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Influence of ultrasound on chemically induced gelation of micellar casein systems

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Gelation is a significant operation in dairy processing. Protein gelation can be affected by several factors such as temperature, pH, or enzyme addition. Recently, the use of ultrasonication has been shown to have a significant impact on the formation of whey protein gels. In this work, the effect of ultrasonication on the gelation of casein systems was investigated. Gels were formed by the addition of 7·6 mM Tetra Sodium Pyro Phosphate (TSPP) to 5 wt% micellar casein (MC) solutions. Sonication at 20 KHz and 31 W for up to 30 min changed the surface hydrophobicity of the proteins, whereas surface charge was unaltered. Sonication before the addition of TSPP formed a firm gel with a fine protein network and low syneresis. Conversely, sonication after TSPP addition led to an inconsistent weak-gel-like structure with high syneresis. Gel strength in both cases increased significantly after short sonication times, while the viscoelastic properties were less affected. Overall, the results showed that ultrasonication can have a significant effect on the final gel properties of casein systems.

Keywords: Casein micelles, ultrasound, gelation, tetrasodium pyrophosphate.

Gelation is a critical first step in both cheese making and yoghurt manufacturing. Gelation can be induced by rennet action, acidification or the heat treatment of milk. The appearance, microstructure and rheological properties of the resulting gel are important physical attributes that contribute to the overall sensory and functionality of the products (Lucey, 2001, 2002). The structure and rheology of these gels are dependent on the mechanism of aggregation which in turn depends on the nature of the interactions between dairy proteins during the aggregation processes (Eliot & Dickinson, 2003).

The use of high intensity ultrasound to change the rheology of whey protein systems and to create such gels has attracted considerable attention (Kresic et al. 2008; Ashokkumar et al. 2009; Jambrak et al. 2010; Zisu et al. 2011). When an acoustic field is applied to a liquid, micro bubbles are formed, in a process known as cavitation. The collapse of cavitation bubbles results in shockwave formation, turbulent motion of the liquid (micro streaming) and micro jetting (Ashokkumar et al. 2004). These physical processes disrupt whey protein aggregates allowing for more rapid heat-induced gelation, increased gel strengths and changes in gel syneresis.

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The effects of ultrasonication on the formation of casein containing acid and heat induced gels have also been considered (Vercet et al. 2002; Riener et al. 2009, 2010; Nguyen & Anema, 2010; Zisu et al. 2011). Acid gel firmness (G') was found to be altered when skim milk was ultrasonically treated prior to acidification, although the effect was attributed largely to denaturation of whey caused simply by the temperature increase resulting from sonication (Nguyen & Anema, 2010). The simultaneous application of heat and ultrasound under moderate pressure (manothermosonication) was shown to increase the strength of yoghurt gels (Vercet et al. 2002). Other researchers also found that thermosonication improved the rheological properties of yoghurt gels (Riener et al. 2009, 2010). Madadlou et al. (2010) studied acid gels made from sonicated casein solutions at 24 and 130 kHz and found that sonication altered the gelation point to lower pH values and increased the elasticity of freshly formed gels. Furthermore, they obtained gels with a more interconnected structure and smaller non distinguishable protein aggregates.

These studies have clearly shown the benefits of sonication to industry in terms of reducing gelation times in cheese making and reducing syneresis in yoghurt preparations. However, the separate roles of casein micelles and whey proteins in response to such sonication have not been clearly elucidated. In this paper, we aim to understand the effects of ultrasound on the gelation of casein micelles in

isolation. We use Tetra Sodium Pyro Phosphate (TSPP) as a laboratory technique to induce gelation of the micelles. This approach has been described by Mizuno & Lucey (2007). The TSPP disrupts the casein micelles by sequestering calcium as calcium pyrophosphate complexes. These complexes then associate with the dispersed caseins. Reducing charge repulsion, which further facilitates interactions between hydrophobic segments of the casein proteins. These aggregation mechanisms lead to the formation of chains of micelles that are linked together to give a 3D network (Schorsch et al. 2001). The approach was chosen in place of rennet or starter culture addition, as many workers have already shown that ultrasound can influence the behaviour of such enzymes (Mawson et al. 2011; Chandrapala et al. 2012b). Similarly, heat induced gelation was avoided as casein micelles are very stable to this approach, and any observed gelation would likely arise from the impact of residual whey protein.

Materials and methods

Materials

A Micellar Casein (MC) powder was obtained from MG Nutritionals (Cobram, Victoria, Australia). The MC powder contained 85·5% protein, 1·6% fat, 0·6% lactose, 5·3% moisture and 7·3% ash. Scanning electron microscopic analysis confirmed that the casein was in a micellar form (results not shown). However, the powder still contained some residual whey protein (around 6% of the total protein content). TSPP was obtained from Sigma-Aldrich Pty Ltd (Castle Hill, NSW 1765, Australia). Ultrapure (MilliQ) water was used in all experiments.

Reconstitution of MC powder

MC powders were reconstituted in MilliQ water to obtain 50 g/kg Solids Non Fat solutions. The solutions were continuously stirred for ½ h at 50 °C and were stirred further for 1 h at room temperature. The solutions were then equilibrated overnight at 4 °C. This reconstitution process, showed maximum dispersion and hydration of the powders after preliminary experiments exploring various stirring speeds, hydration times and temperatures (results not shown). On the next day, the solutions were equilibrated at 25 °C for 1 h prior to further analysis.

Sonication

Solutions (60 ml) were sonicated in a glass vessel equipped with a cooling jacket using a 20 kHz ultrasonic horn (19 mm diameter, Branson Sonifier 450 (450 W),) at an amplitude of 50% for 0, 1, 5, 10 and 30 min. The actual power delivered to the solution was 31 W as determined by calorimetry. During sonication, chilled water was continuously circulated through the cooling jacket to maintain the sample temperature at 6 ± 4 °C.

Gel preparation

Two procedures were used; the first procedure involved the addition of TSPP after sonication of MC solutions and the second procedure involved TSPP addition to MC solutions prior to sonication. Stock 500 mM-TSPP solution was prepared and diluted to 7·6 mM in the final MC solutions. The pH was adjusted to pH 6·0 using 1 M HCl. These pH and TSPP concentration were selected based on preliminary results to obtain a gel with maximum strength (results not shown). Solutions were allowed to gel at 25 °C for 24 h after TSPP addition. The gels were then kept at 4 °C for another 24 h.

Surface hydrophobicity

Surface hydrophobicity measures the relative hydrophobicity of the surface of proteins, protein aggregates and casein micelles. Measurements were carried out using a fluorometric assay as described by Chandrapala et al. (2011). The excitation and emission slits and wavelengths were set at 5 nm/5 nm and 380 nm/465 nm, respectively. The Relative Fluorescence Intensity (RFI) of each solution was measured starting from buffer blank and then the lowest to highest protein concentration. Surface hydrophobicity was determined by taking an average of 3 individual measurements.

Surface charge measurements

A Malvern Zetasizer (Malvern Instruments Ltd., Malvern, Worcestershire, UK) was used to similarly measure the surface charge of proteins, aggregates and micelles. MC solution (1 µl) was diluted in 3 ml water. The diluted solutions were injected to the cell through the automated injection system to measure the charge.

Gel strength

Gel strength was measured using a TA-XT2 texture analyser (Stable Microsystems, Godalming, UK), fitted with a 5 kg load cell and a P/5–5 mm diameter stainless steel cylinder probe attachment. Samples were prepared in containers of diameter 4 cm and sample height of 3 cm and samples were allowed to equilibrate to 4 °C prior to testing. A two stage compression test was performed at 2 mm/s with a compression distance of 15 mm directly on the gels in their containers. The process was repeated three times with fresh samples and an average of these readings was recorded as the gel strength.

Gel syneresis

Gel syneresis was determined as the ratio of the liquid mass expelled during centrifugation (Heraeus Biofuge Primo, Germany) at 1449 ***g*** for 20 min to the weight of the original sample (Zisu et al. 2011).

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Viscoelastic properties

Gels are viscoelastic and their rheological properties can be determined by low amplitude dynamic oscillation resulting in the measurements of G′ and loss tangent (Tan δ). The viscoelastic properties were measured as described in Madadlou et al. (2010). An ARES Rheometer with a cup and vane geometry (4°, 40 mm) was used (TA Instruments Ltd, Fleming Way, Crawley, West Sussex RH10 9NB). Frequency sweep measurements were performed on freshly formed gels in the range of 0·01 to 10 Hz. To assure working conditions remained within the linear viscoelastic region, strain sweep measurements were performed and 1·5% strain amplitude was used.

Confocal scanning laser microscopy

The microstructure of gels was visualised by a Leica TCS SP2 confocal microscope (Leica Microsystems Pty Ltd, 112–118, Talavera Road, North Ryde, 2113 Australia). Fast green (300 µl) was added to 30 ml MC solutions after the addition of TSPP. Once the gels were set, a portion was cut carefully and placed onto a microscope slide. A 100X oil immersion objective lens was used for observation of the gels.

Statistical analyses

When necessary, one way ANOVA with a 95% confidence interval was used. The ANOVA data with $P<0·05$ were considered statistically significant. Furthermore, the relationships between all variables were analysed using a correlation matrix.

Results and discussion

Surface hydrophobicity and surface charge

The results by Mizuno & Lucey (2007) suggested that the aggregation of proteins is governed by a balance between attractive and repulsive forces. The attractive forces in casein are hydrogen bonds, calcium phosphate crosslinks and hydrophobic associations, whereas the repulsive forces can involve electrostatic repulsions which are affected by the net charge of caseins or the ionic strength of the solution. Hence, it is important to look at the changes in surface hydrophobicity and charge of the particles as they can act as the main contributors to aggregation processes.

Figure 1(a) shows the surface hydrophobicity of MC solutions with or without sonication and TSPP addition. Sonication alone significantly ($P<0·05$) increased the hydrophobicity of MC solutions within 5 min of treatment. These results concur with the observations of our prior work, where such affects are attributed to the disruption of whey or casein-whey protein aggregates (Zisu et al. 2010; Chandrapala et al. 2011). Sonication at these intensities is not sufficiently strong to actively disrupt the micelle structure (Chandrapala et al. 2012a, b). The addition of TSPP to

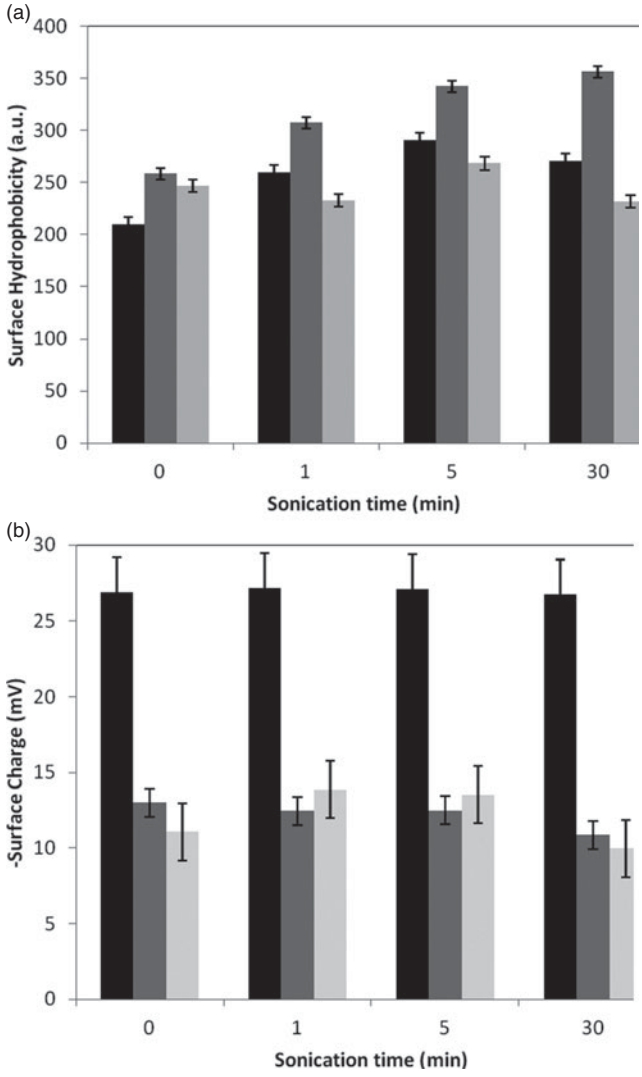

Fig. 1. The surface hydrophobicity (a) and surface charge (b) of MC solutions with/without addition of TSPP as a function of sonication time; ■ Sonicated, ■ Sonicated + Addition, ■ Addition + Sonicated

sonicated MC solutions increased the hydrophobicity even further ($P<0·05$). This is probably due to the disruption of casein micelles which release individual caseins with greater hydrophobicites. The magnitude of the hydrophobicity increase is proportional to sonication time. This may again be due to the disruption of whey or casein-whey protein aggregates. Alternatively, the ultrasound may loosen the casein micelles without any disruption and thereby enhance the penetration of TSPP to chelate with calcium within the micelle, leading to disintegration. The alternative approach involving the additon of TSPP followed by sonication did not change the surface hydrophobicity with an increase in sonication time to a statistically significant level. This may reflect the inability of casein micelles which have already been disrupted by TSPP to become further dissociated by sonication.

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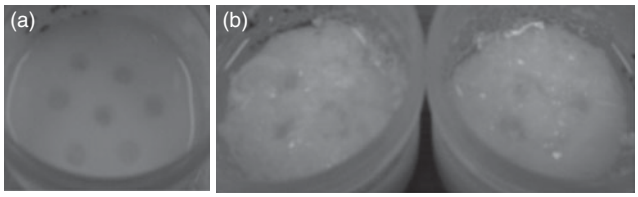

Fig. 2. Appearance of gels set by (a) sonication prior to TSPP addition & (b) TSPP addition after sonication

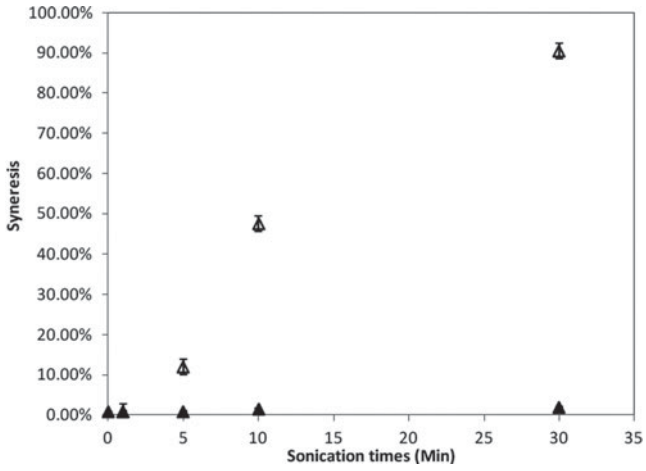

Fig. 3. Syneresis of gels prepared by two approaches as a function of sonication time at pH 6·0 and 5·8; ▲ – Sonication, then TSPP addition △ – TSPP addition, then sonication at pH 6·0

Figure 1(b) represents the surface charge of MC particles in solutions with or without sonication and TSPP addition. The surface charge of the native casein micelles was ∼ 27 mV. A similar value has been reported in the literature (McKinnon & Chandrapala, 2006). Sonication alone did not change this charge to a statistically significant level. However, the TSPP addition reduced the charge as the casein micelles are disrupted and the κ caseins no longer form an external negatively charged layer. The surface charge remained unchanged with any increase in sonication time independent of the approach taken.

Effect of sonication on the visual and microscopic characteristics of chemically induced MC gels

Figure 2 shows the appearances of gels prepared by the two approaches. Each sonication approach resulted in the formation of quite different gel networks. Gels created by sonication prior to TSPP addition had a more uniform protein matrix and exhibited low syneresis (left). Individual gel particles could not be clearly observed through confocal images because of the fineness of the protein network. However, a uniform structure formation was seen (results not shown). Gels formed by sonication after TSPP addition showed a weak and inconsistent gel like structure and had more syneresis (right). This syneresis increased steadily with an increase in sonication time (Fig. 3).

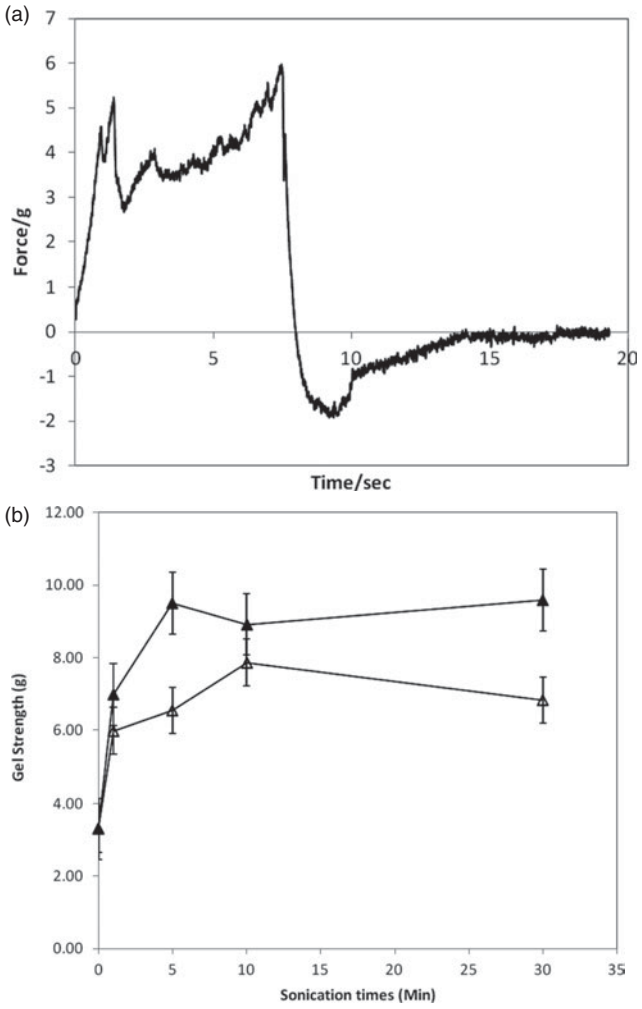

Fig. 4. (a) A typical texture profile graph and (b) gel strength of gels prepared by two approaches as a function of sonication time; ▲ – Sonication, then TSPP addition △ – TSPP addition, then sonication

A typical texture profile is shown in Fig. 4(a). The maximum point after yield stress was taken as the gel strength and graphed in Fig. 4(b). For both systems, a large increase in gel strength is observed with sonication time up to 5 or 10 min ($P < 0{\cdot}05$). Beyond this time, the gel strength stabilises. While TSPP reduces electrostatic repulsion, these differences between the sonicated and non sonicated gel strengths can only be explained through increased hydrophobic bonding. Chandrapala et al. (2011) have recently shown that sonication does not change the thiol groups of the proteins and the micellar caseins used in this work have very few thiol groups. Hence S–S interactions can be discounted as the cause of increased gel structure following sonication.

Rheological properties

Frequency sweep rheology tests were performed to determine whether the sonication of MC solutions before gelation

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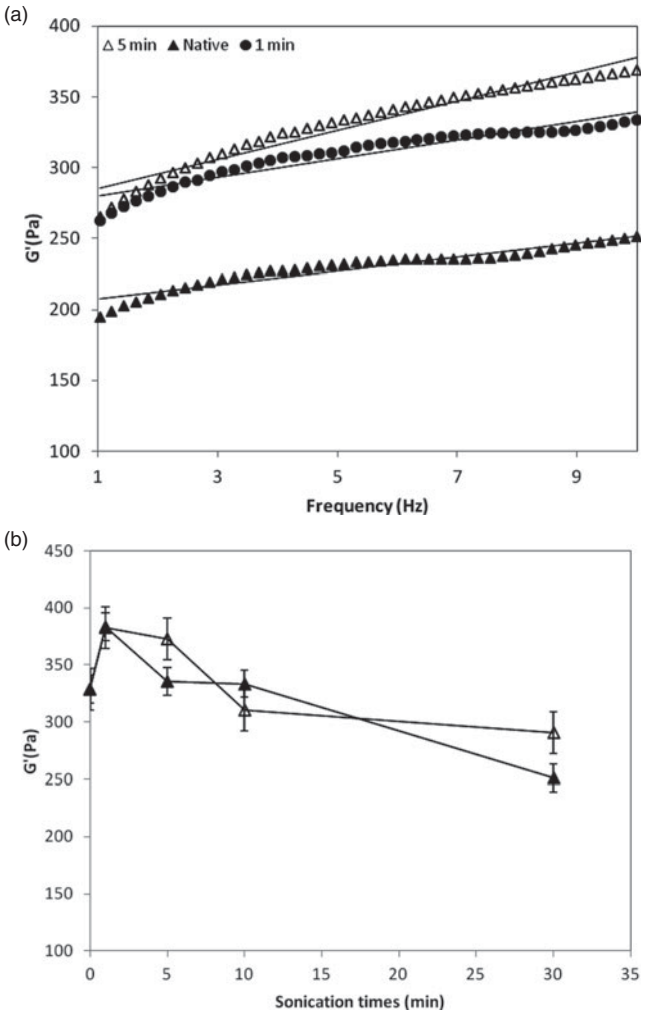

Fig. 5. (a) A typical frequency sweep graph and (b) final G′ at the frequency of 10 Hz for gels prepared by two approaches as a function of sonication time ▲ – Sonication, then TSPP addition △ – TSPP addition, then sonication

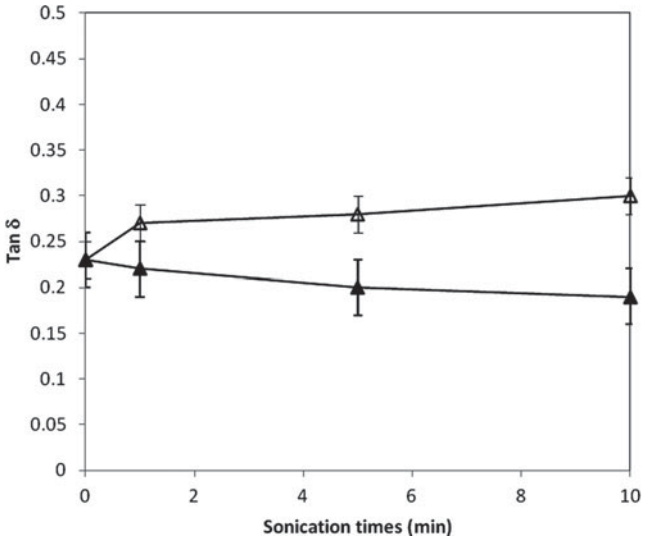

Fig. 6. Tan δ at a frequency of 4 Hz for gels prepared by two approaches as a function of sonication time; ▲ – Sonication, then TSPP addition △ – TSPP addition, then sonication

influenced the resulting textural characteristics (Figs. 5 & 6). The *G*′ values are greater than G′ (results not shown) at any frequency for all gels indicating the domination of the elastic nature of the gels and reflecting the typical behaviour of a solid viscoelastic material.

Figure 5(a) represents a typical *G*′ vs. frequency graph, while Fig. 5(b) summarises the G′ values at 10 KHz frequency for all gels prepared and Fig. 6 shows the loss tangent recorded at 10 KHz. Overall, changes in these values are small and not statistically significant. However, an initial increase in *G*′ does appear to occur in a comparable manner to the gel strength (Fig. 4)

These results are different from those observed by Madadlou et al. (2010) who investigated the properties of casein gels made from solutions sonicated at 24 and 130 kHz ultrasound for 0, 60 and 120 min followed by acidification with Glucono-Delta-Lactone (GDL) at 30 °C. They showed that the gels prepared with sonicated casein solutions led to higher *G*′ values compared with unsonciated solutions. However, Nguyen & Anema (2010) found that ultrasonication of skim milk for moderate times had only small effects on the acid gel properties. They concluded that ultrasonication itself had only a small effect on the gelation of milk systems. Our results are more in accordance with this latter work indicating that sonication affects the gelation of casein micellar systems through changes to hydrophobicity and charge of the proteins. Madadlou et al. (2010) used reconstituted casein powders in their study. These systems may have contained big casein aggregate particles, where sonication may act more efficiently in disrupting the aggregates and thereby influence the functionality to a significant extent. Hence, the different results may be due to the differences in systems and processing conditions used.

Conclusions

The TSPP-induced gelation process involved the dispersion of caseins followed by the association of these dispersed caseins via hydrophobic and electrostatic interactions. Sonication prior to TSPP addition acted synergistically to increase the dispersion of the caseins, while sonication after TSPP led to an inconsistent gel with a high degree of syneresis. In this latter case, the sonication probably acted to disrupt the protein network formation. The final gel properties were affected by sonication in both cases, with a significant increase in gel strength at short sonication times, but little change to viscoelasticity.

These findings provide an understanding of how ultrasound can affect the behaviour of casein micelle systems. These results, in combination with similar data for pure whey protein systems (Chandrapala et al. 2011; Zisu et al. 2011)

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can lead to a greater understanding of how protein mixtures may behave when rennet or acid induced gels are prepared in combination with sonication.

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