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University College Cork

School of Food and Nutritional Sciences




# Controlling the denaturation and aggregation of whey proteins using κ-casein and caseinomacropeptide

Thesis presented to the National University of Ireland

for the degree of Doctor of Philosophy by

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December 2019

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# Declaration

This thesis is my work and it has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents.

I have read and understood the regulations of University College Cork concerning plagiarism.

Date:

Sophie Gaspard

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# Abbreviations:

| | |
|---|---|
| AEC: | Anion-exchange chromatography |
| AFM: | Atomic force microscopy |
| ATR: | Attenuated total reflectance |
| BCA: | Bicinchoninic assay |
| BSA: | Bovine serum albumin |
| CAC: | Critical aggregation concentration |
| $C_g$: | Critical concentration for gelation |
| CMP: | Caseinomacropeptide |
| d-CMP: | Desialylated-caseinomacropeptide |
| DSC: | Differential scanning calorimetry |
| DSC: | Differential scanning calorimetry |
| FPLC: | Fast protein liquid chromatography |
| FTIR: | Fourier-transform infrared spectroscopy |
| Gal: | Galactose |
| GalNAc: | N-acetylgalactosamine |
| HPLC: | High-performance liquid chromatography |
| Ig: | Immunoglobulin |
| Lf: | Lactoferrin |
| MPC: | Milk protein concentrate |
| NeuAc: | N-acetylneuraminic acid |
| OD: | Optical density |
| pI: | Isoelectric point |
| RP: | Reversed-phase |
| SEC: | Size-exclusion chromatography |
| SEM: | Scanning electron microscopy |
| WP: | Whey proteins |
| WPI: | Whey protein isolate |
| α-la: | α-lactalbumin |
| β-lg: | β-lactoglobulin |

## Abstract

Whey proteins ingredients are extensively used in a variety of product formulations such as dairy beverages, infant formula and sport nutritional beverages, due to their nutritional and functional properties. Dairy protein-containing beverages are thermally processed, typically to ensure microbiological safety. However, whey proteins denature and aggregate at temperatures greater than 60°C, which can lead to fouling of industrial equipment and/or uncontrolled gelation, depending on formulation and heating conditions. The presence of caseins has been previously reported to limit the extent of aggregation of whey proteins. The objective of this study was to investigate the effect of κ-casein and caseinomacropeptide (CMP) on the denaturation and aggregation of whey proteins, with a view to developing practical strategies for controlling whey protein denaturation and aggregation for ingredient applications. This study demonstrated that both κ-casein and CMP have the ability to improve the heat stability of whey proteins. The inclusion of κ-casein reduced the size of the aggregates of whey protein after a first heat treatment (90°C for 25 min at pH 7.2) and enhanced their solubility during subsequent heating (90°C for 1 h at pH 7.2). The presence of CMP during heating increased the temperatures of denaturation and gelation of whey proteins and prevented the formation of solid whey protein gels when combined with a low heating rate. The presence of CMP also resulted in a lower turbidity of whey protein solutions after heating and an enhanced solubility of whey protein aggregates. The effect of glycosylation of CMP on the denaturation and aggregation of whey proteins was pH-dependent; a transition occurred at pH 6, below which the glycosylation of CMP reduced its stabilizing properties. This thesis provides new insights into the interactions of whey proteins with κ-casein and CMP, with potential for novel applications in improving the

heat-stability and solubility of whey proteins. The outcomes of this study have applications for the manufacture of clear, heat-stable beverages containing whey proteins.

# Chapter 1

## Literature review

# 1. Major proteins in milk

## 1.1 Protein fraction in bovine milk

Milk is a secretion intended for the nutrition of the new-born mammals. Bovine milk is the most consumed and the most studied type of milk (Fox, 2001). Cow's milk contains 87% (w/v) water, 4.5% (w/v) fats and 4% (w/v) lactose (O'Mahony & Fox, 2013). The proteins represent around 3 to 4% (w/v) of the total volume and citrate, calcium, potassium are the major minerals in milk (Fox, 2001) with respective concentration of 0.18%, 0.15% and 0.12% (w/v). The composition of proteins varies as a function of many factors, including bovine genetics and lactation period. In mature milk, there are two families of proteins: caseins and whey proteins, which represent around 80 and 20% (w/w) of the total amount of protein, respectively (Fig. 1.1).

Caseins and whey proteins can be distinguished by their very different structures, global composition and technological properties (Fox, 2001). Caseins are traditionally defined as the milk proteins that precipitate at pH 4.6, whereas native whey proteins are soluble at all pH. Caseins are phosphorylated, whereas whey proteins are richer in sulphur, which affects the nature of their intermolecular interactions during heat treatment (Sections 2 and 3). Whey proteins are bioactive carriers, thanks to their very compact globular structure, which allows them to bind other molecules. In contrast, individual molecules of casein present little order but associate in micelles. The flexibility of the molecules of casein contributes to their heat-stability, whereas the elaborate structures of the whey proteins make them prone to unfolding during heat treatment (Section 2). More details on the caseins and whey proteins are provided in Sections 1.2 and 1.3.

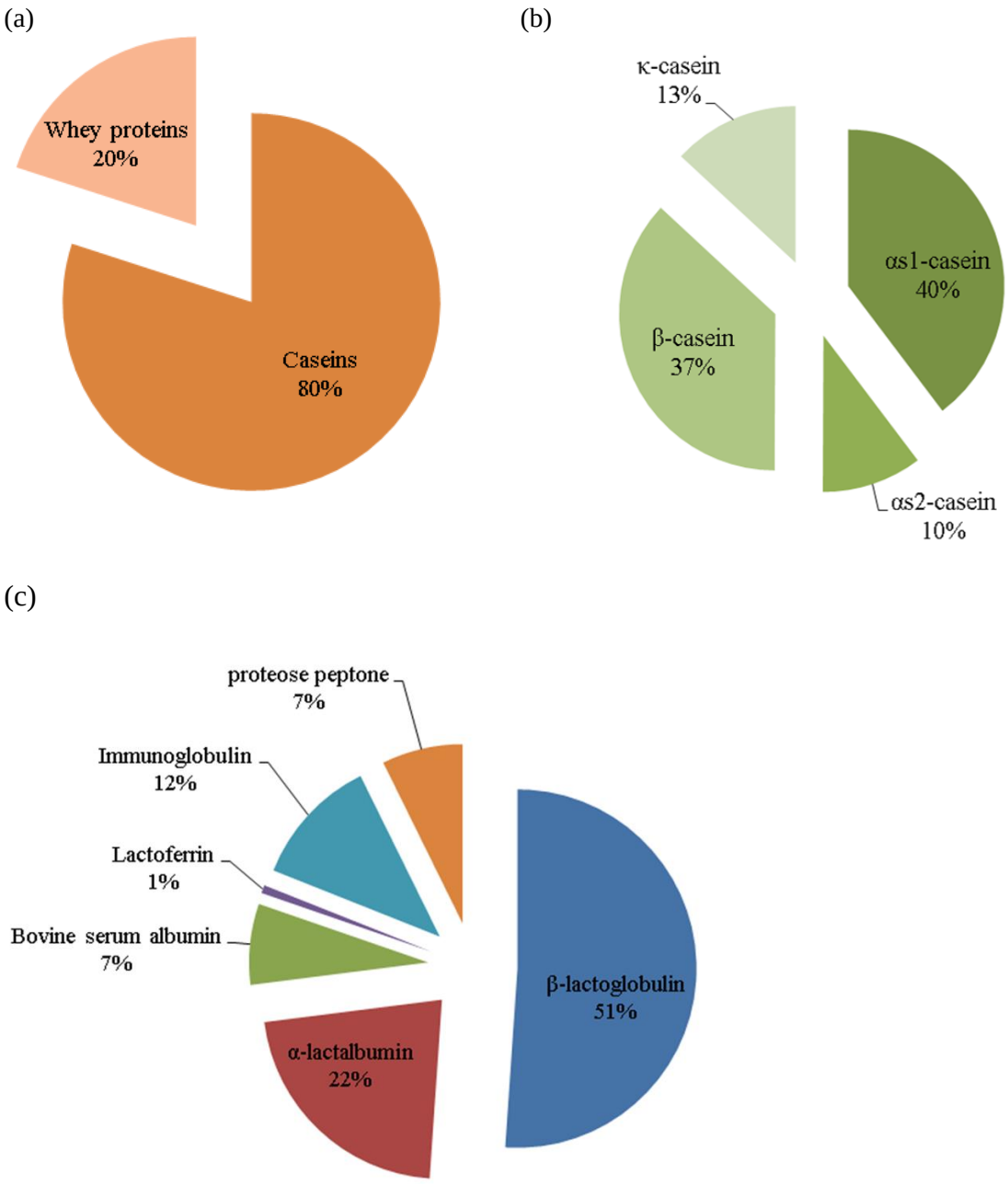



**Fig. 1.1** Major proteins in (a) the total protein fraction, (b) the casein fraction and (c) the whey protein fraction in bovine milk, adapted from Bansal and Bhandari (2016) and Broyard and Gaucheron (2015).

## 1.2 Caseins

### *1.2.1 Casein micelle and dissociation*

In milk, caseins are naturally present in a micellar form (Fig. 1.2) and a minor amount can dissociate from the micelle as a function of the temperature and the pH as shown in Fig. 1.3. In particular, κ-casein dissociates in a greater amount and in a continuous manner above pH 6.7 and from 20 to 90°C (Fig. 1.4). $\alpha_s$- and β-casein are located in the heart of the micelle and κ-casein is located on its surface. According to the nanoclusters model, the micelle is held together by hydrophobic and electrostatic interactions between phosphoserines, with sizes ranging from 10 to over 300 nm and an average hydrodynamic size of 150-200 nm (Dalgleish & Corredig, 2012). The presence of κ-casein at the surface of the casein micelle provides an additional stabilization against coagulation, thanks to its C-terminus pole of negative charges (Cases, Vidal, & Cuq, 2003). Much effort has been made in the past decades to separate the caseins individually, in order to understand and use their individual properties. The characteristics of the caseins are presented in the sections below and are summarized in Table 1.1.

(a) (b)

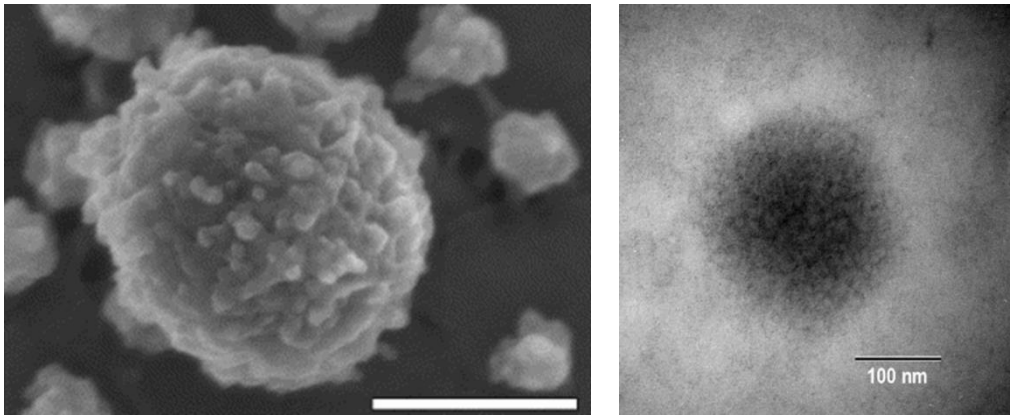



**Fig. 1.2** Images of the casein micelle in milk by (a) field emission scanning electron microscopy and (b) transmission electron. The bar length in the image (a) equals to 200 nm (McMahon & Oommen, 2008; Spagnuolo, Dalgleish, Goff, & Morris, 2005).

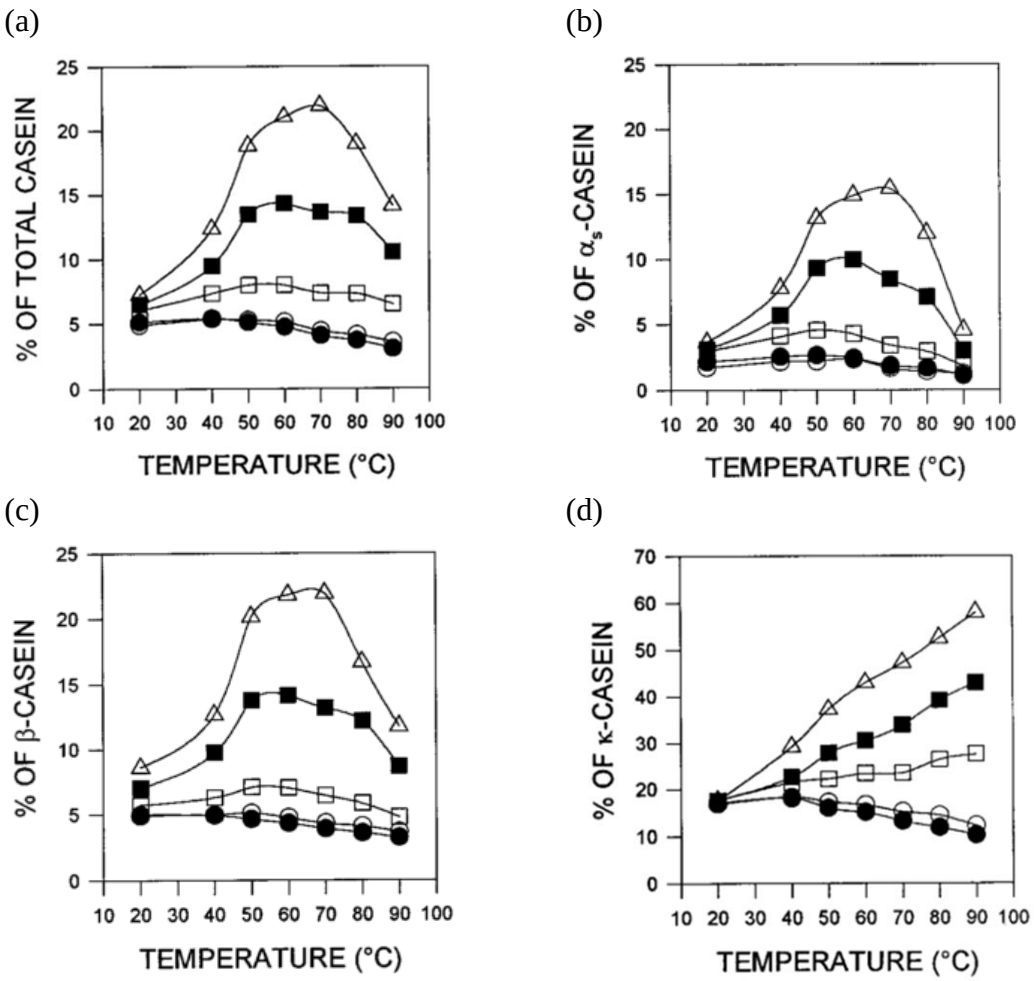



**Fig. 1.3** Dissociation of (a) the casein fraction (b) the $\alpha_s$-casein fraction, (c) the β-casein fraction and (d) the κ-casein fraction from the micelle in skim milk, as a function of temperature (20-90°C) and pH (○) 6.3, (●) 6.5, (□) 6.7, (■) 6.9 or (△) pH 7.1 (Anema & Klostermeyer, 1997).

**Table 1.1** Summary of the properties of the main caseins in bovine milk, adapted from Huppertz (2013) and Holland (2008).

| | $\alpha_{s1}$-casein (variant B-8P) | $\alpha_{s2}$-casein (variant A-11P) | β-casein (variant $A^2$-5P) | κ-casein (variant A-1P) |
|---|---|---|---|---|
| Number of amino acids | 199 | 207 | 209 | 169 |
| Molecular weight (kDa)[1] | ≈ 23.6 | ≈ 25.2 | ≈ 24.0 | ≈ 19.1 |
| Disulphide bonds | 0 | 1 | 0 | 1 |
| Free thiol | 0 | 0 | 0 | 0 |
| Isoelectric point | 4.4 | 5.0 | 4.7 | 5.6/3.5 [2] |
| Self-association[3] | oligomers | dimers | micellization | oligomers |
| Calcium sensitivity | ++ | +++ | + | - |
| Hydrophobicity (kJ/res)[4] | 4.9 | 4.6 | 5.6 | 5.1 |
| Phosphorylation | 8 | 11 | 5 | 1 |
| Glycosylation | 0 | 0 | 0 | up to 6 [5] |

[1] The calculation of the molecular weight takes the phosphorylation into account.

[2] The value of 5.6 corresponds to the monophosphorylated and non-glycosylated κ-casein (variant A), whereas the value of 3.5 corresponds to the lowest isoelectric point measured for κ-casein, which is correlated with its glycosylation and phosphorylation degree.

[3] Highest structure order observed in pure solutions

[4] These values are calculated from the primary amino acid sequence and do not take into account post-translational modifications of caseins.

[5] Up to six glycosylations were observed in the variant B of κ-casein.

### *1.2.2 $\alpha_{s1}$-casein*

$\alpha_{s1}$-Casein is a highly phosphorylated protein and the major casein in bovine milk (Fig. 1.1). There are six genetic variants reported for $\alpha_{s1}$-casein, with additional heterogeneities in the degree of phosphorylation. $\alpha_{s1}$-casein B-8P (genetic variant B with 8 phosphorylations) is the major genetic variant of $\alpha_{s1}$-casein in bovine milk (Holland, 2008). A summary of the major properties of $\alpha_{s1}$-casein is summarized in Table 1.1. The molecular weight increases and isoelectric point (pI) decreases with a higher degree of phosphorylations and glycosylation of caseins (Huppertz, 2013). The molecular weight of $\alpha_{s1}$-casein B is 23.6 kDa and its pI is as low as 4.4. It is noteworthy that phosphorylation and glycosylation of caseins also contribute to making the molecules more hydrophilic. Despite the phosphorylations and the carboxylated residues of $\alpha_{s1}$-casein that bind calcium and stabilize the protein, $\alpha_{s1}$-casein is sensitive to calcium precipitation. $\alpha_{s1}$-Casein is capable of self-association and can form large rod-like oligomers through hydrophobic interactions (Farrell, Brown, & Malin, 2013). This polymerization also depends on the concentration and the ionic strength (Huppertz, 2013). The monomeric form is favoured around neutral pH. $\alpha_{s1}$-Casein is moderately hydrophobic and its charge distribution is heterogeneous, as illustrated in Fig. 1.4.

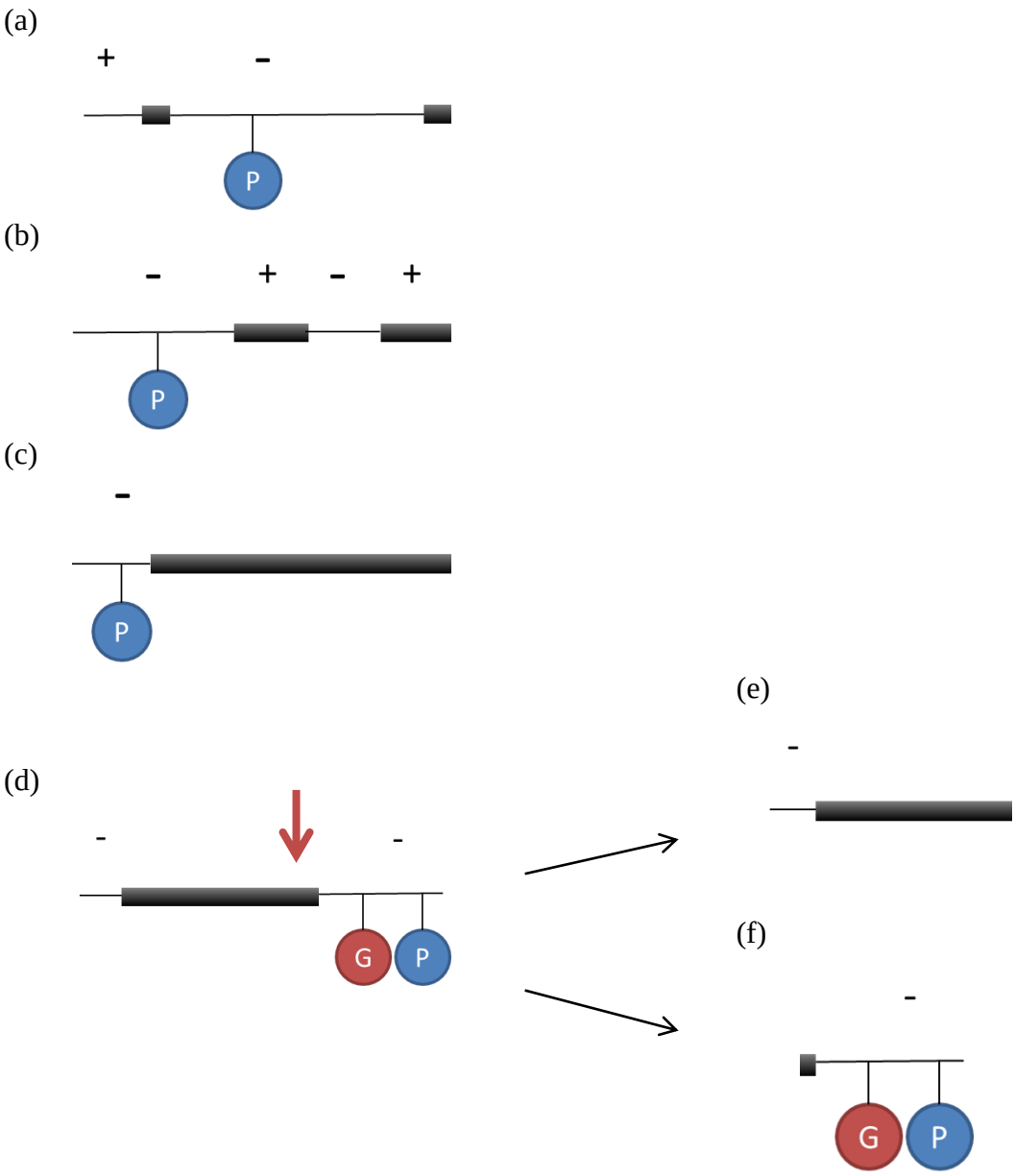



**Fig 1.4** Simplified schematic of the charge and hydrophobicity distribution in (a) $\alpha_{s1}$-casein, (b) $\alpha_{s2}$-casein, (c) β-casein, (d) κ-casein, (e) caseinomacropeptide and (f) para-κ-casein, adapted from Huppertz (2013). The approximate locations of the phosphorylation (P) and glycosylation sites (G) are also indicated. The patches of significant hydrophobicity are indicated by the black bars. The red arrow indicates the cleavage site of chymosin on κ-casein (Phe105-Met106).

### *1.2.3 $\alpha_{s2}$-casein*

$\alpha_{s2}$-Casein is the most phosphorylated casein and one of the only two caseins containing a disulphide bond (Table 1.1). There are four genetic variant of $\alpha_{s2}$-casein, with $\alpha_{s2}$-casein A being the major form in bovine milk (Huppertz, 2013), and the amino acid sequence contains up to 16 potential phosphorylation sites (Holland & Boland, 2014). Both monomers and dimers of $\alpha_{s2}$-casein have been observed and the dimers are formed through intermolecular disulphide bonds (Huppertz, 2013). The self-association of $\alpha_{s2}$-casein also depends on the ionic strength of the environment. As for all caseins, the charge and hydrophobicity distribution is heterogeneous (Fig 1.5). $\alpha_{s2}$-Casein is considered to be the most hydrophilic casein, thanks to its high number of charged residues.

### *1.2.4 β-casein*

β-Casein is the second major casein in milk and is present in twelve genetic variations (Huppertz, 2013), with β-casein $A^2$ being the major β-casein in bovine milk, and five phosphates are generally attached to the molecule (Holland & Boland, 2014). β-Casein is a very amphipathic molecule (Fig. 1.4), with a highly negatively-charged and hydrophilic N-terminus, whereas the rest of the molecule is hydrophobic and contains little charge (Huppertz, 2013). Above 4-5°C, β-casein self-associates in micelles containing 15 to 60 monomers, formation of which depends on a balance of hydrophobic and electrostatic interactions. The micellization of β-casein depends on β-casein concentration, pH, temperature and ionic strength (Li et al., 2019).

### *1.2.5 κ-casein*

κ-Casein is the only glycosylated casein and one of the two caseins containing disulphide bonds (Huppertz, 2013). Those features have implications for the heat-stability of whey protein when they are co-heated with κ-casein (Section 3.3). Contrary to other caseins, κ-casein has a low degree of phosphorylation and is the casein the least sensitive to calcium precipitation (Table 1.1). There are two main genetic variants of κ-casein (A and B), with monophosphorylated κ-casein A being the major variant observed. The heterogeneity of κ-casein is amplified by the various degrees of glycosylation and phosphorylation of the protein, with fourteen glycosylated forms of κ-casein A being observed, in addition to the non-glycosylated forms (Recio, Moreno, & López-Fandiño, 2009). As for the other caseins, the pI of κ-casein decreases with a higher degree of glycosylation and phosphorylation. The pI of κ-casein is around 5.6 when the molecule is phosphorylated, whereas it is as low as 3.5 when the molecule is glycosylated (Huppertz, 2013). The carbohydrate chain is attached to the threonine residues, located in the C-terminus region of κ-casein, by O-linkage. Galactose (Gal), N-acetylgalactosamine (GalNAc) and N-acetyl neuraminic acid (NeuAc) are the units of the carbohydrate chain, organized from monosaccharides to tetrasaccharides (Fig 1.6). NeuAc is the most abundant member of the sialic acid family, and plays an important physiological role of cell stabilization and transport of positively-charged ions (Traving & Schauer, 1999; Varki, 2008). The very negative charges carried by NeuAc at neutral pH arise from its low pKa (2.6). In milk, the negative charges carried by the glycosylation of the κ-casein provide additional stabilization to the casein micelle (Cases et al., 2003).

Phosphorylation or glycosylation of caseins occurs as post-translational modifications and do not take place on every synthetized casein (Holland, 2008). It depends, for example, on the presence of a specific sequence motif, the presence of the protein and the accessibility of the site by the enzymes. In milk, 50 % of κ-casein is glycosylated (Recio et al., 2009) and Martín, Martín-Sosa, García-Pardo, and Hueso (2001) observed that the amount of sialic acid is around 0.01% (w/w) in mature milk. This value varies as a function of the stage of lactation, with a peak concentration in the colostrum (Martín et al., 2001). There are six potential sites of glycosylation ($Thr_{142}$, $Thr_{152}$, $Thr_{154}$, $Thr_{163}$, $Thr_{166}$ and $Thr_{186}$) and three potential sites of phosphorylation ($Ser_{170}$, $Ser_{148}$, $Thr_{166}$) on κ-casein (Holland, 2008). The major forms of phosphorylated κ-casein are mono- and di-phosphorylated and up to six glycosylations have been identified on the variant B of κ-casein. The charge and hydrophobicity distribution on κ-casein is heterogeneous (Fig. 1.4). The C-terminus region, containing all the phosphorylation and glycosylation sites, is very negatively-charged and hydrophilic. The rest of the molecule is globally hydrophobic and interacts with other caseins during the formation of the casein micelle (Farrell et al., 2013). κ-Casein has little secondary structure and the very negatively-charged C-terminus part is flexible due to the high net charge and high hydration of this region (Huppertz, 2013). The secondary structures reported are 10-20% α-helix, 20-30% β-structure and 15-25% turns. κ-Casein contains a disulphide bond that can be intramolecular or intermolecular, forming octamers and larger species.

(a) $GalNac_{OH}$ **1%**

(b) $Gal\beta1\text{-}3GalNac_{OH}$ **6%**

(c) $NeuAc\alpha2\text{-}3Gal\beta1\text{-}3GalNac_{OH}$ **18%**

(d) NeuAc
2
6 α
$Gal\beta1\text{-}3GalNac_{OH}$ **19%**

(e) NeuAc
2
6 α
$NeuAc\alpha2\text{-}3Gal\beta1\text{-}3GalNac_{OH}$ **56%**

**Fig. 1.5** Chemical structure and frequency of the (a) monosaccharide, (b) disaccharide, (c) trisaccharides, (d) branched trisaccharides and (e) tetrasaccharide of galactose (Gal), N-acetyl galactosamine (GalNac) and N-neuraminic acid (NeuAc) in κ-casein from bovine milk (Saito & Itoh, 1992).

### *1.2.6 Caseinomacropeptide*

The caseinomacropeptide (CMP) is a 64-amino acid peptide resulting from the enzymatic cleavage of κ-casein by chymosin between residues $Phe_{105}$ and $Met_{106}$. κ-Casein contains a hydrophobic and hydrophilic part. After cleavage, CMP contains most of the hydrophilic and negatively-charged part of κ-casein (Fig. 1.4). However, CMP contains few hydrophobic amino acids in its N-terminus region. In cheese whey, CMP represents around 20-25% of the protein and peptide fraction (Thomä-Worringer, Sørensen, & López-Fandiño, 2006). CMP is also present in raw milk and colostrum to a minor extent (Furlanetti & Prata, 2003). The molecular weight of CMP ranges from 7,500 to 9,631, as a function of the degree of glycosylation and phosphorylation of the molecule. CMP can self-associate and form up to 50 kDa oligomers (Deeth & Bansal, 2018; O'Riordan, Kane, Joshi, & Hickey, 2014). The

polymerization of CMP has been described as a pH-driven phenomenon. However, some authors found that the oligomers of CMP are the result of pH-induced interactions between molecules of κ-casein on the surface of the casein micelle, prior to its enzymatic cleavage (Mikkelsen et al., 2005). Non-glycosylated molecules of CMP, or glycosylated and non-glycosylated CMP, can form even larger aggregates, likely through electrostatic and hydrophobic interactions (Kreuß, Strixner, & Kulozik, 2009; Villumsen et al., 2015). Interactions between CMP molecules are pH-, temperature- and concentration-dependent (Kreuß et al., 2009; Loria, Aragón, Torregiani, Pilosof, & Farías, 2018; Martín-Diana, Gomez-Guillén, Montero, & Fontecha, 2006; Villumsen et al., 2015). Fig. 1.6 shows the electrical potential created by the negative charge of a non-glycosylated molecule of CMP and the hydrophobic areas that are not shielded by the negative charges and are available for interactions at pH 7.0. The amount of sialic acid bound to CMP is around 4.5% (w/w) as reported by Taylor and Woonton (2009). CMP has been reported to be mainly disordered with little secondary structure, and its glycosylation has very little effect on the secondary structure (Smith, Edwards, Palmano, & Creamer, 2002).

CMP is a bioactive peptide thanks to both its backbone chain and the presence of the sialic acid. For example, the immunomodulatory effect of CMP depends both on its backbone chain and on the sialic acid (Brody, 2000; O'Riordan et al., 2014; Thomä-Worringer et al., 2006). NeuAc is responsible for the promotion of microbial gut growth and the improvement of learning abilities. Moreover, the use of CMP presents interesting properties from a technological point of view. In particular, CMP is very heat-stable and hydrophilic, thanks to its disordered structure and the negative charges carried by the charged amino acid residues and the glycosylation and phosphorylation at neutral pH. It is noteworthy that NeuAc can be degraded by

combination of acid and heat treatment (Kilic-Akyilmaz & Karimidastjerd, 2018; Siegert, Tolkach, & Kulozik, 2012; Taylor & Woonton, 2009). The interactions between CMP and whey proteins during heat-treatment and the impact on the heat-stability of whey proteins are discussed in Section 3.4.

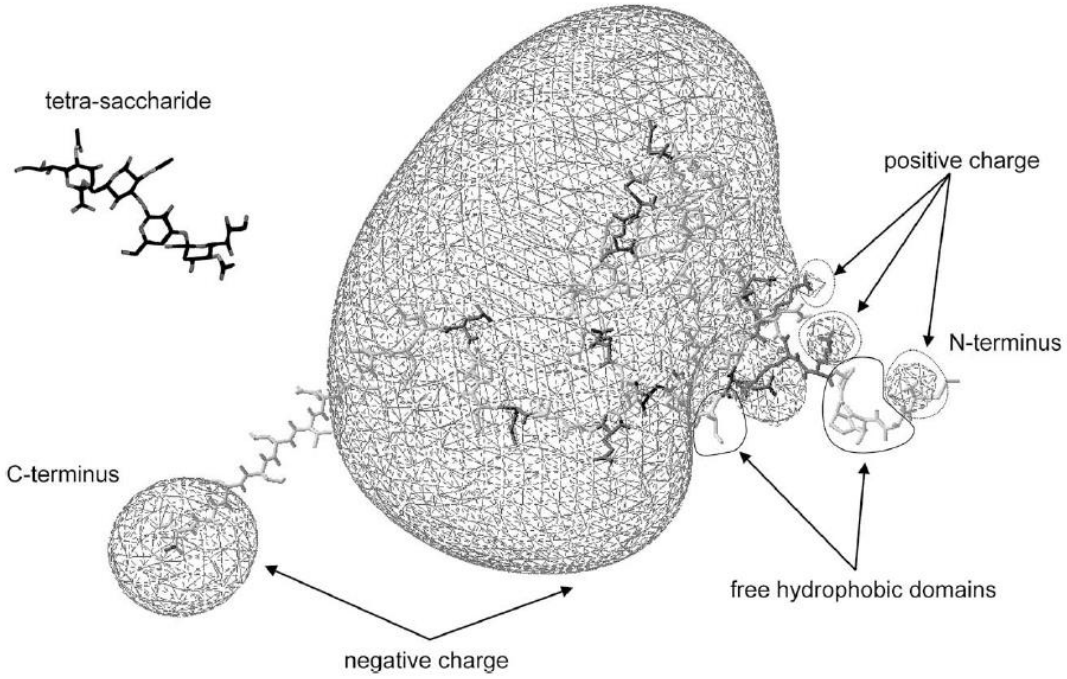



**Fig. 1.6** Three-dimensional structure of non-glycosylated caseinomacropeptide (genetic variant A) and a tetrasaccharide, at pH 7.0 and zero ionic strength, modelled with PDB protein modelling software. The lattices indicate the electrical potential of the charged amino acids as well as the N- and C-terminus. The free hydrophobic domains, i.e. not shielded by the negative charges at pH 7.0, are also indicated (Kreuß et al., 2009).

## 1.3 Whey proteins

Table 1.2 presents the major properties of β-lactoglobulin (β-lg) and α-lactalbumin (α-la), the two major whey proteins in milk.

**Table 1.2** Properties of the two major whey proteins in bovine milk, β-lactoglobulin and α-lactalbumin, adapted from Deeth and Bansal (2019) and Sawyer (2013).

| | β-lactoglobulin | α-lactalbumin |
|---|---|---|
| Number of amino acids | 162 | 123 |
| Molecular weight (kDa) | 18.3 | 14.2 |
| Disulphide bonds | 2 | 4 |
| Thiol group | 1 | 0 |
| Isoelectric point | 5.1 | 4.2-4.5 |
| Molecules or ions transported* | Retinol, SDS, Oleic acid | Ca, Zn, Mg |

* Non-exhaustive list

### *1.3.1 β-lactoglobulin*

β-lg is the major whey protein in bovine milk (Fig**.** 1.1). β-lg A is the major genetic variant of β-lg in milk and can self-associate in octamers at low pH (Fig. 1.7). The dimer of β-lg dissociates in monomer at low β-lg content, low ionic strength and high pH (Mehalebi, Nicolai, & Durand, 2008). Fig. 1.8 shows the interaction area for dimerization of β-lg. As a member of the lipocalin family, β-lg binds small molecules (Table 1.2); this is allowed by the presence of eight anti-parallel β-sheets, which forms a binding site in the shape of a calyx, lined with hydrophobic amino acids (Fig. 1.8 and 1.9), although another site of binding has also been identified at the surface of the protein (Wijayanti, Brodkorb, Hogan, & Murphy, 2019). At the entrance of the calyx, a loop connecting β-strands, acts like a gateway, allowing the

access to the calyx around neutral pH (Kontopidis, Holt, & Sawyer, 2004). This phenomenon is part of the Tanford transition. In addition, located in the core of β-lg (Fig. 1.8), four cysteine are involved in a disulphide bonds ($Cys_{66}$-$Cys_{160}$ and $Cys_{160}$-$Cys_{119}$) and one of them is free ($Cys_{121}$). This is of particular importance in the irreversible aggregation of β-lg during heat treatment, as further described in Section 2. In total, native β-lg structure counts 8% α-helix, 45% β-helix and 47% random coils in its native form (Deeth & Bansal, 2018).

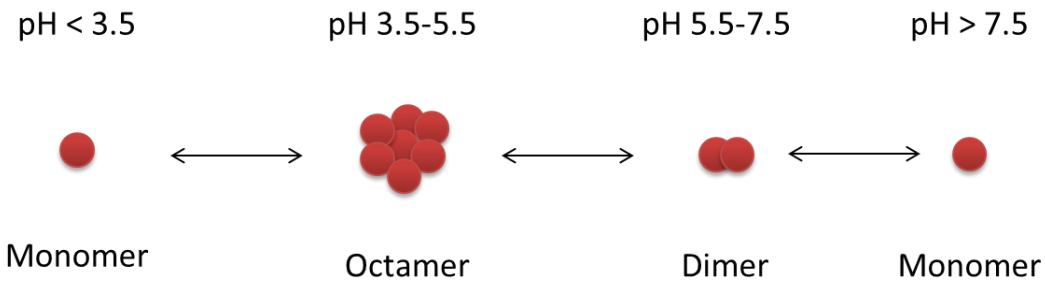



**Fig. 1.7** Polymerization of β-lactoglobulin as a function of the pH, adapted from Wijayanti et al. (2019).

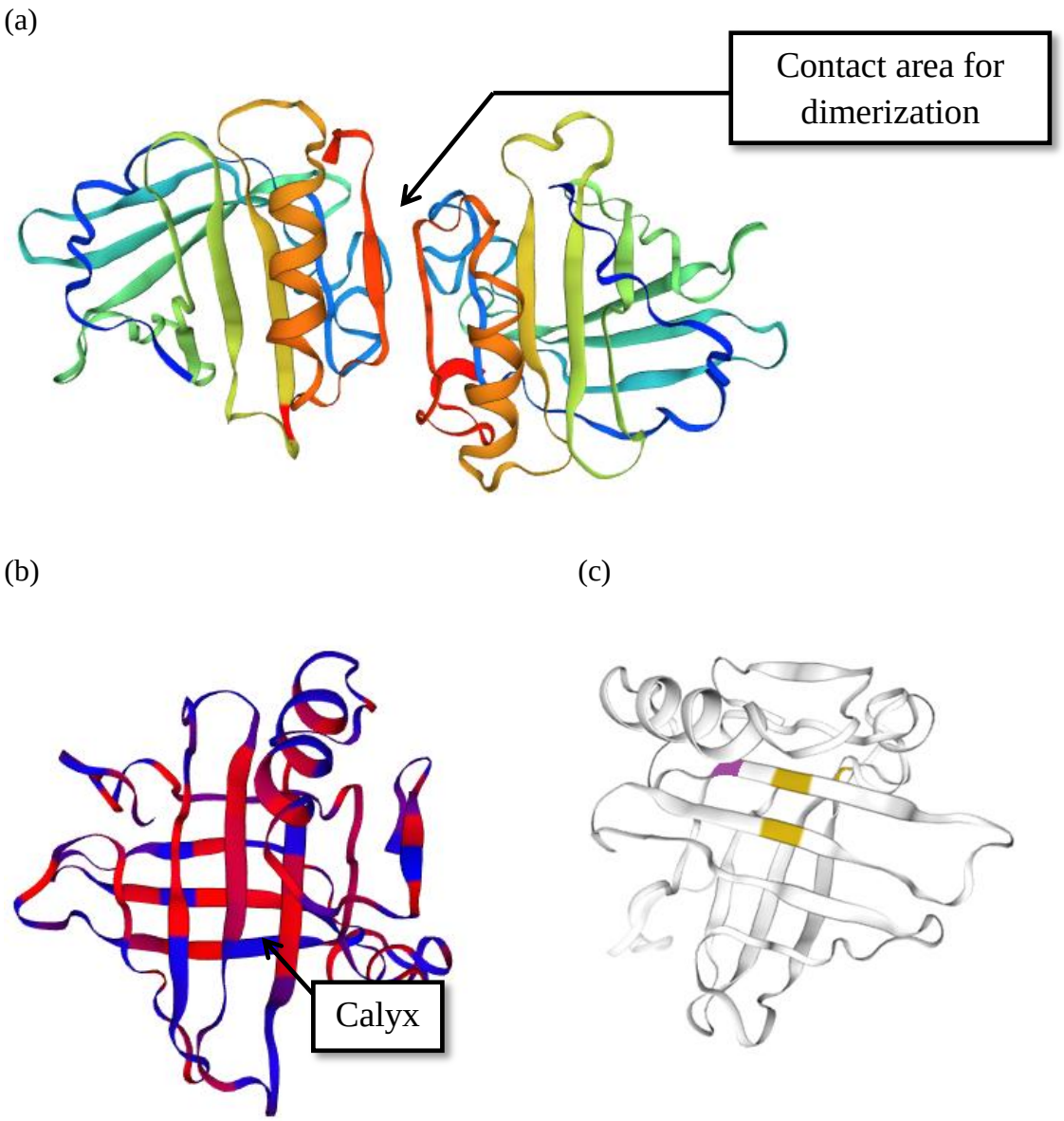



**Fig. 1.8** Three-dimensional image of (a) a dimer of β-lactoglobulin (β-lg) B, (b) the hydrophobic (red) and hydrophilic (blue) area of a molecule of β-lg B and (c) β-lg B with disulphide bond cysteine in yellow and the free $Cys_{121}$ in purple, from the Swiss model repository with the UniProtKB AC name P02754 (LACB_BOVIN).

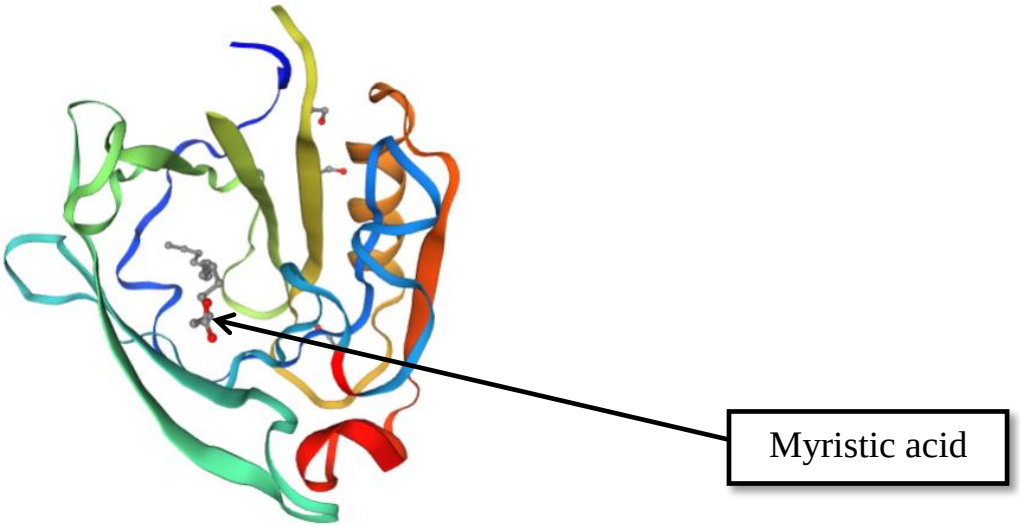



**Fig. 1.9** Three-dimensional image of β-lactoglobulin B with a molecule of myristic acid bound to its calyx. This illustration was obtained from the Swiss model repository with the UniProtKB AC name P02754 (LACB_BOVIN).

### *1.3.2 α-lactalbumin*

α-la is the second major whey protein in bovine milk (Fig. 1.1) and the major genetic variant is α-la B (Farrell Jr et al., 2004). The predominance of α-la in the whey fraction of human milk explains the great interest of the industry for the enrichment of infant formula with bovine α-la. The consequences of this enrichment on the functionality of these beverages are being studied (Barone, O'Regan, & O'Mahony, 2019; Buggy, McManus, Brodkorb, McCarthy, & Fenelon, 2016). Major differences in the properties between α-la and β-lg (Table 1.2) result in different denaturation behaviour. α-la contains 123 amino acids and eight cysteine, which are engaged in four disulphide bonds (Deeth & Bansal, 2018). α-la does not have a free thiol group, in contrast to β-lg. The structure of α-lactalbumin is comprised of 60% of random coils, 26% α-helix and 50% β-sheets. The stability of the molecule is highly dependent on the fixation of one calcium ion on the Asp residues located in the

hydrophobic core of its structure (Fig. 1.10). The fixation of calcium (holo form) aids in the refolding and the correct formation of intramolecular disulphide bonds (Edwards & Jameson, 2014). Although the protein does not unfold completely upon removal of the calcium ion (apo form), the structure is more sensitive to thermal denaturation and the denaturation temperature is lowered to around 30°C, whereas the temperature of denaturation of the holo form is around 60°C (Relkin, Eynard, & Launay, 1992). α-La unfolds partially, in a so-called molten-globule state at acidic or alkaline pH, or with an increase in temperature (Brew, 2013). The apo form is in this molten-globule state at neutral pH and low ionic strength.

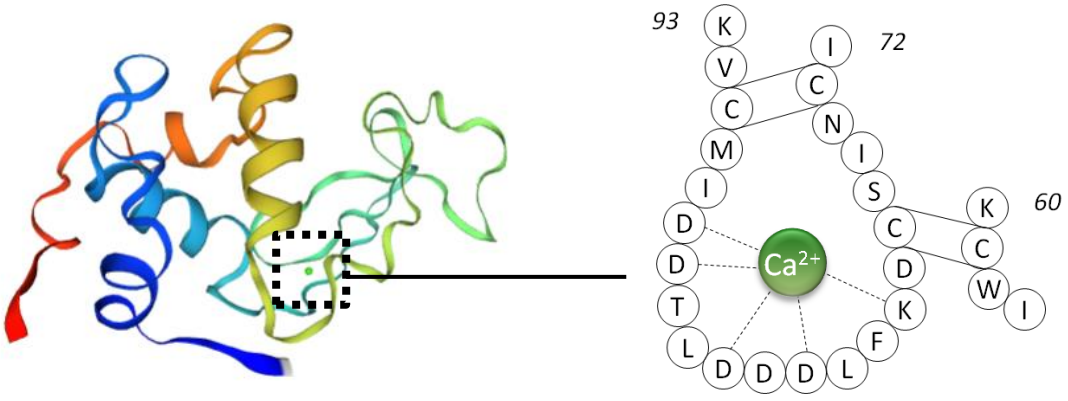



**Fig. 1.10** Three-dimensional image of holo alpha-lactalbumin A and structure of the binding site for calcium, adapted from Edwards and Jameson (2014). The three-dimensional image was obtained from the Swiss model repository with the UniProtKB AC name P00711(LALBA_BOVIN).

### *1.3.3 Minor whey proteins*

*a. Immunoglobulin*

Immunoglobulins (Ig) are composed of four polypeptides in a Y-shape. The polypeptides are assembled by disulphide bonds (Farrell Jr et al., 2004). The main Ig (IgG, IgA and IgM) are present in greater levels in colostrum than in mature milk (Deeth & Bansal, 2019). The total molecular weight of IgG, the major form of Ig in milk and colostrum, is around 160 kDa. Ig are glycosylated and contain sialic acids, which are important inflammatory agent for the new-born (O'Riordan et al., 2014). The Ig were reported to be the most heat-labile whey protein in skim milk (Law & Leaver, 2000).

*b. Bovine serum albumin*

Also contained in blood stream, bovine serum albumin (BSA) is a molecule carrier binding fatty acids, metal ions and flavour compounds (Deeth & Bansal, 2019). The presence of fatty acid increases the heat-stability of BSA. BSA is particularly rich in cysteine residues with thirty-four of them engaged in disulphide bonds and one free thiol group. The structure of BSA consists of a three homologous-domain protein, mainly with α-helical structures (Edwards & Jameson, 2014). Lengthy loops connect the different domains and are held together by disulphide bonds.

*c. Lactoferrin*

Lactoferrin (Lf) is a 708 amino acid protein in bovine milk (Fig. 1.1), single-chain, ferrous ion carrier and glycosylated with sialic acids (Farrell Jr et al., 2004). The molecular weight of Lf is around 80 kDa (Lönnerdal & Suzuki, 2013). Lf is a bioactive peptide, the continuous presence of which in colostrum stresses its role in the nutrition and protection of the new-born (O'Riordan et al., 2014). In particular, Lf

presents anti-microbial, prebiotic, anti-viral, anti-inflammatory and immunomodulatory activities.

*d. Proteose peptone*

Proteose peptones are minor proteins and peptides in bovine milk, some being the product of the proteolytic degradation of caseins and some being indigenous to milk (Deeth & Bansal, 2019). They are defined as the soluble fraction of whey, after heating at 95-100°C for 30 min and subsequent adjustment at pH 4.6 (Mulvihill & Donovan, 1987). Some proteose peptones are bioactive peptides (Wynn & Sheehy, 2013).

e. *Non-protein nitrogen fraction*

Around 5% of the nitrogen in milk does not originate from proteins and is referred as non-protein nitrogen fraction (Karman & Van Boekel, 1986). It is defined as the fraction of nitrogen that does not precipitate in 12% trichloroacetic acid and is thought to contain small molecules, such as urea, orotic acid, amino acids and some peptides. Recent experiments have shown that around 24% (w/w) of CMP does not precipitate in 8% trichloroacetic acid (Thomä, Krause, & Kulozik, 2006).

# 2. Mechanism of whey protein denaturation and control of the aggregation by heating parameters

## 2.1 Forces involved in the conformational stability of proteins and intermolecular interactions

Whey proteins fold in an elaborate 3-dimensional structure, arranged from secondary to quaternary structure (Mathews & Van Holde, 1990). The information for the proper folding of proteins is mostly contained in their amino acid sequence, and their conformational stability is guaranteed by intramolecular and solvent-protein interactions. Due to external stress such as heat, proteins unfold, i.e. lose at least partially their secondary and tertiary structure. Then, the molecule can either refold or aggregate with other proteins. The intermolecular interactions involve the same forces as those that initially stabilize the native structure of the proteins. These forces and their involvement in both conformational stability and intermolecular interactions are described below.

### *2.1.1 Disulphide bonds*

Some milk proteins, such as β-lg, α-la, BSA, and κ-casein, have in their amino acid sequence a certain number of cysteine residues (Table 1.3). Cysteine residues contain a thiol group that react with another thiol group to form a disulphide bond (Fig. 1.11). This interaction can occur within the same protein (intramolecular disulphide bond) or between proteins (intermolecular disulphide bond). The intermolecular disulphide bonds can also be reduced to form new intra- or intermolecular bonds. The presence of intramolecular disulphide bonds contributes to the thermodynamic stabilization of the protein folding by reducing the number of conformations possible for the unfolded molecule (Mathews & Van Holde, 1990).

The high pKa of the thiol group (≈8.5) limits its reactivity at mild acidic conditions. In addition, the reactivity of the thiol group also depends on the tertiary structure of the protein, the redox state of the environment and the protonation state of the cysteine (Visschers & de Jongh, 2005). Cysteine are generally located in the core of the proteins (Poole, 2015); therefore, the formation of intermolecular disulphide bond requires the unfolding of the whey proteins. The formation of disulphide bonds between proteins causes the irreversibility of the heat-induced aggregation process. This has consequences for the aggregation pattern of each individual whey protein. When α-la is heated on its own, denaturation tends to be reversible, as it does not include free cysteine interacting with other molecules (Relkin et al., 1992).

**Table 1.3** Number of free thiol groups, position of the cysteine containing the free thiol group and number of intramolecular disulphide bonds in the amino acid sequence of κ-casein, $\alpha_{s2}$-casein, β-lactoglobulin (β-lg), α-lactalbumin (α-la), bovine serum albumin (BSA) and lactoferrin (Brew, 2013; Huppertz, 2013; Sawyer, 2013).

| | Number of free sulphydryls | Position of the free sulfhydryls | Number of disulphide bonds |
|---|---|---|---|
| κ-casein | 0 | NA | 1 |
| $\alpha_{s2}$-casein | 0 | NA | 1 |
| β-lg | 1 | $Cys_{121}$ | 2 |
| α-la | 0 | NA | 4 |
| BSA | 1 | $Cys_{34}$ | 17 |
| Lactoferrin | 0 | NA | 1 |

(a) (b)

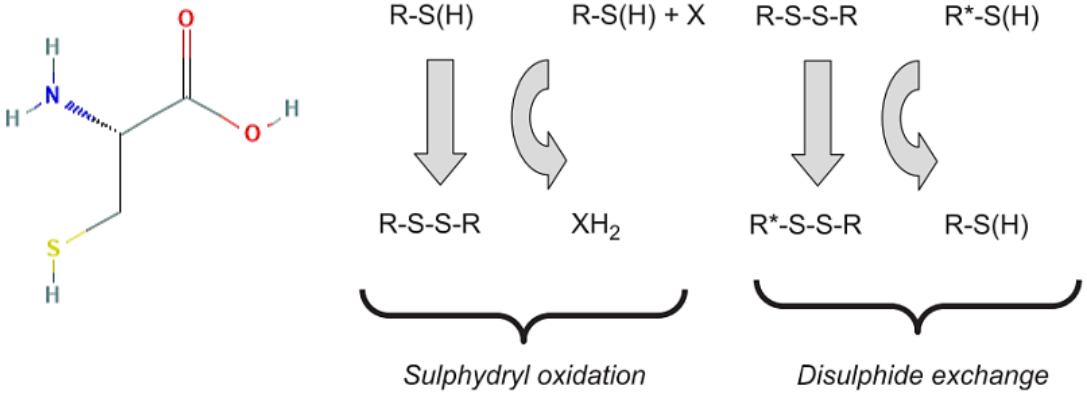



**Fig. 1.11** (a) Two-dimensional chemical structure of the amino acid cysteine (PubChem CID 5862) and (b) representation of the thiol oxidation and disulphide exchange reaction (Visschers & de Jongh, 2005).

### *2.1.2 Electrostatic interactions*

In an aqueous environment, molecules and ions carry charges or electronegativity. The different types of electrostatic interactions involved in protein stability and interactions are described below.

*a. Charge-charge interactions*

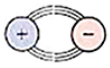
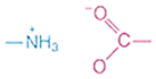
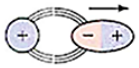
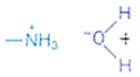
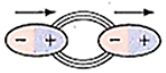
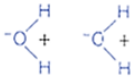
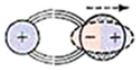
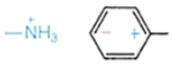
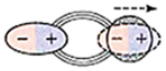
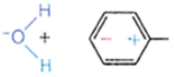
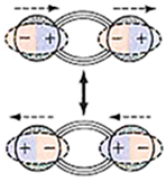
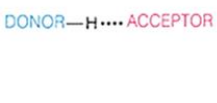
Charge-charge interactions are attractive forces that are formed between molecules or ions carrying opposite net charges. For example, the opposite charges of the side-chain of amino acids, arising from their ionization as a function of pH (Table 1.4), can stabilise the folding of distant regions of the polypeptide chain (Sheehan, 2013). It is noteworthy that the binding of polyvalent ions such as calcium ions to the carboxylate group of proteins are also a type of charge-charge interaction (Metzler, 1977). This is of particular importance when considering the gelation of proteins in the presence of salts (Bryant & McClements, 1998).

Coulomb's law allow the calculation of the force F of interactions between two charges, $q_1$ and $q_2$, separated by a distance, r:

$$F = k\frac{q_1 q_2}{\varepsilon r^2} \qquad (1)$$

The screening effect of the molecules of water between the charges is expressed by the dielectric constant ε. k is a constant, the value of which depends on the units used. A negative F means that the interactions are attractive, whereas a positive F means that the interactions are repulsive. The charge-charge interaction depends on the distance between the two molecules or ions and is non-directional, in contrast to other electrostatic interactions described below. The charge-charge interaction is the longest-range electrostatic force (Table 1.4).

**Table 1.4** Types of electrostatic interactions. The induced-dipole (d,e) and the dispersion force (f) depend on a distortion (⟿) of the electron distribution in a non-polar atom or molecule (Mathews & Van Holde, 1990).

| Type of interaction | Model | Example | Dependence of energy on distance | Comment |
|---|---|---|---|---|
| (a) Charge-charge |  |  | $1/r$ | Longest-range force; nondirectional |
| (b) Charge-dipole |  |  | $1/r^2$ | Depends on orientatio of dipole |
| (c) Dipole-dipole |  |  | $1/r^3$ | Depends on *mutual* orientation of dipoles |
| (d) Charge-induced dipole |  |  | $1/r^4$ | Depends on polarizab of molecule in which dipole is induced |
| (e) Dipole-induced dipole |  |  | $1/r^5$ | Depends on polarizab of molecule in which dipole is induced |
| (f) Dispersion |  |  | $1/r^6$ | Involves mutual synchronization of fluctuating charges |
| (g) Hydrogen bound |  | Hydrogen bond length | Length of bond fixed | Depends on donor–acceptor pair |

*b. Permanent dipole and induced dipole interactions*

Molecules presenting an asymmetric internal distribution of charges (e.g., charge distribution between the atom of oxygen and the atoms of hydrogen in the molecule of water) are called dipoles and are said to have a permanent dipole moment (Mathews & Van Holde, 1990). As a result, dipoles can interact with each other or with ions (Table 1.4). Under the influence of a close charged molecule or a dipole, a dipole moment can also be induced in polarizable molecules. The movement of electrons around the atoms can even allow attractive interactions between two molecules that do not have net charges or permanent dipole moment. These interactions are called induced dipole interactions (Table 1.4). Both permanent dipole and induced dipole interactions are short-range interactions.

*c. Hydrogen bonds*

Hydrogen bonds are a special case of dipole-dipole interactions, involving very strongly electronegative atoms, such as nitrogen (hydrogen bond donor) and oxygen (hydrogen bond acceptor). Much stronger than the other van der Waals interactions, hydrogen bonds have a major role in the formation of secondary structures in proteins (Mathews & Van Holde, 1990). During the folding process, hydrogen bonds are formed between C=O and N-H groups of the polypeptide chain or the side chain group (Table 1.4), which contribute to the desolvation of the polypeptide backbone. At the surface of the protein, hydrogen bonds between hydrophilic amino acids on the surface of the protein and the solvent water are formed, creating a solvation shell at the surface of the protein.

*d. Maximum molecular packing and colloidal stability*

Molecules or atoms attract each other through permanent, induced dipoles interactions or hydrogen bonds, as described in the previous sections. These attractive interactions all belong to the van der Waals type of interactions. When the molecules or atoms are too close and their electron orbitals or electrical layers overlap, this creates a strong electrostatic repulsion that exceeds the attractive forces. The energy of interaction between two atoms or molecules is the sum of the electrostatic repulsions and the attractive van der Waals interactions (Fig. 1.12). An optimal distance exists for minimising the energy required for the two atoms or molecules to get closer. The optimal distance between the atoms of a protein defines its maximum packing of a protein during the folding process (Sheehan, 2013). Although van der Waals interactions are weak, their effect is additive.

An electrical double layer made of hydration and ions forms at the surface of charged proteins (Fig. 1.13), affecting the net charge around the molecule. Proteins are amphoteric, i.e., contain both acidic and basic groups, and their net charge depends on the pH. The pI of a protein is the pH at which the migration of a protein under an electric field stops. The pI generally corresponds to the pH at which the protein carries no net charge (isoionic or isoelectric point). At their pI, the proteins have patches of both negative and positive charges. Therefore, the intermolecular interactions increase and the proteins tend to precipitate (Fig. 1.13). However, the presence of the double layer of ions protects the protein from precipitation by modifying the apparent charges around the molecules (salting-in phenomenon). Then, the interactions between proteins are the result of the balance of attractive and repulsive forces, as described above (Fig. 1.12). However, at lower ionic strength, the double layer is ineffective, whereas at higher ionic strength, the solvation of the

native protein decreases, leading to instability, due to the mobilisation of the water forming the solvation shell of the protein by the ions (salting-out).

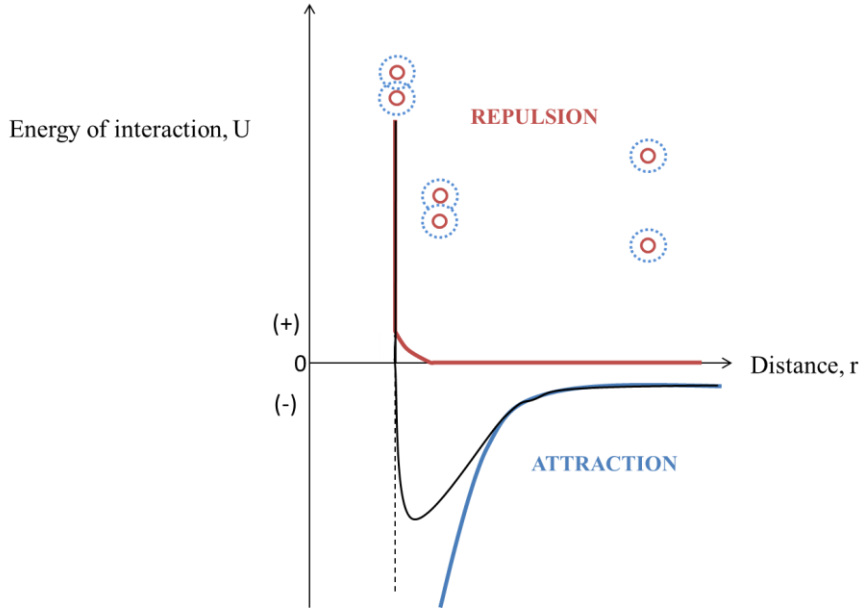



**Fig. 1.12** Schematic representation of the van der Waals interactions between two atoms, molecules or proteins.

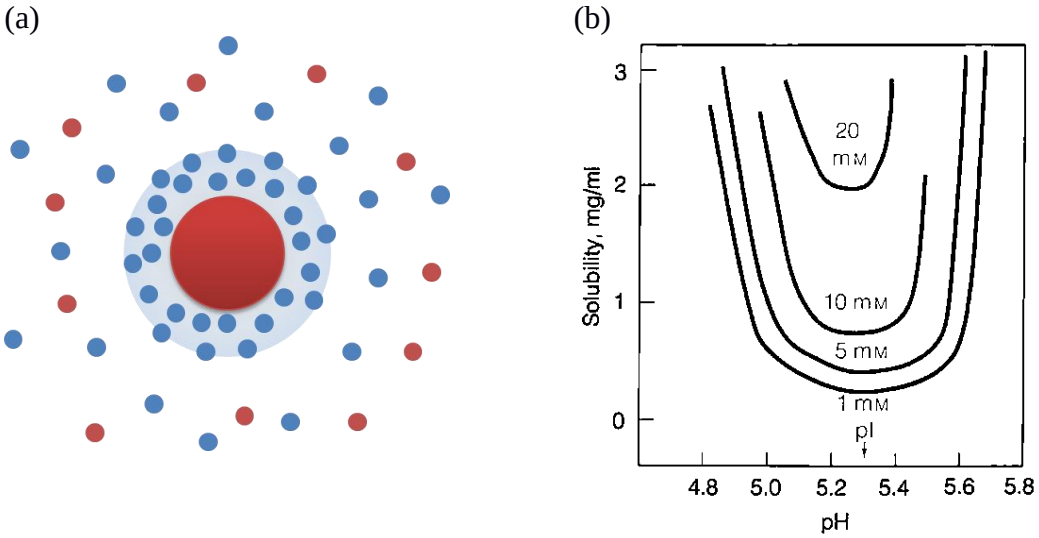



**Fig. 1.13** (a) Double electrical layer surrounding a negatively-charged protein in solution with (●) positively-charged ions at the surface of the protein and (●) negatively-charged ions beyond the effective counter ions atmosphere. (b) solubility of β-lactoglobulin as a function of the pH and the concentration in sodium chloride (Mathews & Van Holde, 1990).

### *2.1.3 Hydrophobic interactions*

The side chains of amino acids influence their properties and affect their intra- and intermolecular interactions. In particular, polar amino acids are able to form some of the electrostatic interactions listed in the previous section because they are charged or able to form a dipole moment. Therefore, these amino acids can form hydrogen bonds with water. In contrast, non-polar molecules are unable to form those interactions; these are called hydrophobic amino acids and their contact with water is minimized (Metzler, 1977). They are preferentially buried in the core of the secondary structure of the protein, closely associated with each other. This phenomenon is called hydrophobic effect. Hydrophobic effect requires the breaking of hydrogen bonds between polar amino acids and water, which reorganize around the hydrophobic sites. Hydrophobic effect is also involved in intermolecular interactions and is referred to as hydrophobic interactions. It requires the unfolding of the molecule, *i.e.*, exposure of the previously-buried amino acids to the solvent. Hydrophobic interactions are long-range, attractive interactions increasing in strength with increasing temperature up to 60-70°C (Bryant & McClements, 1998; Metzler, 1977).

### *2.1.4 Thermodynamics of protein unfolding*

Denaturation is generally defined as an alteration to the secondary or tertiary structure of a protein that does not affect its primary structure (Mulvihill & Donovan, 1987). Therefore, whey proteins, whose secondary and tertiary structures are particularly complex, are labile to heat. The loss of their native conformation has a major impact on the functionalities and nutritional properties of whey proteins (Pellegrino, Masotti, Cattaneo, Hogenboom, & De Noni, 2013; Schmidt, Packard, &

Morris, 1984). The denaturation mechanism of the whey proteins has been partly elucidated and modelled; in the solvent water, native and unfolded forms of the proteins are in equilibrium (Sheehan, 2013). The stabilising forces in favour of the native structure are only slightly thermodynamically favoured over the destabilising forces. The energy cost in maintaining the protein in a small number of conformations (rather than the large number possible if the protein was unfolded), also referred as chain entropy, contributes to the destabilization of the native protein structure. The other main destabilization force is the presence of local unfavourable folding in the protein, such as electrostatic repulsion between the side-chains of amino acids in close contact. Hydrogen bonds, disulphide bonds, hydrophobic and van der Waals interactions are stabilizing factors (Table 1.5) for the reasons presented in the Section 2.1 of this chapter.

**Table 1.5** Contributions to the stabilization of the folded form of proteins with the free energy of folding, ΔG. The stabilising forces contribute to a negative ΔG, while destabilising forces ΔG values are positive (Sheehan, 2013).

| Interaction (protein of 100 residues) | $\Delta G$ at 25°C (kcal/mole) |
|---|---|
| ***Destabilising:*** | |
| Chain entropy | 330-1000 |
| Unfavourable folding interactions | 200 |
| ***Stabilising:*** | |
| Increased van der Waals bonds due to close packing | -227 |
| Hydrogen bonds | -49 to -719 |
| Hydrophobic effect | -264 |
| Disulphide bridges | -4 |

Therefore, relatively marginal alterations to the environment of the protein, such as an increase in temperature, contribute to overcoming those stabilizing forces. In this particular case, transition to the denatured form of the protein occurs within a narrow range of temperature. Table 1.6 presents the temperatures of denaturation of some of the major whey proteins, i.e., the temperatures at which 50% of the molecules are denatured. It is clear from these values that the denaturation mechanisms of the whey proteins vary with solvent and pH. The high values of activation energy, calculated from the Arrhenius relationship for the denaturation of β-lg, provide additional evidence for the dependence of the denaturation phenomenon on temperature (Section 2.4).

**Table 1.6** Denaturation temperature of whey proteins and activation energy for the denaturation of β-lactoglobulin. From Mulvihill and Donovan (1987).

| Protein | Dispersion conditions | Temperature of denaturation (°C) | Activation energy (kJ/mole) |
|---|---|---|---|
| β-lactoglobulin | SMUF*, pH 6.7 | 72.8 | *n.a.*** |
| | $H_2O$, pH 3-9 | 58-85 | *n.a.* |
| | Phosphate, pH 6.75 | 70.5 | 306/341 |
| | $H_2O$, pH 3.5 | 85/140 | *n.a.* |
| | $H_2O$, pH 6.8 | 65-75/125-140 | *n.a.* |
| | Phosphate, pH 6.0 | 78 | *n.a.* |
| | Various buffer, pH 4-9 | 81.5-85.5 | 465-568 |
| | SMUF, pH 2.5-6.5 | 75.9-81.9 | *n.a.* |
| α-lactalbumin | SMUF, pH 6.7 | 65.2 | *n.a.* |
| | Phosphate, pH 6.0 | 62 | *n.a.* |
| | SMUF, pH 2.5-6.5 | 58.6-61.5 | *n.a.* |
| Bovine serum albumin | SMUF, pH 6.7 | 62.2 | *n.a.* |
| | Phosphate, pH 6.0 | 64 | *n.a.* |
| | SMUF, pH 2.5-6.5 | 71.9-74 | *n.a.* |
| Whey protein concentrate | Phosphate, pH 6.0 | 62/78 | *n.a.* |
| | SMUF, pH 2.5-6.5 | 76.9-88 | *n.a.* |

*Simulated milk ultrafiltrate (SMUF)

** Data unavailable (*n.a.*: not applicable)

## 2.2 Heat-induced denaturation of whey proteins

The denaturation mechanism of β-lg, the major whey protein in milk, has been studied in detail and is known to affect considerably the heat-induced coagulation of milk and whey protein systems. At pH 6-9, and at room temperature, β-lg is present in solution as a dimer. At temperatures greater than 40°C, β-lg undergoes a reversible change in conformation, i.e., β-lg dissociates into monomers and the intramolecular repulsions between its charged amino acids increase. From around 60°C, the hydrophobic sites of the protein and the thiol group initially located in the heart of the structure ($Cys_{121}$) are exposed to the solvent and are available for intermolecular reactions (Hoffmann & van Mil, 1997; Kehoe, Wang, Morris, & Brodkorb, 2011; Rowland, 1933; Tolkach & Kulozik, 2007). The reaction of $Cys_{121}$ in the formation of disulphide bonds enhanced the dissociation of β-lg into a monomer (Kontopidis et al., 2004). These covalent and non-covalent interactions occur between molecules of β-lg, or within a molecule of β-lg, leading to a variety of species formed, from non-native monomers (Croguennec, Bouhallab, Mollé, O'Kennedy, & Mehra, 2003) to high molecular weight aggregates (Hoffmann & van Mil, 1997). The formation of intermolecular disulphide bonds results in the irreversibility of the aggregation of β-lg (Kitabatake, Wada, & Fujita, 2001; Mounsey & O'Kennedy, 2007). The size of the aggregates varies, from nanoparticles to macro gels, as a function of the heating conditions and the starting material. Baussay, Bon, Nicolai, Durand, and Busnel (2004) reported that β-lg only aggregates once the concentration of β-lg is greater than the critical association concentration (CAC). A higher concentration ($C_g$) is required for the gelation of the system. In certain conditions, the unfolding of the whey proteins is only partial and the unfolded structure is referred to as molten-globule state. The individual monomers of

partially unfolded whey proteins at pH 7.0 are still visible after aggregation (Fig. 1.14). The intermolecular interaction of β-lg involved the disruption of its intramolecular β-sheet and the formation of intermolecular β-sheets (Lefèvre & Subirade, 2000).

It is noteworthy that some peptides and amphiphilic molecules affect the denaturation and aggregation of β-lg, possibly through binding to its calyx or the surface of the protein (Barbeau, Gauthier, & Pouliot, 1996; Donovan & Mulvihill, 1987; Kosters, Wierenga, de Vries, & Gruppen, 2011; Patel, Kilara, Huffman, Hewitt, & Houlihan, 1990; Wijayanti et al., 2019).

Although all whey proteins are described as thermolabile, they present important differences in structure, which affect the conditions for their denaturation. α-La, the second major whey protein in milk, contains four disulphide bonds and no free thiol group. For this reason, α-la, heated on its own, does not polymerize and has been shown to renature upon cooling under certain conditions, by reforming its intramolecular disulphide bonds (Relkin et al., 1992). However, α-la aggregates with β-lg when heated together in whey protein isolate (Kehoe et al., 2011). The contribution of the non-covalent bonds to the denaturation of α-la was greater than for β-lg (Crowley, Dowling, Caldeo, Kelly, & O'Mahony, 2016). Greater sensitivity to denaturation was reported for Ig, BSA and β-lg compared to α-la in skim milk, acid whey and cheese whey (Havea, Singh, & Creamer, 2002; Law & Leaver, 2000). In addition, the stability of α-la is directly related to the binding of one ionic calcium (Section 1). The holo-form, (one calcium ion bound) is more stable than the apo-form (no calcium bound), with denaturation temperatures of ≈60°C and ≈30°C, respectively (Relkin et al., 1992). The characteristics of α-la affect its interactions

with β-lg and the heat-induced aggregates formed (Buggy et al., 2016; Crowley et al., 2016).

The formation of heat-induced particles of whey proteins can be simplified in two steps: denaturation and aggregation. The kinetics of denaturation and aggregation are calculated as a function of the initial concentration of protein and the disappearance of native protein over time. Denaturation and aggregation are first-order and second-order processes respectively (Verheul, Roefs, & de Kruif, 1998). Therefore, depending on the conditions of heating, either the first step is rate-limiting, with an overall reaction order of 1, or the aggregation step is rate-limiting, whereby the reaction order is closer to 2. The conditions of heating promoting aggregation as rate-limiting are high temperature, pH above the pI of the protein and low ionic strength (Section 2.4). In general, the overall reaction order of β-lg and α-la are reported to be around 1.5 and 1, respectively (Havea et al., 2002; Kehoe et al., 2011). The conditions of heating promoting the denaturation step as rate-limiting are low heating temperature, pH close to the pI of the proteins and high NaCl concentration for example.

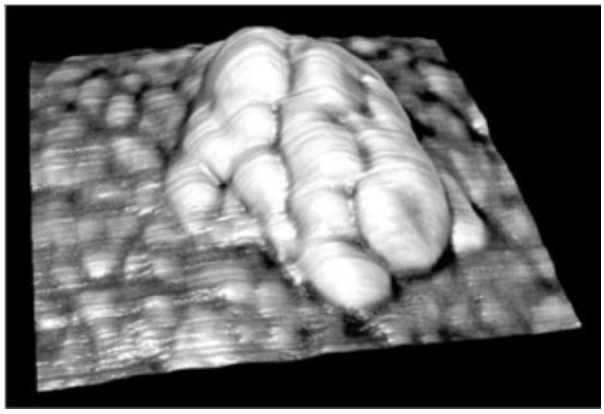


**Fig. 1.14** Atomic force microscopy image of heat-induced whey protein aggregates, with an overlaid phase image (Kehoe et al., 2011). The individual monomers of whey proteins are still visible after aggregation. The solution of whey proteins (5%, w/v) was heated at 78°C for 30 min at pH 7.0.

## 2.3 Controlling denaturation and aggregation of β-lactoglobulin by modulating heating conditions

Conditions of heating can promote closer contact between proteins and destabilize the native whey proteins by favouring their polymerization. Other conditions of heating can stabilise the whey proteins or contribute to the formation of soluble aggregates. Although they do not constitute an exhaustive list of parameters affecting the heat-induced denaturation and aggregation of whey proteins, pH, ionic strength, protein concentration and temperature are key to controlling the aggregation of whey proteins (Fig. 1.15). The interdependence of these parameters on the denaturation and aggregation of the whey proteins requires caution when interpreting results. β-Lg is the major whey protein in milk. This section presents some findings on the effect of these parameters and their combination in β-lg systems, with the objective of a better comprehension of the phenomena presented in the experimental chapters. Section 3 is solely dedicated to the effect of casein molecules on the denaturation and aggregation of whey proteins, with a particular emphasis on the effects of κ-casein and caseinomacropeptide.

At the pI, the native conformation of a protein is more stable to denaturation, thanks to minimal internal repulsive charges (Hillier, Lyster, & Cheeseman, 1979). However heating close to the pI also lowers the electrostatic repulsion between proteins, thus favouring their aggregation (Fig 1.17) and promotes the formation of larger-size aggregates (Mehalebi, Nicolai, & Durand, 2008). At pH less than the pI of a molecule, β-lg stability is at its maximum (Fig. 1.16), likely due to the formation of extra internal hydrogen bonding through the titration of carboxyl groups (Lefèvre & Subirade, 2000). The loss of localized unfavourable electrostatic interactions could also contribute to the increased stability of the protein (Kella & Kinsella, 1988;

Verheul et al., 1998). On the contrary, an increase in pH leads to greater intramolecular charge repulsion. In addition to the exposure of the thiol group of β-lg at pH 7, its reactivity increases from pH 6, promoting the formation of intermolecular disulphide bonds. At pH 6.0, the aggregates have been shown to be mainly formed by non-covalent interactions (Hoffmann & van Mil, 1997). At pH greater than pH 6.0, the denaturation and aggregation involved intermolecular disulphide bonds.

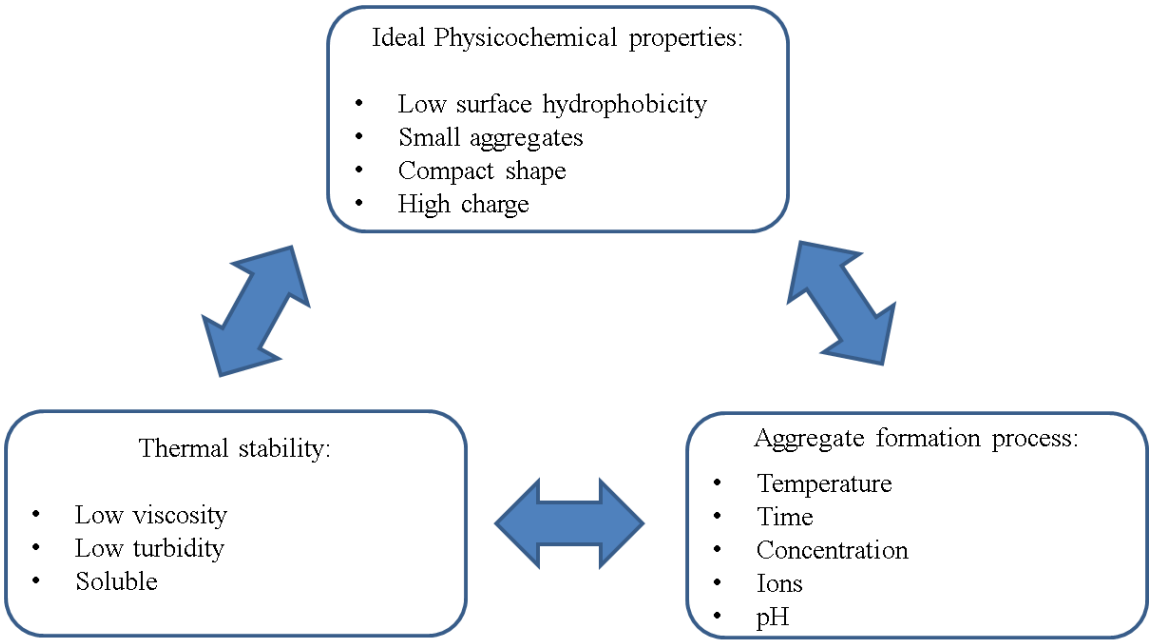



**Fig. 1.15** Intertwined relationships between the conditions of heating, the physico-chemical properties of the resulting heat-induced aggregates and their thermal stability (Ryan, Zhong, & Foegeding, 2013).

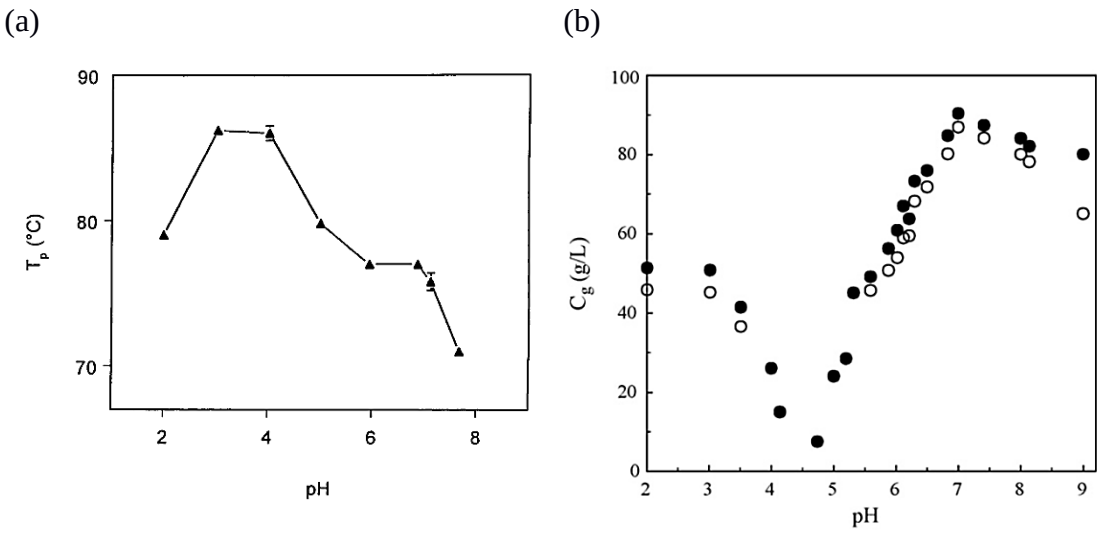



**Fig. 1.16** (a) Temperature of denaturation (Tp) of β-lactoglobulin (β-lg) measured by differential scanning calorimetry as a function of pH. The concentration of β-lg was 4.6% (w/v) and the heating rate was 5°C/min (Verheul et al., 1998). (b) Dependence of the critical concentration of gelation ($C_g$) on the pH after heating at 80°C for 15 h (Mehalebi et al., 2008). The isoelectric point of β-lg is 5.2.

With pH 6.0 as a transition point, the structure of β-lg gels differs considerably as influenced by pH (Fig. 1.17). Particulate gels are formed between pH 4.0 and 6.0, whereas fine-stranded networks are formed at pH greater than pH 6.0 when the electrostatic repulsion is strong between proteins (Stading & Hermansson, 1990). At very acidic pH (pH≈2), different types of fine-stranded structures are found and the structure of the protein network influences its firmness and its elastic properties.

The pH and the ionic strength of a solution of β-lg have a combined effect on the aggregation behaviour and the resulting structure of the heat-induced aggregates. As described in Section 2.1.2, ions in solution interacts with native proteins and can even improve their solubility. During heat treatment, whey proteins unfold and intermolecular interactions take place. In that new configuration, salts affect both the heat-induced denaturation and the aggregation of whey proteins. Around neutral pH,

the native conformation of β-lg is stabilised by the addition of sodium chloride (NaCl) and the reaction order in the initial stage of denaturation decreases, i.e. the denaturation becomes the rate-limiting step (Verheul et al., 1998). However, the charge screening, at this pH, decreases the electrostatic interactions between proteins and promotes physical aggregation, involving non-covalent interactions, and chemical aggregation, involving disulphide bonds. Larger aggregates are formed and the CAC and Cg of β-lg at pH 7.0 is lowered in presence of NaCl (Baussay, Bon, Nicolai, Durand, & Busnel, 2004; Mahmoudi, Mehalebi, Nicolai, Durand, & Riaublanc, 2007). The effect of calcium is stronger than that of NaCl. Baussay et al. (2004) reported a value of Cg at 0.1 M of NaCl of 0.7% (w/v), while the Cg in the presence of calcium (1 mM) was less than 0.1% (w/v). The required amount of calcium added to decrease the temperature of gelation of β-lg was 5.5 times lower than that of added NaCl (Gault & Fauquant, 1992). In particular, calcium is thought to screen the charges of the protein by binding specifically to the surface of the protein through the carboxylate groups with a threshold affinity, facilitating intermolecular interactions between whey proteins (Simons, Kosters, Visschers, & de Jongh, 2002). The presence of calcium was shown to accelerate the loss of native whey proteins, to promote the formation of large aggregates and to favour the formation of clusters of proteins rather than the fine-stranded network expected at pH greater than pH 6 (Havea et al., 2002). The effect of calcium on β-lg denaturation and aggregation was particularly strong at pH 7, where β-lg is negatively charged (O'Kennedy & Mounsey, 2009). In some extreme cases of combined extended heating, low pH and low ionic strength, β-lg formed long fibrils (Nicolai & Durand, 2013).

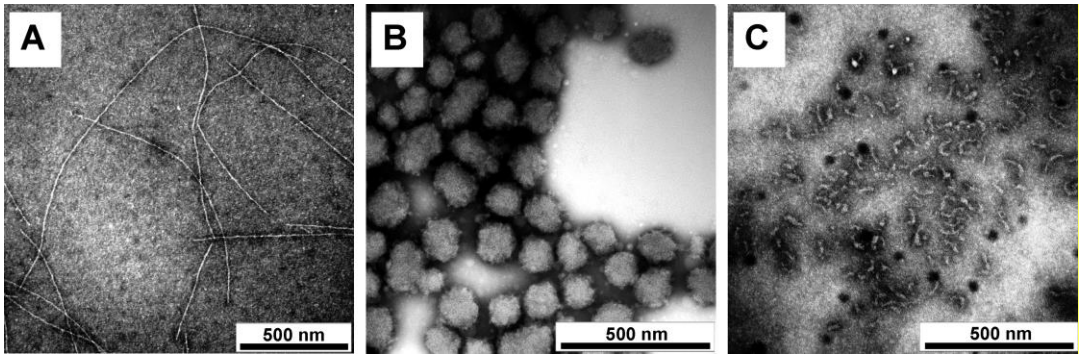



**Fig. 1.17** Transmission electron micrographs of heat-induced aggregates of β-lactoglobulin (1% w/v) at (A) pH 2.0, (B) pH 5.8 and (C) pH 7.0 (Jung, Savin, Pouzot, Schmitt, & Mezzenga, 2008).

As reported in Section 2.3, the formation of aggregates and the gelation of the system depend on the concentration of protein. In addition, the rate of whey protein aggregation depends on the ratio of protein to calcium (Sherwin & Foegeding, 1997). In general, an increase in β-lg content yields larger molecular weight aggregates and promotes the aggregation of the protein, by increasing the probability of contact between the unfolded molecules (Baussay et al., 2004; Hillier et al., 1979; Mehalebi et al., 2008). Kehoe et al. (2011) reported that the number of aggregates increased with the protein concentration and the heating time. In 2-9% (w/v) β-lg solutions heated at 78°C and pH 7.0, the rate of denaturation of β-lg increased with the protein concentration in a linear relationship (Kehoe et al., 2011). However, the increase in concentration resulted in a decrease of the reaction order. A further increase in whey protein concentration (25%, w/v) was reported to lower the rate of denaturation, which could be caused by a reduced molecular mobility (Dissanayake, Ramchandran, Donkor, & Vasiljevic, 2013).

The heat load is defined as the intensity of heat treatment, and is a function of its duration and temperature. Tolkach and Kulozik (2007) reported that the denaturation rate of β-lg increased with temperature (85-120°C). Increasing temperature from 75

to 90°C was reported to increase the number and size of whey protein aggregates (Ndoye, Erabit, Flick, & Alvarez, 2013). At 90°C, the partial unfolding of β-lg in a molten-globule state, was complete. Under the conditions of the study, the unfolding was rate-limiting at temperature below 90°C, i.e., the unfolding of β-lg was slower than aggregation. In contrast, at temperatures greater than 90°C, the aggregation was the rate-limiting process (Tolkach & Kulozik, 2007). However, other heating conditions, in particular the ones described in this section, must be taken into consideration (Fig. 1.18). In general, longer heating time allows the denaturation process of whey proteins to continue and the aggregates to grow until a steady-state is reached, whose duration depends on the heating conditions (Tolkach & Kulozik, 2007).

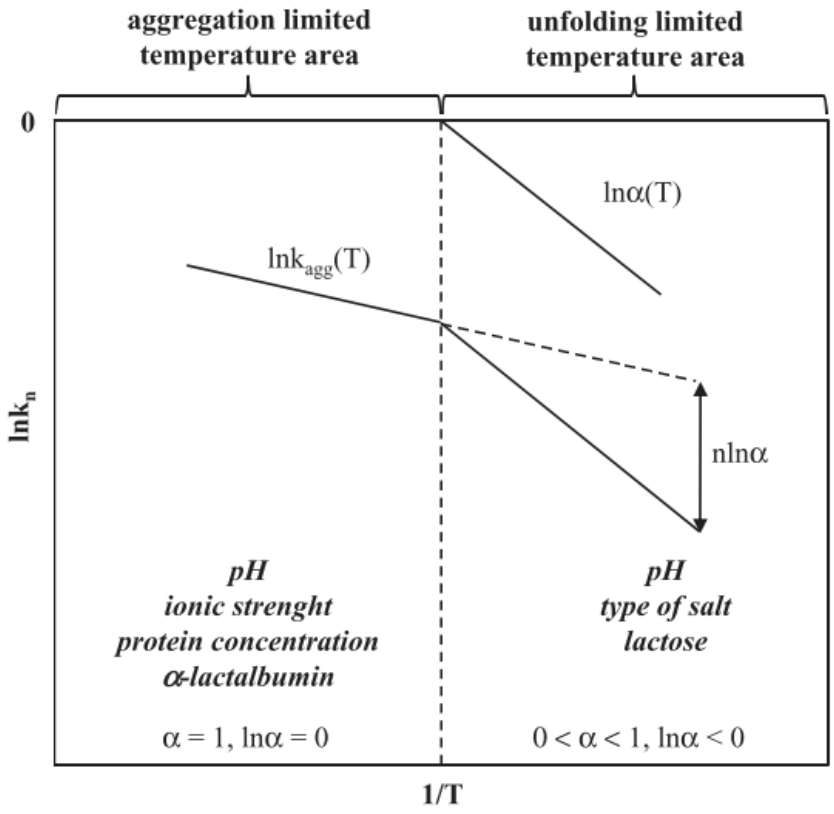



**Fig. 1.18** Arrhenius plot showing the dependence of the denaturation rate of β-lactoglobulin on the temperature and other heating conditions, with α, the degree of unfolding, T, the temperature, $k_n$, the velocity constant for the denaturation of β-lg, n, the reaction order and $k_{agg}$, the aggregation rate constant. $k_n$ depends on α and $k_{agg}$. In the temperature range where aggregation is rate-limiting, all molecules are unfolded (α=1) and $k_n$ takes the value of $k_{agg}$. In the temperature range where denaturation is rate-limiting, the degree of unfolding decreases (0<α<1), therefore, $k_n$ decreases and its value deviates from the one calculated at temperature where the aggregation was rate-limiting. The conditions of heating influence $k_n$ both in the unfolding- and the aggregation-limiting area (Nicolai, Britten, & Schmitt, 2011).

# 3. Chaperone-like activity of caseins

## 3.1 Introduction

In biology, molecules with the ability to act as chaperones participate in the folding and refolding of other proteins, and can protect them against unfolding, aggregation and precipitation when subjected to various stresses, such as heat, oxidation and reduction. Caseins display chaperone-like activity, as detailed in the section below, which may be used to explain the enhanced solubility of whey proteins when heated together with caseins. However, the activity of caseins does not necessarily comply with the strict definition of chaperone activity in biological conditions as conventionally applied in biological systems. In food science, a major concern for food processing is the denaturation, aggregation and gelation of protein ingredients during heat treatment. Therefore, the term "chaperone-like activity" will be used instead, and will apply to molecules that prevent or limit the extent of denaturation or aggregation specifically of the heat-labile whey proteins. In addition, the following section focuses solely on the chaperone like activity of individual casein molecules, in contrast to the gelation behaviour of whey proteins in systems containing casein micelles.

## 3.2 Chaperone-like activity of $\alpha_s$- and β-casein

Both $\alpha_s$- and β-casein have been reported to reduce the extent of the aggregation of heat-labile proteins. $\alpha_s$-Casein and β-lg form heat-induced aggregates of molecular weight around 1.8 MDa and $\alpha_s$-casein prevents the precipitation of β-lg at 70°C and pH 7.1, as illustrated in Fig. 1.19 (Morgan, Treweek, Lindner, Price, & Carver, 2005). This effect is pH-dependent, with a lesser chaperone-like activity at pH > 7.0. This could be due to a shift towards the monomeric form of β-lg at higher pHs or due

to the deprotonation of $\alpha_s$-casein. $\alpha_s$-Casein also prevents the heat-induced precipitation at pH 7.1 of α-la in its holo form and after reduction of its disulphide bonds, in a ratio $\alpha_s$-casein to α-la of 6:1. At pH 5.8 to 6.7, β-casein aggregated with β-lg to form nano-sized particles with a narrower size distribution than that of aggregates comprised of β-lg only (Kehoe & Foegeding, 2014). β-Casein can even interrupt the on-going aggregation of a target protein during heating (Zhang et al., 2005). The chaperone-like activity of $\alpha_s$-casein on β-lg at pH 6.0 was observed up to 75°C, whereas β-casein was an effective chaperone for β-lg at 90°C, as shown on Fig. 1.20 (Yong & Foegeding, 2008). Both caseins are thought to bind β-lg at pH 6.0 and to promote the formation of transparent, fine-stranded gels, rather than turbid, particulate gels. In addition, $\alpha_s$-casein has been reported to decrease the temperature of denaturation of β-lg, α-la and BSA at pH 6.6 (Paulsson & Dejmek, 1990).

The typical characteristics of a molecule capable of chaperone-like activity include a flexible structure, exposed regions of high hydrophobicity and an amphipathic nature (Koudelka, Hoffmann, & Carver, 2009). As detailed in Section 1, caseins do not possess a high degree of secondary structure and present heterogeneity of structures, with distinct hydrophobic domains and highly-negatively charged regions (Fig. 1.5). In particular, the negative charges arising from the phosphorylated residues on $\alpha_s$- and β-casein are thought to play an important role in the reduction of the aggregation of target proteins, and to solubilise the resulting aggregates (Koudelka et al., 2009). It is noteworthy that the dephosphorylation of $\alpha_s$- and β-casein results in an increase in their degree of the secondary structure and a decrease of the exposure of their hydrophobic regions.

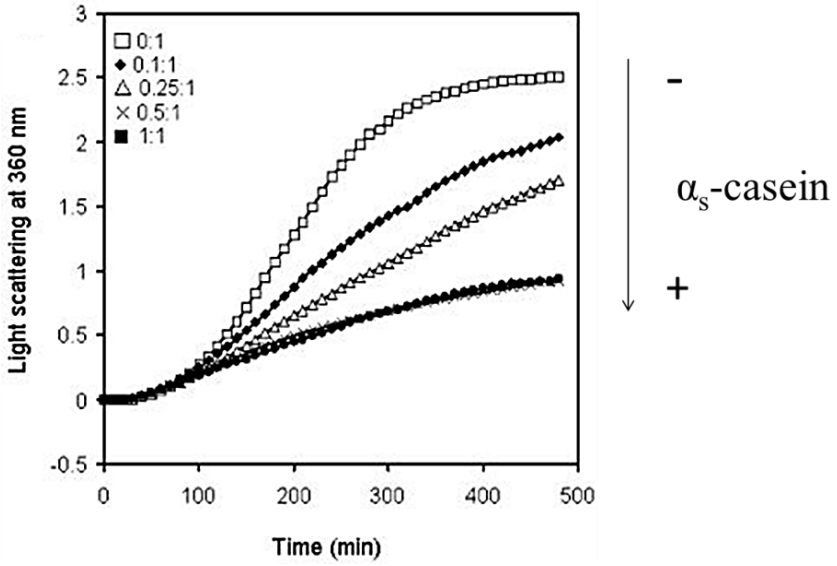



**Fig. 1.19** Measurement of the light scattering at 360 nm as an indication of the extent of β-lactoglobulin aggregation at pH 7.1 during heating at 70°C for 500 min, with $\alpha_s$-casein in a ratio of β-lg to $\alpha_s$-casein from 0:1 to 1:1. Modified from Morgan et al., 2005.

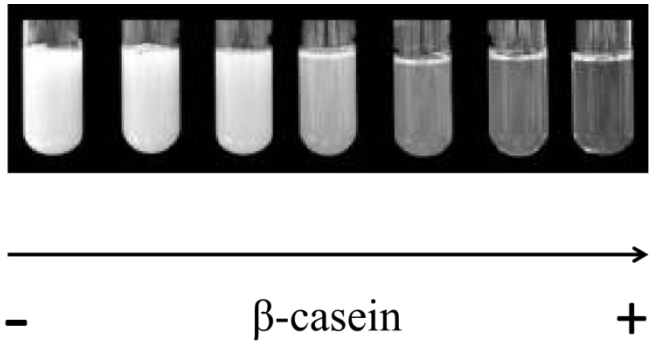



**Fig. 1.20** Appearance of mixtures of 6% (w/v) β-lactoglobulin and β-casein (0-2%, w/v) after heating at 90°C for 20 min with an increasing proportion of β-casein, modified from Yong and Foegeding (2008).

### 3.3 Chaperone-like activity of κ-casein

In contrast to $\alpha_s$- and β-casein, κ-casein is able to form intermolecular disulphide bonds with whey proteins, which results in the irreversible formation of aggregates (Guyomarc'h, Law, & Dalgleish, 2003). In heated milk, these aggregates are present in the serum phase or attached to the surface of the casein micelle (Donato & Dalgleish, 2006). The concentration of the aggregates of κ-casein and whey proteins in the serum increases with the pH of heating.

The aggregates of κ-casein and whey proteins have a net charge of approximately -18 mV at pH 6.7 and a size ranging from 30 to 100 nm, with a molecular weight around $4.10^6$-$2.10^7$ g/mol (Guyomarc'h et al., 2003; Jean, Renan, Famelart, & Guyomarc'h, 2006). Their pI is around 4.5 but they precipitate at pH 5.3 to 5.5 (Morand, Guyomarc'h, & Famelart, 2011). The size of the aggregates decreases with an increasing proportion of κ-casein in the aggregates, up to an optimum ratio of whey protein to κ-casein (1:0.5), and the aggregates have been described as roughly spherical (Guyomarc'h et al., 2003; Guyomarc'h, Nono, Nicolai, & Durand, 2009).

Whey proteins precipitate in milk ultrafiltrate, whereas aggregates of κ-casein and whey proteins remain soluble (Parker, Donato, & Dalgleish, 2005). Adding κ-casein to a whey protein solution restricts the development of turbidity during heat treatment at pH 7.0, resulting in the formation of a transparent system rather than highly turbid solutions (Guyomarc'h et al., 2009). Doi, Ibuki, and Kanamori (1981) reported that the precipitation of β-lg, induced by its heating at 90°C for 20 min, was limited by the presence of κ-casein. In addition, the chaperone-like activity of κ-casein increased with its carbohydrate content. As discussed in Section 1, the

glycosylation of κ-casein decreases its pI and increases the net negative charge on the molecule. The charge density of κ-casein, co-aggregated with whey proteins, is likely to limit the extent of aggregation by electrostatic repulsion.

The presence of κ-casein does not modify the shape of the whey protein aggregates; although the aggregates are less dense than that of the aggregates of whey protein only (Guyomarc'h et al., 2009). However, at lower ratio of whey protein to κ-casein (1:2), the aggregates are more linear (Fig. 1.21), possibly due to a favoured head-to-tail aggregation of the whey proteins in the presence of the highly negative charges of κ-casein (Morand et al., 2011). It is noteworthy that κ-casein also influences the denaturation of β-lg, with an increase in the ratio of κ-casein to β-lg resulting in a decrease of the denaturation temperature of β-lg (Paulsson & Dejmek, 1990).

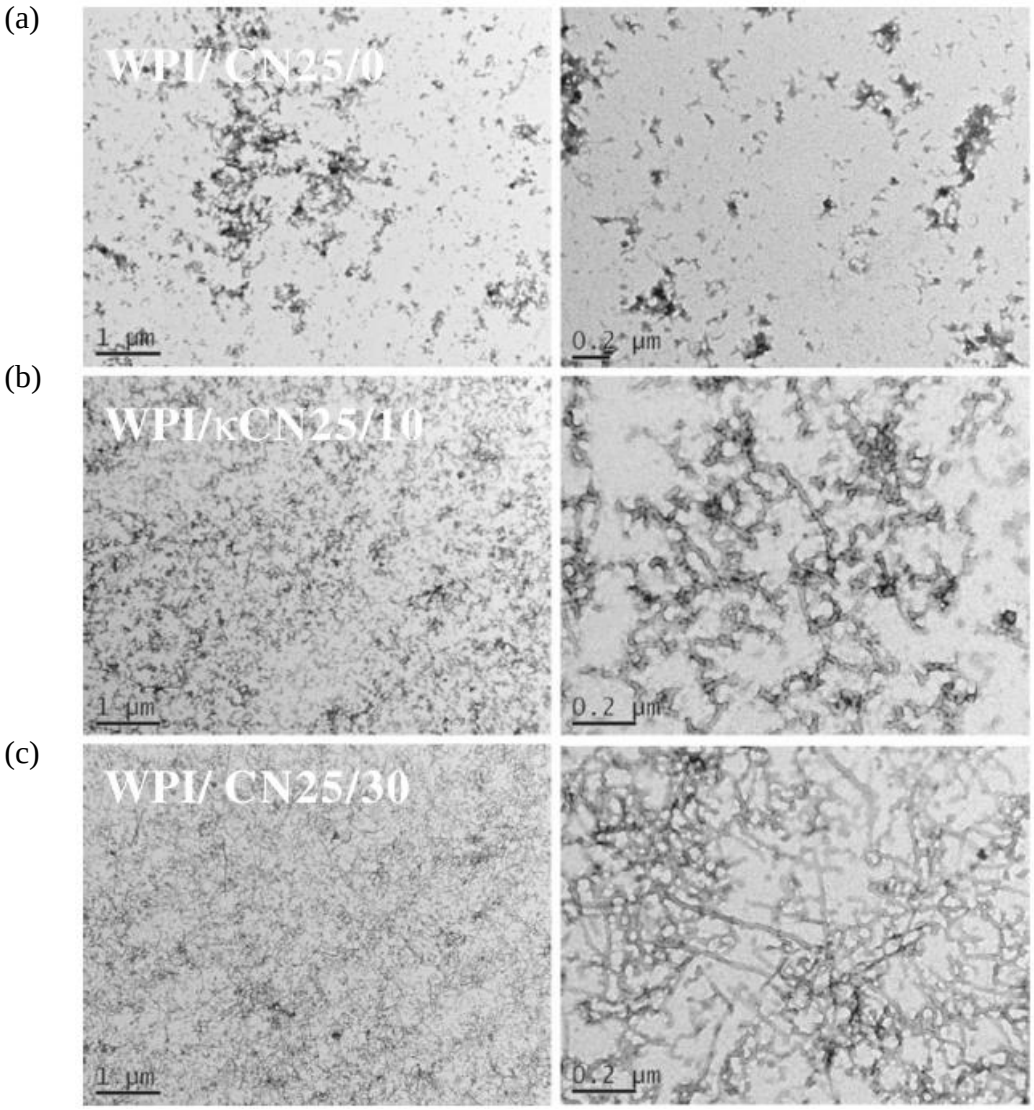



**Fig. 1.21** Transmission electron microscopy images of heat-induced aggregates of whey protein (2.5%, w/w) and κ-casein (0-3%, w/w) in ratios of whey protein to κ-casein of (a) 25:0, (b) 25:10 and (c) 25:30, formed after heating at 80°C for 24 h at pH 7.0 in the presence of 0.1 M NaCl (Morand et al., 2011).

### 3.4 Chaperone-like activity of caseinomacropeptide

CMP is a peptide derived from the enzymatic cleavage of κ-casein and is glycosylated and phosphorylated to the same degree as κ-casein, as discussed in Section 1.2.6. Therefore, CMP is a very hydrophilic and negatively-charged peptide. However, it lacks the ability to form disulphide bonds with whey proteins due to the absence of cysteine residues. It also contains hydrophobic amino acids available for intermolecular interactions that are mainly located at the N-terminus end of the peptide (Fig. 1.6). CMP and β-lg interact spontaneously at room temperature and form aggregates of approximately 6 nm at pH 7.0 and up to 1 μm at pH 3.5, through non-covalent interactions (Martinez, Farías, & Pilosof, 2010). From pH 3.0 to 6.7, CMP accelerates the formation of aggregates of β-lg in the temperature range 65 to 95°C (Croguennec et al., 2014). During heat treatment at pH 6.7, the negative charges carried by CMP are thought to destabilise the native conformation of β-lg, whereas the hydrophobic amino acids of CMP interact with the hydrophobic regions of the unfolded β-lg. The negative charges of CMP limit the extent of aggregation of β-lg by providing additional electrostatic repulsions (Fig. 1.22). The effect of CMP on β-lg denaturation is dependent on the pH and the ratio of whey protein to CMP. The aggregates of CMP and whey proteins had sizes ranging from 180 to 200 nm at pH 6.7 and over 5 μm at pH 4.0. At pH 6.7, heated solutions of CMP and β-lg were clear, whereas precipitation occurred at pH 4.0. The attractive electrostatic interactions between the negatively-charged CMP and the positively-charged β-lg at pH 4.0 are thought to initially stabilise the native conformation of β-lg, and then to promote interactions between the unfolded protein and CMP. In addition, CMP hinders the formation of disulphide bonds between whey proteins during heating (Xianghe, Pan, Peilong, Ismail, & Voorts, 2012). Data on the thermal unfolding of

whey proteins in the presence of CMP and on the characteristics of the gels of whey protein and CMP vary considerably depending on the study. However, both types of data depend on the pH of heating and the ratio of whey protein to CMP (Martinez et al., 2010; Svanborg, Johansen, Abrahamsen, Schüller, & Skeie, 2016; Xianghe et al., 2012).

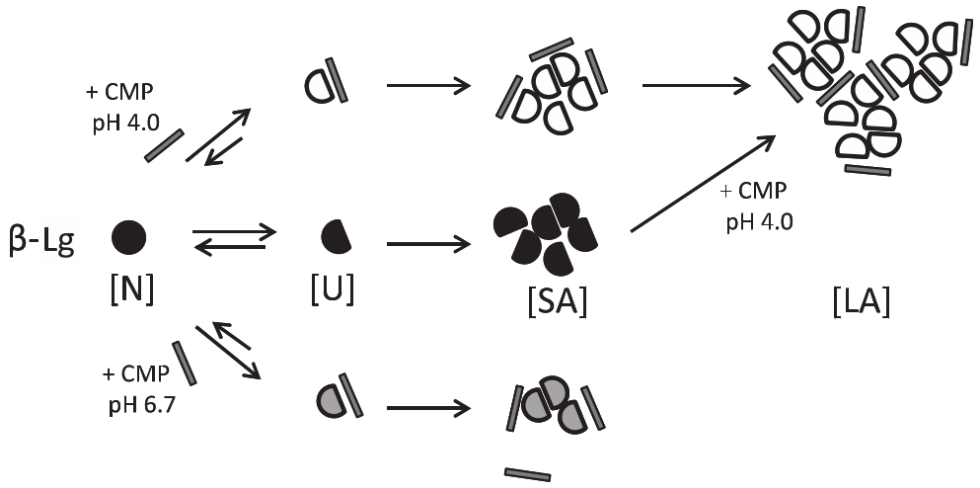



**Fig. 1.22** Mechanisms of interactions between CMP and β-lactoglobulin (β-lg) during heat treatment at pH 4.0 and 6.7, with the native (N) and unfolded (U) form of β-lg. SA and LA are the small and large heat-induced aggregates of β-lg, respectively. The negatively-charged β-lg are in grey and the positively-charged β-lg are in white. At pH 4.0, CMP and β-lg are oppositely-charged and interact by attractive electrostatic interactions to form large aggregates. At pH 6.7, CMP and β-lg interact by hydrophobic interactions and the negative charges carried by CMP prevent the extensive aggregation of β-lg. (Croguennec et al., 2014)

## 4. Conclusion

Whey proteins denature and aggregate irreversibly at temperatures greater than 60°C. The aggregation of whey proteins is driven by a balance of attractive and repulsive forces and interactions, which can be modulated by the heating parameters, such as salt, pH, temperature, protein composition and concentration, etc. In particular, the presence of caseins can limit the extent of aggregation and precipitation of whey proteins during heat treatment, with the negative charges carried by the phosphorylated residues of caseins playing an important role in their chaperone-like activity. In contrast to other caseins, κ-casein is also glycosylated, which makes the molecule strongly negatively-charged. In addition, κ-casein can form intermolecular disulphide bonds with whey proteins. CMP is a 64 amino acid peptide derived from κ-casein that is not able to form disulphide bonds with whey proteins, however it is also glycosylated and phosphorylated to the same degree as κ-casein. These specificities are assumed to play an important role in modulating the denaturation and aggregation behaviour of whey proteins in the presence of κ-casein and CMP.

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## Research objectives

Thermal processes, such as pasteurisation and ultra-high temperature processing, are applied to ingredients and products in the dairy industry to ensure the microbiological safety, aligned with the storage conditions of such products. However, these processes can result in the turbidity development, sedimentation and sensory challenges in the products and fouling of the heat treatment equipment used, due to the denaturation and aggregation of the heat-labile whey proteins.

In contrast to other caseins, κ-casein can form disulphide bonds with whey proteins during heat-treatment and when heated in combination with whey proteins (e.g, in milk-based systems), the negative charges of the glycosylated residues of κ-casein at neutral pH are thought to be responsible for the relatively small size of the whey protein aggregates. Caseinomacropeptide (CMP), a peptide derived from the enzymatic cleavage of κ-casein during cheese and rennet manufacture and present at significant levels in sweet whey (~25%, w/w of total protein). Although CMP lacks the ability to form disulphide bonds, it has the same degree of phosphorylation and glycosylation as κ-casein and has been shown to display chaperone-like activity with β-lactoglobulin. However, the effect of such glycosylated residues on the denaturation and aggregation of whey proteins has not been fully elucidated yet. In particular, the role of the glycosylation in the mechanism of the chaperone-like activity is poorly understood to date. Therefore, κ-casein and CMP could be included in whey protein-based ingredients and products to enhance the heat-stability of such systems.

The objectives of this study were:

1) To determine the effect of κ-casein on the heat-induced aggregation of whey proteins and the effect of the inclusion of κ-casein on the heat-stability and solubility of whey protein aggregates.

2) To assess the ability of CMP to limit the extent of aggregation of whey proteins and increase their solubility after heat-treatment.

3) To determine the effect of the CMP and the role of glycosylation of CMP on the mechanism of denaturation and aggregation of whey proteins.

This research sought to gain a better understanding of the interactions between such negatively-charged molecules and whey proteins to assist the development and production of highly heat-stable ingredients.

κ-Casein has been shown to display chaperone-like activity on the aggregation of whey proteins, as reviewed in Chapter 1. However, the mechanism of chaperone-like activity of κ-casein has not been fully elucidated yet. The following chapter aimed to determine the effect of κ-casein on the heat induced aggregation of whey proteins and the effect of the inclusion of κ-casein on the heat-stability and solubility of whey protein aggregates.

# Chapter 2

## Isolation and characterization of κ-casein/whey protein particles from heated milk protein concentrate and role of κ-casein in whey protein aggregation

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SJG contributed to the experimental design, determined the protein content of the samples, the detail of their composition in protein, their hydrophobicity, their particle size distribution by light scattering and size-exclusion chromatography, analysed the data and wrote the manuscript. MAEA provided the electron microscopy images. AB provided the atomic force microscopy images and reviewed the manuscript. JOM and AK reviewed the manuscript.

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**Abstract**

Milk protein concentrate (MPC, 79% protein) reconstituted at 13.5% (w/v) protein was heated at 90°C for 25 min (pH 7.2) with or without added calcium chloride (2.5 or 5 mM). After fractionation of the casein and whey protein aggregates by fast protein liquid chromatography (FPLC), the heat stability (90°C for up to 1 h) of the fractions (0.25%, w/v, protein) was assessed. The heat-induced aggregates were composed of whey protein and casein, in whey protein:casein ratios ranging from 1:0.5 to 1:9. The heat stability was positively correlated with the casein concentration in the samples. The samples containing the highest proportion of caseins were the most heat-stable, and close to 100% (w/w) of the aggregates were recovered post-heat treatment in the supernatant of such samples (centrifugation for 30 min at 10,000×*g*). κ-Casein appeared to act as a chaperone controlling the aggregation of whey proteins, and this effect was stronger in the presence of $\alpha_s$- and β-casein.

# 1. Introduction

Dairy protein-based ingredients are widely used in the food industry due to their high nutritional value (Hambræus & Lönnerdal, 2003) and their functional properties, such as emulsification, foaming and encapsulation (Buggy et al., 2016; Doherty et al., 2011; Morr, 1982; Ryan et al., 2013; Tavares, Croguennec, Carvalho, & Bouhallab, 2014). Today, they are common ingredients in sport drinks, meal replacers and infant formula (Early, 2012; Smithers, 2015).

Heat treatment is widely applied in the dairy industry, normally to increase the shelf-life of products, but also to improve functional properties. Nevertheless, intensive thermal treatment can lead to undesirable outcomes such as gelation, Maillard reactions, and precipitation (i.e., fouling and sedimentation) of proteins. Bovine milk protein contains 80% (w/w) caseins and 20% (w/w) whey protein. However, the heat-induced coagulation of milk is a process dominated by the chemistry and reactivity of β-lactoglobulin (β-lg), the major whey protein in milk. Inducing the aggregation of whey proteins into nano- to micro-sized particles, by pre-heating, is known to increase their heat stability (Joyce, Brodkorb, Kelly, & O'Mahony, 2016; Ryan et al., 2012) and has been extensively applied to whey proteins solution and skim milk (Laiho, Ercili-Cura, Forssell, Myllärinen, & Partanen, 2015; Ryan & Foegeding, 2015; Ryan et al., 2012; Sağlam, Venema, de Vries, & van der Linden, 2014). Milk protein solutions with high thermal stability are characterized by low viscosity, low turbidity and high solubility after heating. These conditions are influenced by physico-chemical properties of the particles, such as surface hydrophobicity, aggregate size, shape and charge (Joyce et al., 2016; Ryan et al., 2013; Wijayanti, Bansal, & Deeth, 2014). As a result, the heat stability of proteins

varies greatly with the pH at heating, the ionic strength of the dispersion, and the heat load applied.

Combined aggregates of whey proteins and caseins show higher heat stability than whey protein aggregates. In fact, a solution of acid whey protein prepared by ultrafiltration, containing around 3.2% (w/w) protein, gelled immediately when heated at 93°C; however, a blend of whey protein and caseins, in the proportion 1:1 and heated under the same conditions was stable for up to 30 min of heating at 93°C, i.e., at least 97% of the total protein initially present in solution were still soluble after heating (Patocka, Jelen, & Kalab, 1993). It is generally accepted that this is due to a chaperone-like activity of the caseins. Chaperone activities can stabilize proteins from unfolding, aggregation and precipitation (Morgan et al., 2005). In dairy science and technology, aggregation is probably the more important phenomenon, as uncontrolled aggregation can lead to destabilisation. $\alpha_s$-, β- and κ-casein have been shown to exhibit chaperone activity against aggregation (Mounsey & O'Kennedy, 2010). In fact, experiments carried out in a tubular heat exchanger at 95°C (Guyomarc'h et al., 2003) and in Teflon tubes (4.6 $cm^3$) in an oil bath at 145°C (Kehoe & Foegeding, 2011) showed that the size of whey protein aggregates is reduced when the whey proteins are heated in the presence of casein micelles or β-casein.

Hydrophobic interactions, ionic interactions, Van der Waals interactions and disulphide bonding are responsible for the formation of reversible and irreversible aggregates between caseins and whey proteins (Guyomarc'h et al., 2003). The aggregates in the serum phase of heated milk are mainly composed of κ-casein, β-lg, and α-lactalbumin (α-la). Bovine serum albumin (BSA), lactoferrin, β-casein and $\alpha_s$-

caseins are also involved in these aggregates, albeit to a minor extent (Donato & Dalgleish, 2006). Even though the formation of casein-whey protein aggregates has been shown (Jang & Swaisgood, 1990), part of the aggregates analysed may also be polymers of κ-casein (Farrell Jr, Wickham, & Groves, 1998) or aggregates of whey proteins only (Boye, Alli, Ismail, Gibbs, & Konishi, 1995). In the serum phase of skim milk heated at 90°C for 10 min (pH 6.7), the ratio of whey protein to κ-casein in the aggregates is in the range 1:0.2 to 1:0.7 (Donato & Dalgleish, 2006). The whey protein and casein aggregates appear to be roughly spherical with a size ranging from 50 to 70 nm, which increases with the whey protein content of the solution (Beaulieu, Pouliot, & Pouliot, 1999; Liyanaarachchi, Ramchandran, & Vasiljevic, 2015). The molecular weight of the aggregates was estimated to be $2\times10^7$ Da, the apparent isoelectric point of the aggregates was 4.5 in milk permeate, and the surface charge at pH 7.0 was 17 mV (Jean et al., 2006). The mechanism leading to the chaperone-like activity of caseins on whey proteins is still poorly understood and little research has been done on the heat stability of milk protein concentrates and isolated aggregates of caseins and whey proteins. However, some authors have proposed that the aggregates exhibit a higher charge density than the native whey proteins, limiting the interactions with other proteins (Guyomarc'h et al., 2009; Kehoe & Foegeding, 2014). The internal structure of the heat-induced aggregates is also affected by the presence of κ-casein; aggregates are less dense, and have a more porous structure, when they include κ-casein (Guyomarc'h et al., 2009).

Calcium chloride greatly influences the mineral equilibrium in milk and favours aggregation of whey protein during heating (On-Nom, Grandison, & Lewis, 2012). Calcium is naturally present in milk (31 mM) and is present at elevated concentration in milk protein concentrates when reconstituted at 13.5% protein w/w

(84 mM calcium); it also plays a major role in the heat stability of proteins (Crowley et al., 2014). Heating conditions, calcium content and pH influence the aggregation of whey proteins and caseins, the morphology of the resulting aggregates, and the extent of aggregation (Nicolai & Durand, 2013). Calcium ions can bind to the carboxylate groups of the proteins, thereby shielding their repulsive charges. Thus, aggregates are more dense and less porous when calcium is added prior to heating. Heating at high ionic strength allows the proteins to get closer and interact initially *via* hydrophobic interactions, followed by the gradual formation of disulphide bonds (Mounsey, O'Kennedy, Fenelon, & Brodkorb, 2008; Ndoye et al., 2013).

In the present study, the formation of heat-induced whey protein aggregates in MPC80, as influenced by calcium chloride concentration was investigated. In addition, the heat stability of these aggregates with different casein profiles, as well as aggregates formed from mixtures of κ-casein and whey proteins, were examined. The aim of this study was to understand the relationship between heat stability and the physico-chemical characteristics of aggregates of caseins and whey proteins in high-protein milk ingredients.

## 2. Material and Methods

### 2.1 Materials

The milk protein concentrate (MPC) powder used in this study was produced on-site (Bio-functional Food Engineering Facility (BFE) at the Teagasc Food Research Centre Moorepark, Fermoy, Co. Cork, Ireland). The skim milk was pre-heated in a temperature of the range of 40-50°C and concentrated by ultrafiltration as described previously (Huffman & Harper, 1999; Renner & Abd-El-Salam, 1991). The concentrate was dried, giving a powder with a total protein content of 79% (w/w, Kjeldahl analysis, nitrogen to protein conversion factor of 6.38) of which 73% (w/w) was casein. The resulting MPC powder contained 8.9% (w/w) lactose, 2.1% (w/w) calcium and 1.4% (w/w) phosphorus.

Whey protein isolate (WPI) Bipro® was purchased from Davisco Foods International (Eden Prairie, Minnesota, U.S.A.) and contained 93.7% (w/w) protein. Freeze-dried κ-casein was purchased from Sigma Aldrich (Saint-Louis, Missouri, U.S.A.); the purity of the κ-casein powder was greater than 70% (w/w). All reagents were purchased from Sigma Aldrich (Saint-Louis, Missouri, U.S.A.) unless stated otherwise.

### 2.2 Production and purification of the casein and whey protein aggregates

Fig. 2.1 summarizes the steps of purification and analysis carried out on the reconstituted MPC. MPC was reconstituted at 13.5% (w/v) protein in distilled water, and sodium azide (0.05%, w/v) was added to prevent microbial growth. When reconstituted at 13.5% (w/v) protein, the concentrate was 3.6-fold more concentrated in protein and 2.8-fold more concentrated in calcium than skim milk, assuming a skim milk density of 1.035 $kg.m^{-3}$ (Nutting, 1970). During the first step of

rehydration, the reconstituted MPC was incubated at 45°C for 2 h under constant stirring using an impeller, and calcium chloride was added at 0, 2.5 or 5 mM. The MPC solution was then stirred overnight at 4°C to ensure complete rehydration. On the following day, the solution was equilibrated to 22 °C and the pH was adjusted to 6.7 or 7.2 using 0.5 M NaOH and 0.5 M HCl, as required. Varying the pH of heating influences the casein dissociation and consequently the amount of caseins reacting with whey proteins in the serum phase (Donato & Guyomarc'h, 2009). After 1 h of equilibration at 22°C, re-adjustment of the pH was performed, if needed. Half of the samples did not undergo a heat treatment, and were used as controls.

Aliquots (22 mL) were filled into 25-mL glass bottles (Pyrex, Greencastle, Pennsylvania, U.S.A.) and heated at 90°C for 25 min (15 min hold time) in a water bath, which allowed heating of several samples of large volume simultaneously. Higher temperature may have caused the degradation of the negatively-charged residues on κ-casein (Alais, Kiger, & Jollès, 1967; Villumsen et al., 2015). After heating, the samples were cooled for 7 min in ice water and warmed for 20 min at 22°C. Weighted aliquots of unheated and heated samples (20 mL) were then centrifuged for 1 h at 38,360×$g$ and 20°C in a centrifuge (Sorvall Lynx 6000) using the rotor Fiberlite F21-8x50y (Thermo Fisher Scientific, Waltham, Massachusetts, USA). After centrifugation, the fat layer was discarded and the supernatants were filtered through 0.45 μm hydrophilic filters (Sartorius, Gottingen, Germany).

To further purify and analyse the aggregates, the method developed by Parker, Donato et al. (2005) was followed. Briefly, supernatant (0.8 mL) was fractionated by fast protein liquid chromatography (FPLC) on a size-exclusion column HiPrep 16/60 (GE Healthcare, Little Chalfont, Buckinghamshire, U.K.) containing Sephacryl

S-500 HR beads (fractionation range $4\times10^4$-$2\times10^7$ Da). The absorbance was monitored at 280 nm by an AKTA Purifier 10 system (GE Healthcare, Little Chalfont, Buckinghamshire, U.K.), at a flow rate of 1 mL.min$^{-1}$. The buffer was a solution of Bis-Tris Propane 20 mM and 0.02% $NaN_3$ at pH 6.7 or 7.2, depending on the original pH of the samples. Fractions (5 mL) were automatically collected using a Frac950 and the total elution time for all samples was 120 min. The separation by FPLC was carried out at least in duplicate. The physico-chemical properties of the κ-casein/whey protein aggregates were measured on the FPLC fractions. Separately, the WPI and κ-casein powder were mixed overnight at 4°C to reach a ratio of whey proteins to κ-caseins of 1:1 or 1:0.7, and were called respectively mixture 1 and mixture 2, respectively.

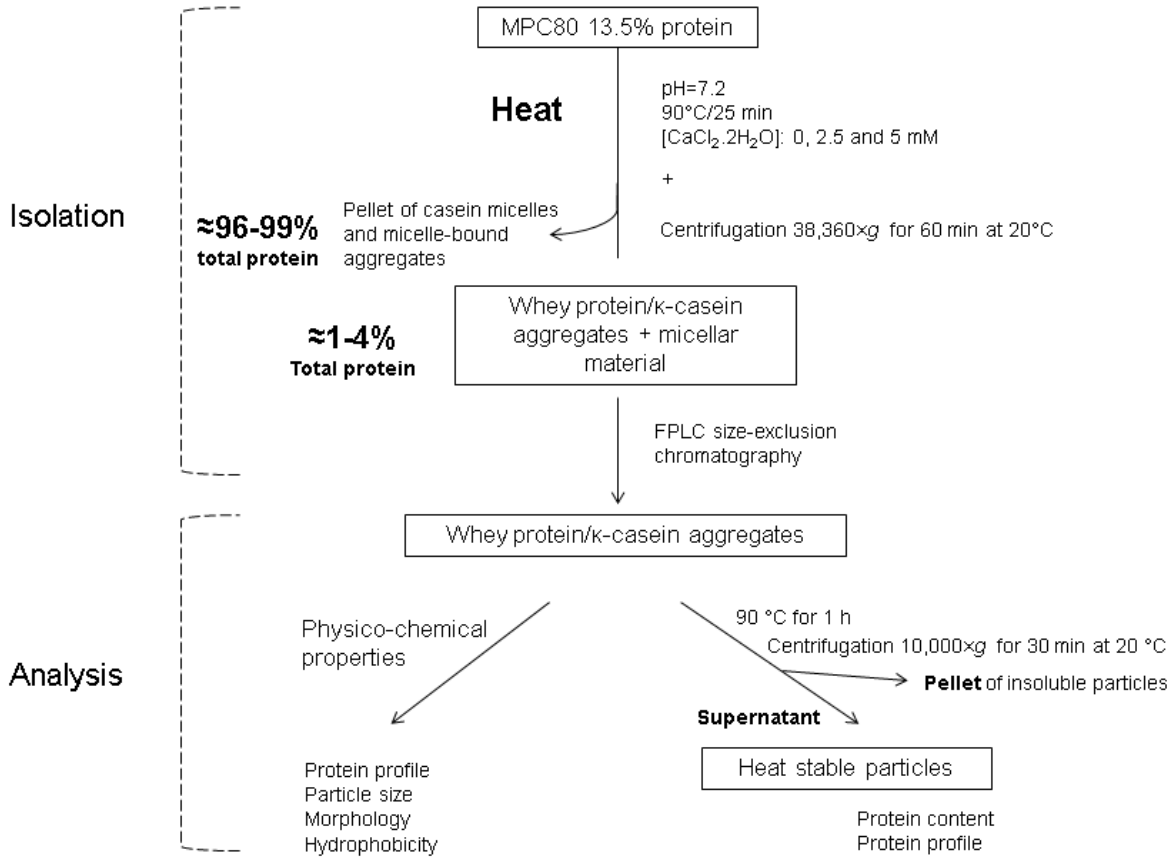



**Fig. 2.1** Flowchart of isolation and analysis of whey protein/κ-casein aggregates.

### 2.3 Protein content measurement

Protein content of liquids and powder were determined by Kjeldahl (IDF Standard, 26, 2001); the protein to nitrogen conversion factor used was 6.38. Due to the very low protein content of the fractions collected by chromatography, the protein content of those samples was determined using a Bicinchoninic acid assay (BCA) kit (Thermo Fisher Scientific, Waltham, U.S.A.); BSA was used as standard. The protein content of the centrifugal supernatants was also determined using this assay. All measurements were made in duplicate.

### 2.4 Protein profile analysis

The protein profile of all samples were analysed by SDS-PAGE electrophoresis under reducing or non-reducing conditions following a modified method of Laemmli (Laemmli, 1970). LDS (lithium dodecyl sulfate) was used instead of SDS (sodium dodecyl sulfate). NuPage Bis-Tris gels at 12% (w/w) acrylamide were used with the NuPage cells and the NuPage power supply (Life Technologies, Carlsbad, California, U.S.A.), in line with the instructions for this system. The samples were first dissolved in the sample buffer at a ratio LDS:protein 200:1. A volume of 2 μL dithiothreitol (DTT) at 500 mM was added to reduce the disulphide bonds between proteins, while addition of DTT was omitted in the case of non-reducing samples. Following the manufacturer's recommendations, the mixture was heated at 70°C for 10 min in a water bath. Sample (10 μL) containing 1.8 μg of protein was loaded in each well and a constant voltage of 200 V was applied for 50 min. The gels were stained in a solution of 0.5% Coomassie Blue R250, 25% isopropanol and 10% acetic acid. Two stages of destaining were performed; the gels were first left for 1 h in a solution of 10% isopropanol and 10% acetic acid, and then held overnight in

distilled water. Commercially sourced BSA, $\alpha_s$-casein ($\alpha_{s1}$- and $\alpha_{s2}$-casein were quantified together), β-casein, κ-casein, β-lg and α-la were used for calibration. The purified proteins were dissolved in the sample buffer, with or without reducing agent, and 10 μL of this mixture of standard proteins was loaded per well. In total, five calibration points ranging from 0.06 to 2 μg of each protein standard per well were included for every gel. Because of unavoidable variations in staining, the calibration points were run on the same gels as the samples. All gels were scanned using an Epson V700 film scanner (Epson, Suwa, Nagano, Japan) and analysed using the software ImageQuant TL (GE Healthcare, Little Chalfont, Buckinghamshire, U.K.). The scanner was not calibrated for optical density. Therefore, the range of protein content of the samples and standards was chosen to be in the linear, and thus unsaturated, region of the scanner. The quantification of the samples was deduced by plotting the known protein content of the purified proteins as a function of the integrated intensity of the standard bands.

### 2.5 Measurement of hydrodynamic diameter

The hydrodynamic diameter of the aggregates was determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments, Malvern, Worcestershire, U.K.). These measurements were carried out at 20°C on the freshly-collected fractions. All samples were equilibrated at room temperature for 120 s in the instrument prior to measurement. The refractive index of the material was considered to be 1.450 and the absorption was 0.001. Considering the low protein content of the FPLC fractions, the refractive index and the viscosity of the dispersant were assumed the same as that of water, i.e., 1.330 and 1.0031 cP respectively. Measurements were carried out at a backscattering angle of 173° and at a wavelength of 633 nm using disposable polystyrene cuvettes. The average diameter was

expressed as zeta-average, though the z-average values can be affected by particle characteristics like shape, compressibility, polydispersity, especially if the z-average values are greater than the inverse scattering vector 1/q (here 38 nm). In this case, the measured particle size is expected to be very close to the real particle size for the afore-mentioned reasons. Each sample was measured three times. Each measurement consisted of 12 separate readings, and the zeta-average and volume diameter recorded were the means of these readings. The attenuation value was between 6 and 10.

### 2.6 Measurement of hydrophobicity

The protocol used in this study was a modification of the method of Hussain et al. (2012). The probe 8-anilinonaphthalene-1-sulfonic acid ammonium salt (8-ANS) was used to determine the surface hydrophobicity of the κ-casein/whey protein aggregates. The FPLC fractions were diluted to 0.002% (w/v) protein in 20 mM Bis-Tris propane at pH 7.2, and 8-ANS was added to 4 mL of sample to obtain a final concentration in the range 5-120 μM 8-ANS. The mixtures were kept in the dark for 30 min before measurement in a Cary Eclipse fluorescence spectrophotometer (Agilent, Santa Clara, California, USA). The excitation wavelength was 350 nm for the casein/whey protein aggregates and 360 nm for the β-lg standard, and the fluorescence spectrum ranged from 400 to 600 nm. The excitation/emission slits were set at 5 nm each. The fluorescence intensity was plotted against the concentration of 8-ANS, and the maximum relative fluorescence intensity (RFI) was used as an index of hydrophobicity.

### 2.7 High-resolution scanning electron microscopy (SEM)

High-resolution scanning electron microscopy (SEM) was used to evaluate the size and shape of the protein aggregates. Protein particle suspensions (10 μL) were pipetted onto a freshly cleaved mica surface attached to an SEM stub. After air drying at 20°C, the samples were sputter coated with chromium prior to examination in a field emission scanning electron microscope (Supra 40VP; Carl Zeiss Ltd., Oberkochen, Baden-Württemberg, Germany). Images (8 bit, TIFF) were acquired at 2 kV accelerating voltage using the in-lens secondary electron detector.

### 2.8 Atomic force microscopy (AFM)

Casein and whey protein aggregates were imaged by atomic force microscopy, using an Asylum Research MFP-3DAFM (Asylum Research UK Ltd., Oxford, UK) in AC-Mode as previously described (Kehoe et al., 2011). All samples were deposited undiluted onto a freshly cleaved mica surface and subsequently dried in a desiccator. Images were processed using AFM imaging software Igor 6.12A and Argyle light for 3D images.

### 2.9 Heat stability

The FPLC fractions were concentrated using centrifugal concentration (Vivaspin 20 100,000 MWCO, Sartorius, Gottingen, Lower Saxony, Germany) to 0.25% (w/v) protein. The heat stability of the concentrated FPLC fractions, MPC, WPI and the mixtures of whey proteins and κ-caseins were assessed by heating the samples in a water bath at 90°C for 1 h. All samples contained 10 mM Bis-Tris propane and 0.01% (w/v) $NaN_3$, without mineral or lactose standardization. Glass tubes (120 mm length, 7 mm diameter and 1.5 mm wall thickness) were filled with 2.5 g of sample at 0.25% (w/v) protein. After heating, 2 mL of each heated sample was centrifuged

at 10,000×g for 30 min using a 5417R Eppendorf centrifuge with rotor F45-30-11 (Eppendorf, Hamburg, Hamburg, Germany). In these conditions, the proteins present in the supernatants were considered as soluble. The supernatants were then analysed by SDS-PAGE as described in Section 2.4.

### 2.10 Statistical data analysis

All experiments were carried out using the same batch of powder. The data are expressed as means with standard deviations of data from two independent replicates.

## 3. Results and discussion

### 3.1 Influence of calcium chloride on the solubility of caseins and whey proteins in MPC

Milk protein concentrate reconstituted at 13.5% (w/v) protein was heated at 90°C for 25 min and the casein micelles were removed by centrifugation, together with the whey proteins attached to the micelles and the large and dense whey protein aggregates. Table 2.1 shows the protein concentration in the supernatant as a function of the pH at heating and the addition of calcium. At pH 6.7, none of the samples gelled after 25 min of heating at 90°C. However, the protein concentration recovered in the supernatants was significantly lower in the samples heated at pH 6.7 (1-2%) than in the samples heated at pH 7.2 (4%). At pH 6.7, the addition of calcium significantly reduced the concentration of protein recovered in the supernatant. The protein content in the supernatant, when heated at pH 7.2, did not change significantly with the calcium content.

**Table 2.1** Protein content of supernatants after heating milk protein concentrate, reconstituted at 13.5% (w/v) protein, at 90°C for 25 min and centrifugation at 38,360×*g* for 1 h at different pH and calcium chloride contents.

| pH | Calcium addition (mM) | Protein content of the supernatants (%, w/v) |
|---|---|---|
| 6.7 | 0.0 | 1.9 ± 0.8 |
| 6.7 | 2.5 | 1.4 ± 0.3 |
| 6.7 | 5.0 | 0.9 ± 0.1 |
| 7.2 | 0.0 | 4.2 ± 0.1 |
| 7.2 | 2.5 | 4.2 ± 0.3 |
| 7.2 | 5.0 | 3.8 ± 0.4 |

The proteins in the supernatants from the heated MPC were separated by FPLC into three fractions; the composition and volume of each fraction are presented in Fig. 2.2 and Table 2.2. Three fractions from the sample at pH 7.2 were collected between 50 and 60 min, 60 and 75 min, 75 and 90 min elution time from the size-exclusion column, equivalent to 42-50%, 50%-63% and 63-76% column volumes, respectively. The fractions collected had increasing amount of casein and increased ratios of κ-casein to whey protein as a function of the elution time, illustrating the heterogeneity of the aggregates within one sample of heated MPC. Little or no absorbance at 280 nm was measured for the samples heated at pH 6.7 with 2.5 or 5 mM calcium chloride. The protein content in the supernatants may have been too low to be detected by the absorbance detector, while the aggregates larger than 450 nm may have been removed by centrifugation or filtration prior to separation by FPLC. High calcium activity has been identified as a major factor influencing the heat stability of concentrated milk (Jeurnink & De Kruif, 1995; Rattray & Jelen, 1996; Rose, 1961; Zittle & Dellamonica, 1956). Decreasing pH promotes a shift in the mineral equilibrium of milk, causing the release of ionic calcium into the serum. The high calcium content, coupled with a low pH, contributes to the formation of large aggregates, which sediment easily during the centrifugation step. This explains the lower absorbance on FPLC (Fig. 2.2) and lower protein recovery in the supernatants for the samples containing calcium (Table 2.1). The addition of calcium also affected the distribution of the aggregates in the samples; without addition of calcium, there were 1.4 times more aggregates in fraction A than in fraction C (integrated area of each fraction on the absorbance signal of FPLC), while the opposite was observed when 5 mM calcium was added before heating.

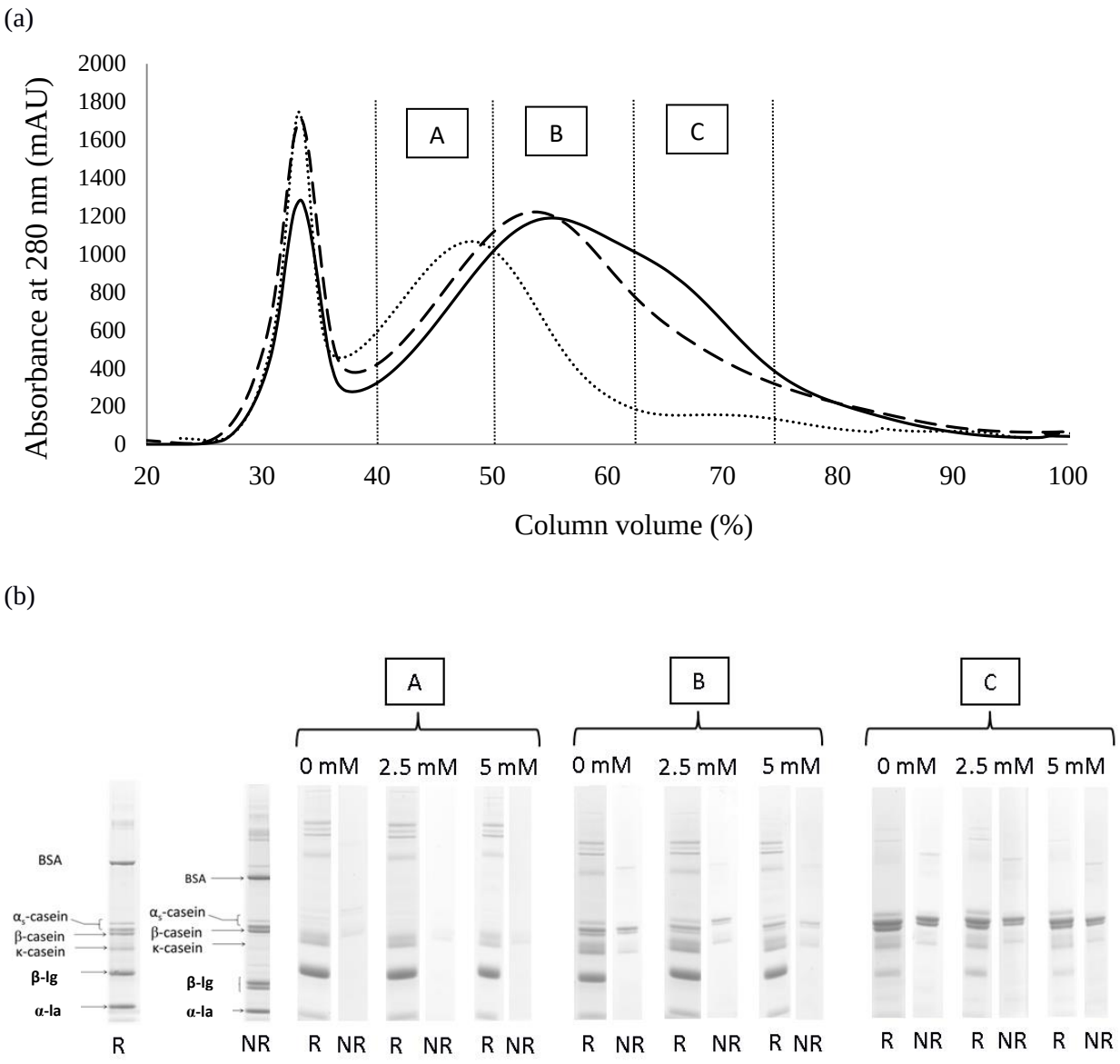



**Fig. 2.2** (a) Size-exclusion chromatography (SEC-FPLC) profiles of MPC heated at 90C for 25 min, pH 7.2 with 2.5 mM (--), 5 mM (…) or without the addition of $CaCl_2$ (—), with (b) the corresponding SDS-PAGE profiles under reducing (R) and non-reducing (NR) conditions, with bovine serum albumin (BSA), $\alpha_s$-casein, β-casein, κ-casein, β-lactoglobulin (β-lg) and α-lactalbumin (α-la).

**Table 2.2** Characteristics and composition of the aggregates in fractions A, B and C (see Fig. 2.2) separated from milk protein concentrate heated at pH 7.2 with up to 5 mM added calcium chloride. The relative amount of protein corresponds to the area under the chromatogram for each fraction compared to the total amount of eluted protein (from 35 to 100% of the column volume).

| **FRACTION** | **A** | | | **B** | | | **C** | | |
|---|---|---|---|---|---|---|---|---|---|
| Calcium chloride addition (mM) | **0** | **2.5** | **5** | **0** | **2.5** | **5** | **0** | **2.5** | **5** |
| Whey protein:κ-casein ratio in the aggregates | 1:0.4 | 1:0.5 | 1:0.5 | 1:0.5 | 1:0.6 | 1:0.6 | 1:1.2 | 1:1.3 | 1:2.0 |
| Whey protein:κ-casein ratio in the FPLC fraction | 1:0.5 | 1:0.6 | 1:0.7 | 1:0.7 | 1:0.8 | 1:0.8 | 1:1.7 | 1:2.2 | 1:3.0 |
| $\alpha_s$- and β-casein (%, w/w total protein) | 1 ± 2 | 0 ± 0 | 0 ± 0 | 14 ± 7 | 12 ± 1 | 11 ± 2 | 60 ± 18 | 65 ± 2 | 61 ± 2 |
| Relative amount of proteins (% of the total eluted proteins) | 27 ± 2 | 31 ± 1 | 40 ± 3 | 48 ± 4 | 44 ± 1 | 37 ± 3 | 19 ± 1 | 16 ± 1 | 10 ± 2 |

3.2 Composition of the fractions from size-exclusion chromatography

SDS-PAGE under reducing conditions (Fig. 2.2) showed the presence of proteins in each fraction; by comparing the reducing and non-reducing conditions, the extent of disulphide bond formation was evaluated. The bands for BSA, α-la and β-lg appeared stronger in reducing conditions, confirming that these proteins were mainly involved in aggregate formation through disulphide bonding. However, the major whey protein involved in the covalent aggregates was β-lg. κ-Casein was also involved in the aggregates by disulphide bonding (58-87%, w/w, of κ-casein in the fractions). Fractions B and C contained $\alpha_s$- and β-casein, while fraction C contained the highest proportion of κ-casein, β-casein and $\alpha_s$-casein, and the lowest ratio of whey protein to κ-casein. With the addition of calcium chloride, the whey protein/κ-casein ratio and the percentage of $\alpha_s$- and β-caseins in each fraction remained unchanged.

Table 2.3 presents the physico-chemical characteristics of each fraction. The aggregate size ranged from 29 to 59 nm, corresponding to the size range observed in previous studies (del Angel & Dalgleish, 2006). The size of the aggregates decreased with the concentration of caseins. Thus, the ratio of whey proteins to caseins positively affected the size of the aggregates, as observed in previous studies (Guyomarc'h et al., 2009; Liyanaarachchi et al., 2015). The chaperone activity of caseins has been reported to reduce whey protein aggregation (Kehoe & Foegeding, 2010; Mounsey & O'Kennedy, 2010); the chaperone activity of a biomolecule refers to its ability to protect another biomolecule against unfolding, aggregation and precipitation. Thus, the difference in particle size between fractions A and B (Table 2.3) may be the result of the chaperone-like activity of the non-covalently bound caseins ($\alpha_s$-, β- and κ-casein) to whey proteins in fraction B (Fig. 2.2).

**Table 2.3** Particle size and hydrophobicity, measured as 8-ANS-relative fluorescence intensity, of the aggregates formed in MPC heated at 90°C for 25 min at pH 7.2 with up to 5 mM added calcium chloride and collected in fractions A, B and C (see Fig. 2.2).

| Calcium chloride addition (mM) | Fractions | Average particle size (nm) | Hydrophobicity (-) |
|---|---|---|---|
| 0.0 | A | 56 ± 2 | 1.3 ± 0.0 |
| 0.0 | B | 42 ± 2 | 1.1 ± 0.1 |
| 0.0 | C | 29 ± 2 | 0.6 ± 0.1 |
| 2.5 | A | 56 ± 1 | 1.3 ± 0.2 |
| 2.5 | B | 44 ± 0 | 1.1 ± 0.1 |
| 2.5 | C | 32 ± 1 | 0.7 ± 0.2 |
| 5.0 | A | 59 ± 1 | 1.3 ± 0.2 |
| 5.0 | B | 47 ± 1 | 1.1 ± 0.1 |
| 5.0 | C | 36 ± 4 | 0.5 ± 0.1 |

Previous studies have shown that micellar material can probably associate into small micelles of size 10-20 nm, which elute after the maximum of the aggregate peak on the FPLC profile (Guyomarc'h et al., 2003; Ono & Takagi, 1986), corresponding to fractions B and C in our study. The size range reported for these “mini-micelles” (10-20 nm) is close to that of the aggregates in fractions B and C (Ono & Takagi, 1986). The formation of such small, dispersed micelles amongst the aggregates of whey proteins and caseins may have caused a shift in the hydrodynamic size measurement. The ratio of κ-casein:[$\alpha_s$-casein + β-casein] which was not covalently bound to whey proteins in our study was up to 1:9. This ratio was comparable to those found by previous authors (Donnelly, McNeill, Buchheim, & McGann, 1984) for skim milk fractionated by size-exclusion chromatography. The authors found that casein micelles in skim milk had sizes ranging from 62 to 154 nm and ratios of κ-casein:[$\alpha_s$-casein + β-casein] ranging from 1:6 to 1:21. These results indicate that,

in fractions B and C, the amount of κ-casein that was not covalently bound to the whey proteins or self-aggregated was sufficient to stabilize $\alpha_s$- and β-casein in the form of "mini-micelles". However, the presence of "mini-micelle" in the fractions and their effect in this study cannot be dissociated from those of the aggregates containing whey proteins.

SEM images (Fig. 2.3) show the morphology and the size distribution of the largest and the smallest aggregates. The smaller aggregates had a narrower size distribution than the larger aggregates, as measured by DLS (Table 2.3). SEM micrographs indicated that the smaller aggregates (Fig. 2.3) were rounded and more spherical than the larger aggregates, which had a slightly angular shape. The sphericity of the heat-induced particles made of caseins and whey proteins are less likely to increase the viscosity of the solutions to which they would be added; thus, they may be a potential candidate for use as ingredients in food applications (Ryan et al., 2013).

Some fractions were also analysed using Atomic Force Microscopy (AFM) in air (Fig. 2.4). The images of aggregates show a near-spherical shape. The cross-section of the height image showed a particle size of approximately 25 to 40 nm, which would correspond to a relatively low polydispersity. Due to tip broadening in AFM, the height of the recorded particles is generally used for estimation of size. However, size measurements by AFM have to be considered with extreme caution as the protein samples have been dehydrated and deposited on mica, which may lead to a complete collapse of the protein particles. However, dynamic light-scattering analysis of the sample shown in Fig 2.4 gave a surprisingly similar particle size (z-average) of 53 nm.

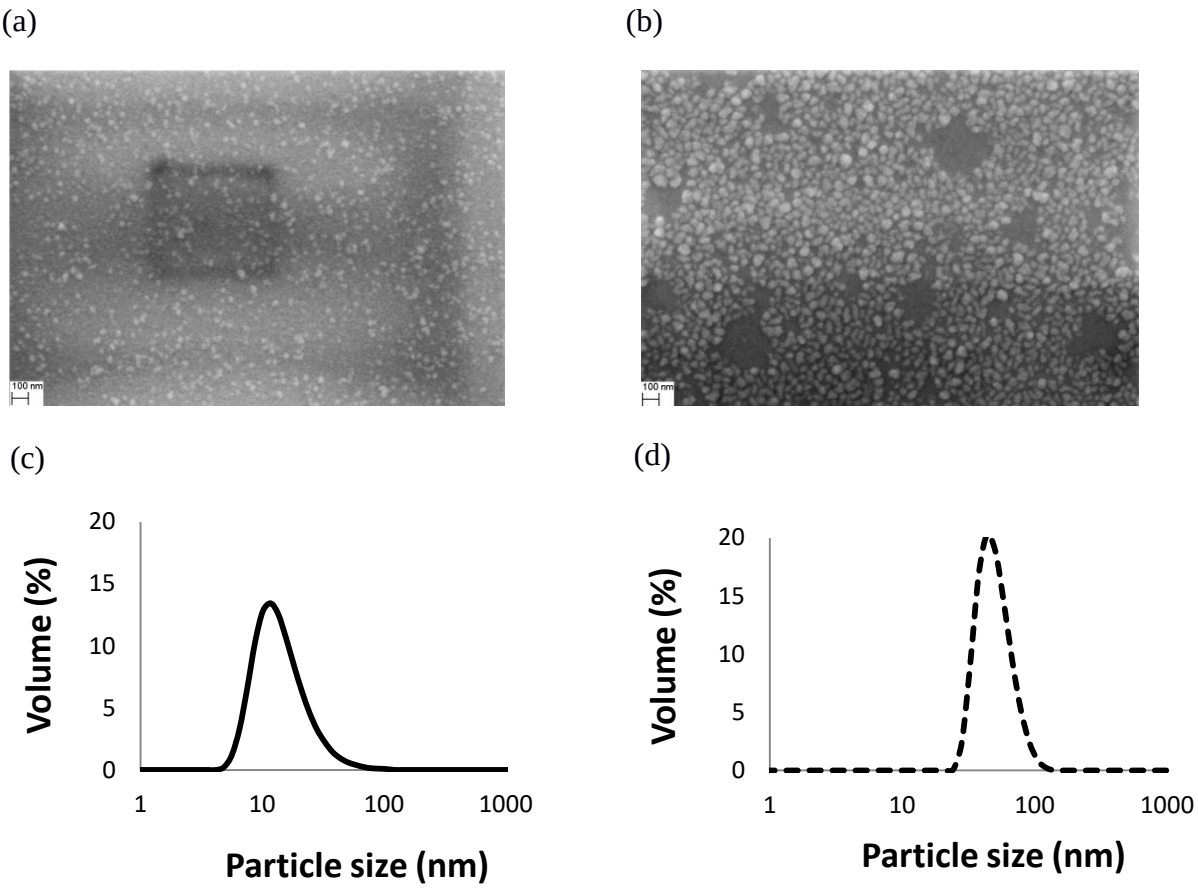



**Fig. 2.3** Scanning electron micrographs from SEC-FPLC fractions; Fraction C (a) and fraction A (b), were dried on mica at 20°C. Fig. 2.3 (c) and 2.3 (d) show the size distributions by volume measured by dynamic light scattering for fractions C and A, respectively.

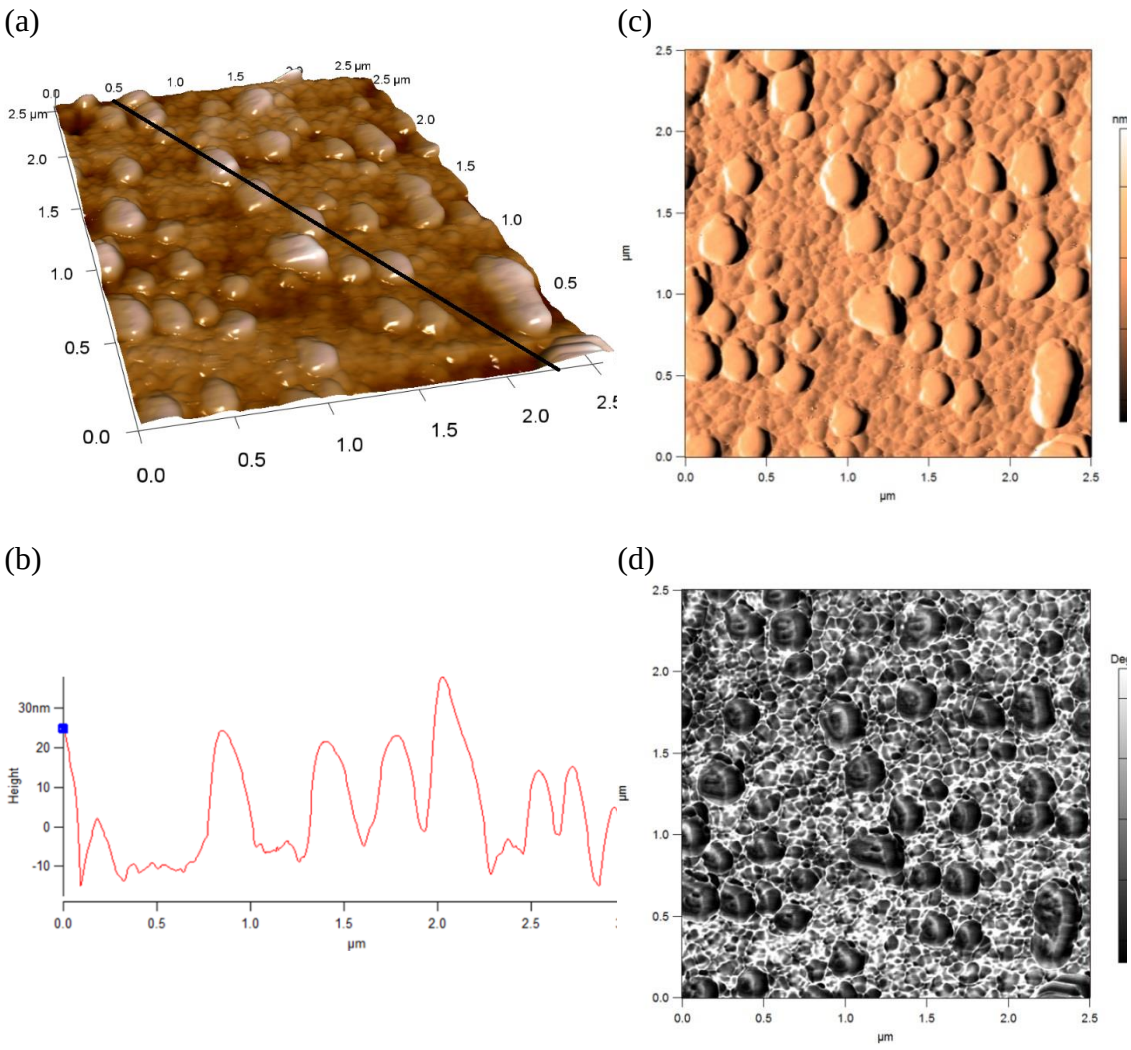



**Fig. 2.4** AFM images showing (a) 3D height, (b) height across the cross-section marked in the 3D height image, (c) amplitude, and (d) phase for a representative sample of the casein and whey protein aggregates in fraction A.

### 3.3 Effect of κ-casein content and pre-heat treatment on the heat stability of whey proteins during heating

The heat stability of the isolated fractions after heating at 90°C for 1 h (pH 7.2) at 0.25% (w/v) protein was assessed (Fig. 2.5 and Table 2.4). In order to compare the stability of the nanoparticles in buffer after heating, the samples were centrifuged at 10,000×*g* for 30 min. The soluble protein content after heating and centrifugation (10,000×*g* for 30 min) and the visual properties of the samples after heating were the criteria used to determine the heat stability of the samples.

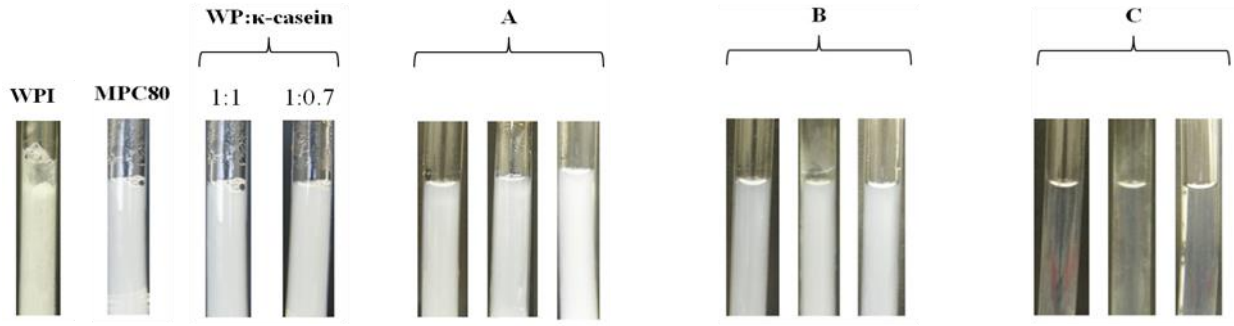



**Fig. 2.5** Heat stability (90°C for 1 h) of SEC-FPLC fractions A, B and C. WPI, MPC80 and mixtures of whey protein and κ-casein (Mixes 1 and 2 had whey protein:κ-casein ratios of 1:1 and 1:0.7, respectively) were also measured for comparison.

**Table 2.4** Composition of size exclusion chromatography-fast protein liquid chromatography fractions A, B and C after heat stability test at 90°C for 1 h and centrifugation at 10,000×*g* for 30 min. WPI, MPC80 and mixtures of whey protein and κ-casein (Mixes 1 and 2 had whey protein:κ-casein ratios of 1:1 and 1:0.7, respectively) were also measured for comparison. The samples that coagulated during the heat stability test, preventing further analysis, are marked with an asterisk.

| | WPI | MPC 80 | Mix 1 | Mix 2 | A | | | B | | | C | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Calcium chloride addition (mM) | 0 | 0 | 0 | 0 | 0 | 2.5 | 5 | 0 | 2.5 | 5 | 0 | 2.5 | 5 |
| % soluble fraction (w/w, total protein) | 0* | 50 ± 12 | 43 ± 11 | 47 ± 20 | 10 ± 5 | 9 ± 7 | 13 ± 10 | 80 ± 28 | 87 ± 8 | 89 ± 16 | 94 ± 7 | 98 ± 3 | 90 ± 4 |
| % Whey protein (w/w, soluble fraction) | 0* | 4 ± 1 | 48 ± 13 | 54 ± 18 | 31 ± 1 | 60 ± 19 | 53 ± 6 | 39 ± 0 | 53 ± 7 | 50 ± 2 | 6 ± 0 | 10 ± 3 | 15 ± 8 |
| % Casein (w/w, soluble fraction) | 0* | 96 ± 1 | 52 ± 13 | 46 ± 18 | 69 ± 1 | 40 ± 19 | 47 ± 6 | 61 ± 0 | 47 ± 7 | 50 ± 2 | 94 ± 0 | 90 ± 3 | 85 ± 8 |
| Whey protein/κ-casein ratio in the soluble phase | 1:0 | 1:0.2 | 1:1.0 | 1:1.4 | 1:1.5 | 1:0.8 | 1:0.8 | 1:1.1 | 1:0.7 | 1:0.9 | 1:5.8 | 1:2.8 | 1:2.9 |

The heat stability of the fractions did not vary significantly with the addition of calcium. With the exception of the unheated MPC sample, all samples were transparent before the test and no visible differences in opacity were observed. Fraction A, containing aggregates with mean diameter of 57 nm and an initial whey protein to κ-casein ratio in the range 1:0.4 to 1:0.5, became opaque within a few seconds of heating. After 1h heating and centrifugation at 10,000×*g* for 30 min, around 10-13% (w/w) of the initial proteins was recovered in the supernatant. In comparison, a pure whey protein isolate at the same concentration coagulated during heating; therefore, the aggregates of whey proteins and κ-casein in fraction A were more heat-stable than the whey proteins in WPI that did not undergo any pre-heat treatment.

After heating of fraction A, 4-11% (w/w) of the whey proteins were recovered in the supernatant. The comparison of the sample composition before and after the heat stability test (Fig. 2.6) shows a significant loss in whey proteins and κ-casein in fraction A. For comparison, a mix of whey protein and κ-casein (at a ratio 1:0.7 or 1:1), that did not undergo any pre-heat treatment, showed a protein recovery of 43-47% (w/w), and around 36-49% (w/w) of the whey proteins were recovered after heat stability test. The mixture containing whey proteins and κ-casein at a ratio 1:0.7 and fraction A had a similar initial composition of caseins and whey proteins; the only difference between these two samples was the pre-heat treatment for fraction A. Thus, the unheated mixture of whey proteins and κ-casein was more stable than aggregates of whey proteins and κ-casein at a ratio of 1:0.7. Therefore, regardless of whether κ-casein involved in aggregates or not, κ-casein apparently exhibited a stabilising effect on the whey proteins.

When comparing the two mixtures of whey proteins and κ-casein at ratios 1:1 and 1:0.7, no difference was observed in protein recovery or composition after the heat stability test. Therefore, the maximum amount of native whey proteins that κ-casein can stabilize may have been reached at a whey protein to κ-casein ratio of 1:0.7. The compositions of the two mixtures may also be too close to exhibit a significant difference in heat stability.

### 3.4 Effect of casein profile on stability of whey proteins during heating

The total protein recovered after heating in fraction B was around 80 to 89% (w/w). Fraction B contained aggregates of mean diameter 44 nm and with a whey protein to κ-casein ratio of 1:0.5 to 1:0.6, containing around 11-14% (w/w, total proteins) of $\alpha_s$- and β-caseins. At equal ratios of whey protein to κ-casein, fraction B showed significantly higher heat stability than fraction A and the mixtures of unheated whey protein and κ-casein. The non-negligible amount of $\alpha_s$- and β-casein may have provided an additional stabilization to the aggregates.

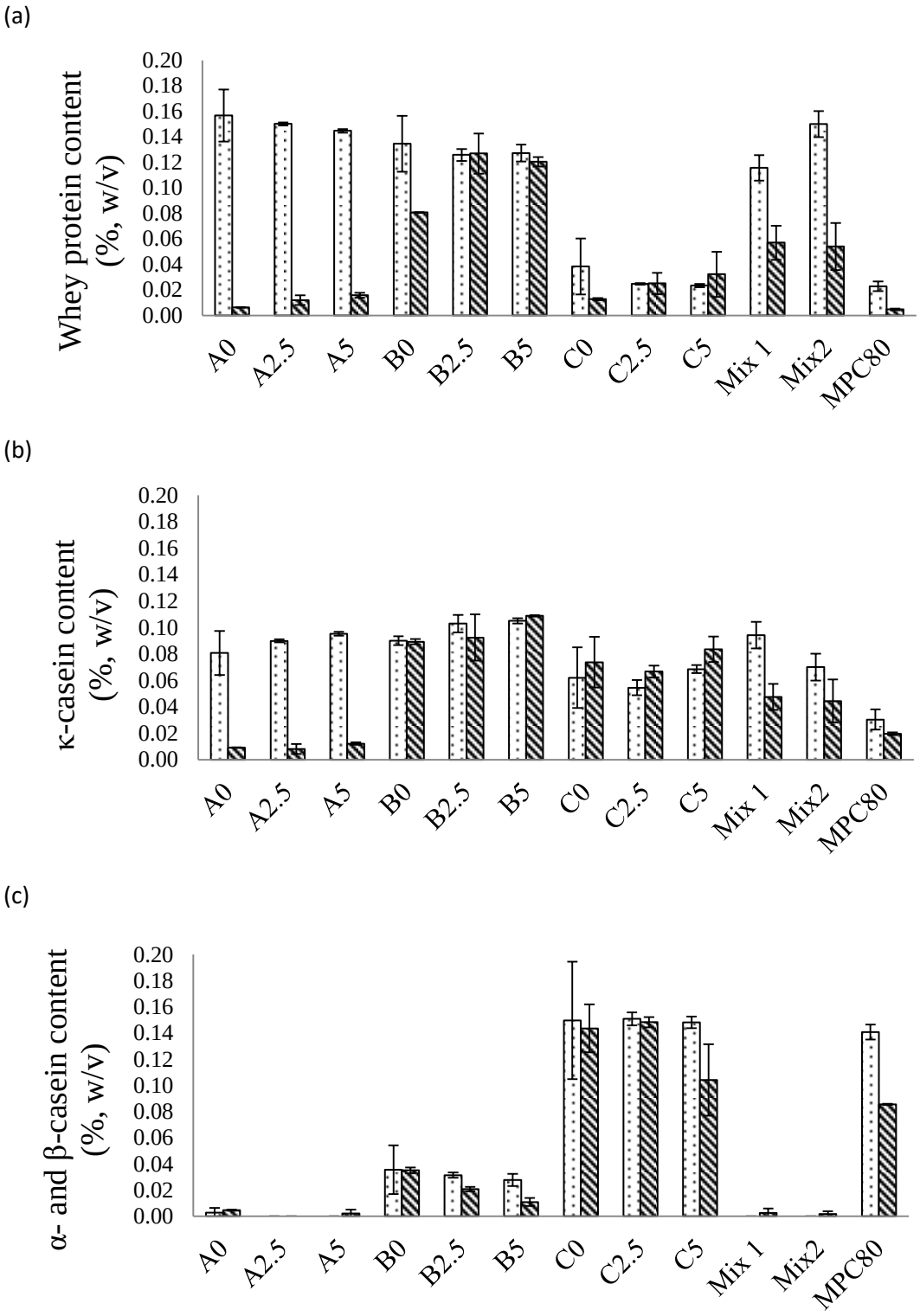



**Fig. 2.6** Protein profile ((a) whey protein, (b) κ-casein and (c) $\alpha_s$- and β-casein) of the SEC-FPLC fractions A, B and C with 0, 2.5 or 5mM $CaCl_2$ addition (see Fig. 2.2 a) before (▨) and after (▩) heat stability testing (90°C for 1 h). MPC80 and mixtures of whey protein and κ-casein (Mix 1 and 2 with whey protein:κ-casein ratios of 1:1 and 1:0.7, respectively) were also measured for comparison.

### 3.5 Effect of casein structure on whey protein stability during heating

Fraction C, made up of aggregates of mean diameter 32 nm, had the highest content of κ-casein and a whey protein to κ-casein ratio of 1:1.2 to 1:2.0. This fraction also contained the highest amount of $\alpha_s$- and β-caseins (60-65% of the total proteins). After one hour of heating, sample C was still transparent and the recovery of protein aggregates in the supernatant was approximately 90-98% (w/w, total protein). Therefore, the amount of soluble whey protein and κ-casein in fraction C did not change significantly after heating (Fig. 2.6). In agreement with the previous observations on fractions A and B, the high casein content may explain this greater heat stability. The whey proteins were still soluble after heating, indicating that caseins may have a chaperone-like activity and protect whey proteins against sedimentation. The same test performed on MPC without pre-heat treatment gave a lower soluble protein content, of 50% (w/w, total protein). Fig. 2.6 also illustrates the significant loss in $\alpha_s$- and β-casein (39% w/w, initial $\alpha_s$- and β-casein), of whey proteins (79% w/w, initial whey protein), and κ-casein (35%, w/w initial κ-casein) in MPC after heat-stability testing at 90°C. In the mixtures and in the fractions, the caseins are present either in individual and soluble form, associated with the whey proteins or present in mini-micelles. Caseins in MPC are likely to be organized in micelles with average size 150-200 nm (Dalgleish & Corredig, 2012), and consequently are less available for association with whey proteins than the soluble casein of the heated supernatant of MPC. The dissociation of κ-casein at pH 7.2, together with the prolonged heating, could have destabilized the casein micelles, leading to the precipitation of most proteins.

### 3.6 Hydrophobicity of casein and whey protein aggregates

The aggregates in fraction C were significantly less hydrophobic than those in fractions A and B. Caseins are relatively hydrophobic and κ-casein is the second most hydrophobic casein after β-casein, with an average hydrophobicity of 5.1 kJ per residue (Bigelow, 1967). However, κ-casein is glycosylated by negatively charged hydrophilic groups, which protrude at the surface of the casein micelle and ensure its stability in the aqueous phase of milk. Thus, in the case of formation of mini-micelles, κ-casein would help in solubilising them, possibly explaining the lower hydrophobicity in fraction C and the enhanced heat stability of this fraction. In the same way, the hydrophilic part of κ-casein could stay at the surface of the aggregates, stabilizing the denatured whey proteins. This mechanism would be similar to that of heat-shock proteins, which are intracellular proteins that prevent the complete unfolding, aggregation and precipitation of proteins denatured by heat, oxidation or reduction (Richter, Haslbeck, & Buchner, 2010). After binding to non-native proteins by hydrophobic interactions and forming high molecular weight complexes, the mobile hydrophilic regions of the heat-shock proteins help solubilizing the complex (Guyomarc'h et al., 2009; Treweek, Thorn, Price, & Carver, 2011). A similar mechanism has also been postulated for the chaperone-like activity of $\alpha_s$- and β-casein against the heat-induced aggregation of whey proteins (Morgan et al., 2005; Zhang et al., 2005).

By comparison, for fractions A and C, the hydrophobicity of the aggregates (Table 2.3) seemed to follow the same trend as the heat stability of the aggregates and the ratio of whey protein:casein. The higher the proportion of casein in the aggregates, the lower the resulting hydrophobicity and the higher their heat stability. Fraction B contained a greater proportion of caseins and was more heat-stable than fraction A;

however, no significant difference in hydrophobicity was observed. As noted earlier, heat stability is correlated with the charge of the aggregates. In addition, the differences between the SDS-PAGE under reducing and non-reducing conditions do not facilitate understanding of whether $\alpha_s$- and β-casein are associated with the aggregates of whey proteins and κ-casein by hydrophobic interactions. It is possible that $\alpha_s$- and β-casein associate or dissociate from the whey protein and κ-casein aggregates during heat treatment, and thus the hydrophobicity of the κ-casein and whey protein aggregates may change, which would influence the heat stability of the aggregates.

The addition of calcium before heating did not have a significant effect on the hydrophobicity of the aggregates in fraction A. This may indicate that the morphology of the κ-casein/whey protein aggregates within a fraction was not significantly affected by the addition of calcium chloride to the starting material. The high molecular weight of the aggregates and the salt concentration of the buffer made the use of isoelectric focusing or zeta potential technique for the determination of the charge of the aggregates difficult. However, measuring the charge of the aggregates may be useful for further examination of the chaperone-like mechanism of κ-casein. Studying mixtures of κ-casein and whey proteins should also be considered in the future to gain a better understanding of the changes in hydrophobicity during heating.

## 4. Conclusions

The presence of caseins provided stabilization of whey protein aggregates during heating. In particular, κ-casein exhibited a chaperone-like activity at a whey protein to κ-casein ratio of 1:0.7, for both heated and unheated mixtures of whey proteins and κ-casein. Pre-heat treatment reduced the chaperone-like activity of κ-casein. The presence of $\alpha_s$- and β-casein in solution contributed to an enhanced heat stability of the whey proteins. These results are a starting point for a better understanding of the heat stability of milk protein and casein aggregates. However, the mechanism of chaperone-like activity of κ-casein needs further investigation.

**Acknowledgments**

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The inclusion of κ-casein increased the heat-stability of whey proteins aggregates (Chapter 2). Measuring the effect of κ-casein on the structure of the whey protein aggregates can develop understanding the molecular interactions involved in the aggregation process. The following chapter aimed at determining the effect of κ-casein on the density and internal protein content of whey protein aggregates using rheometry.

# Chapter 3

## Measurement of the internal density and internal protein content of whey protein and κ-casein aggregates by rheometry

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SJG contributed to the experimental design, measured the protein content, density and viscosity of the samples, analysed the data and wrote the manuscript. AB, JOM and ALK reviewed the manuscript.

**Abstract**

The co-heating of whey proteins with κ-casein improves their heat stability and results in the formation of nanometre-sized, covalently-bonded aggregates. In order to understand further the interactions between whey proteins and κ-casein, a method for the measurement of internal density and internal protein content of the aggregates was evaluated. A solution of milk protein concentrate (13.5% protein, w/v) at pH 7.2 was heated at 90°C for 25 min. Three fractions containing aggregates of whey protein and κ-casein, in whey protein:κ-casein ratios ranging from 1:0.4 to 1:1.2 and containing 1-60% (w/w, total protein) $\alpha_s$- and β-casein, were then isolated by centrifugation (38,360×*g*) and size-exclusion chromatography using fast-protein liquid chromatography. The internal density and internal protein content of the particles were deduced from Einstein's, Eiler's and Lee's equations after measurement of their viscosity and density. The internal protein content of the aggregates was estimated to be around 9-12% (w/w) and the internal density was around 1.02 to 1.05 g/cm$^3$. These results demonstrate the challenges in using such approaches, and possible improvement of the method and alternatives are presented.

# 1. Introduction

Heat-treatment of whey proteins at temperatures greater than 60°C results in the irreversible aggregation of whey proteins, through formation of disulphide bonds and non-covalent interactions (Hoffmann & van Mil, 1997). Modulating the heating conditions allows for control of the extent of aggregation (Mehalebi, Nicolai, & Durand, 2008; Ryan et al., 2012; Simons, Kosters, Visschers, & de Jongh, 2002; Tolkach & Kulozik, 2007). Heating whey proteins with $\alpha_s$-, β- or κ-casein has been shown to limit the extent of whey protein aggregation (Guyomarc'h, Nono, Nicolai, & Durand, 2009; Kehoe & Foegeding, 2014; Morgan, Treweek, Lindner, Price, & Carver, 2005). In particular, κ-casein forms irreversible disulphide bonds with whey proteins during heat-treatment (Jang & Swaisgood, 1990).

Using light-scattering techniques, previous studies reported that the density of aggregates of whey proteins and κ-casein was lower and the structure more porous than that of aggregates containing whey proteins only (Guyomarc'h et al., 2009). Other authors were able to quantify the internal density and internal protein content of micrometre-sized aggregates of whey proteins using rheometry, through the measurement of the volume fraction of the particles in solution (Sağlam, Venema, de Vries, & van der Linden, 2014). Rheometry has also been used for the calculation of the volume fraction of food proteins, such as whey proteins, soy proteins and casein micelles (Boulet, Britten, & Lamarche, 1998). Measuring the effect of κ-casein on the structure of soluble whey protein aggregates can develop understanding of the molecular interactions involved in the aggregation process. In Chapter 2, we characterized soluble aggregates of whey protein and κ-casein, and observed that the heat stability of these aggregates varied as a function of their whey protein:κ-casein ratio and the concentration of $\alpha_s$- and β-casein in the fractions. In the present study,

we attempted to use rheometry for the measurement of the internal density and internal protein content of such aggregates.

The aggregates were produced by heating milk protein concentrate (90°C, 25 min), reconstituted at 13.5% (w/v) protein, at pH 7.2. The particles of heat-induced aggregates were then isolated using centrifugation and size-exclusion chromatography on a fast-protein liquid chromatography system. Three fractions were collected with increasing ratio of whey protein:κ-casein (1:0.4, 1:0.5 and 1:1.2) and increasing content of $\alpha_s$- and β-casein (1-60%, total protein, w/w). Challenges encountered during this measurement, potential improvements, and alternatives are discussed.

## 2. Principles

Einstein assumed that the volume fraction Φ (dimensionless), i.e., the place occupied by the particles in solution, is a function of the viscosity of the dispersion, $\eta_{dispersion}$, (mPa/s) and the viscosity of the buffer, $\eta_{buffer}$, (mPa/s). This relation is described in equation (1). The measurement of $\eta_{dispersion}$ and $\eta_{buffer}$ is detailed in Section 3.

$$\lim_{\Phi \to 0} \frac{\eta_{dispersion}/\eta_{buffer} - 1}{\Phi} = 2.5 \qquad (1)$$

The value of 2.5 in equation (1) corresponds to the viscosity increment for solid spheres (Alexander & Block, 2014). This value increases when the shape of the particles deviate from that of a sphere. The term ($\eta_{dispersion}/\eta_{buffer}$ -1) is called specific viscosity and relates to the intrinsic ability of a polymer to increase the viscosity of a particular solvent at a given temperature. Equation (1) is valid at very dilute concentrations of particles. In practice, the sample is diluted in series to very low concentrations and Φ, calculated from equation (1), is plotted against $C_{dispersion}$ (%, w/v), the protein content in the dispersion. When the dilution is the only treatment applied to the molecule and it behaves similarly to a neutral particle in the solvent, $C_{dispersion}$ and Φ are then related by a constant ratio V (mL/g), the voluminosity (Boulet et al., 1998).

Internal density of the particles $\rho_{particle}$ (g/cm$^3$) and internal protein content $C_{particle}$ (%, w/w) are deduced from equations (2) and (3), respectively, where $\rho_{dispersion}$ (g/cm$^3$) and $\rho_{buffer}$ (g/cm$^3$) are the density of the dispersion and the buffer. The measurements of $C_{dispersion}$, $\rho_{dispersion}$ and $\rho_{buffer}$ are detailed in Section 3.

$$\rho_{particle} = \frac{\rho_{dispersion} - \rho_{buffer}.(1 - \Phi)}{\Phi} \quad (2)$$

$$C_{particle} = \frac{C_{dispersion}}{\Phi \times \rho_{particle}} \quad (3)$$

To apply Einstein's equation, three conditions must be satisfied, which are that the particles must be solid, spherical and non-interacting. Therefore, it is assumed that the buffer must be inert. However, the conditions required by Einstein's equation are rarely applicable to food proteins, for several reasons. Firstly, the protein content of food is relatively high and the proteins are colloidal molecules that interact with the solvent and other proteins. Secondly, proteins are hydrated, not strictly spherical and their size distribution is very polydisperse. To overcome these strict conditions, authors adapted Einstein's equation to more practical conditions. Eiler's equation (4) corrects for the effect of interactions between proteins and solvent (Dewan, Bloomfield, Chudgar, & Morr, 1973):

$$\frac{\eta_{dispersion}}{\eta_{buffer}} = \left(1 + \frac{1.25\Phi}{1 - 1.35\Phi}\right)^2 \quad (4)$$

Lee's equation (5) allows for the calculation of the volume fraction in more concentrated samples, up to a volume fraction around 0.5. The interactions between the protein particles and the aqueous solvent are taken into account (Boulet et al., 1998).

$$\frac{\eta_{dispersion}}{\eta_{buffer}} = (1 + 2.5\Phi + 7.031\Phi^2 + 37.371\Phi^3) \quad (5)$$

# 3. Materials and Methods

## 3.1 Sample preparation

The experimental flowchart (Fig. 3.1) summarizes the rehydration, isolation and analyse performed on the samples. All reagents were purchased from Sigma Aldrich (St. Louis, MO, USA), unless stated otherwise. The samples were prepared according to the method described in Chapter 2. Briefly, milk protein concentrate (MPC) containing 79% (w/w) protein, was produced in the Bio-functional Engineering Facility of the Food Research Centre Moorepark (Fermoy, Co. Cork, Ireland) and reconstituted to 13.5% (w/v) protein in distilled water, with 0.05% (w/v) sodium azide added to prevent microbial growth. To ensure complete rehydration of the proteins, the MPC solutions were heated for 2 h at 45˚C and were stirred at 4˚C overnight to ensure complete rehydration of the proteins. The pH was adjusted to 7.2 on the following day with minor readjustment after 1 h stirring at 22°C. The solution was then heated at 90˚C for a holding time of 25 min in a water bath in 22 mL aliquots, and then cooled to room temperature.

To isolate the whey protein/κ-casein aggregates from the micellar phase, the samples were centrifuged at 38,360×$g$ for 1 h at 20˚C using a Sorvall Lynx 6000 centrifuge equipped with a rotor Fiberlite F21-8x50y (Thermo Fisher Scientific, Waltham, MA, USA). The supernatant (0.8 mL) was fractionated by size-exclusion chromatography (SEC) on the fast protein liquid chromatography (FPLC) system Akta Purifier 10 (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The column was a HiPrep 16/60 containing Sephacryl S-500 HR beads (GE Healthcare), and the buffer was Bis-Tris propane 20 mM at pH 7.2 with 0.02% (w/v) sodium azide. Three fractions were collected, the size and protein composition of which were characterized in Chapter 2, and the results are summarized in Table 2.2 and 2.3.

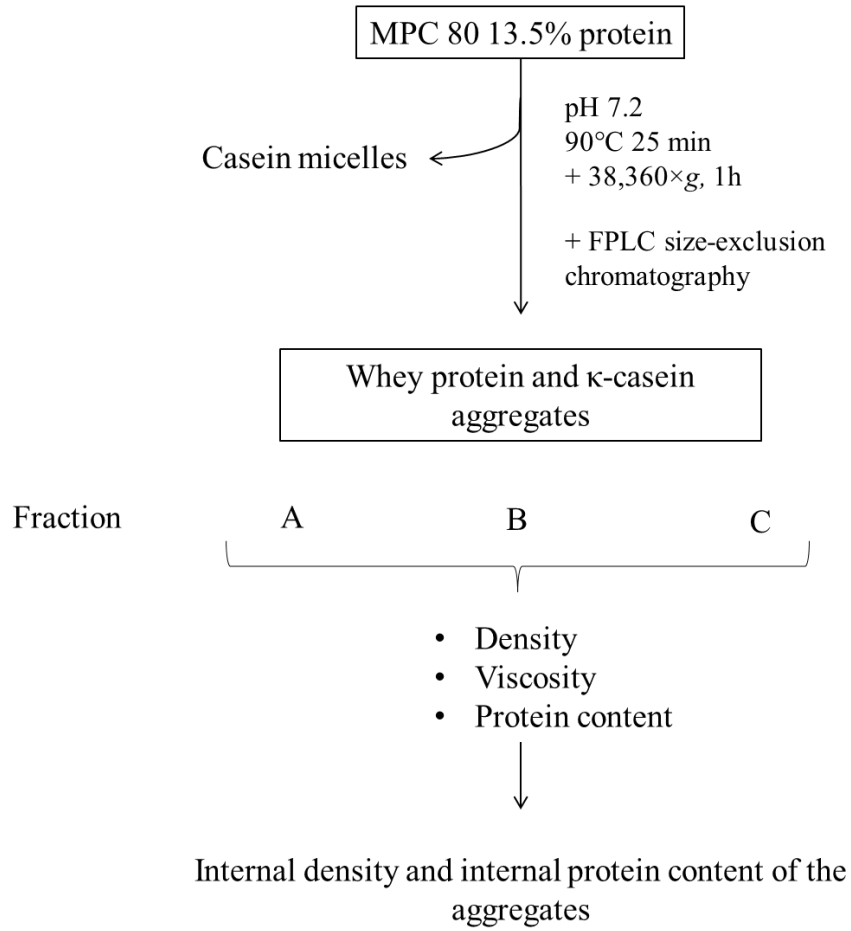



**Fig. 3.1** Flowchart of the preparation and analysis of whey protein/κ-casein aggregates. The preparation and isolation of whey protein and κ-casein aggregates is described in more detail in Chapter 2.

### 3.2 Sample concentration and protein content measurement

The very dilute fractions collected after SEC were concentrated before the density and viscosity measurements. Volumes of 5 mL were concentrated using centrifugal concentration (Vivaspin 20, MWCO 100,000 Da, Sartorius Göttingen, Germany) at 5,000×*g* at 20°C. Samples were collected at three centrifugation times, 20, 50 and 60 min, and the total protein content of each of these samples was measured using bicinchoninic acid assay (BCA) kit (Thermo Fisher Scientific), using BSA as standard. This method was preferred to a series of dilution in the SEC buffer to avoid destabilization of the most concentrated aggregates and limit the foaming caused by homogenization of the sample upon mixing the buffer and the protein solutions.

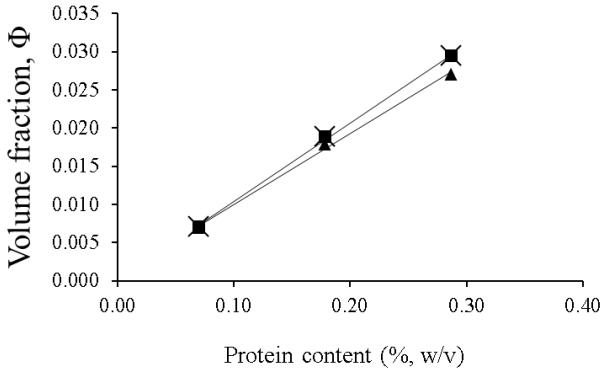
### 3.3 Viscosity and density measurements

The densitometer/viscometer DMA5000M/Lovis2000ME (Anton Paar, Graz, Austria) allowed simultaneous measurement of the density and dynamic viscosity of the samples. The measurements were carried out at 20°C on the buffer (20mM Bis-Tris propane, pH 7.2) and on the three fractions at three different concentrations of protein. The viscosity was determined using a rolling-ball viscometer at a fixed angle of 70°C containing a gold ball with a density of 7.99 g/cm$^3$. In order to avoid the formation of air bubbles, the samples were very gently mixed prior to the measurement and were degassed using a syringe, the opening of which was closed by a blocker. The experiment was carried once on each fraction and the results presented here were obtained after optimization of the procedure.

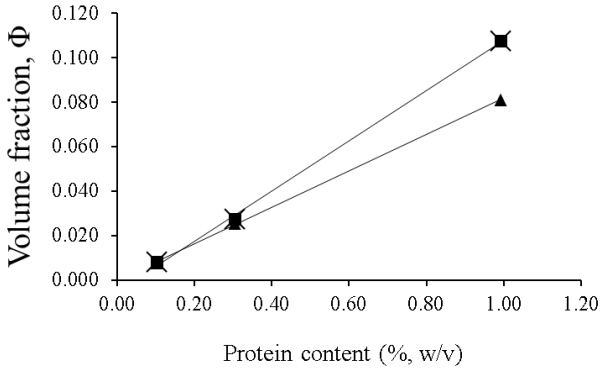
## 4. Results and discussion

The volume fraction of whey protein and κ-casein aggregates was determined in fractions A, B and C collected after fractionation of the aggregates using SEC. Einstein's, Eiler's and Lee's equations were used to convert the viscosity of the buffer and the viscosity of the protein dispersions into volume fractions. The aggregates varied in size and protein composition (Table 2.2 and 2.3). On the basis of the microscopy images (Fig. 2.3), the particles were assumed to be spherical, and therefore the viscosity increment was assumed to be 2.5. Prior to the measurement of internal density and internal protein content, the occurrence of protein-protein or protein-solvent interactions was assessed by plotting the calculated volume fractions against the protein concentrations (Fig. 3.2). All plots exhibited linear relationships, though only three concentrations were measured. Hence, interactions between proteins or interactions between the proteins and the buffer changes with concentration had a negligible effect on viscosity in this range of concentrations (Boulet et al., 1998). The voluminosity was deduced from these plots, as described in Section 2, and was around 10 mL/g in the fractions A, B and C (Table 3.1).

(a)




(b)




(c)

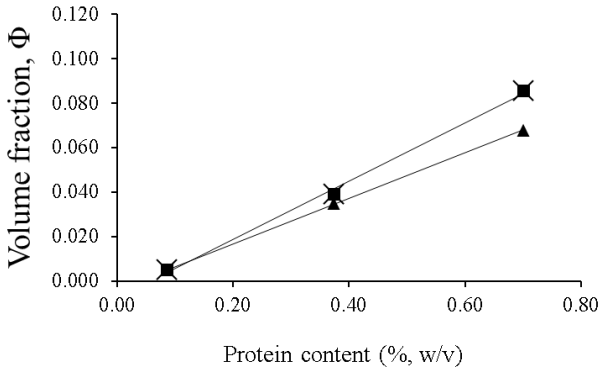



**Fig. 3.2** Variation of the volume fraction of the aggregates, calculated using (■) Einstein's equation, (×) Lee's equation or (▲) Eiler's equation, as a function of the protein concentration in fractions (a) A, (b) B and (c) C. The straight lines represent the linear trend for each equation used.

Previous studies reported that the voluminosity of non-heated whey proteins at pH 6.8 and casein micelles at pH 7.8 were 1.4-2 mL/g and 7-9 mL/g, respectively, using Lee's equation and a viscosity increment of 2.5 (Boulet et al., 1998). The voluminosity of the casein micelle is close to that of the aggregates of whey protein and κ-casein in this study. Donato and Guyomarc'h (2009) calculated a crude estimate of the density of heat-induced aggregates of whey protein and κ-casein (70 nm) by centrifugation and reported a value around 1.080 g/cm$^3$, which is close to the average density of the casein micelles (1.063 g/cm$^3$) reported by O'Mahony and Fox (2013). This supports our results and the hypothesis that whey protein and κ-casein aggregates are as voluminous as casein micelles.

Interestingly, in fractions B and C, the volume fraction calculated by Eilers equation deviated from those calculated by Einstein's and Lee's equations with increasing protein concentration (Fig. 3.2) and the voluminosity of the aggregates was lower (Table 3.1). These are small differences that could be due to the lower protein concentrations used for analysis in fraction A (0.1-0.3%, w/v), compared to the range of concentration in fractions B and C (0.1-1%, w/v). However, if more experimental data with higher protein content confirm the findings, it could also indicate that some equations correct or over-correct for protein-protein and protein-solvent interactions in fraction B and C, and that such a correction is not needed in fraction A. Differences in surface properties of the particles could explain this phenomenon, as they are likely to cause variations in protein-protein or protein-solvent interactions. In particular, the aggregates in fraction C were reported to be more hydrophilic than the aggregates in fraction A or B (Chapter 2). It is noteworthy that, due to the large amount of $\alpha_s$- and β-casein in fractions B and C, caseins may form small micelles with sizes close to those of the whey protein and κ-casein aggregates (Chapter 2).

Consequently, calculated volume fractions would partly reflect the characteristics of small casein micelles in fractions B and C.

Internal protein content and internal density of particles were deduced from equations (2) and (3). These data did not vary greatly with the protein concentration; therefore, an average of these values is presented in Table 3.2. All fractions contained particles with an internal protein content of 9-12% (w/w) and an internal density of 1.02-1.05 g/cm$^3$. This range of internal densities is relatively close to that calculated by Donato and Guyomarc'h (2009).

A deviation from the ideal hard sphere model results in a greater viscosity increment. It is claimed that Mendoza's equation provides a more accurate volume fraction for particles that are not perfect spheres (Santamarı´a-Holek & Mendoza, 2010). In addition to the viscosity increment, Mendoza's equation contains a factor, the critical packing volume fraction, which is dependent on the shape of the particles. To our knowledge, these data are not available for whey protein and κ-casein aggregates and would require a deeper study of the shape of the aggregates by microscopy or light-scattering, for example.

**Table 3.1** Voluminosity of the aggregates of whey protein and κ-casein in fractions A, B and C, calculated using Einstein's, Lee's and or Eiler's equation.

| | **Voluminosity (mL/g)** | | |
|---|---|---|---|
| **Fractions** | **Einstein** | **Lee** | **Eiler** |
| A | 10.3 | 10.3 | 9.3 |
| B | 11.3 | 11.3 | 8.2 |
| C | 13.1 | 13.1 | 10.2 |

Other authors induced the sphericity of the particles by heating a high concentration of whey proteins (25%, w/w) in an oil-continuous phase (Sağlam, Venema, de Vries, Sagis, & van der Linden, 2011). This could be the most practical approach to improve the accuracy of the volume fraction calculation using rheometry. However, in this case it is assumed that the particles formed are equivalent to those formed in aqueous solution. Light-scattering techniques also provide useful information on shape, density and molecular weight of whey protein aggregates, over a wide range of pH and ionic strength conditions (Baussay, Bon, Nicolai, Durand, & Busnel, 2004; Mehalebi et al., 2008). However, one of the advantages of using rheometry techniques for the measurement of internal density and internal protein content of whey proteins is the availability of the equipment in most research laboratories, provided that the geometry is sensitive enough for samples with a low density and a low viscosity. Light-scattering could be used as a validation technique for the measurement of internal density of such aggregates.

Technical difficulties encountered in this study included the time necessary for the isolation of the aggregates by SEC (two hours run per sample injected, 9 fractionation steps necessary) and for the subsequent concentration. Despite the concentration step, the protein content was still relatively low (less than 1%, w/v), due to the extensive dilution of the aggregates in the buffer during SEC. In addition, the volume of sample obtained after concentration was relatively low and limited the range of concentrations that could be studied, therefore reducing the accuracy of the measurement.

**Table 3.2** Internal density and internal protein content of the aggregates of whey protein and κ-casein in fractions A, B and C, calculated by Einstein's, Lee's and Eiler's equations. These results were the mean of two to three concentrations tested on the same sample and the resulting standard deviations are also indicated.

| **Fraction** | **Internal density of the particles (g/cm$^3$)** | | | **Internal protein content of the particles (%, w/w)** | | |
|---|---|---|---|---|---|---|
| | Einstein | Lee | Eilers | Einstein | Lee | Eilers |
| **A** | 1.028 ±0.008 | 1.029 ±0.009 | 1.029 ±0.009 | 9.4 ±0.3 | 9.9 ±0.3 | 9.8 ±0.2 |
| **B** | 1.019 ±0.015 | 1.023 ±0.021 | 1.022 ±0.019 | 11.0 ± 2.1 | 12.3 ±1.0 | 11.9 ±1.4 |
| **C** | 1.040 ± 0.008 | 1.047 ±0.006 | 1.045 ±0.007 | 8.6 ±0.9 | 10.1 ±0.2 | 9.5 ±0.5 |

## 5. Conclusion

The present study highlights the challenges in measuring the internal density and the internal protein content of aggregates of whey protein and κ-casein. The internal protein content of the aggregates was estimated to be 9-12% (w/w) and the internal density was 1.02-1.05 g/cm$^3$. Possible improvements were suggested for the determination of internal density and internal protein content of aggregates using rheometry. Therefore the results obtained in this study could be the starting point for a deeper analysis of the effect of κ-casein on the density of the whey protein aggregates.

**Acknowledgements**

The authors would like to thank Dr Vitaly Buckin and Dr Margarida Caras Altas for their assistance with the densitometer and the viscometer, and Prof Thom Huppertz for his help with the interpretation of the results. This work was supported by Dairy Levy Research Trust (project MDDT6261 "ProPart"). S. J. Gaspard was funded under the Teagasc Walsh Fellowship Scheme (reference number 2012211).

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The inclusion of κ-casein increased the heat stability of whey proteins aggregates (Chapter 2). Caseinomacropeptide (CMP), a peptide derived from the enzymatic cleavage of κ-casein, has also been shown to display chaperone-like activity on β-lactoglobulin aggregation, as reviewed in Chapter 1. The following chapter aimed to assess the potential chaperone-like activity of CMP on whey protein aggregation and to determine the nature of their interactions before, during and after heating.

# Chapter 4

## 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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SJG contributed to the experimental design, did all the experiments, analysed the data and wrote the manuscript. PS provided advices on the rheological methods and reviewed the manuscript. CF and JT provided the caseinomacropeptide powder. JOM, ALK and AB reviewed the manuscript.

Chapter 4 is a manuscript in preparation for publication.

**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

Chaperone-like activity is defined as the ability of a molecule to protect another biomolecule against unfolding, 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 (Chapter 2). 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 (NeuAc), 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 NeuAc generally located at the end of the carbohydrate chain (Saito & Itoh, 1992). The low pKa (2.6) of NeuAc 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 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. 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 dry matter content from the moisture, fat, lactose and ash 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. 4.1). 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 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.

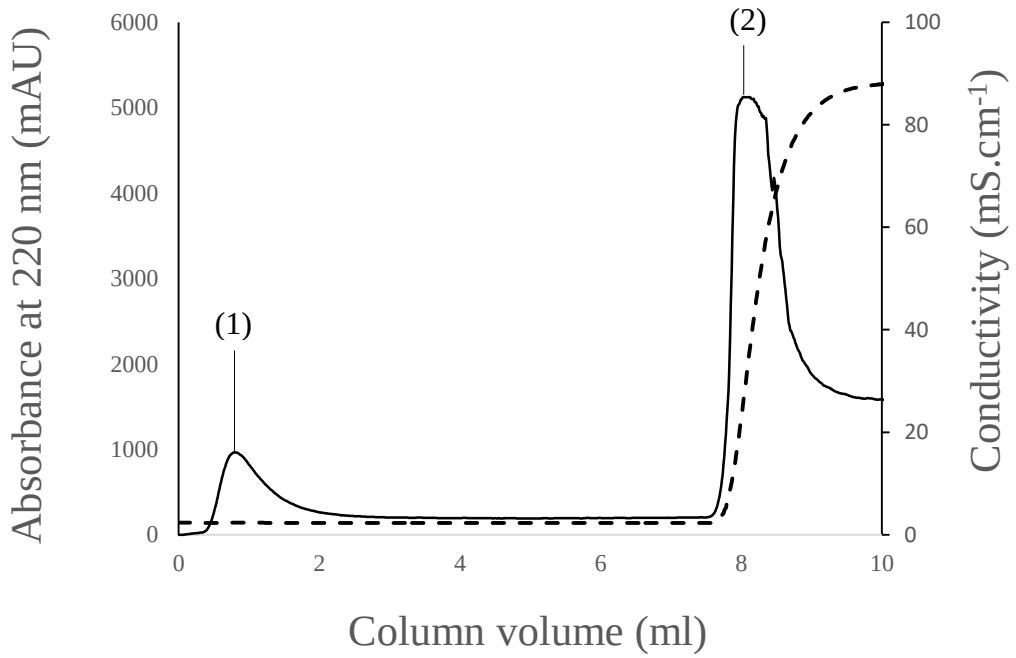



**Fig. 4.1** Anion-exchange chromatography on a fast protein liquid chromatography system of (—) a solution of 0.125 % (w/v) caseinomacropeptide (CMP). The peak labelled (1) corresponds to the non-glycosylated CMP and the peak labelled (2) corresponds to the glycosylated CMP. The equilibration buffer 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.

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 5% (w/v) total protein, 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 10% (w/v) total protein, 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. 4.2 summarizes the steps of rehydration, heating and analysis carried out on the solutions of whey proteins and CMP.

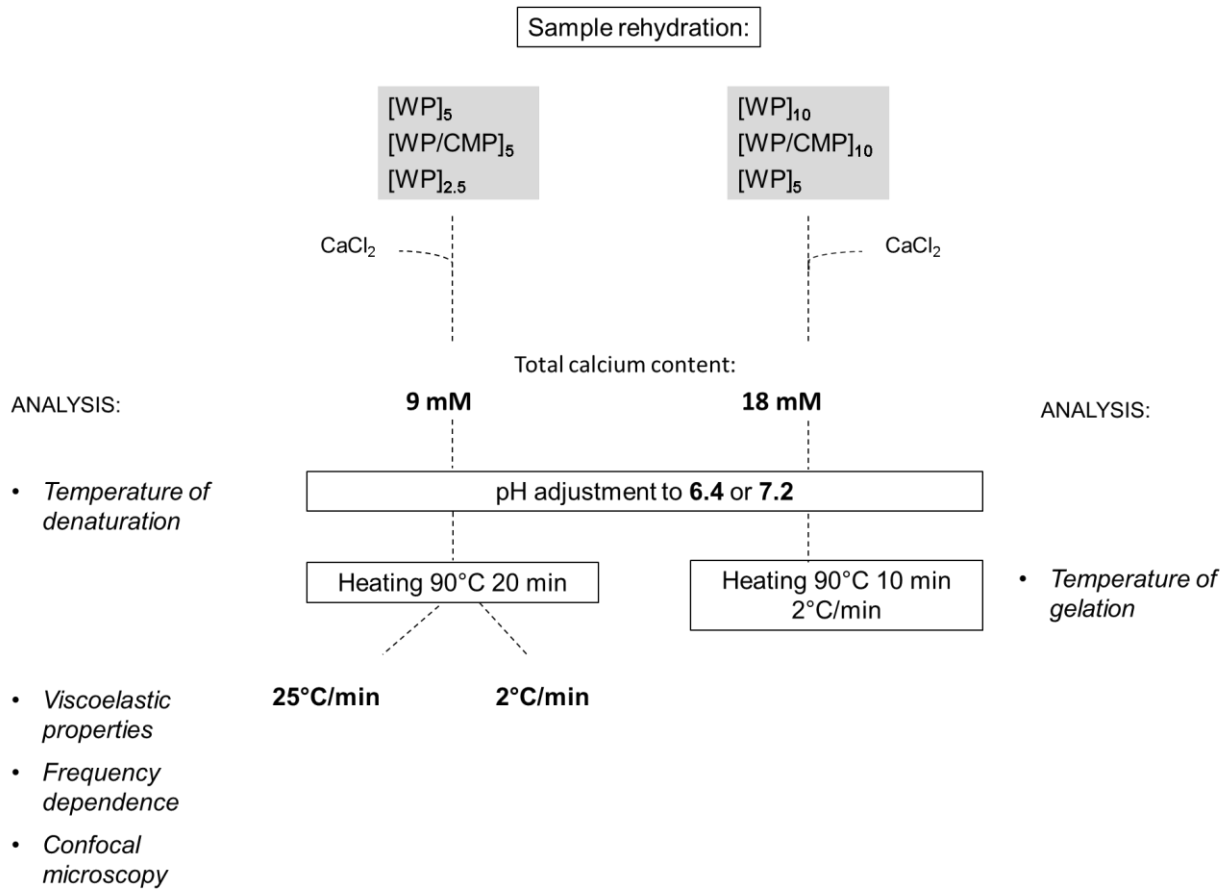



**Fig. 4.2** Flowchart for the preparation and analysis of whey protein and caseinomacropeptide (CMP) mixtures. Solutions of 2.5, 5 or 10% (w/v) whey protein (abbreviated $[WP]_{2.5}$, $[WP]_5$ or $[WP]_{10}$, respectively) and mixtures of whey proteins and CMP containing 5 or 10% (w/v) total protein (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).

## 2.3 Anion-exchange chromatography

A solution of 1 ml of 0.125% (w/v) CMP was analysed by anion-exchange chromatography by fast protein liquid 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 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 and the heating rate to 90°C was 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 to 50 Hz on heated samples containing 2.5-5% (w/v) protein and 9 mM calcium, and 0.1 to 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 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. One-way ANOVA post-hoc Tukey tests were used and the results are presented as 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. 4.3 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).

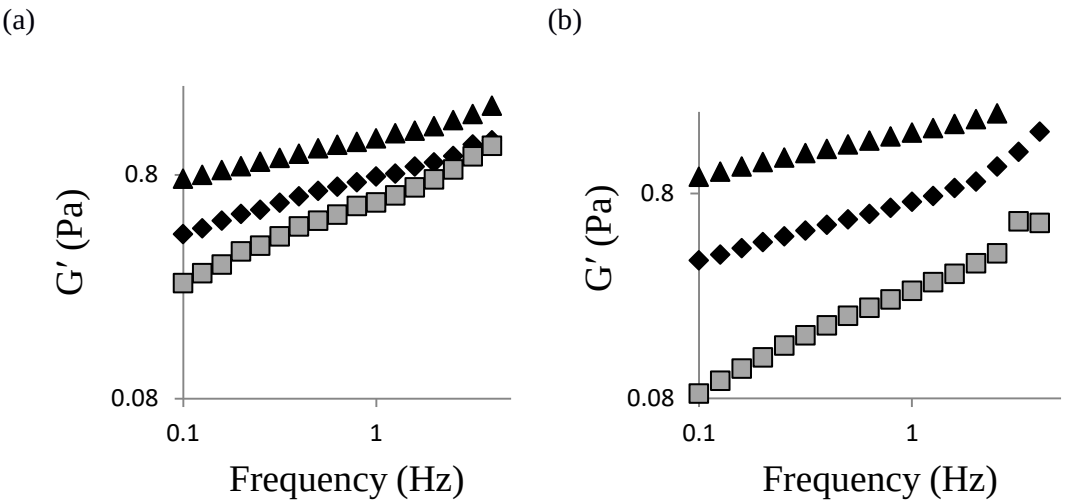



**Fig. 4.3** Storage modulus (G′) as a function of the frequency at (a) pH 7.2 or (b) 6.4 for non-heated samples containing (◆) 5% (w/v) whey protein, (▲) 2.5% (w/v) whey protein or (▣) a mixture of whey proteins and caseinomacropeptide (CMP) at a total protein of 5% (w/v). The whey protein:CMP ratio in the mixtures was 1:0.9 (w/w). The experiment was performed in three independent replicates.

For all non-heated samples, G′ was higher than G″ (results not shown) on most of the frequency range, representative of a dominant elastic behaviour, which 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. 4.3. 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. 4.3), 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. 4.3). 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.

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. 4.4 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 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

bonds and van der Waals interactions during cooling (Lefèvre & Subirade, 2000; Martinez et al., 2010).

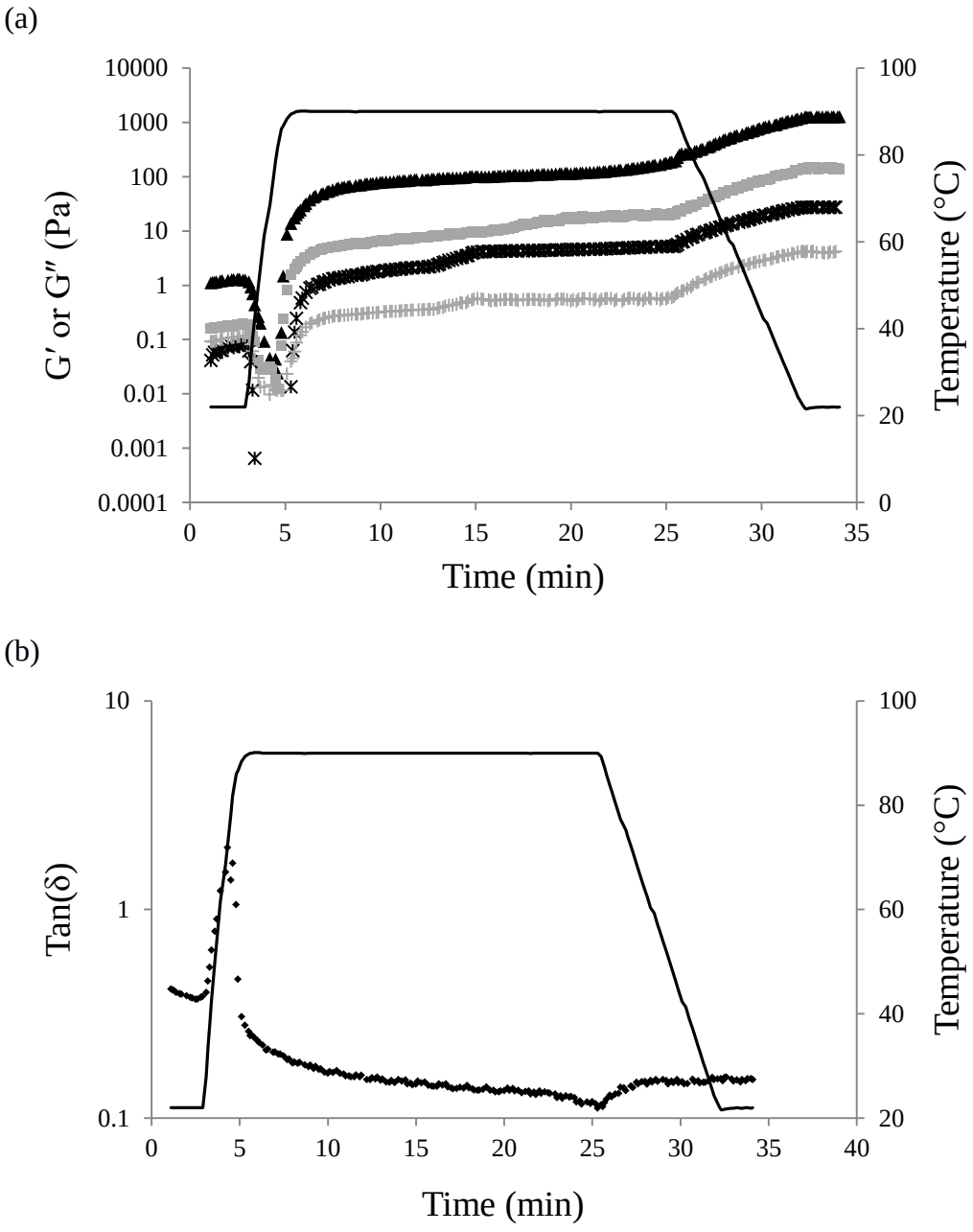



**Fig. 4.4** Typical profile of (a) (▲) storage modulus (G′) and (■) loss modulus (G″) of a 5% (w/v) whey protein solution and (✳) G′ and (+) G″ of a mixture of whey proteins and caseinomacropeptide (CMP) at a total protein content of 5% (w/v) during heat treatment. All samples were heated at 90°C for 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 4.1 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′ value of the mixture of whey protein and CMP was considerably lower in the presence of CMP, compared to those of the controls, before and after cooling (Table 4.1 and Fig. 4.4). 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 one minute, compared to that of the controls containing whey protein only (Table 4.1 and Fig. 4.4). 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 4.1). 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.

**Table 4.1** 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 and are presented as mean ± SD. 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$^{d}$ | 1117 ±137$^{a}$ | 4.8 ±0.1$^{a}$ | 0.09 ±0.01$^{a}$ |
| $[WP/CMP]_5$ | 7.2 | 7 ±3$^{a}$ | 38 ±9$^{b}$ | 5.7 ±0.1$^{b}$ | 0.15 ±0.04$^{b}$ |
| $[WP]_{2·5}$ | 7.2 | 66 ±33$^{b}$ | 253 ±91$^{c}$ | 4.9 ±0.3$^{a}$ | 0.09 ±0.01$^{ab}$ |
| $[WP]_5$ | 6.4 | 339 ±113$^{e}$ | NA | 4.5 ±0.2$^{a}$ | 0.10 ±0.00$^{ab}$ |
| $[WP/CMP]_5$ | 6.4 | 124 ±4$^{c}$ | NA | 4.9 ±0.1$^{a}$ | 0.09 ±0.01$^{a}$ |
| $[WP]_{2·5}$ | 6.4 | 22 ±8$^{ab}$ | 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.

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. 4.5).

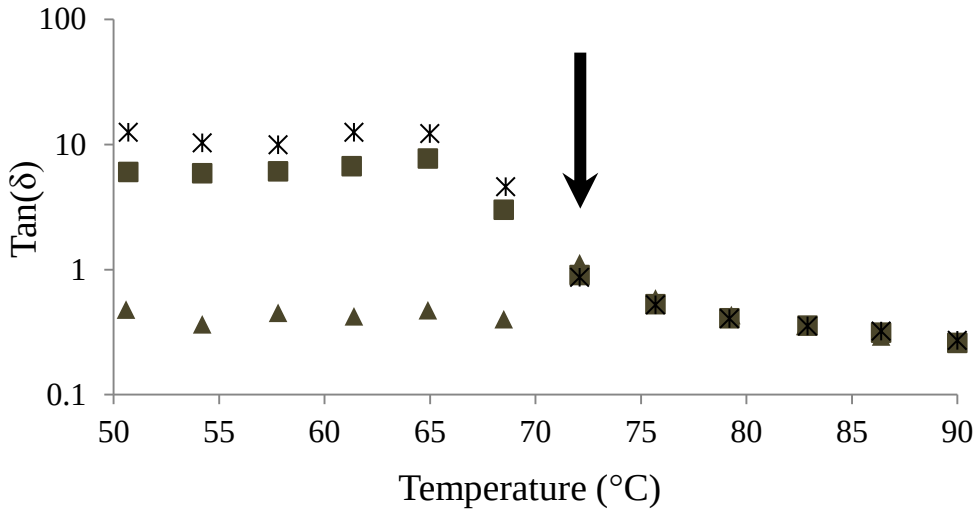



**Fig. 4.5** Typical profile of the loss tangent (δ) of a solution of 10% (w/v) whey protein as a function of the temperature, measured at (▲) 5.1, (■) 7.5 and (✳) 10.0 Hz. The arrow indicates the gel point of the sample, i.e., the collapse of the loss tangent values at the temperature of gelation of the sample.

The viscoelastic properties of these samples are reported in Table 4.2. 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 4.2). 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 difference in G′ between the mixture at 5% (w/v) total protein and the sample containing 5% (w/v) whey protein only at pH 7.2 (Table 4.1) was not observed at higher protein content (Table 4.2). This 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.

**Table 4.2** 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 and are presented as mean ± SD. The superscripts indicate the statistical significance with $p < 0.05$.

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

* G′ is the storage modulus 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. 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.

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 4.1 and 4.2; 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. 4.6).

The microscopy images revealed a fine-stranded gel structure in the samples that contained whey protein only (Fig. 4.6 a,c, d and f), and an even finer gel structure for the samples containing CMP (Fig. 4.6 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 4.1). At pH 7.2, a finer structure of the strands could explain the lower G′ of the mixtures after heating (Table 4.1), 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 4.1), despite a clear difference in gel structure (Fig. 4.6 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).

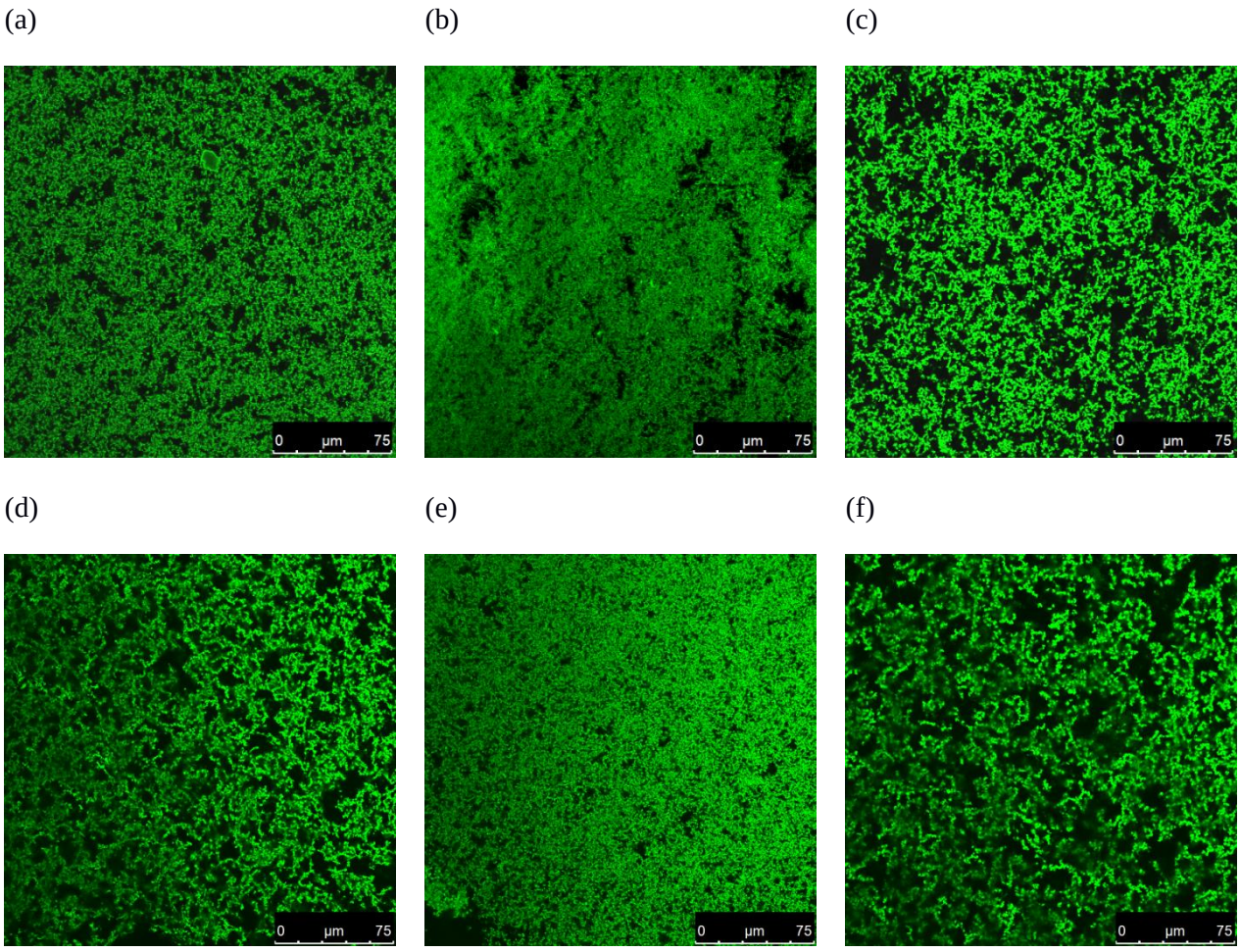



**Fig. 4.6** Confocal microscopy images of gels formed after heating (a,d) 5% whey protein solution, (b,e) a mixture of whey proteins and caseinomacropeptide (CMP) at 5% (w/v) total protein (w/v) and (c,f) 2.5% (w/v) whey protein solution at 90°C for 20 min, and at (a,b,c) pH 7.2 or (d,e,f) pH 6.4. The 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).

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. 4.7 and 4.8). One endothermic peak was observed, with an onset of denaturation around 60°C and a maximum at 71-79°C (Fig. 4.7), 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).

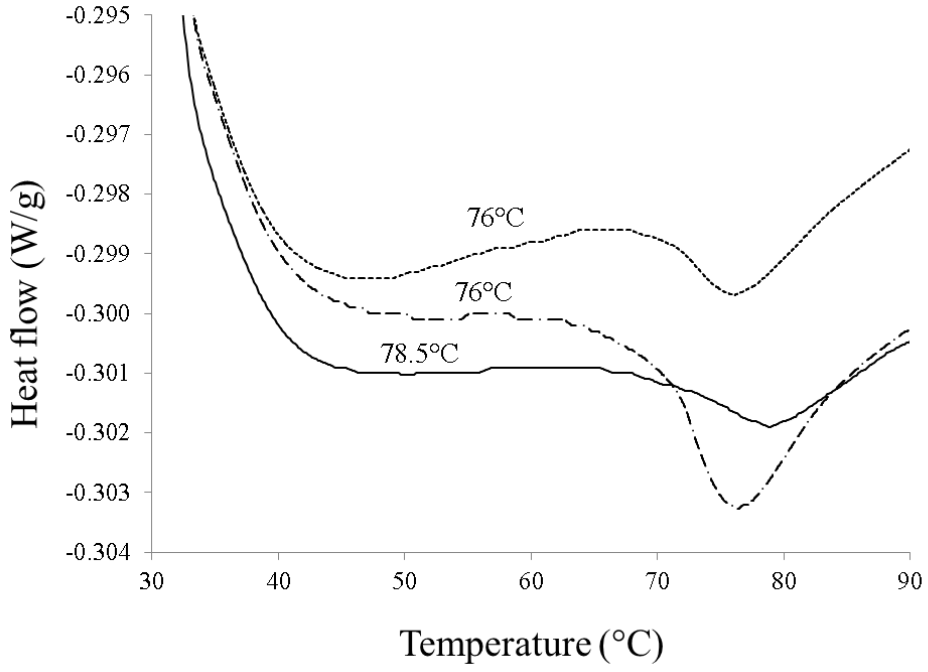



**Fig. 4.7** Typical thermograms of (- - -) 5% (w/v) whey protein, (——) a mixture of whey proteins and caseinomacropeptide (CMP) at 5% (w/v) total protein and (·····) 2.5% (w/v) whey protein containing 9 mM calcium at pH 6.4. The whey protein:CMP ratio in the mixtures was 1:0.9 (w/w). Above the lines, the temperature of denaturation is indicated for each endothermic peak.

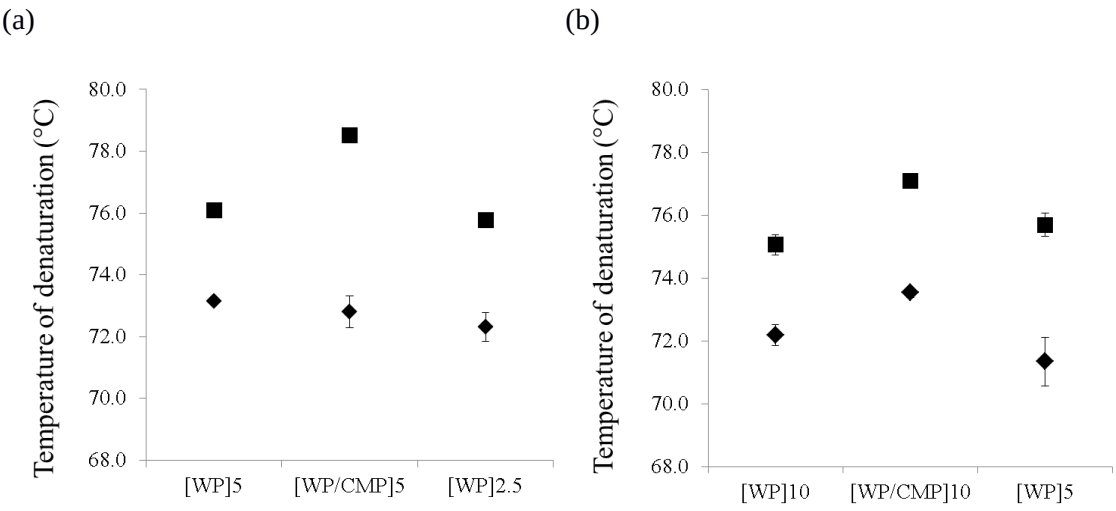



**Fig. 4.8** Temperature of denaturation of β-lactoglobulin measured by differential scanning calorimetry at (◆) pH 7.2 or (■) 6.4 in (a) solutions containing 9 mM calcium and 2.5 or 5% (w/v) whey protein (abbreviated $[WP]_{2.5}$ or $[WP]_5$, respectively) or a mixture of whey proteins and caseinomacropeptide (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.

The temperature of denaturation for all samples decreased with increasing pH at heating (Fig. 4.8). 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. 4.8 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. 4.8 b).

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. 4.9).

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 4.1). 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.

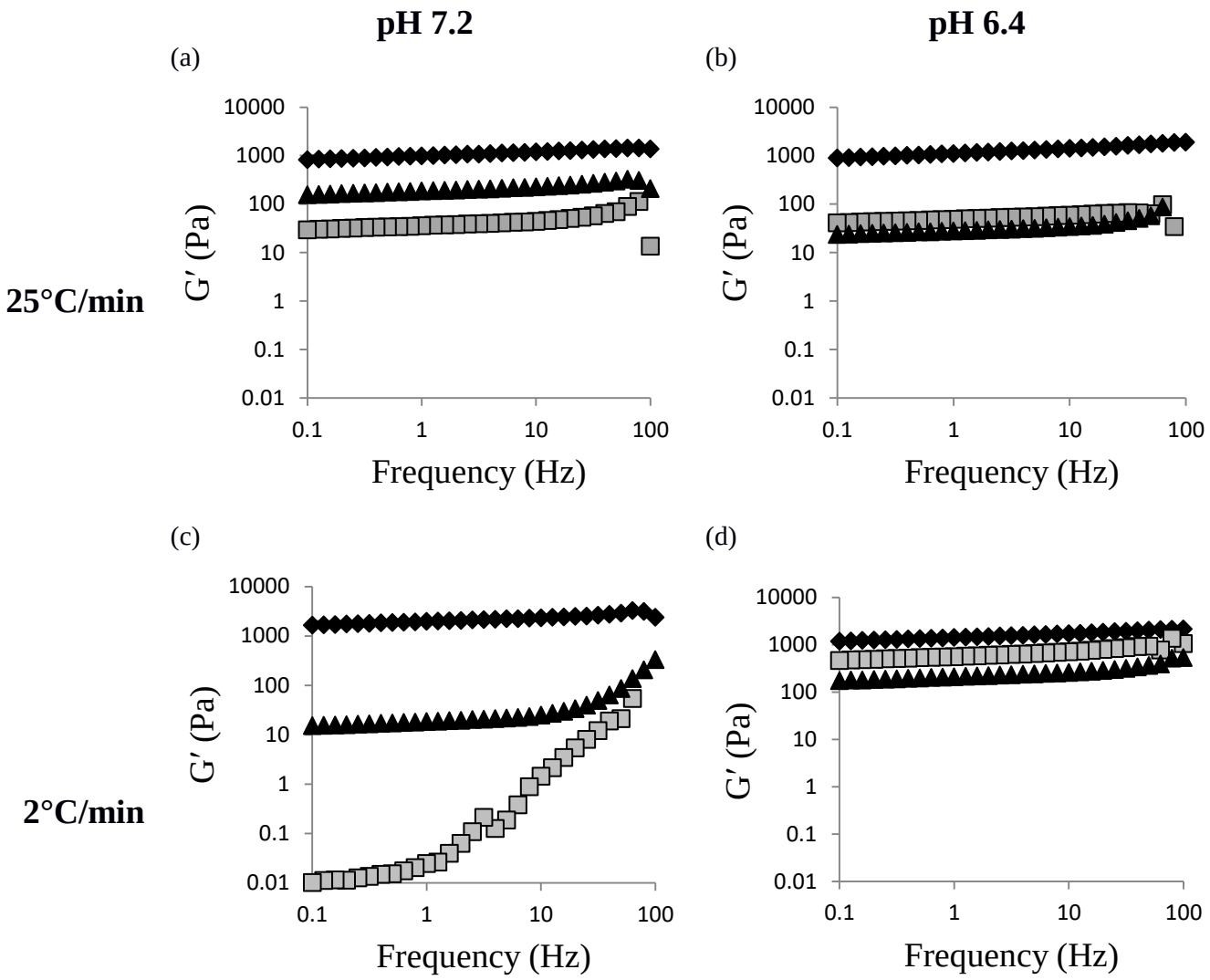



**Fig. 4.9** Storage modulus G′ as a function of frequency for samples containing (◆) 5% (w/v) whey protein, (□) a mixture of caseinomacropeptide and whey proteins at 5% total protein (w/v) or (▲) 2.5% (w/v) whey protein, after heating at 90°C for 20 min at a heating rate of (a,b) 25°C/min and (c,d) 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.

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 7.5°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. 4.9). 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. 4.9), likely due to the differences in protein and mineral composition and heating conditions.

## 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 the 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.

**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 (Project MDDT6261 “ProPart”). S.J. Gaspard was funded under the Teagasc Walsh Fellowship Scheme (reference number 2012211).

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Caseinomacropeptide (CMP) displayed a chaperone-like activity on the denaturation and aggregation of whey proteins (Chapter 4). The following chapter aimed to determine the role of the glycosylation of CMP on its chaperone-like activity.

# Chapter 5

## Influence of desialylation of caseinomacropeptide on the denaturation and aggregation of whey proteins

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SJG contributed to the experimental design, measured the temperature of denaturation of whey proteins, the amount of denatured proteins, the size distribution of the protein aggregates, the turbidity and ξ-potential of the samples, and the conformational changes of the proteins by FTIR, analysed the data and wrote the manuscript. LGGM provided the electron microscopy images. AVS, LBL and NAP provided the caseinomacropeptide powders and reviewed the manuscript. AB provided the atomic force microscopy images and reviewed the manuscript. JOM and ALK reviewed the manuscript.

Chapter 5 is a modified version of the following publication:

Gaspard, S. J., Sunds A. V., Larsen L. B., Poulsen L. A., O'Mahony, J. A., Kelly, A. L., & Brodkorb, A. (2020). Influence of desialylation of caseinomacropeptide on the denaturation and aggregation of whey proteins. *Journal of Dairy Science*

**Abstract**

The effect of the addition of caseinomacropeptide (CMP) or desialylated-CMP on the heat-induced denaturation and aggregation of whey proteins was investigated in the pH range 3 to 7 after heating at 80°C for 30 min. The rate and temperature of denaturation, the extent of aggregation and the changes in secondary structure of the whey proteins heated in presence of CMP or desialylated-CMP were measured. The sialic acid bound to CMP favored the denaturation and aggregation of the whey proteins when the whey proteins were oppositely charged to CMP at pH 4. A transition occurred at pH 6, below which the removal of sialic acid enhanced the stabilizing properties of the CMP against the denaturation and aggregation of the whey proteins. At pH > 6, the interactions between desialylated-CMP and the whey proteins led to more extensive denaturation and aggregation. Sialic acid bound to CMP influenced the denaturation and aggregation behaviour of whey proteins in a pH-dependent manner and this should be considered in future studies on the heat stability of such systems containing CMP.

## 1. Introduction

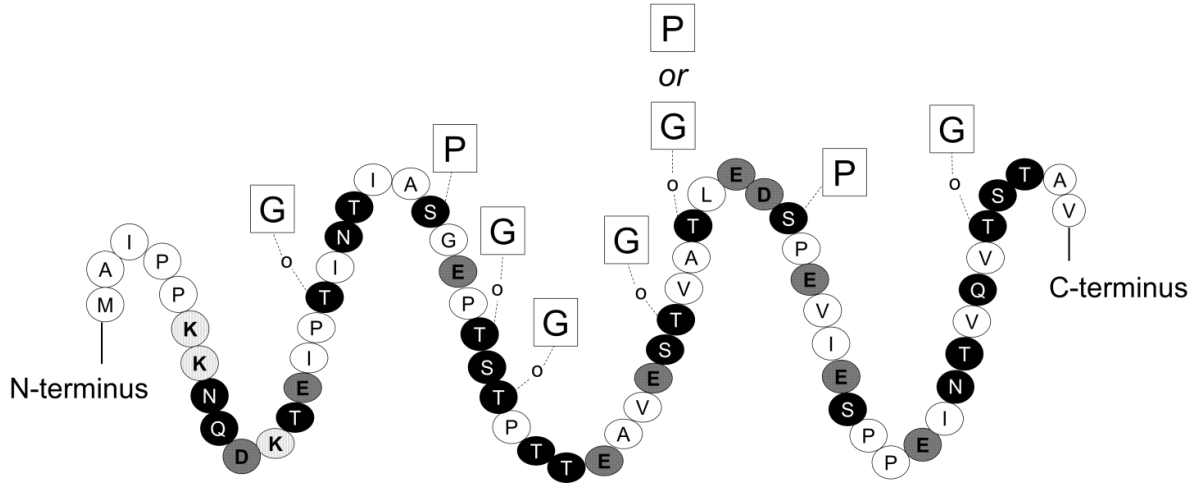
Bovine whey proteins are known for their nutritional and bioactive properties, which make them ideal ingredients for nutritional beverages such as infant milk formula and protein drinks for athletes and the elderly. However, these ingredients need to be able to withstand thermal treatments such as pasteurization and ultra-high heat treatment applied for reasons of microbiological safety control. Whey proteins are thermolabile and form soluble aggregates, undesirable large visible gel particles or continuous gel networks depending on the conditions of heat treatment.

In contrast to this, bovine caseinomacropeptide (CMP), commonly referred as glycomacropeptide (GMP) when glycosylated, is a 64 amino acid peptide resulting from the enzymatic cleavage of κ-casein into two peptides (CMP and *para*-κ-casein) and is very heat stable. Glycosylated proteins, such as CMP, present bioactive properties specific to their carbohydrate side chains, sometimes also referred to as prosthetic groups (Nagel, Dellweg, & Gierasch, 1992). N-acetyl Neuraminic acid (NeuAc) is the most abundant member of the sialic acid family in mammalians and is responsible for many bioactive properties of bovine CMP, for example, the promotion of gut microbial growth, the improvement of learning abilities and the modulation of the immune system response (Brody, 2000; O'Riordan, Kane, Joshi, & Hickey, 2014; Thomä-Worringer, Sørensen, & López-Fandiño, 2006). Amongst other functions, sialic acids play roles in stabilization of cells and proteins, and participate to the transport of positively-charged ions (Cases, Vidal, & Cuq, 2003; Traving & Schauer, 1999; Varki, 2008).

CMP represents up to 25% (w/w) of the total protein in cheese whey (Thomä-Worringer et al., 2006). In this fraction, around 50% of CMP is glycosylated, with

the peptide containing up to six glycosylation and three phosphorylation sites in its C terminal part (Fig. 5.1 a).

(a)




(b)

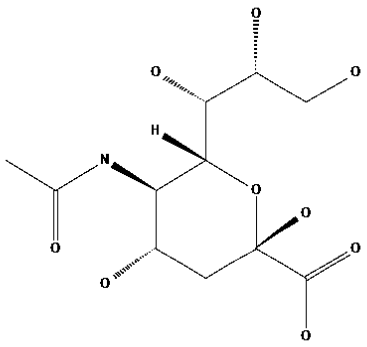


**Fig. 5.1** (a) Amino acids sequence of caseinomacropeptide (CMP), derived from κ-casein A, with potential sites for post-translational modifications; potential glycosylation site: ○–o–G ; potential phosphorylation site: ○–P). The amino acids with (○) non-polar, (●) polar, (▩) negatively-charged and (▨) positively-charged side chains are also indicated on the figure, as reviewed by Holland (2008). (b) 2D structure of N-acetyl-beta-D-Neuraminic acid (PubChem CID: 445063).

In mature cow milk, NeuAc is generally located at the end of a glycosylation chain, which apart from NeuAc, contains galactose and N-acetyl galactosamine, organized from monosaccharide to tetrasaccharide (Saito & Itoh, 1992). Thanks to its carboxylic functional group (Fig. 5.1 b), NeuAc exhibits a pKa of 2.6 and lowers the overall isoelectric point (pI) of the glycoproteins. The estimated pI of κ-casein based on the primary sequence is 5.93; phosphorylation lowers the pI to 5.6, while the glycosylation lowers the pI of κ-casein down to 3.5 (Huppertz, 2013). The pI of glycosylated and non-glycosylated CMP were reported to be 3.2 and 4.2, respectively (Kreuß, Strixner, & Kulozik, 2009). The degree of phosphorylation and glycosylation of CMP varies widely, and is illustrated by multiple peaks in the elution profile of reversed-phase HPLC (Thomä, Krause, & Kulozik, 2006) and LC-MS (Sunds, Poulsen, & Larsen, 2019), as well as by separation of CMP spots by 2-dimensional electrophoretic analysis (Le et al., 2016). The negative charges carried by the charged amino acid residues, the post-translational modifications at neutral pH and the disordered structure of the peptide, all result in a very hydrophilic and heat stable polypeptide. However, NeuAc is sensitive to acid and heat treatment (Kilic-Akyilmaz & Karimidastjerd, 2018; Siegert, Tolkach, & Kulozik, 2012). Therefore not all CMP contains the same amount of NeuAc due to heat-induced losses (Taylor & Woonton, 2009).

These properties can improve the solubility of other proteins when heated in the presence of CMP. The post-translational modifications of the heat-stable proteins $\alpha_s$-, β- and κ-casein are thought to be involved in the control of the aggregation of whey proteins during heat treatment, by limiting the size of the aggregates or the extent of aggregation (Guyomarc'h, Nono, Nicolai, & Durand, 2009; Kehoe & Foegeding, 2014; Morgan, Treweek, Lindner, Price, & Carver, 2005). In Chapter 2, the inclusion

of κ-casein was shown to improve the heat stability of whey protein aggregates. In addition, Doi, Ibuki, and Kanamori (1981) showed a correlation between degree of glycosylation of κ-casein and improved heat stability of β-lg. Croguennec et al. (2014) studied the effect of CMP on the denaturation and aggregation of whey proteins and demonstrated that CMP increased the rate of denaturation of β-lactoglobulin (β-lg) *via* hydrophobic and electrostatic interactions at pH 4.0 and 6.7. However, it could limit the aggregation of β-lg at pH 6.7 due to the negative charges carried by CMP around the neutral pH. In Chapter 4, CMP displayed a chaperone-like activity for whey protein aggregation, giving a higher temperature of denaturation and temperature of gelation of the whey protein solutions at pH 6.4 and 7.2 in the presence of CMP. Therefore, it is possible that the glycosylation of CMP is involved in the control of aggregation of whey proteins.

To the author's knowledge, the effect of the sialic acid content of CMP on the denaturation and aggregation of a mixture of β-lg and α-lactalbumin (α-la), such as in whey protein isolate (WPI), has not been the subject of any studies yet. The aim of this study is to bring new insights to the effect of the negatively-charged NeuAc on the denaturation and aggregation behavior of β-lg and α-la in WPI in a wide pH range (3 to 7) during heat treatment (80°C for 2-30 min) with a view to developing strategies for enhancement of the heat stability of whey protein systems.

## 2. Materials and Methods

### 2.1 Materials

All reagents were purchased from Sigma Aldrich (St. Louis, MO, USA) unless stated otherwise. Denatured whey proteins were removed from native whey proteins by isoelectric precipitation. Briefly, a solution of 10% (w /v) WPI (whey protein isolate) Davisco Bipro®, Eden Prairie, MN, USA) was rehydrated in Milli-Q® water, heated at 40°C for 2 h and stirred overnight at 4°C. The pH of the solution was adjusted to 4.6 and centrifuged at 4,000×*g* for 40 min to separate aggregated material from soluble whey proteins. The supernatant, containing the native whey proteins, was adjusted to pH 7.0 and dialyzed against 10 mM sodium phosphate (pH 7.0) for 24 h with 2 changes of buffer, then for 24 h in distilled water with two changes of water. The dialyzed solutions were freeze-dried. The protein content was measured using reversed-phase high-performance liquid chromatography (RP-HPLC) using a modification of the method of Beyer and Kessler (1989).

A solution of CMP (Lacprodan cGMP-20®, Arla Foods Ingredients, Viby J, Denmark) was enzymatically desialylated following the method described initially by Villumsen et al. (2015) and modified by Sunds et al. (2019). Briefly, the sialidase was added in a ratio of 1:57,000 (w/w) to the protein solution rehydrated at 7% at pH 5.8. The sample was incubated at 37°C overnight and freeze-dried.

The desialylation of CMP resulted in a shift in its pI from 3.0 for the untreated CMP to around 3.7 for the desialylated CMP (d-CMP) after analysis by 2-dimensional SDS-PAGE as analyzed and reported elsewhere on the same batch of powder (Sunds et al., 2019). The chromatogram presented in Fig. 5.2 shows the effect of desialylation on the chromatogram of CMP and d CMP. The CMP and the d-CMP

powders were rehydrated, dialyzed and freeze-dried following the same process as for the WPI powders.

2.2 Reconstitution

Mixtures of WPI with CMP or d-CMP were rehydrated in Milli-Q® water. The concentration of whey protein in the mixtures was 0.5% (w/v). However, the protein content of the freeze-dried CMP and d-CMP powders could not be accurately estimated by Kjeldahl due to the unknown nitrogen-to-protein conversion factor of CMP and d-CMP used in this study, which varies from 6.71 to 7.37 depending on the variant and as a function of the degree of glycosylation (Karman & Van Boekel, 1986). Attempt to quantify CMP and d-CMP by RP-HPLC by measuring the sum of the peak areas at 214 nm shows that both powders contained the peptide in comparable amounts. Therefore, the CMP or d-CMP powders were added to the whey protein sample in a concentration of 0.5% (weight of CMP or d-CMP powder/v). As control samples, solutions of 0.5 and 1% (w/v) whey protein were rehydrated in Milli-Q® water.

Higher proteins concentrations were required for the differential scanning calorimetric (DSC) and the Fourier transform infrared spectroscopy (FTIR) measurements. Hence, solutions of 2.5% (w/v) whey proteins were also prepared, with CMP or d-CMP (2.5%, w/v). As control samples, solutions of 2.5 and 5% (w/v) whey proteins were rehydrated in Milli-Q® water. Solutions of 2.5% (weight of powder/v) CMP or d-CMP were also rehydrated in Milli-Q® water and were used as controls for the FTIR measurements.

## 2.3 Heating of protein solutions

For the measurement of the degree of denaturation of the whey proteins, the ξ-potential, the molecular weight distribution, the turbidity measurement and the microscopy images, the pH of the 5 mL-solutions was adjusted to 3, 4, 5, 6 and 7 and subsequently heated in a water bath at 80°C and aliquots of 0.7 mL were removed at 2, 5, 10, 15, 20 and 30 min for analysis. The aliquots were immediately cooled to room temperature.

For the FTIR measurement, the pH of the samples was adjusted to 4, 5, 6 or 7 and a volume of 200 µl was heated for 30 min at 80°C in a water bath. The samples were heated for only 5 min at pH 5 to avoid the gelling of the samples. The samples were immediately cooled to room temperature. For the DSC measurement, the pH of the samples was adjusted to 4, 5, 6 or 7 and heated in the equipment as described below.

## 2.4 Degree of Denaturation

The residual content of native whey proteins after heating was measured by RP-HPLC. The samples were diluted in a sodium acetate/acetic acid buffer at pH 4.6 with a ratio 1:1 to precipitate all denatured and subsequently aggregated proteins (Kehoe, Wang, Morris, & Brodkorb, 2011; Tolkach, Steinle, & Kulozik, 2005). The samples were centrifuged at 14,000×*g* for 30 min at 20°C and the supernatant was filtered through 0.45 µm hydrophilic filters (PES membrane filter type, Sartorius, Göttingen, Germany). A C5 PolymerX RP1 column from Phenomenex (Torrance, California, USA) was used. Buffer A contained 0.1% (v/v) TFA in water and buffer B contained 90% (v/v) ACN and 0.1% (v/v) TFA. The gradient of buffer B was 20% for 3 min, 20 to 40% in 10 min, 40 to 60% in 20 min, 60 to 100% in 2 min, 100% for 5 min, 100 to 20% in 0.5 min. The temperature of the column was maintained at

28°C during the run and the flow rate was 1 mL/min. The absorbance was measured at 280 nm and 214 nm. The whey protein standards were β-lg, α-la and CMP. The injection volume was 20 µl. The peaks were integrated and the ratio $C_t/C_0$ was plotted against the heating time, with $C_t$ the residual amount of native whey proteins at a time point t between 0 and 30 min, and $C_0$ the initial amount of native whey proteins. The rate of denaturation was estimated to be the slope of the tangent line along the first 5 min of heating, during which the amount of native whey proteins decreased the most. The amount of denatured and subsequently aggregated protein after 30 min heating was also reported. A typical chromatogram obtained after mixing whey protein and CMP is shown in Fig. 5.2.

### 2.5 Differential Scanning Calorimetry

For the DSC measurements, 20-30 mg of sample were placed into an aluminium pan and heated in parallel to an empty reference pan. Despite the starting concentration of the samples (2.5%, w/v) being relatively low, the denaturation peak of β-lg could still be identified, while the denaturation peak for α-la could not be identified in this study. The DSC used for this experiment was a DSC Q2000 (TA Instrument, Newcastle, Delaware, USA) equipped with a refrigerator and a computer. The thermograms were analyzed by the software TA Universal Analysis (TA Instrument, New Castle, DE, USA). The temperature of denaturation of β-lg at pH 3 was not tested as measurements using RP-HPLC showed that there was no loss of native β-lg and formation of aggregates after heating for 30 min at 80°C (Fig. 5.4 b).

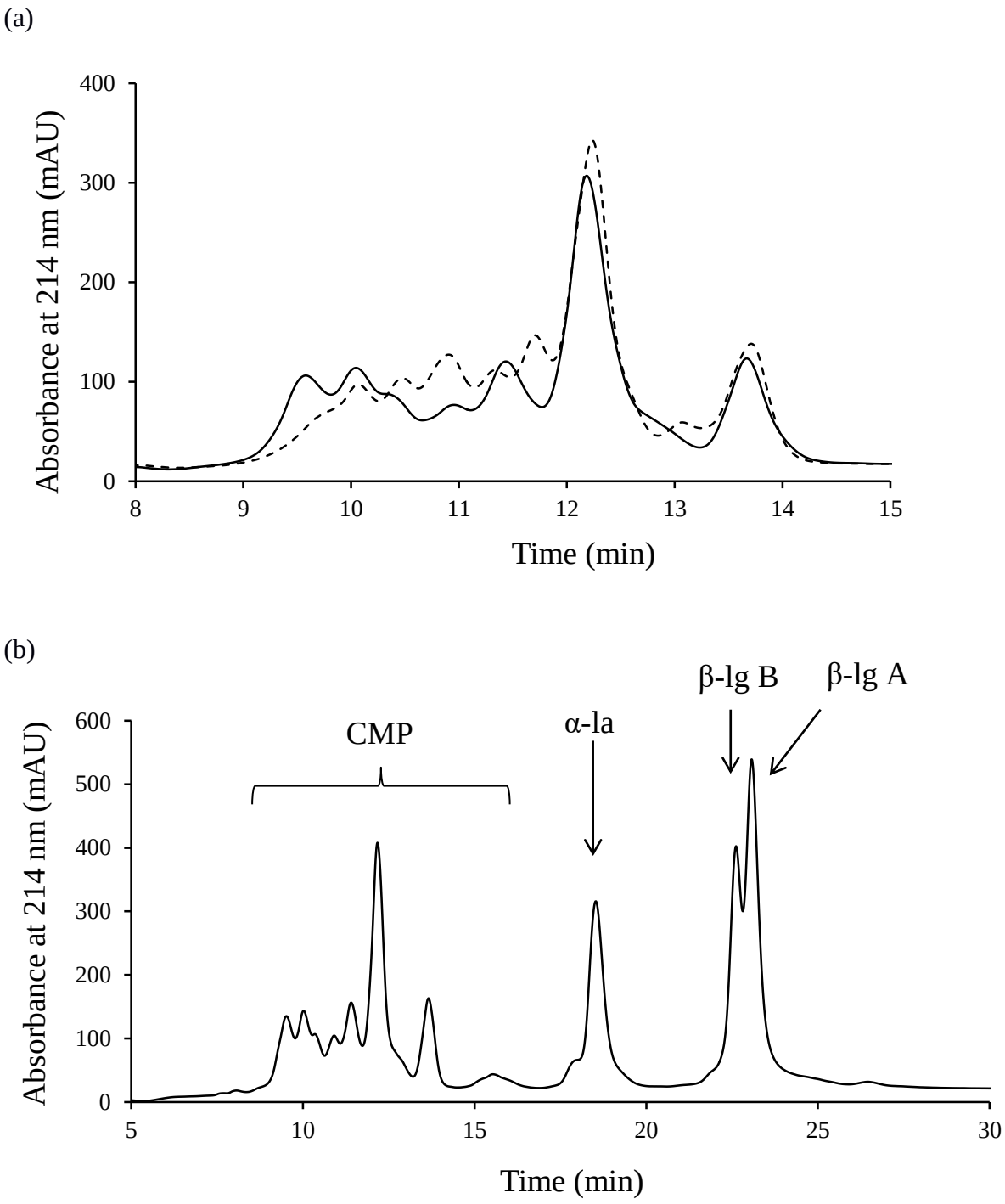



**Fig. 5.2** Chromatogram of (a) solutions of (——) caseinomacropeptide (CMP) and ( – –) desialylated CMP and (b) a mixture of CMP and whey proteins, containing α-lactalbumin (α-la) and β-lactoglobulin A and B (β-lg) resolved using reversed-phase high performance liquid chromatography.

2.6 Attenuated Total Reflection – Fourier Transform Infrared Spectroscopy

Measurements of the FTIR were carried before and after heating using a Bruker Tensor 27 instrument (Billerica, MA, USA) equipped with a thermally controlled attenuated total reflection cell BioATRcell II (Harrick Scientific, New York, NYS, USA). An average of 120 scans by samples was taken. The spectra were analyzed using the OPUS 7.5 software (Bruker) after atmosphere compensation, vector normalization and substraction to non-heated samples or samples containing whey proteins only. At pH 3, the whey proteins exhibited very little denaturation and aggregation after heating (Fig. 5.4 and 5.8 a), therefore this condition was not tested here.

2.7 Turbidity and ξ-potential

The turbidity of the samples was measured in polystyrene micro-cuvettes (Sarstedt, Nümbrecht, Germany) in a standard UV/vis-spectrophotometer (Cary100 scan, Varian, Palo Alto, CA, USA) at 20°C. The turbidity was expressed as the optical density at 600 nm. At pH 3, the whey proteins exhibited very little denaturation and aggregation after heating, therefore this condition was not presented here (Fig 5.4 and 5.8 a).

The ζ-potential of each sample was measured before and after heating for 30 min. The ζ-potential was determined using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). The measurements were carried out at 20°C after an equilibration time of 120 s at room temperature. The refractive index and the viscosity of the dispersant were assumed the same as that of water, i.e., 1.330 and 1.0031 cP, respectively. The attenuation values were between 5 and 11. After heating

at pH 5, all samples exhibited microscopic aggregation during heating and the ζ-potential could not be measured.

### 2.8 Molecular Weight Distribution

The molecular weight distribution of the aggregates was measured by size-exclusion chromatography on a HPLC system (Waters Alliance e2695, Milford, MA, USA) equipped with a UV/visible detector (2489, Waters Alliance) and the analysis software Empower (Waters Alliance). Two columns in series, TSKgel G2000$SW_{XL}$ and TSKgel G3000$SW_{XL}$ (Tosoh Bioscience GmbH, Griesheim, Germany) with a guard column were used for the separation and analysis of the proteins. The dimension of the columns was 7.8 x 300 mm each and the exclusion volume was equivalent to $5\times10^5$ Da. The absorbance was recorded at 280 nm. The buffer was 20 mM sodium phosphate (pH 7.0). The flow rate was 0.5 mL/min and the total duration of each run was 60 min. The coefficient of partition was calculated for the standards (thyroglobulin, aldolase, bovine serum albumin, β-lg and α-la) and the whey protein aggregates using the elution volume of blue dextran ($2\times10^6$ Da) as exclusion volume.

### 2.9 Scanning electron microscopy

High-resolution scanning electron microscopy was used to evaluate the size of the protein aggregates. Protein particle suspensions (20 μL) were diluted to 0.1% protein (w/v) and pipetted onto microscope stubs. After air drying, the microscope stubs were coated with chromium (K550X, Emitech, Ashford, UK). Scanning electron microscopy images were acquired at 2.00 kV using a Zeiss Supra 40P field emission SEM (Carl Zeiss SMT Ltd.,Cambridge, UK). Representative micrographs were taken at 10,000× and 20,000× magnification.

## 2.10 Atomic Force Microscopy

Whey proteins and d-CMP aggregates were imaged by atomic force microscopy (AFM) using an Asylum Research MFP-3DAFM (Asylum Research UK Ltd., Oxford, UK) in AC-Mode, as previously described (Kehoe et al., 2011). All samples were diluted to 0.1% protein (w/v) and deposited onto a freshly cleaved mica surface. The samples were subsequently dried in a desiccator. Images were processed using AFM imaging software Igor 6.12A (Wavemetrics, Portland, OR) and Argyle light (Asylum Research, Goleta, CA) for 3D images.

## 2.11 Statistical Analysis

All measurements were done, at least, in three independent replicates. DSC and FTIR measurements were done in two independent replicates due to the higher protein concentration required for these experiments and the limited amount of sample available after the enzymatic treatment. The FTIR measurements on solutions of CMP or d-CMP were performed only once. The distribution of the rates of denaturation and the turbidity were presented as medians, with quartiles and whiskers representing, respectively, the $25^{th}$ and $75^{th}$ mark and the minimum and maximum values. Percentage of denatured proteins, $\xi$-potential and molecular weight distribution and peak temperature of denaturation were presented as the mean ±SD.

## 3. Results and discussion

### 3.1 Denaturation of β-lg and α-la in the presence of CMP and d-CMP

At temperature greater than 60°C, whey proteins are known to unfold, exposing their hydrophobic sites and making the thiol groups accessible for new intra- and intermolecular interactions. This results in the irreversible aggregation of the whey proteins, which is strongly dependent on the heating conditions. In their native form, β-lg and α-la are soluble at all pH values, including at their pI, 5.1 and 4.2-4.5 respectively (Eigel et al., 1984). The denaturation and aggregation of whey proteins causes their precipitation at pH 4.6 (Okuda & Zoller, 1921). This allowed the measurement of the native proteins during heating, by precipitation of the denatured and subsequently aggregated proteins at pH 4.6, and thus, the estimation of a rate of denaturation in the very early stage of heating. The rate of denaturation of β-lg and α-la, i.e. the estimated rates of denaturation in the first 5 min of heating, are presented in Fig. 5.3. The amounts of denatured α-la and β-lg after 30 min heating are presented in Fig. 5.4. The temperature of denaturation, at which half of the β-lg in the samples has lost their native conformation, was measured by DSC and presented in Table 5.1.

As expected, due to its unordered, flexible and highly stable structure (Smith, Edwards, Palmano, & Creamer, 2002), CMP did not exhibit any denaturation at any pH tested (results not shown). Both β-lg and α-la had a higher rate of denaturation at pH 5 than at all other pH, with over 88% (w/w) of β-lg and 66% (w/w) of α-la denatured after 30 min heating at pH 5.0, regardless of the protein composition and concentration (Fig. 5.3 and 5.4). In contrast, at pH 3.0, there was little denaturation of whey proteins observed after 30 min heating (Fig. 5.4). Stable particles of

partially unfolded whey proteins from pH 2.5 were previously observed (Harwalkar, 1980). However, for experimental conditions used in this study, only native proteins could be measured by RP-HPLC as explained above (Kehoe et al., 2011). Therefore, if any changes in conformation happened to the whey proteins during heating at pH 3 in this study, these modifications had to be reversible to be undetectable by RP-HPLC. Moreover, Verheul, Roefs, and de Kruif (1998) found a decrease of the initial reaction rate and an increase in the temperature of denaturation of β-lg when the protein was heated at pH 3, which supports our findings and suggests that the heating temperature in this study may have been below the temperature of denaturation of whey proteins.

Above pH 4, α-la had generally a lower rate of denaturation and relative amount of denatured material than β-lg (Fig. 5.3 and 5.4). This is in agreement with previous studies reporting the greater sensitivity to denaturation of β-lg compared to α-la (Law & Leaver, 2000). However, at pH 4, and in particular in the presence of CMP or d-CMP, the rate of denaturation (Fig. 5.3) was higher, regardless of the protein composition and concentration of the samples, than that of β-lg. Although the conformation of proteins is more stable around their pI (4.2-4.5 for α-la), non-covalent interactions are promoted, resulting in greater precipitation of α-la. A higher protein content (1%, w/v, whey protein) also favored α-la denaturation, as reported in previous studies (Hillier, Lyster, & Cheeseman, 1979), which could be another reason for the greater rate of denaturation of α-la in the presence of CMP or d-CMP (Fig. 5.3).

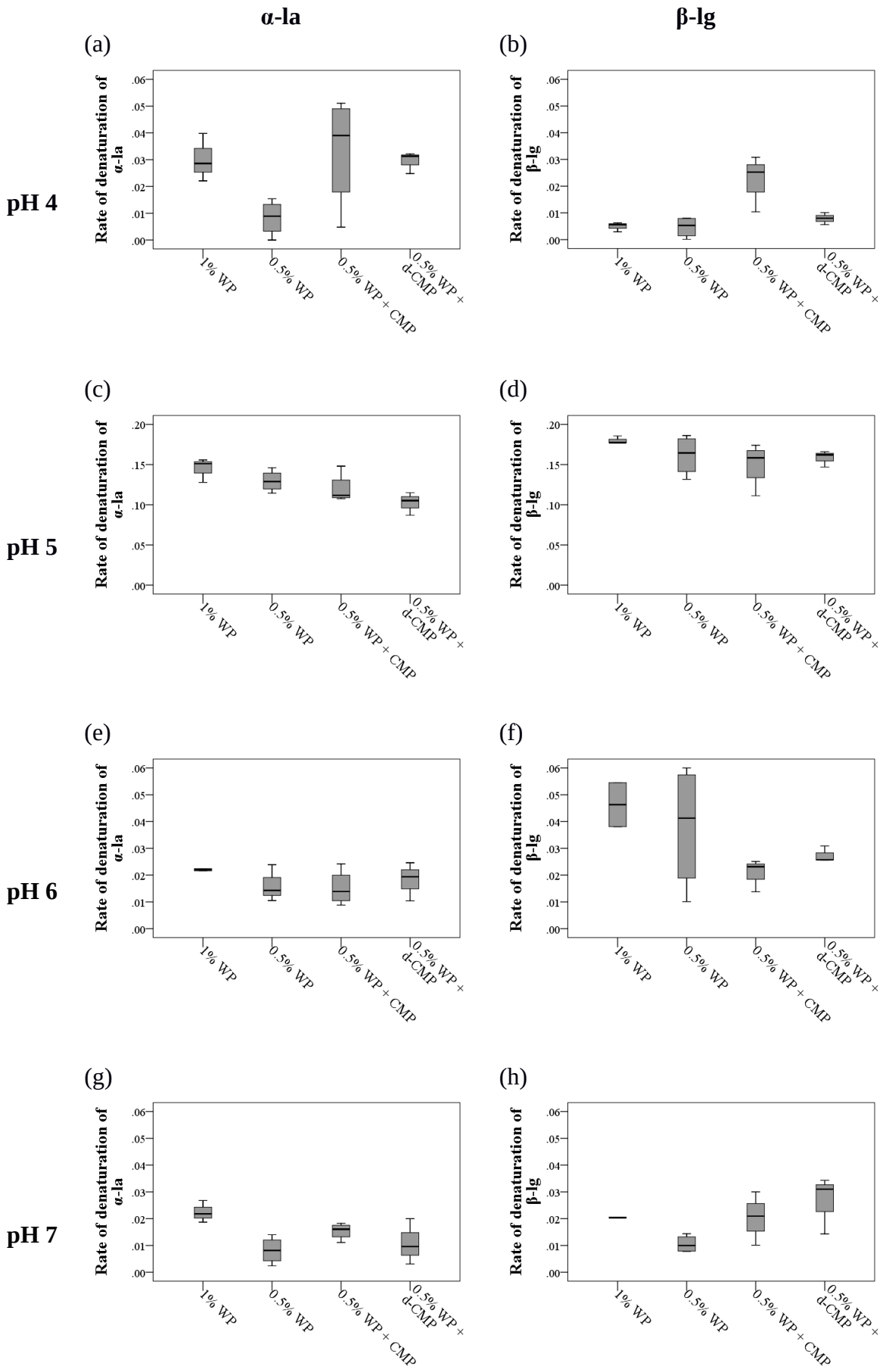
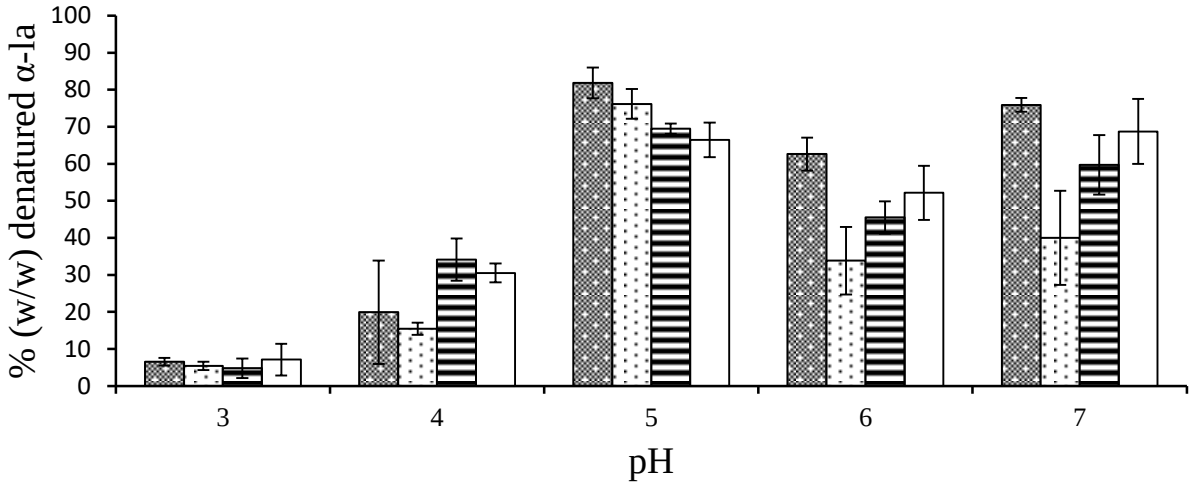

α-la
β-lg
(a)
(b)
pH 4
(c)
(d)
pH 5
(e)
(f)
pH 6
(g)
(h)
pH 7
Rate of denaturation of α-la
Rate of denaturation of β-lg
1% WP
0.5% WP
0.5% WP + CMP
0.5% WP + d-CMP

**Fig. 5.3** Rates of denaturation ($min^{-1}$) of (a, c, e, g) α-lactalbumin (α-la) and (b, d, f, h) β-lactoglobulin (β-lg) in the first 5 min of heating at 80°C at (a, b) pH 4, (c, d) pH 5, (e, f) pH 6 and (g, h) pH 7 of 1% (w/v) whey protein (WP) solution, 0.5% (w/v) WP solution, a mixture of 0.5% (w/v) WP and caseinomacropeptide (CMP) and a mixture of 0.5% (w/v) WP and desialylated CMP (d-CMP). The experimental points were the average of data from at least three independent trials. The results were presented as medians, with quartiles and whiskers representing the $25^{th}$ and $75^{th}$ mark and the minimum and maximum values, respectively. Fig. 5.4 showed that the whey proteins exhibited very little denaturation and aggregation after heating at pH 3 for 30 min at 80°C, therefore the rates of denaturation of α-la and β-lg at pH 3.0 were not presented here.

The presence of CMP or d-CMP resulted in a lower temperature of denaturation (Table 5.1) and a greater amount of denatured β-lg after 30 min heating at pH 4 than those of 0.5% (w/v) whey protein only (Fig. 5.4). However, CMP had a stronger effect on the rate of denaturation of β-lg at pH 4 compared to d-CMP (Fig. 5.3). At pH 4, β-lg is positively charged, however CMP is strongly negatively charged, whereas d-CMP is close to its pI (3.7), thus the attractive electrostatic interactions are stronger with CMP than with d-CMP, leading to faster denaturation of β-lg. A greater decrease of the ξ-potential before heating was observed for the mixture containing 0.5% (w/v) whey proteins with CMP, which could be a result of the attractive interactions between the whey proteins and CMP or an average of their respective ξ-potential at pH 4 (Fig. 5.5 a). Previous authors found that CMP and β-lg interacted at pH 3.5 to form aggregates from few nanometers to 1 μm in diameter before heating, most likely via electrostatic interactions or hydrogen bonding (Martinez, Farías, & Pilosof, 2010).

(a)




(b)

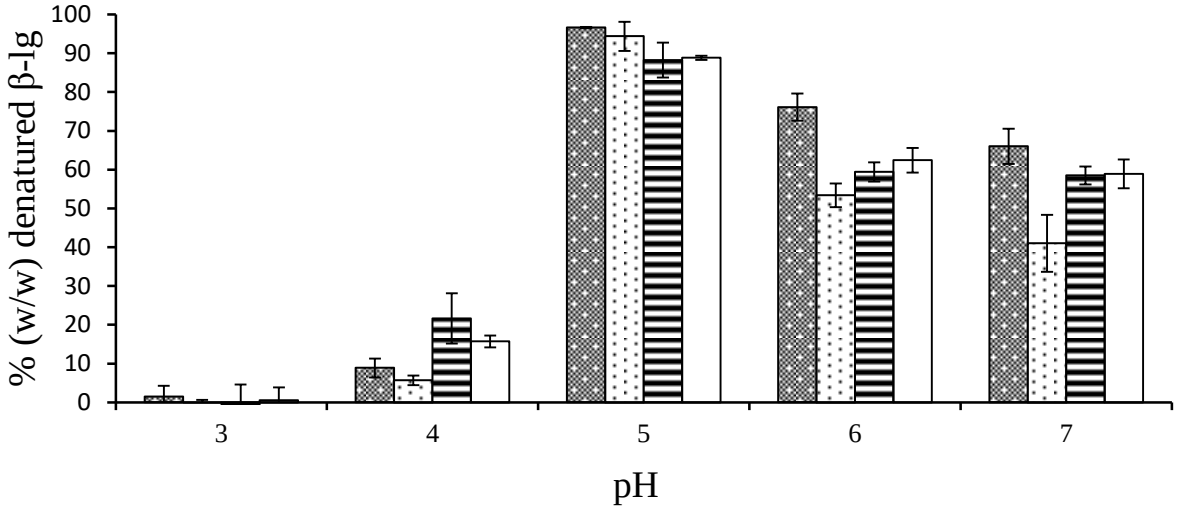



**Fig. 5.4** Percentage of denatured (a) α-lactalbumin (α-la) and (b) β-lactoglobulin (β-lg) after heating (▩) 1% (w/v) whey protein, (▨) 0.5% (w/v) whey protein, (▤) a mixture of 0.5% (w/v) whey protein and caseinomacropeptide (CMP) and (▢) a mixture of 0.5% (w/v) whey protein and desialylated CMP (d-CMP) at 80°C for 30 min after adjustment at pH 3, 4, 5, 6 and 7. The annotation w/w refers to weight of denatured protein per total of the corresponding protein. Experimental points were the average of data from at least 3 independent trials and the error bars correspond to the standard deviations.

**Table 5.1.** Temperature of denaturation of β-lactoglobulin (β-lg) by differential scanning calorimetry (DSC) for samples containing 2.5-5% (w/v) whey protein (WP) and a mixture of 2.5% (w/v) WP and CMP or desialylated CMP (d-CMP). The samples were heated up to 100°C, at pH 4 to 7, and the heating rate was 5°C/min. The temperature of denaturation of β-lg at pH 3 was not tested as previous measurements (Fig. 5.4) showed that β-lg did not denature after heating for 30 min at 80°C. The experimental points were the average of data from two independent trials ±SD.

| | Temperature of denaturation of β-lg (°C) | | | |
|---|---|---|---|---|
| pH | 4 | 5 | 6 | 7 |
| 5% WP | 87.5 ±0.0 | 80.3 ±0.2 | 79.8 ±0.1 | 77.8 ±0.1 |
| 2.5% WP | 88.3 ±0.3 | 80.2 ±0.2 | 80.9 ±0.1 | 73.2 ±0.5 |
| 2.5% WP + CMP | 85.0 ±0.5 | 82.4 ±0.4 | 81.3 ±0.0 | 77.0 ±0.2 |
| 2.5% WP + d-CMP | 86.3 ±0.8 | 83.6 ±0.2 | 82.1 ±0.4 | 75.1 ±1.0 |

Changes in the secondary structure of the unheated proteins upon addition of CMP or d-CMP are presented in Fig. 5.6. At pH 4, a decrease around 1655 $cm^{-1}$, indicated a loss of α-helix and disordered structures in the mixture of whey proteins and CMP or d-CMP, compared to the sample containing whey proteins only (Barth, 2007). Bovine β-lg and α-la have 8% and 26% of α-helix, and 47% and 60% of random coils in their native form, respectively (Deeth & Bansal, 2018). CMP was reported to be mainly disordered with little secondary structure and its glycosylation has very little effect on the secondary structure (Smith et al., 2002). The spectra of amide I bands of solutions of CMP or d-CMP are presented in Fig. 5.7. The peak of absorbance around 1645 $cm^{-1}$ indicates that these peptides contain mainly α-helix and disordered structures. Therefore, the change in secondary structure at pH 4

before heating could be attributed to either the whey proteins or the CMP, or both, and provides evidence for interactions between whey proteins and CMP or d-CMP before heating. Our results showing a higher rate of denaturation of the whey proteins in the presence of CMP (Fig. 5.3) and a greater extent of denaturation in presence of CMP or d-CMP (Fig. 5.4) were in agreement with recent studies that highlighted that the temperature for the onset of denaturation and the temperature of denaturation of β-lg decreased with the ratio of β-lg to CMP at pH 3.5 (Martinez et al., 2010) and the denaturation of β-lg accelerated in the presence of CMP at pH 4.0 (Croguennec et al., 2014).

At pH 5, β-lg was very close to its native pI of 5.2 and its global surface charge was minimal, which promoted non-covalent aggregation. Under these circumstances, the rate of denaturation and the amount of denatured β-lg were higher than those of α-la (Fig. 5.3 and 5.4). The rates of denaturation of β-lg and α-la were lowered at pH 5 and 6 in the presence of CMP or d-CMP (Fig. 5.3), with the exception of α-la in presence of d-CMP and at pH 6. These lower rates of denaturation could be caused by the stabilization of the whey proteins by additional electrostatic repulsion provided by CMP or d-CMP. These results are supported by a higher temperature of denaturation of β-lg at pH greater than 4 in the presence of CMP or d-CMP (Table 5.1). The ξ-potential was closer to zero in 0.5% (w/v) whey protein sample in the presence of d-CMP than in the presence of CMP at pH 5 (Fig. 5.5 a). This could be the average of the surface charges of the peptide and the whey proteins, or the proteins could interact more readily by attractive electrostatic and possibly hydrophobic interactions when the sialic acid NeuAc was removed. At pH 5 and 6, the temperature of denaturation of β-lg in the presence of d-CMP was higher than

that in the presence of CMP (Table 5.1).

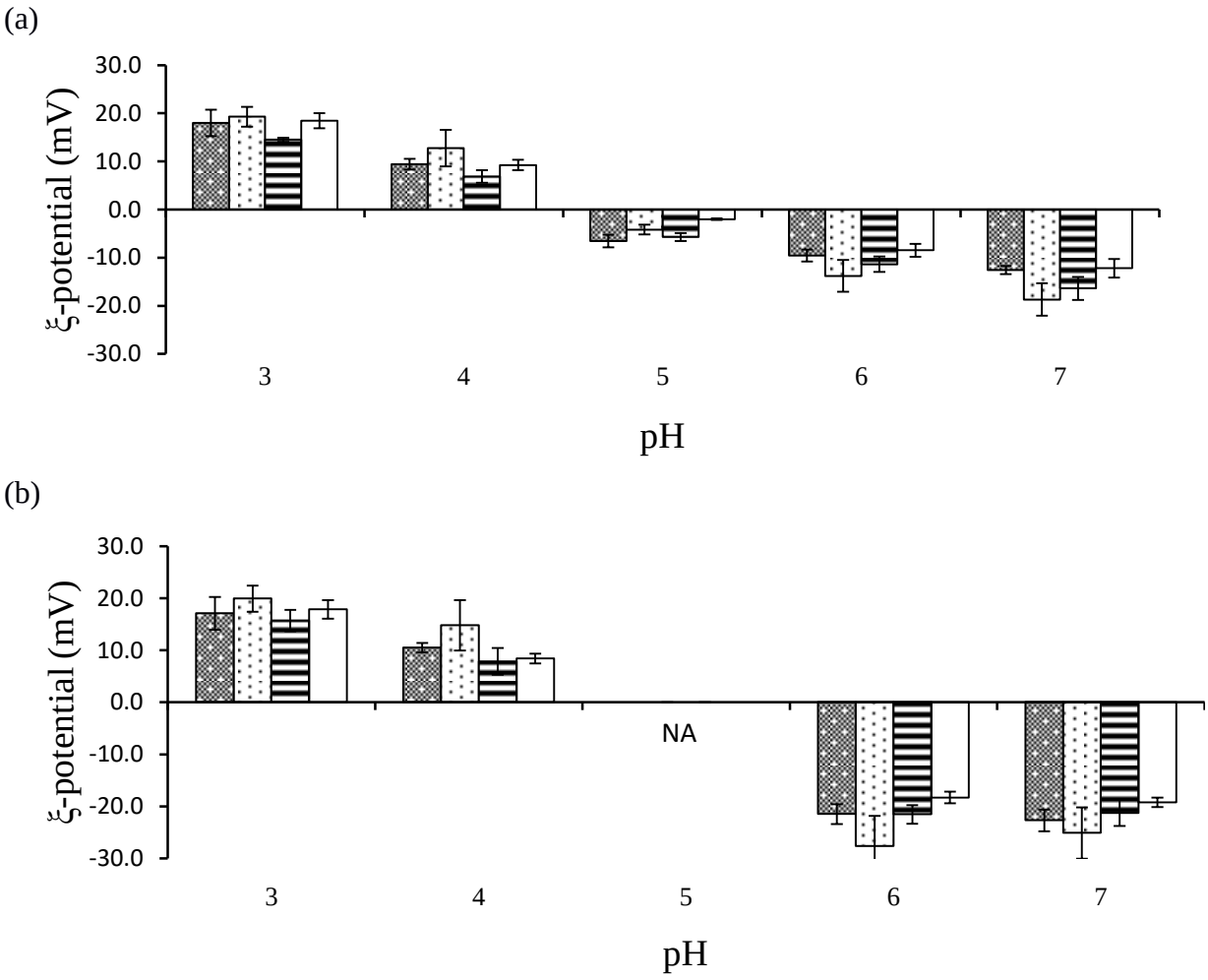



**Fig. 5.5** ξ-potential of (▩) 1% (w/v) whey protein solution, (▨) 0.5% (w/v) whey protein solution, (▤) a mixture of 0.5% (w/v) whey protein and caseinomacropeptide (CMP) and (▢) a mixture of 0.5% (w/v) whey protein and desialylated CMP (d-CMP) (a) before and (b) after heating at 80°C for 30 min at pH 3, 4, 5, 6 and 7. Experimental points were the average of data from at least 3 independent trials and the error bars correspond to the standard deviations. NA indicates that the samples exhibited microscopic aggregation during heating and no measurements were taken.

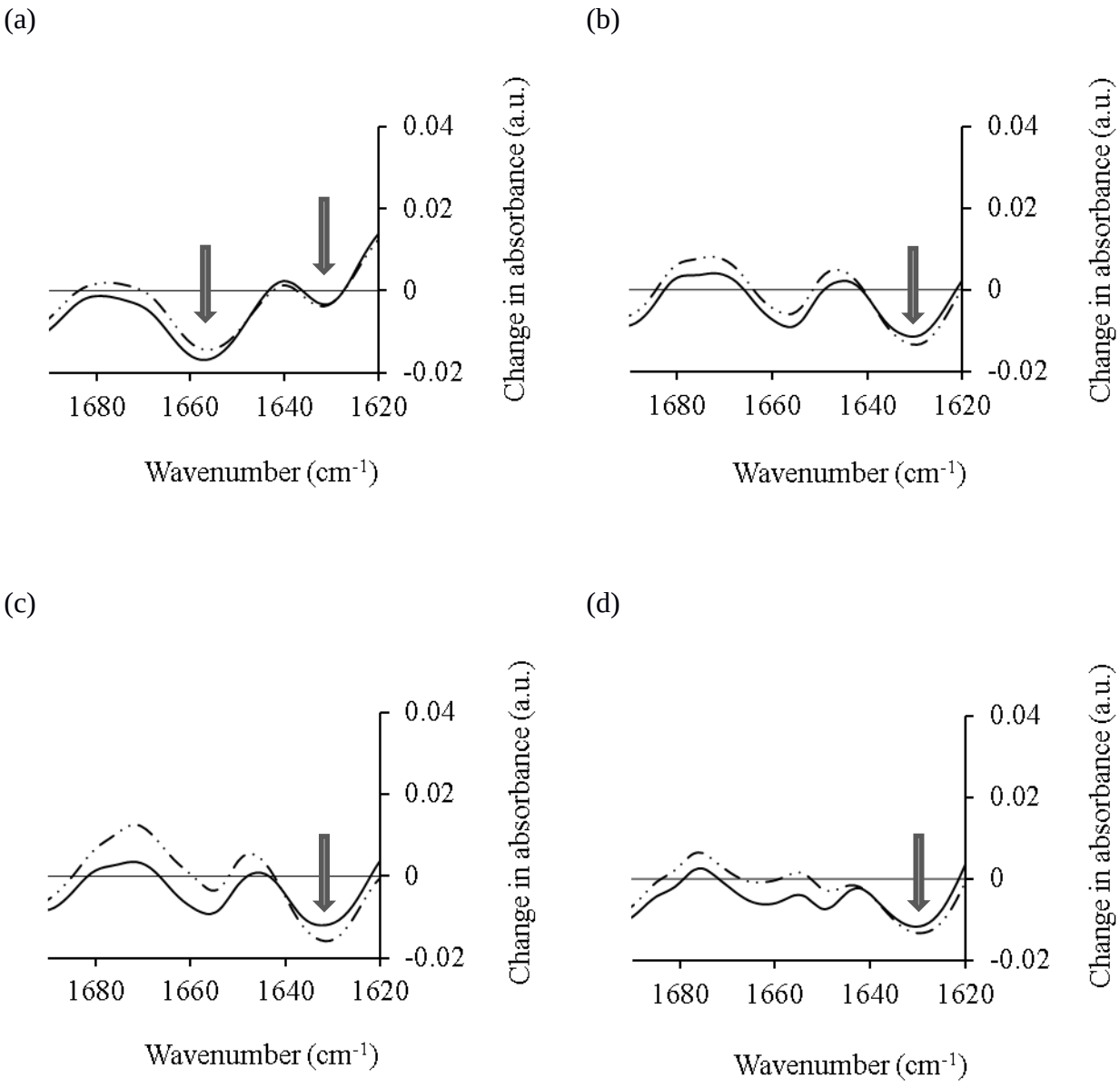



**Fig. 5.6** Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) spectra of amide I bands of non-heated samples at (a) pH 4, (b) 5, (c) 6 and (d) 7 containing (——) 2.5% (w/v) whey protein with caseinomacropeptide (CMP) or (– ·· –) 2.5% (w/v) whey protein with desialylated CMP (d-CMP), with the spectra of the non-heated solution of 2.5% (w/v) whey protein substracted. The arrows highlighted the deviations in the region 1655 $cm^{-1}$ and 1630 $cm^{-1}$ upon addition of CMP or d-CMP from the whey protein solutions, represented by a straight line through the origin. The experimental points were the average of data from two independent trials. The FTIR measurements were carried on samples exhibiting some protein denaturation after heating for 30 min at 80°C. At pH 3, the whey proteins exhibited very little denaturation and aggregation after heating, therefore this condition was not tested here (Fig. 5.4).

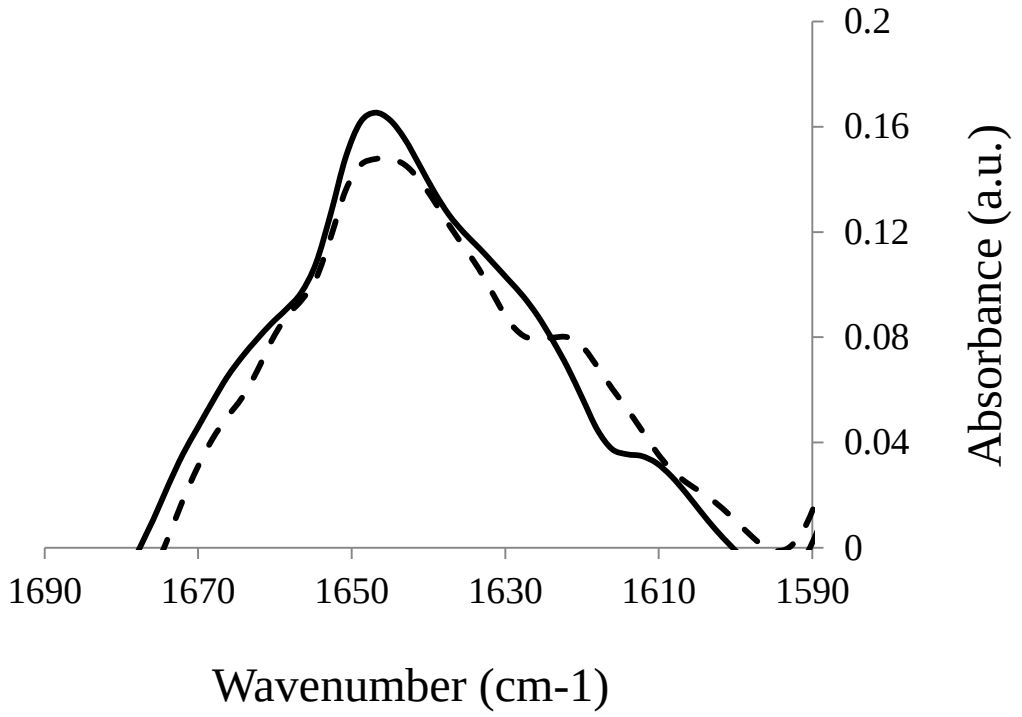



**Fig. 5.7** Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) spectra of amide I bands of (——) 2.5% (weight of powder/v) caseinomacropeptide or (– –) desialylated-CMP.

Haque and Khalifa (1992) found that the hydrophobicity of κ-casein fractions decreased with their content of NeuAc. In addition, the glycosylation of CMP caused steric hindrance, electrostatic repulsion and less hydrophobic interaction, which prevented interaction with oil at the interface of emulsions (Kreuß et al., 2009). Therefore, the highly negatively charged glycosylation of CMP may prevent hydrophobic interactions by increasing the electrostatic repulsions between CMP and whey proteins. The negative charges carried by the amino acids and the negatively charged phosphorylated residues of d-CMP could also have contributed to the stability of the whey proteins on their own. Koudelka, Hoffmann, and Carver (2009) showed that the phosphorylated residues, the amphipathic nature and the flexibility of the caseins, $\alpha_{s1}$- and β-casein, are key features of their chaperone activity on proteins.

At pH 5, the amount of denatured whey proteins after 30 min of heating in the presence of d-CMP was reduced as compared to that of 0.5% (w/v) whey protein only (Fig. 5.4). However, at pH 6, the stabilization of β-lg by d-CMP was only effective in the initial stage of heating (up to 5 min) and the amount of denatured β-lg increased after 30 min heating with d-CMP (Fig 5..3 and 5.4). The conformation of whey proteins is more stable at pH values close to their pI, and less stable at pH greater than 5, due to increased intramolecular repulsion leading to unfolding and increased reactivity of the thiol groups (Hoffmann & van Mil, 1997). This was illustrated by a decrease of the temperatures of denaturation (Table 5.1) for all samples at pH greater than 4. Thus, although the presence of d-CMP initially stabilized the native conformation of β-lg, the interactions between β-lg and d-CMP were affected by the heat induced unfolding of the whey proteins and the formation of new intermolecular disulphide bonds, and promoted the denaturation of β-lg on prolonged heating at pH 6. Croguennec et al. (2014) also observed that the pH affected the heat-induced interactions between CMP and β-lg with a stabilizing effect on the native conformation of β-lg at pH 4.0 and a destabilizing effect at pH 6.7. The mixture of whey proteins and d-CMP had a lower ξ-potential than the sample with 0.5% (w/v) whey protein only at pH 6 (Fig. 5.5 a), which could be due to greater electrostatic interactions or could be the average of the ξ-potential of all proteins in solution. However, interactions between CMP or d-CMP and the whey proteins above pH 5 were evident from a strong decrease in the intramolecular β-sheet signal (Fig. 5.6) around 1630 $cm^{-1}$. This contrasts with the changes in secondary structure obtained at pH 4, where a loss of α-helix or random coil structure was observed (Fig. 5.6), and highlights the effect of pH on the nature of the interaction between the whey proteins and CMP or d-CMP.

At pH 7, CMP, and more particularly d-CMP, promoted the denaturation of β-lg (Fig. 5.3 and 5.4). Thus, from this method based on the precipitation of the aggregates at their pI, there was no evidence for stabilization of the whey proteins, even during the initial stages of heating. In contrast to this, the temperature of denaturation of β-lg, i.e., the temperature measured by DSC at which 50% of β-lg is denatured, was higher in the presence of CMP or d-CMP at pH 7 (Table 5.1). However, the temperature of denaturation of β-lg heated in the presence of d-CMP was close to that of the control containing 0.5% (w/v) whey protein only. Therefore, the sialic acid hindered the denaturation of β-lg at pH 7, and its removal resulted in lower electrostatic interactions, which could have facilitated the hydrophobic interactions between d-CMP and the unfolded β-lg. Other authors have previously demonstrated the role of hydrophobic and electrostatic interactions on stabilizing the native conformation of β-lg in the presence of peptides from hydrolyzed whey proteins (Barbeau, Gauthier, & Pouliot, 1996). The higher charge density of the peptides and the hydrophobic interactions of β-lg with the peptides were assumed to induce a more compact form of β-lg. Above pH 6.8, the protective effect of the negatively-charged peptides was lower, which is in agreement with our results. Other authors emphasized that CMP accelerated the denaturation rate of β-lg and promoted the unfolding of β-lg at pH 6.7 due to an increase in negative charges, which destabilizes the native state of β-lg (Croguennec et al., 2014). The authors concluded that CMP interaction is stronger with the unfolded form of β-lg than with the compact native form. In addition, Martinez, Sanchez, Patino, and Pilosof (2009) reported a decrease in the temperature of denaturation and the onset temperature of denaturation of β-lg measured by DSC in the presence of CMP. However, other authors found that CMP increased the temperature of denaturation when β-lg was

heated with other whey proteins (Svanborg, Johansen, Abrahamsen, Schüller, & Skeie, 2016). The contrasting results between studies could be explained by the differences in the composition of the starting materials.

The presence of CMP or d-CMP did not affect the early stage of denaturation of α-la at pH greater than 5 to the same extent as it affected the early denaturation of β-lg (Fig. 5.3). The differences in chemical composition between the two whey proteins, in particular the absence of a free thiol group on α-la and the ability of β-lg to bind small hydrophobic molecules, could explain the differences observed (Muresan, van der Bent, & de Wolf, 2001).

3.2 Aggregation behaviour of whey proteins in the presence of CMP or d-CMP

The molecular weight distribution of the protein solutions after heating is presented in Fig. 5.8 and the turbidity after heating at 80°C for 30 min is presented in Fig. 5.9 and 5.10. The optical density (OD) at 600 nm was a sensitive indicator of the extent of whey protein aggregation (Li et al., 2019).

As expected, the proteins did not form large aggregates at pH 3 (Fig. 5.8 a). At pH 5, the turbidity increased greatly within 2 min of heating, due to a greater instability of β-lg during heating as electrostatic repulsion was at its minimum around its pI (results not shown); all samples gelled after 30 min of heating at pH 5. At pH 4 and within 5 min of heating, the samples containing CMP developed a higher turbidity than the samples containing only whey proteins (Fig. 5.9 a and 5.10 a). The greater extent of aggregation at this pH could be the result of attractive electrostatic interactions between the whey proteins and CMP. Most of those aggregates had likely been removed after filtration through 0.45 μm filters, prior to size exclusion chromatography, a very small amount of aggregates greater than 500 kDa were

present in the filtrate (Fig. 5.8 b), which is in contradiction with the high turbidity developed in the samples containing CMP (Fig. 5.10 a). At pH 4, the aggregates in the samples containing CMP presented a higher turbidity than the ones in the samples heated at pH greater than 5 (Fig. 5.10 a), although the amount of denatured protein was lower (Fig. 5.4). Croguennec et al. (2014) observed the formation of aggregates with diameter greater than 5 μm, and a phase separation in a solution of β-lg and CMP heated at pH 4.0.

At pH 6, the largest aggregates were formed when 1% (w/v) whey protein was heated on its own (Fig. 5.8 c). This is in agreement with the higher amount of denatured protein (Fig. 5.4) and the high OD of the samples after 30 min heating (Fig. 5.10 b). The OD was much lower in the samples containing CMP or d-CMP (Fig. 5.9 b and 5.10 b). The amount of denatured β-lg in the samples containing CMP or d-CMP at pH 6 was higher than that containing 0.5% (w/v) whey protein only (Fig. 5.4). This confirmed that the effect of CMP and d-CMP on the denaturation of the whey proteins also affected the aggregation behaviour of the whey proteins. In spite of the interactions between β-lg and CMP or d-CMP leading to more denatured β-lg after 30 min of heating, its stabilization within the first 5 min of heating could have had a durable effect on the structure of the aggregates. The aggregates in the sample containing CMP after heating at pH 6, appeared visually smaller on the scanning electron micrographs than those in the samples containing whey protein only or whey proteins and d-CMP (Fig. 5.11). An example of a three-dimension AFM image of the aggregates of whey proteins and d-CMP at pH 6 is also presented in Fig. 5.12. The height across section (Fig. 5.12 b) shows that the aggregates are polydisperse, with sizes ranging from 5 to 20 nm. High resolution

phase and amplitude image (Fig. 5.12 a and c) show that the aggregates consist of individual monomers of proteins, presumably β-lg.

At pH 7, the aggregate size range and the OD were lower than at pH 6 for all samples (Fig. 5.8 and 5.10). The OD of the samples containing 0.5% whey protein, with or without CMP, or d-CMP, were very close (Fig. 5.10 c). However, the relative amount of medium size aggregates was lower and the relative amount of monomers, dimers or trimers smaller than 60 kDa was higher in 0.5% (w/v) whey protein than in the samples containing d-CMP (Fig. 5.8 d). This is in agreement with more denatured β-lg being measured in the case of the samples containing d-CMP (Fig. 5.4), and can be explained by stronger interactions between the whey proteins in the presence of d-CMP. It was previously reported that, at pH 7.0, the heat-induced gelation of β-lg would only occur in the presence of CMP, while a solution of β-lg on its own would not gel, at least under the experimental conditions of this study (Martinez et al., 2010); the authors highlighted that the temperature required for the gelation of the protein systems was lowered in the presence of CMP. In contrast, Croguennec et al. (2014) found smaller particle size of aggregates and a decrease in turbidity after heating β-lg at pH 6.7 in the presence of CMP, although the corresponding activation energy in the aggregation-limited temperature range (above 80°C) decreased in the presence of CMP. Both of these studies hypothesized that the negative charges of β-lg and CMP around pH 7 destabilized the native form of β-lg. The main difference between the studies was the resulting type of protein gel. This could be due to the variation in heating conditions and starting materials. Overall, the differences in molecular weight of the aggregates were minor at pH 7 in the present study.

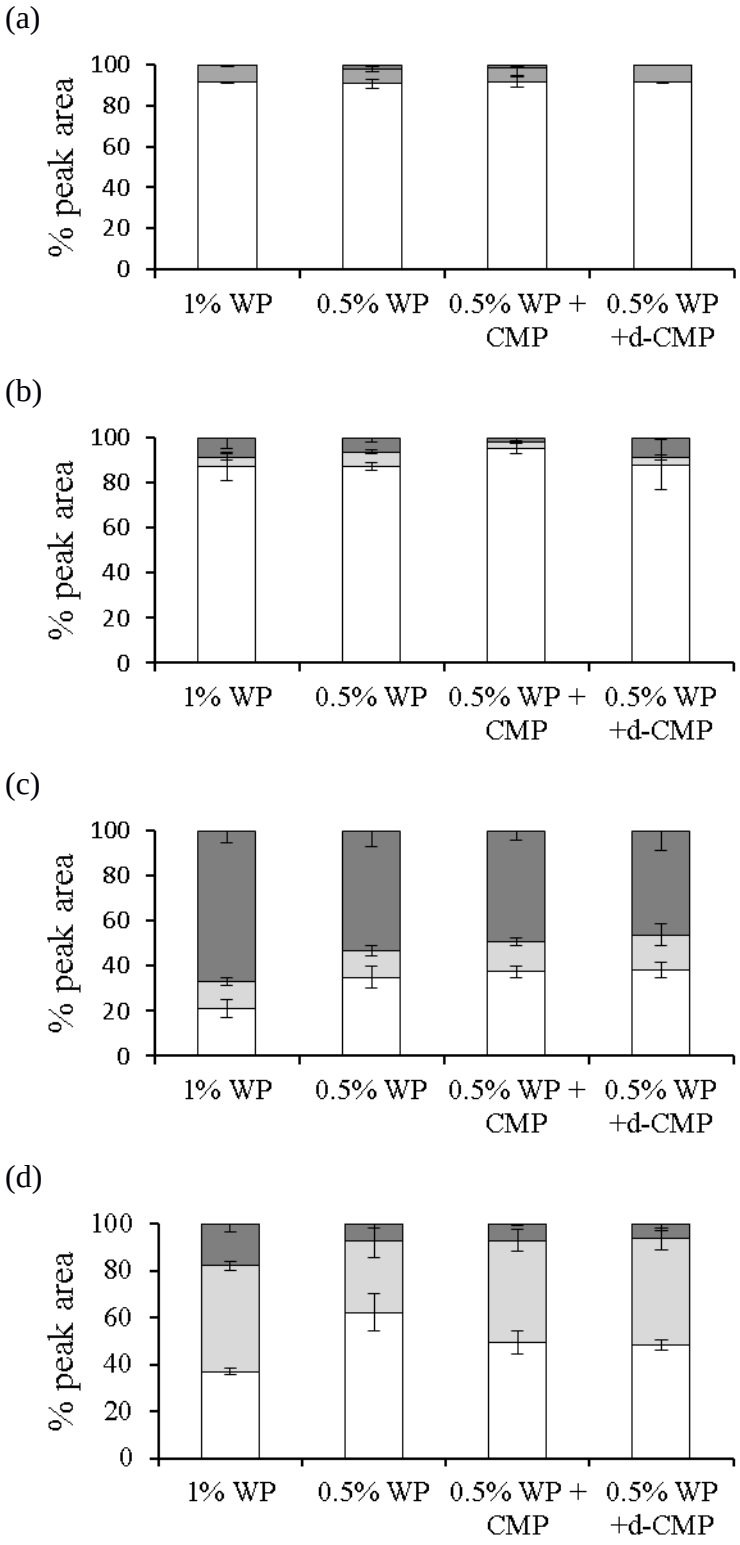



**Fig. 5.8** Molecular weight distribution of soluble whey proteins (WP) by size-exclusion chromatography (SEC-HPLC) in 1% (w/v) WP solution, 0.5% (w/v) WP solution, a mixture of 0.5% (w/v) WP and caseinomacropeptide (CMP) and a mixture of 0.5% (w/v) WP and desialylated CMP (d-CMP) after heating at 80°C for 30 min at (a) pH 3.0, (b) 4.0, (c) 6.0 and (d) 7.0. Distribution represented as: (□) 8 to 60 kDa, (□) 60 to 500 kDa and (□) >500 kDa. The experimental points were the

average of data from at least three independent trials and the error bars correspond to the standard deviation. All samples displayed microscopic aggregates at pH 5 after few minutes of heating at 80°C and most of the aggregates formed during heating were filtered out through 0.45 μm, therefore the results were not presented here.

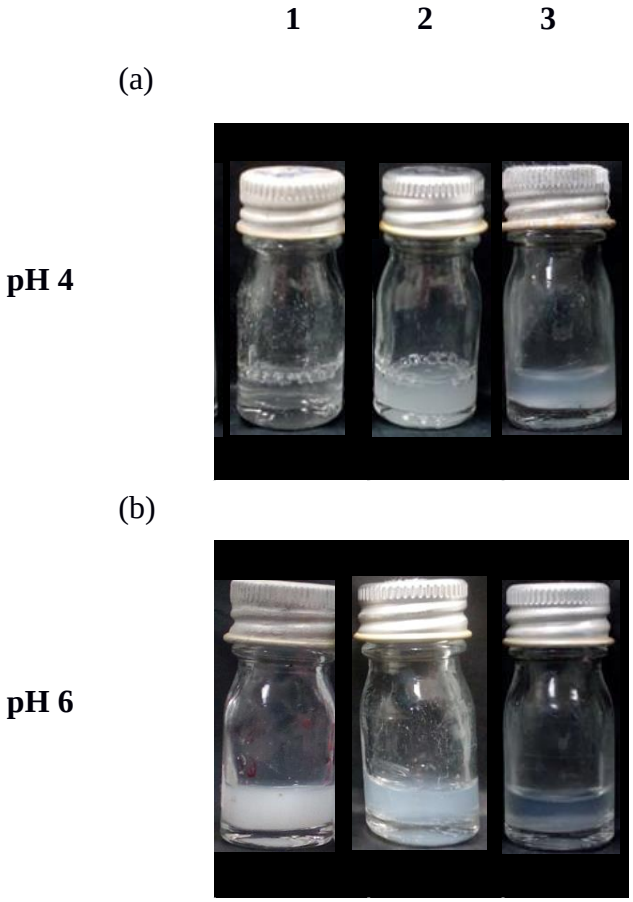
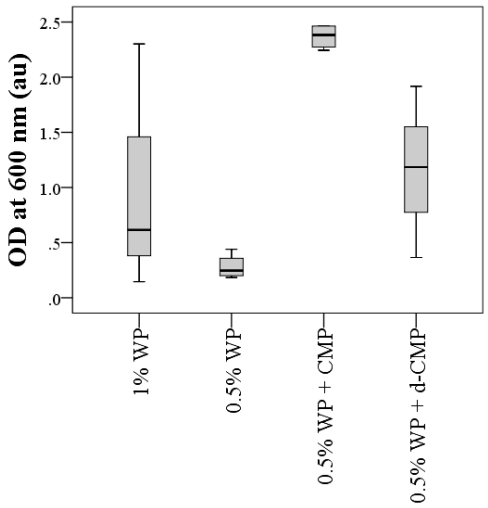



**Fig. 5.9** Photographic images of a solution of (1) 0.5% (w/v) whey protein solution, (2) a mixture of 0.5% (w/v) whey protein and CMP and (3) a mixture of 0.5% (w/v) whey protein and desialylated CMP (d-CMP) after heating at 80°C for 30 min at (a) pH 4 and (b) pH 6.

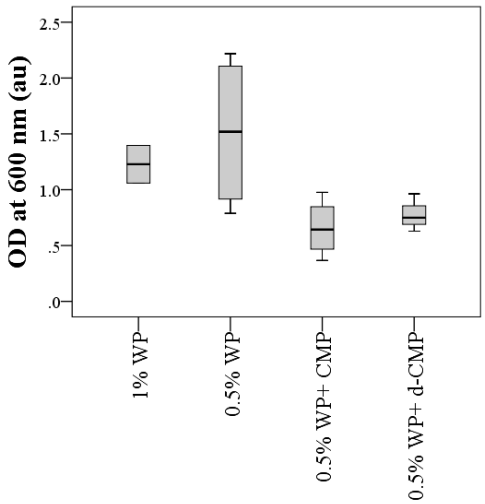
(a)

**pH 4**




(b)

**pH 6**




(c)

**pH 7**

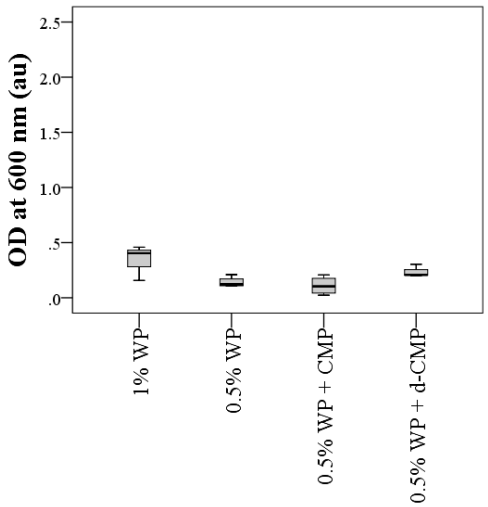


**Fig. 5.10** Turbidity expressed as optical density (OD) at 600 nm of 1% (w/v) whey protein (WP) solution, 0.5% (w/v) WP solution, a mixture of 0.5% (w/v) WP and caseinomacropeptide (CMP) and a mixture of 0.5% (w/v) WP and desialylated CMP (d-CMP) after heating at 80°C for 30 min at (a) pH 4, (b) 6 and (c) 7. The results were presented as medians, with quartiles and whiskers representing, respectively, the $25^{th}$ and $75^{th}$ mark and the minimum and maximum values. The experimental points were the average of data from at least three independent trials. All samples displayed microscopic aggregates at pH 5 after a few minutes of heating, and measurements could not be taken. At pH 3, the whey proteins exhibited very little denaturation and aggregation after heating (Fig. 5.4), therefore the results at this pH condition were not presented here.

After heating, all aggregates formed in solutions at pH greater than 5, with or without CMP, exhibited a more negative zeta potential (Fig. 5.5 b). This is an effect of heating on whey proteins that is well documented (Kehoe & Foegeding, 2014; Ryan et al., 2012). The changes in secondary structure of the proteins after heating give further insight on the effect of CMP or d-CMP on whey protein aggregation as a function of pH (Fig. 5.13). At pH 4, very little change in the β-sheets structure was observed (Fig. 5.13). As explained above, the changes in β-sheets are mainly attributed to β-lg and α-la, containing respectively 45% and 14% β-sheets in their native form (Deeth & Bansal, 2018). Intramolecular β-sheets absorb around 1630 $cm^{-1}$, and the heat-induced formation of intermolecular β-sheets causes a shift in their absorption to 1620 $cm^{-1}$ (Kehoe, Remondetto, Subirade, Morris, & Brodkorb, 2008; Lefèvre & Subirade, 2000). From pH 4 to 6, the presence of CMP or d-CMP prevented the formation of intermolecular β-sheet in heat-induced aggregates. At pH 7, CMP or d-CMP did not prevent the formation of intermolecular β-sheets (Fig. 5.13), which is in agreement with the rate of denaturation and denatured material results (Fig. 5.3 and 5.4).

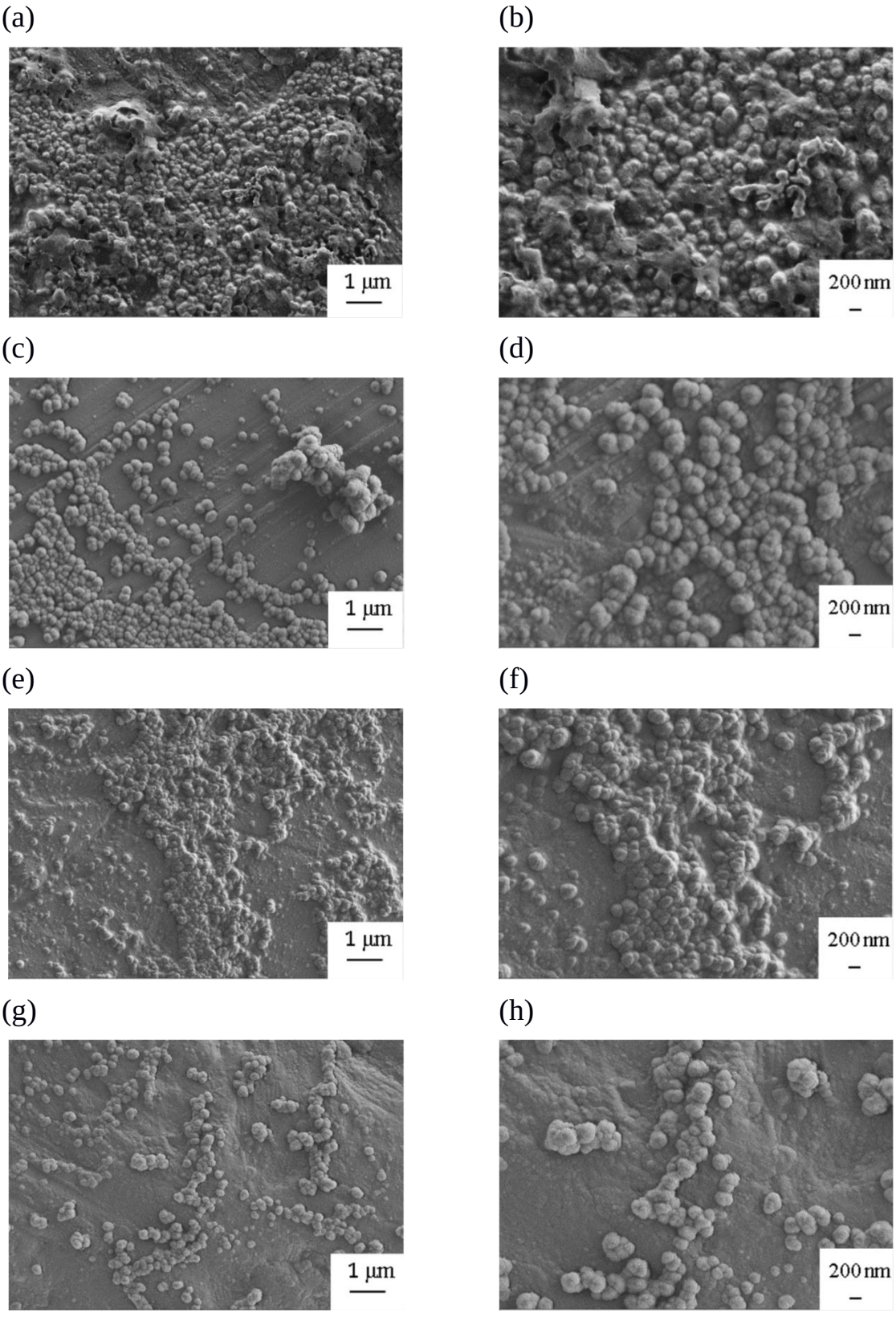
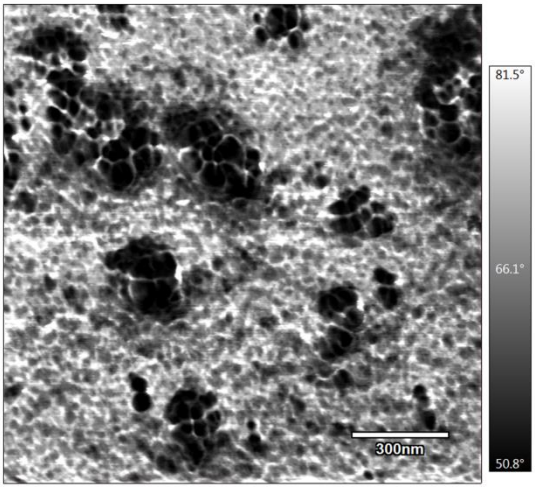
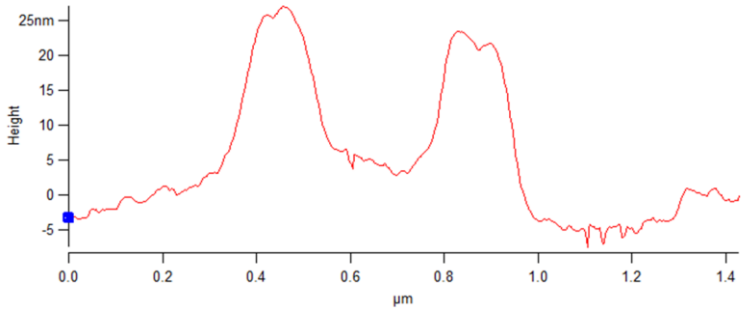



**Fig. 5.11** Scanning electron micrographs from (a,b) 1% (w/v) whey protein solution, (c,d) 0.5% (w/v) whey protein solution, (e,f) a mixture of whey proteins and caseinomacropeptide (CMP) and (g,h) a mixture of whey proteins and desialylated-CMP dried on microscope stubs at 20°C.

(a)




(b)




(c)

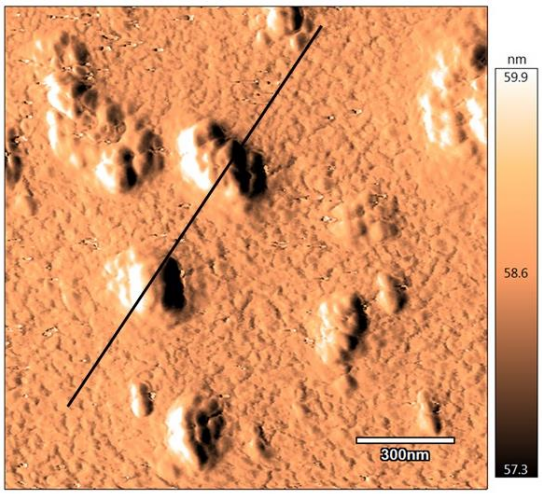



**Fig. 5.12** Atomic force microscopy images showing (a) phase, (b) height across the cross-section marked in the 3D height image and (c) amplitude for a representative sample of 0.5% (w/v) whey protein and desialylated-CMP after heating at 80°C for 30 min at pH 6.

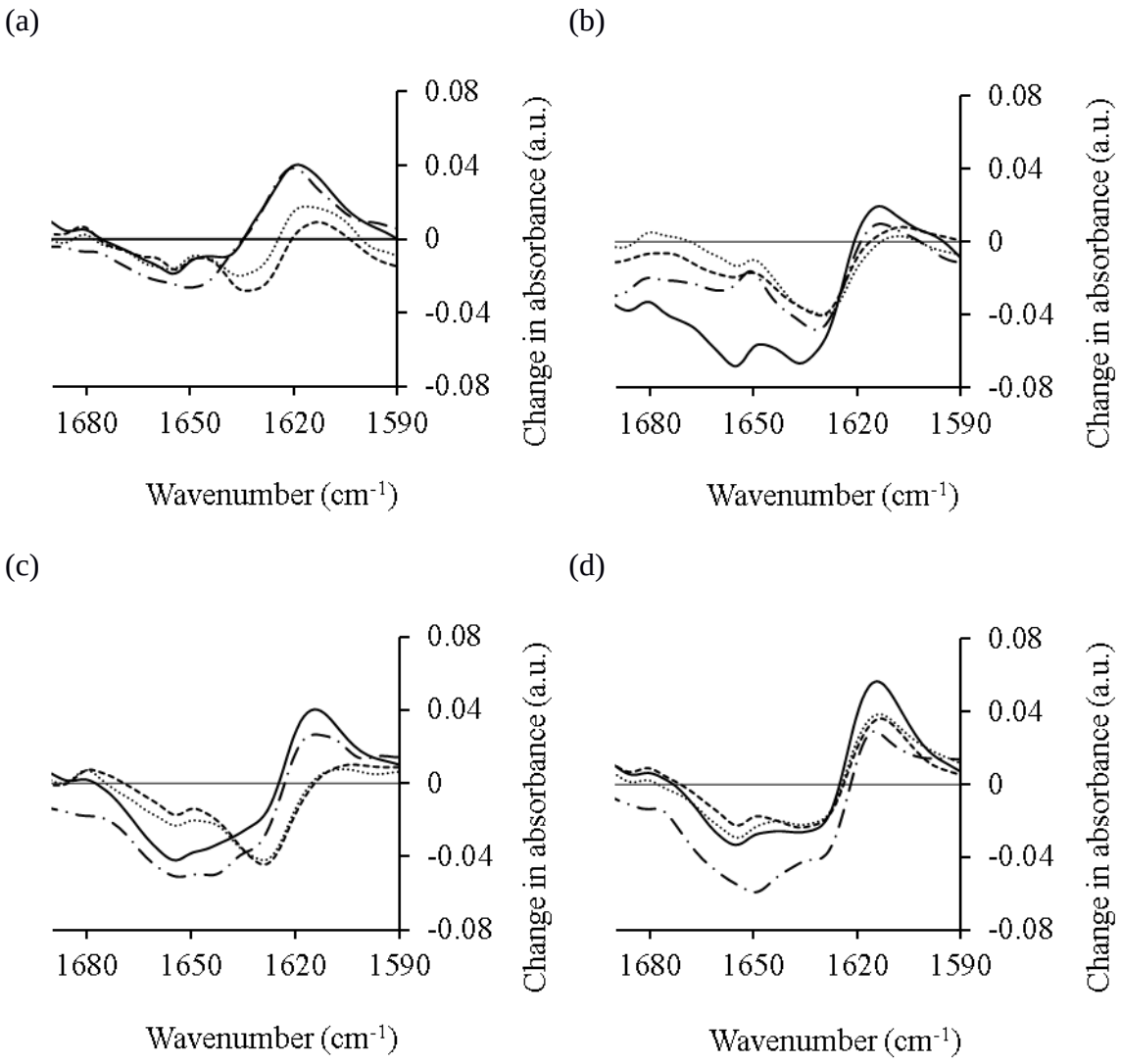



**Fig. 5.13** Attenuated total reflectance - Fourier transform infrared spectroscopy (ATR-FTIR) spectra of amide I bands of heated samples at (a) pH 4, (b) 5, (c) 6, (d) 7 containing (——) 5% (w/v) whey protein, (— ·) 2.5% (w/v) whey protein, (- - -) 2.5% (w/v) whey protein with caseinomacropeptide (CMP) or (.....) 2.5% (w/v) whey protein with desialylated CMP (d-CMP), with the spectra of the corresponding non-heated samples substracted. The straight line through the origin highlighted the deviations in the spectra upon heating. The samples were heated at pH 4, 5, 6 or 7 for 30 min at 80°C and at pH 5 for 5 min. The experimental points were the average of data from two independent trials. The FTIR measurements were carried out on samples exhibiting some protein denaturation after heating for 30 min at 80°C. At pH 3, the whey proteins exhibited very little denaturation and aggregation after heating, therefore this condition was not tested here (Fig. 5.4).

## 4. Conclusion

The desialylation of CMP modified the electrostatic interactions between the peptide and major whey proteins β-lg and α-la during heating as a function of pH. Above the pI of the proteins, the removal of the sialic acid facilitated interactions between CMP and the major whey proteins, particularly β-lg, likely through enhanced hydrophobic interactions. The presence of CMP led to a greater extent of denaturation and aggregation of the whey proteins when they were heated at around neutral pH (i.e., pH favoring their unfolding). In the initial stages of heating and at pH 5-6 (i.e., near their pI), the whey proteins were in a more stable conformation and the interactions with CMP led to an enhanced stability of the whey proteins against denaturation and aggregation. These results contribute to a better understanding of the mechanism of interaction between the major whey proteins and CMP. Advantage should be taken of this knowledge and the innate CMP content of cheese whey to enhance the heat-stability of whey proteins. In particular, any pre-processes resulting in the loss of sialic acid are likely to affect the heat-induced denaturation and aggregation of whey proteins.

**Acknowledgements**

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κ-Casein and caseinomacropeptide (CMP) displayed a chaperone-like activity on whey protein aggregation (Chapters 2, 4 and 5). In particular, the inclusion of κ-casein increased the heat-stability and solubility of the aggregates of whey proteins (Chapter 2). The following chapter aimed to determine the effect of the presence of CMP during and after heat treatment on the solubility of whey protein aggregates.

# Chapter 6

## Effect of caseinomacropeptide and heating rate on the solubility of heat-induced aggregates of whey proteins

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SJG contributed to the experimental design, measured the absorbance of the samples, the CMP content of the samples, and the conformational changes of the proteins by FTIR, analysed the data and wrote the manuscript. YC analysed the composition in whey proteins of the samples. PS provided advice in the experimental design. CF and JT provided the caseinomacropeptide powder. AB, JOM and ALK reviewed the manuscript.

Chapter 6 is a manuscript in preparation for publication.

**Abstract**

Caseinomacropeptide (CMP) has been shown to affect the denaturation and aggregation behaviour of whey proteins. Soluble aggregates of whey proteins only, or mixtures of whey proteins and CMP, at a total protein content of 2.5-5% (w/v), were produced by heat treatment at 90°C for 20 min. The influence of including CMP, in a ratio of whey protein:CMP of 1:0.9 (w/w), and the effect of heating rate (2-25°C/min) on the solubility of the aggregates were determined from pH 5.8 to 7.2. The presence of CMP during heating improved the solubility of the whey protein aggregates in the pH range and heating rate tested. Lowering the heating rate improved the solubility of β-lg in the pH range 6.0 to 7.2. The addition of CMP before heat treatment and the control of the heating rate should be taken into consideration for enhancing the solubility of whey proteins.

# 1. Introduction

Caseinomacropeptide (CMP) is a 64 amino acid peptide derived from the enzymatic cleavage of κ-casein during cheese manufacture. CMP is very hydrophilic and heat-stable due to its disordered structure, its high proportion of negatively-charged amino acids and its post-translational modifications (Siegert, Tolkach, & Kulozik, 2012). CMP can contain up to three phosphorylation sites and up to six glycosylation sites (Holland, 2008). Glycosylation, in particular, strongly reduces the isoelectric point (pI) of CMP, with a pI of 3.2 and 4.2 for the glycosylated and non-glycosylated CMP, respectively (Kreuß, Strixner, & Kulozik, 2009).

In contrast to CMP, the major whey proteins are very labile to heat. At temperatures greater than 60°C, whey proteins unfold and aggregate through covalent and non-covalent interactions (Hoffmann & van Mil, 1997). The heating parameters can be monitored to control the extent of aggregation of the whey proteins, and form continuous gels, precipitates or soluble aggregates (Ryan, Zhong, & Foegeding, 2013). CMP interacts with whey proteins before and during heat treatment through hydrophobic bonds and electrostatic interactions (Croguennec et al., 2014; Martinez, Farías, & Pilosof, 2010).

In Chapter 4, we reported that CMP increased the temperature of denaturation and the temperature of gelation of whey proteins, which affected the structure and the viscoelastic properties of whey protein gels. In Chapter 5, glycosylation and phosphorylation were shown to play an important role in the heat-induced denaturation and aggregation behaviour of the whey proteins. In addition, in Chapter 2, the glycosylation of κ-casein was proposed to enhance the solubility of whey proteins after a first and a second heat-treatment.

To our knowledge, the effect of the presence of CMP during and after heat treatment on the solubility of the whey protein aggregates has not been investigated yet. In the present study, we were able to produce soluble aggregates of whey proteins and CMP by decreasing the ratio of calcium to proteins used in Chapter 4. The aim of this study was to evaluate the effect of the presence of CMP during the formation of the heat-induced aggregates on their solubility at pH 5.8 to 7.2. The heating rate was previously reported in Chapter 4 to greatly influence the aggregation behaviour of the mixtures of CMP and whey proteins at pH 7.2. In the present study, whey proteins were heated at 90°C for 20 min, at pH 7.2, and in the presence of CMP in a ratio of whey protein to CMP 1:0.9. Three heating rates (2, 15 and 25°C/min) were tested and the solubility of the resulting aggregates was tested from pH 5.8 to 7.2.

## 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 was composed of 87.4% CMP (w/w), 5.4% (w/w) moisture, 4.8% (w/w) ash, of which 0.72% (w/w) was calcium. Whey protein isolate (WPI) was purchased from Davisco Bipro® (Davisco Food International, U.S.A.) and contained 91.9% (w/w) of protein (Kjeldahl analysis, nitrogen-to-protein conversation factor: 6.38). 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 5% (w/v) total protein, 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. The mixtures of whey proteins and CMP contained 5.5 mM calcium, due to the higher calcium content of the CMP powder. Therefore, calcium chloride ($CaCl_2.2H_2O$) was added to the samples containing whey protein only to reach 5.5 mM total calcium content. Fig. 6.1 summarizes the steps of rehydration, heating and analysis carried out on the solutions of whey proteins and CMP.

The pH was then adjusted in all samples to 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.

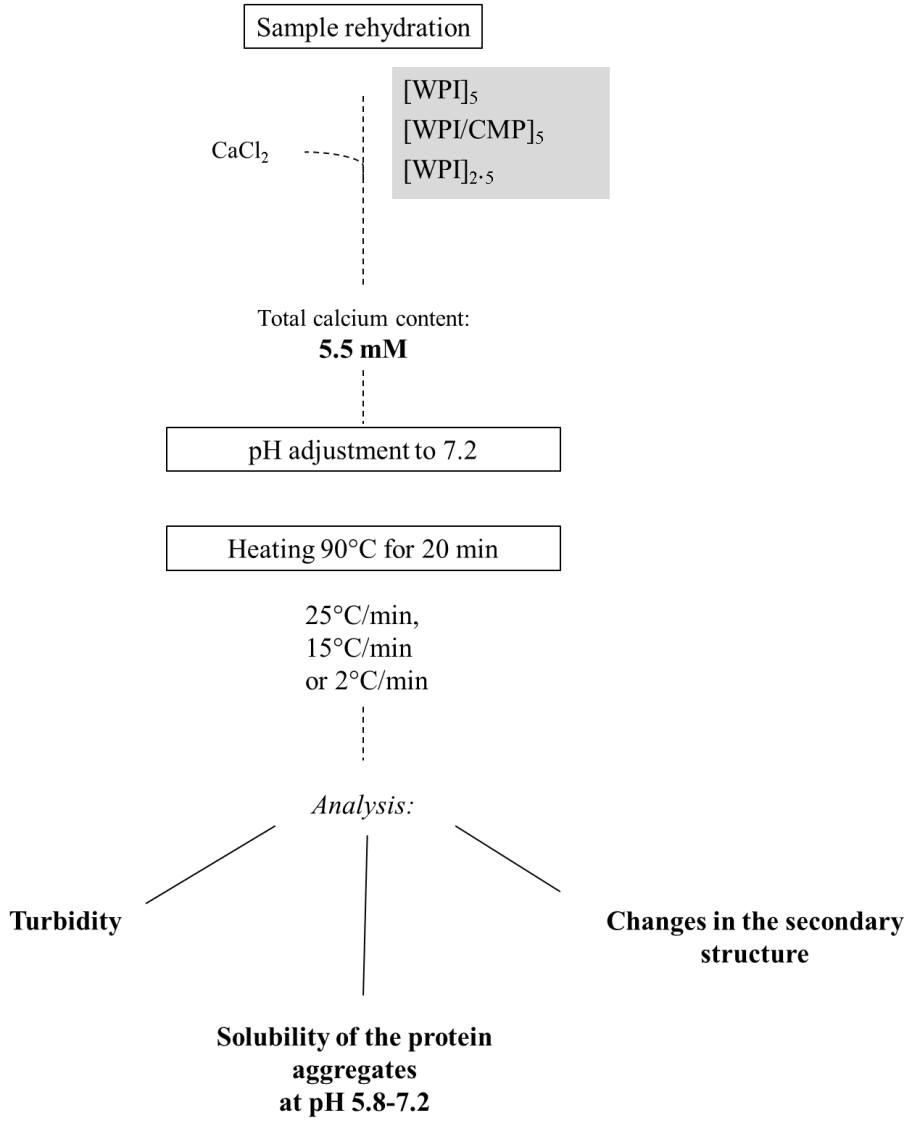



**Fig. 6.1** Flowchart for the preparation and analysis of whey protein and caseinomacropeptide (CMP) mixtures. Solutions of 2.5-5% (w/v) whey protein (abbreviated $[WP]_{2.5}$ or $[WP]_5$, respectively) and mixtures of whey proteins and CMP at 5% (w/v) total protein (abbreviated $[WP/CMP]_5$), were reconstituted in MilliQ® water. The mixtures contained CMP and whey proteins at a whey protein:CMP ratio of 1:0.9 (w/w).

### 2.3 Heating

Aliquots of 5 mL of samples were heated in the starch pasting cell of a rheometer (AR2000ex, TA Instrument, New Castle, Delaware, USA) equipped with the software Rheology Advantage (TA Instrument). This equipment was solely used for ensuring a good control of the heating rate and no rheological measurements were taken. Aluminium foil covered the starch pasting cell to avoid evaporation during heating. The samples were equilibrated for 1 min at 22°C, and then the samples were heated to 90°C with a heating rate of 2, 15 or 25°C/min. After a holding time of 20 min at 90°C, the samples were cooled in water to room temperature.

### 2.4 Turbidity

The turbidity of the heated samples was expressed as the optical density at 600 nm and was measured using polystyrene cuvettes (Sarstedt, Nümbrecht, Germany) and a UV/vis-spectrophotometer (Cary100 scan, Varian, Palo Alto, CA, USA) at 20°C. The measurement was not carried out on the samples containing 2.5% (w/v) whey protein, as these samples gelled during heating.

### 2.5 Solubility of whey proteins

The heated samples were diluted to 1% (w/v) whey protein and the pH was adjusted to 5.8, 6.0, 6.4, 6.8 or 7.2. Volumes of 1 mL of the samples were centrifuged at 20,817×$g$ for 1 h at 22°C. The absorbance at 280 nm of the centrifuged and non-centrifuged samples was measured using a quartz cuvette (Hellma Analytics, Müllheim, Germany) and a UV/vis-spectrophotometer (Cary100 scan, Varian) at 20°C. The samples were diluted in water for their absorbance at 280 nm to be linearly correlated with the whey protein content, i.e. an absorbance between 0.2 and 0.7. The solubility of whey proteins was reported as a percentage ratio of the

absorbance at 280 nm of the supernatant to the absorbance of the non-centrifuged samples. The measurement was not carried out on the samples containing 2.5% (w/v) whey protein, as these samples gelled during heating.

2.6 Composition of the soluble aggregates in whey proteins

The detailed whey protein content of the supernatants obtained after centrifugation for the solubility test and the whey protein content of the non-centrifuged samples were analysed by reversed-phase high-performance liquid chromatography (RP-HPLC) according to a method by Visser, Slangen, and Rollema (1991) and described in detail in Gaspard and Brodkorb (2019). For the analysis of the whey protein content, a Poroshell 300SB-C18 column (Agilent, Santa Clara, CA, USA) and an HPLC system (1200 series, Agilent, Palo Alto, CA, USA) were used. Volumes of 200 µL of the samples were mixed in 3.8 mL of sample buffer containing β-mercaptoethanol and urea in a ratio 1:20 (v/v). The samples were left to stain at room temperature for 1 h and were mixed every 15 min. Then, the samples were filtered through 0.22-µm low-protein-binding hydrophilic filters (Sartorius, Göttingen, Germany). The injection volume was 5 µL and the flow rate was 0.5 mL/min. The temperature during the run was fixed at 35°C. The mobile phase A contained 10% (v/v) acetonitrile and 0.1% (v/v) trifluoroacetic acid in water. The mobile phase B contained 90% (v/v) acetonitrile and 0.1% (v/v) trifluoroacetic acid in water, and the absorbance was read at 280 nm. The gradient of buffer B was 26 to 37% in 10 min, 37 to 45% in 13 min, 45% to 100% in 3 min, 100% for 3.5 min, 100% to 26% in 2 min and 26% for 2.5 min. The protein standards were α-lactalbumin (α-la) and β-lactoglobulin (β-lg) A and B. The peaks were integrated and the solubility of each protein was calculated as a percentage ratio of the protein content in the supernatant to the protein content before centrifugation. The

measurement was not carried out on the samples containing 2.5% (w/v) whey protein, as these samples gelled during heating.

2.7 Caseinomacropeptide content of the soluble aggregates

The CMP content of the supernatants obtained after centrifugation for the solubility test and the CMP content of the non-centrifuged samples were measured using RP-HPLC, according to a method by Beyer and Kessler (1989) and described in detail in Gaspard and Brodkorb (2019), using a C5 PolymerX RP1 column (Phenomenex, Torrance, CA, USA) and a HPLC system (Waters Alliance e2695, Milford, MA, USA) equipped with a UV/Vis detector (2489, Waters Alliance) and the analysis software Empower (Waters Alliance). The samples were diluted in a sodium acetate/acetic acid buffer at pH 4.6 in a ratio 1:4 (v/v), and centrifuged at 14,000×$g$ for 30 min at 20°C, to precipitate all denatured proteins and aggregates. Then, the supernatants were filtered through 0.45 µm hydrophilic and low protein binding filters (Sartorius) and a volume of 20 µL was injected in the column. The absorbance was read at 214 nm and the flow rate was 1 mL/min. The temperature of the column was maintained at 28°C during the run. The mobile phase A contained 0.1% (v/v) trifluoroacetic acid in water and the mobile phase B contained 90% (v/v) acetonitrile and 0.1% (v/v) trifluoroacetic acid. The gradient of buffer B was 20% for 3 min, 20 to 40% in 10 min, 40 to 60% in 20 min, 60 to 100% in 2 min, 100% for 5 min, 100 to 20% in 0.5 min. The standard was a non-heated CMP solution. The peaks were integrated and the solubility of CMP was calculated as a percentage ratio of the CMP content in the supernatant to the CMP content before centrifugation. The measurement was not carried out on the samples containing 2.5% (w/v) whey protein, as these samples gelled during heating.

### 2.8 Attenuated Total Reflection – Fourier Transform Infrared Spectroscopy

Measurements of attenuated total reflection – Fourier transform infrared spectroscopy (ATR-FTIR) were carried out on the heated and non-heated samples using a Bruker Tensor 27 instrument (Bruker Optik GmbH, Ettlingen, Germany) equipped with a thermally controlled attenuated total reflection cell BioATRcell II (Harrick Scientific, adapted for Bruker Optik GmbH) and the software OPUS 7.5 (Bruker). An average of 120 scans per sample was taken and the spectra of the non-heated samples were substracted from the heated samples after atmosphere compensation and vector normalisation. The measurement was not carried out on the samples containing 2.5% (w/v) whey protein, as these samples gelled during heating.

### 2.9 Statistical data analysis

All the experiments were carried out using the same batch of powder. The CMP content was measured in two independent replicates. All other measurements were carried out in three independent replicates. 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

The visual appearance of the samples after heating at 90°C for 20 min with a heating rate from 2 to 25°C/min are presented in Fig. 6.2. The corresponding turbidity values are shown in Table 6.1. After 20 min heating at 90°C, and regardless of the heating rate, the control containing 2.5% (w/v) whey proteins formed a white gel (Fig. 6.2). In contrast, the mixture of whey proteins and CMP and the control containing 5% (w/v) whey protein were still liquid after heating at 90°C for 20 min. However, the mixture of whey proteins and CMP was in general less turbid than that of the control containing 5% (w/v) whey protein (Table 6.1), except at the lowest heating rate (2°C/min). The control containing 5% (w/v) whey protein was less turbid when heated at a rate of 2°C/min than at 15 or 25°C/min. Due to the gelation of the control containing 2.5% (w/v) whey protein, further analyse on this sample were not performed. Therefore, references to a control are only addressed to the sample containing 5% (w/v) whey protein for the rest of the study, unless stated otherwise.

**Table 6.1** Turbidity expressed as optical density (OD) at 600 nm of samples containing 5% whey protein, abbreviated $[WP]_5$, or mixtures of whey proteins and caseinomacropeptide (CMP) at 5% (w/v) total protein, abbreviated $[WP/CMP]_5$, after heating at 90°C for a holding time of 20 min and at a heating rate of 25°C/min, 15°C/min or 2°C/min. The samples containing 2.5% (w/v) whey protein gelled after heating, thus the turbidity could not be measured. The mean was the result of three independent triplicates and presented as mean ±SD. Means with different superscripts are statistically different at $p < 0.05$

| | 25°C/min | 15°C/min | 2°C/min |
|---|---|---|---|
| $[WP]_5$ | 2.39 ±0.57 [a] | 2.28 ±0.49 [a] | 0.62 ± 0.13 [b] |
| $[WP/CMP]_5$ | 0.67 ± 0.07 [b] | 0.62 ± 0.08 [b] | 0.49 ± 0.06 [b] |
| $[WP]_{2.5}$ | Gel | Gel | Gel |

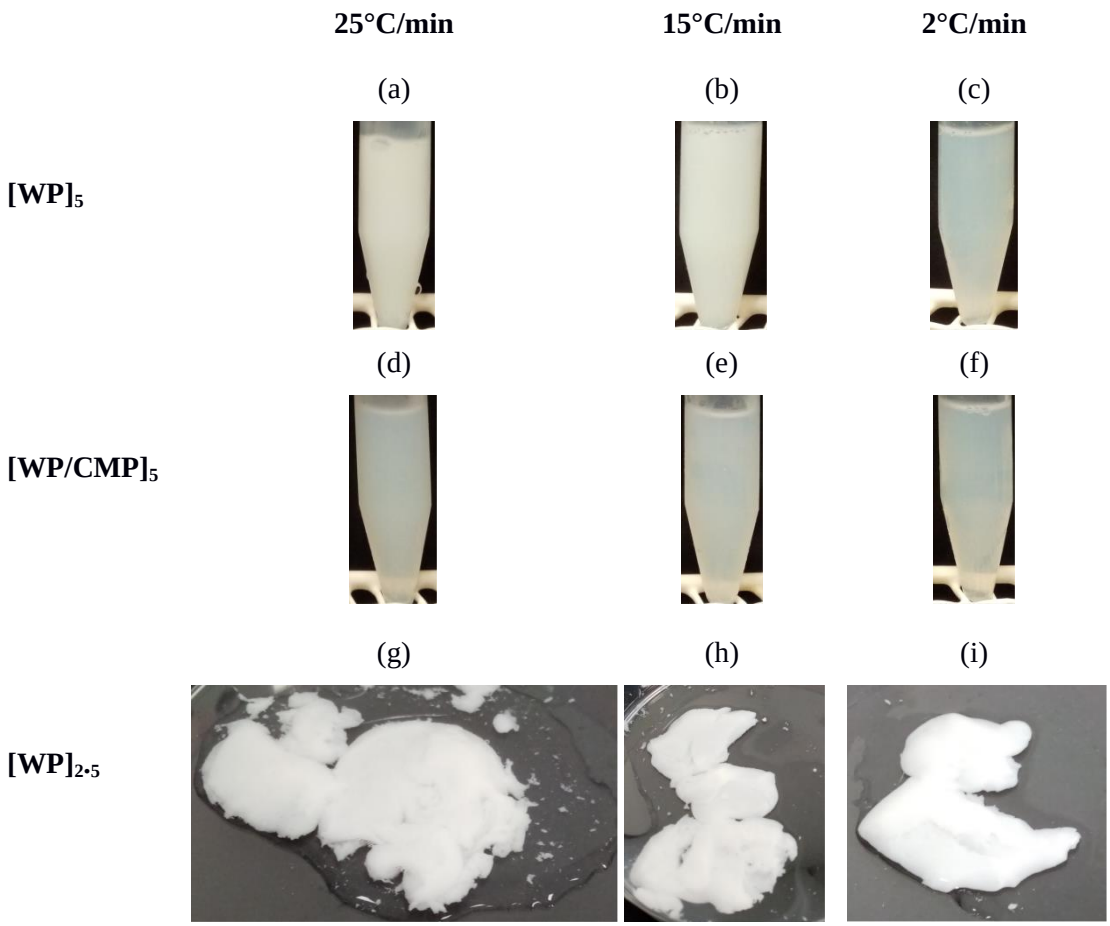



**Fig. 6.2** Photographs of samples containing (a, b, c) 5% whey protein, abbreviated $[WP]_5$, (d, e, f) mixtures of whey proteins and caseinomacropeptide (CMP) at 5% (w/v) total protein, abbreviated $[WP/CMP]_5$, or (g, h, i) 2.5% whey protein, abbreviated $[WP]_{2.5}$, after heating at 90°C for a holding time of 20 min and at a heating rate of (a, d, g) 25°C/min, (b, e, h) 15°C/min or (c, f, i) 2°C/min. The mixtures contained CMP and whey proteins at a whey protein:CMP ratio of 1:0.9 (w/w).

The solubility of the whey proteins at pH 5.8 to 7.2 was measured by their absorbance at 280 nm after centrifugation and the results are presented in Fig. 6.3. The whey proteins heated in the presence of CMP were very soluble (57-87%), regardless of the pH or heating rate, in contrast to the whey proteins in the control at 15 or 25°C/min (7-20%). With the exception of pH 5.8, the whey proteins in the control were more soluble after they were heated at a rate of 2°C/min than those heated at 15 or 25°C/min. Between pH 6.0 and 7.2, the whey proteins in the control heated at a rate of 2°C/min were as soluble as the whey proteins in the mixture of whey proteins and CMP.

The composition of whey proteins and CMP of the soluble fractions after heating at a rate of 2 or 25°C/min and centrifugation at pH 5.8 or 7.2 was analysed by RP-HPLC. All of the CMP was found to be soluble, regardless of the pH or heating rate (results not shown). The details of the composition in whey proteins of the soluble fractions are presented in Fig. 6.4. The analysis showed that 32-47% (w/w) of the whey proteins in the heated samples could not be analysed by RP-HPLC. This may be due to a higher degree of insolubility of these aggregates, which could not be reduced by addition of β-mercaptoethanol and urea.

Regardless of the heating rate and the pH tested, the solubility of the heated α-la and the solubility of β-lg were not statistically different (Fig. 6.4). However, some differences in how pH, CMP and heating rate influenced the solubility of the two proteins were observed. The solubility of α-la was not affected by the heating rate. In general, α-la was as soluble in the mixture of whey proteins and CMP as in the control containing whey protein only. However, the solubility of α-la in the control after heating at a rate of 2°C/min was reduced when the pH decreased from 7.2 to

5.8. This loss of solubility was not observed when the whey proteins were heated in the presence of CMP.

β-Lg A and B displayed the same solubility trend (results not shown); therefore the results are presented as the solubility of the total β-lg. In general, the solubility of β-lg was greater in the presence of CMP, at all pH and heating rates. However, the solubility of β-lg at pH 7.2 in the control increased with a lower heating rate (2°C/min) and was similar to that of β-lg heated in the presence of CMP. The solubility of β-lg at pH 5.8 did not increase with a slower heating rate.

The effect of heating at different rates (2, 15 or 25°C) on the secondary structure of the proteins is presented in Fig. 6.5. In all samples, increases in the region of 1611 $cm^{-1}$ and 1618 $cm^{-1}$ were observed, which correspond to the vibration of side-chain residues and the formation of intermolecular β-sheets during heating, respectively (Lefèvre & Subirade, 2000). In the control, a loss in the region of 1635 $cm^{-1}$ and 1645-1655 was observed, which corresponds to a loss of intramolecular β-sheet and α-helix or random coil with heating. Lesser changes were observed in the mixture as compared to the control in the range of wavelength numbers 1630 to 1700 $cm^{-1}$.

However, CMP was reported to be mainly disordered with little secondary structure (Smith, Edwards, Palmano, & Creamer, 2002); therefore these results should mainly reflect the changes in the secondary structure of the whey proteins, in particular in the region of the intra- and intermolecular β-sheets. Thus, the lesser changes in secondary structure could be due to the lower whey protein content in the mixture. In the mixture of whey protein and CMP, two peaks at 1611 and 1618 $cm^{-1}$ were observed when the mixture was heated at a rate of 2 or 15°C/min, whereas only one peak was formed at 1615 $cm^{-1}$ for the mixture heated at 25°C. The shift from 1618

$cm^{-1}$ to lower wavelength numbers has been observed before and may correspond to the strengthening of hydrogen bond during cooling (Lefèvre & Subirade, 2000).

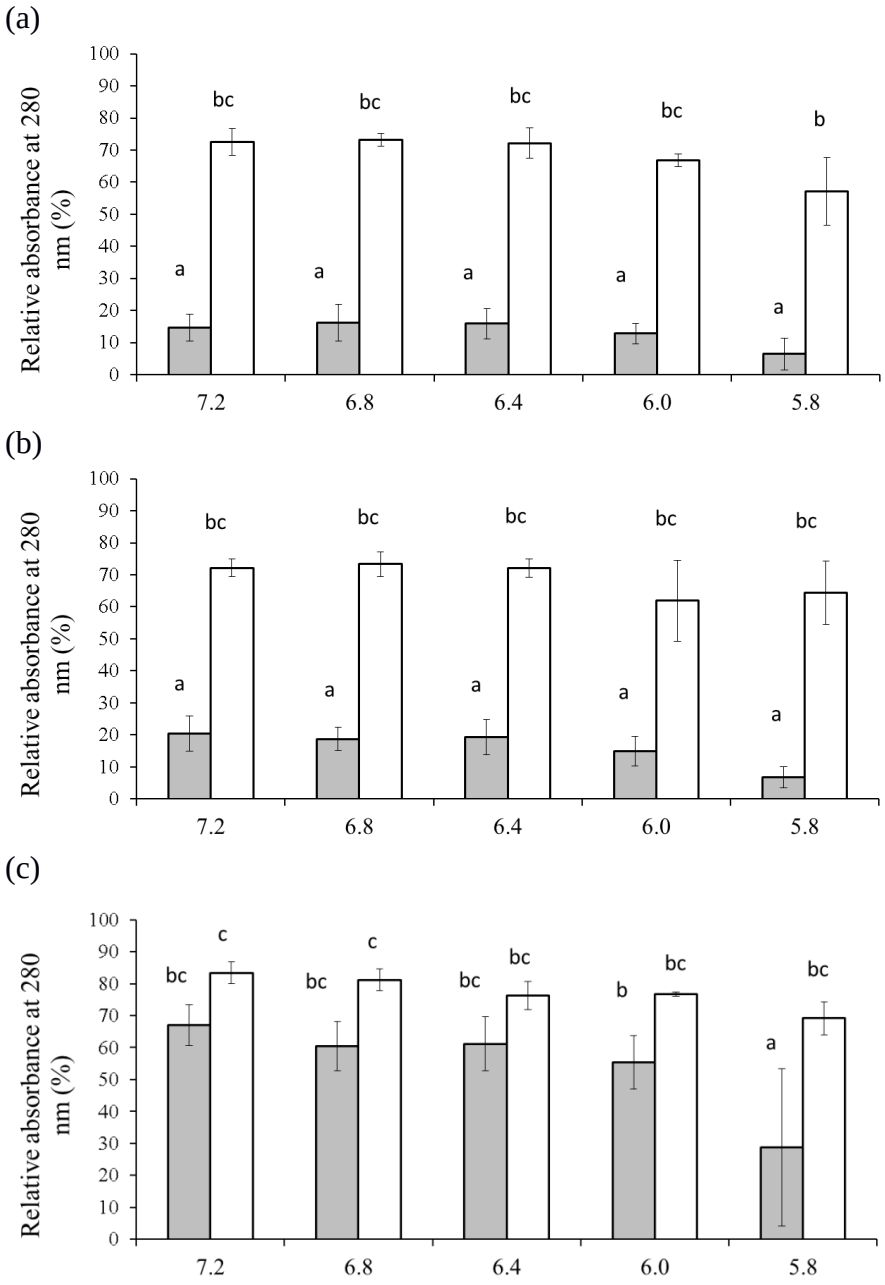



**Fig. 6.3** Solubility of the whey proteins at pH 5.8 to 7.2 in (■) 5% (w/v) whey protein or (□) or mixtures of whey proteins and caseinomacropeptide (CMP) at 5% (w/v) total protein, abbreviated $[WP/CMP]_5$, heated at 90°C for a holding time of 20 min at pH 7.2 and at a rate of (a) 25°C/min, (b) 15°C/min or (c) 2°C/min. The solubility of the whey proteins was expressed as the percentage ratio of the absorbance at 280 nm of the supernatants to the absorbance of the heated sample. The samples were centrifuged at 20,817×g for 1 h at 22°C after dilution to 1% (w/v) whey proteins and pH adjustment from 5.8 to 7.2. The samples containing 2.5% (w/v) whey protein gelled during heating and could not be measured. The measurements were done in three independent triplicates. Means are the result of three independent triplicates and presented as mean ±SD. Means with different superscripts are statistically different at $p < 0.05$.

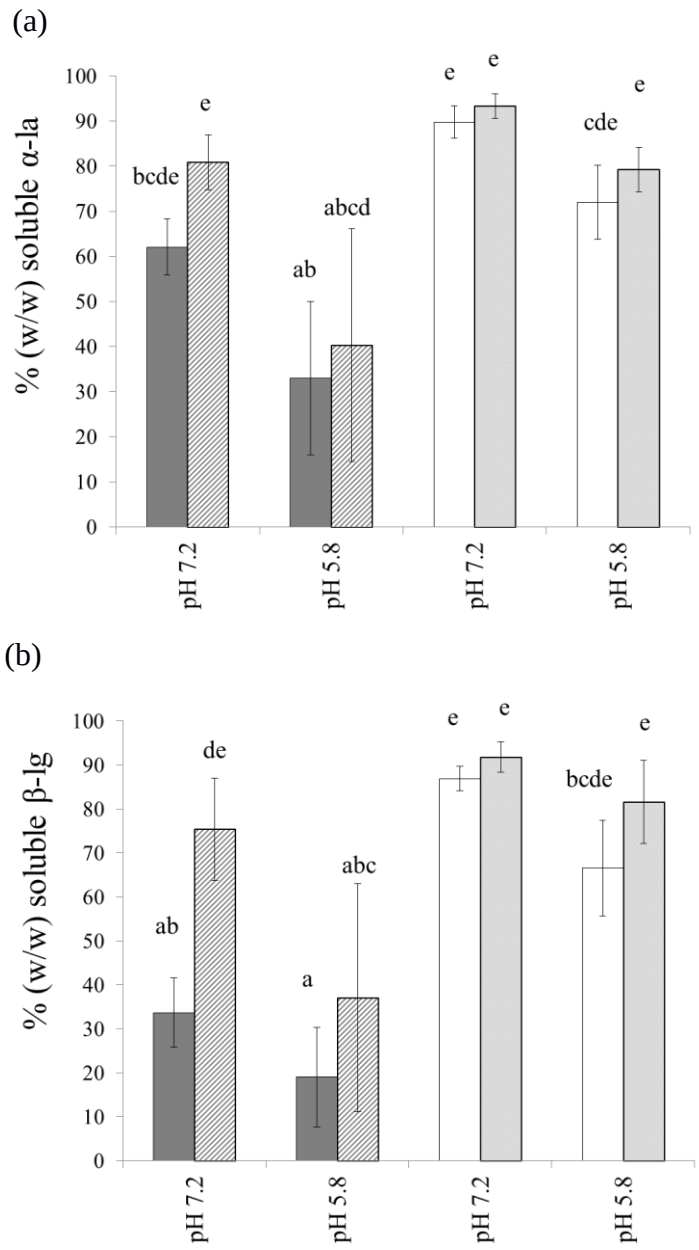
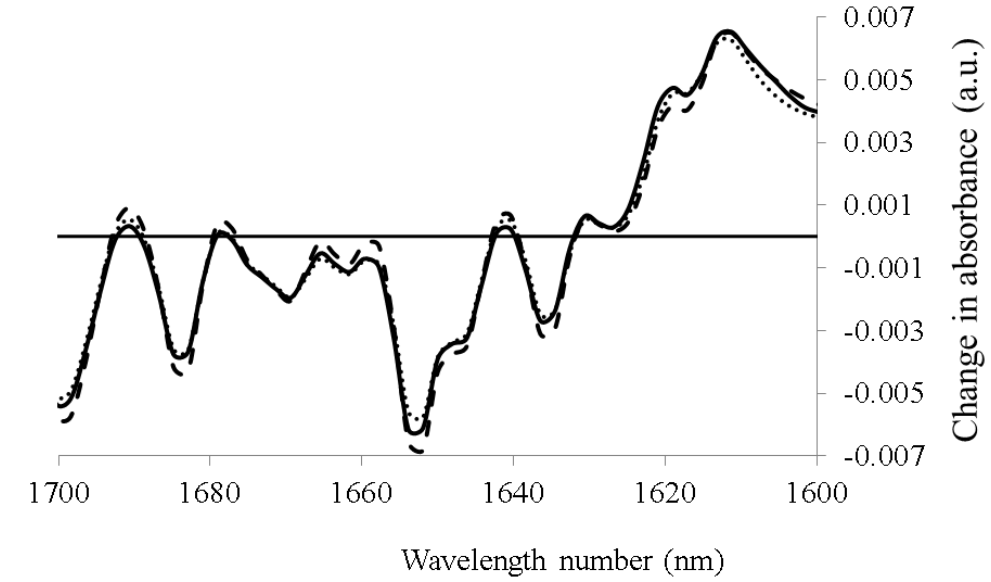



**Fig. 6.4** Solubility of (a) α-lactalbumin (α-la) and (b) β-lactoglobulin (β-lg) involved in the heat-induced whey protein aggregates at pH 7.2 or 5.8. Solutions of (■,▨) 5% (w/v) whey protein or (□,▣) mixtures of whey proteins and caseinomacropeptide (CMP) at 5% (w/v) total protein, abbreviated $[WP/CMP]_5$, were heated at 90°C for 20 min, at a rate of (■,□) 25°C/min or (▨,▣) 2°C/min. The samples were centrifuged at 20,817×g for 1 h at 22°C after dilution to 1% (w/v) whey proteins and pH adjustment to 5.8 or 7.2. The solubility was expressed as the percentage ratio of the protein content in the supernatant to the protein content before centrifugation. The sample containing 2.5% (w/v) whey protein could not be measured as it gelled after heating. The mean was the result of three independent triplicates and presented as mean ±SD. Means with different superscripts are statistically different at $p < 0.05$.

(a)




(b)

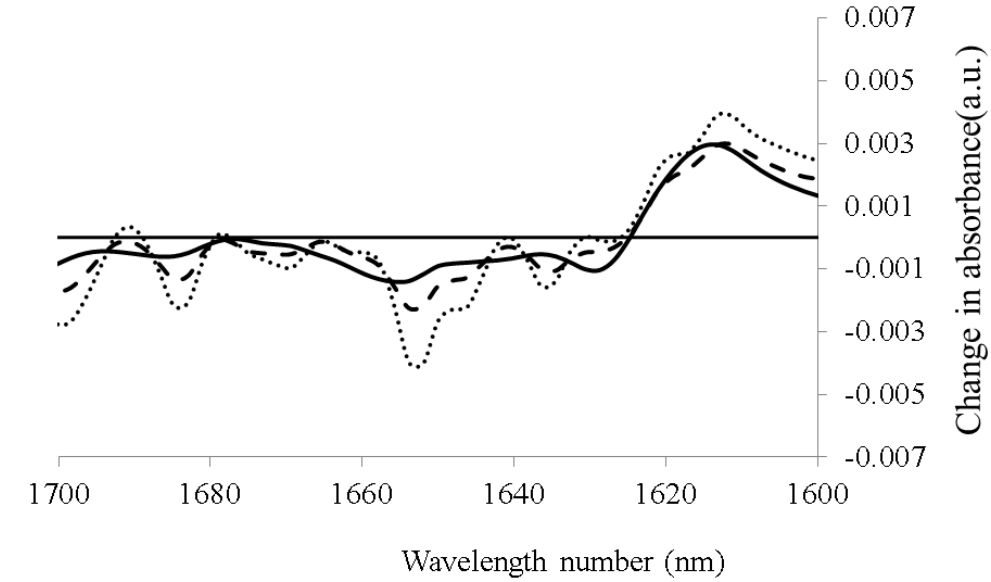



**Fig. 6.5** Attenuated total reflectance - Fourier transform infrared spectroscopy (ATR-FTIR) spectra of amide I bands of (a) 5% (w/v) whey protein or (b) or mixtures of whey proteins and caseinomacropeptide (CMP) at 5% (w/v) total protein, abbreviated $[WP/CMP]_5$, heated at a rate of (——) 25°C.min$^{-1}$, (– –) 15°C.min$^{-1}$ or (·····) 2°C.min$^{-1}$ to 90°C for a holding time of 20 min and at pH 7.2. The spectra of the non-heated samples were substracted. The straight line through the origin highlights the deviations in the spectra upon heating. The measurements were done in three independent triplicates. The sample containing 2.5% (w/v) whey protein was not measured.

## 4. Discussion

The turbidity is an indication of the extent of aggregation of the proteins in solution (Li et al., 2019). The solutions of whey proteins formed a clear liquid in the presence of CMP instead of a white gel or a turbid liquid (Fig. 6.2 and Table 6.1). Therefore, CMP enhanced the heat-stability of whey proteins by limiting the extent of their aggregation. This could be due to increased electrostatic repulsion between proteins during heating provided by the highly negatively-charged CMP. Xianghe, Pan, Peilong, Ismail, and Voorts (2012) reported that the formation of disulphide bonds by whey proteins during heating decreased in the presence of CMP, which supports our hypothesis.

In Chapter 4, heat-induced gels of whey protein had a lower storage modulus when the whey proteins were heated in the presence of CMP at pH 7.2, which could be explained by increased electrostatic repulsions. However, in Chapter 5, a decrease in the turbidity of the whey protein solution in the presence of CMP was only observed at pH 6. In the present study, the samples contained calcium (5.5 mM) which is known to promote the gelation of whey proteins, in particular around neutral pH (O'Kennedy & Mounsey, 2009).

CMP enhanced the solubility of α-la and β-lg in the pH range of 5.8 to 7.2, regardless of the rate at which the whey proteins were heated (Fig. 6.3 and 6.4). In addition, all of the CMP was found in the supernatant with the soluble aggregates, after heating and centrifugation. Croguennec et al. (2014) reported a decrease in the turbidity of whey protein solution heated in the presence of CMP at pH 6.7. The authors assumed that CMP and whey proteins interacted by hydrophobic interactions during heating, while the negative charges carried by CMP protected the whey

proteins against extensive aggregation. Our results support this hypothesis and show that the higher negative charges of the aggregates of whey proteins and CMP prevented their precipitation at lower pH.

Lowering the heating rate from 25 to 2°C/min had a very positive effect on the heat-stability of whey proteins, with a lesser extent of aggregation in the control containing 5% (w/v) whey protein. However, the calcium to protein ratio had a stronger impact on the gelation than the heating rate, as illustrated by the continuous gel formed by a solution containing 2.5% (w/v) protein at all heating rates. Previous authors reported that the heat-induced denaturation of β-lg at pH 3.5 was only irreversible at heating rates below 10°C/min, and assumed that a lower heating rate allowed the disulphide bond exchanges to complete (Relkin, Eynard, & Launay, 1992).

In addition, the mechanism of disulphide exchange of β-lg is known to differ from that of α-la, due to the presence of a free thiol group that contributes to the greater sensitivity of β-lg to heat and the irreversibility of the aggregation (Law & Leaver, 2000). This would explain the greater effect of the heating rate on the solubility of β-lg in contrast to that of α-la. Stading, Langton, and Hermansson (1992) reported that heat-induced gels of β-lg formed at pH 7.5 and at a rate of less than 5°C/min had a lower storage modulus than those formed at higher heating rates. In addition, the strands of β-lg observed by electron microscopy were shorter and thicker. Therefore, the heating rates modified the structure of the aggregates of β-lg around neutral pH.

In the present study, a slower heating rate could have promoted the formation of smaller aggregates of β-lg, which is in agreement with the lower turbidity in the control containing 5% (w/v) whey protein. A smaller size of the aggregates could

influence their propensity to sediment during the solubility test. The greater precipitation of α-la and β-lg at pH 5.8 indicates that the charge of the aggregates of whey proteins was not modified by the heating rate; therefore, a slower heating rate could not provide electrostatic stabilization in the way as CMP did.

The formation of intermolecular β-sheets during heating is a marker of the aggregation of β-lg (Lefèvre & Subirade, 2000). In Chapter 5, we observed that heating the whey proteins with CMP can prevent the formation of intermolecular β-sheet up to pH 6. In the present study, CMP did not prevent the formation of intermolecular β-sheets, as expected at pH 7.2 (Fig. 6.5). However, after heating at the fastest heating rate (25°C/min) and in the presence of CMP, a shift in the wavelength number of the intermolecular β-sheet towards lower number indicated a strengthening of the hydrogen bonds that was not observed at the lower heating rates (2 and 15°C/min). In Chapter 4, we observed a lower degree of frequency dependence and a higher storage modulus of the gels of whey proteins and CMP with an increase in the heating rate from 2 to 25°C/min. A greater strengthening of the hydrogen bonds at 25°C/min could partly explain the results obtained. The shift in the wavelength number of intermolecular β-sheet was only observed when the whey proteins were heated in the presence of CMP. Thus, CMP could induce a change in the conformation of the whey proteins that would promote the formation of hydrogen bonds, or CMP could be involved in the formation of these hydrogen bonds. The very negatively charged N-acetyl neuraminic acid located at the end of the carbohydrate chain of CMP could be involved in the formation of hydrogen bonds through its carboxylic group.

## 5. Conclusion

Heating whey proteins in the presence of CMP or lowering the heating rate limited the extent of aggregation of whey proteins and enhanced the solubility of the resultant aggregates. However, adding CMP increased the solubility of the aggregates on a wider pH range than the lowest heating rate used in this study alone (2°C/min). These results contribute to a better understanding of the effect of CMP on the aggregation behaviour of whey proteins and the properties of whey protein aggregates. The findings of this work are also of relevance to end-users of whey protein ingredients in beverage applications looking for parameters allowing better control of the aggregation and resulting solubility of the whey proteins.

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# General discussion and suggestions for future work

## 1. General discussion and conclusions

Whey proteins are nutritional and functional ingredients used in dairy beverages and products, such as infant formula and sport nutritional drinks. However, whey proteins are prone to denaturation and aggregation during the heat treatment of such products, resulting in the formation of undesirable, visible aggregates, resulting in turbidity and sedimentation, in addition to associated processing challenges such as fouling. Greater heat stability of whey proteins can be achieved by modulating the heating conditions and/or the protein profile of the beverages.

The objectives of this work were to investigate the effect of κ-casein and CMP on the heat-induced denaturation and aggregation of whey proteins and their resulting solubility. In addition, this work aimed at determining the role of glycosylation of CMP on the mechanism of denaturation and aggregation of whey proteins. To achieve this, the chaperone-like activity of κ-casein and CMP on whey proteins was assessed, i.e. their ability to prevent or limit the extent of denaturation or aggregation of whey proteins during heat treatment. Key features of the interactions between whey proteins and κ-casein or CMP were identified at a molecular level; in particular, the effect of glycosylation of CMP on its interactions with α-lactalbumin (α-la) and β-lactoglobulin (β-lg) during heating was examined. Moreover, the effect of CMP addition on denaturation and aggregation of the major whey proteins was assessed in whey protein solutions with protein content of 5%, w/v, to be representative of commercial dairy-protein-based products.

κ-Casein prevented the formation of a continuous gel of whey proteins during heat treatment at 90°C for 1 h (Chapter 2). The inclusion of κ-casein, together with an increasing content of $\alpha_s$- and β-casein in solution, was associated with a decrease in the particle size of the heat-induced aggregates of whey proteins (Chapter 2). Furthermore, an increase in the ratio of caseins to whey proteins enhanced the solubility of whey proteins after a second heat-treatment. The strongly negatively-charged nature of glycosylated κ-casein is thought to provide additional stabilization to the aggregates through greater electrostatic repulsions. Such whey protein particles could possibly be used as heat-stable ingredients in dairy beverages. However, our preliminary attempts to separate the heat-induced aggregates from the casein micelles by membrane filtration after heat treatment was not successful. The size range of the aggregates (30-60 nm) is close to that of the casein micelles (10 to over 300 nm), which could explain the challenges associated with separation (Dalgleish & Corredig, 2012; Ono & Takagi, 1986). In addition, κ-casein had a greater chaperone-like activity towards whey proteins when κ-casein was not already involved in covalently-bond aggregates with the whey proteins. Finally, it should be noted that κ-casein represents only 13% (w/w) of the caseins in milk; therefore, its isolation from the casein micelle may not be a very cost-effective process.

CMP, the peptide derived from the cleavage of κ-casein by chymosin during cheese manufacture, has been recently shown to considerably restrict aggregation of β-lg during heat treatment (Croguennec et al., 2014). However, little research has been done on this topic and the mechanism of interaction between the whey proteins and CMP is still poorly understood. Caseinomacropeptide represents around 20 to 25% (w/w) of proteins in cheese whey (Thomä-Worringer, Sørensen, & López-Fandiño, 2006) hence, it is more readily available than κ-casein isolate. It contains all the

glycosylation sites that are thought to contribute to the chaperone-like activity of κ-casein assessed in Chapter 2. The presence of CMP protected the whey proteins against unfolding and the formation of a continuous gel during heat treatment, with an increase in temperature of denaturation by up to 3°C and the temperature of gelation by up to 7°C (Chapter 4). In addition, aggregates of whey proteins formed in the presence of CMP were more soluble in the pH range 5.8 to 7.2 than whey proteins heated on their own (Chapter 6). The role of glycosylation in the chaperone-like activity of CMP has been assessed in Chapter 5. It was shown that the glycosylation of CMP reduced its stabilizing effect against the denaturation and aggregation of whey proteins at pH < 6.0. Therefore, other types of interactions are also involved in the chaperone-like activity of CMP, such as hydrophobic interactions and electrostatic interactions provided by the negative charges of the phosphorylations and the peptide backbone of CMP. Glycosylations and phosphorylations are very heat-labile structures (Khalifa, Niki, & Arima, 1985; Taylor & Woonton, 2009); therefore, the chaperone-like activity of CMP will depend on the conditions of the manufacture of CMP and the conditions of the subsequent heat treatment of the whey proteins.

The extent of aggregation of whey protein was lower at slower heating rate and the solubility of the resultant heat-induced aggregates of whey proteins increased (Chapter 6). Combined with the presence of CMP, a rate of heating of 2°C/min prevented the formation of a continuous whey protein gel (Chapter 4). Typical rates of heating for the sterilisation of milk by UHT treatment in industry are much higher than those tested in this thesis, with ~60°C/min for direct heating and ~40°C/min for indirect heating (Deeth & Lewis, 2016). However, in-container sterilization involves heating at much slower rates (~5°C/min) and could be a more suitable industrial

thermal processing application for our novel findings (Beliciu, Sauer, & Moraru, 2012; Deeth & Lewis, 2016).

In Chapter 6, the heating rate and the presence of CMP affected the extent of aggregation of whey proteins at pH 7.2 in solutions containing 5.5 mM calcium. However, at higher calcium concentration (9 mM), a lower heating rate and the presence of CMP did not prevent the formation of a continuous gel, unless these two parameters were combined (Chapter 4). In addition, preliminary experiments showed that whey proteins gelled at lower pH (6.4), independently of the calcium concentrations tested in Chapters 4 and 6. This indicates that the ratio of calcium to proteins and the pH of heating were the major factors determining the heat stability of whey protein in the present work.

Havea, Singh, and Creamer (2002) demonstrated that the effect of CMP on the heat-induced aggregation of whey protein in cheese whey was negligible compared with the effect of calcium content. Therefore, the dominant effect of calcium on the denaturation and aggregation of whey proteins could explain why the effect of CMP has not received as much attention until recent years. In addition, the whey protein to CMP ratio in the study of Havea et al. (2002) was much lower (1:0.2) than that used in this thesis (1:0.9). There may be an optimal ratio of whey protein to CMP for the chaperone-like activity of CMP on the heat-induced denaturation and aggregation of whey proteins in the presence of calcium.

In Chapter 5, the rate of denaturation of α-la in the initial stages of heating was less affected by the presence of CMP than that of β-lg. In Chapter 6, the solubility of α-la was not enhanced by a lower heating rate at pH 7.2, in contrast to β-lg. Therefore, a different behaviour in response to heat and resulting solubility of the whey proteins

heated in the presence of CMP or at slower heating rates should be expected in beverages containing high α-la content.

The main result of this thesis is the chaperone-like activity of κ-casein and CMP on the heat-induced denaturation and aggregation of the whey proteins. In view of these results and considering that CMP is more readily available than κ-casein, it could be used to enhance the heat stability of whey proteins and produce clear beverages, or to modify the viscoelastic properties of gels of whey proteins. With the purpose of producing protein-enriched ingredients for dairy-based beverages in the future, a method for the measurement of the internal density and protein content of soluble aggregates of whey proteins and κ-casein by rheometry was also tested, and some options for further improvements were discussed.

## 2. Recommendations for future studies

With a view to improving the heat-stability of whey proteins using CMP, the results presented in this thesis raise new research questions which are listed here, followed by a more detailed elaboration of how these questions could be the basis of developing new areas of research:

1. Is there an optimal ratio of whey protein to CMP for the chaperone-like activity of CMP on the heat-induced denaturation of whey proteins? What are the mechanisms of interactions between the whey proteins and CMP taking place before heating and how do they affect the chaperone-like activity of CMP?
2. How do the heating rate and the chaperone-like activity of CMP affect the structure of the continuous gels and soluble aggregates of whey proteins?

3. Is the chaperone-like activity of CMP, observed on a lab-scale, applicable to industrial conditions?

*1. Optimal ratio of whey protein to CMP and interactions between CMP and whey proteins before heating*

Previous authors studied the effect of the ratio of whey protein to CMP on the heat-induced denaturation and gelation of whey proteins (Croguennec et al., 2014; Martinez, Farías, & Pilosof, 2010). However, the combination of methods used in the present work has provided new information on the unfolding and aggregation of whey proteins in the presence of CMP. Therefore, we recommend quantifying the denaturation of whey proteins in the presence of CMP at different ratios by reversed-phase chromatography. In addition, the temperature of denaturation and the temperature of gelation could be measured by differential scanning calorimetry and oscillatory rheology, respectively. Isothermal titration calorimetry could also provide more information on the interactions between CMP and whey proteins, while the effect of CMP on the conformation of whey proteins as a function of the pH and temperature could be measured by circular dichroism, Fourier-transform infrared spectroscopy and fluorescence spectroscopy (tryptophan, intrinsic and hydrophobicity).

*2. Structure of the aggregates of whey protein produced in presence of CMP*

We observed, using confocal microscopy, that the structure of the aggregates was influenced by CMP during heating (Chapter 4). We also noted a reduction of the extent of aggregation of whey proteins at lower heating rate (Chapter 6). To further study the structure of the aggregates as a function of the heating rate and the

presence of CMP, thorough confocal and electron microscopy analysis could be carried out. In addition, previous authors observed a decrease in the formation of intermolecular disulphide bonds in the presence of CMP (Xianghe, Pan, Peilong, Ismail, & Voorts, 2012), which is very likely to affect the final structures of the aggregates of whey proteins. Therefore, we recommend the measurement of disulphide bond formation as a function of the heating rate and the ratio of whey protein to CMP. Moreover, the internal density of the soluble aggregates of whey protein and CMP could be measured by rheometry or light-scattering, as discussed in Chapter 3.

*3. Industrial application*

The application of our findings on an industrial scale presents some challenges considering the restrictions in pH and mineral content involved by a transition to an industrial scale, and the differences in shear rates and temperatures applicable. In order to use the chaperone-like activity of CMP for the production of clear and stable beverages of whey proteins, model beverages could be tested on pilot-scale equipment. The ratio of whey protein to CMP, the total protein concentration and the mineral content would vary in the model beverages. The turbidity and solubility of the proteins after heating and storage could be used as validation criteria for the use of CMP in the production of heat-stable beverages of whey proteins. The correlation between the turbidity and solubility of the whey proteins and the amount of protein-bound sialic acid and phosphorus after heat treatment should be assessed.

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# List of publications

**Refereed publications:**

Gaspard, S. J., Auty, M. A., 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*.

Gaspard, S. J., & Brodkorb, A. (2019). The Use of High Performance Liquid Chromatography for the Characterization of the Unfolding and Aggregation of Dairy Proteins. In J. J. McManus (Ed.), *Protein Self-Assembly: Methods and Protocols* (pp. 103-115). New York, NY: Springer New York.

Gaspard S. J., Sunds A. V., Larsen L. B., Poulsen N. A., Kelly A. L., O'Mahony, J. A., and Brodkorb A. (2020). Influence of desialylation of caseinomacropeptide on the denaturation and aggregation of whey proteins. *Journal of Dairy Science*.

**Manuscript in preparation:**

Gaspard S. J, Sharma P., Fitzgerald C., Tobin J.T., Kelly A. L., O'Mahony, J. A., and Brodkorb A. (2020). Influence of chaperone-like activity of caseinomacropeptide on the gelation behaviour of whey proteins at pH 6.4 and 7.2.

Gaspard S. J, Chen Y., Sharma P., Fitzgerald C., Tobin J.T., Kelly A. L., O'Mahony, J. A., and Brodkorb A. (2020). Effect of caseinomacropeptide and heating rate on the solubility of heat induced aggregates of whey proteins.

# Conference proceedings

- Poster presentations:

Sophie J. Gaspard, James A. O'Mahony, Alan L. Kelly, Mark Fenelon and André Brodkorb, Characterization of protein aggregates in milk protein concentrate (MPC). *International Symposium on Food Rheology and Structure,* 7th-11th June, 2015, Zurich, Switzerland.

Sophie J. Gaspard, James A. O'Mahony, Alan L. Kelly, Mark Fenelon and André Brodkorb, Thermal stability of heat-induced aggregates of κ-casein/whey proteins isolated from Milk Protein Concentrate (MPC), *44th Annual Food Science and Technology Conference,* 14th December, 2015, Fermoy, Ireland.

Sophie J. Gaspard, Mark A.E. Auty, Alan L. Kelly, James A. O'Mahony, André Brodkorb, Thermal stability of heat-induced aggregates of κ-casein/whey proteins isolated from Milk Protein Concentrate (MPC), *International Dairy Federation conference*, 11th-13th April 2016, Dublin, Ireland.

Sophie J. Gaspard, Alan L. Kelly, James A. O'Mahony, André Brodkorb, Effect of the addition of caseinomacropeptide on the heat stability of whey protein isolate, *Society of Dairy Technology Spring conference*, 10-12th April 2017, Cork, Ireland.

- Oral presentations:

Sophie J. Gaspard, Mark A.E. Auty, Alan L. Kelly, James A. O'Mahony, André Brodkorb, Isolation and characterization of κ-casein/whey protein particles from heated milk protein concentrate, *10th NIZO Dairy conference, Innovations in Dairy Ingredients*, 1st-3rd October, 2017. Papendal, The Netherlands.

Sophie J. Gaspard, Mark A.E. Auty, Alan L. Kelly, James A. O'Mahony, André Brodkorb, Isolation and characterization of κ-casein/whey protein particles from heated milk protein concentrate, *46th Annual Food Science and Technology Conference*, December 6th-7th, 2017, Dublin, Ireland.

## Awards

Prize for best poster presentation. 44th Annual Food Science and Technology Conference. December 14th, 2015. Hosted at Teagasc Food Research Centre, Moorepark, Fermoy, Ireland.

Runner-up prize for oral presentation. 46th Annual Food Science and Technology Conference. December 6th-7th, 2017. Hosted at Teagasc Food Research Centre, Ashtown, Dublin, Ireland.