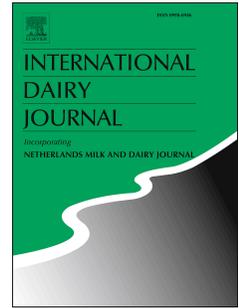


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Isolation and characterisation of  $\kappa$ -casein/whey protein particles from heated milk protein concentrate and role of  $\kappa$ -casein in whey protein aggregation

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1 **Isolation and characterisation of  $\kappa$ -casein/whey protein particles from heated milk**  
2 **protein concentrate and role of  $\kappa$ -casein in whey protein aggregation.**

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22

23

24 ABSTRACT

25

26 Milk protein concentrate (79% protein) reconstituted at 13.5% (w/v) protein was heated (90  
27 °C, 25 min, pH 7.2) with or without added calcium chloride. After fractionation of the casein  
28 and whey protein aggregates by fast protein liquid chromatography, the heat stability (90 °C,  
29 up to 1 h) of the fractions (0.25%, w/v, protein) was assessed. The heat-induced aggregates  
30 were composed of whey protein and casein, in whey protein:casein ratios ranging from 1:0.5  
31 to 1:9. The heat stability was positively correlated with the casein concentration in the  
32 samples. The samples containing the highest proportion of caseins were the most heat-stable,  
33 and close to 100% (w/w) of the aggregates were recovered post-heat treatment in the  
34 supernatant of such samples (centrifugation for 30 min at 10,000 × g).  $\kappa$ -Casein appeared to  
35 act as a chaperone controlling the aggregation of whey proteins, and this effect was stronger  
36 in the presence of  $\alpha_s$ - and  $\beta$ -casein.

37

38

39

## 40 1. Introduction

41

42 Dairy protein-based ingredients are widely used in the food industry due to their high  
43 nutritional value (Hambræus & Lönnerdal, 2003) and their functional properties, such as  
44 emulsification, foaming and encapsulation (Buggy, McManus, Brodkorb, Carthy, & Fenelon,  
45 2016; Doherty et al., 2011; Morr, 1982; Ryan, Zhong, & Foegeding, 2013; Tavares,  
46 Croguennec, Carvalho, & Bouhallab, 2014). Today, they are common ingredients in sport  
47 drinks, meal replacers and infant formula (Early, 2012; Smithers, 2015).

48 Heat treatment is widely applied in the dairy industry, normally to increase the shelf-  
49 life of products, but also to improve functional properties. Nevertheless, intensive thermal  
50 treatment can lead to undesirable outcomes such as gelation, Maillard reactions, and  
51 precipitation (i.e., fouling and sedimentation) of proteins. Bovine milk protein contains 80%  
52 (w/w) caseins and 20% (w/w) whey protein. However, the heat-induced coagulation of milk  
53 is a process dominated by the chemistry and reactivity of  $\beta$ -lactoglobulin, the major whey  
54 protein in milk. Inducing the aggregation of whey proteins into nano- to micro-sized  
55 particles, by pre-heating, is known to increase their heat stability (Joyce, Brodkorb, Kelly, &  
56 O'Mahony, 2016; Ryan et al., 2012) and has been extensively applied to whey proteins  
57 solution and skim milk (Laiho, Ercili-Cura, Forssell, Myllärinen, & Partanen, 2015; Ryan &  
58 Foegeding, 2015; Ryan et al., 2012; Sağlam, Venema, de Vries, & van der Linden, 2014).

59 Milk protein solutions with high thermal stability are characterised by low viscosity,  
60 low turbidity and high solubility after heating. These conditions are influenced by physico-  
61 chemical properties of the particles, such as surface hydrophobicity, aggregate size, shape  
62 and charge (Joyce et al., 2016; Ryan et al., 2013; Wijayanti, Bansal, & Deeth, 2014). As a  
63 result, the heat stability of proteins varies greatly with the pH at heating, the ionic strength of  
64 the dispersion, and the heat load applied.

65 Combined aggregates of whey proteins and caseins show higher heat stability than  
66 whey protein aggregates. In fact, a solution of acid whey protein prepared by ultrafiltration,  
67 containing around 3.2% (w/w) protein, gelled immediately when heated at 93 °C; however, a  
68 blend of whey protein and caseins, in the proportion 1:1 and heated under the same  
69 conditions was stable for up to 30 min of heating at 93 °C, i.e., at least 97% of the total  
70 protein initially present in solution were still soluble after heating (Patocka, Jelen, & Kalab,  
71 1993). It is generally accepted that this is due to a chaperone-like activity of the caseins.  
72 Chaperone activities can stabilise proteins from unfolding, aggregation and precipitation  
73 (Morgan, Treweek, Lindner, Price, & Carver, 2005). In dairy science and technology,  
74 aggregation is probably the more important phenomenon, as uncontrolled aggregation can  
75 lead to destabilisation.  $\alpha$ <sub>S</sub>-,  $\beta$ - and  $\kappa$ -casein have been shown to exhibit chaperone activity  
76 against aggregation (Mounsey & O'Kennedy, 2010). In fact, experiments carried out in a  
77 tubular heat exchanger at 95 °C (Guyomarc'h, Law, & Dalglish, 2003) and in Teflon tubes  
78 (4.6 cm<sup>3</sup>) in an oil bath at 145 °C (Kehoe & Foegeding, 2014) showed that the size of whey  
79 protein aggregates is reduced when the whey proteins are heated in the presence of casein  
80 micelles or  $\beta$ -casein.

81 Hydrophobic interactions, ionic interactions, Van der Waals interactions and  
82 disulphide bonding are responsible for the formation of reversible and irreversible aggregates  
83 between caseins and whey proteins (Guyomarc'h et al., 2003). The aggregates in the serum  
84 phase of heated milk are mainly composed of  $\kappa$ -casein,  $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin  
85 (Guyomarc'h et al., 2003). Bovine serum albumin (BSA), lactoferrin,  $\beta$ -casein and  $\alpha$ <sub>S</sub>-caseins  
86 are also involved in these aggregates, albeit to a minor extent (Donato & Dalglish, 2006).

87 Even though the formation of casein-whey protein aggregates have been shown (Jang  
88 & Swaisgood, 1990), part of the aggregates analysed may also be polymers of  $\kappa$ -casein  
89 (Farrell, Wickham, & Groves, 1998) or aggregates of whey proteins only (Boye, Alli, Ismail,

90 Gibbs, & Konishi, 1995). In the serum phase of skim milk heated at 90 °C for 10 min (pH  
91 6.7), the ratio of whey protein to  $\kappa$ -casein in the aggregates is in the range 1:0.2 to 1:0.7  
92 (Donato & Dalgleish, 2006). The whey protein and casein aggregates appear to be roughly  
93 spherical with a size ranging from 50 to 70 nm, which increases with the whey protein  
94 content of the solution (Beaulieu, Pouliot, & Pouliot, 1999; Liyanaarachchi, Ramchandran, &  
95 Vasiljevic, 2015). The molecular mass of the aggregates was estimated to be  $2 \times 10^7$  Da, the  
96 apparent isoelectric point of the aggregates was 4.5 in milk permeate, and the surface charge  
97 at pH 7.0 was 17 mV (Jean, Renan, Famelart, & Guyomarc'h, 2006).

98 The mechanism leading to the chaperone-like activity of caseins on whey proteins is  
99 still poorly understood and little research has been done on the heat stability of milk protein  
100 concentrates and isolated aggregates of caseins and whey proteins. However, some authors  
101 have proposed that the aggregates exhibit a higher charge density than the native whey  
102 proteins, limiting the interactions with other proteins (Guyomarc'h, Nono, Nicolai, & Durand,  
103 2009; Kehoe & Foegeding, 2014). The internal structure of the heat-induced aggregates is  
104 also affected by the presence of  $\kappa$ -casein; aggregates are less dense, and have a more porous  
105 structure, when they include  $\kappa$ -casein (Guyomarc'h et al., 2009).

106 Calcium chloride greatly influences the mineral equilibrium in milk and favours  
107 aggregation of whey protein during heating (On-Nom, Grandison, & Lewis, 2012). Calcium  
108 is naturally present in milk (31 mM) and is present at elevated concentration in milk protein  
109 concentrates when reconstituted at 13.5% (w/w) protein (84 mM calcium); it also plays a  
110 major role in the heat stability of proteins (Crowley et al., 2014). Heating conditions, calcium  
111 content and pH influence the aggregation of whey proteins and caseins, the morphology of  
112 the resulting aggregates, and the extent of aggregation (Nicolai & Durand, 2013). Calcium  
113 ions can bind to the carboxylate groups of the proteins, thereby shielding their repulsive  
114 charges. Thus, aggregates are more dense and less porous when calcium is added prior to

115 heating. Heating at high ionic strength allows the proteins to get closer and interact initially  
116 via hydrophobic interactions, followed by the gradual formation of disulphide bonds  
117 (Mounsey, O’Kennedy, Fenelon, & Brodkorb, 2008; Ndoye, Erabit, Flick, & Alvarez, 2013).

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

124

## 125 **2. Material and methods**

126

### 127 *2.1. Materials*

128

129 The milk protein concentrate (MPC) powder used in this study was produced on-site  
130 (Bio-functional Food Engineering Facility, Teagasc Food Research Centre Moorepark,  
131 Fermoy, Co. Cork, Ireland). The skim milk was pre-heated at a temperature in the range 40–  
132 50 °C and concentrated by ultrafiltration as described previously (Huffman & Harper, 1999;  
133 Renner & Abd-El-Salam, 1991). The concentrate was dried, giving a powder with a total  
134 protein content of 79% (w/w; Kjeldahl analysis, nitrogen to protein conversion factor of 6.38)  
135 of which 73% (w/w) was casein. The resulting MPC powder contained 8.9% (w/w) lactose,  
136 2.1% (w/w) calcium and 1.4% (w/w) phosphorus.

137 Whey protein isolate (WPI) Bipro<sup>®</sup> was purchased from Davisco Foods International  
138 (Eden Prairie, MN, USA) and contained 93.7% (w/w) protein. Freeze-dried  $\kappa$ -casein was  
139 purchased from Sigma Aldrich (St. Louis, MO, USA); the purity of the  $\kappa$ -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  $1.035 \text{ kg m}^{-3}$   
150 (Nutting, 1970). During the first step of rehydration, the reconstituted MPC was incubated at  
151  $45 \text{ }^{\circ}\text{C}$  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  $4 \text{ }^{\circ}\text{C}$  to ensure complete  
153 rehydration. On the following day, the solution was equilibrated to  $22 \text{ }^{\circ}\text{C}$  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  $22 \text{ }^{\circ}\text{C}$ , 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  $90 \text{ }^{\circ}\text{C}$  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  $\kappa$ -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  $22 \text{ }^{\circ}\text{C}$ . Weighed aliquots of unheated and heated samples (20 mL) were

165 then centrifuged for 1 h at  $38,360\times g$  and  $20\text{ }^{\circ}\text{C}$  in a centrifuge (Sorvall Lynx 6000) using the  
166 rotor Fiberlite F21-8x50y (Thermo Fisher Scientific, Waltham, MA, USA). After  
167 centrifugation, the fat layer was discarded and the supernatants were filtered through  $0.45\text{ }\mu\text{m}$   
168 hydrophilic filters (Sartorius, Gottingen, Germany).

169 To further purify and analyse the aggregates, the method developed by Parker,  
170 Donato, and Dalgleish (2005) was followed. Briefly, supernatant (0.8 mL) was fractionated  
171 by fast protein liquid chromatography (FPLC) on a size-exclusion column HiPrep 16/60 (GE  
172 Healthcare, Little Chalfont, Buckinghamshire, UK) containing Sephacryl S-500 HR beads  
173 (fractionation range  $4\times 10^4$ – $2\times 10^7$  Da). The absorbance was monitored at 280 nm by an  
174 AKTA Purifier 10 system (GE Healthcare), at a flow rate of  $1\text{ mL}\cdot\text{min}^{-1}$ . The buffer was a  
175 solution of bis-Tris propane 20 mM and 0.02%  $\text{NaN}_3$  at pH 6.7 or 7.2, depending on the  
176 original pH of the samples. Fractions (5 mL) were automatically collected using a Frac950  
177 and the total elution time for all samples was 120 min. The separation by FPLC was carried  
178 out at least in duplicate. The physico-chemical properties of the  $\kappa$ -casein/whey protein  
179 aggregates were measured on the FPLC fractions. Separately, the WPI and  $\kappa$ -casein powder  
180 were mixed overnight at  $4\text{ }^{\circ}\text{C}$  to reach a ratio of whey proteins to  $\kappa$ -caseins of 1:1 or 1:0.7,  
181 and were called mixture 1 and mixture 2, respectively.

182

### 183 2.3. *Protein content measurement*

184

185 Protein content of liquids and powder were determined by Kjeldahl (IDF, 2014); the  
186 protein to nitrogen conversion factor used was 6.38. Due to the very low protein content of  
187 the fractions collected by chromatography, the protein content of those samples was  
188 determined using a bicinchoninic acid assay (BCA) kit (Thermo Fisher Scientific); bovine

189 serum albumin was used as standard. The protein content of the centrifugal supernatants was  
190 also determined using this assay. All measurements were made in duplicate.

191

#### 192 2.4. Protein profile analysis

193

194 The protein profile of all samples were analysed by sodium dodecylsulphate-  
195 polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis under reducing or non-  
196 reducing conditions following a modified method of Laemmli (1970). Lithium  
197 dodecylsulphate (LDS) was used instead of SDS. NuPage bis-Tris gels at 12% (w/w)  
198 acrylamide were used with the NuPage cells and the NuPage power supply (Life  
199 Technologies, Carlsbad, California, USA), in line with the instructions for this system. The  
200 samples were first dissolved in the sample buffer at a ratio LDS:protein 200:1. A volume of 2  
201  $\mu\text{L}$  dithiothreitol (DTT) at 500 mM was added to reduce the disulphide bonds between  
202 proteins, while addition of DTT was omitted in the case of non-reducing samples. Following  
203 the manufacturer's recommendations, the mixture was heated at 70 °C for 10 min in a water  
204 bath. Sample (10  $\mu\text{L}$ ) containing 1.8  $\mu\text{g}$  of protein was loaded in each well and a constant  
205 voltage of 200 V was applied for 50 min. The gels were stained in a solution of 0.5%  
206 Coomassie Blue R250, 25% isopropanol and 10% acetic acid. Two stages of destaining were  
207 performed; the gels were first left for 1 h in a solution of 10% isopropanol and 10% acetic  
208 acid, and then held overnight in distilled water. Commercially sourced bovine serum albumin  
209 (BSA),  $\alpha_{\text{S1}}$ -casein ( $\alpha_{\text{S1}}$  and  $\alpha_{\text{S2}}$ -casein were quantified together),  $\beta$ -casein,  $\kappa$ -casein,  $\beta$ -  
210 lactoglobulin and  $\alpha$ -lactalbumin were used for calibration. The purified proteins were  
211 dissolved in the sample buffer, with or without reducing agent, and 10  $\mu\text{L}$  of this mixture of  
212 standard proteins was loaded per well. In total, five calibration points ranging from 0.06 to 2  
213  $\mu\text{g}$  of each protein standard per well were included for every gel. Because of unavoidable

214 variations in staining, the calibration points were run on the same gels as the samples. All  
215 gels were scanned using an Epson V700 film scanner (Epson, Suwa, Nagano, Japan) and  
216 analysed using the software ImageQuant TL (GE Healthcare). The scanner was not calibrated  
217 for optical density. Therefore, the range of protein content of the samples and standards was  
218 chosen to be in the linear, and thus unsaturated, region of the scanner. The quantification of  
219 the samples was deduced by plotting the known protein content of the purified proteins as a  
220 function of the integrated intensity of the standard bands.

221

### 222 2.5. *Measurement of hydrodynamic diameter*

223

224 The hydrodynamic diameter of the aggregates was determined by dynamic light  
225 scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments, Malvern, Worcestershire,  
226 UK). These measurements were carried out at 20 °C on the freshly-collected fractions. All  
227 samples were equilibrated at room temperature for 120 s in the instrument prior to  
228 measurement. The refractive index of the material was considered to be 1.450 and the  
229 absorption was 0.001. Considering the low protein content of the FPLC fractions, the  
230 refractive index and the viscosity of the dispersant were assumed the same as that of water,  
231 i.e., 1.330 and 1.0031 cP respectively. Measurements were carried out at a backscattering  
232 angle of 173° and at a wavelength of 633 nm using disposable polystyrene cuvettes. The  
233 average diameter was expressed as zeta-average, though the z-average values can be affected  
234 by particle characteristics like shape, compressibility, polydispersity, especially if the z-  
235 average values are greater than the inverse scattering vector  $1/q$  (here 38 nm). In this case, the  
236 measured particle size is expected to be very close to the real particle size for the afore-  
237 mentioned reasons. Each sample was measured three times. Each measurement consisted of

238 12 separate readings, and the zeta-average and volume diameter recorded were the means of  
239 these readings. The attenuation value was between 6 and 10.

240

#### 241 2.6. *Measurement of hydrophobicity*

242

243 The protocol used in this study was a modification of the method of Hussain et al.  
244 (2012). The probe 8-anilinonaphthalene-1-sulfonic acid ammonium salt (8-ANS) was used to  
245 determine the surface hydrophobicity of the  $\kappa$ -casein/whey protein aggregates. The FPLC  
246 fractions were diluted to 0.002% (w/v) protein in 20 mM bis-Tris propane at pH 7.2, and 8-  
247 ANS was added to 4 mL of sample to obtain a final concentration in the range 5–120  $\mu$ M 8-  
248 ANS. The mixtures were kept in the dark for 30 min before measurement in a Cary Eclipse  
249 fluorescence spectrophotometer (Agilent, Santa Clara, California, USA). The excitation  
250 wavelength was 350 nm for the casein/whey protein aggregates and 360 nm for the  $\beta$ -  
251 lactoglobulin standard, and the fluorescence spectrum ranged from 400 to 600 nm. The  
252 excitation/emission slits were set at 5 nm each. The fluorescence intensity was plotted against  
253 the concentration of 8-ANS, and the maximum relative fluorescence intensity (RFI) was used  
254 as an index of hydrophobicity.

255

#### 256 2.7. *High-resolution scanning electron microscopy*

257

258 High-resolution scanning electron microscopy (SEM) was used to evaluate the size  
259 and shape of the protein aggregates. Protein particle suspensions (10  $\mu$ L) were pipetted onto a  
260 freshly cleaved mica surface attached to an SEM stub. After air drying at 20 °C, the samples  
261 were sputter coated with chromium prior to examination in a field emission scanning electron  
262 microscope (Supra 40VP; Carl Zeiss Ltd., Oberkochen, Baden-Württemberg, Germany).

263 Images (8 bit, TIFF) were acquired at 2 kV accelerating voltage using the in-lens secondary  
264 electron detector.

265

## 266 2.8. *Atomic force microscopy*

267

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

273

## 274 2.9. *Heat stability*

275

276 The FPLC fractions were concentrated using centrifugal concentration (Vivaspin 20  
277 100,000 MWCO, Sartorius, Gottingen, Lower Saxony, Germany) to 0.25% (w/v) protein.  
278 The heat stability of the concentrated FPLC fractions, MPC, WPI and the mixtures of whey  
279 proteins and  $\kappa$ -caseins were assessed by heating the samples in a water bath at 90 °C for 1 h.  
280 All samples contained 10 mM bis-Tris propane and 0.01% (w/v)  $\text{NaN}_3$ , without mineral or  
281 lactose standardisation. Glass tubes (120 mm length, 7 mm diameter and 1.5 mm wall  
282 thickness) were filled with 2.5 g of sample at 0.25% (w/v) protein. After heating, 2 mL of  
283 each heated sample was centrifuged at  $10,000 \times g$  for 30 min using a 5417R Eppendorf  
284 centrifuge with rotor F45-30-11 (Eppendorf, Hamburg, Germany). Under these conditions,  
285 the proteins present in the supernatants were considered as soluble. The supernatants were  
286 then analysed by SDS-PAGE as described in Section 2.4.

287

288 2.10. *Statistical data analysis*

289

290 All experiments were carried out using the same batch of powder. The data are

291 expressed as means with standard deviations of data from two independent replicates.

292

293 **3. Results and discussion**

294

295 3.1. *Influence of calcium chloride on the solubility of caseins and whey proteins in MPC*

296

297 Milk protein concentrate reconstituted at 13.5% (w/v) protein was heated at 90 °C for

298 25 min and the casein micelles were removed by centrifugation, together with the whey

299 proteins attached to the micelles and the large and dense whey protein aggregates. Table 1

300 shows the protein concentration in the supernatant as a function of the pH at heating and the

301 addition of calcium. At pH 6.7, none of the samples gelled after 25 min of heating at 90 °C.

302 However, the protein concentration recovered in the supernatants was significantly lower in

303 the samples heated at pH 6.7 (1–2%) than in the samples heated at pH 7.2 (4%). At pH 6.7,

304 the addition of calcium significantly reduced the concentration of protein recovered in the

305 supernatant. The protein content in the supernatant, when heated at pH 7.2, did not change

306 significantly with the calcium content.

307 The proteins in the supernatants from the heated MPC were separated by FPLC into

308 three fractions; the composition and volume of each fraction are presented in Fig. 2 and Table

309 2. Three fractions from the sample at pH 7.2 were collected between 50 and 60 min, 60 and

310 75 min, 75 and 90 min elution time from the size-exclusion column, equivalent to 42–50%,

311 50%–63% and 63–76% column volumes, respectively. The fractions collected had increasing

312 amount of casein and increased ratios of  $\kappa$ -casein to whey protein as a function of the elution

313 time, illustrating the heterogeneity of the aggregates within one sample of heated MPC. Little  
314 or no absorbance at 280 nm was measured for the samples heated at pH 6.7 with 2.5 or 5 mM  
315 calcium chloride. The protein content in the supernatants may have been too low to be  
316 detected by the absorbance detector, while the aggregates larger than 450 nm may have been  
317 removed by centrifugation or filtration prior to separation by FPLC. High calcium activity  
318 has been identified as a major factor influencing the heat stability of concentrated milk  
319 (Jeurnink & De Kruif, 1995; Rattray & Jelen, 1996; Rose, 1961; Zittle & Dellamonica,  
320 1956). Decreasing pH promotes a shift in the mineral equilibrium of milk, causing the release  
321 of ionic calcium into the serum. The high calcium content, coupled with a low pH,  
322 contributes to the formation of large aggregates, which sediment easily during the  
323 centrifugation step. This explains the lower absorbance on FPLC (Fig. 2) and lower protein  
324 recovery in the supernatants for the samples containing calcium (Table 1). The addition of  
325 calcium also affected the distribution of the aggregates in the samples; without addition of  
326 calcium, there were 1.4 times more aggregates in fraction A than in fraction C (integrated  
327 area of each fraction on the absorbance signal of FPLC), while the opposite was observed  
328 when 5 mM calcium was added before heating.

329

### 330 3.2. *Composition of the fractions from size-exclusion chromatography*

331

332 SDS-PAGE under reducing conditions (Fig. 2) showed the presence of proteins in  
333 each fraction; by comparing the reducing and non-reducing conditions, the extent of  
334 disulphide bond formation was evaluated. The bands for BSA,  $\alpha$ -lactalbumin and  $\beta$ -  
335 lactoglobulin appeared stronger in reducing conditions, confirming that these proteins were  
336 mainly involved in aggregate formation through disulphide bonding. However, the major  
337 whey protein involved in the covalent aggregates was  $\beta$ -lactoglobulin.  $\kappa$ -Casein was also

338 involved in the aggregates by disulphide bonding (58–87%, w/w, of  $\kappa$ -casein in the fractions).  
339 Fractions B and C contained  $\alpha_S$ - and  $\beta$ -casein, while fraction C contained the highest  
340 proportion of  $\kappa$ -casein,  $\beta$ -casein and  $\alpha_S$ -casein, and the lowest ratio of whey protein to  $\kappa$ -  
341 casein. With the addition of calcium chloride, the whey protein/ $\kappa$ -casein ratio and the  
342 percentage of  $\alpha_S$ - and  $\beta$ -caseins in each fraction remained unchanged.

343 Table 3 presents the physico-chemical characteristics of each fraction. The aggregate  
344 size ranged from 29 to 59 nm, corresponding to the size range observed in previous studies  
345 (del Angel & Dalgleish, 2006). The size of the aggregates decreased with the concentration of  
346 caseins. Thus, the ratio of whey proteins to caseins positively affected the size of the  
347 aggregates, as observed in previous studies (Guyomarc'h et al., 2009; Liyanaarachchi et al.,  
348 2015). The chaperone activity of caseins has been reported to reduce whey protein  
349 aggregation (Kehoe & Foegeding, 2010; Mounsey & O'Kennedy, 2010); the chaperone  
350 activity of a biomolecule refers to its ability to protect another biomolecule against unfolding,  
351 aggregation and precipitation. Thus, the difference in particle size between fractions A and B  
352 (Table 3) may be the result of the chaperone-like activity of the non-covalently bound caseins  
353 ( $\alpha_S$ -,  $\beta$ - and  $\kappa$ -casein) to whey proteins in fraction B (Fig. 2).

354 Previous studies have shown that micellar material can probably associate into small  
355 micelles of size 10–20 nm, which elute after the maximum of the aggregate peak on the  
356 FPLC profile (Guyomarc'h et al., 2003; Ono & Takagi, 1986), corresponding to fractions B  
357 and C in our study. The size range reported for these “mini-micelles” (10–20 nm) is close to  
358 that of the aggregates in fractions B and C (Ono & Takagi, 1986). The formation of such  
359 small, dispersed micelles amongst the aggregates of whey proteins and caseins may have  
360 caused a shift in the hydrodynamic size measurement. The ratio of  $\kappa$ -casein:[ $\alpha_S$ -casein +  
361  $\beta$ -casein] that was not covalently bound to whey proteins in our study was up to 1:9. This  
362 ratio was comparable with those found by Donnelly, McNeill, Buchheim, and McGann

363 (1984) for skim milk fractionated by size-exclusion chromatography. These authors found  
364 that casein micelles in skim milk had sizes ranging from 62 to 154 nm and ratios of  $\kappa$ -  
365 casein:[ $\alpha_S$ -casein +  $\beta$ -casein] ranging from 1:6 to 1:21. These results indicate that, in fractions  
366 B and C, the amount of  $\kappa$ -casein that was not covalently bound to the whey proteins or self-  
367 aggregated was sufficient to stabilise  $\alpha_S$ - and  $\beta$ -casein in the form of “mini-micelles”.  
368 However, the presence of “mini-micelle” in the fractions and their effect in this study cannot  
369 be dissociated from those of the aggregates containing whey proteins.

370 SEM images (Fig. 3) show the morphology and the size distribution of the largest and  
371 the smallest aggregates. The smaller aggregates had a narrower size distribution than the  
372 larger aggregates, as measured by DLS (Table 3). SEM micrographs indicated that the  
373 smaller aggregates (Fig. 3) were rounded and more spherical than the larger aggregates,  
374 which had a slightly angular shape. The sphericity of the heat-induced particles made of  
375 caseins and whey proteins are less likely to increase the viscosity of the solutions to which  
376 they would be added; thus, they may be a potential candidate for use as ingredients in food  
377 applications (Ryan et al., 2013).

378 Some fractions were also analysed using atomic force microscopy (AFM) in air (Fig.  
379 5). The images of aggregates show a near-spherical shape. The cross-section of the height  
380 image showed a particle size of approximately 25 to 40 nm, which would correspond to a  
381 relatively low polydispersity. Due to tip broadening in AFM, the height of the recorded  
382 particles is generally used for estimation of size. However, size measurements by AFM have  
383 to be considered with extreme caution as the protein samples have been dehydrated and  
384 deposited on mica, which may lead to a complete collapse of the protein particles. However,  
385 dynamic light-scattering analysis of the sample shown in Fig. 5 gave a surprisingly similar  
386 particle size (z-average) of 53 nm.

387

388 3.3. *Effect of  $\kappa$ -casein content and pre-heat treatment on the heat stability of whey*  
389 *proteins during heating*

390

391 The heat stability of the isolated fractions after heating at 90 °C for 1 h (pH 7.2) at  
392 0.25% (w/v) protein was assessed (Fig. 4 and Table 4). To compare the stability of the  
393 nanoparticles in buffer after heating, the samples were centrifuged at 10,000 × g for 30 min.  
394 The soluble protein content after heating and centrifugation (10,000 × g for 30 min) and the  
395 visual properties of the samples after heating were the criteria used to determine the heat  
396 stability of the samples.

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

407 After heating of fraction A, 4–11% (w/w) of the whey proteins were recovered in the  
408 supernatant. The comparison of the sample composition before and after the heat stability test  
409 (Fig. 6) shows a significant loss in whey proteins and  $\kappa$ -casein in fraction A. For comparison,  
410 a mix of whey protein and  $\kappa$ -casein (at a ratio 1:0.7 or 1:1), that did not undergo any pre-heat  
411 treatment, showed a protein recovery of 43–47% (w/w), and around 36–49% (w/w) of the  
412 whey proteins were recovered after heat stability test. The mixture containing whey proteins

413 and  $\kappa$ -casein at a ratio 1:0.7 and fraction A had a similar initial composition of caseins and  
414 whey proteins; the only difference between these two samples was the pre-heat treatment for  
415 fraction A. Thus, the unheated mixture of whey proteins and  $\kappa$ -casein was more stable than  
416 aggregates of whey proteins and  $\kappa$ -casein at a ratio of 1:0.7. Therefore, regardless of whether  
417  $\kappa$ -casein involved in aggregates or not,  $\kappa$ -casein apparently exhibited a stabilising effect on  
418 the whey proteins.

419 When comparing the two mixtures of whey proteins and  $\kappa$ -casein at ratios 1:1 and  
420 1:0.7, no difference was observed in protein recovery or composition after the heat stability  
421 test. Therefore, the maximum amount of native whey proteins that  $\kappa$ -casein can stabilise may  
422 have been reached at a whey protein to  $\kappa$ -casein ratio of 1:0.7. The compositions of the two  
423 mixtures may also be too close to exhibit a significant difference in heat stability.

424

#### 425 3.4. *Effect of casein profile on stability of whey proteins during heating*

426

427 The total protein recovered after heating in fraction B was around 80–89% (w/w).  
428 Fraction B contained aggregates of mean diameter 44 nm and with a whey protein to  $\kappa$ -casein  
429 ratio of 1:0.5 to 1:0.6, containing around 11–14% (w/w, total proteins) of  $\alpha$ <sub>S</sub>- and  $\beta$ -caseins.  
430 At equal ratios of whey protein to  $\kappa$ -casein, fraction B showed significantly higher heat  
431 stability than fraction A and the mixtures of unheated whey protein and  $\kappa$ -casein. The non-  
432 negligible amount of  $\alpha$ <sub>S</sub>- and  $\beta$ -casein may have provided an additional stabilisation to the  
433 aggregates.

434

#### 435 3.5. *Effect of casein structure on whey protein stability during heating.*

436

437 Fraction C, made up of aggregates of mean diameter 32 nm, had the highest content  
438 of  $\kappa$ -casein and a whey protein to  $\kappa$ -casein ratio of 1:1.2 to 1:2.0. This fraction also contained  
439 the highest amount of  $\alpha_s$ - and  $\beta$ -caseins (60–65% of the total proteins). After one hour of  
440 heating, sample C was still transparent and the recovery of protein aggregates in the  
441 supernatant was approximately 90–98% (w/w, total protein). Therefore, the amount of  
442 soluble whey protein and  $\kappa$ -casein in fraction C did not change significantly after heating  
443 (Fig. 6). In agreement with the previous observations on fractions A and B, the high casein  
444 content may explain this greater heat stability. The whey proteins were still soluble after  
445 heating, indicating that caseins may have a chaperone-like activity and protect whey proteins  
446 against sedimentation. The same test performed on MPC without pre-heat treatment gave a  
447 lower soluble protein content, of 50% (w/w, total protein). Fig. 6 also illustrates the  
448 significant loss in  $\alpha_s$ - and  $\beta$ -casein (39%, w/w, initial  $\alpha_s$ - and  $\beta$ -casein), of whey proteins  
449 (79%, w/w, initial whey protein), and  $\kappa$ -casein (35%, w/w, initial  $\kappa$ -casein) in MPC after  
450 heat-stability testing at 90 °C. In the mixtures and in the fractions, the caseins are present  
451 either in individual and soluble form, associated with the whey proteins or present in mini-  
452 micelles. Caseins in MPC are likely to be organised in micelles with average size 150-200 nm  
453 (Dalglish & Corredig, 2012), and consequently are less available for association with whey  
454 proteins than the soluble casein of the heated supernatant of MPC. The dissociation of  $\kappa$ -  
455 casein at pH 7.2, together with the prolonged heating, could have destabilised the casein  
456 micelles, leading to the precipitation of most proteins.

457

### 458 3.6. *Hydrophobicity of casein and whey protein aggregates*

459

460 The aggregates in fraction C were significantly less hydrophobic than those in  
461 fractions A and B. Caseins are relatively hydrophobic and  $\kappa$ -casein is the second most

462 hydrophobic casein after  $\beta$ -casein, with an average hydrophobicity of 5.1 kJ per residue  
463 (Bigelow, 1967). However,  $\kappa$ -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,  $\kappa$ -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  $\kappa$ -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  $\alpha$ <sub>S</sub>- and  $\beta$ -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  $\alpha$ <sub>S</sub>- and  $\beta$ -casein are associated  
485 with the aggregates of whey proteins and  $\kappa$ -casein by hydrophobic interactions. It is possible  
486 that  $\alpha$ <sub>S</sub>- and  $\beta$ -casein associate or dissociate from the whey protein and  $\kappa$ -casein aggregates

487 during heat treatment, and thus the hydrophobicity of the  $\kappa$ -casein and whey protein  
488 aggregates may change, which would influence the heat stability of the aggregates.

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

#### 500 **4. Conclusions**

501  
502 The presence of caseins provided stabilization of whey protein aggregates during  
503 heating. In particular,  $\kappa$ -casein exhibited a chaperone-like activity at a whey protein to  $\kappa$ -  
504 casein ratio of 1:0.7, for both heated and unheated mixtures of whey proteins and  $\kappa$ -casein.  
505 Pre-heat treatment reduced the chaperone-like activity of  $\kappa$ -casein. The presence of  $\alpha_S$ - and  $\beta$ -  
506 casein in solution contributed to an enhanced heat stability of the whey proteins. These  
507 results are a starting point for a better understanding of the heat stability of milk protein and  
508 casein aggregates. However, the mechanism of chaperone-like activity of  $\kappa$ -casein needs  
509 further investigation.

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512

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517

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## 1 **Figure legends**

2 **Fig. 1.** Flowchart of isolation and analysis of whey protein/ $\kappa$ -casein aggregates.

3 **Fig. 2.** Size-exclusion chromatography profiles (a) of MPC heated at 90 °C for 25 min, pH  
4 7.2 with 2.5 mM (---), 5 mM (...) or without the addition of CaCl<sub>2</sub> (—), with the corresponding  
5 sodium dodecylsulphate-polyacrylamide gel electrophoresis profiles (b) of the derived  
6 fractions A, B and C under reducing (R) and non-reducing (NR) conditions.

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

10 **Fig. 4.** Heat stability (90 °C for 1 h) of size-exclusion chromatography fractions A, B and C.  
11 WPI, MPC80 and mixtures of whey protein and  $\kappa$ -casein (Mixes 1 and 2 had whey protein: $\kappa$ -  
12 casein ratios of 1:1 and 1:0.7, respectively) were also measured for comparison.

13 **Fig. 5.** Atomic force microscopy images showing (a) 3D height, (b) height across the cross-  
14 section marked in the 3D height image, (c) amplitude and (d) phase for a representative  
15 sample of the casein and whey protein aggregates in fraction A.

16 **Fig. 6.** Whey protein (a),  $\kappa$ -casein (b) and  $\alpha$ <sub>S</sub>- and  $\beta$ -caseins (c) profiles of the size-exclusion  
17 chromatography fractions A, B and C with 0, 2.5 or 5 mM CaCl<sub>2</sub> addition (see Fig. 2a) before  
18 (☐) and after (▣) heat stability testing (90 °C for 1 h). MPC80 and mixtures of whey protein  
19 and  $\kappa$ -casein (Mix 1 and 2 with whey protein: $\kappa$ -casein ratios of 1:1 and 1:0.7, respectively)  
20 were also measured for comparison.

21

**Table 1**

Protein content of heated milk protein concentrate supernatants at different pH and calcium chloride contents. <sup>a</sup>

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

<sup>a</sup> 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. <sup>a</sup>

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 <sub>S</sub> - 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

<sup>a</sup> 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).<sup>a</sup>

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

<sup>a</sup> 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.<sup>a</sup>

Component	WPI	MPC 80	Mix 1	Mix 2	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
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

<sup>a</sup> 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.

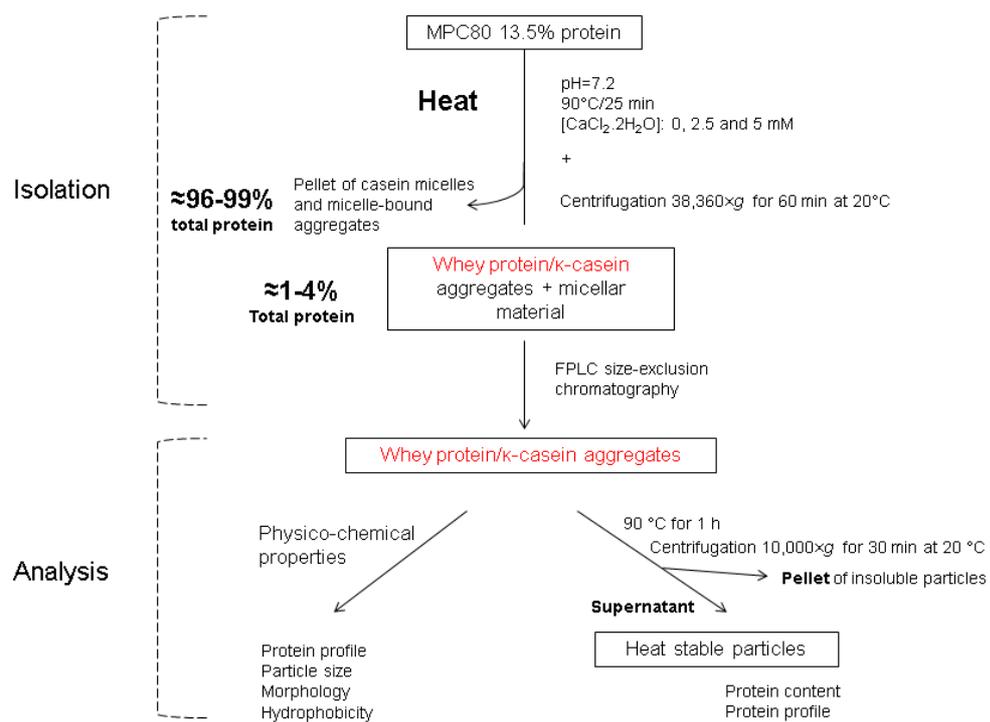


Figure 1

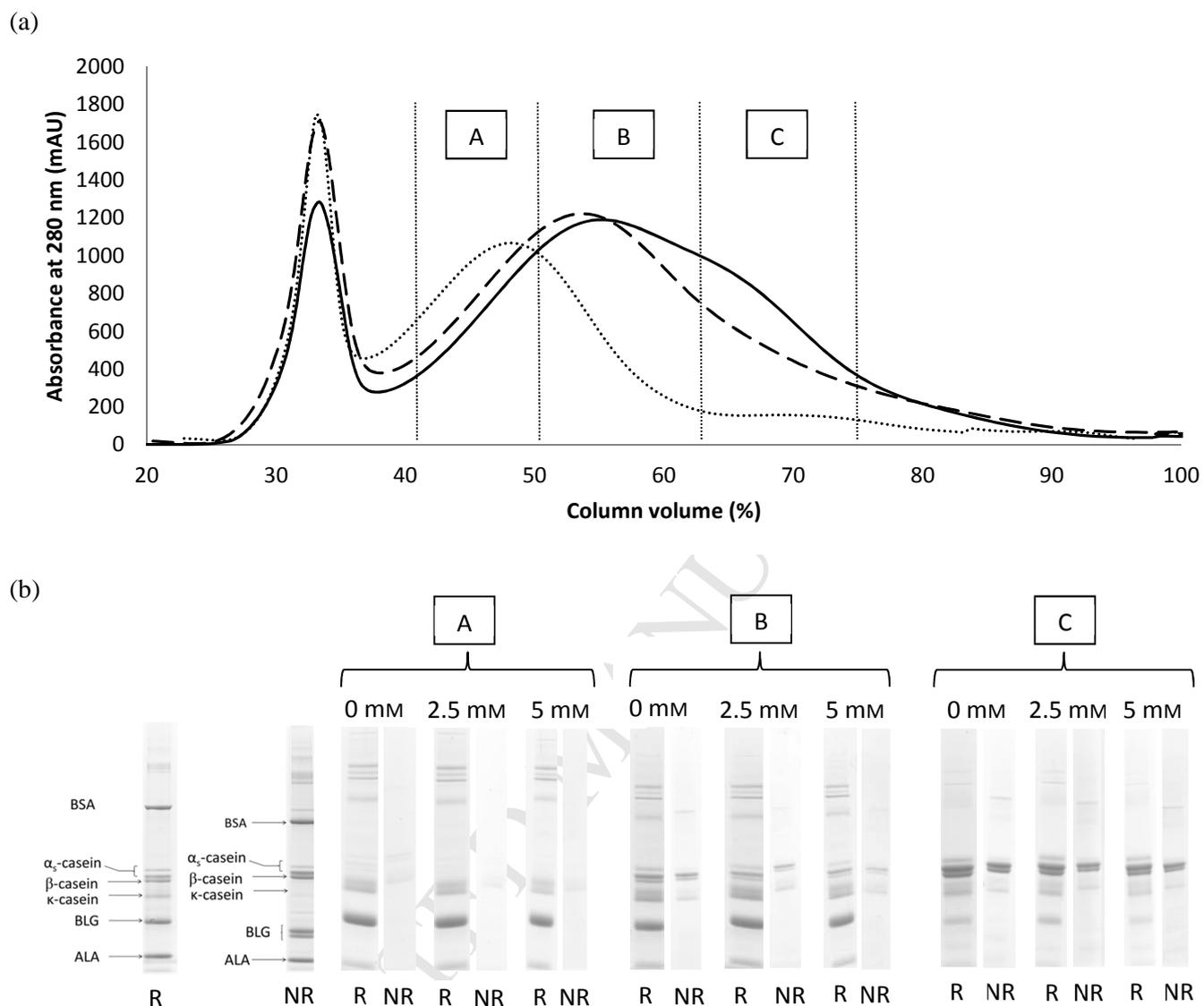


Figure 2

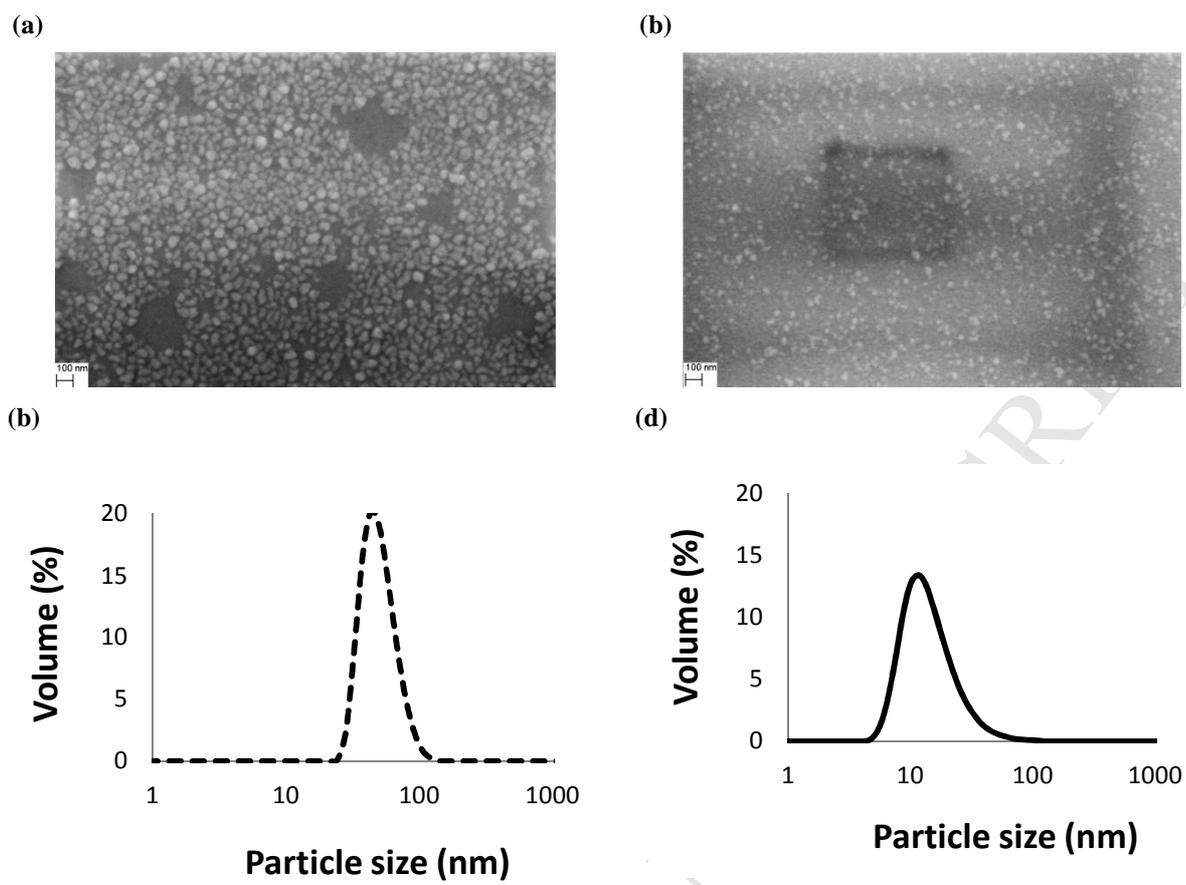


Figure 3

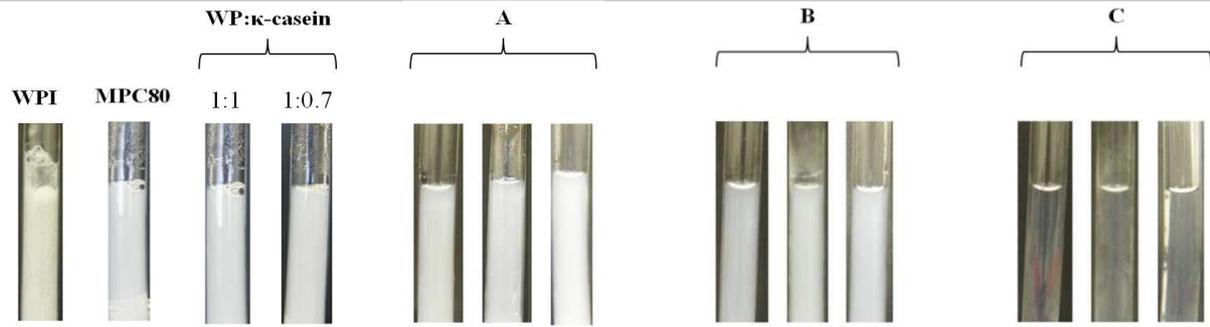


Figure 4

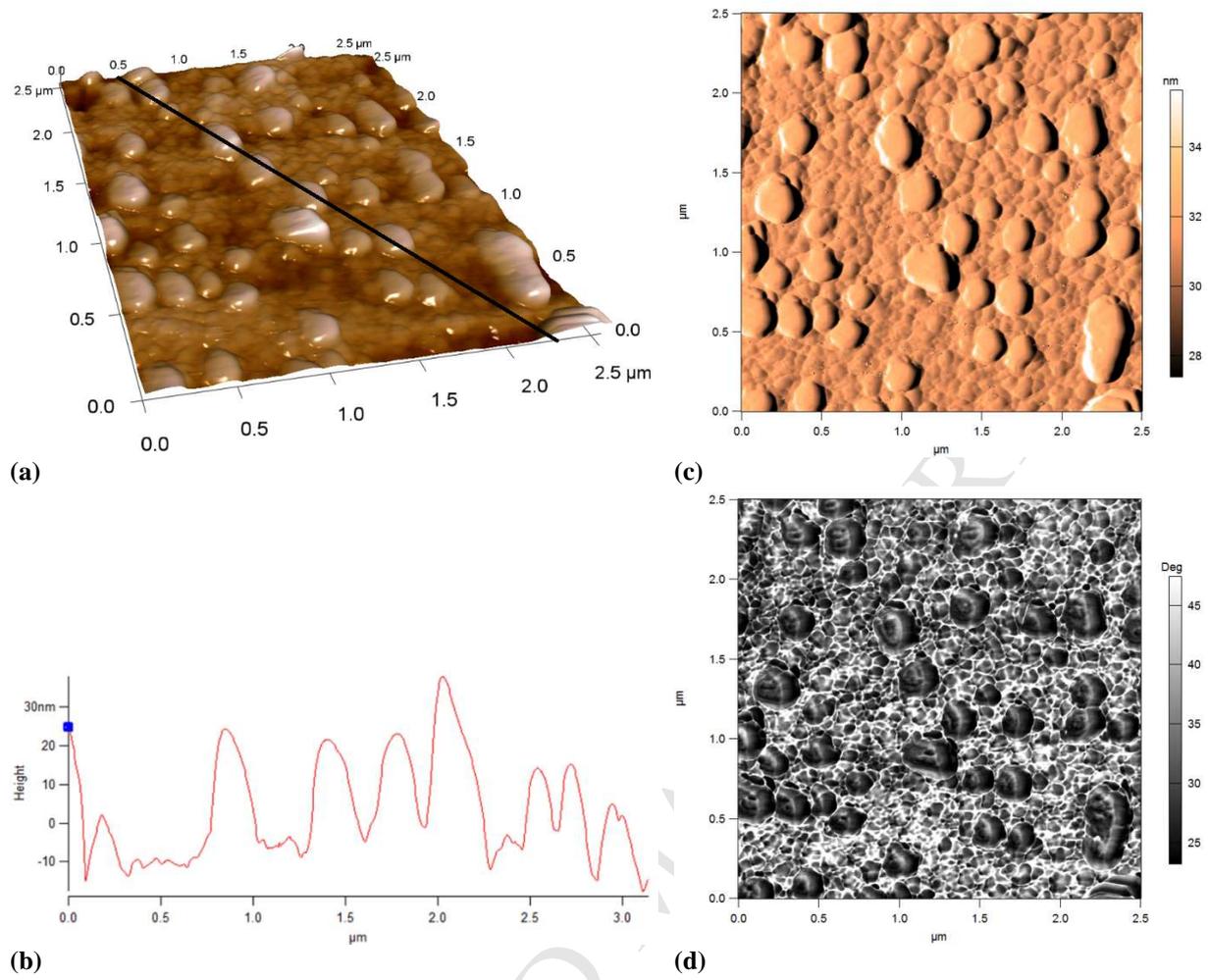


Figure 5

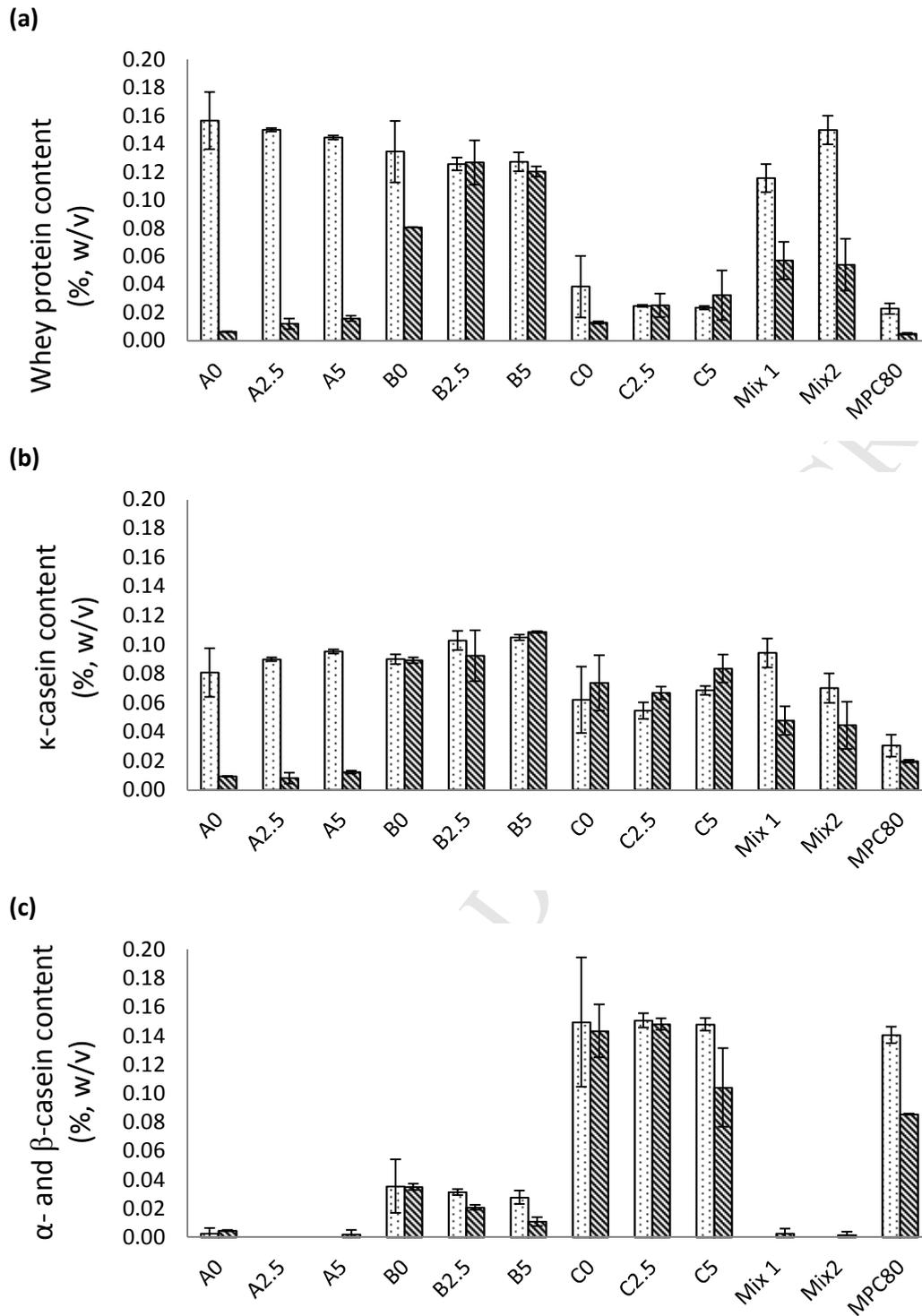


Figure 6