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Effect of the desialylation of caseinomacropeptide on its impact on the
denaturation and aggregation of whey proteins.

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Keywords: caseinomacropeptide, desialylation, whey protein, denaturation, aggregation

Abstract:
The effect of caseinomacropeptide (CMP) and desialylated-CMP (d-CMP) on the heat-induced denaturation and aggregation of whey proteins was investigated in the pH range 3 and 7 after heating at 80°C for 30 min. The presence of sialic acid favoured 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 stabilising properties of the CMP against the denaturation and aggregation of the whey protein. Above pH 6, the interactions between d-CMP and the whey proteins led to greater extent of denaturation and aggregation. Sialic acid influenced the denaturation and aggregation behaviour of whey proteins in a pH-dependent manner and this should be taken into account for future studies on the heat stability of samples containing the caseinomacropeptide.
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, ingredients need to withstand thermal treatment for safety reasons. Whey proteins are thermolabile and increased temperature alters the conformational stability of the whey proteins by disrupting hydrogen bonding, hydrophobic and electrostatic interactions. Above 60°C, whey proteins unfold, exposing their hydrophobic sites and making the thiol groups accessible for new intra- and intermolecular interactions (Mulvihill & Donovan, 1987). Depending on the conditions of heat treatment, the whey proteins can form soluble aggregates, undesirable large visible gel particles or continuous gel network.

In contrast to this, bovine caseinomacropeptide (CMP), commonly referred as glycomacropeptide (GMP) when glycosylated, is a 64 amino acids peptide resulting from the enzymatic cleavage of κ-casein into two peptides (CMP and paracasein) and is very heat-stable. CMP is present in cheese whey and in minor quantities in bovine milk and colostrum (Furlanetti & Prata, 2003; Martín, Martín-Sosa, García-Pardo, & Hueso, 2001; O'Riordan, Kane, Joshi, & Hickey, 2014). Glycosylated proteins, such as caseinomacropeptide (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 widespread member of the sialic acid family in mammalians and is responsible for many bioactive properties of bovine CMP, for example, the promotion of microbial gut growth, the improvement of learning abilities and the modulation of the immune system response (Brody, 2000; O'Riordan et al., 2014; Thomä-Worringer, Sørensen, & López-Fandiño, 2006). Amongst other functions, sialic acids play roles in stabilisation of cells and proteins. Negatively charged at physiological pH, sialic
acids are, for example, involved in the attraction/repulsion of cells and the transport of positively charged ions (Traving & Schauer, 1999; Varki, 2008). In milk, the negative charges carried by NeuAc and the amphiphilic amino acid sequence of κ-casein stabilise the casein micelle (Cases, Vidal, & Cuq, 2003; Fiat & Jollès, 1989; Hill & Wake, 1969). Glycosylation also stabilises the native conformation of glycoproteins during heat treatment and participate to refolding after denaturation (Tani, Shirai, Nakanishi, Yasumoto, & Kitabatake, 2004; Wang, Eufemi, Turano, & Giartosio, 1996).

CMP represents up to 25% (w/w) of the proteins 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. 1). In mature cow milk, NeuAc is generally located at the end of a glycosylation chain, which apart from NeuAc is containing galactose and N-acetyl galactosamine, organised from monosaccharide to tetrasaccharide (Saito & Itoh, 1992). Thanks to its carboxylic function (Fig. 1), 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 isoelectric point to 5.6, while the glycosylation lowers the pI of κ-casein down to 3.5 (Huppertz, 2013). The pI of CMP was reported to be between 3.2 and 4.2 (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 gels (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. In CMP isolated from raw milk, the
total amount of NeuAc, was reported to be around 4.5% on a weight for weight basis (Taylor
& Woonton, 2009).

These properties can improve the solubility of other proteins when heated in presence of
CMP. It was demonstrated that αs-, β- and κ-casein, also heat-stable proteins with
post-translational modifications, are able to limit 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). 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 7.0. However, it could limit the aggregation of β-lg at pH 6.7 thanks to the negative
charges carried by CMP around the neutral pH. In addition, Doi, Ibuki, and Kanamori (1981)
showed a correlation between degree of glycosylation of κ-casein and improved heat stability
of β-lg.

To the author’s knowledge, the effect of the sialic acid 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 studied yet. The aim of this study is to bring new insights of the effect of
the negatively charged NeuAc on the denaturation and aggregation behaviour 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).
2. Material and Methods

2.1 Materials

All reagents were purchased from Sigma Aldrich unless stated otherwise. Denatured whey proteins were removed from native whey proteins by isoelectric precipitation. Briefly, a solution of 10 (w/v) % WPI (Davisco Bipro®) was rehydrated in Milli-Q® water, heated at 40°C for two hours 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 dialysed against 10 mM sodium phosphate (pH 7.0) for 24 hours with 2 changes of buffer, then for 24 hours in distilled water with two changes of water. The dialysed solutions were freeze-dried. The protein content was measured using reversed-phase chromatography (RP-HPLC) by a modification of a method of Beyer and Kessler (1989).

A solution of CMP (Lacprodan cGMP-20®, Arla Foods Ingredients, 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% and 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 isoelectric point from 3.0 for the untreated CMP to around 3.7 for d-CMP after analysis by 2-dimensional SDS-PAGE as reported elsewhere (Sunds et al., 2019). The CMP and the d-CMP powders were rehydrated, dialysed 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 CMP and d-CMP freeze-dried 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 as a function of the degree of glycosylation (Karman & Van Boekel, 1986). The attempt of quantification of CMP and d-CMP by reversed-phase chromatography (RP-HPLC) by measuring the absorbance at 214 nm shows that both powders contained the peptide in comparable amount. As control samples, solutions of 0.5 and 1% (w/v) WPI were also rehydrated in Milli-Q® water.

2.3 Heating

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 0.7 mL was collected at 2, 5, 10, 15, 20 and 30 min for analysis. The aliquots were immediately cooled down to room temperature.

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 of 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 read 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 drastically. 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. 2.

2.5 Differential scanning calorimetry (DSC)

For calorimetric measurements, 20-30 mg of sample containing 2.5-5% whey protein were placed into an aluminium pan and heated in parallel to an empty reference pan. Despite the starting concentration of the samples (2.5%) being relatively low, the denaturation peak of β-lg could still be identified. The peak of denaturation of 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 analysed 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. 4).

2.6 Fourier transform infrared spectroscopy – Attenuated total reflection (FTIR-ATR)

Volume of 200 μl mixtures of WPI, with or without CMP or-d-CMP reconstituted in the same way as described in section 2.2, were heated in a water bath at 80°C for 30 min at pH 4, 6 or 7. The samples were heated for 5 min only at pH 5 to avoid the gelling of the samples.
Measurements 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 analysed by the OPUS 7.5 software (Bruker) after atmosphere compensation, vector normalization and substraction to non-heated samples or samples containing WPI only. At pH 3, the whey proteins exhibited very little denaturation and aggregation after heating (Fig. 4 and 9), therefore this condition was not tested here.

2.7 Turbidity and ζ-potential

The turbidity of the samples was measured in polystyrene micro-cuvettes in a standard UV/vis-spectrophotometer 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 4 and 9).

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 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 7 and 9. 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 an HPLC (High Performance Liquid Chromatography) 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 G2000SW\textsubscript{XL} and TSKgel G3000SW\textsubscript{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 and the exclusion volume was equivalent to 5×10^5 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 the run was 60 min. The coefficient of partition was calculated for the standards and the whey protein aggregates using the elution volume of blue dextran (2×10^6 Da) as exclusion volume.

2.9 Atomic force microscopy (AFM)

Whey proteins and d-CMP aggregates were imaged 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% (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 and Argyle light for 3D images.

2.10 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 larger protein concentration required for these experiments and the limited amount of powder available after the enzymatic treatment. The distribution of the rates of denaturation and the turbidity were presented as medians, with quartiles and whiskers representing, respectively, the 25\textsuperscript{th} and 75\textsuperscript{th} mark and the minimum and maximum values. Percentage of denatured proteins, ξ-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

Whey proteins are known to denature and aggregate above 60°C. In their native form, β-lg and α-la are soluble at all pH, including at their isoelectric point, 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. 3. The amounts of denatured α-la and β-lg after 30 min heating are presented in Fig. 4. The temperature of denaturation (Tm), at which half of the β-lg in the samples has lost their native conformation, was measured by DSC and presented in Table 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 90% (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. 3 and 4). In contrast, at pH 3.0, there was little denaturation of whey proteins observed after 30 min heating (Fig. 4). Partial unfolding of the whey proteins from pH 3.5 were previously observed which resulted in stable end product (Mulvihill & Donovan, 1987). 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 $\beta$-lg when the protein was heated at pH 3, which emphasizes our findings.

Above pH 4, $\alpha$-la had a lower rate of denaturation and relative amount of denatured material than $\beta$-lg (Fig. 3 and Fig. 4). This is in agreement with previous studies reporting the greater sensitivity to denaturation of $\beta$-lg compared to $\alpha$-la (Law & Leaver, 2000). However, at pH 4, and in particular in the presence of CMP or d-CMP, the rate of denaturation and the amount of denatured $\alpha$-la (Fig. 3 and Fig. 4) was higher (15%, w/w, in 0.5% WPI), regardless of the protein composition and concentration of the samples, than that of $\beta$-lg (6%, w/w in 0.5% WPI). Although the conformation of proteins is more stable around their isoelectric point (4.2-4.5 for $\alpha$-la), non-covalent interactions are promoted, resulting in greater precipitation of $\alpha$-la. A higher protein content (1% WPI) also favoured $\alpha$-la denaturation, as reported in previous studies (Hillier, Lyster, & Cheeseman, 1979), which could be another reason for the higher extent of denaturation of $\alpha$-la in presence of CMP or d-CMP (Fig. 3 and 4). Overall, CMP had a stronger effect on the denaturation of $\beta$-lg, i.e. on the rate of denaturation (Fig. 3), on the amount of denatured material (Fig. 3 and 4) and on its temperature of denaturation compared to d-CMP (Table 1). At pH 4, $\beta$-lg is positively charged, however CMP is very negatively charged, whereas d-CMP is close to its isoelectric point (3.7), thus the attractive electrostatic interactions are stronger with CMP than with d-CMP, leading to greater denaturation of $\beta$-lg. Both CMP and d-CMP promoted the denaturation of $\alpha$-la through attractive electrostatic interactions, although to a lesser extent than for $\beta$-lg. A greater decrease of the $\xi$-potential before heating was observed for the mixture containing 0.5% WPI with CMP, which could be a result of the attractive interactions between the whey proteins and CMP or an average of their respective $\xi$-potential at pH 4 (Fig. 5 a). Previous authors
found that CMP and β-lg interacted at pH 3.5 to form aggregates from few nanometres to 1 μm before heating, most likely via electrostatic interactions or hydrogen bonding (Martinez, Farías, & Pilosof, 2010). Changes in the secondary structure of the non-heated proteins upon addition of CMP or d-CMP are presented in Fig. 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 0.5% WPI 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). 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 on the greater denaturation degree of the whey proteins in presence of CMP were in agreement with recent studies that highlighted that the temperature for the onset of denaturation and the temperature of denaturation of denaturation of β-lg decreased with the addition of 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 closer to its native isoelectric point 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. 2 and 3). At pH 5 and 6, the rates of denaturation of α-la and β-lg were lowered in presence of the CMP or d-CMP (Fig. 3), which could be caused by the stabilisation 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 above pH 4 in presence of CMP or d-CMP.
The ξ-potential were more negative in 0.5% WPI sample in presence of CMP and closer to zero in presence of d-CMP (Fig. 5a). 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 presence of d-CMP was slightly higher than that in presence of CMP (Table 1). Haque and Khalifa (1992) found that the hydrophobicity of κ-casein increased with the removal 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). 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, αs1- 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 presence of CMP or d-CMP was reduced as compared to that of 0.5% WPI only (Fig. 4). However, at pH 6, the stabilisation of β-lg by CMP or 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 CMP or d-CMP (Fig. 3 and 4). The conformation of whey proteins is more stable at pH values close to their isoelectric point, and less stable at pH above 5, due to increased intra-molecular repulsion leading to unfolding and increased reactivity of the thiol groups (Hoffmann & van Mil, 1997). The rate of denaturation and the amount of denatured β-lg and α-la increased above pH 4 (Fig. 3 and 4). Thus, as the whey proteins unfold and new intermolecular disulphide bonds are formed, the interactions between β-lg and CMP or d-CMP that stabilised β-lg
initially and potentially even allowed β-lg to return to a native state during cooling and before analysis, promoted its denaturation on prolonged heating at pH 6. The mixture of whey proteins and CMP or d-CMP had a lower ζ-potential than the sample with 0.5% whey protein only (Fig. 5 a), which could be due to greater electrostatic interactions or could be the average of the ζ-potential of all proteins in solution. However, interactions between CMP or d-CMP and the whey proteins above pH 5 were evident from a decrease in the intramolecular β-sheet signal (Fig. 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 was observed (Fig. 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. 3 and 4). Thus, from this method based on the precipitation of the aggregates at their isoelectric point, there was no evidence for stabilisation 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 presence of CMP or d-CMP at pH 7 (Table 1). However, the temperature of denaturation of β-lg heated in presence of d-CMP was close to that of the control containing 0.5% WPI only. Therefore, the sialic acid hindered the denaturation of β-lg at pH 7 and its removal, resulting in lesser electrostatic interactions, facilitated the interactions between CMP and the unfolded β-lg, likely through hydrophobic interactions. Previous authors demonstrated the role of hydrophobic and electrostatic interactions on stabilising the native conformation of β-lg in presence of peptides from hydrolysed whey proteins. The higher charge density and hydrophobic interactions was assumed to induce a more compact form of β-lg in presence of the peptides. Above pH 6.8, the protective effect of the negatively charged peptides was much
lesser, which is in agreement with our results (Barbeau, Gauthier, & Pouliot, 1996). 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 destabilises the native state of β-lg. The authors concluded that CMP interaction is stronger with the unfolded form of β-lg than with the compact native form (Croguennec et al., 2014). In addition, Martinez, Sanchez, Patino, and Pilosof (2009) reported a decrease in the temperature of denaturation of denaturation and the onset temperature of denaturation of β-lg measured by DSC in the presence of CMP. However, other groups found that CMP increased the temperature of denaturation of denaturation when β-lg was heated with other whey proteins (Svanborg, Johansen, Abrahamsen, Schüller, & Skeie, 2016). These results could be explained by differences in the composition of the starting materials, such as the presence of fat or minerals.

The presence of CMP or d-CMP did not affect the early stage of denaturation of α-la to the same extent as it affected the early denaturation of β-lg (Fig. 2). 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

Fig. 7 and 8 present the turbidity after heating at 80°C for 30 min and Fig. 9 illustrates the molecular weight distribution of the samples after heating. 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 aggregates at pH 3 (Fig. 9 a). At pH 5, the turbidity increased greatly within 2 minutes of heating, due to a greater instability of β-lg during heating as electrostatic repulsion was at their minimum around its isoelectric point (results not shown); after 30 min of heating at pH 5, all samples gelled. At pH 4 and within 5 min of heating, the samples containing CMP developed a higher turbidity than the samples containing WPI only (Fig. 7 a and 8 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 above 70 kDa were present in the filtrate (Fig. 9 b), which is in contradiction with the high turbidity developed in the samples containing CMP (Fig. 7 a). At pH 4, the aggregates in the samples containing CMP were larger than the ones in the samples heated above pH 5, although the amount of denatured protein was smaller (Fig. 4). In heat-induced gels of WPI and CMP at pH 4.0, the gel strength was reported to be higher than those at neutral pH due to strong electrostatic interactions (Svanborg et al., 2016). Croguennec et al. (2014) observed the formation of over 5 μm aggregates, 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% WPI was heated on its own (Fig. 9 c). This is in agreement with the higher amount of denatured protein (Fig. 4) and the high OD of the samples after 30 min heating (Fig 7 b). The OD was much lower in the samples containing CMP or d-CMP (Fig. 7 b and 8 b), and fewer large aggregates were formed in the samples containing CMP (Fig. 9 c). The amount of denatured whey proteins in the samples containing CMP or d-CMP at pH 6 was higher than that containing 0.5% whey protein only (Fig. 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; more small aggregates were formed in presence of CMP and d-CMP, which were potentially denser. In spite of the
interactions between β-lg and CMP or d-CMP leading to more denatured β-lg after 30 min heating, its stabilisation within the first 5 min of heating could have had a durable effect on the structure of the aggregates. An example of a three-dimension AFM image of the aggregates of whey proteins and d-CMP at pH 6 is presented in Fig. 10. The height across section (Fig. 10 b) shows that the aggregates are polydisperse, with sizes ranging from 5 to 20 nm. High resolution phase and amplitude image (Fig. 10 c) show that the aggregates are made of individual monomers of proteins, presumably β-lg.

At pH 7, the OD and the aggregate size range were lower than at pH 6 for all samples (Fig. 7 and 9). The OD of the samples containing 0.5% whey protein, with or without CMP, or d-CMP, were very close (Fig. 7 c). However, the relative amount of medium size aggregates was lower and the relative amount of monomers, dimers or trimers smaller than 70 kDa was higher in 0.5% WPI than in the samples containing d-CMP (Fig. 9 d). This is in agreement with more denatured β-lg being measured in the case of the samples containing CMP (Fig. 4), and can be explained by stronger interactions between the whey proteins in presence of CMP or d-CMP. It was previously reported that, at pH 7.0, the heat-induced gelation of β-lg would only occur in 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 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 hypothesised that the negative charges of β-lg and CMP around pH 7 destabilised 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.

After heating, all aggregates formed in solutions above pH 5, with or without CMP, exhibited
a more negative zeta potential (Fig. 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. At pH 4, very little change in the β-sheets
structure was observed (Fig. 11). 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). Intra-molecular β-sheets absorb around 1630 cm⁻¹, and the
heat-induced formation of inter-molecular 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 inter-molecular
β-sheet in heat-induced aggregates. At pH 7, CMP or d-CMP did not prevent the formation of
inter-molecular β-sheets (Fig. 11), which is in agreement with the rate of denaturation and
denatured material results (Fig. 3 and 4).
4. Conclusion

The desialylation of CMP modified the electrostatic interactions between the peptide and the whey proteins during heating as a function of the pH. Above the isoelectric point of the proteins, the removal of the sialic acid facilitated the interactions between CMP and the whey proteins, more 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 around neutral pH, i.e. at a pH favouring their unfolding. In the initial stage of heating and at pH 5 or 6, i.e. around their isoelectric point, 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 from this knowledge and the non-negligible amount of CMP in cheese whey, in contrast to acid whey, to enhance the heat-stability of whey proteins in food products.
5. Acknowledgement

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Fig. 1

Holland (2008).
Chromatogram of a mixture of caseinomacropeptide (CMP) and WPI, containing α-lactalbumin (α-la) and β-lactoglobulin A and B (β-lg), by reversed-phase HPLC (RP-HPLC). The absorbance is detected at 214 nm.
\[ \alpha-\text{la} \]

**pH 4**

Rate of denaturation of α-la (min⁻¹)

- Y% WPI
- 0.5% WPI
- 0.5% WPI + CMP

**pH 5**

Rate of denaturation of α-la (min⁻¹)

- Y% WPI
- 0.5% WPI
- 0.5% WPI + CMP

**pH 6**

Rate of denaturation of α-la (min⁻¹)

- Y% WPI
- 0.5% WPI
- 0.5% WPI + CMP

**pH 7**

Rate of denaturation of α-la (min⁻¹)

- Y% WPI
- 0.5% WPI
- 0.5% WPI + CMP

\[ \beta-\text{lg} \]

**pH 4**

Rate of denaturation of β-lg (min⁻¹)

- Y% WPI
- 0.5% WPI
- 0.5% WPI + CMP

**pH 5**

Rate of denaturation of β-lg (min⁻¹)

- Y% WPI
- 0.5% WPI
- 0.5% WPI + CMP

**pH 6**

Rate of denaturation of β-lg (min⁻¹)

- Y% WPI
- 0.5% WPI
- 0.5% WPI + CMP

**pH 7**

Rate of denaturation of β-lg (min⁻¹)

- Y% WPI
- 0.5% WPI
- 0.5% WPI + CMP
Fig. 3

Rates of denaturation (min⁻¹) of (a, c, e, g) α-lactalbumin (α-la) and β-lactoglobulin (β-lg) (b, d, f, h) 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) WPI solution, 0.5% (w/v) WPI solution, a mixture of WPI and caseinomacropeptide (CMP) and a mixture of WPI and desialilated CMP (d-CMP). The experimental points were the average of at least three independent trials. The results were presented as medians, with quartiles and whiskers representing the 25th and 75th mark and the minimum and maximum values, respectively. The annotation w/v refers to weight of proteins per volume. Fig. 4 showed that the whey proteins exhibited very little denaturation and aggregation after heating for at pH 3 for 30 min at 80°C, therefore the rate of denaturation of α-la and β-lg at pH 3.0 was not presented here.
Fig. 4

Percentage of denatured (a) α-lactalbumin (α-la) and (b) β-lactoglobulin (β-lg) after heating ( ) 1% WPI, ( ) 0.5% WPI, ( ) a mixture of WPI and CMP and ( ) a mixture of WPI 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.

The experimental points were the average of at least three independent trials.
Fig. 5
Table 1

Temperature of denaturation of β-lactoglobulin (β-lg) by differential scanning calorimetry (DSC) for samples containing WPI, a mixture of CMP 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. 4) showed that β-lg did not denature after heating for 30 min at 80°C. The experimental points were the average of two independent trials.

<table>
<thead>
<tr>
<th>pH</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% WPI</td>
<td>87.5 ±0.0</td>
<td>80.3 ±0.2</td>
<td>79.8 ±0.1</td>
<td>77.8 ±0.1</td>
</tr>
<tr>
<td>2.5% WPI</td>
<td>88.3 ±0.3</td>
<td>80.2 ±0.2</td>
<td>80.9 ±0.1</td>
<td>73.2 ±0.5</td>
</tr>
<tr>
<td>2.5% WPI + CMP</td>
<td>85.0 ±0.5</td>
<td>82.4 ±0.4</td>
<td>81.3 ±0.0</td>
<td>77.0 ±0.2</td>
</tr>
<tr>
<td>2.5% WPI + d-CMP</td>
<td>86.3 ±0.8</td>
<td>83.6 ±0.2</td>
<td>82.1 ±0.4</td>
<td>75.1 ±1.0</td>
</tr>
</tbody>
</table>
Fig. 6

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(a) pH 4

(b) pH 6

(c) pH 7
Turbidity expressed as optical density (OD) at 600 nm of 1% WPI solution, 0.5% WPI solution, a mixture of WPI and CMP and a mixture of WPI 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 25th and 75th mark and the minimum and maximum values. The experimental points were the average of at least three independent trials. All samples displayed microscopic aggregates at pH 5 after few minutes heating, and measurements could not be taken. At pH 3, the whey proteins exhibited very little denaturation and aggregation after heating (Fig. 4), therefore the results at this pH condition was not presented here.
Fig. 8

Pictures of the samples after heating of (1) 0.5% WPI solution, (2) a mixture of WPI and CMP and (3) a mixture of WPI and desialylated CMP (d-CMP) after heating at 80°C for 30 min at (a) pH 4 and (b) pH 6.
Fig. 9
and >500 kDa. The experimental points were the average of at least three independent trials. All samples displayed microscopic aggregates at pH 5 after few minutes heating at 80°C, most of the aggregates formed during heating were filtered out through 0.45 μm, therefore the results were not presented here.
Fig. 10

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% WPI and desialylated CMP (d-CMP) after heating at 80°C for 30 min at pH 6.
Fig. 11
6. References


