formed at faster heating rates. In that study, the cross-links of proteins observed by electron microscopy appeared weaker and the strands of β-lg were shorter and thicker than those formed at faster heating rates. The storage modulus of the networks formed in these conditions was frequency-dependent. In contrast, in a later study, it was reported that the gels of β-lg formed at pH 5.3 developed a higher storage modulus when the heating rate was slowed down, but the frequency dependence of the gels was not affected (Stading, Langton, & Hermansson, 1993). Thus, the pH-dependent nature of the whey protein interactions played a major role in controlling the interactions between the molecules of β-lg at slower heating rates.

This is in agreement with the results of the present study, as the frequency-dependence of the mixtures of whey proteins and CMP was only affected by the heating rate at pH 7.2 (Fig. 9). These results suggest that the interactions taking place between CMP and whey proteins are modified by a slower heating rate. Higher electrostatic repulsion provided by CMP could be enhanced by the changes induced by a slow heating rate around neutral pH, in particular conformational changes in whey proteins and the nature of protein-protein interactions. Croguennec et al. (2014) highlighted the role of electrostatic interactions in the denaturation and aggregation of β-lg in the presence of CMP. In contrast to the results presented by Stading et al. (1992), the frequency dependence of the samples containing whey protein only was not affected by a slower heating rate in the present study (Fig. 9), likely due to the differences in protein and mineral composition and heating conditions.

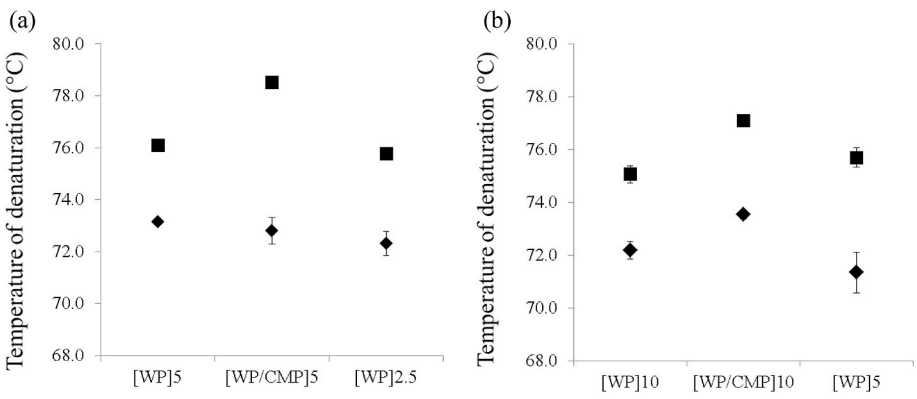


Fig. 8. Temperature of denaturation of β-lactoglobulin measured by differential scanning calorimetry at (◆) pH 7.2 or (■) 6.4 in (a) solutions containing 9 mM calcium and 2.5 or 5% (w/v) whey protein (abbreviated $[WP]_{2.5}$ or $[WP]_5$, respectively) or a mixture of whey proteins and caseinomacropeptide (CMP) at 5% (w/v) total protein (abbreviated $[WP/CMP]_5$) and (b) solutions containing 18 mM calcium and 5 or 10% (w/v) whey protein (abbreviated $[WP]_5$ or $[WP]_{10}$, respectively) or a mixture of whey proteins and CMP at 10% (w/v) total protein (abbreviated $[WP/CMP]_{10}$). The whey protein:CMP ratio in the mixtures was 1:0.9 (w/w). The heating rate was 5 °C/min. The experimental points were the average of at least two independent replicates.

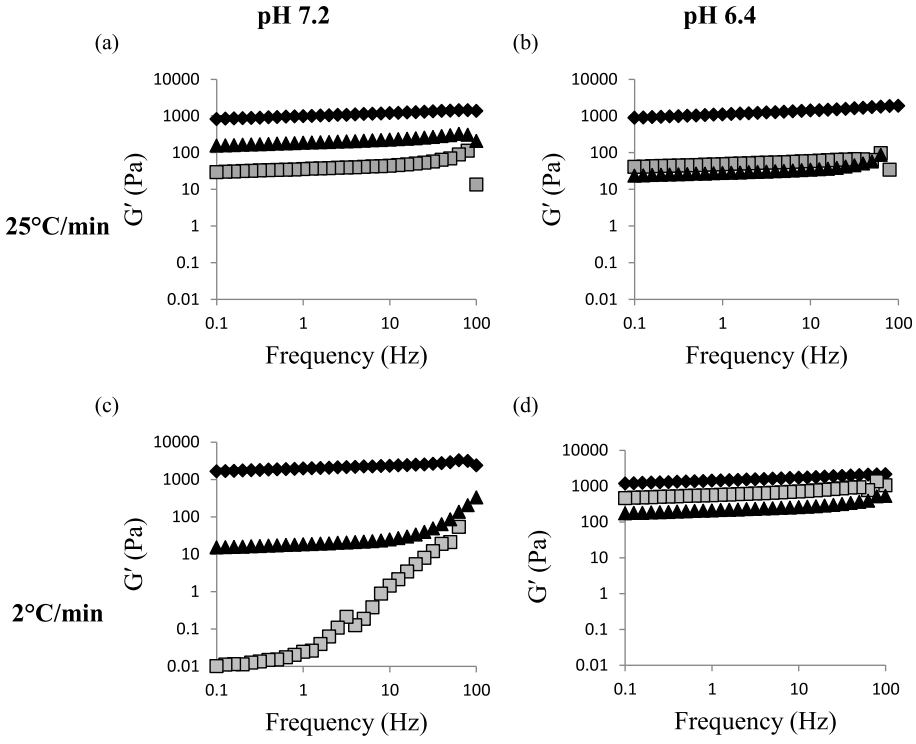


Fig. 9. Storage modulus G′ as a function of frequency for samples containing (◆) 5% (w/v) whey protein, (▣) a mixture of caseinomacropeptide and whey proteins at 5% total protein (w/v) or (▲) 2.5% (w/v) whey protein, after heating at 90 °C for 20 min at a heating rate of (a,b) 25 °C/min and (c,d) 2 °C/min at pH 7.2 (a,c) or 6.4 (b,d). The whey protein:CMP ratio in the mixtures was 1:0.9 (w/w). The experiment was performed in at least three independent replicates.

4. Conclusion

CMP displayed a chaperone-like activity for whey protein aggregation, giving a higher temperature of gelation of whey protein solutions at pH 6.4 and 7.2 in the presence of CMP. At pH 7.2, the presence of CMP decreased the storage modulus of the gels, and modulation of the heating rate further influenced the interactions between whey proteins and CMP, interrupting the formation of a solid gel. These modifications of the rheological properties of whey proteins, combined with the health-promoting properties of CMP, could be particularly useful for the formulation of heat stable dairy beverages, or protein gels, with tailored physicochemical, health benefiting and sensory characteristics. From that perspective, the influence of protein composition and concentration, heat-load and salt environment on the mechanism of interaction of CMP and whey proteins still need further investigation.

CRediT authorship contribution statement

Sophie J. Gaspard: Conceptualization, Investigation, Methodology, Data curation, Writing - original draft, Writing - review & editing. **Prateek Sharma:** Conceptualization, Methodology, Data curation, Writing - review & editing. **Ciarán Fitzgerald:** Methodology. **John T. Tobin:** Conceptualization, Methodology, Writing - review & editing. **James A. O'Mahony:** Supervision, Conceptualization, Writing - review & editing. **Alan L. Kelly:** Supervision, Conceptualization, Writing - review & editing. **André Brodkorb:** Supervision, Project administration, Funding acquisition, Writing - review & editing.

Declaration of competing interest

None.

Acknowledgements

The authors would like to thank Helen Slattery, Bernard Corrigan and Yihong Chen for carrying out protein and mineral analysis on the powders and Laura G. Gomez-Mascaraque for her help with confocal microscopy. This work was supported by the Dairy Levy Research Trust (Dairy Research Ireland, funded by Irish Dairy Farmers, Project MDDT6261 "ProPart"). S.J. Gaspard was funded under the Teagasc Walsh Fellowship Scheme (reference number 2012211). P. Sharma received funding from the EU Horizon 2020 Research and Innovation Programme through Enterprise Ireland under the Marie Skłodowska-Curie grant agreement No. 713654.

References

Bourbon, A. I., Pinheiro, A. C., Carneiro-da-Cunha, M. G., Pereira, R. N., Cerqueira, M. A., & Vicente, A. A. (2015). Development and characterization of lactoferrin-GMP nanohydrogels: Evaluation of pH, ionic strength and temperature effect. *Food Hydrocolloids, 48*, 292–300.

Brody, E. P. (2000). Biological activities of bovine glycomacropeptide. *British Journal of Nutrition, 84*(S1), 39–46.

Croguennec, T., Leng, N., Hamon, P., Rousseau, F., Jeantet, R., & Bouhallab, S. (2014). Caseinomacropeptide modifies the heat-induced denaturation–aggregation process of β-lactoglobulin. *International Dairy Journal, 36*(1), 55–64.

Gaspard, S. J., Auty, M. A. E., Kelly, A. L., O'Mahony, J. A., & Brodkorb, A. (2017). Isolation and characterisation of κ-casein/whey protein particles from heated milk protein concentrate and role of κ-casein in whey protein aggregation. *International Dairy Journal, 73*, 98–108.

Guyomarc'h, F., Nono, M., Nicolai, T., & Durand, D. (2009). Heat-induced aggregation of whey proteins in the presence of κ-casein or sodium caseinate. *Food Hydrocolloids, 23*(4), 1103–1110.

Hoffmann, M. A. M., & van Mil, P. J. J. M. (1997). Heat-induced aggregation of β-lactoglobulin: role of the free thiol group and disulfide bonds. *Journal of Agricultural and Food Chemistry, 45*(8), 2942–2948.

Karman, A. H., & Van Boekel, M. A. J. S. (1986). Evaluation of the Kjeldahl factor for conversion of the nitrogen content of milk and milk products to protein content. *Netherlands Milk and Dairy Journal, 40*, 315–336.

Kreuß, M., Krause, I., & Kulozik, U. (2008). Separation of a glycosylated and non-glycosylated fraction of caseinomacropeptide using different anion-exchange stationary phases. *Journal of Chromatography A, 1208*(1–2), 126–132.

Lefèvre, T., & Subirade, M. (2000). Molecular differences in the formation and structure of fine-stranded and particulate β-lactoglobulin gels. *Biopolymers, 54*(7), 578–586.

Liyanaarachchi, W. S., Ramchandran, L., & Vasiljevic, T. (2015). Controlling heat induced aggregation of whey proteins by casein inclusion in concentrated protein dispersions. *International Dairy Journal, 44*, 21–30, 0.

Mahmoudi, N., Mehalebi, S., Nicolai, T., Durand, D., & Riaublanc, A. (2007). Light-scattering study of the structure of aggregates and gels formed by heat-denatured whey protein isolate and β-lactoglobulin at neutral pH. *Journal of Agricultural and Food Chemistry, 55*(8), 3104–3111.

Martinez, M. J., Farías, M. E., & Pilosof, A. M. R. (2010). The dynamics of heat gelation of casein glycomacropeptide – β-lactoglobulin mixtures as affected by interactions in the aqueous phase. *International Dairy Journal, 20*(9), 580–588.

Martinez, M. J., Sanchez, C. C., Patino, J. M., & Pilosof, A. M. (2009). Interactions in the aqueous phase and adsorption at the air-water interface of caseinoglycomacropeptide (GMP) and β-lactoglobulin mixed systems. *Colloids and Surfaces B: Biointerfaces, 68*(1), 39–47.

Mehalebi, S., Nicolai, T., & Durand, D. (2008). Light scattering study of heat-denatured globular protein aggregates. *International Journal of Biological Macromolecules, 43*(2), 129–135.

Meza, B. E., Verdini, R. A., & Rubiolo, A. C. (2009). Viscoelastic behaviour of heat-treated whey protein concentrate suspensions. *Food Hydrocolloids, 23*(3), 661–666.

Mezger, T. G. (2006). *The rheology handbook: For users of rotational and oscillatory rheometers*. Vincentz Network GmbH & Co KG.

Mikkelsen, T. L., Frøkiær, H., Topp, C., Bonomi, F., Iametti, S., Picariello, G., … Barkholt, V. (2005). Caseinomacropeptide self-association is dependent on whether the peptide is free or restricted in κ-casein. *Journal of Dairy Science, 88*(12), 4228–4238.

Morand, M., Guyomarc'h, F., & Famelart, M.-H. (2011). How to tailor heat-induced whey protein/kappa-casein complexes as a means to investigate the acid gelation of milk-a review. *Dairy Science & Technology, 91*(2), 97–126.

Neeser, J.-R. (1991). *United States Patent No, 4*, 992,420.

Ney, D. M., Gleason, S. T., van Calcar, S. C., MacLeod, E. L., Nelson, K. L., Etzel, M. R., … Wolff, J. A. (2009). Nutritional management of PKU with glycomacropeptide from cheese whey. *Journal of Inherited Metabolic Disease, 32*(1), 32–39.

Pasin, G., & Miller, S. (2000). *US whey products and sports nutrition. Applications monograph*. USA: US Dairy exports council. www.usdec.org.

Patel, M. T., Kilara, A., Huffman, L. M., Hewitt, S. A., & Houlihan, A. V. (1990). Studies on whey protein concentrates. 1. Compositional and thermal properties. *Journal of Dairy Science, 73*(6), 1439–1449.

Reid, M., O'Donovan, M., Elliott, C. T., Bailey, J. S., Watson, C. J., Lalor, S. T. J., … Lewis, E. (2015). The effect of dietary crude protein and phosphorus on grass-fed dairy cow production, nutrient status, and milk heat stability. *Journal of Dairy Science, 98*(1), 517–531.

Relkin, P., Eynard, L., & Launay, B. (1992). Thermodynamic parameters of β-lactoglobulin and α-lactalbumin. A DSC study of denaturation by heating. *Thermochimica Acta, 204*(1), 111–121.

Saito, T., & Itoh, T. (1992). Variations and distributions of O-glycosidically linked sugar chains in bovine κ-casein. *Journal of Dairy Science, 75*(7), 1768–1774.

Sharma, P., Munro, P. A., Dessev, T. T., & Wiles, P. G. (2016). Shear work induced changes in the viscoelastic properties of model Mozzarella cheese. *International Dairy Journal, 56*, 108–118.

Sherwin, C. P., & Foegeding, E. A. (1997). The effect of $CaCl_2$ on aggregation of whey proteins. *Milchwissenschaft, 52*(2), 93–96.

Siegert, N., Tolkach, A., & Kulozik, U. (2012). The pH-dependent thermal and storage stability of glycosylated caseinomacropeptide. *Lebensmittel-Wissenschaft & Technologie, 47*(2), 407–412.

Smith, M. H., Edwards, P. J., Palmano, K. P., & Creamer, L. K. (2002). Structural features of bovine caseinomacropeptide A and B by ^{1}H nuclear magnetic resonance spectroscopy. *Journal of Dairy Research, 69*(1), 85–94.

Stading, M., & Hermansson, A.-M. (1990). Viscoelastic behaviour of β-lactoglobulin gel structures. *Food Hydrocolloids, 4*(2), 121–135.

Stading, M., Langton, M., & Hermansson, A.-M. (1992). Inhomogeneous fine-stranded β-lactoglobulin gels. *Food Hydrocolloids, 6*(5), 455–470.

Stading, M., Langton, M., & Hermansson, A.-M. (1993). Microstructure and rheological behaviour of particulate β-lactoglobulin gels. *Food Hydrocolloids, 7*(3), 195–212.

Svanborg, S., Johansen, A.-G., Abrahamsen, R. K., Schüller, R. B., & Skeie, S. B. (2016). Caseinomacropeptide influences the functional properties of a whey protein concentrate. *International Dairy Journal, 60*, 14–23.

Swaisgood, H. E. (2003). Proteins, parts A. In P. F. Fox, & P. L. H. McSweeney (Eds.), *Advanced dairy chemistry: Volume 1* (p. 1346). Springer US.

Thomä-Worringer, C., Sørensen, J., & López-Fandiño, R. (2006). Health effects and technological features of caseinomacropeptide. *International Dairy Journal, 16*(11), 1324–1333.

Tunick, M. H. (2010). Small-strain dynamic rheology of food protein networks. *Journal of Agricultural and Food Chemistry, 59*(5), 1481–1486.

Verheul, M., Roefs, S. P. F. M., & de Kruif, K. G. (1998). Kinetics of heat-induced aggregation of β-lactoglobulin. *Journal of Agricultural and Food Chemistry, 46*(3), 896–903.

Winter, H. H., & Chambon, F. (1986). Analysis of linear viscoelasticity of a crosslinking polymer at the gel point. *Journal of Rheology, 30*(2), 367–382.

Xianghe, M., Pan, Q., Peilong, S., Ismail, A. A., & Voorts, F. R.v. d. (2012). Impact of caseinomacropeptide on heat-induced gel strength of neutral whey protein concentrates: Model system study. *Milchwissenschaft, 67*(1), 47–51.

Zhang, Y. P., & Gaffar, A. (2001). *United States of America patent No. US* (Vol. 6, p. 207). W. I. P. Organization.