Published in final form as:

Loveday, S. M., Su, J., Rao, M. A., Anema, S. G., & Singh, H. (2012). Whey protein nanofibrils: Kinetic, rheological and morphological effects of group IA and IIA cations. *International Dairy Journal, 26*, 133-140.

Whey protein nanofibrils: kinetic, rheological and morphological effects of group IA and IIA cations

Simon M. Loveday*	Riddet Institute, New Zealand[1]
Jiahong Su	Riddet Institute, New Zealand[1]
M. Anandha Rao	Cornell University, USA[2]
Skelte G. Anema	Fonterra Research Centre, New Zealand[3]
Harjinder Singh	Riddet Institute, New Zealand[1]

1: Riddet Institute
Massey University
Private Bag 11 222
Palmerston North
NEW ZEALAND

2: Department of Food Science
Cornell University
Geneva
NY 14456
USA

3: Fonterra Research Centre
Dairy Farm Road
Private Bag 11029
Palmerston North
NEW ZEALAND

* Corresponding author: email s.loveday@massey.ac.nz , fax: +64 6 350 5655, phone: +64 6 356 9099

Abstract

Self-assembly of whey proteins into amyloid-like fibrils during heating at pH 2 and low ionic strength is sensitive to the presence of NaCl and $CaCl_2$. Our earlier work established that at 10 - 120 mM of these salts speeds up self-assembly and favours short, flexible fibrils over long semiflexible fibrils in a way that depends on cation concentration and cation type. Here we explored how other mono- and divalent salts affected fibril morphology and the rheology of fibril dispersions. Divalent salts ($MgCl_2$, $CaCl_2$, $BaCl_2$) had much stronger effects than monovalent salts (LiCl, NaCl, KCl) on gelation kinetics, and differences between salts of the same type were not large. No marked effects of salt type on fibril morphology were evident, but there were subtle differences in the size and extent of fibril networks with mono- *vs.* divalent salts, which may explain differences in bulk rheology.

Keywords: bovine whey protein, self-assembly, amyloid-like nanofibril, heating, ionic strength, salts, pH

1 Introduction

Many globular food proteins will self-assemble into semiflexible amyloid-like fibrils when heated at low ionic strength and low pH, e.g. lysozyme (Mishra, et al., 2007), ovalbumin and bovine serum albumin (Pearce, Mackintosh, & Gerrard, 2007), and soy glycinin (Tang & Wang, 2010). Fibrils can form entanglement networks that increase bulk viscosity with relatively little protein, which makes them potentially economical ingredients for modifying texture in food and biomedical products. Whey proteins from bovine milk have drawn the most attention from the food industry (Loveday, Rao, Creamer, & Singh, 2009). Globular proteins in whey include α-lactalbumin, bovine serum albumin and β-lactoglobulin; the last forms fibrils most readily at pH 2 and low ionic strength, and gives fibril dispersions with the highest viscosity (Bolder, Hendrickx, Sagis, & van der Linden, 2006a, 2006b).

A number of studies have examined the effect of NaCl on β-lactoglobulin fibril morphology (Arnaudov & de Vries, 2006; Durand, Gimel, & Nicolai, 2002; Ikeda & Morris, 2002; Kavanagh, Clark, & Ross-Murphy, 2000; Veerman, Ruis, Sagis, & van der Linden, 2002), and some have looked at its influence on the rate of self-assembly (Arnaudov & De Vries, 2007; Aymard, Nicolai, Durand, & Clark, 1999). Adding NaCl to β-lactoglobulin solutions heated at low pH leads to the appearance of short, flexible worm-like fibrils rather than the long-semiflexible fibrils formed at very low ionic strength. With 50-100 mM NaCl the two fibril types coexist, and the proportions of each depend on the amount of NaCl present (Arnaudov & de Vries, 2006). NaCl speeds up self-assembly during the growth phase (Arnaudov & de Vries, 2006; Aymard, et al., 1999), but has little effect on the duration of the lag phase (Loveday, et al., 2010).

Despite large effects of NaCl on fibril morphology and self-assembly kinetics, there is almost no information in the literature about the effect of NaCl on the rheological properties on β-lactoglobulin fibril dispersions, or the effect of salts other than NaCl. We reported that $CaCl_2$ had similar effects on fibril morphology to NaCl but at lower concentrations, and that $CaCl_2$ was much more effective than NaCl at speeding up fibril formation at 80°C and pH 2, even accounting for ionic strength differences (Loveday, et al., 2010). However NaCl and $CaCl_2$ had similar effects on the rate of self-assembly under conditions where salt concentration was apparently not the limiting factor (Loveday, Wang, Rao, Anema, & Singh, 2011). We found that adding 20 - 40 mM $CaCl_2$ to a whey protein solution before heating at pH 2 and 80°C produced fibril dispersions that were less viscous than the control sample without added $CaCl_2$, whereas 60 - 120 mM $CaCl_2$ gave more viscous dispersions (Loveday, Su, Rao, Anema, & Singh, 2011). We proposed that $CaCl_2$ facilitates the interaction of fibril building blocks with nuclei or growing fibrils, and that it affects rheological properties *via* its influence on fibril morphology, but does not form an ongoing part of fibril structure or gel structure (Loveday, Su, et al., 2011).

There is also little published information about how pH affects the self-assembly of fibrils during heating, and almost all investigations of β-lactoglobulin heat-induced fibrillar self-

assembly have been carried out at pH 2. Self-assembly occurs slightly faster at pH 1.5-1.6 (Loveday, et al., 2010; Loveday, Wang, et al., 2011), but surprisingly, self-assembly at pH 3 with 50 mM NaCl and 40 mM $CaCl_2$ is substantially faster again (Loveday, Wang, et al., 2011). We have previously noted that rapid self-assembly does not necessarily result in high viscosity, because fibril characteristics such as length, flexibility and alignment are more important than simply mass or volume fraction of fibrils (Loveday, Su, et al., 2011; Loveday, Wang, Rao, Anema, & Singh, 2012).

Our previous work indicates that the effects of salts on whey protein fibril formation depend on pH, ionic strength and cation identity. This is not surprising when one considers that calcium-specific effects are known to influence β-lactoglobulin aggregation under other conditions (Simons, Kosters, Visschers, & de Jongh, 2002), and cation-specific effects on amyloid fibril formation by other proteins have been known for over 10 years (Uversky, Li, & Fink, 2001).

Cations can potentially influence the intermolecular interactions of macromolecules in several different ways:

1. screening side-chain charges such as COO^-, i.e. reducing the Debye length
2. site-specific salt bridging by polyvalent cations
3. competing with protein for preferential interactions with water, so-called 'specific ion effects' (Kunz, 2010).

The terminology is somewhat confusing here, in that both 2 and 3 are 'specific', but 2 pertains to a specific location on the macromolecule surface, whereas 3 pertains to a specific chemical element. The first two can occur at both low concentration (<0.1 M) and higher concentrations, whereas specific ion effects occur only at higher concentrations (Kunz, 2010).

Moving down periods in a given group of the periodic table sees elements becoming larger in size, and outer shell electrons becoming less strongly attracted to the nucleus due to the screening effect of inner-shell electrons. Ionisation potentials decrease and polarisability increases (Marcus, 1997). Smaller ions with higher charge density, i.e. 'hard' ions, have a tendency to interact strongly with similarly 'hard' species such as water molecules (Collins, 2004), leading to more negative entropy of hydration and higher Jones-Dole B coefficient (Marcus, 1997), which measures the impact of ions on water viscosity.

Uversky *et al.* (2001) reported that the ability of metal ions to induce fibrillation-related structural change in α-synuclein at pH 7.5 was directly correlated with the ionic charge density and ionic radius (Uversky, et al., 2001), i.e. small, highly-charged cations catalysed structural change more efficiently than large ions with low charge. Moreover, di- and trivalent cations were much more efficient than monovalent cations, leading those authors to suggest that polyvalent cations could bridge between two or more carboxylate groups (Uversky, et al., 2001). Simons *et al.* (2002) investigated the role of Ca^{2+} in β-lactoglobulin aggregation during heating at neutral pH, and ruled out intermolecular bridging, preferring instead a scenario in which Ca^{2+} ions bind to single carboxylate groups on the protein surface, screening their negative charges and thus minimizing intermolecular repulsion.

Heat-induced β-lactoglobulin fibril formation is usually carried out at pH 2, where most Asp and Glu residues will be predominantly protonated (98.1% of Asp and 99.3% of Glu according to the Henderson-Hasselbalch equation), so cations are unlikely to fulfil bridging (Uversky, et al., 2001) or screening (Simons, et al., 2002) roles *via* carboxylate groups at such acidic pH. Site-specific interactions of cations with non-charged regions of a macromolecule may also occur, for example cation-π interactions (Dougherty, 1996). Lund *et al.* (2008) demonstrated the energetic favourability of specific interactions between large, soft, poorly-hydrated anions and nonpolar patches on the surface of proteins.

This investigation set out to address gaps in the literature relating to cation-specific effects of salts on β-lactoglobulin self-assembly. Our previous findings about NaCl and $CaCl_2$ were extended by trialling a range of other salts and by focusing on their effects on the rheological properties of fibril dispersions. The rheological consequences of surprisingly rapid cation-catalysed self-assembly at pH 3 (Loveday, Wang, et al., 2011) were also explored.

2 Materials and Methods

2.1 Chemicals

$CaCl_2$ (anhydrous, ≥93% pure), $MgCl_2$ (anhydrous, >99% pure) and KCl (>99% pure) were purchased from Sigma (St Louis, MO). $BaCl_2$ (analytical grade) was purchased from Labserv (Auckland, New Zealand), LiCl (>99%) was purchased from Merck (Whitehouse Station, NJ) and NaCl (>99.5%) was purchased from BDH (BDH Chemicals, PA). WPI was supplied by Fonterra Cooperative Ltd. (Auckland, New Zealand) and Milli-Q® water was used throughout the study.

2.2 Sample Preparation

WPI was dissolved in Milli-Q water with 0.02% sodium azide, pH was adjusted to 2 with 6M HCl and the sample was stirred overnight at 4°C. The pH was checked the next day and adjusted if necessary. Salts were prepared as 1M stock solutions. WPI solutions were heated at 80 ± 0.2 °C in a Lab Companion BS-11 water bath (Jeio Tech, Seoul, Korea).

2.3 Negative Stain Transmission Electron Microscopy

Fibril samples were filtered using a method reported previously (Bolder, Vasbinder, Sagis, & van der Linden, 2007; Loveday, et al., 2010) to reduce the background in TEM images. Fibrils were transferred to TEM grids and negatively stained as described earlier (Loveday, et al., 2010). Negatively-stained grids were examined with a Philips CM10 electron microscope (Eindhoven, The Netherlands). Image contrast was improved by contrast-stretching with Adobe Photoshop Elements 2.0 (Adobe Systems Inc., San Jose, CA).

2.4 Rheometry

Continuous rotational flow data on the fibril dispersions were collected at 20°C using an AR-G2 rheometer (TA Instruments, New Castle, DE) fitted with a 60 mm diameter stainless steel cone with angle 4° and truncation length 112 μm. Sample preparation and measurement were done as previously described (Loveday, Su, et al., 2011).

In addition, *in situ* rheometer heating experiments were conducted as described previously (Loveday, Su, et al., 2011), except that silicone heat transfer paste (Unick Chemical Corp, Taipei, Republic of China), was applied to the underside of the protective base plate to improve thermal contact with the rheometer base. The protective plate induced a small thermal lag, and rheometer software settings were adjusted to compensate for this. The rheometer 'zero gap' position was re-set daily with the cover plate in place and pre-equilibrated at the heating temperature.

WPI solutions were degassed with a water aspirator then 1.3 mL was transferred onto the coverplate, which was preheated to 58°C, and the cone was lowered into place. Silicone oil (200 Fluid, 1000 CST, Dow Corning, Midland, MI) was applied to the exposed edges of the sample and a close-fitting steel moisture trap (TA Instruments) was put in place over the sample. A collar on top of the cone was filled with silicone oil, and the top edges of the moisture trap dipped into the oil, forming an air-tight seal.

After the sample was heated to 80°C, oscillatory shear measurements were conducted at a frequency of 1 rad.s^{-1} and strain of 0.1%. The temperature history of samples was tested with a thermocouple placed in the centre of a water sample, with the gap between cone and plate set at 1000 μm to accommodate the thermocouple. Heating from 58°C to 75°C took 122.2 ± 2.7 s, and final equilibration to 80°C took approximately 3 min, but timing began once the rheometer temperature control system reached the set temperature, which took 104 ± 0.1 s.

After the required heating period, the sample was cooled at approximately 8 °C.min^{-1} to 30°C, and at approximately 4 °C.min^{-1} below 30°C. Once the temperature control reached 20°C, the sample was allowed to equilibrate for 1 min before undergoing a frequency sweep (0.01-100 Hz at 0.1% strain) and a strain sweep (0.01-50% strain at a frequency of 1 rad.s^{-1}).

Linear regression of oscillatory shear data was done with Excel 2007 (Microsoft, Redmond, WA).

3 Results

Addition of $BaCl_2$ and $MgCl_2$ increased the viscosity of 2% WPI solutions slightly, even before heating, but 50 mM of either salt produced little or no further increase in viscosity with heating (Figure 1A). Adding 50 mM of LiCl or KCl produced a slight increase in viscosity with heating, but final viscosity was lower than the control without added salts. With 100 mM of added salts, a different picture emerged (Figure 1B): $BaCl_2$ and $MgCl_2$ caused a large increase in viscosity between 1 h and 2 h, and gave final viscosity approximately a decade higher than the control. KCl produced a smaller increase in viscosity between 1 h and 3 h, but the sample with LiCl had viscosity approximately the same as the control.

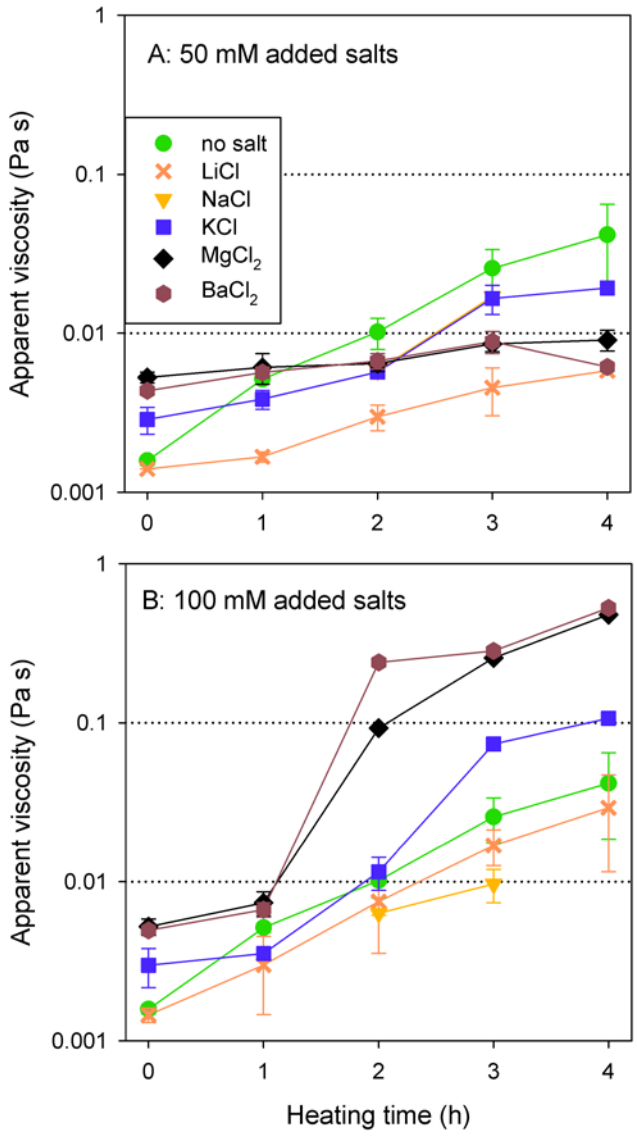
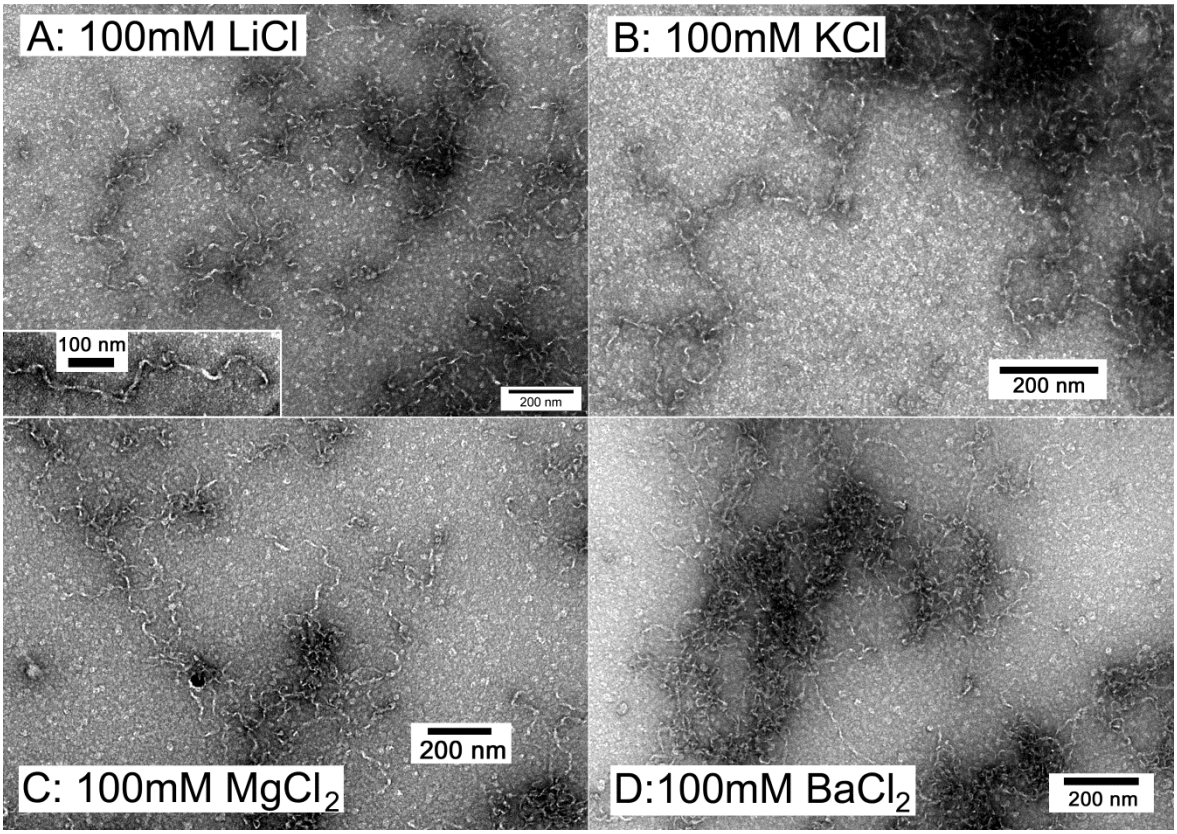


Figure 1. Apparent viscosity at 10 s^{-1} of 2% w/w WPI during heating at 80°C, showing the effect of adding 50 mM (A) or 100 mM (B) of various salts. Note that in pane A the NaCl data at 2 h and 3 h are obscured by KCl data, which were almost identical.

Fibrils formed in the presence of 100 mM LiCl, KCl, $MgCl_2$ or $BaCl_2$ (Figure 2) were quite similar in appearance to those made with NaCl or $CaCl_2$ (Loveday, et al., 2010). They were typically highly flexible, and sometimes had alternating thick and thin segments suggestive of a twisted ribbon, as reported earlier for both salt-free semiflexible fibrils (Adamcik, et al., 2010) and highly flexible fibrils made with NaCl and $CaCl_2$ (Loveday, et al., 2010). Fibrils were mostly entangled in local networks, and networks were larger and more dense with $MgCl_2$ and $BaCl_2$. The high viscosity of $MgCl_2$ and $BaCl_2$ fibril dispersions with ≥2 h heating prevented TEM imaging, so samples heated for 1 h were imaged for these salts (Figure 2C and D). The morphology of $MgCl_2$ and $BaCl_2$ fibrils formed after 1 h heating was very close to that of $CaCl_2$ fibrils formed after 6 h heating (Loveday, et al., 2010), so we believe that the final morphology was reached after 1 h.

Figure 2. TEM images of fibrils made with various salts. Fibrils were made by heating 2% w/w WPI at pH 2 and 80°C. Heating times were A: 4 h, B: 3 h, C and D: 1 h.



Similar experiments were carried out with 120 mM added salts to allow comparison with earlier work (Loveday, Su, et al., 2011). Maximum viscosity was generally attained within 3 h (data not shown), and final viscosities at a range of shear rates after 3 h are shown in Figure 3. For these experiments, LiCl was excluded due to its poor viscosity enhancement. At 120 mM, all salts increased viscosity, and $MgCl_2$ and $CaCl_2$ produced the largest increase over the control: nearly two decades. When $BaCl_2$ was added to WPI solutions, turbidity was observed, suggesting that some protein aggregation had occurred. All fibril dispersions were shear-thinning to approximately the same extent, as shown by consistent vertical spacing of points in Figure 3.

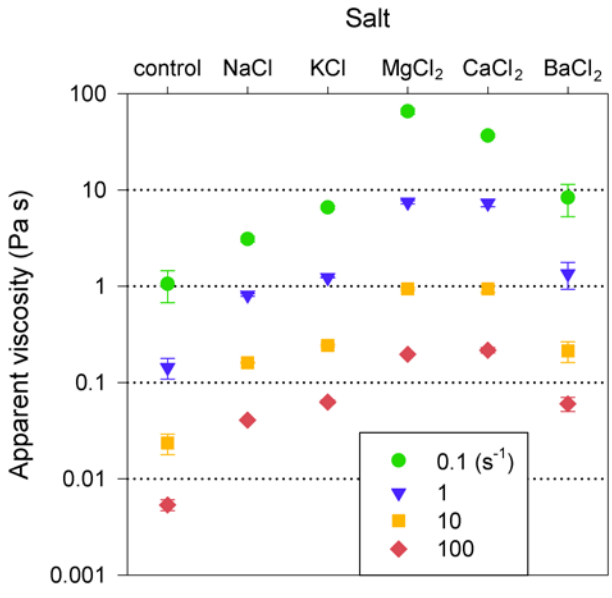


Figure 3. Viscosity of 2% w/w WPI solutions at pH 2 after heating for 3 h at 80°C with no salt (control) or 120 mM of various salts. Different symbols indicate viscosity at different shear rates, and vertical bars are the minimum and maximum of duplicates.

Different salts produced marked differences in the kinetics of gel formation with 5% WPI (Figure 4). G' values were initially low, and varied randomly until a well-defined time, after which G' increased sharply. Gel cure data for samples without added salts (Figure 4A) initially varied randomly then began to increase and become less noisy, although the beginning of gelation was not as well-defined as in samples with salts.

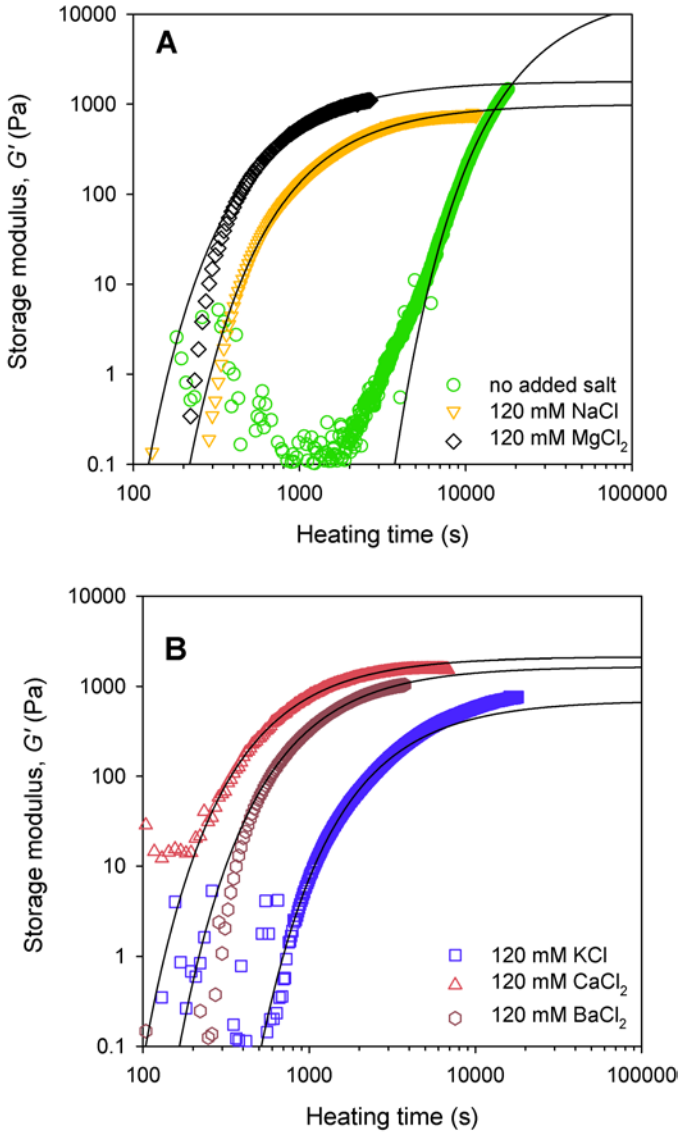


Figure 4. Gel cure data for 5% w/w WPI solutions with various salts added. Samples were heated at 80°C on the rheometer with oscillatory measurement *in situ*.

Gel cure time curves were fitted with the exponential model of Scott Blair and Burnett (1963), using Eq 1 (and its linear form, Eq 2), in which G' is the storage modulus, G'_{inf} is G' at infinite time, B is the time taken for G' to reach G'_{inf} / e , and t is time. A linear regression analysis of ln(G') against inverse time, using only the range of data points over which the plot was linear, gave a y-intercept of ln(G'_{inf}) and slope of $-B$, as shown in Eq 2. The gel time, t_{gel}, was taken to be the x-intercept of Eq 2, as discussed previously (Loveday, Su, et al., 2011). The rate at which G' changed, dG'/dt was calculated by differentiating Eq 1 with respect to time, yielding Eq 3 (Loveday, Su, et al., 2011). The maximum rate of G' increase, $(dG'/dt)_{max}$, was calculated with Eq (4), which was derived analytically from Eq (3) (Loveday, Su, et al., 2011).

$$G' = G'_{\inf} \exp(-B/t) \quad (1)$$

$$\ln(G') = \ln(G'_{\inf}) - B(1/t) \quad (2)$$

$$\frac{dG'}{dt} = G'_{\inf} \frac{B}{t^2} \exp(-B/t) \quad (3)$$

$$\left(\frac{dG'}{dt}\right)_{\max} = \frac{4G'_{\inf}}{B} \exp(-2) \quad (4)$$

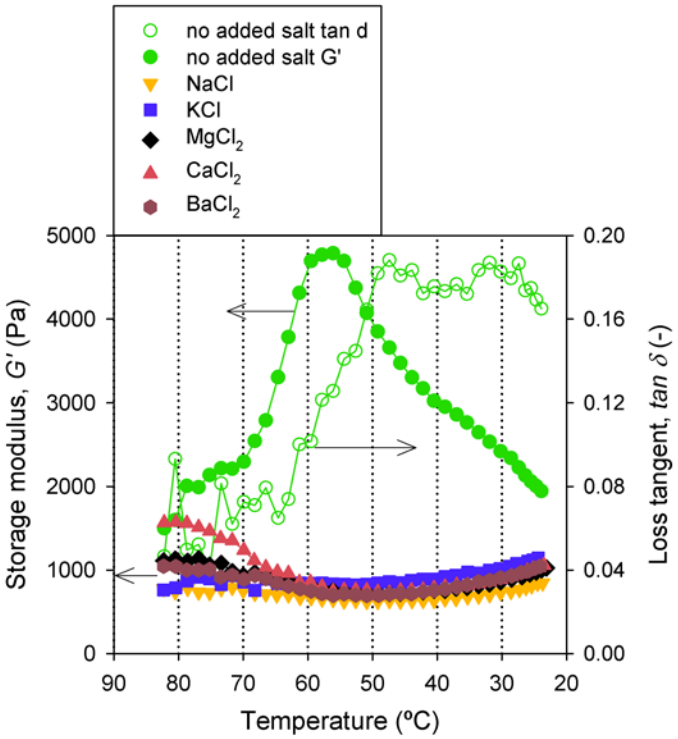
Values of G'_{inf} and t_{gel} from replicate runs agreed quite well in most cases (Table 1). G'_{inf} for samples with no added salts were approximately an order of magnitude higher than all other samples, and divalent salts gave G'_{inf} almost a factor of two higher than monovalent salts. Adding salts decreased t_{gel} by a factor of about 8 for monovalent salts and about 20 for divalent salts. Variation between replicates was higher for $(dG'/dt)_{max}$, but there were clear differences between monovalent and divalent salts. Adding KCl or NaCl decreased $(dG'/dt)_{max}$ slightly, whereas $CaCl_2$, $BaCl_2$ and $MgCl_2$ increased it by approximately 3-fold.

Table 1. Kinetic parameters for gel cure experiments with various salts. Data are from measurements of 5% w/w WPI solutions at pH 2 heated at 80°C on the rheometer, with 120 mM of various salts added before heating. Bold typeface indicates the individual replicates shown in Figure 4 and Figure 5.

G'_{inf} (Pa)						
replicate	no salt	$CaCl_2$	$BaCl_2$	$MgCl_2$	KCl	NaCl
1	**18086**	**2116**	1668	**1793**	1782	1013
2	24544	2092	**1650**	2153	**694**	**992**
3	23408					819
t_{gel} (s)						
replicate	no salt	$CaCl_2$	$BaCl_2$	$MgCl_2$	KCl	NaCl
1	**4620**	**135**	163	**161**	678	634
2	4725	159	**217**	123	**697**	**291**
3	5598					461
dG'/dt_{max} (Pa s^{-1})						
replicate	no salt	$CaCl_2$	$BaCl_2$	$MgCl_2$	KCl	NaCl
1	**0.22**	**1.11**	0.75	**0.80**	0.19	0.12
2	0.28	0.93	**0.55**	1.23	**0.08**	**0.27**
3	0.23					0.14

After heating and gelation at 80°C, oscillatory-shear data at 1 rad.s^{-1} and strain of 0.1% were collected while the sample was cooled in the rheometer to 20°C. Salts had a dramatic effect on the temperature sensitivity of G' (Figure 5). In the absence of added salt, G' increased more than three-fold to reach a maximum at 55-65°C, then decreased correspondingly with further cooling to 20°C. For gels made with salts, G' decreased slightly between the heating temperature and 50°C, then with further cooling it returned to levels close to those at 80°C. All the salts had similar effects on G' during cooling. For all samples, tan δ increased between 80°C and 50°C and was relatively constant with further cooling, as shown by the example data in Figure 5.

Figure 5. Temperature dependence of *G'* during cooling (8 °C.min^{-1}) of heat-induced 5% WPI fibril gels made with 120 mM of various salts. One set of tan δ data is shown; other datasets showed the same trend. Note that temperatures shown are those given by rheometer software, and actual sample temperatures will be slightly higher because of the thermal lag caused by the rheometer base plate cover.



Once samples were cooled to 20°C, frequency sweep and strain sweep data were collected (data shown in Supplementary Material). *G'* was relatively insensitive to frequency or added salts, but G" increased approximately a decade between 1 and 100 Hz and was slightly higher with divalent salts than for other samples. In strain sweeps, the linear viscoelastic region (where *G'* is strain-independent) extended to between 1 and 10% strain, confirming that deformation at 0.1% during heating had a reversible effect on sample structure. The linear viscoelastic region extended to the highest strain (approximately 4%) for gels made with KCl and NaCl, whereas $MgCl_2$, $CaCl_2$ and $BaCl_2$ gels showed nonlinear effects at slightly lower strain, and gels without added salt showed a drop-off in *G'* at even lower strain. However the differences were not large.

During heating at pH 3, the viscosity increased little with 0 or 40 mM $CaCl_2$, and even with 80 mM $CaCl_2$ there was a lag phase of 2 h before viscosity began to increase (Figure 6). With 120 mM $CaCl_2$, viscosity increased slowly during the first 2 h heating, then rapidly between 2 and 4h, after which it continued to increase slowly. Samples became more turbid with increasing $CaCl_2$ concentration at pH 3 (Figure 7), in contrast to pH 2 samples, which were transparent even with 120 mM $CaCl_2$.

Figure 6. Effect of $CaCl_2$ on the viscosity of 2% WPI solutions at pH 3 during heating at 80°C.

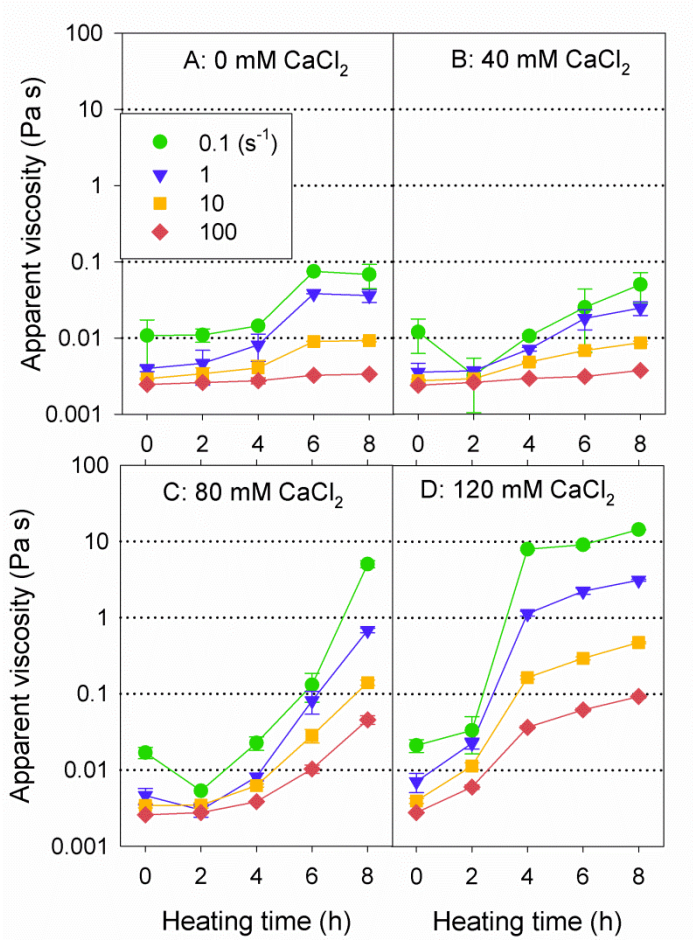
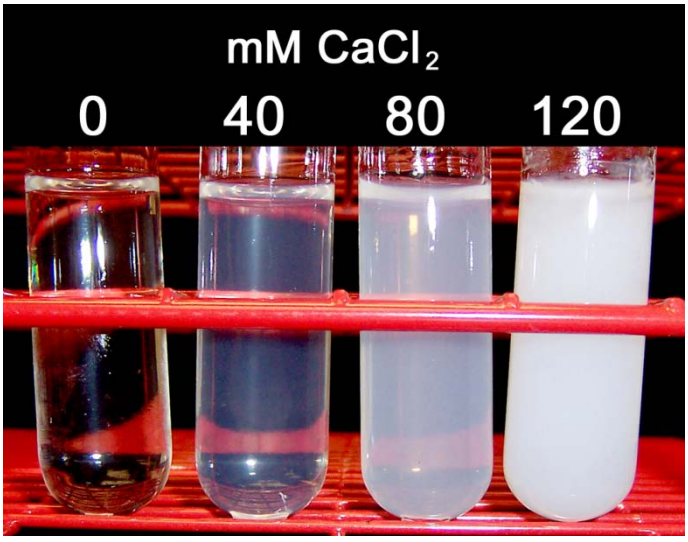


Figure 7. Appearance of 2% WPI solutions at pH 3 with 0-120 mM $CaCl_2$ after heating at 80°C for 8 h.



4 Discussion

We previously showed that $CaCl_2$ has a substantial concentration-dependent impact on the kinetics of β-lactoglobulin fibril self-assembly and the development of viscosity in β-lactoglobulin or WPI heated under fibril-forming conditions (Loveday, et al., 2010; Loveday, Su, et al., 2011). The effect could not be explained by ionic strength or Cl^- concentration, and we proposed that calcium cations can facilitate fibril nucleation by bridging *via* cation-π interactions with electron-rich side chains (Loveday, et al., 2010). Removing calcium by post-heating dialysis produced minimal change in viscosity or morphology, so it appears to play no ongoing role in fibril structure or gel structure (Loveday, Su, et al., 2011). The present study compared the effects of different group IA and group IIA cations under conditions directly comparable with the calcium studies.

5% WPI solutions at pH 2 became slightly turbid as soon as 120 mM $BaCl_2$ was added. Both protein solution and 1M $BaCl_2$ were transparent before mixing, and acidifying to pH 2 did not affect the solubility of $BaCl_2$, so the turbidity was probably due to limited aggregation of protein. Aggregated protein may be less available for self-assembly than monomeric protein, which may partly explain why $BaCl_2$ produced lower G'_{inf}, slightly longer t_{gel} and lower dG'/dt_{max} than $CaCl_2$ or $MgCl_2$. $BaCl_2$ induced protein aggregation but $CaCl_2$ and $MgCl_2$ did not, and the fact that Ca^{2+} and Mg^{2+} have a higher affinity for carboxylate groups than Ba^{2+} (Collins, 2004; Kunz, 2010) rules out specific inter-protein bridging of carboxylate groups by divalent cations. We are unable to explain the cause of the turbidity induced by $BaCl_2$.

The different salts were used here at 50-120 mM, so all three phenomena cited in the introduction (charge screening, salt bridging and specific ion effects) may occur, but at these concentrations the Debye length for even monovalent salts at 50 mM is smaller than the diameter of β-lactoglobulin molecules (Dill, 2011), so protein surface charges will be effectively screened in all cases where a salt was added. Charge screening reduces *intra*molecular repulsion between like charges on the same protein molecule, thus allowing the protein to adopt a more compact configuration, and it also reduces *inter*molecular repulsion, facilitating aggregation (Schokker, Singh, Pinder, & Creamer, 2000).

The ionic radii of Li^+, Na^+ and K^+ are very similar to those of Mg^{2+}, Ca^{2+} and Ba^{2+} respectively (Marcus, 1997). The dramatic differences in fibril formation kinetics between corresponding salts, e.g. LiCl *vs.* $MgCl_2$ (Table 1) may testify to the importance of divalency and/or charge density. Within each group of the same valency, there was no clear relation between ionic radius and kinetic effects on fibril formation, which argues against a mechanism involving ion-binding in sterically-restricted environments such as the interior of the protein.

Anions generally have a stronger impact on protein-water interactions than cations of the same size and charge density (Kunz, 2010), and anion-water interactions were implicated in the acceleration of α-synuclein fibril formation at both pH 7.4 and pH 2.0 by a range of sodium salts (Munishkina, Henriques, Uversky, & Fink, 2004). The effect of cations on α-synuclein fibrillation at pH 7.5 was attributed to conformational change related to charge screening (Uversky, et al., 2001). Bridging by polyvalent cations was also thought to be important, but water-protein interactions were not discussed. On the basis of the data shown here, we cannot distinguish between site-specific salt bridging and specific ion effects on protein-water interactions, and it is likely that both operate with 120 mM of added salts.

Addition of $MgCl_2$ and $BaCl_2$ had similar effects to $CaCl_2$ on the viscosity of 2% WPI solutions, i.e. decreasing viscosity at intermediate concentration (Figure 1A) and increasing viscosity at high concentration (Figure 1B, Figure 3). We postulated a mechanism for this effect in our earlier work with calcium (Loveday, Su, et al., 2011).

Monovalent salts were much less effective at promoting viscosity enhancement than divalent salts, and LiCl even reduced the viscosity below that of the control (Figure 1). The different rheological effects of monovalent and divalent salts may reflect different volume fractions of fibrils formed after a given heating time, different network-forming behaviours, and/or differences in the mechanical properties of fibrils such as bending or extensional modulus. No clear differences in fibril morphology were seen here, suggesting that mechanical properties of fibrils are likely to be similar on a unit length basis.

The viscosity of fibril dispersions depends on both the volume fraction of fibrils and their degree of entanglement or alignment in solution. In viscous or gelled fibril dispersions, entanglement or alignment becomes quite important in both salt-free long semiflexible fibrils (Akkermans, van der Goot, Venema, van der Linden, & Boom, 2008) and curly fibrils made with $CaCl_2$ (Loveday, Su, et al., 2011). Fibrils made with $MgCl_2$ and $BaCl_2$ appeared to form networks more effectively than those made with LiCl or KCl, which could explain the higher bulk viscosity. Different network-forming abilities could reflect differences in fibril surface properties such as charge and hydrophobicity, or the $MgCl_2$ and $BaCl_2$ fibrils could simply be longer than LiCl and KCl fibrils. It was difficult to trace the entire length of fibrils, which were often entangled and overlaid with others, so length differences cannot be ruled out.

The peak in *G'* on cooling fibril dispersions made without added salt (Figure 5) was unexpected, and has apparently not been reported for β-lactoglobulin fibril gels. Yan *et al.* (2008) presented temperature sweep data (cooling and heating at 1°C/min) for hen egg white lysozyme fibril gels, which showed a temperature-independent region below 50°C (cooling) or 65°C (heating) and a melting/setting region at higher temperature, in which *G'* decreased with increasing temperature. Ipsen, Otte and Qvist. (2001) found a *G'* peak at approximately 35°C during cooling of α-lactalbumin nanotube gels from 50°C to 25°C at 1°C/min, but did not suggest an explanation. Gosal, Clark and Ross-Murphy (2004) reported a consistent though small decrease in *G'* with decreasing temperature for β-lactoglobulin fibril gels equilibrated at 25°C, 45°C, 55°C and 65°C. Here we cooled at approximately 8°C/min - more rapidly than Yan *et al.* (2008) or Ipsen *et al.* (2001) - and we did not equilibrate samples at each temperature like Gosal *et al.* (2004). The *G'* peak we observed may relate to a relaxation phenomenon that occurs over a time frame of several minutes, for example a rearrangement in fibril structure or gel structure resulting from a temperature-dependent change in the relative strength of hydrophobic interactions and hydrogen bonding (Brandts, 1964; Makhatadze & Privalov, 1993; Privalov & Makhatadze, 1993). In such a scenario, cooling rapidly would outstrip relaxation, briefly producing a nonequilibrium state.

The effect of $CaCl_2$ at pH 3 on the viscosity of heated WPI solutions (Figure 6) was similar to its effect at pH 2 (Loveday, Su, et al., 2011), in that 40 mM $CaCl_2$ was detrimental to viscosity, whereas 120 mM at the same pH produced a large and rapid increase in viscosity. The unusually large and rapid increase in Thioflavin T fluorescence we saw on heating 1% w/w β-lactoglobulin at pH 3 with 50 mM NaCl and 40 mM $CaCl_2$ (Loveday, Wang, et al., 2011) suggested rapid self-assembly. Investigations of cation-catalysed protein aggregation at neutral pH proposed putative carboxylate screening (Simons, et al., 2002) or bridging (Uversky, et al., 2001) roles for cations, which are unlikely at pH 2 but more likely at pH 3, where 16.3% of Asp residues and 6.6% of Glu residues (using the Henderson-Hasselbalch equation) will bear negative charges. Although self-assembly in the presence of high concentrations of salts was very rapid at pH 3 (Loveday, Wang, et al., 2011), and viscosity increased sharply between 2 h and 4 h (Figure 6), final viscosity was lower than at pH 2 (Figure 3). Poor viscosity enhancement is probably related to the short and irregular morphology of pH 3 fibrils (Loveday, Wang, et al., 2011). The turbidity of WPI solutions heated at pH 3 with $CaCl_2$ (Figure 7) suggests that fibrils aggregated into flocs large enough to scatter light strongly, whereas corresponding samples at pH 2 were transparent, suggesting that fibrils did not aggregate at pH 2.

5 Conclusions

This work has shown that earlier conclusions about the effects of Na^+ and Ca^{2+} on the rheological and microstructural properties of whey protein fibril dispersions also hold for other group IA and group IIA cations. For example, the nonlinear relation between Ca^{2+} concentration and viscosity was also seen with Mg^{2+} and Ba^{2+}, and the morphology of fibrils formed at high salt concentrations differs little with different salts. However, subtle differences in network-forming ability may result in large differences in bulk viscosity. This work has confirmed that kinetic phenomena measured via Thioflavin T fluorescence cannot predict bulk rheological properties. The latter appear to result from a complex combination of the length and shape of fibrils, as well as the mechanical properties of individual fibrils, which have

recently been measured for the first time (Adamcik, Berquand, & Mezzenga, 2011), and are sure to be a fruitful topic for future study.

6 Acknowledgements

We thank Doug Hopcroft and Dr Jianyu Chen at the Manawatu Microscopy and Imaging Centre, IMBS, Massey University. This work was funded by Fonterra Cooperative Ltd. and the New Zealand Foundation for Research, Science and Technology, contract number DRIX0701.

7 References

Adamcik, J., Jung, J. M., Flakowski, J., De Los Rios, P., Dietler, G., & Mezzenga, R. (2010). Understanding amyloid aggregation by statistical analysis of atomic force microscopy images. *Nature Nanotechnology, 5*, 423-428.

Adamcik, J., Berquand, A., & Mezzenga, R. (2011). Single-step direct measurement of amyloid fibrils stiffness by peak force quantitative nanomechanical atomic force microscopy. *Applied Physics Letters, 98*.

Akkermans, C., van der Goot, A. J., Venema, P., van der Linden, E., & Boom, R. M. (2008). Formation of fibrillar whey protein aggregates: Influence of heat and shear treatment, and resulting rheology. *Food Hydrocolloids, 22*, 1315-1325.

Arnaudov, L. N., & de Vries, R. (2006). Strong impact of ionic strength on the kinetics of fibrilar aggregation of bovine β-lactoglobulin. *Biomacromolecules, 7*, 3490-3498.

Arnaudov, L. N., & De Vries, R. (2007). Theoretical modeling of the kinetics of fibrilar aggregation of bovine β-lactoglobulin at pH 2. *Journal of Chemical Physics, 126*.

Aymard, P., Nicolai, T., Durand, D., & Clark, A. (1999). Static and dynamic scattering of β-lactoglobulin aggregates formed after heat-induced denaturation at pH 2. *Macromolecules, 32*, 2542-2552.

Bolder, S. G., Hendrickx, H., Sagis, L. M. C., & van der Linden, E. (2006a). Fibril assemblies in aqueous whey protein mixtures. *Journal of Agricultural and Food Chemistry, 54*, 4229-4234.

Bolder, S. G., Hendrickx, H., Sagis, L. M. C., & Van der Linden, E. (2006b). Ca^{2+}-induced cold-set gelation of whey protein isolate fibrils. *Applied Rheology, 16*, 258-264.

Bolder, S. G., Vasbinder, A. J., Sagis, L. M. C., & van der Linden, E. (2007). Heat-induced whey protein isolate fibrils: conversion, hydrolysis, and disulphide bond formation. *International Dairy Journal, 17*, 846-853.

Brandts, J. F. (1964). The thermodynamics of protein denaturation. II. A model of reversible denaturation and interpretations regarding the stability of chymotrypsinogen. *Journal of the American Chemical Society, 86*, 4302-4314.

Collins, K. D. (2004). Ions from the Hofmeister series and osmolytes: Effects on proteins in solution and in the crystallization process. *Methods, 34*, 300-311.

Dill, K. A. (2011). Salt ions shield charged objects in solution. In S. Bromberg (Ed.), *Molecular driving forces : statistical thermodynamics in biology, chemistry, physics, and nanoscience* (2nd ed., pp. 455-470). London, UK: Garland Science.

Dougherty, D. A. (1996). Cation-pi Interactions in Chemistry and Biology: A New View of Benzene, Phe, Tyr, and Trp. *Science, 271*, 163-168.

Durand, D., Gimel, J. C., & Nicolai, T. (2002). Aggregation, gelation and phase separation of heat denatured globular proteins. *Physica A: Statistical Mechanics and its Applications, 304*, 253-265.

Gosal, W. S., Clark, A. H., & Ross-Murphy, S. B. (2004). Fibrillar β-lactoglobulin gels: Part 2. Dynamic mechanical characterization of heat-set systems. *Biomacromolecules, 5*, 2420-2429.

Ikeda, S., & Morris, V. J. (2002). Fine-stranded and particulate aggregates of heat-denatured whey proteins visualized by atomic force microscopy. *Biomacromolecules, 3*, 382-389.

Ipsen, R., Otte, J., & Qvist, K. B. (2001). Molecular self-assembly of partially hydrolysed α-lactalbumin resulting in strong gels with a novel microstructure. *Journal of Dairy Research, 68*, 277-286.

Kavanagh, G. M., Clark, A. H., & Ross-Murphy, S. B. (2000). Heat-induced gelation of globular proteins: part 3. Molecular studies on low pH β-lactoglobulin gels. *International Journal of Biological Macromolecules, 28*, 41-50.

Kunz, W. (2010). Specific ion effects in colloidal and biological systems. *Current Opinion in Colloid & Interface Science, 15*, 34-39.

Loveday, S. M., Rao, M. A., Creamer, L. K., & Singh, H. (2009). Factors affecting rheological characteristics of fibril gels: The case of β-lactoglobulin and α-lactalbumin. *Journal of Food Science, 74*, R47-R55.

Loveday, S. M., Wang, X. L., Rao, M. A., Anema, S. G., Creamer, L. K., & Singh, H. (2010). Tuning the properties of β-lactoglobulin nanofibrils with pH, NaCl and $CaCl_2$. *International Dairy Journal, 20*, 571-579.

Loveday, S. M., Su, J., Rao, M. A., Anema, S. G., & Singh, H. (2011). Effect of Calcium on the Morphology and Functionality of Whey Protein Nanofibrils. *Biomacromolecules, 12*, 3780-3788.

Loveday, S. M., Wang, X. L., Rao, M. A., Anema, S. G., & Singh, H. (2011). Effect of pH, NaCl, $CaCl_2$ and temperature on self-assembly of β-lactoglobulin into nanofibrils: a central composite design study. *Journal of Agricultural and Food Chemistry, 59*, 8467-8474.

Loveday, S. M., Wang, X. L., Rao, M. A., Anema, S. G., & Singh, H. (2012). β-Lactoglobulin Nanofibrils: effect of temperature on fibril formation kinetics, fibril morphology, and the rheological properties of fibril dispersions. *Food Hydrocolloids, 27*.

Lund, M., Vrbka, L., & Jungwirth, P. (2008). Specific ion binding to nonpolar surface patches of proteins. *Journal of the American Chemical Society, 130*, 11582-11583.

Makhatadze, G. I., & Privalov, P. L. (1993). Contribution of Hydration to Protein Folding Thermodynamics. I. The Enthalpy of Hydration. *Journal of Molecular Biology, 232*, 639-659.

Marcus, Y. (1997). *Ion properties*. New York: Marcel Dekker.

Mishra, R., Sorgjerd, K., Nystrom, S., Nordigarden, A., Yu, Y.-C., & Hammarstrom, P. (2007). Lysozyme Amyloidogenesis Is Accelerated by Specific Nicking and Fragmentation but Decelerated by Intact Protein Binding and Conversion. *Journal of Molecular Biology, 366*, 1029-1044.

Munishkina, L. A., Henriques, J., Uversky, V. N., & Fink, A. L. (2004). Role of protein-water interactions and electrostatics in α-synuclein fibril formation. *Biochemistry, 43*, 3289-3300.

Pearce, F. G., Mackintosh, S. H., & Gerrard, J. A. (2007). Formation of Amyloid-like Fibrils by Ovalbumin and Related Proteins under Conditions Relevant to Food Processing. *Journal of Agricultural and Food Chemistry, 55*, 318-322.

Privalov, P. L., & Makhatadze, G. I. (1993). Contribution of Hydration to Protein Folding Thermodynamics. II. The Entropy and Gibbs Energy of Hydration. *Journal of Molecular Biology, 232*, 660-679.

Schokker, E. P., Singh, H., Pinder, D. N., & Creamer, L. K. (2000). Heat-induced aggregation of β-lactoglobulin AB at pH 2.5 as influenced by ionic strength and protein concentration. *International Dairy Journal, 10*, 233-240.

Scott Blair, G. W., & Burnett, J. (1963). An equation to describe the rate of setting of blood and milk. *Biorheology, 1*, 183-191.

Simons, J.-W. F. A., Kosters, H. A., Visschers, R. W., & de Jongh, H. H. J. (2002). Role of calcium as trigger in thermal β–lactoglobulin aggregation. *Archives of Biochemistry and Biophysics, 406*, 143-152.

Tang, C.-H., & Wang, C.-S. (2010). Formation and Characterization of Amyloid-like Fibrils from Soy Î²-Conglycinin and Glycinin. *Journal of Agricultural and Food Chemistry, 58*, 11058-11066.

Uversky, V. N., Li, J., & Fink, A. L. (2001). Metal-triggered structural transformations, aggregation, and fibrillation of human α-synuclein - a possible molecular link between

Parkinson's disease and heavy metal exposure. *Journal of Biological Chemistry, 276*, 44284-44296.

Veerman, C., Ruis, H., Sagis, L. M. C., & van der Linden, E. (2002). Effect of electrostatic interactions on the percolation concentration of fibrillar β-lactoglobulin gels. *Biomacromolecules, 3*, 869-873.

Yan, H., Nykanen, A., Ruokolainen, J., Farrar, D., Gough, J. E., Saiani, A., & Miller, A. F. (2008). Thermo-reversible protein fibrillar hydrogels as cell scaffolds. *Faraday Discussions, 139*, 71-84.

Supplementary Material

Figure S1. Frequency sweep data for 5% w/w WPI with 120 mM of various salts added after heating at 80°C on the rheometer then cooling to 20°C.

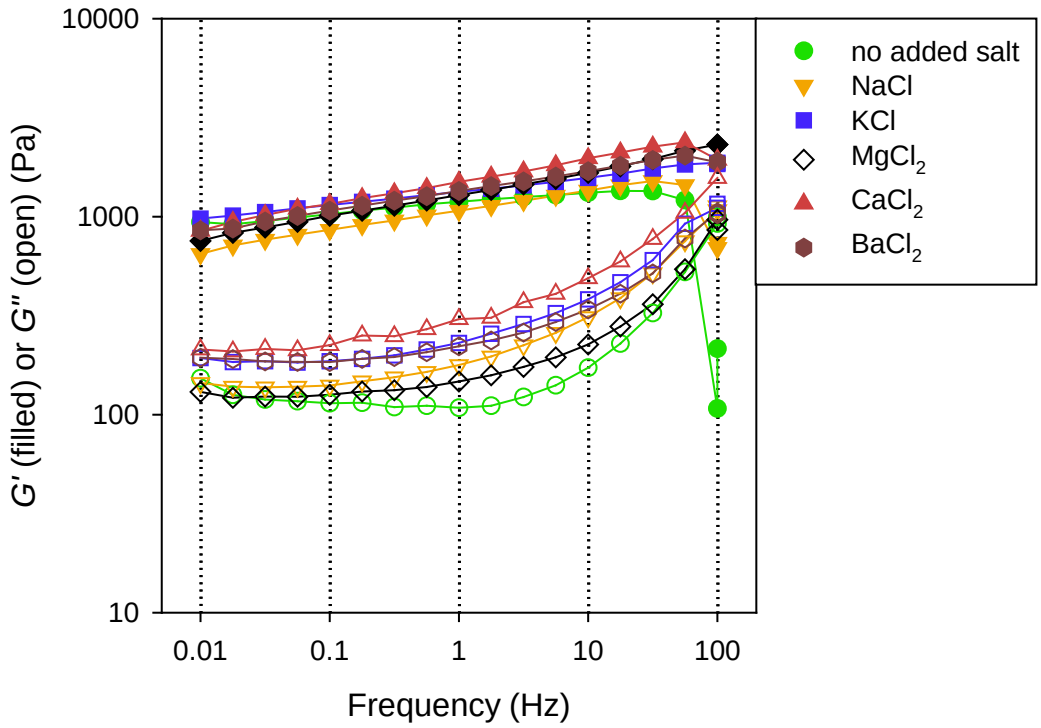


Figure S2. Strain sweep data for 5% w/w WPI with 120 mM of various salts added after heating at 80°C on the rheometer, cooling to 20°C and running a frequency sweep