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Effect of addition of gelatin on the rheological and microstructural properties of acid milk protein gels

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Abstract

To gain an understanding of the gelation mechanism of mixtures of milk proteins and gelatin, rheological and microstructural properties of the mixtures were characterized following four stages. During the acidification stage (at 45 °C), the presence of gelatin at sufficient concentration (higher than 1%) led to a lower storage modulus ($G'$) than that of the pure milk protein gels and a more heterogeneous microstructure with larger milk protein clusters was formed. During the cooling (from 45 to 10 °C) and annealing stage (at 10 °C), the $G'$ of the gels increased because of both milk gel enhancement and gelatin gelation. Higher concentrations of gelatin led to earlier formation of strand-like structures, seen in the micrographs. The gelation of gelatin changed the microstructure of whey protein isolate (WPI) gel dramatically, while gels of milk protein concentrate (MPC) and skim milk powder (SMP) maintained the typical milk gel network and gelatin formed strands and films without destroying the existing gels. During the heating stage (from 10 to 45 °C), gelatin strands were melted and the $G'$ of the mixed gels tended to revert to the value at the end of the acidification stage, indicating that the changes caused by gelatin in the microstructure of milk protein gels after acidification are reversible. Additionally, gelatin enhanced the water holding capacity (WHC) of the gels (no serum expulsion was observed for gels containing ≥1% gelatin), without increasing gel firmness significantly.

Key words

Gelatin, acid milk gels, gelation, rheology, microstructure, texture, water holding capacity

1. Introduction

Gel formation by milk proteins is the crucial stage in the manufacture of acid gels such as yogurt and many other dairy-based products. To understand the gelation mechanism of milk proteins, considerable research has been carried out using a range of dairy ingredients such as skim milk powder (SMP), milk protein concentrate (MPC), whey protein isolate (WPI) and sodium caseinate

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(Cavallieri & Da Cunha, 2008; Cooney, Rosenberg, & Shoemaker, 1993; Graveland-Bikker & Anema, 2003; Hashizume & Sato, 1988). In addition to milk proteins, hydrocolloids are important ingredients in yogurt manufacture for producing a variety of mechanical and textural properties to cater for consumers’ preferences and to improve product stability. Among the hydrocolloids used, gelatin, an animal protein produced from collagen (Boran, Mulvaney, & Regenstein, 2010), is still widely used to modify the texture of yogurt. It has high flexibility of the polypeptide chains and a non-random occurrence of imino acids (i.e., proline or hydroxyproline) in its sequence, which is unusual among gel-forming agents (Karim & Bhat, 2009). The intermolecular contacts in gelatin gels are hydrogen bonds, which make the gels thermally reversible. Specifically, a gelatin gel melts below human body temperature, which gives it the well-known “melt-in-mouth” property (Djabourov, 1988). The effects of added gelatin on the microstructure and rheology of acid milk gels have been previously reported (Fiszman & Salvador, 1999; Koh, Merino, & Dickinson, 2002; Walkenstrom & Hermansson, 1996). However, most of these studies were focused on the properties of the final mixed gel and some concerned heat-set milk gels. Therefore, the mechanism of interactions occurring in milk protein–gelatin systems during gelation (both of milk and gelatin) and melting of gelatin, which would be valuable to understand in relation to the function of gelatin during the manufacture process of yogurt and also consumption of yogurt, is still not clear.

Moreover, few studies have been published on the microstructure of milk protein–gelatin acid gels. In the 1970s, Kalab, Emmons, and Sargant (1975) reported that the gelatin in yogurt could not be detected by either scanning electron microscopy (SEM) or transmission electron microscopy (TEM), even at a very high concentration (10%). Fiszman, Lluch, and Salvador (1999) carried out a study using cryo-SEM, in which 1.5% gelatin was added to both a reconstituted milk gel and yogurt. They found that gelatin formed flat sheets or surfaces which interacted with the milk gel matrix and connected the granules and chains of milk proteins. Cryo-SEM is a powerful technique for observing samples which are difficult to observe by conventional SEM; however, attention must be paid to the possible formation of artifacts by this method. The formation of ice crystals can displace structural elements and destroy the original structure (Kalab, Allanwojtas, & Miller, 1995). SEM has been a very useful technique for determining the microstructure of milk protein gels with simple specimen preparation and to provide a three-dimensional image (Kalab & Harwalkar, 1973). The microstructure of milk gels with polysaccharides has been studied widely using SEM (Cavallieri & Cunha, 2009; Hood, Seifried, & Meyer, 1974; Sanchez, Zuniga-Lopez, Schmitt, Despond, & Hardy, 2000). The specimens in the study of Kalab, et al. (1975) were prepared by freeze drying after fixation and observed at accelerating voltage of 20 kV. The relatively high accelerating voltage may induce structure damage, especially with the technique of freeze drying during specimen preparation, while at a low acceleration voltage, the freeze drying technique was reported to produce poor resolution (Trieu & Qutubuddin, 1994). Critical-point drying (CPD) has been used widely to produce dried specimens
for SEM, which can provide distortion-free images (Bray, Bagu, & Koegler, 1993). Therefore, CPD was used in the present SEM study.

To understand how gelatin and milk components interact in yogurt, acid gelation of reconstituted WPI, MPC and SMP was studied individually, with and without gelatin. These products, with different compositions represent the ingredients in yogurt. Two important yogurt manufacturing stages were followed in this study: firstly, the solutions of gelatin and milk protein were heated at 95°C for 10 minutes, by which gelatin was melted and whey proteins were denatured, and, secondly, fermentation of yogurt was simulated by using glucono-delta-lactone (GDL) for acidification. The convenience and reproducibility of the GDL method has already been proved (Kim & Kinsella, 1989; Vlahopoulou & Bell, 1995).

The objectives of this work were to determine the ability of gelatin to alter the physical and microstructural properties of acid-induced milk protein gels and to gain an understanding of the mechanism of gelation of milk protein–gelatin mixed systems.

2. Materials and Methods

2.1. Materials

The gelatin used in this study was supplied by Gelita (Beaudesert, Australia). It was a light coloured edible beef skin (type B) gelatin powder with bloom 200, mesh 20 and isoelectric point of ~5.0, which is a commercial product commonly used in the food industry. The milk protein ingredients, whey protein isolate (WPI, protein 93.9%, moisture 4.7%, fat 0.3%, lactose 0.4% and ash 1.5%), milk protein concentrate (MPC, protein 85%, moisture 7%, fat 2.5%, lactose 5.5% and ash 8.5%) and skim milk powder (SMP, protein 33%, moisture 3.6%, fat 0.9%, lactose 54.7% and ash 7.8%) were obtained from Murray Goulburn Co-Operative Ltd (Melbourne, Australia). The chemical composition of these ingredients was provided by the supplier. The acidulant glucono-delta-lactone (GDL) was purchased from Sigma Chemical Co. (St. Louis, USA).

2.2. Methods

2.2.1. Preparation of protein solutions and acid protein gels

Milk protein solutions were prepared by dispersing the required amount of powders (WPI, SMP or MPC) in distilled water under continuous stirring for 30 min to obtain a milk protein concentration of 4.5% (w/w). To prepare the mixed solutions, milk ingredients were dispersed in water with gelatin. Three concentrations of gelatin (0.4, 1.0 and 2.5% [w/w]) were investigated. All solutions were stored at 4 °C overnight before use. The solutions were heated in a 95 °C water bath for 10 min at their natural unadjusted pH and then cooled to 45 °C immediately using cold water. For gel formation, an
appropriate amount (0.6% for WPI, 1.2% for MPC and 1.5% for SMP [w/w]) of GDL was added to
the solutions to decrease the pH to 4.6 in 4 h at 45 °C. During acidification the change in pH was
monitored with a pH meter.

2.2.2. Small deformation rheological measurement

Dynamic oscillatory measurements were performed on a stress-controlled rheometer (Model AR-G2,
TA Instruments, USA). Aliquots of protein solutions with or without gelatin were poured onto the
bottom plate of the rheometer equipped with a 4 cm, 2° cone-plate measuring system immediately
after GDL was added. The measurements were performed in a four-stage process as described by
Pang, Deeth, Sopade, Sharma, and Bansal (2013), with some modifications:

Acidification stage: Measurement commenced at 45 °C and this temperature was maintained for 4 h,
promoting formation of the milk protein gel;

Cooling stage: the temperature was lowered from 45 to 10 °C at a constant rate of 1 °C/min promoting
gelatin gel formation;

Annealing stage: the oscillatory tests were performed at 10 °C for 2.5 h to observe the maturation of
the gelling samples;

Heating stage: the melting characteristics of the gels were determined by increasing the temperature
from 10 to 45 °C at 1 °C/min.

Preliminary experiments for strain sweep showed that a strain of 0.5% was within the linear
viscoelastic region for all samples at a frequency of 1Hz. The gelation point was defined as the point
when a sharp increase in $G'$ from the baseline occurred, according to a previous milk gel study
(Matia-Merino, Lau, & Dickinson, 2004). Two independent repetitions were conducted for each
sample.

2.2.3. Microstructure

Scanning Electron Microscopy (SEM)

Milk protein solutions with or without gelatin were prepared as described above. After addition of
glucono-delta-lactone (GDL), samples were transferred to a temperature-programmable water bath
(Thermo Haake, C25P, Karlsruhe, Germany). The temperature profile was set as follows:
Acidification: 45 °C for 4 h; Cooling: 45 to 10 °C, 1 °C/min; Annealing: 10 °C for 2.5 h and Heating:
10 to 45 °C, 1 °C/min. Six samples were taken for microscopy at the following points: Point 1-end of
acidification; Point 2-10 min from the start of annealing; Point 3-30 min from the start of annealing;
Point 4- end of annealing; Point 5- at 35 °C during heating; Point 6- at 45 °C during heating.
The microstructure of the gels was determined by SEM as described by Pang, et al. (2013). Gel at each point as listed above was fixed immediately with glutaraldehyde at room temperature, dehydrated with ethanol at room temperature and then dried with a CO₂ critical point dryer (Tousimis Automatic). This procedure removed the soluble substances in the gels, such as lactose (Kalab, et al., 1973). Dried samples were platinum-coated and observed with a scanning electron microscope (JEOL 6610) at an acceleration voltage of 10 kV.

Confocal Laser Scanning Microscopy (CLSM)

Rhodamine B (0.1% [w/w]) was added to milk protein/gelatin solutions to dye the protein prior to acidification (20 µL per mL sample). After GDL addition and stirring, a drop of solution was transferred to a microscope slide, covered with a glass cover slip and sealed with nail polish to prevent evaporation. The slide was then kept at 45 °C for 4 h before observing the microstructure corresponding to Point 1 in SEM. CLSM was performed using an inverted microscope (Zeiss LSM 700), equipped with an Ar/Kr laser. A wavelength of 568 nm was used to excite the Rhodamine-labeled proteins. Images were taken using a 60x oil immersion objective (de Jong, Klok, & van de Velde, 2009).

2.2.4. Texture analysis

Texture measurements were performed using a TA–XT2 Texture Analyser (Godalming, Surrey, UK). Samples after acidification, as prepared in 2.2.1, were transferred to an incubator at 10 °C, and kept for 2.5 h before measurement. Three independent repetitions were conducted for each sample at 10 °C. The probe used was cylindrical with a flat base of 12.7 mm diameter, operating at a speed of 1 mm/s. The sample height was 30 mm in a cylindrical container of about 40mm diameter. The probe penetrated the gel during a total displacement of 10 mm. Two parameters were obtained from the force–time curves: (a) Fracture force (N/mm), defined as the force at the first significant break in the curve; (b) firmness (N/mm), defined as the initial slope of the penetration curve within the first 2 s (Fiszman & Salvador, 1999).

2.2.5. Water holding capacity

The serum expelled (SE) was quantified using a centrifugation technique according to (Farnsworth, Li, Hendricks, & Guo, 2006) with some modifications. Milk protein gels (MG), with or without gelatin, were formed in 15 ml centrifuge tubes using the steps described in 2.2.1. After 2.5 h storage at 10 °C, samples were centrifuged at 2500 rpm (1000 g) for 10 min at 10 °C. The water holding capacity (WHC) was defined as WHC (%) = 100 (MG weight – SE weight) / MG weight. Three independent repetitions were conducted for each sample and the measurements were conducted in duplicate.

2.2.6. Statistical Analysis
Minitab ver. 16 software (Minitab Inc., USA) was used for analysis of variance (ANOVA), test of significance (p < 0.05).

3. Results and Discussion

3.1. Rheology

Fig. 1 and 2 show representative results of rheological analysis of WPI and SMP gels, respectively. Results for MPC gels are shown in supplementary data (Fig. S1). For each sample, two trials were conducted and similar trends were observed in both trials.

3.1.1. Acidification stage

During acidification, for all WPI samples, with and without gelatin, $G'$ showed a steep increase at the beginning, indicating rapid gelation (Fig. 1A). This increase in $G'$ was accompanied by a sharp decrease of pH. Then $G'$ became more stable at pH around 5.5. Gelation of whey proteins started only in the acidification step (Fig. 1A). The protein concentration used in this work was lower than the minimum required for thermal gelation of whey proteins. So the heat treatment (95 °C for 10 min) prior to acidification did not cause gelling of WPI, although the whey proteins would have been denatured with partial unfolding and subsequent aggregation into fine strands (Cavallieri, et al., 2009; Cavallieri, et al., 2008; Ju & Kilara, 1998). More than 90% whey proteins could be denatured with the heat treatment conditions used in this study (Vasbinder, Alting, Visschers, & de Kruijf, 2003). The decrease of pH towards the pI of whey proteins (~ 5.3) reduces the electrostatic repulsion among the protein aggregates and promotes hydrophobic interactions and interchange reactions between sulphhydryl groups and disulfide bonds (Graveland-Bikker, et al., 2003; Hashizume, et al., 1988), resulting in gelation. This process is known as cold-gelation (Cavallieri, et al., 2008). The maximum $G'$ of the WPI gel without gelatin reached as high as 2000 Pa.

The mixed gels showed much lower $G'$ values than pure WPI gels except for the gel with 0.4% gelatin, which had $G'$ value similar to that of pure WPI gel (Fig. 1A). The decrease of $G'$ by addition of gelatin increased with increasing concentration of gelatin. At the end of acidification, the maximum $G'$ of the WPI gel with 1 and 2.5% gelatin was ~ 1400 and 55 Pa, respectively. Thus, addition of gelatin at 2.5% concentration inhibited the gelation of WPI dramatically. During this stage, gelatin could not form a gel due to the high temperature. Therefore, it is clear that WPI is the only gelling agent during acidification. Gelatin seemed to interrupt the cold-set acid gelation of whey proteins in its coiled (ungelled) form. As reported by Pang, et al. (2013), no interaction was observed in gelatin/WPI system. Nevertheless, the presence of gelatin molecules and aggregates may cause some steric interference to the formation of whey protein network, resulting in lower elastic modulus. Simultaneously, bicontinuous phase separation possibly happened in this system (Loren, Langton, &
Hermansson, 1999). Phase separation has been acknowledged in mixed systems of milk proteins and hydrocolloids that do not have strong electrostatic interactions with milk proteins (Ye, 2008). Heat induced gelatin A/WPC mixed gel was also reported as bicontinuous gel (Walkenstrom, et al., 1996). It was reported that phase separation in whey protein gels with polysaccharides only occurs when the pH approaches the isoelectric point of the proteins where the gelation of proteins occurs. But phase separation only happens when the system is liquid and not in a gel. The gelation of milk proteins decreases the mobility of gelatin molecules and inhibits phase separation. The kinetics of phase separation and the kinetics of gelation will determine the morphology of the system at the end of acidification (Lofgren, Walkenstrom, & Hermansson, 2002).

For all the SMP gels, the $G'$ increased rapidly in the first 50 min of acidification (Fig. 2A) because of micellar fusion, and casein dissociation and rearrangement due to solubilization of the colloidal calcium phosphate (Gastaldi, Lagaude, & DelAFuente, 1996). The formation of disulphide cross-linkages between denatured whey proteins and casein chains during the preheat treatment also plays an important role in the gelation (Sadeghi, 2012). After the first 50 min, the $G'$ showed small change for the next hour, corresponding to the pH decrease from 5.2 to 4.8. This could be due to the reincorporation of casein into the micelle structure (Gastaldi, et al., 1996) and Lucey, Tamehana, Singh, and Munro (1998) attributed this to solubilisation of colloidal calcium phosphate that has been in the gel network. After 100 min, when the pH was around 4.6, the $G'$ increased linearly till the end of the acidification step for all samples (Fig. 2A). At pH 4.6, chains and clusters of casein particles are formed to constitute the final network (Gastaldi, et al., 1996). The MPC gels showed a trend similar to the SMP gels (Fig. S1A) except that MPC gels started gelling earlier than SMP gels. The difference might be due to the different lactose composition of the two powders. SMP contains larger amounts of lactose than MPC, which may delay the gelation of SMP.

As observed in WPI mixed gels, addition of gelatin reduced the $G'$ of SMP and MPC gels (Fig. 2A, S1A). Gelatin has been reported to lower the gel strength of acid caseinate gel during the acidification step (Koh, et al., 2002) and similar results have been observed for MPC gels containing low methoxy pectin and $\lambda$-carrageenan. The authors attributed the reduction of gel strength to inhibition of casein network rearrangements due to interactions between casein and hydrocolloids (Everett & McLeod, 2005; Matia-Merino, et al., 2004; Matia-Merino & Singh, 2007). The steric interference as well as electrostatic repulsions is reduced when the pH is below the pI of casein. The negatively charged polysaccharides could adsorb onto the casein micelle surface, by interacting with net positively charged patches on $\kappa$-casein. If the concentration of the polysaccharides is higher than that required for saturation coverage of the casein micelles, depletion flocculation may take place and phase separation may occur (Everett, et al., 2005; Matia-Merino, et al., 2004; Matia-Merino, et al., 2007; Snoeren, 1975). However, gelatin is negatively charged at pH higher than 5.0 and slightly positively charged at pH lower than 5.0. Therefore, one possibility is that in the MPC (SMP)/ gelatin system,
gelatin interacted with the positively charged patch of κ-casein during the acidification step when the pH was higher than 5.0 and decreased the $G'$. As can be seen from Fig. 2A, and S1A, $G'$ was higher for the pure SMP and MPC gel than mixed gels from the beginning of the acidification step, where the pH was higher than 5.0. With high gelatin concentration (2.5%), depletion flocculation may have taken place and phase separation occurred.

3.1.2. Cooling and annealing stage

In the cooling stage, all gels showed an increase in $G'$ (Fig. 1B, 2B, S1B), because of the swelling of the milk protein particles and junctions (Lucey, van Vliet, Grolle, Geurts, & Walstra, 1997) and also gelation of gelatin in mixed gels. The gel with 2.5% gelatin showed an obvious change in the trend of the $G'$ value at 15 °C, which is the gelling temperature of gelatin at that concentration (Pang, et al., 2013). The gels with 0.4 and 1% gelatin showed linear change in this stage. The results were in agreement with our previous study on pure gelatin gelation, which showed that gelatin concentration of $\leq 1\%$ was insufficient for gelation during the cooling stage (Pang, et al., 2013).

During the annealing stage, $G'$ of gels containing 0, 0.4 and 1% gelatin showed little change, while $G'$ of the gel with 2.5% gelatin showed an increase (Fig. 1C, 2C, S1C). It should be noted that $G'$ of the MPC gel with 2.5% gelatin surpassed the value of the pure MPC gel during the annealing stage. This could be because the gelation of 2.5% gelatin increased the elasticity of the MPC gel, which supplemented and exceeded the decrease in $G'$ during the acidification step; while for other gels, the increase in gel elasticity by gelation of gelatin was insufficient to negate the decrease in $G'$ during acidification.

3.1.3. Heating stage

In the heating stage, the $G'$ of all the gels with and without 0.4% gelatin decreased linearly, as well as WPI gel with 1% gelatin (Fig. 1D). It has been reported that higher temperatures lead to more or stronger hydrophobic bonds in milk gels, which could cause the protein particles to shrink and consequently interactions and contact junctions between particles would be weakened (Lucey, et al., 1997). Unlike WPI gels, SMP and MPC gels with 1% gelatin showed an inflection at around 25°C (Fig. 2D, S1D), which corresponded to the melting temperature of pure gelatin gels at 1% (Pang, et al., 2013). The difference could be attributed to the much higher $G'$ of the WPI gels than the MPC and SMP gels, therefore the small inflection by 1% gelatin melting could not be detected in the profiles of the WPI gels. The WPI gels with 2.5% gelatin showed a dramatic decrease of $G'$ between 20 and 27°C (Fig. 1D), which was in agreement with the melting profiles of pure gelatin gel (Pang, et al., 2013). Similar results have been reported for mixed gels of sodium caseinate and β-glucan, an inflection corresponding to the melting point of β-glucan was observed in gels containing sufficient β-glucan (Kontogiorgos, Ritzoulis, Biliaderis, & Kasapis, 2006). In addition, the gels did not melt completely,
indicating a continuous milk protein gel. It seems that during heating, melting of gelatin does not disrupt the continuity of the milk gels, which is still preserved in the form of a continuous matrix.

Similar results have been reported for a heat-induced whey protein gel with added κ-carrageenan; the protein gel remained after the κ-carrageenan network melted (Turgeon & Beaulieu, 2001). The final G' of all gels at the end of the heating step reached a value similar to that at the end of the acidification step, suggesting gelation and melting of gelatin have little influence on the continuity of the gels. Similar results were found in heat-set whey protein gels with gelatin (Cooney, et al., 1993; Walkenstrom & Hermansson, 1994; Walkenstrom, et al., 1996).

Nevertheless, a subtle difference could be observed among the WPI, MPC and SMP gels when gelatin started melting. In the WPI and MPC gels, melting of gelatin caused a smooth decrease in G' (Fig. 1D, S1D). Two trends of decrease of G' could be seen: one caused by gelatin and milk proteins together and the other caused by milk proteins only. In the SMP gels (Fig. 2D), the G' increased again after gelatin had melted and became stable after 30 °C. It seems that the melting of gelatin at 2.5% in the mixed SMP gel affected the cohesion of the system, and after the gelatin had totally melted, the SMP gel structure rearranged and G' increased. This difference could be due to the different degrees of interaction between gelatin and milk proteins and further study needs to be done to understand this more thoroughly.

From the results of the four stages, it can be seen that the negative effect of addition of gelatin on the G' of the gels occurred in the acidification stage. It is suggested that during gelatin gelation, the gelatin strands form in the pores of gels and this gelation does not affect the continuity of existing network. Gelation of the main component of whey protein isolate, β-lactoglobulin, has been studied together with κ-carrageenan (Eleya & Turgeon, 2000). It was found that a bicontinuous gel was formed by independent conformational changes in β-lactoglobulin during heating and cooling; no interactions occurred between the two components, as indicated by identical DSC profiles for pure β-lactoglobulin gel and κ-carrageenan mixed gel. The authors suggested that upon cooling, the κ-carrageenan gelled in the pores of the protein network and also formed a continuous network (Eleya, et al., 2000). For all three types of protein gels, the formation of mixed gels with gelatin could be divided into two stages. One stage is when the temperature is above the coil-to-helix transition temperature of gelatin and the gelatin molecules are free or bound to proteins in the random coil conformation interfering with the formation of milk protein gels. The second stage is when the temperature is below the transition temperature of gelatin, which indicates gelation of the gelatin. This stage can improve the gel strength of protein gels. A similar gelation mechanism has been reported for sodium caseinate/κ-carrageenan mixed gels (Ribeiro, Rodrigues, Sabadini, & Cunha, 2004). Therefore, the final G' of the gel after annealing is considered to be due to a balance between the decrease of gel strength by interference of gelatin in the acidification stage and an increase of gel strength by gelatin gelation during the cooling stage. Different results could be obtained with different hydrocolloids.
Gellan gum, κ-carrageenan and pectin were reported to increase G' of WPI gel to varying degrees (de Jong, et al., 2009; de Jong & van de Velde, 2007).

3.2. Microstructure

At least two independent replicates of each sample were analysed for microstructure. Similar results were obtained for the replicates. Hence, representative results are included in this section. Since pure milk protein gels did not change much during the whole process after acidification, results for those gels are only shown at the end of acidification (point 1) and annealing (point 4) for comparison. Gels containing 0.4% gelatin showed micrographs similar to pure gels (data was not shown).

3.2.1. Acidification stage

The effect of the coil form of gelatin on microstructure formation of the milk protein gels was observed from the results at point 1 (Fig. 3). Gelatin was not observed in any of these micrographs, since it existed in liquid form and did not form a gel at this temperature.

The micrographs of pure WPI gels at point 1 revealed a porous, homogeneous structure (Fig. 3A). Protein aggregates were evenly distributed among the protein network, connected by some thin strands. Pure MPC and SMP gels showed that milk proteins formed a branched network of chains and clusters (Fig. 3B, C). Round clusters were distributed in a well-organized network. The diameter of the casein particles was 0.2-0.3 µm and the average pore size was about 1-2 µm. Similar results have been previously reported (Aichinger, et al., 2003; Cavallieri, et al., 2009; Walkenstrom, et al., 1996). Further information about the surface of the particles and how these particles connect was not obtained in our study because of the resolution limitation. It has been reported that some degree of fusion between casein micelle particles or between casein and whey proteins can be seen from SEM (Kalab, et al., 1973). However, TEM micrographs revealed that the casein particles were connected by some filamentous structures or aggregates located on the surface of casein particles, rather than being fused (Sanchez, et al., 2000). Also, it was reported that heat treatment before acidification plays an important role in the formation of filaments and aggregates. The denatured whey proteins form a covalent complex with the κ-casein located at the surface of casein micelles. These complexes connect with other denatured whey proteins associated with micelles, acting as bridges (Kalab & R., 1974; Modler & Kalab, 1983).

At this point, the microstructures of mixed gels with 0.4 and 1% gelatin were very similar to that of corresponding pure milk protein gels and no gelatin network could be observed (data not shown). However, with increasing gelatin concentration, the gel network appeared increasingly heterogeneous with thicker chains and larger clusters, except for the SMP gel (Fig. 3F) in which no clear differences could be seen. This change could be seen more clearly in the gels with 2.5% gelatin (Fig. 3D, E). In
WPI/2.5% gelatin gel (Fig. 3D), highly compacted whey protein particles and larger pores (~ 1 µm) than in the pure WPI gel (~ 0.5 µm) were observed. In MPC/2.5% gelatin gel (Fig. 3E), the casein particles were grouped in thicker chains and larger clusters than in the pure gel.

To confirm these observations, confocal microscopy was used to examine the three milk protein gels with 2.5% gelatin at point 1. Results are shown in Fig. 4. In gelatin-containing gels (Fig. 4D-F), the networks were more heterogeneous and the contrast between the milk protein phase (red) and the serum phase (black) was substantially increased, compared with pure milk gels. It appears that the presence of 2.5% gelatin increased the porosity of milk gels. This observation explains well the rheological results, where gelatin reduced the strength of the network in the acidification stage. Similar results with confocal microscopy have been reported for cold-set gels of WPI/‘low charge density’ gums (LBG, gellan gum, κ-carrageenan and HM pectin) (de Jong, et al., 2009). In caseinate/pectin mixed gels, a more open microstructure and larger pore size was found with increasing pectin concentration (Matia-Merino, et al., 2004; Matia-Merino, et al., 2007). It should be mentioned that gelatin was indistinguishable from other proteins in the micrographs in Fig. 4. This could be because at this temperature gelatin did not form any structures that could be resolved using confocal microscopy. In addition, the presence of gelatin, regardless of concentration, only changed the density of the gel network, not the size of the primary casein particles and whey protein particles. The large clusters formed in gels with gelatin could still be seen to be comprised of small particles (Fig. 3). Similar results have been found for milk gels with certain polysaccharides (Sanchez, et al., 2000). The similarities between the micrographs of SEM (Fig. 3) and confocal microscopy (Fig. 4) also suggest that protein structures obtained by SEM are not artefacts. The un-gelled coil form of gelatin was not observed under the experimental conditions used in this study. The un-gelled gelatin could have been lost during sample preparation or not resolved by the magnification for SEM used in this study. 3.2.2. Cooling and annealing stages

In the cooling and annealing stages, gelatin was expected to start gelling and the changes in the microstructure of the milk protein gels were followed. However, no gelatin structures were observed in any samples during the cooling stage (data not shown), which differed from the rheological observations. This could be attributed to the methods used to prepare the samples. For the rheology study, the sample was a thin layer on a rheometer plate, which ensured the sample temperature was very close to the set temperature of the machine. For the microstructure study, the sample was prepared in bulk, so there was some delay in the samples reaching the set temperature. Therefore, gelatin gelation in the acid milk protein gels was only observed in the annealing stage. Comparing the pure and mixed gels, the interpretation of the micrographs is that the thicker strands and larger aggregates are from milk proteins and the thinner strands and smaller aggregates are from gelatin. The possibility of occurrence of artefacts due to sample preparation techniques used cannot be ruled out, as they may induce changes in the distribution of the gel network. However, it is unlikely that the
formation/appearance of structures attributable to gelatin can be solely due to the sample preparation techniques used in this study. All the samples were treated and prepared for SEM in exactly the same manner. While this does not preclude the possibility of artefacts, it does suggest that artefacts should have been observed even in samples that did not contain gelatin. Also, gelatin structures were only observed in samples where the gelatin was in gel form. The transition of the gel microstructure during the four stages correlated very well with the rheological results. These facts strongly suggest that the strand-like structures observed in this study were due to the presence of gelatin in the samples, rather than being artefacts.

After 10 min at 10 °C (point 2) (Fig. 5), the gels with 1% gelatin showed similar structure to those taken at point 1 and no gelatin strands could be seen. But, in the MPC and SMP gels, some small particles located on the surface of casein aggregates could be observed (Fig. 5B, C) and these could be gelatin aggregates that formed before the strands were formed. In the gels with 2.5% gelatin, gelatin strands can be seen clearly, except in SMP gel (Fig. 5F) which cannot be explained by current knowledge. In WPI/2.5% gelatin gel, it seems that gelatin strands repelled the existing whey protein gel strands and the protein phase became heterogeneous with large clusters and voids (Fig. 5D). The interpenetrating network was composed of two different sub-phases: one rich in protein aggregates (solid line arrow, w) and one formed by gelatin filaments (dash line arrow, gs), while in both MPC and SMP gels, the existing milk gel structure was not changed; the apparent diameter of pores remained the same as in the pure gels; some thin gelatin strands were seen connecting the casein particles (Fig. 5E). After 30 min at 10 °C (point 3) (Data not included, see Supplementary Fig. S2), more gelatin networks were developed, especially in WPI gel with 2.5% gelatin where gelatin strands became very dense and no clear single strand could be easily observed. Therefore, it can be inferred that as the 2.5% gelatin started gelling as early as 10 min in annealing (except in the SMP gel), the 1% gelatin started gelling at 30 min and the 0.4% gelatin did not show any gelation in the entire annealing process. These results were in agreement with our rheological results, high gelatin concentration leading to earlier gelation during annealing. This is also in agreement with the report that gelatin does not gel when the concentration is less than 1% (Djabourov, Lechaire, & Gaill, 1993; Pang, et al., 2013).

The results for the gels at the point 4, which was the final gel network at the end of the annealing stage, are shown in Fig. 6. In gels with 1% gelatin, more strands were formed than at the point 3 and were distributed throughout the entire gel network (Fig. 6D-F). The WPI gel was highly changed and very large voids were observed. In the MPC gel, gelatin formed a film which covered the milk protein network. However, the organization of the MPC and SMP gels did not change much and the size of the casein particles was not modified by the gelatin. In gels with 2.5% gelatin (Fig. 6G-I), very dense and solid structures were observed; almost no voids could be seen in the network. The WPI gel had lost its original porous network and became over-aggregated. This observation correlated well with
the rheological results that at 2.5% concentration gelatin was more dominating in WPI gels than in MPC and SMP gels. The MPC and SMP gels still maintained the typical casein gel network and the apparent diameter of the casein particles did not change. Microstructures of milk gels with polysaccharides have been studied widely and similar results have been obtained (Cavallieri, et al., 2009; de Jong, et al., 2009; Sanchez, et al., 2000; van den Berg, Rosenberg, van Boekel, Rosenberg, & van de Velde, 2009). The gelation of gelatin induces solvent redistribution between the phases as a result of the conformational changes that accompany gelation (Beaulieu, Turgeon, & Doublier, 2001). Therefore, with high gelatin concentration, a large solvent redistribution would be expected and the existing milk gel structure would be affected. This could be seen clearly in the WPI/gelatin gels in which gelatin strands displaced the existing WPI gel network and the gel became heterogeneous, even at 1% gelatin concentration (Fig. 6D). It seems that the WPI network was easier to disrupt by gelatin strands than the MPC and SMP networks, even though higher gel strength was observed for the WPI gel from the rheology and texture study.

3.2.3. Heating stage

During the heating stage, gelatin was expected to melt and the effect of its melting on milk protein gels was followed by microstructure observation at two temperature points. At 35 °C (point 5) (see Supplementary Fig.S3), most of the gelatin strands had melted in the gels with 1% gelatin, except in the SMP gel, where several gelatin strands could still be clearly seen. In gels with 2.5% gelatin, the structure was not changed much compared to point 4, except in the WPI gel. At 45 °C (the point 6) (Fig. 7), no gelatin strands could be seen in any of the gels. Gels with both concentrations of gelatin showed clear milk gel network similar to those at the point 1. For the WPI gels (Fig. 7A, D), the network continued to reorganize since gelatin melted at the point 5 and the size of the protein clusters became closer to those at point 1. Higher temperature was required to melt all the gelatin at higher concentration, which was in agreement with our rheological study (Pang, et al., 2013). The results that the structure of milk protein gels could revert to the structures at point 1 after melting of gelatin also agreed with previous rheological results, which showed that after melting of gelatin, the strength of the gel was almost the same as before gelatin gelation. Therefore, it seems that the gelatin strands were formed without destroying the original gel network and that gelatin reversibly changes the milk gel microstructure during its gelation by only displacing and concentrating the existing milk protein particles, but not changing the size of the particles. No more particle fusion occurred during this change. Similar results were reported by Sanchez, et al. (2000).

3.3. Texture analysis

The representative penetrometry profiles of the milk protein gels with different concentrations of gelatin were shown in supplementary data (Fig. S4). Very different profiles were obtained from the three types of milk protein gels. For pure gels, WPI gels showed a sharp peak at fracture, which
indicated that strong gels were formed. Profiles of MPC and SMP gels indicated a moderately firm gel, which broke during analysis at comparatively lower fracture force and lower displacement (the distance at fracture) than the WPI gels. SMP gels seemed more deformable than MPC gels, as lower fracture force was observed from SMP gels. Also, the difference between pure gels and mixed gels could be observed from the shape of profiles. WPI mixed gels were WPI-dominated gels at low gelatin concentration, as the shape of the profiles were very similar to that of pure WPI gels and only changed at high gelatin concentration (2.5%) (Fig. S4A). In MPC gels at any concentrations of gelatin and SMP at \( \geq 1\% \) gelatin, the curves became very smooth after fracture (no small peaks observed until the end of the compression), which may indicated an improvement of textural smoothness of the gels by gelatin (Fig. S4B, C).

Specific to each kind of gel, gel firmness was calculated as the initial slope of the penetrometry curves and the results were compared between different concentrations of gelatin (Table 1). The pure WPI and MPC gels had significantly higher firmness than their respective mixed gels. Similar results have been reported for a range of polysaccharides on cold-set WPI gels (de Jong, et al., 2007; Li, Eleya, & Gunasekaran, 2006). Unlike other gels, SMP gels with gelatin showed higher firmness than pure SMP gels with 2.5% gelatin being the firmest. In addition, the fracture force of gels was recorded (Table 1). Low breaking force at fracture indicates high fracturability. Gelatin lowered the fracture force in both WPI and MPC gels and an increase was only seen in the SMP gel with 2.5% gelatin. It seemed that a positive effect of gelatin on the texture of milk protein gels could only be seen in the SMP gel, which may indicate that gelatin was more compatible with SMP in textural construction of gels than with WPI and MPC.

Comparing different protein gels at fixed gelatin concentration, it was found that at 2.5% gelatin concentration the differences between gel firmness and fracturability of different protein gels were negligible. It seemed that at higher gelatin concentrations, the textural characteristics of all milk protein gels became more gelatin-dominated. This is in agreement with the study on gelatin type A by Fiszman and Salvador (1999) and similar results have been reported on whey protein gels with \( \kappa \)-carrageenan (Turgeon, et al., 2001).

### 3.4. Water holding capacity

Whey separation happens during the rearrangements of clusters and particles in milk gel systems as pH of milk decreases (Lucey, 2001). Water holding capacity (WHC) results for all gels, with and without gelatin, are shown in Table 1. WPI gels showed no serum expulsion after centrifuging with or without gelatin, therefore 100% WHC was obtained. Both MPC and SMP gels showed some serum expulsion without gelatin after centrifuging. From rheology and texture analysis of the gels, WPI formed much firmer gels than MPC and SMP, which could result in higher WHC. The structure of acid casein gels, especially made with heated milk, was reported to show large pores and dense
aggregates, due to the rearrangements of protein clusters and particles (Lucey, 2001). This could lead to low WHC in MPC and SMP gels (Unal, Metin, & Isikli, 2003). Also, WHC was higher for SMP than for MPC gels, which could be attributed to the higher total solids content of the SMP gel (Remeuf, Mohammed, Sodini, & Tissier, 2003). The WHC of both MPC and SMP gels was improved by adding gelatin. With ≥1% gelatin, no serum expulsion was observed and for the MPC gels, 0.4% gelatin significantly increased its WHC. The similar effect of gelatin on WHC in yogurt has been previously reported (Fiszman, Lluch, et al., 1999).

Interestingly, comparing SMP gels with 0.4 and 1% gelatin, it was found that a higher WHC (100%) was obtained with 1% gelatin, but higher gel firmness and storage modulus were observed for 0.4% gelatin containing gel during annealing. Therefore, with the appropriate concentration, gelatin could effectively increase the WHC of milk protein gels while not increasing the gel strength; the critical concentration could also depend on the gelatin type and bloom. It has been reported that gelatin stabilized stirred yogurt showed a lower susceptibility to syneresis with relatively low gel strength (Modler, et al., 1983). They attributed this to the efficient immobilization of aqueous phase in yogurt network. This is a unique property of gelatin and could be useful in applications, since high WHC normally correlates well with high gel strength (Lucey, 2001).

4. Conclusions

According to the results of this study, the interference in milk gel formation by gelatin most likely occurs in the acidification step. During the cooling and annealing stages, gelatin gelled but the milk protein gels were affected little by the gelatin gelation. This process was expected to enhance the strength of the milk protein gels. If the enhancement exceeded the interference effect, the final G’ at the end of annealing stage for the mixed gel would be higher than that for the pure gel; this was the case of the MPC gel with 2.5% gelatin. Otherwise, the final G’ values of the mixed gels, such as 1% gelatin containing gels, were lower than those of the pure protein gels. Addition of 0.4% gelatin did not affect the gels significantly. In the heating stage, gelatin melted and the G’ returned to the value at the end of the acidification step. This again proved that the negative interference by gelatin occurred only in the first (acidification) step; otherwise the G’ at the end of the heating step would have been even lower. Addition of gelatin decreased the firmness and increased the fracturability of all gels except SMP gels. Micrographs proved the hypothesis that gelatin forms strands and films without destroying the existing milk gel. The form of the gelatin structures in the micrographs was dependent on the temperature of sampling and concentration of gelatin. When the gelatin in the mixed gels is totally melted, the microstructure of the gels reverts to that of the gels before gelation of the gelatin. Therefore, gelatin only causes changes to the structure of acid milk protein gels during the acidification, while during the cooling and annealing only the density of existing milk gel network is changed and the change is reversible. The results help to explain the gelling and melting properties of
gelatin in acid milk protein gels and the effect of gelatin on gel strength. Gelatin can enhance the water holding capacity of milk protein gels without increasing their firmness, and that WPI gels have very high water holding capacity, which could be valuable in yogurt manufacture.

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References


Table 1. Effect of gelatin on penetration parameters and water holding capacity (WHC) of acid milk protein gels

<table>
<thead>
<tr>
<th>Milk protein</th>
<th>Gelatin concentration (%)</th>
<th>Firmness (N/mm)</th>
<th>Fracture Force (N/mm)</th>
<th>WHC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WPI</td>
<td>0</td>
<td>0.20±0.01 a</td>
<td>2.78±0.19 a</td>
<td>100±0 a</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>0.14±0 c</td>
<td>1.47±0.17 b</td>
<td>100±0 a</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.18±0.01 b</td>
<td>1.80±0.02 b</td>
<td>100±0 a</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>0.12±0 c</td>
<td>0.87±0.03 c</td>
<td>100±0 a</td>
</tr>
<tr>
<td>MPC</td>
<td>0</td>
<td>0.20±0 a</td>
<td>1.07±0.03 a</td>
<td>82.8±0.54 c</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>0.10±0 c</td>
<td>0.81±0.02 b</td>
<td>91.7±0.81 b</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.13±0 a</td>
<td>0.50±0.02 d</td>
<td>100±0 a</td>
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<tr>
<td></td>
<td>2.5</td>
<td>0.13±0.01 b</td>
<td>0.62±0.03 e</td>
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</tr>
<tr>
<td>SMP</td>
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<td>0.07±0 d</td>
<td>0.31±0.01 b,c</td>
<td>98.5±0.15 b</td>
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<tr>
<td></td>
<td>0.4</td>
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<td>0.40±0.04 b</td>
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<td>0.22±0.02 f</td>
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</tr>
<tr>
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<td>2.5</td>
<td>0.16±0 a</td>
<td>0.58±0.011 a</td>
<td>100±0 a</td>
</tr>
</tbody>
</table>

Means at different gelatin concentrations are compared only within a column and only within a milk protein type; mean values with different letters are significantly different (p < 0.05)
Fig. 1

A) Temperature (°C) vs. G' (Pa)
B) pH vs. G' (Pa)
C) Time (min) vs. G' (Pa) with different gelatin concentrations
D) Temperature (°C) vs. G' (Pa) with different gelatin concentrations

Without gelatin
With 0.4% gelatin
With 1% gelatin
With 2.5% gelatin
Fig. 2

A. Time (min) vs. G' (Pa) for different pH levels (4.4 to 6.4) and temperatures (10 to 40°C).

B. pH vs. G' (Pa) at different temperatures (10 to 40°C).

C. G' (Pa) vs. time (0 to 140 min) for samples without and with different concentrations of gelatin (0.4%, 1%, 2.5%).

D. G' (Pa) vs. temperature (10 to 40°C) for samples with different concentrations of gelatin.
Fig. 5
Fig. 7
Highlights

• Gelation and melting of gelatin in milk protein/gelatin gels can be observed from both rheology and microstructure results
• The interference on milk protein gels by addition of gelatin happens during acidification stage
• Gelation of gelatin does not destroy the continuity of existing milk gels
• Rheological and microstructural properties of mixed gels revert to that at the end of acidification after gelatin melts
• Gelatin can enhance the water holding capacity of SMP gels without increasing the firmness
Fig. S2
Fig. S4

A

Distance (mm)

Force (N)

B

Distance (mm)

Force (N)

C

Distance (mm)

Force (N)