

1 **The inhibitory roles of native whey protein on the rennet gelation of bovine**  
2 **milk**

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15

16 **Abstract**

17 Rennet gelation is used to produce many types of cheese. The effect of native whey protein on  
18 rennet gelation kinetics was investigated. Milks with a wide range of whey protein:casein  
19 (WP:CN) ratios (with standardised casein concentrations) were made from powders produced  
20 by microfiltration. Measurements of casein macro peptide release showed that native whey  
21 protein inhibited the enzymatic action of chymosin, which delayed the onset and reduced the  
22 subsequent rate of gelation. Experiments in which increased chymosin concentrations  
23 compensated for the inhibition, demonstrated that other factors also contributed to the reduced  
24 gelation rate. Neither an increase in viscosity nor a reduction in soluble calcium was  
25 responsible, leading to the conclusion that in addition to inhibiting chymosin, native whey

26 proteins present a physical barrier to para-casein aggregation. This study demonstrates and  
27 explains how casein-enriched retentates from microfiltration gel faster than regular cheese milk  
28 that contains higher amounts of native whey protein.

29

30 **Key words:** rennet gelation; whey protein; gelation kinetics; chymosin; inhibition; cheese;  
31 milk; microfiltration

32

33

### 34 **1. Introduction**

35 Rennet gelation is a key process in cheese making that transforms liquid milk to a coagulum.  
36 Much research has been undertaken to understand the mechanisms of rennet gelation (Guinee  
37 & Wilkinson, 1992). This has facilitated improvement of cheese production processes to  
38 manufacture better quality cheese at a lower cost. More recently, the advent of membrane  
39 filtration technology has enabled control over the protein composition of the milk used for  
40 cheese making (Maubois, 2002). While the overall mechanisms of rennet gelation are well  
41 established (Lucey, 2002), a more detailed understanding of the mechanisms in relation to  
42 protein composition is required to take full advantage of membrane technology in the  
43 manufacture of renneted cheese.

44

45 Milk is a complex system, comprising of an emulsion of fat globules, a colloidal suspension of  
46 two distinct types of proteins (caseins & whey proteins), and an aqueous solution of lactose  
47 and various minerals (Jenness, 1999). Whereas, whey proteins are soluble in water, caseins  
48 (CN) are present in milk in the form of large colloidal aggregates (50-200 nm in diameter)  
49 known as casein micelles. The surface of casein micelles is rich in  $\kappa$ -casein, which has a  
50 hydrophilic section that protrudes outwards like a 'hairy layer', stabilising the micelles through

51 electrostatic and steric repulsion (Walstra, 1999). Rennet gelation destabilises the casein  
52 micelles by selectively cleaving the ‘hairy layer’ using a proteolytic enzyme called ‘chymosin’  
53 that is found in rennet (Vasbinder, Rollema, & De Kruif, 2003). As this enzymatic reaction  
54 progresses, the cleaved C-terminal of the  $\kappa$ -CN known as casein macro peptide (CMP)  
55 solubilises in the milk serum. The casein micelles become progressively more hydrophobic  
56 allowing them to aggregate to form a coagulum (Fox, Guinee, Cogan, & McSweeney, 2017;  
57 Vasbinder, Rollema, & De Kruif, 2003; Walstra, 1990).

58

59 Casein micelles are central to the rennet gelation process and the rate of coagulation increases  
60 with increasing casein concentration (Fox, Guinee, Cogan, & McSweeney, 2017).  
61 Ultrafiltration can be used to concentrate milk proteins in cheese milk (Nelson & Barbano,  
62 2005), which increases the rate of gelation as well as the volumetric productivity of the cheese  
63 vats. Whereas UF concentrates all of the milk proteins, microfiltration can separate larger  
64 casein micelles from the smaller whey proteins (Maubois, 2002; Papadatos, Neocleous, Berger,  
65 & Barbano, 2003; Zulewska, Newbold, & Barbano, 2009). This enables the production of  
66 cheese milks from MF retentates with increased casein:whey protein (CN:WP) ratios.

67

68 Although the overall mechanisms of rennet gelation are well established, the role of whey  
69 proteins has yet to be fully elucidated. As separate soluble proteins, the whey proteins are not  
70 directly involved in the rennet gelation process. However, when combined with heat treatment  
71 above the denaturation temperature ( $\sim 65\text{-}70\text{ }^{\circ}\text{C}$ ), whey proteins have been found to impair  
72 rennet gelation leading to elongated gelation time and weaker gels (Vasbinder, Rollema, & De  
73 Kruif, 2003). This behaviour was attributed to the formation disulphide bridging between thiol  
74 groups of the  $\kappa$ -CN and denatured  $\beta$ -lactoglobulin ( $\beta$ -LG), the most abundant of the whey  
75 proteins (Jang & Swaisgood, 1990). Although it was initially thought that this disulphide

76 bridging blocked the  $\kappa$ -casein hairs from being cleaved (Hinrichs, 2001), a more recent study  
77 has shown that heat treatment has a more significant effect on the aggregation of the renneted  
78 casein micelles (para-casein micelles) than on the actual  $\kappa$ -casein hydrolysis (Vasbinder,  
79 Rollema, & De Kruif, 2003). In a contrasting study, supplementation of reconstituted skim  
80 milk powder with whey protein prior to heating at 65 °C for 30 min and subsequent rennet  
81 gelation was found to result in firmer gels (Meza-Nieto, Vallejo-Cordoba, González-Córdova,  
82 Félix, & Goycoolea, 2007). Although this degree of heat treatment was shown not to result in  
83 extensive attachment of denatured whey protein to the casein micelles, the increased gel  
84 strength was attributed to the cross-linking between the casein micelles by whey proteins  
85 (Meza-Nieto, Vallejo-Cordoba, González-Córdova, Félix, & Goycoolea, 2007). A more likely  
86 explanation, however, is that the addition of whey powder simply increased the soluble calcium  
87 levels, which were not standardised and are otherwise lacking in reconstituted skim milk  
88 powder (Martin, Williams, & Dunstan, 2007), thereby limiting the rate of rennet gelation  
89 (Martin, Williams, Choong, Lee, & Dunstan, 2008).

90

91 In cheese production, milk does not typically undergo severe enough heat treatment for whey  
92 proteins to be denatured. Therefore, it is the absence of native whey protein in MF retentates  
93 that is of interest from a cheese making perspective. However, to date there have been only a  
94 few studies (Lelievre, Creamer, & Tate, 1990) investigating the effect of native whey proteins  
95 on the rennet gelation process focusing mainly on the overall gelation properties of milk  
96 systems. Lelievre and colleagues observed an increased clotting time with added whey protein  
97 isolate (WPI) and an inhibition of  $\alpha_{s1}$ -casein hydrolysis, which develops flavour during the  
98 cheese maturation stage. However, the effects native whey proteins have on the enzyme  
99 hydrolysis of  $\kappa$ -CN and the aggregation phases post-renneting have not yet been distinguished  
100 clearly. Therefore, in this study we investigate for the first time the effect of native whey protein

101 on the kinetics of the enzymatic hydrolysis and the aggregation phases of rennet gelation. This  
102 was done by comparing the gelation of milks prepared with a wide range of WP:CN ratios  
103 (with a constant CN concentration) using casein concentrate and whey protein concentrate  
104 (WPC) powders produced through microfiltration.

105

106 Establishing the role of native whey protein on the kinetics and mechanisms of rennet gelation  
107 will enable the cheese industry to optimise the formulation of cheese milks with different  
108 WP:CN ratios to maximise productivity and reduce the total production cost. Further, a detailed  
109 kinetic study of rennet gelation will provide new insights, especially into the secondary  
110 aggregation stage of casein micelles that is less understood to date.

111

## 112 **2. Materials and Methods**

### 113 *2.1 Production of casein and whey powders*

114 All casein and whey protein solutions were made from reconstituted powders that were isolated  
115 using a microfiltration process. The microfiltration consisted of a 3-stage batch concentration  
116 process adapted from Nelson and Barbano (Nelson & Barbano, 2005), designed to isolate  
117 casein from whey proteins in skim milk. ISOFLUX<sup>TM</sup> microfiltration membranes with a  
118 nominal pore size of ~ 0.15 µm were operated in a membrane filtration pilot plant (GEA, 2012  
119 Model Type R). The microfiltration process was operated with a nominal permeate flux of  
120 between 60-90 kg/hour, feed pressure of 1 bar, and recirculation pressure between 2.5-2.9 bar.

121 700 kg of pasteurized skim milk was processed through the microfiltration pilot plant in stage  
122 1. Casein was concentrated in the retentate as whey proteins were selectively removed in the  
123 permeate. Retentate was bled from the holding tank at a rate of ~ 30 kg/hour. Approximately  
124 3X protein concentration of the retentate occurred at the completion of each stage of  
125 processing. Prior to stage 2 and 3 diafiltration, a volume of filtered water was added to the

126 retentate to return it to ~1X protein concentration. Temperature was maintained at 55 °C  
127 throughout the process.

128 The permeate (native whey protein stream) was collected and separately concentrated using a  
129 multistage ultrafiltration (UF) process. The UF was operated at a nominal permeate flux of  
130 between 240-300 L/hour, feed pressure of 3 bar, and recirculation pressure of between 4.0-4.1  
131 bar. Approximately 20X protein concentration was achieved by the UF process. 2 stages of  
132 diafiltration with 90 kg of water were performed. Temperature was maintained at 10 °C  
133 throughout the ultrafiltration process.

134 The concentrated liquid retentate and permeate streams were collected and spray dried (GEA-  
135 Niro, Mobile Minor). Liquid was fed into the spray dryer via a rotary atomizer set with an  
136 outlet temperature of 80 °C. Water was evaporated at a rate of ~2 kg/hour.

137

## 138 *2.2 Compositional analysis of casein and whey powders*

139 Protein analysis of powders were performed based on the Dumas Combustion method using a  
140 LECO Trumac NCS analyser (LECO Corporation, Michigan, USA) following a method that  
141 complies with the standard ISO 1489. Samples of powder (~ 0.1 g) were weighed onto a  
142 ceramic boat and dried in the oven overnight (104 °C). The boats were then loaded into the  
143 LECO analyser and combusted at a temperature of 1100 °C within the furnace. Protein values  
144 were determined from the measured nitrogen % by multiplying with the ratio 6.38 (Mariotti,  
145 Tomé, & Mirand, 2008). The protein composition of the casein powder and native whey protein  
146 powder were 84.9 %w/w and 76.3 %w/w respectively.

147 The proportion of casein and whey protein were also assessed using the LECO Trumac NCS  
148 analyser, using reconstituted liquid samples after casein and/or whey proteins were precipitated  
149 by acid to determine the non-casein nitrogen and non-protein nitrogen. The protein in the casein

150 powder was determined to be 97.0 %w/w casein and 3.0 %w/w whey protein. The protein in  
151 the native whey protein powder was determined to be 1.5 %w/w casein and 98.5 %w/w whey  
152 protein.

153

### 154 *2.3 Standardised cheese milks*

155 Batches of concentrated casein (10% w/w) and whey protein (10% w/w and 20% w/w) were  
156 prepared by reconstituting ultrafiltered casein and native whey protein concentrate powders  
157 (preparation and composition provided in sections 2.2 and 2.3 respectively). Sodium azide  
158 (Chem-Supply, assay: 99%) was added to each solution at 0.02% w/w to inhibit microbial  
159 action and stored at 4 °C. Four milk systems were prepared by mixing the concentrated  
160 solutions to achieve WP:CN ratios of 0.03:1, 0.25:1 (the ratio in natural milk), 1:1 and 4:1 w/w  
161 while keeping the casein concentration constant at 0.0264 g/g to match the casein content of  
162 skim milk. Calcium chloride (Chem-Supply, assay: 93%) was added to each milk system to  
163 achieve a final concentration of 4.7 mM. The pH (measured from Mettler Toledo Education  
164 Line pH meter) of every milk system was maintained between 6.6 and 6.8. Replicate samples  
165 made from concentrated casein and whey protein batches were tested during each experiment.  
166 The number of replicates of each experiment are denoted in the caption of figures illustrated in  
167 the results and discussion section.

168

### 169 *2.4 Determination of gelation kinetics by rheometry*

170 Samples (20 mL) of each milk system were brought to 31 °C in a water bath and renneted with  
171 0.2 mL of 3.5 IMCU/mL rennet solution (prepared from 200 IMCU/mL Chymax Plus FPC,  
172 Cheeselinks, Australia, batch numbers 3233770 & 3241446). The rate of gelation was  
173 determined by measuring the storage modulus ( $G'$ ) as a function of time using an AR-G2  
174 rheometer (TA Instruments) with a controlled shear strain of 2.5% at 1 Hz using the standard

175 concentric cylinder (996284) tool at 31 °C for 1 hr. The onset of gelation was defined as the  
176 time at which G' first exceeded >0.2 Pa (after normalising the G' at t = 0 to -0.2 Pa).

177

### 178 *2.5 Soluble calcium measurement*

179 Each milk system, before and after the addition of calcium chloride, was ultracentrifuged at  
180 100,000 x g using a Beckman Coulter ultracentrifuge unit (Model: Optima L-100 XP with type  
181 70 Ti rotor) for 1 hr at 4 °C. The supernatant was immediately separated and filtered through  
182 0.45 µm syringe filters. The filtrate was acid precipitated with 100% w/v trichloro acetic acid  
183 (BDH AnalR, assay: 99.5%) (1.2 mL per 0.5 mL sample) and diluted to 10 mL with deionized  
184 and filtered water. The samples were then centrifuged at 839 g for 5 minutes using a  
185 Thermofisher Hereus Megafuge 8 bench top centrifuge with HIGHConic III fixed angle rotor  
186 and the supernatant was filtered using a 0.45 µm syringe filters. 0.3 mL of the filtrate was  
187 diluted with 4.7 mL of deionized and filtered water and the soluble calcium was measured with  
188 inductive coupled plasma (ICP) optical emission spectrometer (Varian 720-ES with SPS 3 auto  
189 sampler, wavelength: 315.887 nm, power: 1 kW, plasma flow: 15 L/min, auxiliary flow: 1.5  
190 L/min, nebulizer flow: 0.75 L/min, pump rate: 15 rpm).

191

### 192 *2.6 Particle size measurement*

193 Freshly prepared samples of each milk system were diluted 2000 times with deionized and  
194 filtered water and analysed under the 'milk protein size analysis' option (particle refractive  
195 index: 1.45, particle absorption 0.001, viscosity of water 0.8872 cP, refractive index of water  
196 1.33, measurement angle: 173° backscatter) of a Malvern Zetasizer (Nano ZS) using disposable  
197 cells (PTS0012) at 25 °C.

198



199 *2.7 Viscosity measurement*

200 The viscosity of each milk system was measured with the same rheometer described above  
201 using a flow procedure with an increasing strain rate (0-500 s<sup>-1</sup>) at 31 °C using a 40 mm 2°  
202 steel cone (988134). The gradient of the linear shear stress vs strain rate graph was recorded as  
203 the viscosity.

204

205 *2.8 Enzymatic kinetics*

206 The kinetics of the hydrolysis of κ-casein by rennet were tracked by measuring the change in  
207 the casein macro peptide (CMP) concentration in the serum using a RP-HPLC (SHIMADZU  
208 controller SCL-10AVP, oven CTO-10ASVP, pump LC-10ATVP, auto injector SIL-10ADVP,  
209 diode array detector APDM10AVP) with eluents A (Water: Acetonitrile: Tri-fluoro acetic acid  
210 900:100:1) and B (Water: Acetonitrile: Tri fluoro acetic acid 100:900:1). The gradient was  
211 started with 15% of B and increased to 28% over 13 min, 32% over 22 min, 70% over 3 min,  
212 kept at 70% for 5 min and returned to the starting condition. A C18 column ( Jupiter 5 μm, 300  
213 A<sup>0</sup>, 250 x 4.6 mm) was used and the flow rate was 0.8 mL at 30 °C (Vasbinder, Rollema, &  
214 De Kruif, 2003). Prepared milk samples were renneted as described above and 1.4 mL of 10%  
215 w/v trichloro acetic acid was added to 2 mL of renneted milk at the desired time intervals to  
216 inhibit rennet action and precipitate the caseins. The samples were immediately refrigerated to  
217 4 °C. The stored samples were centrifuged at 2717 g in a Thermofisher Hereus Megafuge 8  
218 benchtop centrifuge with HIGHConic III fixed angle rotor for 20 minutes and the supernatants  
219 filtered using 0.45 μm syringe filters. 0.2 mL of the permeate was diluted in 2 mL of a 70:30  
220 mixture of A and B and used for analysis. Preliminary analyses were carried out to identify the  
221 peak given by the CMP by renneting a sample of WP:CN 0.03:1 for 3 hr and comparing the  
222 chromatogram with an un-renneted sample pre-treated identically.

223

## 224 2.9 Statistical Analysis

225 A single factor analysis of variance (ANOVA) was carried out for the experimental data of the  
226 particle size measurement using Microsoft Excel. A confidence interval of 99% ( $\alpha = 0.01$ ) was  
227 used for the comparison of the means.

228

## 229 3. Results and Discussion

### 230 3.1 Overall kinetics of gelation

231 Rennet induced gelation of milk proceeds in three overlapping stages: i) hydrolysis of  $\kappa$ -casein,  
232 ii) interaction between para-casein micelles, and iii) the formation of a cross-linked gel  
233 (Carlson & Hill Jr, 1987; Hyslop, 2003). Rheology was used in this study to compare the  
234 overall rate of milk gelation as a function of different ratios of casein and whey protein. Figure  
235 1(a) illustrates the change in  $G'$  of each milk system (at 31 °C) with time after the addition of  
236 rennet. The abrupt increase in  $G'$  indicates the onset of gelation and is the characteristic  
237 behaviour of a gelling system under kinetic control (Meza-Nieto, Vallejo-Cordoba, González-  
238 Córdova, Félix, & Goycoolea, 2007). A comparison between the four milk systems show that  
239 the rate of gelation decreased with increasing whey protein concentration. At or below the  
240 native WP:CN ratio (i.e. 0.25:1 and 0.03:1) the onset of gelation did not change significantly  
241 (Figure 1b). However, at higher WP:CN ratios (1:1 and 4:1) the increase in whey protein  
242 concentration significantly delayed the onset of gelation. The value of  $G'$  reached after 60 min  
243 decreased with increasing whey protein across the range of WP:CN ratios tested. (Figure 1c).  
244 These results show that native whey proteins can inhibit the rennet gelation process.  
245 Subsequent experiments were performed to determine which stages of the gelation process  
246 were impaired, and to help reveal the inhibitory mechanisms of the native whey proteins.

247

248 *3.2 Rate of  $\kappa$ -casein hydrolysis*

249 To investigate whether the presence of native whey protein affects the rate of  $\kappa$ -CN hydrolysis,  
250 experiments were conducted in which the concentration of CMP in the serum was measured at  
251 different time intervals after addition of rennet (Figure 2a). When the systems with WP:CN  
252 ratios of 0.03:1 and 4:1 are compared, a decreased rate of hydrolysis is observed in the milk  
253 system with the higher concentration of whey protein. This is consistent with the results of  
254 Lelièvre et al. (Lelievre, Creamer, & Tate, 1990) and further with the observed delay in the  
255 onset of gelation (Figure 1b). While impaired gelation due to whey addition was previously  
256 attributed to enzymatic inhibition by Lelièvre and colleagues (Lelievre, Creamer, & Tate,  
257 1990), adequate data were not presented to conclude that the  $\kappa$ -CN hydrolysis was impaired.  
258 The results from the current study (Figure 2a) confirm that  $\kappa$ -CN hydrolysis was impaired,  
259 which increased the time taken to sufficiently reduce the electrostatic and steric repulsion  
260 between the micelles.

261

262 The reduction in the rate of  $\kappa$ -CN hydrolysis caused by the presence of native whey proteins  
263 could be attributed to one of two mechanisms: i) a reduction in the diffusivity of the chymosin  
264 molecule, or ii) enzymatic inhibition. According to the Stokes-Einstein equation, the diffusivity  
265 of a molecule in an ideal system is inversely proportional to the viscosity of the medium  
266 (Young, Carroad, & Bell, 1980). Experiments were performed to determine whether the change  
267 in the viscosity (leading to a reduction in diffusivity) caused by the presence of whey protein  
268 contributed to the observed delay in the onset of gelation. For this, the viscosities of the milk  
269 systems were measured (Figure 3a) and rennet gelation experiments were conducted on milk  
270 systems prepared with the same casein concentration as skim milk but substituting xanthan  
271 gum for the whey protein to match the viscosities of the original milk systems. The results  
272 show that the onset of gelation was not affected by the presence of xanthan gum (Figure 3b),

273 indicating that the increased viscosity resulting from the addition of whey protein is not  
274 responsible for the reduced rate of  $\kappa$ -CN hydrolysis. This could be explained by the fact that  
275 the viscosity of the actual aqueous medium through which the enzymes diffuse is not actually  
276 increased by the presence of whey proteins (or xanthan gum), and that the change in bulk  
277 viscosity does not significantly affect the diffusivity of the small chymosin molecules.

278

279 As the change in viscosity could not account for the delayed onset of gelation, the observed  
280 decrease in the rate of hydrolysis must instead indicate enzymatic inhibition. Enzyme inhibition  
281 mechanisms are broadly classified as either competitive, uncompetitive or non-competitive.  
282 Competitive inhibition would mean that one or more of the native whey proteins competes with  
283  $\kappa$ -CN for the chymosin active site. For uncompetitive inhibition, the inhibitory protein(s) binds  
284 exclusively to the enzyme-substrate (chymosin- $\kappa$ -CN) complex. In a non-competitive reaction,  
285 the inhibitor binds to both the substrate and the enzyme-substrate complex (Bisswanger, 2002).  
286 Conceptually it seems plausible that any of the mechanisms could occur, and it is not clear yet  
287 which of the native whey proteins is responsible. While the data presented here indicate that  
288 native whey proteins inhibit chymosin activity, it is worth noting that this could be considered  
289 to be a relatively mild form of inhibition. For instance, chymosin is known to be strongly  
290 inhibited by small amounts of  $\alpha_2$ -Macroglobulin present in blood serum, which can actively  
291 attach to and disable chymosin enzymes (Bansal, Fox, & McSweeney, 2010). In comparison,  
292 the concentrations of whey proteins in the present experiments are very high, indicating that  
293 the native whey protein(s) involved have a relatively low affinity for the enzyme or enzyme-  
294 substrate complex.

295

296 *3.3 Aggregation of para-casein micelles*

297 The observed enzymatic inhibition may not be the only cause for the delayed onset of gelation  
298 and the subsequent decrease in the rate of gelation. To clarify whether the impaired hydrolysis  
299 could fully account for impaired gelation, an experiment was carried out in which twice the  
300 amount of rennet (2%) was added to the WP:CN 4:1 system. This was done to achieve rapid  
301  $\kappa$ -CN hydrolysis in the presence of whey protein, and to test if this could overcome the  
302 previously observed delay in the onset of gelation. The results show that even though faster  $\kappa$ -  
303 CN hydrolysis occurred in the high-rennet/high WP (2% rennet WP:CN 4:1) system (Figure  
304 2a), the onset of gelation occurred later than that of the low rennet/low WP (1% rennet WP:CN  
305 0.03:1) system (Figure 2b). This shows that in addition to inhibiting the action of the chymosin,  
306 the whey protein impaired the aggregation of the para-casein micelles.

307

308 Aggregation of para-casein micelles relies on two factors: i) diffusion through the medium via  
309 Brownian motion so that particles can approach each other; and ii) reaction of the particles so  
310 that they can fuse together. The first factor is governed by the concentration (affecting the  
311 probability of particles coming to the same location) and the diffusivity of the particles  
312 (affected by the particle size and medium viscosity)(Carlson, Hill, & Olson, 1987). Although  
313 the presence of the whey proteins was found not to affect the diffusivity of the chymosin  
314 enzyme sufficiently to affect an appreciable change in the rate of  $\kappa$ -CN hydrolysis, it is possible  
315 that the diffusivity of the much larger (10-50x the diameter) casein micelles may be affected,  
316 as they will 'see' the protein suspension (rather than the aqueous solution) as the continuous  
317 phase through which they must move. However, the experiments using xanthan gum in place  
318 of whey protein to increase the viscosity (Figure 3b) suggest that this is not the case, at least  
319 with respect to the onset of gelation, which was not affected. The experiment performed at a  
320 viscosity of 1.4 mPa.s (equivalent to a WP:CN ratio of ~1.2:1) showed no observable difference  
321 in the onset or rate of gelation compared to the control (WP:CN 0.03:1) (Figure 3b). This is in

322 contrast to the significant delays in the onset and reductions in the rates of gelation observable  
323 for the samples containing whey protein (as opposed to xanthan gum) (Figure 1). Therefore,  
324 the presence of whey protein must inhibit the reaction of the para-casein micelles, rather than  
325 the particle diffusion. This is further confirmed by dynamic light scattering measurements,  
326 which showed that the particle size (and therefore the diffusivity of the particles, which is what  
327 is actually measured), was not significantly altered (analysed by one way ANOVA with  $\alpha =$   
328 0.01 or 99% confidence interval) by the presence of whey protein (Table 1). The experiments  
329 performed with xanthan gum at viscosities of 1.7 mPa.s and 2.1 mPa.s (equivalent to WP:CN  
330 ratios of ~2.3:1 and ~3.6:1 showed some unusual behaviour, with the  $G'$  eventually decaying  
331 after an initial increase (Figure 3b). It has previously been observed that when sufficient  
332 amounts of xanthan gum were added to milk protein solutions, phase separation occurred, or  
333 “thread-like xanthan-rich regions” were developed (Hemar, Tamehana, Munro, & Singh,  
334 2001). Although no macroscopic phase separation was observed in the present study, it is  
335 possible that xanthan-rich regions were present in the casein:gum systems with viscosities of  
336 1.7 mPa.s and 2.1 mPa.s (which contained 0.0074 % and 0.029 % w/w xanthan gum), that  
337 interrupted and weakened the developing gel network.

338

339 Aggregation of colloidal particles, such as para-casein micelles, requires overcoming the  
340 energy barrier presented by electrostatic and steric repulsion. The electrostatic repulsion is due  
341 to the repulsion of like charges (in this case negative) on the surface of the casein micelles. In  
342 some systems, the strength of electrostatic repulsions can be gauged by measurement of the  $\zeta$ -  
343 potential. This was attempted, but unfortunately reliably comparable measurements could not  
344 be obtained across the range of WP:CN ratios because the presence of whey protein in the  
345 medium has a potential to alter the diffusivity of the casein micelles under an electric field,  
346 hence the dynamic light scattering measurement. Nonetheless, further understanding can be

347 gained by considering the fundamentals of the electrostatic repulsive forces, which are  
348 predominantly affected by three factors: the pH (which will affect the net surface charge of the  
349 ionic proteins), the ionic strength (which reduces the depth of the electrical double layer), and  
350 the presence of charge-neutralising calcium ions. As the pH was kept consistent between the  
351 experiments, this is not an explanatory factor. The addition of WPC resulted in an increase in  
352 the ionic strength as a function of increasing WP:CN ratio. As this should in fact decrease the  
353 electrostatic repulsion, it cannot explain the observed impairment in aggregation that occurred  
354 with increasing WP:CN.

355

356 The final factor contributing electrostatic stabilisation is the soluble calcium concentration that  
357 is known to increase the rate of rennet gelation by neutralising the net-negative surface charge  
358 (Dalglish, 1983, 1984). It is believed that the hydrolysis of  $\kappa$ -casein causes bare patches on  
359 the casein micelles exposing negatively charged amino acid residues (Sandra, Ho, Alexander,  
360 & Corredig, 2012) that continue to repel the para-casein micelles leading to elongated gelation  
361 times. The  $\text{Ca}^{2+}$  ions in the milk serum can reduce the overall charge of such residues and being  
362 divalent, help bridge two negatively charged micelles. Higher concentrations of soluble  
363 calcium thereby enable closer approach and faster aggregation (Dalglish, 1984; Hooydonk,  
364 Hagedoorn, & Boerrigter, 1986; Martin, Williams, Choong, Lee, & Dunstan, 2008). Previous  
365 studies (Hooydonk, Hagedoorn, & Boerrigter, 1986; Udabage, McKinnon, & Augustin, 2001)  
366 have shown that the increase in  $\text{Ca}^{2+}$  by addition of  $\text{CaCl}_2$  (up to 50 mmol/kg = 2000 ppm) in  
367 milk results in faster gelation.

368

369 In our experiments, calcium chloride was added (4.7 mM) to each milk system to ensure  
370 sufficient calcium was available for gelation so as to minimise this effect. To investigate the  
371 effect of the addition of whey protein isolate, the soluble calcium in each milk system was

372 measured before and after the addition of calcium chloride (Figure 4). The results show a clear  
373 increase in the soluble calcium as a function of increasing WP:CN ratio. A similar increasing  
374 trend was observed with respect to the conductivity of milk samples (data not shown) as well.  
375 This trend is expected as the WPC powder contains a total calcium concentration of 4.1 mg/g  
376 that contributes to an increase in both the soluble calcium and conductivity. The presence of  
377 additional calcium should neutralise the electrostatic repulsion between casein micelles while  
378 the increased ionic strength should further reduce the thickness of the electric double layer  
379 around the micelles. Although both the above phenomena should result in firmer gels and faster  
380 gelation, the opposite was observed in this study, confirming that neither calcium nor the ionic  
381 strength were responsible for the observed impairment in gelation resulting from an increase  
382 in whey protein concentration. To further confirm this conclusion, two additional experiments  
383 were carried out (data not shown). In the first, WP:CN 0.03:1 systems with increased soluble  
384 calcium concentrations up to 1015 ppm showed a faster aggregation than the WP:CN 0.03:1  
385 systems with only 300 ppm. In the other experiment, WP:CN 4:1 systems without any addition  
386 of CaCl<sub>2</sub> showed no gelation for 1 hour. This observation makes it possible to conclude that  
387 even though soluble calcium helps to neutralise the electrostatic repulsion in casein micelles,  
388 the presence of whey protein has a more significant negative impact in the gelation behaviour  
389 observed in this study.

390

391

392 To summarise, when chymosin is added to a system containing casein micelles and native whey  
393 proteins (Figure 5a), the enzyme is inhibited resulting in slower hydrolysis (Figure 5b). We  
394 have also shown that neither the inhibition of the enzymatic hydrolysis, nor an increase in  
395 viscosity, nor a reduction in the electrostatic repulsion can fully account for the observed  
396 decrease in the rate of gelation in the presence of native whey protein. We therefore propose



397 that this leaves only physical or steric hindrance as the remaining explanatory mechanism.  
398 While there is no evidence to suggest that the native whey proteins become permanently  
399 attached to the para-casein micelles, they are the right size to occupy the gaps in the 'hairy  
400 layer' that arise as the  $\kappa$ -CN is cleaved from the micelles. In this way, the native whey proteins  
401 can provide a physical barrier to intimate contact between cleaved para-casein micelles,  
402 partially substituting for the reduction in steric repulsion caused by removal of the  $\kappa$ -CN 'hairy  
403 layer' (Figure 5c). As hard particles, they would represent a strong physical barrier for the  
404 aggregation of cleaved micelles (Figure 5d), in comparison to the increased viscosity resulting  
405 from the addition of the more fluid xanthan gum. A related observation that is consistent with  
406 this idea, is that in both WP:CN 4:1 systems with 1% and 2% rennet, the onset of gelation  
407 occurred after approximately 70% of the total CMP was released, while in the CN:WP 1:0.03  
408 system, the onset occurred after around 50% of the CMP was released (Figure 2a). Increasing  
409 concentrations of whey protein can more effectively shield collisions between para-casein  
410 micelles thereby increasing the amount of  $\kappa$ -CN that must be removed before gelation can  
411 occur.

412

### 413 **Conclusions**

414 The current study demonstrates that native whey protein inhibits  $\kappa$ -CN hydrolysis by chymosin  
415 and also impairs the rate of aggregation of the para-casein micelles. While the higher  
416 concentrations of whey protein examined in the this study were not practically relevant in a  
417 commercial setting (WP:CN of 1:1 and 4:1), they enabled clear trends to be observed that  
418 helped reveal the underlying mechanisms of the inhibitory roles of whey protein during rennet  
419 gelation of milk. The results confirm that the above inhibitory effects are not caused by the  
420 associated changes in the mineral balance or viscosity, but rather directly by the native whey

421 proteins themselves. More studies are required to determine the exact mechanism of enzyme  
422 inhibition and which of the whey proteins are responsible.

423

424 The milk that was tested with a greatly reduced whey protein content (WP:CN of 0.03:1) is of  
425 practical relevance to the use of MF retentates in cheese making. The results show that the rate  
426 of gelation is increased when native whey protein is not present, which would translate to more  
427 efficient and productive cheese vats. Further investigations into the effect of the removal of  
428 whey proteins on curd microstructure would be beneficial to demonstrating the potential of  
429 using casein retentate from MF in the manufacture of renneted cheese.

430

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437

438 Conflicts of interest: none.

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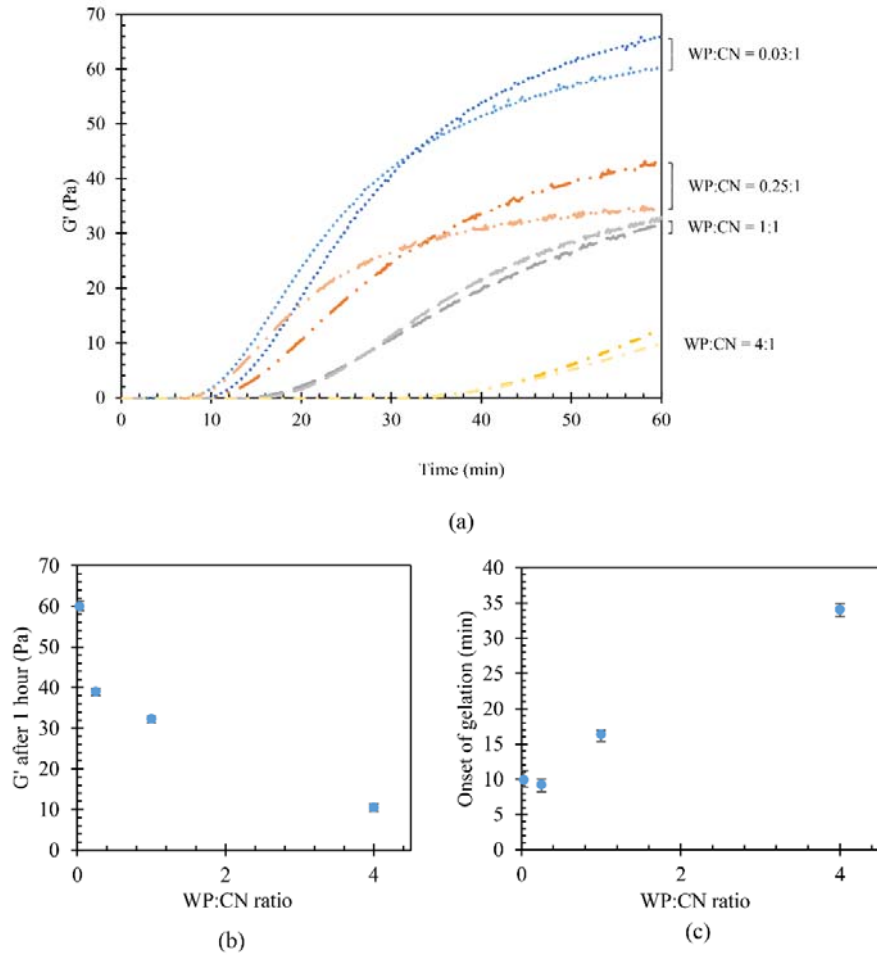
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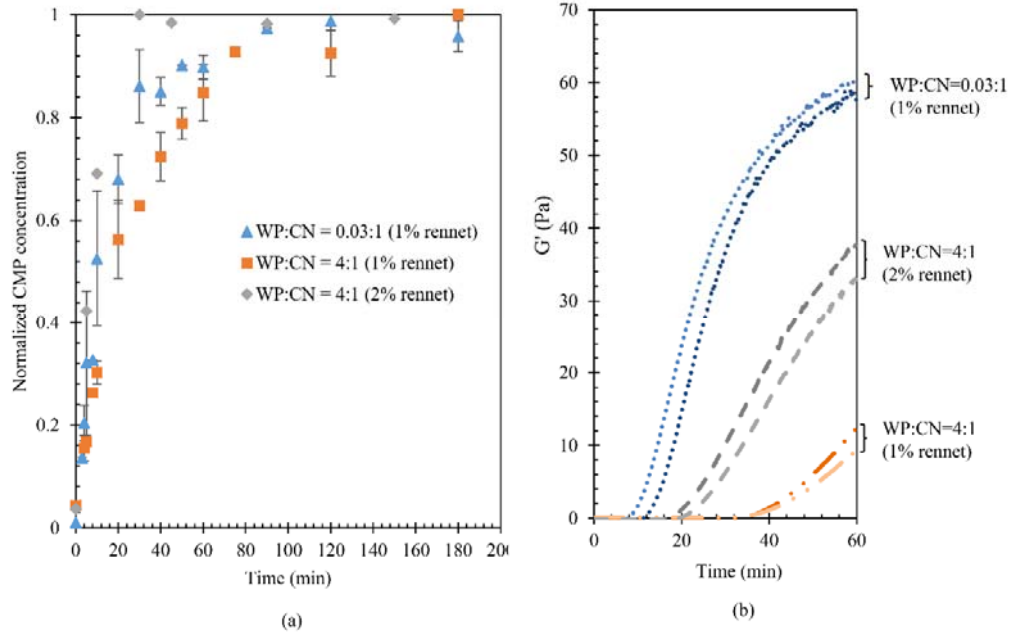
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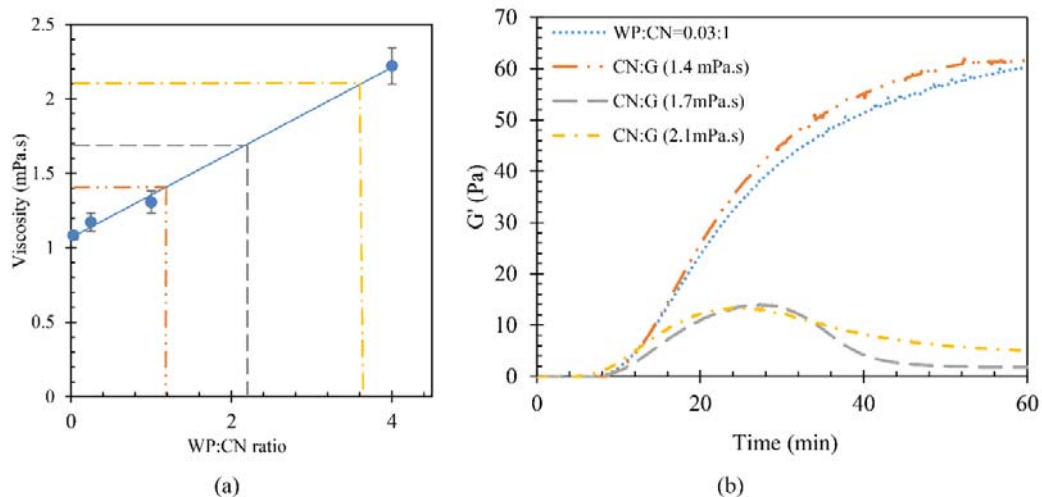
514

515 **Figure 1:** (a)  $G'$  as a function of time after addition of rennet for milk systems with WP:CN  
 516 ratios of 0.03:1 ( ..... ), 0.25:1 ( - . - . - ), 1:1 ( - - - ) and 1:4 ( - . . - ). (b) Onset of gelation as a  
 517 function of the WP:CN ratio in each milk system. (c) Gel strength after 1 hr as a function of  
 518 WP:CN ratio in each milk system. The error bars indicate the standard deviation of  
 519 measurements of duplicate experiments.



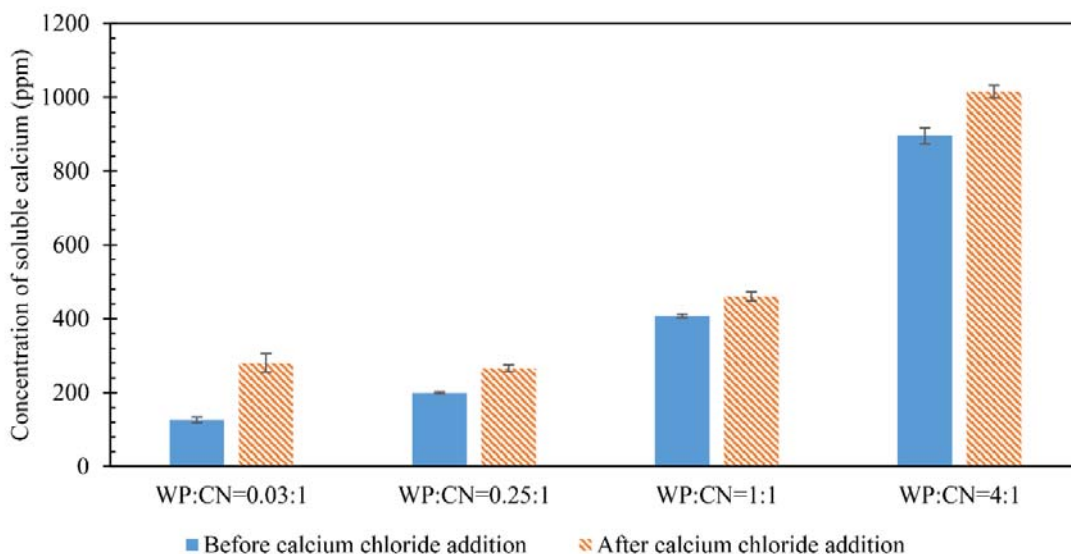
520

521 **Figure 2:** (a) The kinetics of  $\kappa$ -casein hydrolysis based on measurements of casein macro  
 522 peptide (CMP) concentration (normalised to the maximum CMP concentration in each trial)  
 523 with time, in milk systems with WP:CN ratios of 0.03:1 with 1% rennet ( $\blacktriangle$ ), 4:1 with 1%  
 524 rennet ( $\blacksquare$ ), and 4:1 with 2% rennet ( $\blacklozenge$ ). The error bars indicate the standard deviation of  
 525 measurements of quadruplicate experiments for the WP:CN 0.03:1 (1% rennet) system and  
 526 triplicate experiments for the 4:1 (1% rennet) system. (b)  $G'$  as a function of time after addition  
 527 of rennet in milk systems with WP:CN ratios of 0.03:1 with 1% rennet (.....), 4:1 with 1%  
 528 rennet (-----), and 4:1 with 2% rennet (-.-.-.-). Duplicate experiments were performed  
 529 as shown for each condition.



530

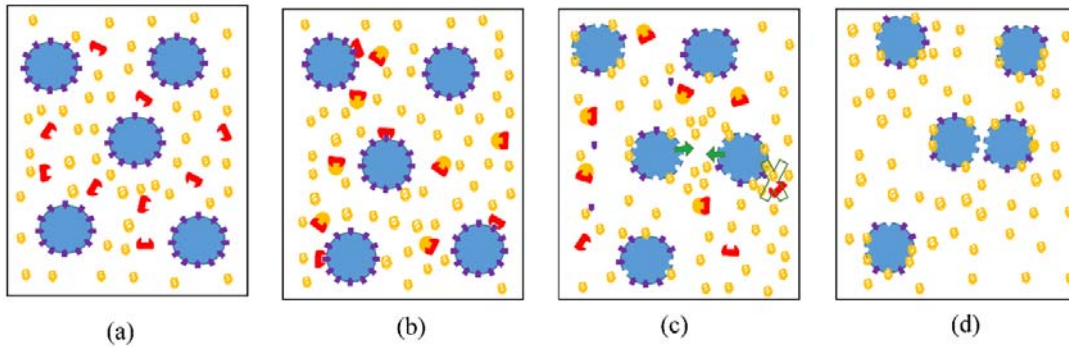
531 **Figure 3:** (a) Change in the viscosity of each milk system with the increase in the concentration  
 532 of whey protein. (b) The gelation behaviour observed in casein-xanthan gum (CN:G) mixtures  
 533 with viscosities 1.4 mPa.s (— · — · —), 1.7 mPa.s (— — —) and 2.1 Pa.s(— · · — ·) compared with the  
 534 original milk system with WP:CN = 0.03:1 (.....). The corresponding WP:CN ratios of milk  
 535 systems with the similar viscosities to gum systems are represented with matching line styles  
 536 in figure (a).The error bars indicate the standard deviation of duplicate measurements.



537

538 **Figure 4:** Soluble calcium in each milk system before (■) and after (▨) the addition of calcium  
 539 chloride. The error bars indicate the standard deviation of measurements of duplicate samples.





540

541 **Figure 5:** Schematic representation of the proposed mechanisms for the contribution of native  
 542 whey proteins to the decreased rate of rennet gelation. (a) Chymosin (👉) is added to a system  
 543 with casein micelles (🔵) and whey proteins (🟡). (b) Whey proteins inhibit (👉) the chymosin  
 544 action and decrease the rate of hydrolysis of  $\kappa$ -CN. (c) Whey proteins occupy the gaps in the  
 545 'hairy layer' (🟣) that arise as the  $\kappa$ -CN is cleaved from the micelles providing a steric hindrance  
 546 for chymosin (👈👈) to reach the remaining  $\kappa$ -CN hairs. This reduces the rate at which attractions  
 547 (👈) between the casein micelles overcome the repulsive forces. (d) Whey proteins cause an  
 548 additional steric hindrance to the intimate contact between cleaved micelles resulting in slower  
 549 aggregation.

550

551 **Table 1:** The particle size of casein in the milk systems with different WP:CN ratios. The  
 552 standard deviation of measurements in triplicate samples is indicated with ( $\pm$ ).

553

Whey Protein:Casein ratio	Z- average diameter (nm)
0.03:1	139.3 $\pm$ 2.9
0.25:1	140.7 $\pm$ 2.9
1:1	133.6 $\pm$ 3.0
4:1	133.3 $\pm$ 2.7

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