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

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ABSTRACT

Milk protein concentrate (79% protein) reconstituted at 13.5% (w/v) protein was heated (90 °C, 25 min, pH 7.2) with or without added calcium chloride. After fractionation of the casein and whey protein aggregates by fast protein liquid chromatography, the heat stability (90 °C, 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 α_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, McManus, Brodkorb, Carthy, & Fenelon, 2016; Doherty et al., 2011; Morr, 1982; Ryan, Zhong, & Foegeding, 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, 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 characterised 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 stabilise proteins from unfolding, aggregation and precipitation (Morgan, Treweek, Lindner, Price, & Carver, 2005). In dairy science and technology, aggregation is probably the more important phenomenon, as uncontrolled aggregation can lead to destabilisation. α_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, Law, & Dalgleish, 2003) and in Teflon tubes (4.6 cm^3) in an oil bath at 145 °C (Kehoe & Foegeding, 2014) 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-lactoglobulin and α-lactalbumin (Guyomarc'h et al., 2003). Bovine serum albumin (BSA), lactoferrin, β-casein and α_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 have been shown (Jang & Swaisgood, 1990), part of the aggregates analysed may also be polymers of κ-casein (Farrell, 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 mass of the aggregates was estimated to be 2×10^7 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, Renan, Famelart, & Guyomarc'h, 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, Nono, Nicolai, & Durand, 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% (w/w) protein (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, Erabit, Flick, & Alvarez, 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, Teagasc Food Research Centre Moorepark, Fermoy, Co. Cork, Ireland). The skim milk was pre-heated at a temperature in the range 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, MN, USA) and contained 93.7% (w/w) protein. Freeze-dried κ-casein was purchased from Sigma Aldrich (St. Louis, MO, USA); the purity of the κ-casein powder was

140 greater than 70% (w/w). All reagents were purchased from Sigma Aldrich unless stated
141 otherwise.
142
143 *2.2. Production and purification of the casein and whey protein aggregates*
144
145 Fig. 1 summarises the steps of purification and analysis carried out on the
146 reconstituted MPC. MPC was reconstituted at 13.5% (w/v) protein in distilled water, and
147 sodium azide (0.05%, w/v) was added to prevent microbial growth. When reconstituted at
148 13.5% (w/v) protein, the concentrate was 3.6-fold more concentrated in protein and 2.8-fold
149 more concentrated in calcium than skim milk, assuming a skim milk density of 1.035 kg m^{-3}
150 (Nutting, 1970). During the first step of rehydration, the reconstituted MPC was incubated at
151 45 °C for 2 h under constant stirring using an impeller, and calcium chloride was added at 0,
152 2.5 or 5 mM. The MPC solution was then stirred overnight at 4 °C to ensure complete
153 rehydration. On the following day, the solution was equilibrated to 22 °C and the pH was
154 adjusted to 6.7 or 7.2 using 0.5 M NaOH and 0.5 M HCl, as required. Varying the pH of
155 heating influences the casein dissociation and consequently the amount of caseins reacting
156 with whey proteins in the serum phase (Donato & Guyomarc'h, 2009). After 1 h equilibration
157 at 22 °C, re-adjustment of the pH was performed, if needed. Half of the samples did not
158 undergo a heat treatment, and were used as controls.
159 Aliquots (22 mL) were filled into 25-mL glass bottles (Pyrex, Greencastle, PA, USA)
160 and heated at 90 °C for 25 min (15 min hold time) in a water bath, which allowed heating of
161 several samples of large volume simultaneously. Higher temperature may have caused the
162 degradation of the negatively charged residues on κ-casein (Alais, Kiger, & Jollès, 1967;
163 Villumsen et al., 2015). After heating, the samples were cooled for 7 min in ice water and
164 warmed for 20 min at 22 °C. Weighed 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, MA, 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, and Dalgleish (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, UK) containing Sephacryl S-500 HR beads (fractionation range 4×10^4–2×10^7 Da). The absorbance was monitored at 280 nm by an AKTA Purifier 10 system (GE Healthcare), 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 mixture 1 and mixture 2, respectively.

2.3. Protein content measurement

Protein content of liquids and powder were determined by Kjeldahl (IDF, 2014); 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); bovine

serum albumin 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 sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis under reducing or non-reducing conditions following a modified method of Laemmli (1970). Lithium dodecylsulphate (LDS) was used instead of SDS. NuPage bis-Tris gels at 12% (w/w) acrylamide were used with the NuPage cells and the NuPage power supply (Life Technologies, Carlsbad, California, USA), 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 bovine serum albumin (BSA), α_S-casein (α_{S1} and α_{S2}-casein were quantified together), β-casein, κ-casein, β-lactoglobulin and α-lactalbumin 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). 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, UK). 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-lactoglobulin 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

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

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, Wang, Morris, & Brodkorb, 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 standardisation. 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, Germany). Under 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 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.

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 and Table 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) and lower protein recovery in the supernatants for the samples containing calcium (Table 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.

3.2. Composition of the fractions from size-exclusion chromatography

SDS-PAGE under reducing conditions (Fig. 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-lactalbumin and β-lactoglobulin 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-lactoglobulin. 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 α_S- and β-casein, while fraction C contained the highest proportion of κ-casein, β-casein and α_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 α_S- and β-caseins in each fraction remained unchanged.

Table 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 3) may be the result of the chaperone-like activity of the non-covalently bound caseins (α_S-, β- and κ-casein) to whey proteins in fraction B (Fig. 2).

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:[α_S-casein + β-casein] that was not covalently bound to whey proteins in our study was up to 1:9. This ratio was comparable with those found by Donnelly, McNeill, Buchheim, and McGann

(1984) for skim milk fractionated by size-exclusion chromatography. These authors found that casein micelles in skim milk had sizes ranging from 62 to 154 nm and ratios of κ-casein:[α_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 stabilise α_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. 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 3). SEM micrographs indicated that the smaller aggregates (Fig. 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. 5). 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. 5 gave a surprisingly similar particle size (z-average) of 53 nm.

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. 4 and Table 4). 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.

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. 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 stabilise 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 80–89% (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 α_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 α_S- and β-casein may have provided an additional stabilisation to the aggregates.

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 α_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. 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. 6 also illustrates the significant loss in α_S- and β-casein (39%, w/w, initial α_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 organised 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 destabilised 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

462 hydrophobic casein after β-casein, with an average hydrophobicity of 5.1 kJ per residue
463 (Bigelow, 1967). However, κ-casein is glycosylated by negatively charged hydrophilic
464 groups, which protrude at the surface of the casein micelle and ensure its stability in the
465 aqueous phase of milk. Thus, in the case of formation of mini-micelles, κ-casein would help
466 in solubilising them, possibly explaining the lower hydrophobicity in fraction C and the
467 enhanced heat stability of this fraction. In the same way, the hydrophilic part of κ-casein
468 could stay at the surface of the aggregates, stabilising the denatured whey proteins. This
469 mechanism would be similar to that of heat-shock proteins, which are intracellular proteins
470 that prevent the complete unfolding, aggregation and precipitation of proteins denatured by
471 heat, oxidation or reduction (Richter, Haslbeck, & Buchner, 2010). After binding to non-
472 native proteins by hydrophobic interactions and forming high molecular weight complexes,
473 the mobile hydrophilic regions of the heat-shock proteins help solubilising the complex
474 (Guyomarc'h et al., 2009; Treweek, Thorn, Price, & Carver, 2011). A similar mechanism has
475 also been postulated for the chaperone-like activity of α_S- and β-casein against the heat-
476 induced aggregation of whey proteins (Morgan et al., 2005; Zhang et al., 2005).

477 By comparison, for fractions A and C, the hydrophobicity of the aggregates (Table 3)
478 seemed to follow the same trend as the heat stability of the aggregates and the ratio of whey
479 protein:casein. The higher the proportion of casein in the aggregates, the lower the resulting
480 hydrophobicity and the higher their heat stability. Fraction B contained a greater proportion
481 of caseins and was more heat-stable than fraction A; however, no significant difference in
482 hydrophobicity was observed. As noted earlier, heat stability is correlated with the charge of
483 the aggregates. In addition, the differences between the SDS-PAGE under reducing and non-
484 reducing conditions do not facilitate understanding of whether α_S- and β-casein are associated
485 with the aggregates of whey proteins and κ-casein by hydrophobic interactions. It is possible
486 that α_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 α_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.

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References

Alais, C., Kiger, N., & Jollès, P. (1967). Action of heat on cow κ-casein. Heat caseino-glycopeptide. *Journal of Dairy Science, 50*, 1738–1743.

Beaulieu, M., Pouliot, Y., & Pouliot, M. (1999). Thermal aggregation of whey proteins in model solutions as affected by casein/whey protein ratios. *Journal of Food Science, 64*, 776–780.

Bigelow, C. C. (1967). On the average hydrophobicity of proteins and the relation between it and protein structure. *Journal of Theoretical Biology, 16*, 187–211.

Boye, J. I., Alli, I., Ismail, A. A., Gibbs, B. F., & Konishi, Y. (1995). Factors affecting molecular characteristics of whey protein gelation. *International Dairy Journal, 5*, 337–353.

Buggy, A. K., McManus, J. J., Brodkorb, A., Carthy, N. M., & Fenelon, M. A. (2016). Stabilising effect of α-lactalbumin on concentrated infant milk formula emulsions heat treated pre- or post-homogenisation. *Dairy Science and Technology*, 1–15.

Crowley, S. V., Megemont, M., Gazi, I., Kelly, A. L., Huppertz, T., & O'Mahony, J. A. (2014). Heat stability of reconstituted milk protein concentrate powders. *International Dairy Journal, 37*, 104–110.

536 Dalgleish, D. G., & Corredig, M. (2012). The structure of the casein micelle of milk and its
537 changes during processing. *Annual Review of Food Science and Technology, 3*, 449–
538 467.
539 del Angel, C. R., & Dalgleish, D. G. (2006). Structures and some properties of soluble
540 protein complexes formed by the heating of reconstituted skim milk powder. *Food*
541 *Research International, 39*, 472–479.
542 Doherty, S. B., Gee, V. L., Ross, R. P., Stanton, C., Fitzgerald, G. F., & Brodkorb, A. (2011).
543 Development and characterisation of whey protein micro-beads as potential matrices
544 for probiotic protection. *Food Hydrocolloids, 25*, 1604–1617.
545 Donato, L., & Dalgleish, D. G. (2006). Effect of the pH of heating on the qualitative and
546 quantitative compositions of the sera of reconstituted skim milks and on the
547 mechanisms of formation of soluble aggregates. *Journal of Agricultural and Food*
548 *Chemistry, 54*, 7804–7811.
549 Donato, L., & Guyomarc'h, F. (2009). Formation and properties of the whey protein/kappa-
550 casein complexes in heated skim milk - A review. *Dairy Science and Technology, 89*,
551 3–29.
552 Donnelly, W. J., McNeill, G. P., Buchheim, W., & McGann, T. C. (1984). A comprehensive
553 study of the relationship between size and protein composition in natural bovine
554 casein micelles. *Biochimica et Biophysica Acta 789*, 136–143.
555 Early, R. (2012). Dairy products and milk-based food ingredients. In D. Baines & R. Seal
556 (Eds.), *Natural food additives, ingredients and flavourings* (pp. 417–445). Cambridge,
557 UK: Woodhead Publishing.
558 Farrell Jr, H. M., Wickham, E. D., & Groves, M. L. (1998). Environmental influences on
559 purified κ-casein: Disulfide interactions. *Journal of Dairy Science, 81*, 2974–2984.

560 Guyomarc'h, F., Law, A. J. R., & Dalgleish, D. G. (2003). Formation of soluble and micelle-
561 bound protein aggregates in heated milk. *Journal of Agricultural and Food*
562 *Chemistry, 51*, 4652–4660.
563 Guyomarc'h, F., Nono, M., Nicolai, T., & Durand, D. (2009). Heat-induced aggregation of
564 whey proteins in the presence of kappa-casein or sodium caseinate. *Food*
565 *Hydrocolloids, 23*, 1103–1110.
566 Hambræus, L., & Lönnerdal, B. (2003). Nutritional aspects of milk proteins. In P. F. Fox &
567 P. L. H. McSweeney (Eds.), *Advanced dairy chemistry* (3rd edn., Vol. 1, pp. 605–
568 645). New York, NY, USA: Springer US.
569 Huffman, L. M., & Harper, W. J. (1999). Maximizing the value of milk through separation
570 technologies. *Journal of Dairy Science, 82*, 2238–2244.
571 Hussain, R., Gaiani, C., Jeandel, C, Ghanbaja, J., & Scher, J. (2012). Combined effect of heat
572 treatment and ionic strength on the functionality of whey proteins. *Journal of Dairy*
573 *Science, 95*, 6260–6273.
574 IDF. (2014). *Milk. Determination of nitrogen content. Part 1/2. Determination of nitrogen*
575 *content using the Kjeldahl method*. Brussels, Belgium: International Dairy Federation.
576 Jang, H. D., & Swaisgood, H. E. (1990). Disulfide bond formation between thermally
577 denatured β-lactoglobulin and κ-casein in casein micelles. *Journal of Dairy Science,*
578 *73*, 900–904.
579 Jean, K., Renan, M., Famelart, M.-H., & Guyomarc'h, F. (2006). Structure and surface
580 properties of the serum heat-induced protein aggregates isolated from heated skim
581 milk. *International Dairy Journal, 16*, 303–315.
582 Jeurnink, T. J. M., & De Kruif, K. G. (1995). Calcium-concentration in milk in relation to
583 heat stability and fouling. *Netherlands Milk and Dairy Journal, 49*, 151–165.

584 Joyce, A. M., Brodkorb, A., Kelly, A. L., & O'Mahony, J. A. (2016). Separation of the
585 effects of denaturation and aggregation on whey-casein protein interactions during the
586 manufacture of a model infant formula. *Dairy Science and Technology*, 1–20.
587 Kehoe, J. J., & Foegeding, E. A. (2010). Interaction between β-casein and whey proteins as a
588 function of pH and salt concentration. *Journal of Agricultural and Food Chemistry, 59*, 349–355.
590 Kehoe, J. J., & Foegeding, E. A. (2014). The characteristics of heat-induced aggregates
591 formed by mixtures of β-lactoglobulin and β-casein. *Food Hydrocolloids, 39*, 264–
592 271.
593 Kehoe, J. J., Wang, L., Morris, E. R., & Brodkorb, A. (2011). Formation of non-native β-
594 lactoglobulin during heat-induced denaturation. *Food Biophysics, 6*, 487–496.
595 Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of
596 bacteriophage T4. *Nature, 227*, 680–685.
597 Laiho, S., Ercili-Cura, D., Forssell, P., Myllärinen, P., & Partanen, R. (2015). The effect of
598 dynamic heat treatments of native whey protein concentrate on its dispersion
599 characteristics. *International Dairy Journal, 49*, 139–147.
600 Liyanaarachchi, W. S., Ramchandran, L., & Vasiljevic, T. (2015). Controlling heat induced
601 aggregation of whey proteins by casein inclusion in concentrated protein dispersions.
602 *International Dairy Journal, 44*, 21–30.
603 Morgan, P. E., Treweek, T. M., Lindner, R. A., Price, W. E., & Carver, J. A. (2005). Casein
604 proteins as molecular chaperones. *Journal of Agricultural and Food Chemistry, 53*,
605 2670–2683.
606 Morr, C. V. (1982). Functional properties of milk proteins and their uses as food ingredients.
607 In P. F. Fox (Ed.), *Developments in dairy chemistry* (Vol. 1, pp. 375–399). London,
608 UK: Appl. Sci. Publ.

Mounsey, J. S., & O'Kennedy, B. T. (2010). Heat-stabilisation of beta-lactoglobulin through interaction with sodium caseinate. *Milchwissenschaft-Milk Science International, 65*, 79–83.

Mounsey, J. S., O'Kennedy, B. T., Fenelon, M. A., & Brodkorb, A. (2008). The effect of heating on β-lactoglobulin–chitosan mixtures as influenced by pH and ionic strength. *Food Hydrocolloids, 22*, 65–73.

Ndoye, F. T., Erabit, N., Flick, D., & Alvarez, G. (2013). In-line characterization of a whey protein aggregation process: Aggregates size and rheological measurements. *Journal of Food Engineering, 115*, 73–82.

Nicolai, T., & Durand, D. (2013). Controlled food protein aggregation for new functionality. *Current Opinion in Colloid and Interface Science, 18*, 249–256.

Nutting, G. C. (1970). The byproducts of milk. In B. H. Webb, & Whittier, E. O. (Eds.), *Byproducts from milk* (pp. 16–17). Westport, USA: The Avi Publishing Company, Inc.

On-Nom, N., Grandison, A. S., & Lewis, M. J. (2012). Heat stability of milk supplemented with calcium chloride. *Journal of Dairy Science, 95*, 1623–1631.

Ono, T., & Takagi, T. (1986). Molecular weights of subunit components of bovine casein micelles. *Journal of Dairy Research, 53*, 547–555.

Parker, E. A., Donato, L., & Dalgleish, D. G. (2005). Effects of added sodium caseinate on the formation of particles in heated milk. *Journal of Agricultural and Food Chemistry, 53*, 8265–8272.

Patocka, G., Jelen, P., & Kalab, M. (1993). Thermostability of skimmilk with modified casein/whey protein content. *International Dairy Journal, 3*, 35–48.

Rattray, W., & Jelen, P. (1996). Thermal stability of skim milk with protein content standardized by the addition of ultrafiltration permeates. *International Dairy Journal, 6*, 157–170.

Renner, E., & Abd-El-Salam, M. (1991). *Application of ultrafiltration in the dairy industry*. London, UK: Elsevier Science Publishers Ltd.

Richter, K., Haslbeck, M., & Buchner, J. (2010). The heat shock response: Life on the verge of death. *Molecular Cell, 40*, 253–266.

Rose, D. (1961). Variations in the heat stability and composition of milk from individual cows during lactation. *Journal of Dairy Science, 44*, 430–441.

Ryan, K. N., & Foegeding, E. A. (2015). Formation of soluble whey protein aggregates and their stability in beverages. *Food Hydrocolloids, 43*, 265–274.

Ryan, K. N., Vardhanabhuti, B., Jaramillo, D. P., van Zanten, J. H., Coupland, J. N., & Foegeding, E. A. (2012). Stability and mechanism of whey protein soluble aggregates thermally treated with salts. *Food Hydrocolloids, 27*, 411–420.

Ryan, K. N., Zhong, Q., & Foegeding, E. A. (2013). Use of whey protein soluble aggregates for thermal stability - A hypothesis paper. *Journal of Food Science, 78*, R1105–R1115.

Sağlam, D., Venema, P., de Vries, R., & van der Linden, E. (2014). Exceptional heat stability of high protein content dispersions containing whey protein particles. *Food Hydrocolloids, 34*, 68–77.

Smithers, G. W. (2015). Whey-ing up the options – Yesterday, today and tomorrow. *International Dairy Journal, 48*, 2–14.

Tavares, G. M., Croguennec, T., Carvalho, A. F., & Bouhallab, S. (2014). Milk proteins as encapsulation devices and delivery vehicles: Applications and trends. *Trends in Food Science and Technology, 37*, 5–20.

657 Treweek, T. M., Thorn, D. C., Price, W. E., & Carver, J. A. (2011). The chaperone action of
658 bovine milk α_{S1}-and α_{S2}-caseins and their associated form α_S-casein. *Archives of*
659 *Biochemistry and Biophysics, 510*, 42–52.
660 Villumsen, N. S., Jensen, H. B., Thu Le, T. T., Møller, H. S., Nordvang, R. T., Nielsen, L. R.,
661 et al. (2015). Self-assembly of caseinomacropeptide as a potential key mechanism in
662 the formation of visible storage induced aggregates in acidic whey protein isolate
663 dispersions. *International Dairy Journal, 49*, 8–15.
664 Wijayanti, H. B., Bansal, N., & Deeth, H. C. (2014). Stability of whey proteins during
665 thermal processing: A review. *Comprehensive Reviews in Food Science and Food*
666 *Safety, 13*, 1235–1251.
667 Zhang, X., Fu, X., Zhang, H., Liu, C., Jiao, W., & Chang, Z. (2005). Chaperone-like activity
668 of β-casein. *International Journal of Biochemistry and Cell Biology, 37*, 1232–1240.
669 Zittle, C. A., & Dellamonica, E. S. (1956). Viscosity and flocculation of heated β-
670 lactoglobulin solutions: Effect of calcium concentration and pH. *Journal of Dairy*
671 *Science, 39*, 514–521.
672

Figure legends

Fig. 1. Flowchart of isolation and analysis of whey protein/κ-casein aggregates.

Fig. 2. Size-exclusion chromatography profiles (a) of MPC heated at 90 °C for 25 min, pH 7.2 with 2.5 mM (--), 5 mM (…) or without the addition of $CaCl_2$ (—), with the corresponding sodium dodecylsulphate-polyacrylamide gel electrophoresis profiles (b) of the derived fractions A, B and C under reducing (R) and non-reducing (NR) conditions.

Fig. 3. Scanning electron micrographs from size-exclusion chromatography fractions A (panel a) and C (panel c) dried on mica at 20 °C; with the size distributions by volume for fractions A (panel b) and C (panel d) as measured by dynamic light scattering.

Fig. 4. Heat stability (90 °C for 1 h) of size-exclusion chromatography 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.

Fig. 5. Atomic force microscopy 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.

Fig. 6. Whey protein (a), κ-casein (b) and α_S- and β-caseins (c) profiles of the size-exclusion chromatography fractions A, B and C with 0, 2.5 or 5 mM $CaCl_2$ addition (see Fig. 2a) 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.

Table 1

Protein content of heated milk protein concentrate supernatants at different pH and calcium chloride contents. [a]

pH	Calcium addition (mM)	Supernatant protein content (%, 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

[a] Milk protein concentrates were reconstituted at 13.5% (w/v) protein and heated at 90 °C for 25 min; centrifugation was at 38,360×*g* for 1 h.

Table 2

Characteristics and composition of the aggregates in fractions A, B and C (see Fig. 2) separated from milk protein concentrate heated at pH 7.2 with up to 5 mM added calcium chloride. [a]

Component	Fraction A			Fraction B			Fraction C		
	Calcium chloride (mM)			Calcium chloride (mM)			Calcium chloride (mM)		
	0	2.5	5	0	2.5	5	0	2.5	5
WP:κ-CN 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
WP:κ-CN 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
α_S- and β-CN (%, w/w, TP)	1 ± 2	0 ± 0	0 ± 0	14 ± 7	12 ± 1	11 ± 2	60 ± 18	65 ± 2	61 ± 2
Relative protein amount (%)	27 ± 2	31 ± 1	40 ± 3	48 ± 4	44 ± 1	37 ± 3	19 ± 1	16 ± 1	10 ± 2

[a] Abbreviations are: WP, whey protein; CN, casein; TP, total protein The relative amount of protein corresponds to the area under the chromatogram for each fraction compared with the total amount of eluted protein (from 35 to 100% of the column volume).

Table 3

Particle size and hydrophobicity 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). [a]

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

[a] Hydrophobicity measured as 8-ANS-relative fluorescence intensity.

Table 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.[a]

Component	WPI	MPC 80	Mix 1	Mix 2	Fraction A Calcium chloride (mM)			Fraction B Calcium chloride (mM)			Fraction C Calcium chloride (mM)		
					0	2.5	5	0	2.5	5	0	2.5	5
SF (% TP)	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
WP (% SF)	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
CN (% SF)	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
WP/κ-CN ratio	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

[a] Abbreviations are: WPI, whey protein isolate; MPC, milk protein concentrate; SF, soluble fraction; TP, total protein; WP, whey protein; CN, casein. Percentages are w/w. WP:κ-CN ratios were measured in the soluble phase. Mix 1 and mix 2: WP:κ-CN ratios of 1:1 and 1:0.7, respectively. WPI, MPC80, Mix 1 and Mix 2 were measured for comparison. The samples that coagulated during the heat stability test, preventing further analysis, are marked with an asterisk.

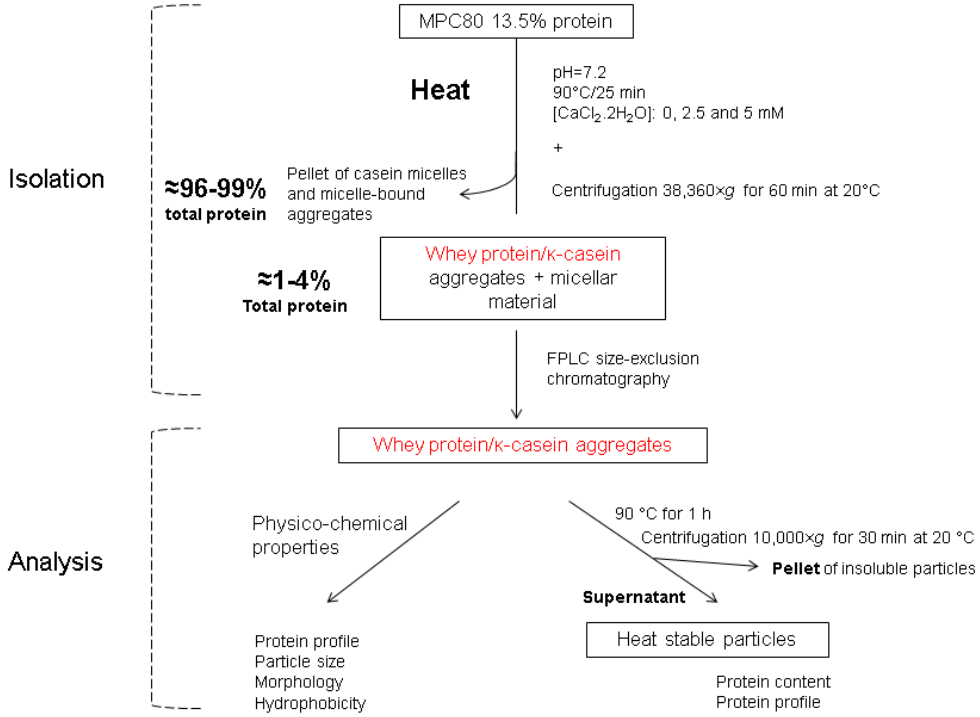
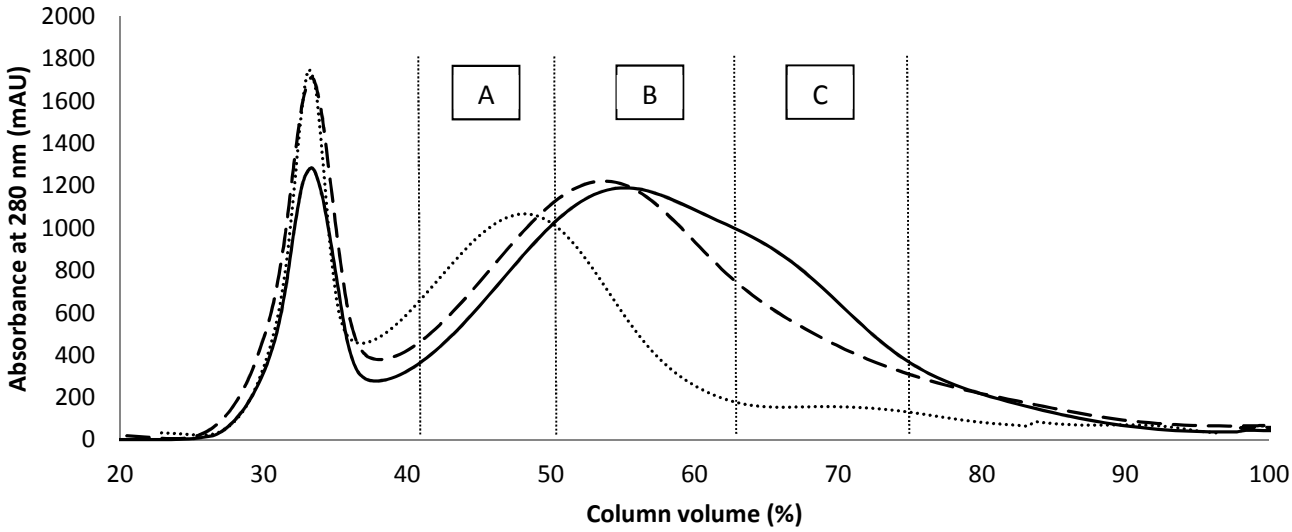

MPC80 13.5% protein
Heat
pH=7.2
90°C/25 min
[CaCl2.2H2O]: 0, 2.5 and 5 mM
+
Centrifugation 38,360×g for 60 min at 20°C
Isolation
≈96-99%
total protein
Pellet of casein micelles and micelle-bound aggregates
≈1-4%
Total protein
Whey protein/κ-casein aggregates + micellar material
FPLC size-exclusion chromatography
Whey protein/κ-casein aggregates
Analysis
Physico-chemical properties
Protein profile
Particle size
Morphology
Hydrophobicity
90 °C for 1 h
Centrifugation 10,000×g for 30 min at 20 °C
Pellet of insoluble particles
Supernatant
Heat stable particles
Protein content
Protein profile

Figure 1

(a)



(b)

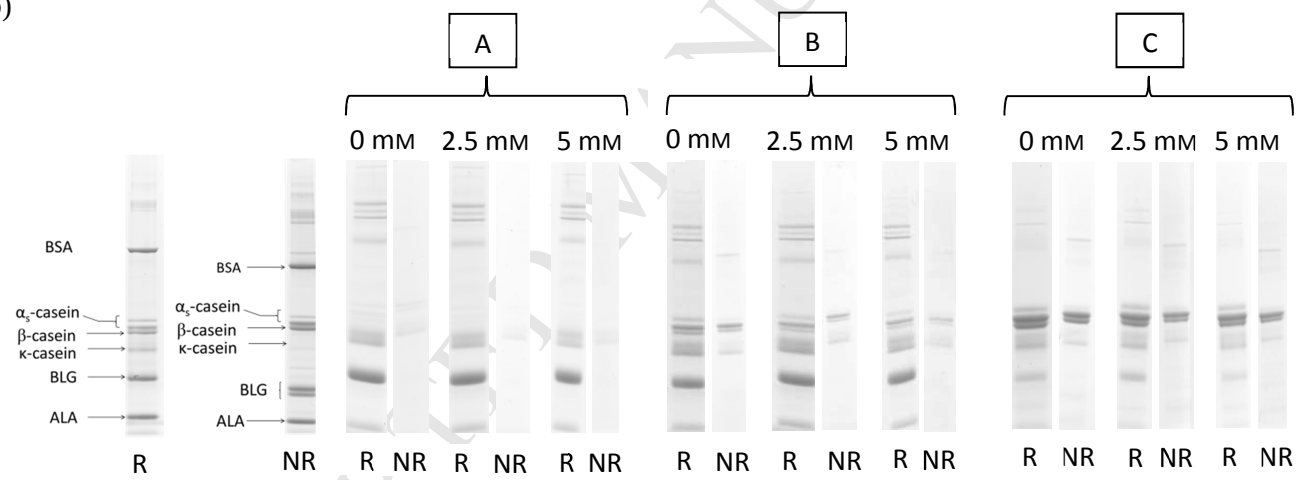


Figure 2

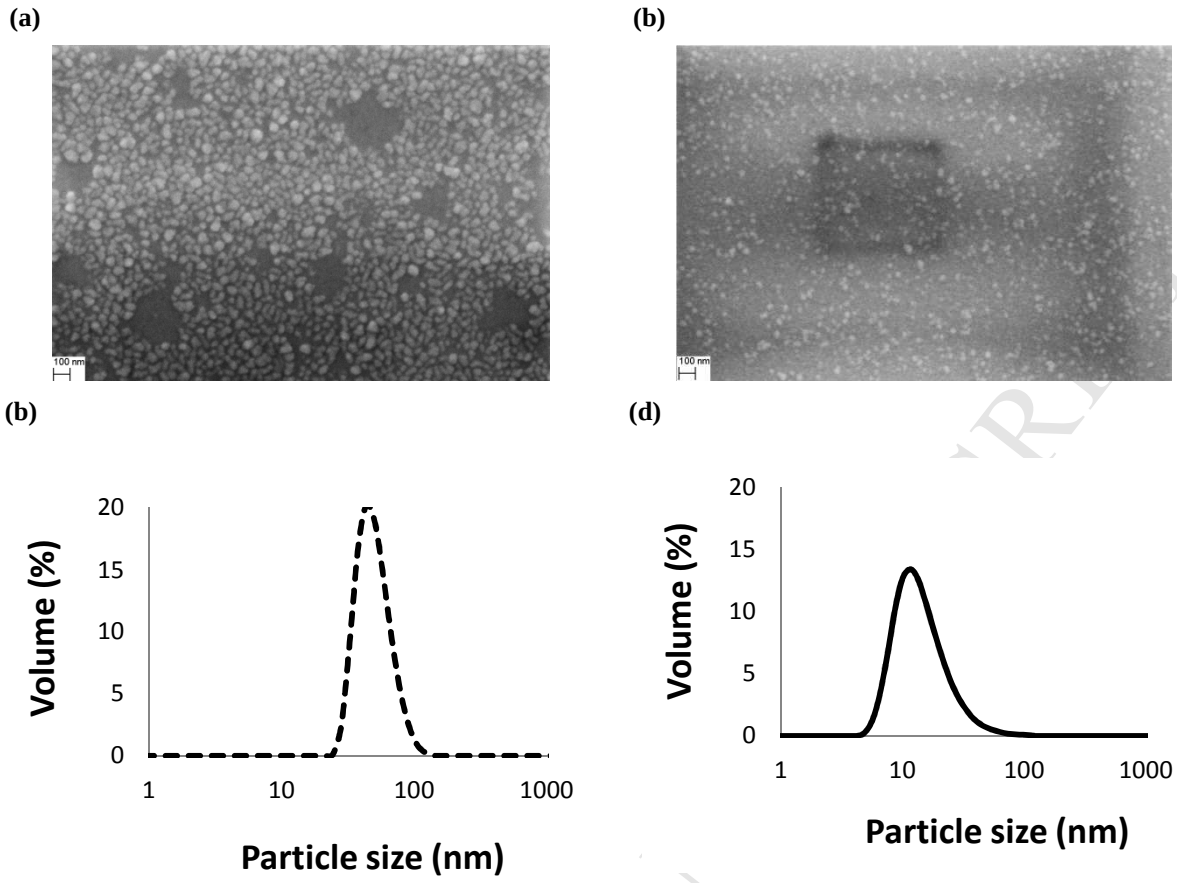


Figure 3

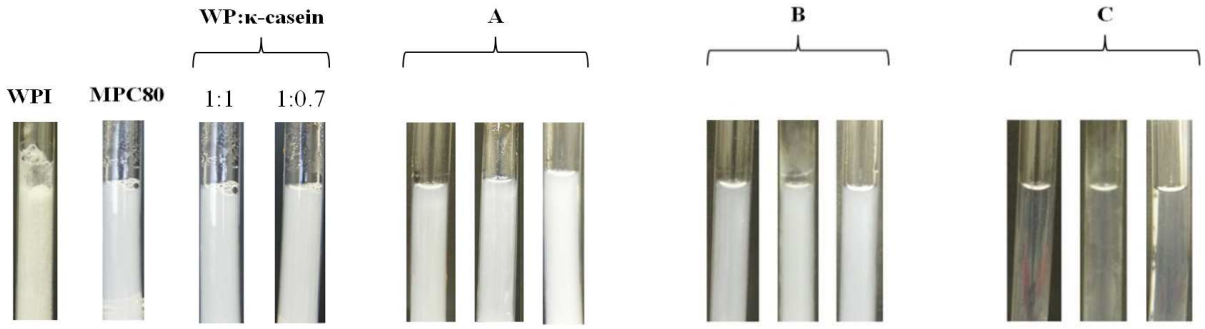
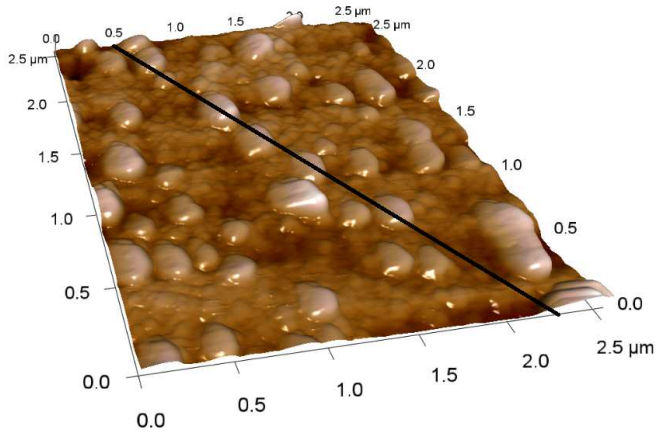
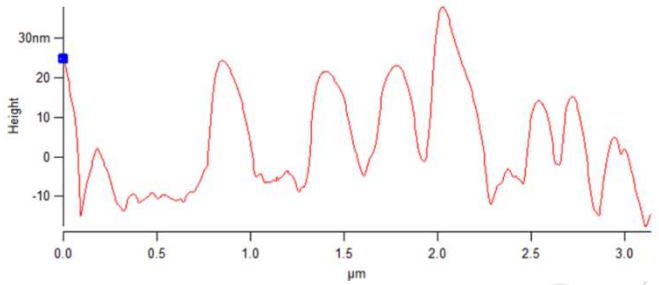


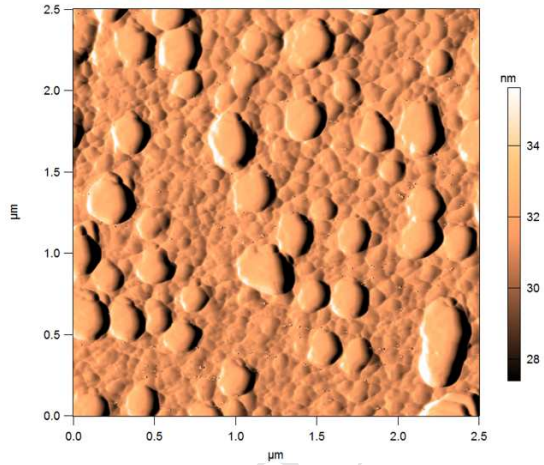
Figure 4



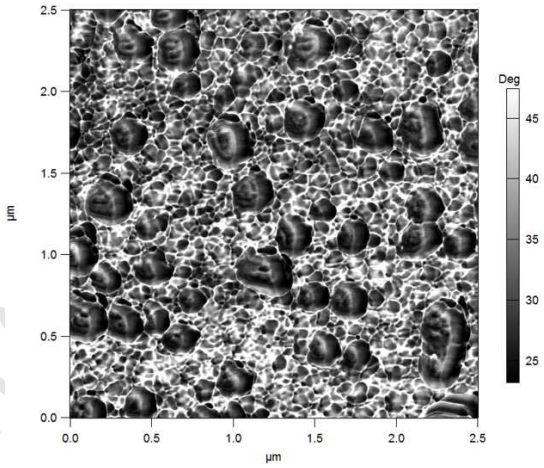
(a)



(b)



(c)



(d)

Figure 5

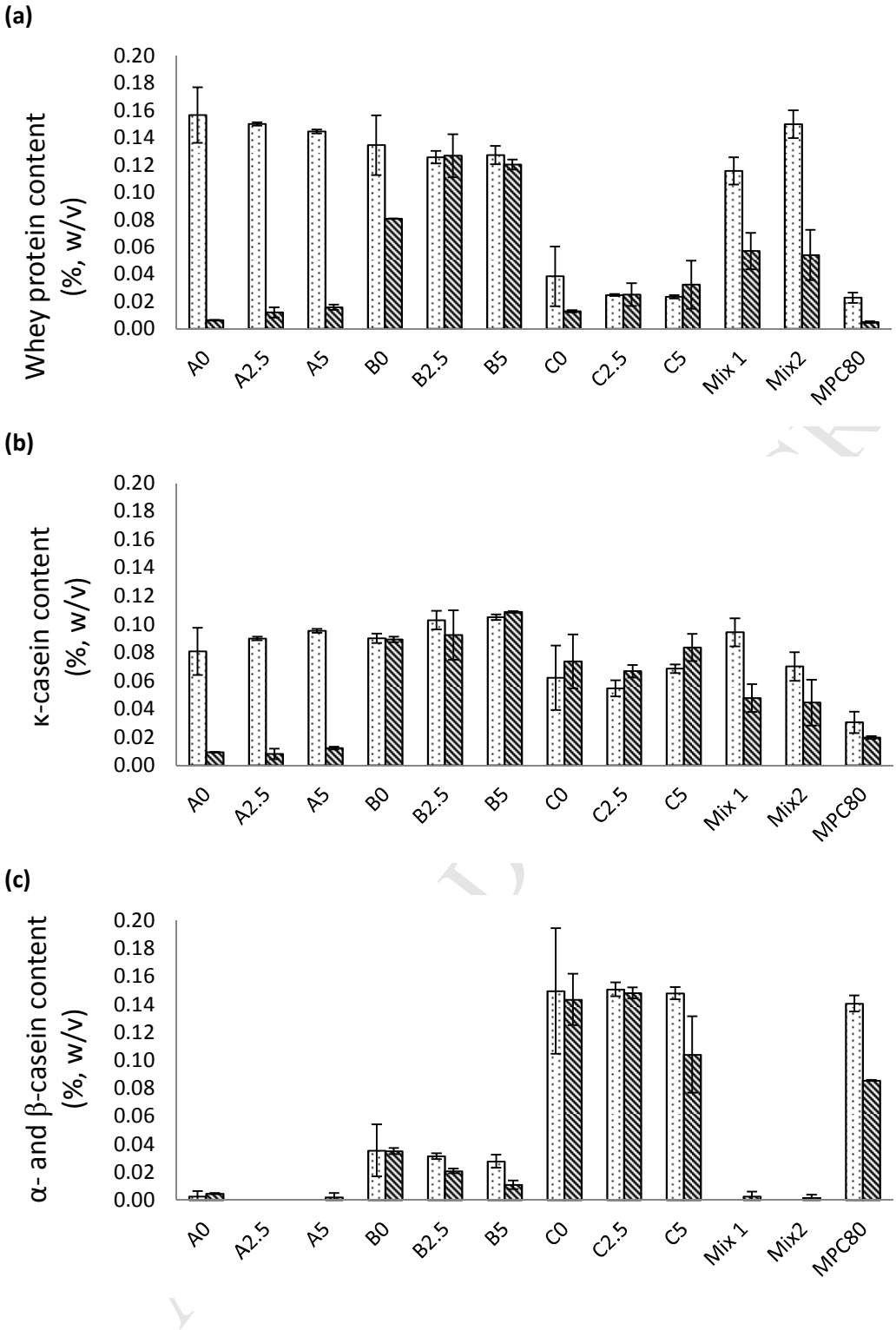

(a)
Whey protein content (%, w/v)
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
A0
A2.5
A5
B0
B2.5
B5
C0
C2.5
C5
Mix 1
Mix2
MPC80
(b)
κ-casein content (%, w/v)
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
A0
A2.5
A5
B0
B2.5
B5
C0
C2.5
C5
Mix 1
Mix2
MPC80
(c)
α- and β-casein content (%, w/v)
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
A0
A2.5
A5
B0
B2.5
B5
C0
C2.5
C5
Mix 1
Mix2
MPC80

Figure 6