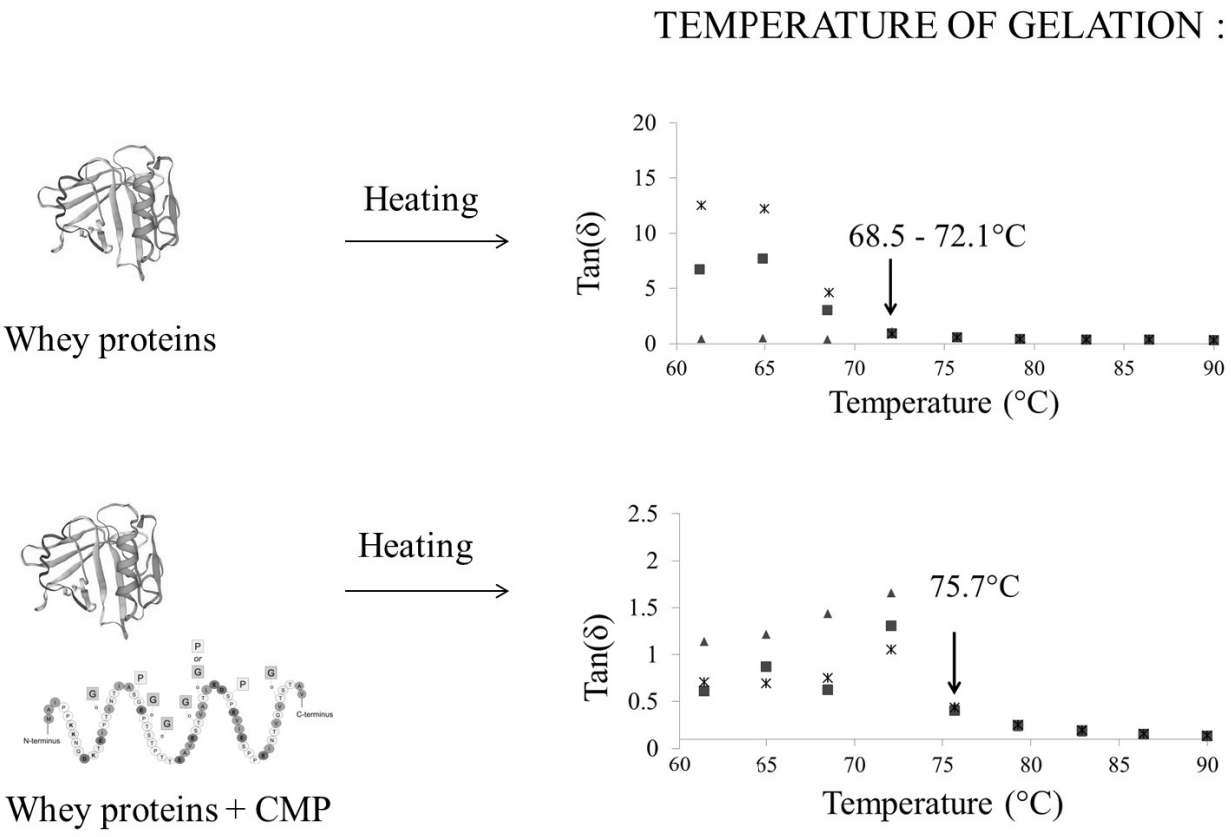
Highlights:

- The presence of CMP increased the temperature of denaturation of β-lactoglobulin by up to 3°C.
- The presence of CMP increased the temperature of gelation of whey proteins by up to 7°C.
- The presence of CMP during heating affected the structure of the heat-induced whey protein gels and resulted in a finer stranded structure.

Graphical abstract:



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

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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, 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 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 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

76 the growth of the gut microbiota and prevention of bacterial and viral adhesion to cells are
77 some of the most cited positive effects of CMP (Brody, 2000; Thomä-Worringer et al., 2006).

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

84

2. Material 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 214 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, and 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.

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 to 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) and 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 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 AR2000ex 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 to 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

157 loss tangent becoming independent of frequency as a function of temperature (Winter &
158 Chambon, 1986).

159 ### *2.4.3 Frequency sweep*

160 Before heating and after measuring the onset of gelation and the temperature of gelation of
161 the samples, a frequency sweep was performed from 0.1 to 4 Hz on non-heated samples, 0.1
162 to 50 Hz on heated samples containing 2.5-5% (w/v) protein and 9 mM calcium, and 0.1 to
163 63 Hz on heated samples containing 5-10% (w/v) and 18 mM calcium. The strain amplitude
164 (0.02) and the temperature (22°C) were kept constant during the measurement. All
165 experiments were conducted within the linear viscoelastic range. The storage modulus and
166 the loss modulus were plotted (log-log plot) against the frequency and the value of the slope
167 (n) of the storage modulus was reported as indices of the strength and nature of the molecular
168 bonds forming the gel (Tunick, 2010).

169 ## *2.5 Differential scanning calorimetry*

170 For calorimetric measurements, 20-30 mg of liquid sample containing 2.5-10% (w/v) protein
171 were placed into an aluminium pan and heated in parallel with an empty reference pan to
172 100°C at 5°C/min. Despite the starting concentration of the samples (2.5%) being relatively
173 low, the denaturation peak for β-lg could still be identified. The peak of denaturation of
174 α-lactalbumin (α-la) could not be identified in this study. The differential scanning
175 calorimetry (DSC) equipment used for this experiment was a DSC Q2000 (TA Instrument,
176 Newcastle, Delaware, USA) equipped with a refrigerator and was computer-interfaced. The
177 thermograms were analysed by the software TA Universal Analysis (TA Instrument).

178 ## *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 AR2000ex 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 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 conditions 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 the 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

219 frequency-dependence of G′ only was presented in Fig. 3. At pH 6.4 or 7.2, the G′ values of
220 the mixtures of CMP and whey protein were more frequency-dependent than those of the
221 control samples containing whey protein only (Fig. 3), with n values of 0.4-0.5 and 0.2-0.3,
222 respectively. In addition, the G′ value of the mixture at pH 7.2 was lower than that of the
223 control samples containing whey protein only across the entire frequency range (Fig. 3). This
224 suggests that whey proteins formed a network at room temperature that was disrupted by the
225 presence of CMP. As the whey proteins and CMP are negatively charged at pH 6.4 and 7.2,
226 additional electrostatic repulsion provided by CMP could explain this result.

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

237 *3.2 Effect of CMP on the gelation of whey proteins*

238 Fig. 4 shows a typical profile of the heat-induced changes in G′, G″ and the loss tangent, δ, in
239 a 5% (w/v) whey protein sample and in a mixture of whey proteins and CMP. G′ and G″
240 values increased on heating from 22 to 90°C and reached a plateau when the temperature was
241 maintained at 90°C for 20 min. The moduli increased further, but to a minor extent, during
242 cooling (from around 200 to 1000 Pa in 5% (w/v) whey protein sample). G′ values were

243 higher than G″ before, during and after heating. The peak of the loss tangent (δ) indicated that
244 the gelation was heat-induced, with a reinforcement of the elastic component during cooling.
245 This phenomenon has been observed previously and has been attributed to the strengthening
246 of hydrogen and van der Waals interactions during cooling (Lefèvre & Subirade, 2000;
247 Martinez et al., 2010).

248 Table 1 presents the viscoelastic properties of the mixtures of whey proteins and CMP (5%,
249 w/v protein) and those of the control samples containing whey protein only (2.5-5%, w/v).
250 The samples were all heated at 90°C for 20 min, at pH 6.4 or 7.2, and at a heating rate of
251 25°C/min. At pH 7.2, the G′ value of the mixture of whey protein and CMP was considerably
252 lower in the presence of CMP, compared to those of the controls, before and after cooling
253 (Table 1 and Fig. 4). This could be due to additional electrostatic repulsion during heating
254 provided by CMP, which could have limited the extent of whey protein aggregation. Previous
255 authors reported that the presence of CMP affected the viscoelastic properties of gels of whey
256 proteins, with a slower increase in the storage modulus of whey protein concentrate gels
257 during heating at pH 7.0, and a significant reduction in gel strength (Svanborg et al., 2016;
258 Xianghe, Pan, Peilong, Ismail, & Voorts, 2012).

259 The onset of gelation of whey proteins was determined at 0.4 Pa, corresponding to a distinct
260 steep increase in G′. At pH 7.2, the onset of gelation of the mixtures of whey proteins and
261 CMP was delayed by one minute, compared to that of the controls containing whey protein
262 only (Table 1 and Fig. 4). This effect of CMP on the onset of gelation was not observed at pH
263 6.4, possibly due to a larger difference in the surface charges between the whey proteins and
264 CMP, leading to greater attractive interactions. This may also be responsible for the higher G′
265 and the lower frequency-dependence of the mixture at pH 6.4 than that at pH 7.2 (Table 1). In
266 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′ 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 were reported in Table 1 and 2; when the samples were heated at 25°C/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 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 (Fig. 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 b). 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 b).

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

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

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

373 Previous authors have reported that decreasing the heating rate can affect the gelation of
374 proteins. Stading and Hermansson (1990) found that the temperature of gelation of β-lg was
375 lower when decreasing the heating rate from 1 to 0.01°C/min, at pH 2.5, 6.5 or 7.5 and
376 assumed that a slower heating rate gives the time necessary for the protein network to
377 develop. Relkin, Eynard, and Launay (1992) reported that the denaturation of β-lg at acidic
378 pH values (3.5) was partially reversible at heating rates above 10°C/min. The authors
379 suggested that only a slower heating rate gives enough time to the proteins to complete the
380 formation of intermolecular disulphide bonds, leading to the irreversibility of their
381 denaturation. Stading, Langton, and Hermansson (1992) reported that β-lg gels formed at pH
382 7.5 on heating at a rate of less than 5°C/min had a lower storage modulus than those formed
383 at faster heating rates. In that study, the cross-links of proteins observed by electron
384 microscopy appeared weaker and the strands of β-lg were shorter and thicker than those
385 formed at faster heating rates. The storage modulus of the networks formed in these
386 conditions was frequency-dependent. In contrast, in a later study, it was reported that the gels
387 of β-lg formed at pH 5.3 developed a higher storage modulus when the heating rate was

388 slowed down, but the frequency dependence of the gels was not affected (Stading, Langton,
389 & Hermansson, 1993). Thus, the pH-dependent nature of the whey protein interactions
390 played a major role in controlling the interactions between the molecules of β-lg at slower
391 heating rates.

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

403

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.

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529

530

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 the statistical significance with $p < 0.05$.

	pH	G′ after 90°C for 20min (Pa)	G′ after cooling to 22°C (Pa)*	Gelation onset (min)**	n value***
$[WP]_5$	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}

* 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).

** 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.

*** 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.

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 the statistical significance with $p < 0.05$.

	pH	Temperature of gelation (°C)	G′ after 90°C for 10min (Pa) *	Frequency slope of G′ *
$[WP]_{10}$	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}

* G′ is the storage modulus of the samples and was measured at 2.575 Hz.

** 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.

Figure captions:

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 1M NaCl and (– –) the change in conductivity is also shown on the chromatogram.

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).

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 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.

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″ of a mixture of whey proteins and

583 caseinomacropeptide (CMP) at a total protein content of 5% (w/v) during heat treatment. All
584 samples were heated at 90°C for 20 min at pH 7.2. The whey protein:CMP ratio in the
585 mixture was 1:0.9 (w/w). (b) Typical profile of the loss tangent δ in all samples tested. The
586 temperature was represented by a continuous line. The heating rate was 25°C/min.

587 Fig. 5

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

592 Fig. 6

593 Confocal microscopy images of gels formed after heating (a,d) 5% whey protein solution,
594 (b,e) a mixture of whey proteins and caseinomacropeptide (CMP) at 5% (w/v) total protein
595 (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
596 (d,e,f) pH 6.4. The heating rate was 25°C/min and the proteins were selectively stained green
597 using 0.1% (w/v) of Fast Green FCF. The whey protein:CMP ratio in the mixtures was 1:0.9
598 (w/w).

599 Fig. 7

600 Typical thermograms of (– · – · –) 5% (w/v) whey protein, (——) a mixture of whey proteins
601 and caseinomacropeptide (CMP) at 5% (w/v) total protein and (·····) 2.5% (w/v) whey
602 protein containing 9 mM calcium at pH 6.4. The whey protein:CMP ratio in the mixtures was
603 1:0.9 (w/w). Above the lines, the temperature of denaturation is indicated for each
604 endothermic peak.

605 Fig. 8

606 Temperature of denaturation of β-lactoglobulin measured by differential scanning calorimetry
607 at (◆) pH 7.2 or (■) 6.4 in (a) solutions containing 9 mM calcium and 2.5 or 5% (w/v) whey
608 protein (abbreviated $[WP]_{2.5}$ or $[WP]_5$, respectively) or a mixture of whey proteins and
609 caseinomacropeptide (CMP) at 5% (w/v) total protein (abbreviated $[WP/CMP]_5$) and (b)
610 solutions containing 18 mM calcium and 5 or 10% (w/v) whey protein (abbreviated $[WP]_5$ or
611 $[WP]_{10}$, respectively) or a mixture of whey proteins and CMP at 10% (w/v) total protein
612 (abbreviated $[WP/CMP]_{10}$). The whey protein:CMP ratio in the mixtures was 1:0.9 (w/w).
613 The heating rate was 5°C/min. The experimental points were the average of at least two
614 independent replicates.

615 Fig. 9
616 Storage modulus G′ as a function of frequency for samples containing (◆) 5% (w/v) whey
617 protein, (□) a mixture of caseinomacropeptide and whey proteins at 5% total protein (w/v) or
618 (▲) 2.5% (w/v) whey protein, after heating at 90°C for 20 min at a heating rate of (a,b)
619 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
620 mixtures was 1:0.9 (w/w). The experiment was performed in at least three independent
621 replicates.

622

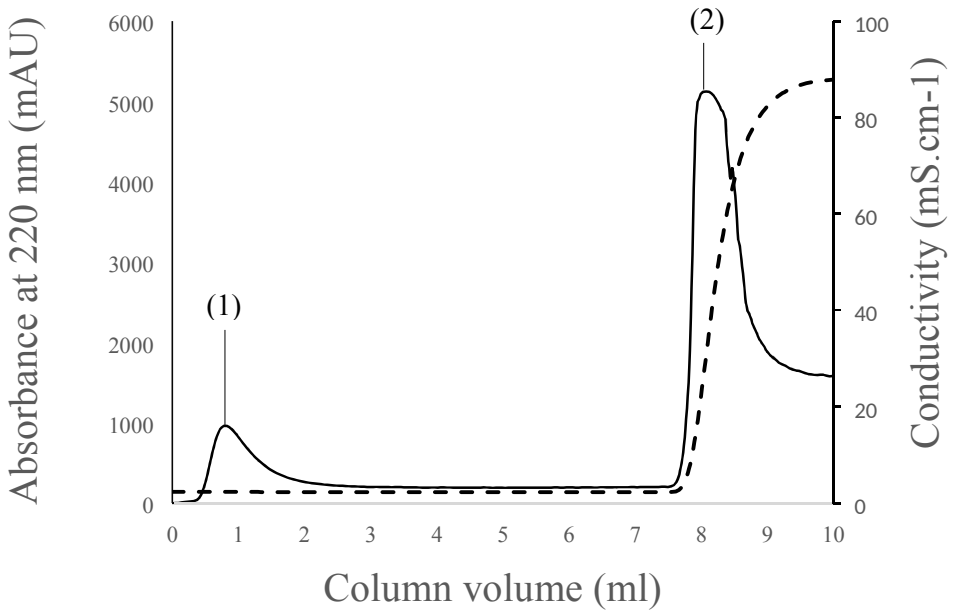

Absorbance at 220 nm (mAU)
6000
5000
4000
3000
2000
1000
0
(1)
(2)
100
80
60
40
20
0
Conductivity (mS.cm-1)
0 1 2 3 4 5 6 7 8 9 10
Column volume (ml)

Fig. 1

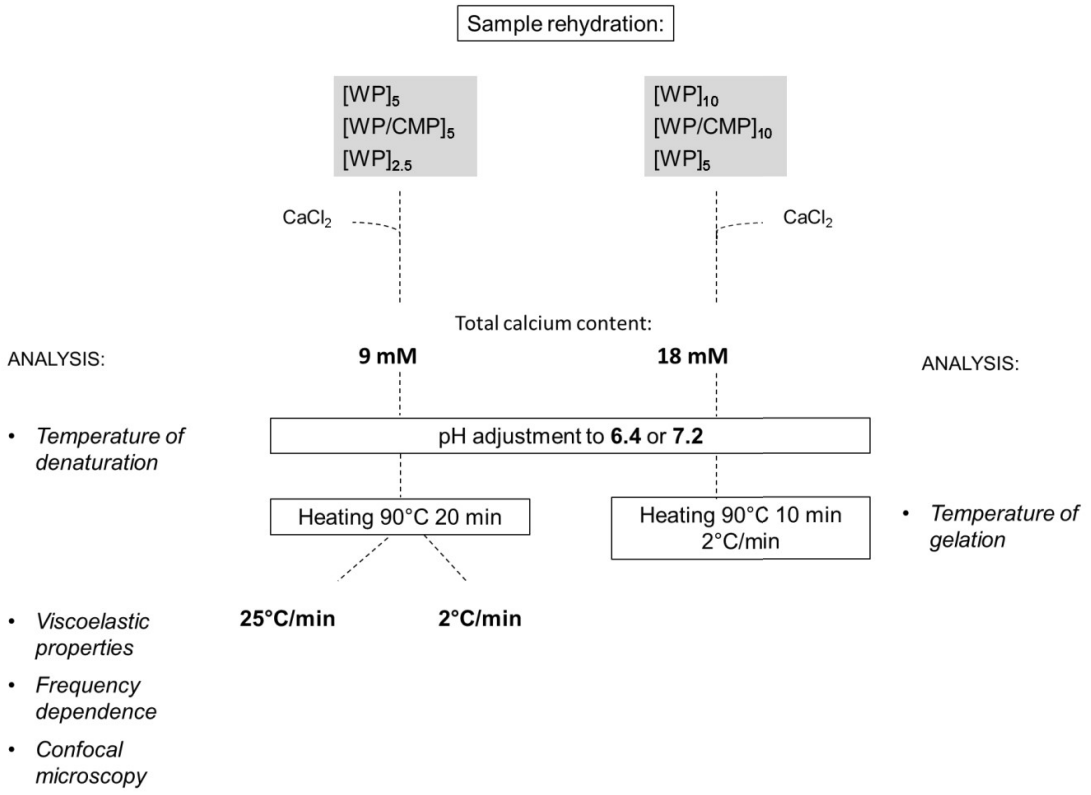
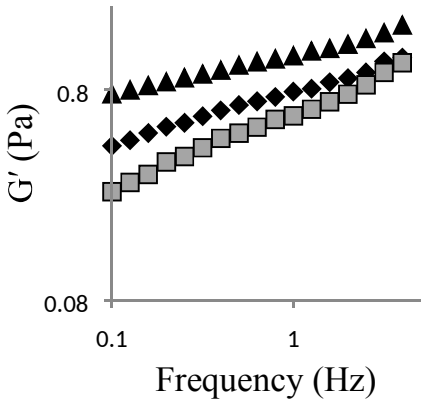

Sample rehydration:
[WP]5
[WP/CMP]5
[WP]2.5
[WP]10
[WP/CMP]10
[WP]5
CaCl2
CaCl2
Total calcium content:
ANALYSIS:
9 mM
18 mM
ANALYSIS:
• Temperature of denaturation
pH adjustment to 6.4 or 7.2
Heating 90°C 20 min
Heating 90°C 10 min
2°C/min
• Temperature of gelation
25°C/min
2°C/min
• Viscoelastic properties
• Frequency dependence
• Confocal microscopy

Fig. 2

(a)



(b)

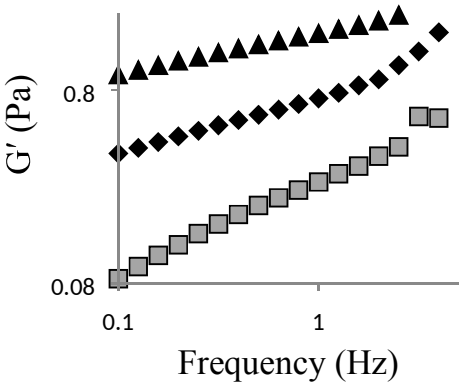
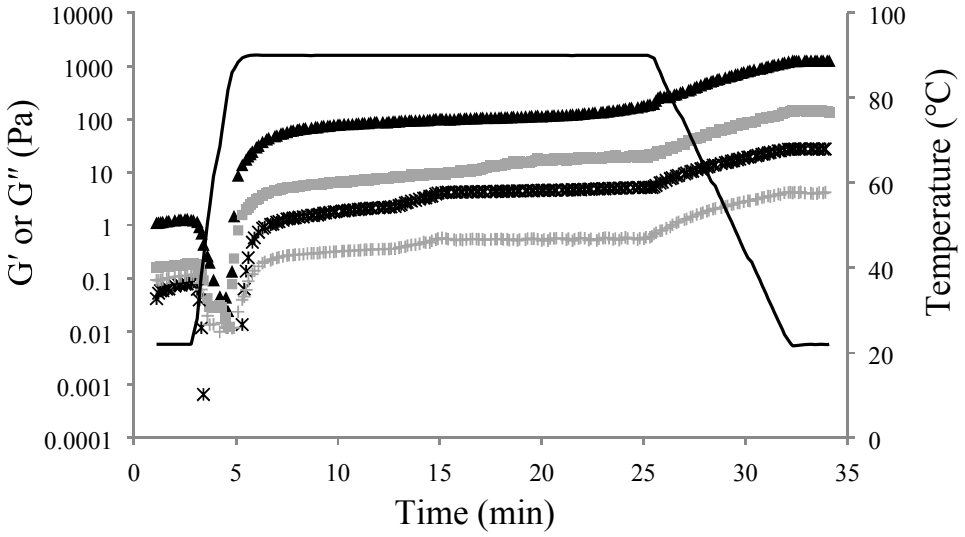


Fig. 3

(a)


10000
1000
100
10
1
0.1
0.01
0.001
0.0001
G′ or G″ (Pa)
0
5
10
15
20
25
30
35
Time (min)
100
80
60
40
20
0
Temperature (°C)

(b)

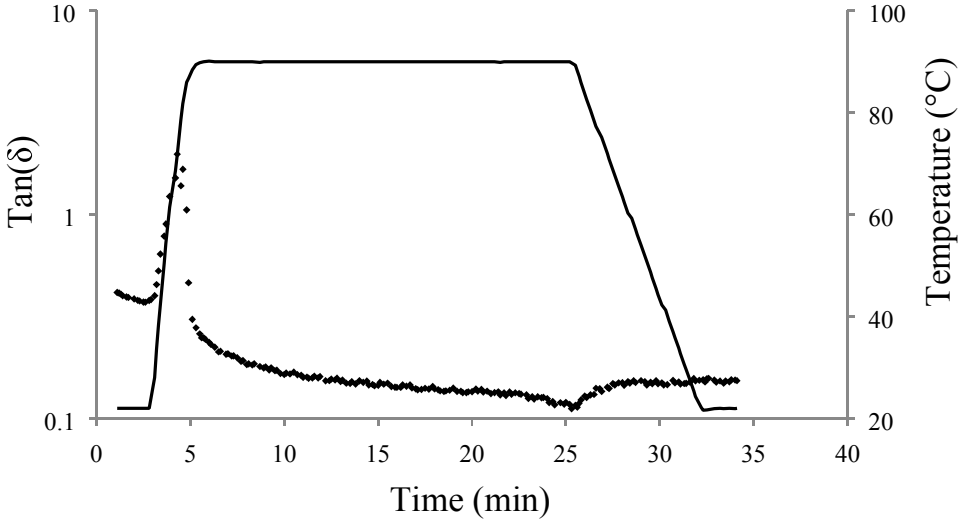

10
1
0.1
Tan(δ)
0
5
10
15
20
25
30
35
40
Time (min)
100
80
60
40
20
Temperature (°C)

Fig. 4

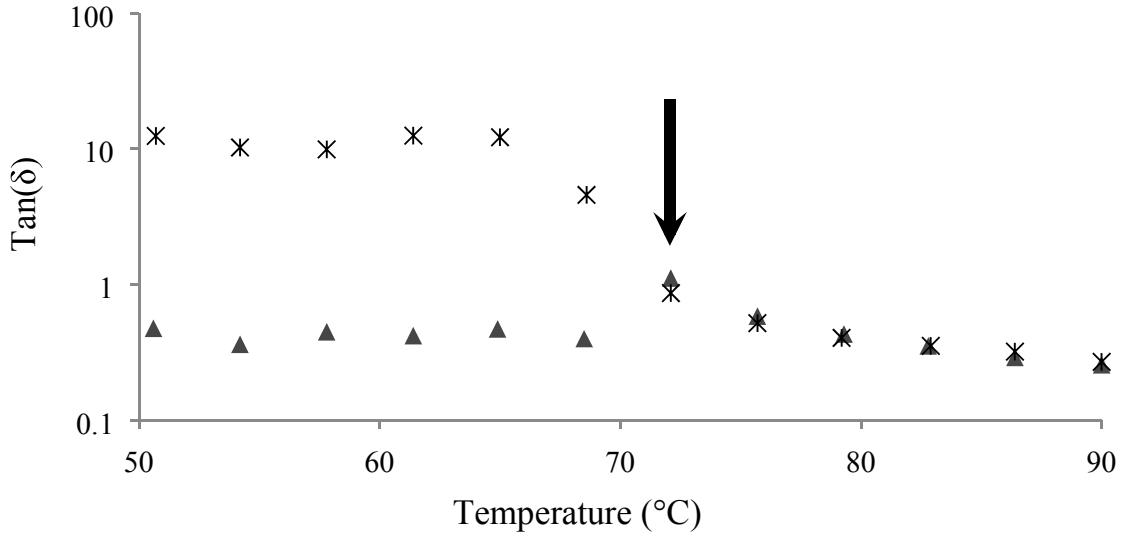


Fig. 5

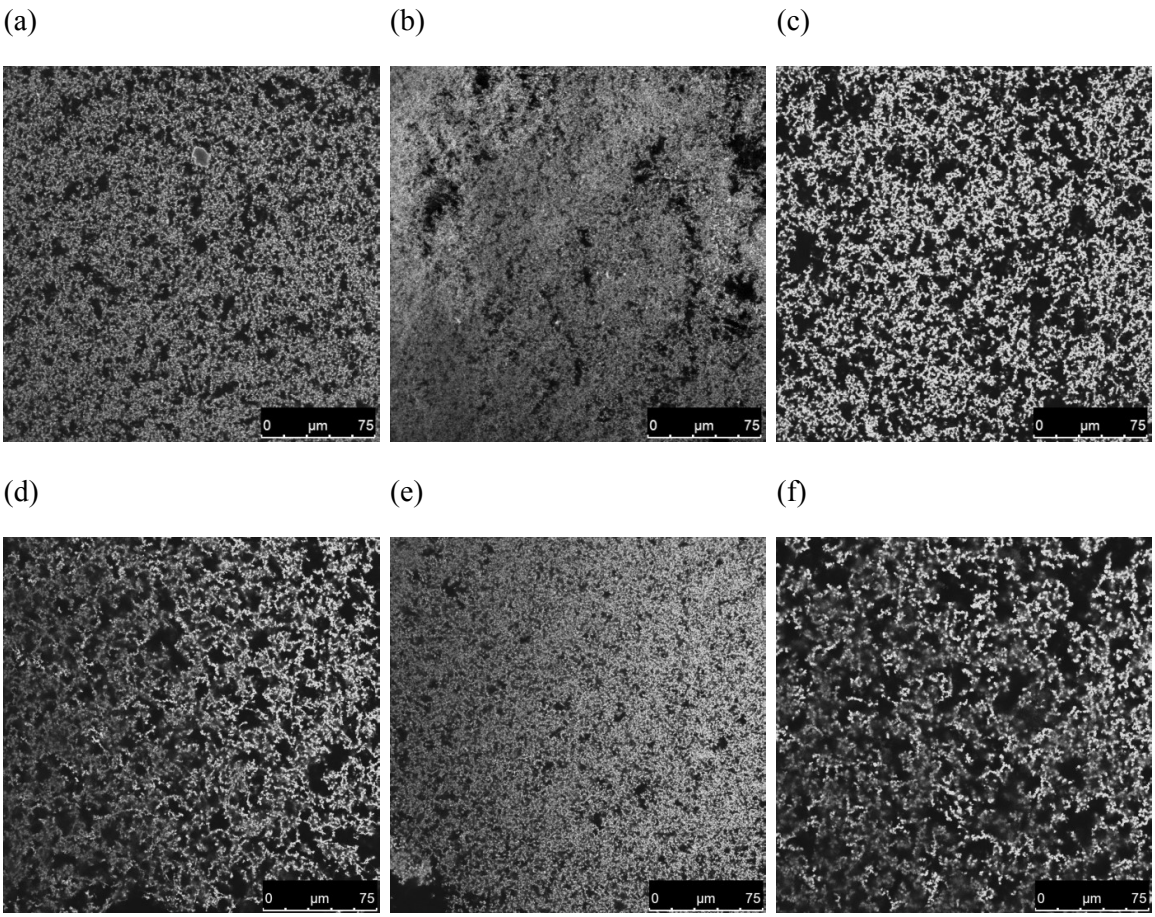


Fig. 6

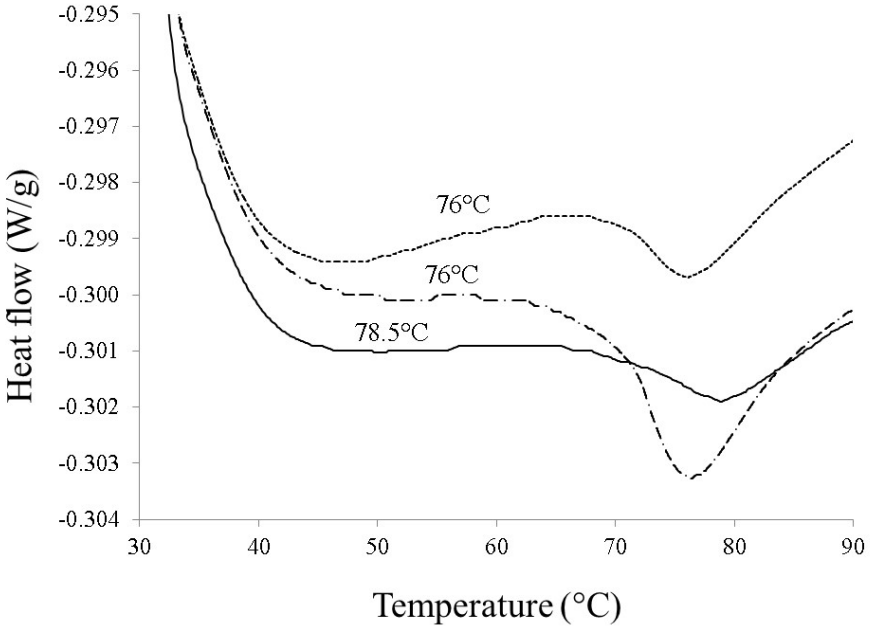
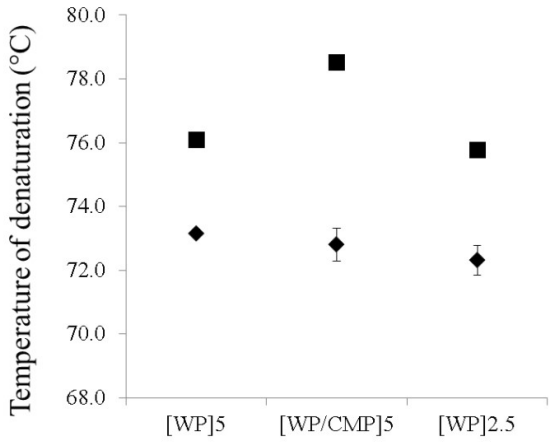

-0.295
-0.296
-0.297
-0.298
-0.299
-0.300
-0.301
-0.302
-0.303
-0.304
Heat flow (W/g)
30
40
50
60
70
80
90
Temperature (°C)
76°C
76°C
78.5°C

Fig. 7

(a)


Temperature of denaturation (°C)
80.0
78.0
76.0
74.0
72.0
70.0
68.0
[WP]5
[WP/CMP]5
[WP]2.5

(b)

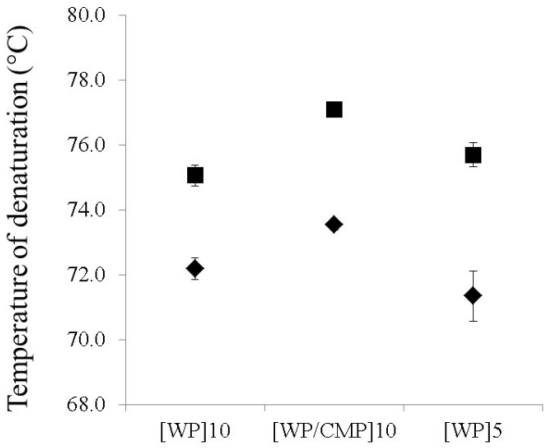

Temperature of denaturation (°C)
80.0
78.0
76.0
74.0
72.0
70.0
68.0
[WP]10
[WP/CMP]10
[WP]5

Fig. 8

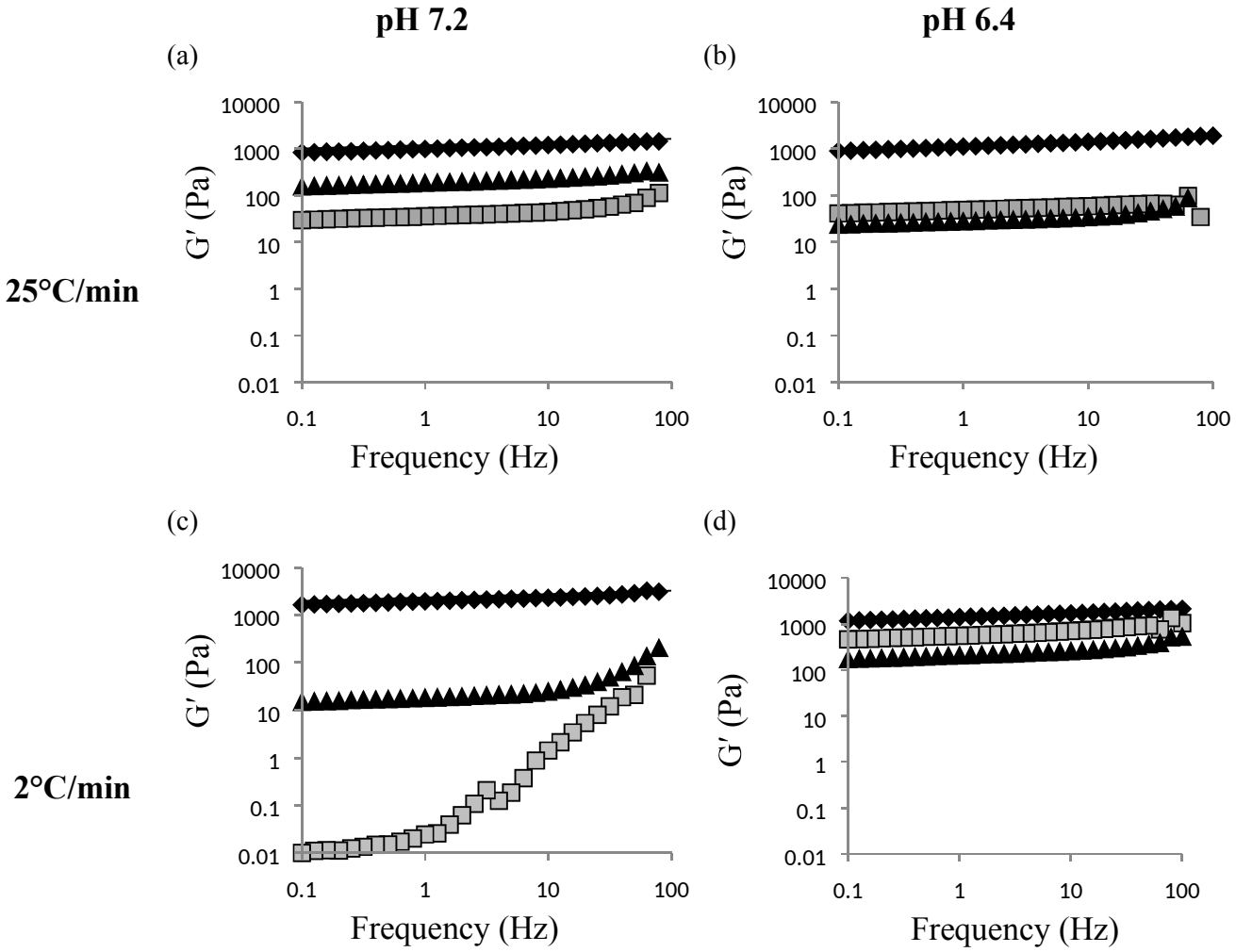

pH 7.2
pH 6.4
(a)
(b)
(c)
(d)
25°C/min
2°C/min
G′ (Pa)
Frequency (Hz)
10000
1000
100
10
1
0.1
0.01
0.1
1
10
100

Fig. 9