



Caseinomacropeptide influences the functional properties of a whey protein concentrate



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ABSTRACT

To investigate the effect of the caseinomacropeptide (CMP) to true protein ratio on the functional properties of a whey protein concentrate powder, heat-induced gels with four different concentrations of CMP (0, 9, 21 and 33% true protein) at three different pH levels (4.0, 5.5, and 7.0) were made from a native whey protein concentrate and a commercial CMP concentrate powder. The microstructure, rheology, and solubility of the whey protein-CMP mixes were evaluated. The microstructures of the gels became porous and coarse as the CMP concentration increased. Gels without CMP were more resistant to stress ($\tau_{LVR,3\%}$) and strain ($\gamma_{LVR,3\%}$), with a 3% decrease in the linear viscoelastic region of G' , than gels containing 9–33% CMP. Solubility was influenced by pH, but not by the protein composition.

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1. Introduction

One strategy to reduce the seasonal variations in milk is to increase the casein content in cheese milk using microfiltration (MF) (Guinee, O'Kennedy, & Kelly, 2006; Papadatos, Neocleous, Berger, & Barbano, 2003). Casein standardisation of cheese milk has been shown to improve the coagulation properties of rennet (Rodríguez, Requena, Fontecha, Goudédranche, & Juárez, 1999; St-Gelais, Roy, & Audet, 1998) and increase the fat and protein recovery (Heino & Uusi-Rauva, 2010; Neocleous, Barbano, & Rudan, 2002). When MF is used to casein-standardise cheese milk, native whey (the skimmed milk MF permeate) is generated. The resulting whey from cheese making using casein-standardised milk may have a higher caseinomacropeptide (CMP) to true protein (TP) ratio (Outinen, Heino, & Uusi-Rauva, 2008, 2010). Native whey, regular cheese whey, and the whey made from cheese making of casein-standardised milk are three different raw materials for further processing to obtain concentrated whey protein products, as they have different chemical compositions. The major difference between the whey protein concentrate (WPC) made from native whey protein concentrate (NWPC) compared with the WPC from cheese

whey is the lack of CMP in the former. Additionally, the NWPC may contain less fat and denatured proteins, depending on the process conditions and type of membrane filtration used during manufacture of the cheese-derived WPCs.

The degree of whey protein denaturation and the fat content influence the functional properties of the WPCs (Morr & Ha, 1993). Undenatured whey proteins are soluble at a wide range of pH values, whereas denatured and/or aggregated whey proteins are insoluble. The loss of solubility indicates that the whey proteins are denatured. The degree of denaturation of the whey proteins is commonly expressed as the degree of solubility at pH 4.6. Very low amounts of denatured proteins are expected in the NWPC, and if found, they are most likely induced during the evaporation (before spray drying). Due to heat treatment of milk and whey, and possibly during evaporation, commercial WPCs will have a significantly higher denatured whey protein content compared with NWPC.

The solubility of globular proteins is strongly correlated to their gel properties (Zayas, 1997b). Gelation is an important functional property of protein ingredients, such as WPCs. The heat-induced gelation mechanism of globular proteins, such as β -lactoglobulin (β -LG), the dominant protein in whey products, is a 3-step process (Sawyer, 1968). The gelation steps are characterised by (i) the unfolding of the protein molecules, exposing free and previously buried sulphhydryl and hydrophobic groups, (ii) the aggregation of denatured protein molecules, and (iii), if the protein concentration

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is sufficiently high, the formation of a three dimensional network that traps water. According to Lorenzen and Schrader (2006), the network of WPC gel at pH values far from the pI of β -LG consists of linear strands ("fine stranded") because electrostatic repulsion stabilises the proteins. Such gels are translucent, rubbery, and deformable. At pH values close to the isoelectric point (pI) (5.1 for β -LG), the net charge of the proteins equals zero, and due to the low electrostatic repulsion, hydrogen bonds and hydrophobic interactions may lead to aggregation. In this situation, gelation may be poor due to a small net charge and a low number of intermolecular bonds, but, when formed, it is described as an intermediate particulate gel (Cornacchia, de la Fortelle, & Venema, 2014). The same authors showed that the net charge of the proteins was reduced when increasing the ionic strength either by adding salts or shifting the pH toward the pI. This resulted in a gel composed of a random association of particulate aggregates ("particulate gels"). The concentration of ions can alter the net charge on the protein molecules and, hence, the types of interactions and aggregates that are involved in the formation of the overall gel network. Havea, Singh, and Creamer (2002) found that the differences in the level and types of salt between different commercial WPCs affected the aggregation of the proteins during heat-induced gelation.

CMP or glycomacropeptide, the C-terminal (f 106–169) segment of κ -casein that is released by chymosin during cheese making, is present in commercial whey protein products obtained from sweet whey. The composition of CMP is variable, depending on the particular whey source (Martin-Diana, Gomez-Guillen, Montero, & Fontecha, 2006).

CMP is the third most abundant peptide fraction found in WPC, accounting for 20–25 (w/w) percentage of the nitrogen fraction in these products (Thomä-Worringner, Sorensen, & López-Fandiño, 2006). CMP has two major genetic variants, A and B (Jensen et al., 2015), and is classified into two major groups with respect to post-translational modifications: the glycosylated and non-glycosylated forms that are evenly distributed in whey. Throughout this paper, CMP is referred to as a mixture of non-glycosylated and glycosylated CMP. The pI of CMP varies from 4.15 for non-glycosylated CMP to 3.15 for glycosylated CMP, due to the presence/absence of sialic acid in the glycan moieties (Kreuss, Strixner, & Kulozik, 2009).

The presence of CMP has been found to have a negative effect on the gel strength of heat-induced WPC or WPI gels (Britten & Pouliot, 1996; Heino, Uusi-Rauva, Rantamäki, & Tossavainen, 2007; Lorenzen & Schrader, 2006; Veith & Reynolds, 2004). However, when comparing functional properties of NWPC with the WPCs, the lack of CMP in NWPC is confounded by the lower concentrations of denatured proteins (Britten, Lamothe, & Robitaille, 2008), lower contents of minerals (Evans, Zulewska, Newbold, Drake, & Barbano, 2009, 2010) and lesser amounts of fat (Heino et al., 2007; Luck et al., 2013; Marcelo & Rizvi, 2008). Recently, Xianghe, Pan, Peilong, Ismail, and van de Voort (2012) studied the effect of increasing the amount of CMP on WPC gels. The WPC produced from acidified (pH 4.6) skimmed milk was filtered, concentrated, neutralised by diafiltration and ultrafiltration, and spray dried. Gels with a fixed protein concentration of 12% were made from neutralised WPC and a CMP concentrate using CMP:TP ratios of 0, 5, 15, 25, and 100. They found that the gel strength decreased as the CMP:WP ratio increased. Even when CMP did not replace any of the whey proteins but was added to a WPC solution containing 12% protein, the gel strength was lost at a similar rate, indicating that the CMP has an important contribution in diminishing the strength of the gels network. However, Xianghe et al. (2012) only studied heat-induced gelation at neutral conditions and not at a low pH.

The objective of this study was to investigate how increasing the CMP:TP ratio from 0 to 33% influenced the microstructure, solubility and rheology of heat-induced WPC gels at pH 4.0, 5.5 and 7.0.

2. Materials and methods

2.1. Experimental design

Four different CMP:TP ratios were tested at three different pH values to investigate the influence of the CMP on the functional properties of the WPC. The mixtures of the NWPC and a commercial CMP concentrate contained 0% (NW0), 9% (NW9), 21% (NW21), and 33% (NW33) CMP and were tested at pH 4.0, 5.5, and 7.0. A commercial WPC (WPC80) made from the whey of Dutch type cheese containing 31% CMP was used as a reference for the NWPC-CMP mixture with the highest concentration of CMP (NW33).

2.2. Protein concentrates

A commercial CMP concentrate powder (Lacprodan® C-GMP 10, Arla Foods, Aarhus, Denmark) with 36% TP was used to adjust the NWPC dispersions to 0–33% CMP:TP. In the CMP powder, 85% of the TP was CMP and the powder contained 1200 mg kg⁻¹ calcium. The NWPC powder was produced from an unpasteurised skimmed milk MF permeate as described by Svanborg, Johansen, Abrahamsen, and Skeie (2014). The MF permeate was further concentrated and diafiltered by UF at 50 °C in a pilot plant unit with a 25 kDa cut-off membrane (GR60PP-6338/48, Alfa Laval, Næskov, Denmark), as previously described by Svanborg, Johansen, Abrahamsen, and Skeie (2015). The UF concentrates from two NWPC products were stored at –20 °C until they were freeze-dried. The frozen UF/DF concentrate was then brought to 4 °C overnight before batch-wise freeze-drying. The commercial WPC80 (TINE Verdal, Oslo, Norway) contained 69% TP, of which 31% was CMP, and served as a standard for comparisons with the NWPC-CMP containing 33% CMP in the TP (NW33).

2.3. Chemical analysis

The total solids were analysed according to IDF 21B:1987 (IDF, 1987). The total nitrogen (TN), casein nitrogen (CN), non-casein nitrogen (NCN), and non-protein nitrogen (NPN) levels were analysed according to IDF 20-1:2001 (IDF, 2001a), IDF 20-4:2001 (IDF, 2001b), IDF 20-5:2001 (IDF, 2001c), and IDF 29-2:2004 (IDF, 2004), respectively. Lactose was analysed using high performance liquid chromatography (HPLC) as described by Moe, Porcellato, and Skeie (2013). The analysis of the fat contents of the powders was performed according to IDF 124-3:2005 (IDF, 2005). The minerals in the milk fractions and powders were analysed using the method described by de La Fuente, Carazo, and Juárez (1997). In short, the samples were digested with 65% concentrated HNO₃ at 250 °C in a Milestone Ultra wave UltraClave III (Milestone, Sorisole, Italy) and diluted to 10% HNO₃ before the mineral composition was determined by Inductive Coupled Plasma Optical Emission Spectrometry (Perkin Elmer Optima 5300 DV, Perkin Elmer, Shelton, CT, USA). The protein profile, including the CMP of the WPCs and the cheese making fractions, were analysed using reverse phase HPLC (RP-HPLC), based on the methods described by Hinz, Huppertz, and Kelly (2012) and Visser, Slangen, and Rollema (1991). The data were analysed with the Chromeleon software version 7.1 (ThermoFisher Scientific, Waltham, MA, USA).

The amount of each protein was determined by dividing the corresponding peak area (obtained by RP-HPLC) for that particular protein by the sum of all the peak areas for the predominant peaks (CMP, α _{S1}-casein, β -casein, α -lactalbumin and β -lactoglobulin). α _{S2}-

Casein and κ -casein were not detected by this method. Finally, the % of each protein was converted to a concentration (g L^{-1}) using equation (1):

$$\text{Protein} \times (\text{g L}^{-1}) = \frac{\% \text{protein} \times \text{protein content}}{100} \quad (1)$$

High-performance liquid chromatography coupled to diode array detection and mass spectrometry (LC-UV-MS) was performed to evaluate the RP-HPLC peaks for CMP at retention times of 6.9 and 6.7 min for CMP A in the CMP concentrate and the NWPC, respectively, and 8.6 min for CMP B. Prior to LC-UV-MS analysis, the WPC, NWPC and CMP samples were dissolved in a reducing buffer containing bis-Tris, urea, trisodium citrate and dithiothreitol (DTT) at pH 7.0. The sample was stirred at room temperature for 60 min, after which it was diluted further by the addition of 4 volumes of a urea solution adjusted to pH 2.5. The samples were filtered through a 0.22- μm filter before being applied to the LC-UV-MS column (Phenomenex, Utrecht, The Netherlands). The MS was comprised of a triple quadrupole equipped with an electro spray ionisation (ESI) source, and the analyses were performed in the positive detection mode. The MS experiments were performed by scanning the parent ions of the proteins in the range from 500 to 1500 m/z . The data were analysed with Xcalibur software version 2.2 (ThermoFisher, Breda, The Netherlands). The semi-quantitative analysis was performed using standards for the caseins and whey-proteins.

2.4. Rheological analysis

Samples containing 12% protein consisting of NWPC powder, CMP concentrate powder, and MilliQ water were mixed to obtain a CMP:TP ratio of 0–33%. The solutions were stirred for 30 min, left at 4 °C overnight and then adjusted to a pH of 4.0, 5.5 or 7.0 shortly before analysis using a Physica MCR301 rheometer (Anton Paar GmbH, Graz, Austria) with a smooth parallel plate–plate system (PP50, Anton Paar). The temperature of the bottom plate was controlled with a Peltier system and brought to 4 °C before an approximately 3 mL sample was added and the top plate was lowered to the measurement position. A guard ring was used to prevent the sample from evaporating and sunflower oil was added to the exposed surfaces for the same reason. The gap between the plates was approximately 1 mm, and, due to swelling, the height of the probe was adjusted by applying a normal force of 0.1 N, allowing the gap to change during the measurement period.

Each sample was heated from 4 to 90 °C at a rate of 17 °C min^{-1} , held at 90 °C for 10 min, and cooled to 20 °C at the same rate as for the temperature increase. The gels were then incubated at 20 °C for 10 min prior to the amplitude sweep. The amplitude sweeps used an angular frequency of 10 rad s^{-1} , with the strain increasing from 0.01% to 100%. A normal force of 0.1 N was applied to the sample, and the temperature was kept constant at 20 °C. G' and G'' (storage modulus and loss modulus, respectively) were continuously recorded at a strain level of 0.02% and an angular frequency of 10 rad s^{-1} . The data were collected from a strain of 0.1% at 20 °C in the linear viscoelastic region (LVR) prior to the amplitude sweep and at the amplitude sweep where the LVR ended ($G' < 3\%$ of the plateau value of $\tau_{\text{LVR},3\%}$, $\gamma_{\text{LVR},3\%}$ and $G'_{\text{LVR},3\%}$, respectively) (Rheoplus/32 V3.40 software, Anton Paar GmbH) as measures of the final gel strength (resistance to stress, $\tau_{\text{LVR},3\%}$, strain ($\gamma_{\text{LVR},3\%}$) and viscoelastic properties ($G'_{\text{LVR},3\%}$). The protein solutions were stored on ice, and at least three replicates per sample were analysed.

2.5. Sample preparation and scanning electron microscopy

Dispersions of the NWPC, the CMP concentrate and MilliQ water containing 0–33% CMP with a total of 12% TP were rehydrated

overnight and the pH was adjusted immediately prior heat-induced gelation. Thirty millilitre samples of aqueous WPC solutions in glass beakers were incubated at 90 °C for 20 min in a water bath and cooled rapidly to 4 °C prior to preparation for the microstructure study. The gels were stored and stabilised at 4 °C for at least 3 h before they were cut in cubes of approximately 0.5 × 0.5 × 0.5 cm and prepared as described by Penna, Subbarao, and Barbosa-Cánovas (2007), with the exception that osmium tetroxide was omitted during fixation. The dry sections were fractured and the fragments were mounted on aluminium stubs and coated with gold-palladium under a vacuum using a Polaron SC 7640 sputter device (Quorum Technologies Ltd, Laughton, UK). The microstructures of the whey protein gels with CMP:TP ratios of 0–33% and of WPC80 at pH 4.0 and 7.0 were examined using a Zeiss EVO-50–EP (Carl Zeiss SMT Ltd., Cambridge, UK) scanning electron microscope (SEM) operating at 20 kV.

2.6. Differential scanning calorimetry

The influence of the CMP:TP ratio on the thermal transition of the protein solutions that had been adjusted to pH 7.0 was studied using a differential scanning calorimeter (DSC) (Mettler Toledo DSC823e/500/335, Mettler Toledo, Greifensee, Switzerland) with Stare software version 8 (Mettler Toledo). The instrument was calibrated against Indium prior to use. Approximately 25 μL of the 12% (w/w) protein solutions were accurately weighed into 40 μL standard aluminium pans (Mettler Toledo) and sealed with a perforated aluminium lid (ME 511408232, Mettler Toledo). The DSC scanning rate was 10 °C min^{-1} from 20 to 90 °C and the sample was incubated at 90 °C for 2 min. An empty pan of equal weight was used as a reference. Each sample solution was measured five times. The temperature at the maximum endothermic peak height (denaturation) and the onset and end-set temperatures were recorded.

2.7. Solubility and particle distribution

Solubility was investigated by measuring the nitrogen solubility of 1% protein dispersions with 0–33% CMP that had been adjusted to pH values of 4.0, 4.6, 5.5, and 7.0 using 1.0 M NaOH and 1.0 or 0.1 M HCl. The samples were centrifuged at 20,000 × g for 25 min at 4 °C. The supernatants were filtered through grade 1 (11 μm) filter paper. The amount of soluble nitrogen in the supernatant after centrifugation was calculated as percentage of the TN content in the corresponding sample prior to centrifugation. The total nitrogen level was determined by the Kjeldahl method, IDF 20-1 (IDF, 2001a). The particle size distribution in the NWPC and WPC80 dry powder samples was measured using a Malvern Mastersizer 3000 (Malvern Instruments Ltd., Malvern, UK), with an Aero S Optic cell and Aero S dry disperser and Mastersizer software version 2.20 (Malvern Instruments Ltd.). Particle distribution was expressed as D_{V10} (the value of the volume diameter where 10% of the powder particles have powder diameters below this value), D_{V50} (the volume median diameter), and D_{V90} (the value of volume diameter where 90% of the powder particles have powder diameters below this value). The analysis was repeated eight times for each powder.

2.8. Data analysis

To test the effect of the whey source on the composition and the functional properties of commercial WPC80 and NW33 (which had a similar CMP:TP ratio) one-way analysis of variance (ANOVA) was performed using the whey source as the variable. Multi-way ANOVA was performed to test the effect of (1) increasing the CMP:TP ratio, (2) the pH, and (3) the CMP:TP ratio × pH interaction

Table 1

Composition of native whey protein concentrate (NWPC) powder made from microfiltration permeate of skimmed milk and commercial whey protein concentrate (WPC80) powder.^a

Component	NWPC	WPC80
Total solids	95.9	97.37
True protein	88.0***	68.94
Total nitrogen	16.10***	18.03
Non-protein nitrogen	0.42***	2.36
Non-casein nitrogen	15.12***	17.56
Casein	6.24***	2.99
Whey protein	93.76**	97.01
β-Lactoglobulin A	21.6	21.7
β-Lactoglobulin B	41.8	29.5
α-Lactalbumin	21.3*	15.1
Caseinomacropeptide	0 ^b	31.0
α _{S1} -Casein	1.2	1.2
β-Casein ^b	1.9	1.8
Lactose	0.0	0.06
Fat	0.00**	0.07
Minerals		
Sodium	192	2.90
Magnesium	65*	115
Phosphorous	182***	638
Potassium	366**	1124
Calcium	486*	885
Iron	15	11
Zinc	7	12
Copper	7	2
Total minerals	1291**	3153

^a Values for total solids and true protein (TP) are in g 100 g⁻¹ sample; all other components other than minerals are given as g 100 g⁻¹ TP (casein concentration was calculated as true protein minus whey protein), minerals are given as mg 100 g⁻¹ TP. Mean values from two batches of NWC and from one batch of WPC80 are presented. Asterisks indicate significant difference between the two whey protein concentrates: **P* < 0.05; ***P* < 0.01; ****P* < 0.001).

^b Compounds eluting in the CMP genetic variant A peak area accounted for 12% of the total peak area; using mass spectrometry, it was confirmed that these compounds were not CMP (Fig. 2).

on the functional properties of NWPC samples with 0–33% CMP. No proper gels were formed at pH 5.5; therefore, the results obtained at pH 5.5 were omitted from the data prior to further statistical analysis. The differences between the four CMP:TP ratios were

analysed by Tukey's pairwise comparison (95% confidence). All statistical analyses were performed using R statistics (version 3.1.1, 2014-07-10, R Development Core Team; <http://www.r-project.org>).

3. Results

3.1. Effects of whey source on the composition of WPC

Table 1 shows the chemical composition of the NWPC and commercial WPC80. The most obvious difference between the powders was the much higher TP content per 100 g⁻¹ in the NWPC compared with the WPC80 (*P* < 0.001), and the presence of CMP in WPC80 compared with its absence in the NWPC. When adjusted for the difference in TP, NWPC contained less TN, whey protein and NPN per 100 g⁻¹ TP than WPC80 (*P* < 0.001). The mineral concentrations were lower in the NWPC (*P* < 0.01), despite the higher (*P* < 0.001) content of casein compared with WPC80. Despite that NWPC contained less whey protein per 100 g⁻¹ TP; the α-LA:TP ratio was higher (*P* < 0.05) in the NWPC than in WPC80, and the β-LG:TP ratios were similarly distributed in the two powders. The semi-quantitative RP-HPLC analysis detected peaks at the CMP retention time area in both the CMP concentrate powder and WPC80. These peaks had a similar profile (Fig. 1), although the peak height was far lower in the WPC80 sample. Fig. 1 also shows that the NWPC has a peak in the area of non-glycosylated CMP (genetic variant A) at a retention time of 6.7 min. However, the profile of the peak did not match the corresponding peak-profile of the CMP concentrate or WPC80 (Fig. 1). Therefore, the compounds in the NWPC samples that eluted in the peak at the CMP retention time were investigated further by LC-UV-MS, and this analysis showed that the profile of the NWPC peak (Fig. 2b) did not match the profile of the non-glycosylated CMP genetic variant A (CMP A) observed in the commercial CMP concentrate (Fig. 2a). Due to the mismatched profiles of the peaks in the area of CMP A in the NWPC sample by LC-UV-MS, the content of the peaks were regarded to be peptides other than CMP.

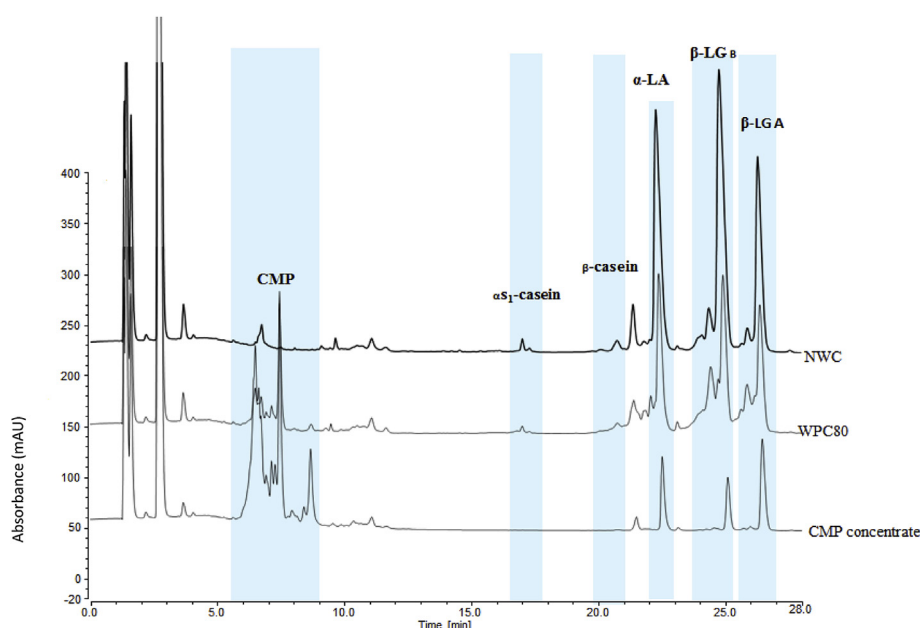


Fig. 1. Reversed-phase high-performance liquid chromatography chromatograms of native whey protein concentrate (NWC), reference WPC80 and caseinomacropeptide (CMP) concentrate showing the major proteins found in whey (α-LA, α-lactalbumin; β-LG A, β-lactoglobulin A; β-LG B, β-lactoglobulin B).

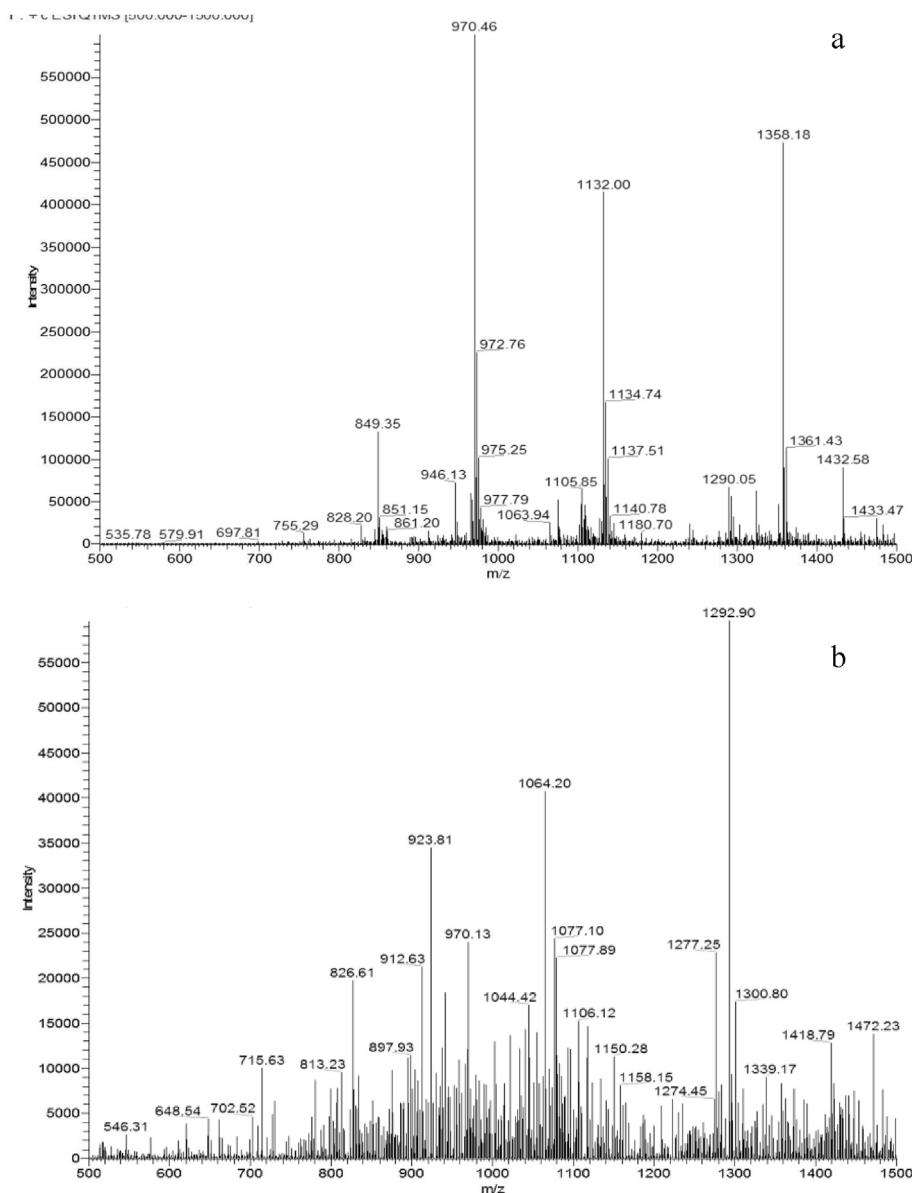


Fig. 2. Identification of peaks in the elution range of caseinomacropeptide (CMP) genetic variant A (retention time 6.9 min) by reversed-phase high-performance liquid chromatography with detection by diode array ultraviolet spectroscopy and mass spectrometry. Representative profiles of peaks obtained from a CMP concentrate (2a) and a native whey protein concentrate (2b).

3.2. Effects of whey source and CMP concentration on WPC particle size distribution and heat denaturation

The two powders had a similar particle size distribution (Table 2), despite the different production methods used. The WPC80 powder was made using an industrial scale spray drier that included an agglomeration step, whereas the NWPC powder was freeze dried at the pilot scale, with manual milling after drying. Denaturation during heat treatment is crucial for the gelling properties of the dispersions. The solubility of the two WPCs was equally high (approximately 85%) at pH 4.6. DSC can also measure the denaturation of whey proteins because the enthalpy changes associated with protein unfolding are proportional to the extent of denaturation (de Wit, 1990). One well-defined endothermic peak was observed for the NWPC and WPC solutions (Table 3). The onset, endset and peak temperature of the NWPC solutions was influenced ($P < 0.001$) by the CMP:TP ratio, but was not affected by the

Table 2

Particle size distribution and nitrogen solubility of native whey protein concentrate (NWPC) powder made from microfiltration permeate of skimmed milk and commercial whey protein concentrate (WPC80) powder.^a

Measurement	WPC80	NWPC
D _{v10} (μm)	26 (0)*	11 (4)
D _{v50} (μm)	82 (1)	46 (16)
D _{v90} (μm)	185 (21)	172 (36)
Solubility at pH 4.6	84.8 (2.6)	86.5 (8)

^a Abbreviations are: D_{v10}, value of volume diameter such that 10% of the powder particles have powder diameters below the value; D_{v50}, median volume diameter; D_{v90} value of volume diameter such that 90% of the powder particles have powder diameters below the value. Mean values with standard deviation (in brackets) from two batches of NWPC and from one batch of WPC80 are presented. An asterisk indicates a significant difference between the two whey protein concentrates from one-way ANOVA using whey source as factor ($P < 0.05$).

Table 3

Effect of caseinomacropeptide (CMP) content of true protein (TP), CMP:TP ratio and whey source on onset and end of transition temperature, transition enthalpy (ΔH) and peak temperature measured by differential scanning calorimetry (DSC) of 12% native whey protein dispersions with 0% (NW0), 9% (NW9), 21% (NW21), 33% (NW33) CMP of TP at pH 7 and WPC80 (with 31% CMP of TP).^a

Parameter	Sample					p-values for effects	
	NW0	NW9	NW21	NW33	WPC80	CMP % of TP	Whey source
Onset (°C)	58.24 (0.21)	63.88 (2.13)	65.85 (1.38)	48.04 (6.46)	55.14 (3.28)	<0.001	<0.01
Peak (°C)	70.34 (0.52)	72.97 (0.23)	73.04 (0.19)	71.87 (0.56)	73.28 (0.48)	<0.001	ns
End (°C)	78.81 (1.67)	80.53 (0.82)	81.25 (0.86)	86.57 (1.08)	84.28 (1.43)	<0.001	<0.001
ΔH (J g ⁻¹ sample)	-0.76 (0.25)	-0.82 (0.25)	-0.77 (0.31)	-0.79 (0.03)	-0.55 (0.13)	ns	ns

^a Values are the mean (with standard deviation in brackets) from five replicates and p-values from one-way ANOVA for the effect of CMP:TP ratio and whey source (NW33 versus WPC80): * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, not significant.

whey source when comparing WPC80 (31% CMP) and the NWPC sample with 33% CMP (NW33). The CMP:TP ratio influenced the onset and end-set temperatures ($P < 0.001$). The sample with no CMP added, NW0, had the lowest peak and end-set temperature; however, NW33 had the lowest onset temperature. Compared with WPC80, NW33 had a higher endset temperature ($P < 0.001$). The transition enthalpy was not influenced by the whey source or the CMP:TP ratio, and showed a somewhat high variation between parallels compared with the other DSC data. WPC80 had the lowest transition enthalpy compared with the samples made from the NWPC and NWPC-CMP concentrate mixtures.

3.3. Effects of CMP concentration on the gel properties of WPC

The rheological analysis revealed a significant effect of the CMP:TP ratio on important gel properties, including gel strain ($\gamma_{LVR,3\%}$) ($P < 0.001$), gel strength ($\tau_{LVR,3\%}$) ($P < 0.001$) and $G'_{LVR,3\%}$ ($P < 0.05$) (Table 4), while the pH had a significant impact on the gel strength ($P < 0.05$) only. In addition, gel strength was influenced by an effect of the interaction between the pH and the CMP:TP: NW0 had a gel strength at pH 4.0 that was higher ($P < 0.05$) than any of the other samples. In general, all gels had a low gel strain, regardless of the pH and CMP:TP ratio, however, NW0 gels were more resistant to strain than the other gels. The sample containing the highest CMP concentration, NW33, had a more than 10 times higher G' value ($P < 0.05$) than NW21 (NWPC with 21% CMP of the TP).

The viscosity of the WPC dispersions was increased at pH 5.5; however, they did not form proper gels. Nevertheless, by evaluating the NWPC gels at 20 °C (in the LVR prior to the amplitude sweep), the results showed that the heat-induced protein dispersions at pH 5.5 had a significantly higher phase angle (δ) (10.5°) and damping factor (0.186) than the gels formed at pH 7.0, but was not different ($P > 0.05$) from the gels formed at pH 4.0. Due to the lack of gelling properties at pH 5.5, the gels formed at pH 5.5 were excluded from the further statistical analysis, and the results from pH 5.5 are not shown in Table 4. When comparing the gels formed at pH 4.0 and 7.0, it was shown that the pH did not influence the phase angle and damping factor, but significant interaction effects were found between the CMP:TP ratio and pH (Table 4). However, an interaction effect of CMP:TP $\times$ pH ($P < 0.05$) was found and showed that the gels formed from NW33 at pH 7.0 had the lowest damping factor and phase angle (0.13 and 7.4, respectively) and those of NW9 at pH 7.0 had the highest damping factor and phase angle (0.18 and 10.6, respectively). The gels with all other CMP:TP ratios and pH combinations were insignificantly different.

Whey source influenced the gel properties: WPC80 gels obtained a higher damping factor ($P < 0.05$) and phase angle ($P < 0.05$) than the NWPC gels containing 33% CMP. The WPC80 gels were resistant to higher strain than the NW33 gels ($P < 0.05$), but whey source did not influence the gel strength. The solubility of the 1% NW0–NW33 samples was influenced by pH, but not by the CMP:TP ratio (Fig. 3a), with a higher mean solubility at pH 7.0 ($P < 0.05$)

Table 4

Influence of caseinomacropeptide (CMP) content of true protein (TP), CMP:TP ratio, pH and the interaction effect CMP:TP ratio $\times$ pH on rheological properties of heat induced whey protein gels containing 0% (NW0); 9% (NW9); 21% (NW21); 31% (WPC80) and 33% (NW33) CMP at pH 4 and 7 on gel strength ($\tau_{LVR,3\%}$), elastic modulus ($G'_{LVR,3\%}$) and gel strain ($\gamma_{LVR,3\%}$) at 3% decrease from linear viscoelastic range (LVR) of G' , and the damping factor and phase angle (°) in the LVR.^a

Sample/effect	pH	$\gamma_{LVR,3\%}$ (%)	$\tau_{LVR,3\%}$ (Pa)	$G'_{LVR,3\%}$ (Pa)	Damping factor	Phase angle (°)
NW0	4	4 ^b	1 571 ^b	44,977 ^a	0.17	9.59
	7	2	523	29,003	0.17	9.60
NW9	4	1 ^a	210 ^a	112,006 ^a	0.15	8.79
	7	1	239	20,740	0.18	10.57
NW21	4	0 ^a	249 ^a	192,038 ^a	0.17	9.90
	7	1	162	32,358	0.17	9.73
NW33	4	0 ^a	241 ^a	166,360 ^b	0.17	9.76
	7	0	231	280,100	0.13	7.43
WPC80	4	1	242	22,787	0.18	10.22
	7	2	187	13,737	0.19	10.48
Effect of CMP:TP ratio ^c		<0.001	<0.001	<0.05	ns	ns
Effect of pH ^c		ns	<0.05	ns	ns	ns
Interaction effect CMP:TP ratio $\times$ pH ^c		ns	<0.005	ns	<0.01	<0.01
Effect of whey source (NW33 versus WPC80) ^d		<0.05	ns	<0.05	<0.05	<0.05

^a Lower case superscript letters of pH 4 values indicate differences between samples with 0–33% CMP by Tukey's pairwise comparison for 95% level in the column for each response. The p-values are for results from multi-way ANOVA of native whey protein concentrate samples with CMP:TP ratio, pH and the interaction CMP:TP ratio $\times$ pH as classification factors (ns, not significant) with the exception of effect of whey source, where the p-values are the results from multi-way ANOVA using whey source as classification factor when comparing commercial WPC80 (31% CMP) and NW33 (ns, not significant).

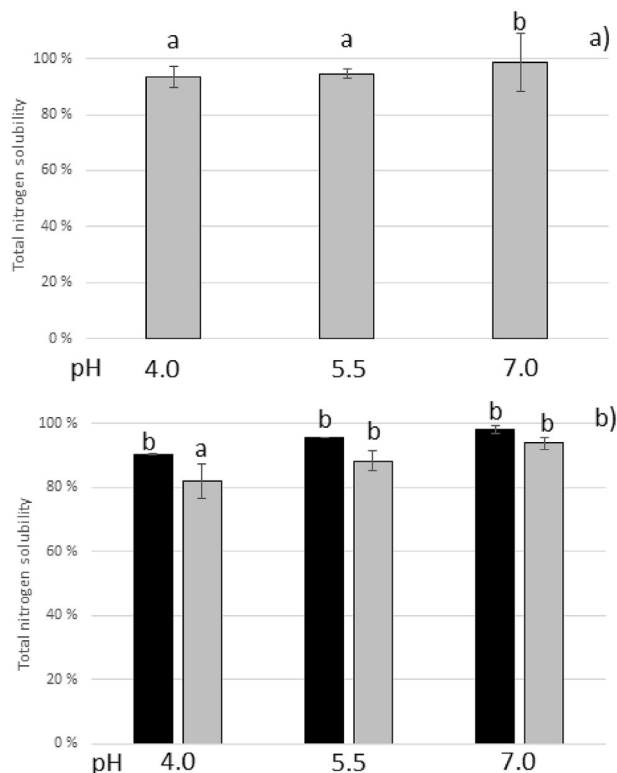


Fig. 3. The effects of (a) pH and (b) the source of whey on solubility. In (a), comparison of the average percentage of total nitrogen in the supernatants of native whey protein concentrate (NWPC) samples containing 0–33% caseinomacropeptide (CMP) and the pH-adjusted sample prior to centrifugation. The letters above the bars indicate a significant effect of pH on solubility. In (b), comparison of the percentage of total nitrogen in the supernatants of NWPC containing 33% CMP (NW33, black bar) and WPC80 (31% CMP, grey bar) at pH levels of 4.0, 5.5 and 7.0, and the pH-adjusted sample prior to centrifugation. The letters above the NW33 and WPC80 bars indicate a significant effect of the source of whey.

compared with the mean solubility at pH 4.0 and 5.5, which had an equally low solubility. Commercial WPC80 was found to have a lower solubility ($P < 0.05$) compared with samples with a similar CMP:TP ratio (NW33) (Fig. 3b). The solubility of the WPC80 samples

at pH 4.0 was significantly lower than the WPC80 samples at pH 5.5 and 7.0, and lower than the NWPC samples at any pH.

3.4. Effects of pH and CMP concentration on the microstructure of WPC gels

The gels studied by SEM were made in a similar manner as the gels formed in the rheometer, except that they had a longer heating time (20 min instead of 10 min) at pH 4.0 and 7.0. After heating, samples adjusted to pH 5.5 were pourable and some of the samples showed extensive precipitation. Because of this, only the gels formed at pH 4.0 and 7.0 were prepared for SEM analysis. The NW0 gel at pH 7.0 was almost translucent compared with the opaque gels made from WPC80 and NWPC with 9–33% CMP. The gels formed at pH 7.0 obtained stronger syneresis after three days at 4 °C compared with the pH 4.0 gels. At pH 4.0, the NW0 gel became slightly more opaque, but none of the NWPC gels became as white as the WPC80 gel. All gels formed at pH 4.0 were generally very stiff and brittle, and cracked during the 4 °C storage prior to fixation. The SEM micrographs (Fig. 4) show an obvious effect of the increasing CMP:TP ratio in the samples, as the structure became more open with larger pores at pH 7.0. This tendency was more pronounced for the gels at pH 7.0 than those at pH 4.0 because the structures of all samples at pH 4.0 were dense and less porous. The NW0 gels had a compact and somewhat less coarse structure at both pH levels compared with the gels with a higher CMP:TP ratio. The differences in microstructure between NW0 and NW33 gels were more pronounced compared with the differences between the NW9 and NW21 gels, which appeared more similar. NW9 was comparable to the reference (WPC80) gel in structure (Figs. 4 and 5). The pH influenced the microstructure of the NWPC and WPC gels, which was reflected in the higher gel strength at pH 4.0. The network structures of the gels formed at pH 7.0 were more swollen and had large cauliflower-like aggregates compared with the denser and less porous networks obtained in the gels formed at pH 4.0. The differences between pH 4.0 and 7.0 were not as pronounced for the NW0 gels compared with the differences obtained for the gels with higher CMP concentrations.

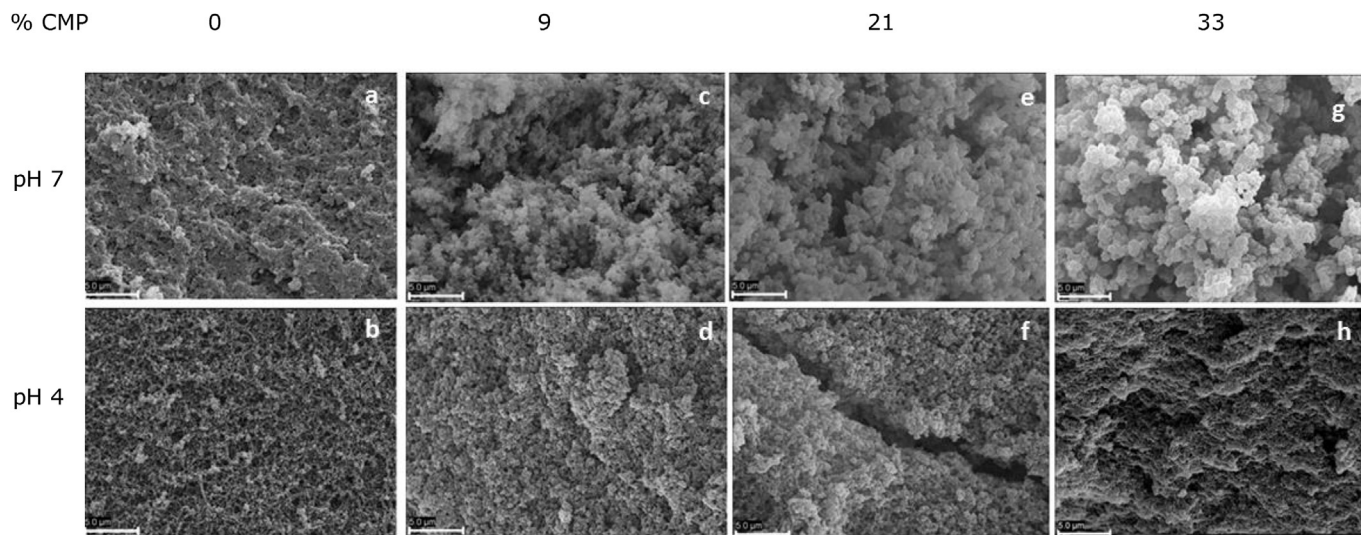


Fig. 4. Scanning electron microscopy micrographs of heat-set WPC gels made from native whey concentrate with various ratios of caseinomacropeptide (CMP) to true protein at pH 7.0 (a, c, e, g) and at pH 4.0 (b, d, f, h). CMP percentages were: 0% (a, b), 9% (c, d), 21% (e, f) and 33% (g, h). Scale bars represent lengths of 50 μm.

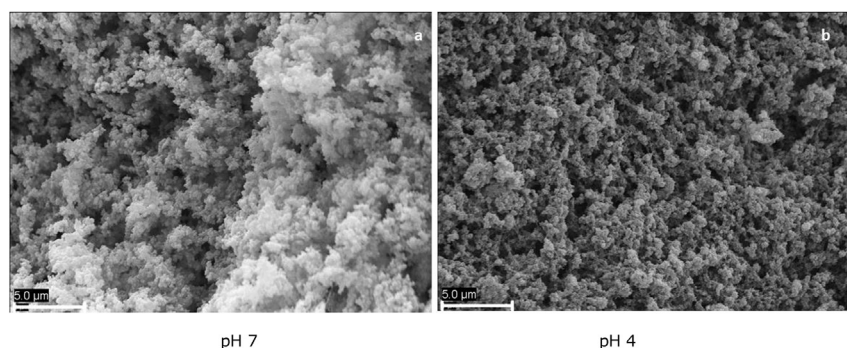


Fig. 5. Scanning electron microscopy micrographs of heat-set WPC gels made from commercial WPC80 at (a) pH 7.0 and (b) pH 4.0. Scale bars represent lengths of 5 μm .

4. Discussion

WPC80 had a somewhat different chemical composition than NWPC that might have influenced the properties of the WPC gels in this study. Some of the compositional differences between the whey sources were the mineral and fat contents. The calcium content in the WPC80 samples was almost twice as high as found in NWPC. The initial mineral content in WPC is critical to gel network structure because ionic calcium is known to contribute to the aggregation and gelation of the whey protein products (Riou, Havea, McCarthy, Watkinson, & Singh, 2011). However, in this study, the dissimilarity in microstructure of WPC80 and NW33 gels, that had a similar CMP content, could be related to the higher minerals content in WPC80. The most obvious difference in the chemical composition of the NWPC and commercial WPC80 is the difference in TP. When adjusting for this difference, the NWPC had a higher casein concentration and a lower NPN concentration, as expected. The CMP:TP ratio in WPC80 was 31%, which was a little higher than had been reported as typical values for cheese whey (Thomä-Worringer et al., 2006). At the point when CMP normally elutes, the chromatographic profile of the eluted compounds in the samples of the NWPC and CMP concentrate differed. The co-eluting peptides that caused a false positive CMP identification may origin from proteolytic activity during NWPC processing, such as activity from heat-resistant proteolytic enzymes from psychrotrophic bacteria. For instance, the composition of pseudo-CMP, which is caused by *Pseudomonas* proteases, differs from CMP in only one amino acid. Moreover, RP-HPLC methods are unable to distinguish between these two varieties of CMP (Campos Motta et al., 2014). The indigenous CMP found in raw milk has been reported to be approximately ten-fold lower than the amount of CMP in cheese whey (Furlanetti & Prata, 2003). Therefore, these ingenious CMPs were not expected to be present in detectable quantities in the NWPC.

For various applications, the solubility is the most important functional property of WPC and is known to have a strong correlation to gelling and foaming (Zayas, 1997a). Solubility at pH 4.6 and the transition enthalpy are closely related to protein denaturation (Li-Chan, 1983; de Wit & Kessel, 1996). However, neither the solubility at pH 4.6 nor the transition enthalpy differed significantly between the two WPCs studied, although the transition enthalpy tended to be higher in the NWPC-CMP concentrate mixtures compared with WPC80. The somewhat low solubility of NWPC at pH 4.6 is likely related to the precipitated casein, (which accounted for more than 6% of the proteins in these samples) rather than denatured whey proteins. The whey protein concentrate made from spray-dried industrially obtained cheese whey normally contains 20–50% denatured whey proteins (Kohnhorst & Mangino, 1985), whereas the NWPC usually has a

very low level of denatured proteins. Only a modest increase in the onset temperature was observed as the CMP content increased up to 21%; however, NW33 had the lowest onset temperature of all of the tested samples. Additionally, NW33 also had the highest end-set temperature. The broader transition boundaries of these samples compared with the samples with a lower CMP content may indicate that the protein aggregation step was somewhat interrupted. In this study the peak temperature at pH 7.0 was slightly increased with increasing CMP:TP ratio, in contrast to Martinez, Carrera Sanchez, Rodriguez Patino, and Pilosof (2009) who found an decrease in peak temperature of CMP – β -LG mixes containing 25–75% CMP compared with pure β -LG samples. No difference in the transition enthalpy (ΔH) with respect to the CMP concentration or the whey origin was detected in this study. However, Croguennec et al. (2014) found that increasing the CMP: β -LG ratio increased the rate of β -LG unfolding, regardless of the pH and heating temperature. CMP is known not to have a defined transition temperature (Xianghe et al., 2012) because it lacks tertiary structure (Kreuss et al., 2009). Xianghe et al. (2012) found that WPCs containing as much as 25% CMP did not have the typical denaturation transition observed for β -LG and α -LA, and at this concentration, the solution behaved more like a CMP concentrate.

The gelling point defines the point at which a fluid is converted to a solid. Reliable gelling points, the G' - G'' -cross over measurements, were not obtained from the rheological analysis, due to a rapid temperature increase that led to a steep G' curve. As shown in the DSC analysis, the tendency of interruption during the gelling of samples with a high CMP:TP ratio was obvious, as the increase in G' and G'' were slower. The G' curve was not smooth during the temperature rising from 4 to 90 $^{\circ}\text{C}$ (data not shown), as also was reported by Martinez, Farias, and Pilosof (2010) when heating CMP – β -LG mixes with a ratios of 75:25. In our study, the broader transition temperature range for the samples with high CMP:TP ratios, and the slower increase in G' and G'' during heating, indicates that CMP interrupted the heat-induced gelation of WPCs. The DSC measurements of β -LG-CMP mixes showed that the formation of β -LG-CMP aggregates were driven by electrostatic interactions and/or by hydrogen bonding (Martinez et al., 2009).

The NW0 gel was more than three times stronger ($\text{higher } \tau_{LVR,3\%}$) at pH 4 compared with any other gel, regardless of pH. The negative influence of the CMP on the gel strength is in accord with several other studies (Heino et al., 2007; Rantamaki et al., 2000; Veith & Reynolds, 2004). Most comparisons of the gel strength between the native whey protein ingredients and WPCs containing CMP have been performed at neutral pH. The shape and size of the aggregates formed from β -LG and CMP is crucial to the properties of the gel network. DSC studies of β -LG–CMP mixes at neutral pH

showed that the formations of β -LG–CMP aggregates were driven by electrostatic interactions and/or by hydrogen bonding (Martinez et al., 2009). At neutral pH, both β -LG and CMP are negatively charged, which may cause a weaker interaction between the CMP and unfolded whey proteins. Croguennec et al. (2014) suggested that CMP possesses a similar chaperone-like behaviour as some caseins (Guyomarc'h, Nono, Nicolai, & Durand, 2009; O'Kennedy & Mounsey, 2006), thus stabilising the β -LG at neutral conditions. Croguennec et al. (2014) suggested that the aggregates in the β -LG–CMP-systems consisted of β -LG surrounded by CMP monomers. The molecular interactions between β -LG and CMP would likely be stronger at pH 4.0 than at pH 7.0, due to the differences in electrical charge. Below the pI (pH 5.1), the net charge of β -LG is positive and that of CMP is negative. In our study, the effect of the CMP:TP ratio on the microstructure seemed to be pH-dependent, as the CMP:TP ratio had a different effect on the gel structure at pH 4.0 and 7.0. The influence of CMP:TP ratio seemed to be pH-dependent with respect to gel strength, as NW0 gels had a far higher gel strength at pH 4.0 than at pH 7.0, whereas the gels containing CMP had an equally low gel strength, regardless of pH. The equally reduced gel strength for NW9-33 may indicate that the presence, rather than the actual level of CMP, is crucial for this gel property. However, the increase in the CMP:TP ratio seemed to influence the microstructure more than the rheological parameters, as the structure became dramatically more porous and swollen as the CMP:TP ratio increased.

A large difference in structural appearance was observed between NW0 and the other samples at pH 7.0. The network became more swollen and the aggregates and pores larger as the CMP:TP ratio increased at pH 7.0. These findings are consistent with the findings of Xianghe et al. (2012). A network with large pores would have a lower water holding capacity than gels with a denser network. The water holding capacity of the gels was not measured in this study, but based on visual observations, the gels formed at pH 7.0 had more syneresis compared with the gels formed at pH 4.0. The effect of increasing CMP:TP ratio was less pronounced in the microstructure of gels formed at pH 4.0, most probably due to the difference in β -LG and CMP charge. The gels formed at pH 4.0 appeared more rigid and cracked more easily compared with the gels formed at pH 7.0. These visual observations were in accord with the rheological results, as the NW0 gels formed at pH 4.0 were significantly stronger and had a higher G' value than the pH 7.0 gels. However, this is in contradiction to the findings of Verheul and Roefs (1998), who reported that whey protein gels at pH > pI are stronger and more elastic, whereas gels formed at pH < pI are weak and brittle.

5. Conclusions

The addition of 9–33% CMP to NWPC did not influence the solubility of the NWPC; however, the solubility was influenced by pH and was higher at pH 7.0 than at pH 4.0 and 5.5. The effect of the CMP:TP ratio on the microstructure and gel strength of the heat-induced gels seemed to be pH-dependent: At pH 7.0, the NWPC gels without CMP had a less porous and more swollen network, with an increasing aggregate and pore size and as the percentage of CMP increased. The gels without CMP had a far higher gel strength at pH 4.0 than at pH 7.0, whereas gels containing CMP had an equally low gel strength, unrelatedly of pH. However, the effect of the CMP:TP ratio was not as pronounced at pH 4.0, where all samples had a similarly dense structure. The gel strain was far higher in the samples that did not contain CMP, and gels with 33% CMP had higher G' values than the gels with less CMP.

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