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

Charitha J. Gamlath[1,2,3], Thomas S. H. Leong[1,2,3], Muthupandian Ashokkumar[1,2], Gregory J. O. Martin[1,3]*

[1] The ARC Dairy Innovation Hub, The University of Melbourne, Parkville, Victoria 3010, Australia

[2] School of Chemistry, The University of Melbourne, Parkville, Victoria 3010, Australia

[3] Department of Chemical Engineering, The University of Melbourne, Parkville, Victoria 3010, Australia

*Corresponding author: Tel.: +61 3 8344 6613; fax: +61 3 8344 4153; email: gjmartin@unimelb.edu.au

Abstract

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

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

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

1. Introduction

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

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

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

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

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

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

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

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

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

2. Materials and Methods

2.1 Production of casein and whey powders

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

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

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

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

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

2.2 Compositional analysis of casein and whey powders

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

The proportion of casein and whey protein were also assessed using the LECO Trumac NCS analyser, using reconstituted liquid samples after casein and/or whey proteins were precipitated 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.

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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.

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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

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

2.5 Soluble calcium measurement

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

2.6 Particle size measurement

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

2.7 Viscosity measurement

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

2.8 Enzymatic kinetics

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

2.9 Statistical Analysis

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

3. Results and Discussion

3.1 Overall kinetics of gelation

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

3.2 Rate of κ-casein hydrolysis

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

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

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

As the change in viscosity could not account for the delayed onset of gelation, the observed decrease in the rate of hydrolysis must instead indicate enzymatic inhibition. Enzyme inhibition mechanisms are broadly classified as either competitive, uncompetitive or non-competitive. Competitive inhibition would mean that one or more of the native whey proteins competes with κ-CN for the chymosin active site. For uncompetitive inhibition, the inhibitory protein(s) binds exclusively to the enzyme-substrate (chymosin-κ-CN) complex. In a non-competitive reaction, the inhibitor binds to both the substrate and the enzyme-substrate complex (Bisswanger, 2002). Conceptually it seems plausible that any of the mechanisms could occur, and it is not clear yet which of the native whey proteins is responsible. While the data presented here indicate that native whey proteins inhibit chymosin activity, it is worth noting that this could be considered to be a relatively mild form of inhibition. For instance, chymosin is known to be strongly inhibited by small amounts of α_2-Macroglobulin present in blood serum, which can actively attach to and disable chyomsin enzymes (Bansal, Fox, & McSweeney, 2010). In comparison, the concentrations of whey proteins in the present experiments are very high, indicating that the native whey protein(s) involved have a relatively low affinity for the enzyme or enzyme-substrate complex.

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 κ-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 κ-
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 κ-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

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

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

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

The final factor contributing electrostatic stabilisation is the soluble calcium concentration that is known to increase the rate of rennet gelation by neutralising the net-negative surface charge (Dalgleish, 1983, 1984). It is believed that the hydrolysis of κ-casein causes bare patches on the casein micelles exposing negatively charged amino acid residues (Sandra, Ho, Alexander, & Corredig, 2012) that continue to repel the para-casein micelles leading to elongated gelation times. The Ca^{2+} ions in the milk serum can reduce the overall charge of such residues and being divalent, help bridge two negatively charged micelles. Higher concentrations of soluble calcium thereby enable closer approach and faster aggregation (Dalgleish, 1984; Hooydonk, Hagedoorn, & Boerrigter, 1986; Martin, Williams, Choong, Lee, & Dunstan, 2008). Previous studies (Hooydonk, Hagedoorn, & Boerrigter, 1986; Udabage, McKinnon, & Augustin, 2001) have shown that the increase in Ca^{2+} by addition of $CaCl_2$ (up to 50 mmol/kg = 2000 ppm) in milk results in faster gelation.

In our experiments, calcium chloride was added (4.7 mM) to each milk system to ensure sufficient calcium was available for gelation so as to minimise this effect. To investigate the 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 $CaCl_2$ 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

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

Conclusions

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

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

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

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Conflicts of interest: none.

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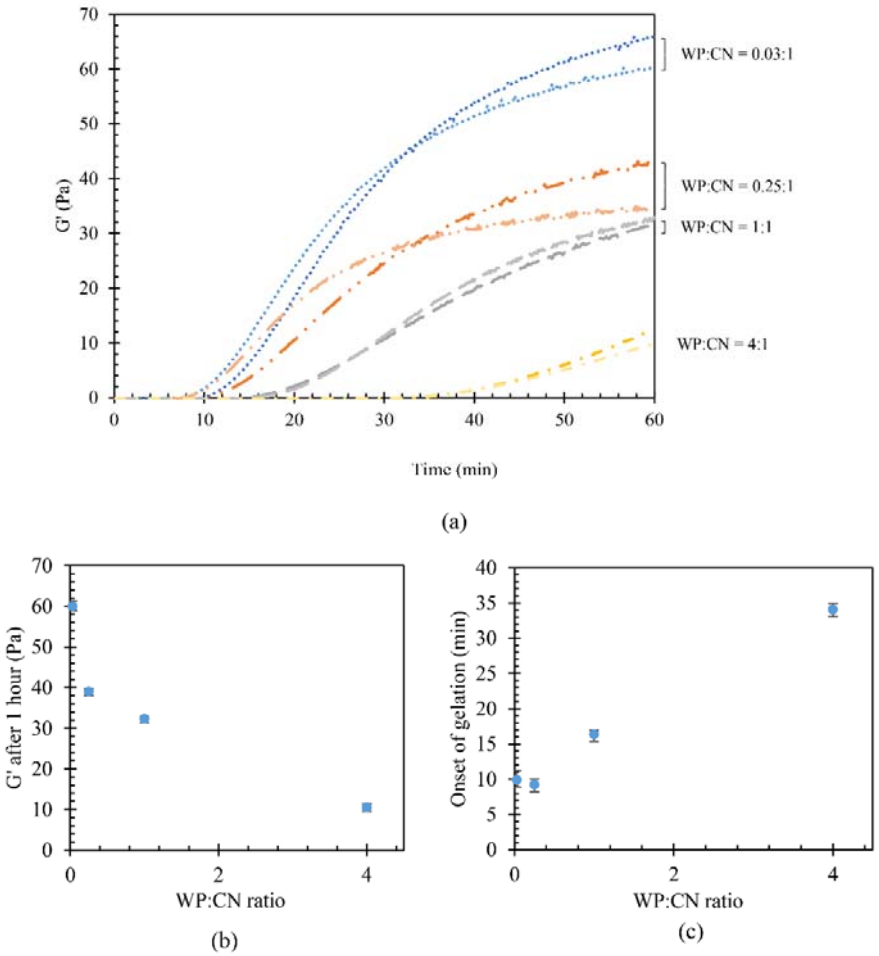
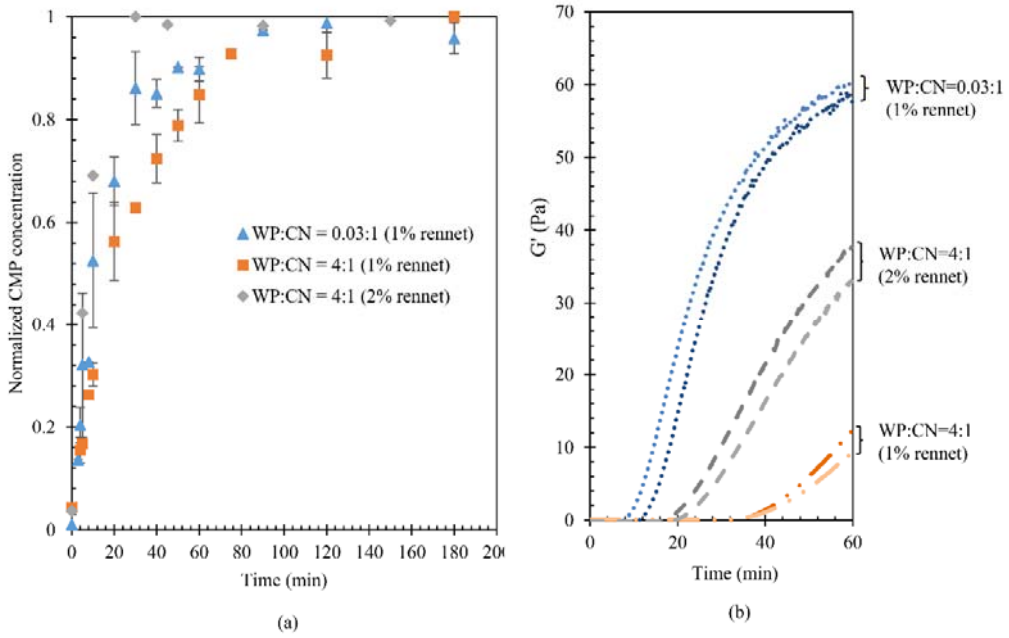


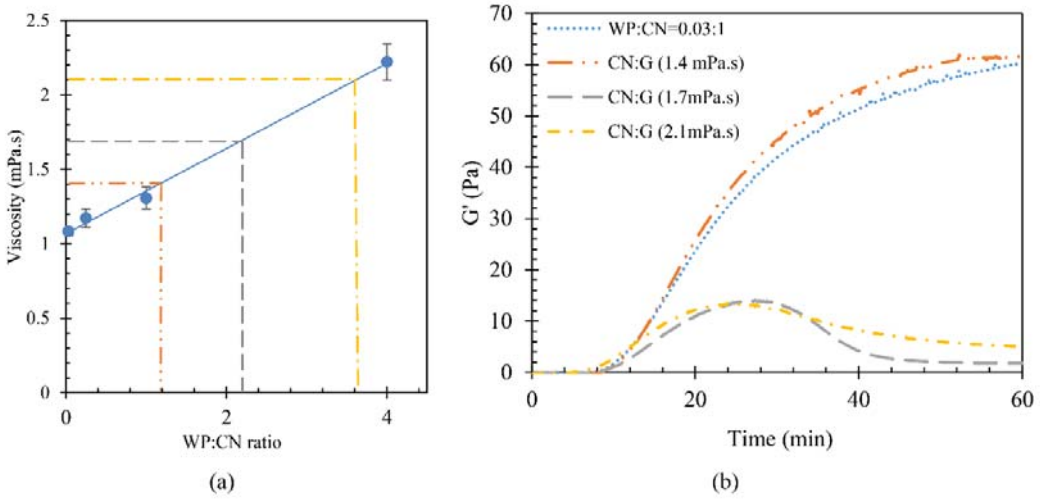
514

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.



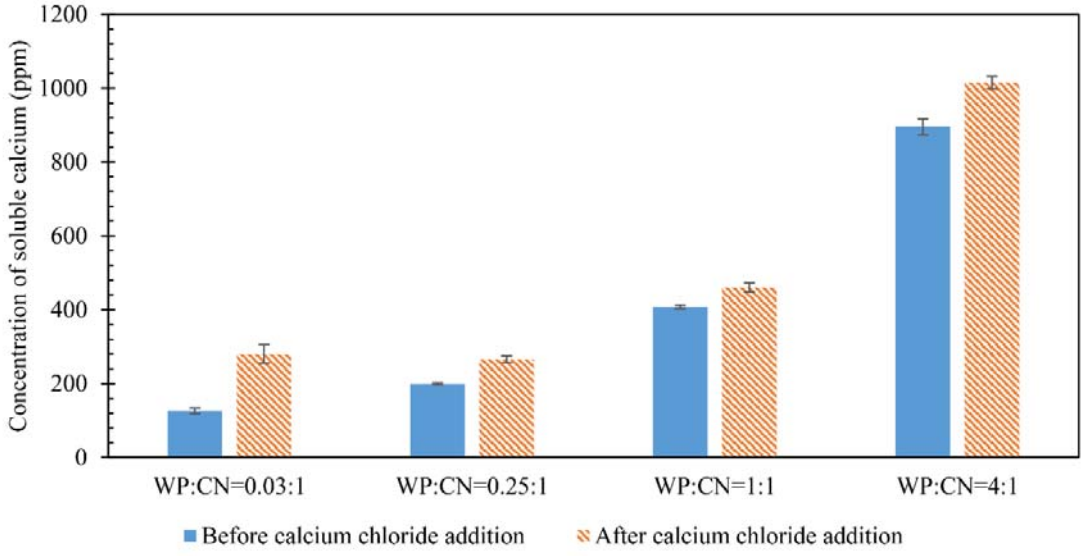
520

521 **Figure 2**: (a) The kinetics of κ-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 (▲), 4:1 with 1%
524 rennet (■), and 4:1 with 2% rennet (◆). 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 2.1Pa.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.

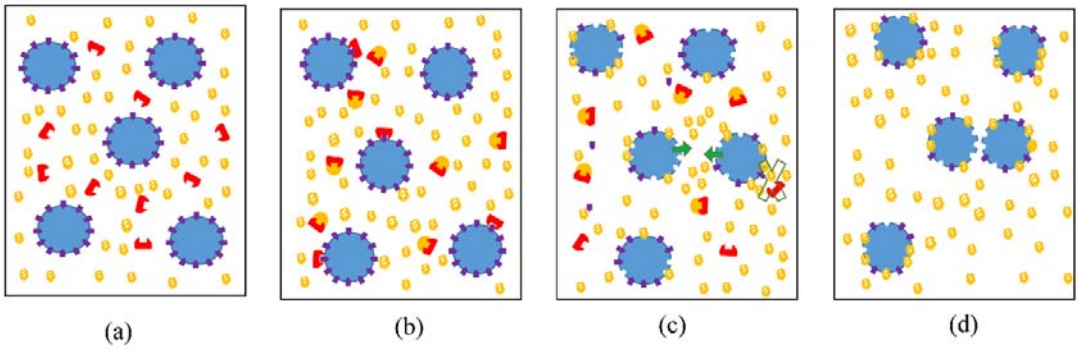


Figure 5: Schematic representation of the proposed mechanisms for the contribution of native whey proteins to the decreased rate of rennet gelation. (a) Chymosin ( ) is added to a system with casein micelles ( ) and whey proteins ( ). (b) Whey proteins inhibit ( ) the chymosin action and decrease the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

Table 1: The particle size of casein in the milk systems with different WP:CN ratios. The standard deviation of measurements in triplicate samples is indicated with (±).

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

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