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Tuning the properties of β-lactoglobulin nanofibrils with pH, NaCl and $CaCl_2$

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Abstract

We investigated the effects of pH (1.6 – 2.4), NaCl and $CaCl_2$ (0 – 100mM) on the kinetics of β-lactoglobulin fibril formation during heating at 80°C. The morphology of fibrils was also examined. At pH 1.8 - 2.4 fibril formation occurred slightly faster with decreasing pH. At pH 1.6 fibril formation during the growth phase occurred much faster than at any other pH. Fibril morphology was unchanged between pH 1.6 and pH 2.0.

Addition of NaCl or $CaCl_2$ accelerated fibril formation during the growth phase, and $CaCl_2$ shortened the lag phase as well. Worm-like fibrils were seen at ≥ 60 mM NaCl or ≥ 33 mM $CaCl_2$, and these had a persistence length which was much shorter than the long semi-flexible fibrils formed without salts.

The efficiency of fibril formation can be substantially enhanced by varying pH and salt concentration.

Introduction

Many proteins are capable of self-assembling into non-random structures. If the ratio of length to width is large, the self-assembled structures are called 'fibrils', although the term 'filaments' is preferred in biochemistry literature. Where fibrils are comprised of β-sheets running perpendicular to the fibril axis, they are termed 'amyloid' or amyloid-like'. 'Amyloid' derives from the association with amyloidosis diseases, such as Alzheimer's and Parkinson's disease, and 'amyloid-like' refers to structurally similar fibrils formed *in vitro* (Fändrich, 2007).

Dairy protein nanofibrils are of interest to the food industry because of their potential to enhance viscosity and form gels at lower protein concentration than with random aggregates (Graveland-Bikker and de Kruif, 2006). They may also have applications in biomedical and materials science (Waterhouse and Gerrard, 2004).

β-Lactoglobulin is a whey protein comprising 0.2 - 0.4 % w/v of skim milk. Recognition that β-lactoglobulin could form fibrils when heated at low pH and low ionic strength came from the finding that β-lactoglobulin gels made at pH < 4 had a 'fine-stranded' microstructure (Langton and Hermansson, 1992). A later study of β-lactoglobulin heated at pH 2 (Aymard et al., 1999) found that aggregates formed at low ionic strength were long, thin and semi-flexible.

Bolder et al. (2007a) proposed that heat-induced conversion of β-lactoglobulin monomers into fibrils involved monomer 'activation', nucleation, polymerisation and termination steps. However, recent evidence indicates that heat-induced β-lactoglobulin fibrils are composed of peptides rather than intact monomers (Akkermans et al. 2008). This is in agreement with reports that limited specific hydrolysis enhances fibril formation with hen egg white lysozyme (Mishra et al. 2007) and α-lactalbumin (de Laureto et al. 2005). Both intact β-lactoglobulin and certain peptides derived from β-lactoglobulin are capable of forming fibrils in 5M urea (Hamada et al. 2009), so it is not clear whether hydrolysis is necessary for fibril formation under such conditions.

The transformation from native protein to amyloid fibril is typically accompanied by an increase in the content of β-sheet, however β-lactoglobulin fibrils appear to be less structurally ordered than other well-known amyloidogenic proteins such as insulin (Bromley et al., 2005, Nielsen et al. 2001a). Some researchers have reported a relatively small increase

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in β-sheet structure as β-lactoglobulin forms fibrils during heating at 80°C or prolonged incubation in solvents (Gosal et al., 2004; Kavanagh et al., 2000b).

Ionic strength and pH affect both the kinetics of fibril formation (Arnaudov and De Vries, 2007; Aymard et al., 1999) and the morphology of fibrils (Arnaudov and de Vries, 2006; Aymard et al., 1999; Durand et al., 2002; Kavanagh et al., 2000b, Mudgal et al. 2009). Macroscopic properties of β-lactoglobulin fibril dispersions/gels such as turbidity (Sagis et al., 2002), gel strength (Kavanagh et al., 2000a) and critical percolation concentration (Sagis et al., 2004) are also affected by ionic strength and pH.

With heat treatments at 80°C, added NaCl substantially accelerated β-lactoglobulin denaturation at pH 2.5 (Schokker et al., 2000) and accelerated fibril formation at pH 2.0 (Arnaudov and De Vries, 2007; Aymard et al., 1999). Fibrils became shorter and more flexible with increasing NaCl concentration (Arnaudov and de Vries, 2006; Aymard et al., 1999; Durand et al., 2002; Kavanagh et al., 2000b), and increasing ionic strength lowered the critical percolation concentration of β-lactoglobulin fibril gels (Sagis et al., 2004).

It is notable that β-lactoglobulin fibril formation has been investigated at pH 2 – 3.35 and in the presence of up to 100 mM NaCl, but not at pH below 2 or in the presence of other salts. Others have reported a strong effect of pH on the rate of amyloid fibril formation with insulin (Nielsen et al., 2001b), egg lysozyme (Arnaudov and de Vries, 2005), and β-amyloid peptide (Naiki and Nakakuki, 1996). However it appears that systematic studies on the effect of pH on β-lactoglobulin fibril formation have not been published. In addition, studies of the 'ionic strength' effect on β-lactoglobulin fibril formation have invariably used only NaCl, so ion-specific effects are unknown.

Here we report on self-assembly of β-lactoglobulin heated at 80°C, at pH 1.6 - 2.4 and in the presence of NaCl or $CaCl_2$, a salt commonly present in processed dairy products.

Materials and Methods

Chemicals. β-Lactoglobulin (90% pure) containing a mixture of genetic variants A and B was purchased from Sigma (St. Louis, MO, product no. L0130-5G). Reverse osmosis filtered water was used throughout. NaCl, $CaCl_2 \cdot 2H_2O$, NaH_2PO_4 and Na_2HPO_4 were AnalaR® grade from BDH (Poole, England). The Thioflavin T powder was purchased from Chroma Corp. (McHenry, IL).

Preparation of β-lactoglobulin solutions. Reverse osmosis filtered water was adjusted to the desired pH (± 0.05) with HCl to make 'HCl buffer'. β-Lactoglobulin was dissolved in HCl buffer to make approximately 1.2% w/v. The solution was stirred overnight at 4°C then centrifuged at 22,600 x g for 30 minutes (Himac CR22G II super speed centrifuge, Hitachi Koki Co., Japan) and filtered (0.2 μm syringe filter, Millex-GS®, Millipore, Billerica, MA).

Residual salts were removed by ultrafiltration using a centrifugal filter with nominal molecular weight cutoff of 10 kDa (Amicon® Ultra-15, Millipore) by centrifuging at 3000 x g for 15 minutes. Filters were rinsed with HCl buffer prior to use. After filtering three times, the conductivity of the protein solution was close to that of the buffer.

Protein concentration in the desalted β-lactoglobulin solution was determined by absorption at 278 nm (Ultrospec 2000 UV spectrophotometer, Pharmacia Biotech, Cambridge, UK), using a β-lactoglobulin standard curve and assuming 90% purity. A small proportion of protein was

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lost during filtering, and an initial concentration of approximately 1.2% w/v before filtering gave a final concentration close to 1% w/v after filtering.

Solutions of β-lactoglobulin were stored at 4°C and used within two days of preparation. Stock solutions of 1 M NaCl and 1 M $CaCl_2$ were used to adjust salt content of β-lactoglobulin solutions.

Heating of β-lactoglobulin solutions. 2 mL aliquots of β-lactoglobulin solution in screw-capped glass tubes (16 mM diameter Kimax® glass, Schott, Elmsford, NY) were heated in a water bath (Lab Companion BS-11, Jeio Tech, Boston, MA) at 80 ± 0.1°C. Following the requisite heating time, a tube was cooled in ice water for 10 min.

Thioflavin T (ThT) fluorescence assay. A stock solution of 3.0 mM ThT in phosphate-NaCl buffer (10 mM phosphate and 150 mM NaCl, pH 7.0) was filtered through a 0.2 μm syringe filter (Millex-GS, Millipore). Stock solution was stored at 4°C in a brown glass bottle covered with aluminium foil. Working solution was prepared by diluting the stock solution 50-fold in phosphate-NaCl buffer (final ThT concentration 60 μM).

In the assay, 48 μL of sample was added to 4 mL of working solution; the mixture was vortexed briefly and held at room temperature for 1 min before measuring fluorescence (RF-1501 spectrofluorimeter, Shimadzu, Kyoto, Japan). Excitation and emission wavelengths were 440 nm and 482 nm, respectively. The fluorescence of unheated protein solution from the same batch was subtracted from all measurements.

Negative stain transmission electron microscopy (TEM). The ultrafiltration method of Bolder et al. (2007b) was used to purify fibrils and reduce the background in TEM images. 100 μL of heated protein solution was added to 2 mL HCl buffer in a centrifuge filter with nominal molecular weight cutoff 100 kDa (Amicon Ultra-4, Millipore) previously washed with 2 mL HCl buffer. The filter was centrifuged at 3000 x g for 15 min and the retentate topped up with 2mL HCl buffer. Filtration was carried out three times in total, and the final retentate was topped up with 1mL HCl buffer, mixed by inversion and transferred to a 1.5 mL plastic tube (Eppendorf, Hamburg, Germany). The final dilution of heated protein was approximately 10-fold.

A Formvar/carbon coated 200 mesh copper grid (Agar Scientific Ltd, Stansted, UK) was placed on a droplet of sample for 5 min. The grid was removed, touched against filter paper to soak away excess sample then placed on a drop of 2% uranyl acetate in water for 5 min. Excess stain was soaked away with filter paper. The negatively-stained grid was examined with a Philips CM10 electron microscope (Eindhoven, The Netherlands).

Image analysis. The software used to measure persistence length and helical pitch was ImageJ 1.42, available from http://rsb.info.nih/gov/ij. For persistence length, a fibril was divided into segments by placing closely-spaced points along its length using the 'point picker' plugin from the MBF plugin collection (www.macbiophotonics.ca/imagej/). The point coordinates were converted from pixels to nm using the scale bar in each image. The persistence length was calculated from the average cosine of the angle made by two adjacent fibril segments, using Eq. (1).

$$L_p = < l > / (1 - < \cos\phi >) \qquad (1)$$

L_p is persistence length, l is segment length, ϕ is the angle made by three adjacent points, and angular brackets denote an average over all pairs of adjacent segments. This approach was explained schematically by Mudgal et al. (2009).

The helical pitch was calculated by measuring the length over which 4 to 8 twists occurred ('measure' function in ImageJ) and dividing by half the number of twists.

Curve-fitting. Thioflavin T fluorescence data were fitted with the Eq. (2), given by Morris et al. (2008). Note that we use this equation as an empirical curve-fitting function, in which f_t is fluorescence at time t and α, β, and γ are arbitrary constants.

$$f_t = \alpha - \frac{\frac{\beta}{\gamma} + \alpha}{1 + \frac{\beta}{\alpha\gamma}\exp[t(\beta + \alpha\gamma)]} \quad (2)$$

The following parameters were derived analytically from Eq. (2) (see supplementary material):

$$t_{0.5\,max} = \frac{\ln\left(2 + \frac{\alpha\gamma}{\beta}\right)}{(\beta + \alpha\gamma)} \quad (3)$$

$$\left(\frac{df}{dt}\right)_{max} = \frac{\left(\frac{\beta}{\gamma} + \alpha\right)(\beta + \alpha\gamma)}{4} \quad (4)$$

$$t_{lag} = \frac{1}{\beta + \alpha\gamma}\left(\ln\left(\frac{\alpha\gamma}{\beta}\right) - 4\frac{\alpha\gamma}{\beta + \alpha\gamma} + 2\right) \quad (5)$$

Where t_{lag} is the lag time, $t_{0.5\,max}$ is the time for fluorescence to reach half its maximal value, and $(df/dt)_{max}$ is the maximal rate of increase in fluorescence. The analytical equation for $t_{max\,rate}$ (the time at which $(df/dt)_{max}$ occurs) differs from the right hand side of Eq. (3) only by lacking the '2' in the numerator (see supplementary material). For the data presented here, the difference between $t_{0.5\,max}$ and $t_{max\,rate}$ was at most 0.1 h.

Data Analysis. SigmaPlot 10.0 (Systat software, Chicago, IL) was used for nonlinear regression. Persistence length calculations were done in Excel 2007 (Microsoft, Redmond, WA), and Minitab 15 (Minitab Inc., State College, PA) was used for statistical analysis.

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Results and Discussion

Kinetics of fibril formation

Effect of pH. When β-lactoglobulin adjusted to pH 1.6 - 2.4 was heated at 80°C, ThT fluorescence increased sigmoidally with heating time (Figure 1). Fluorescence changed little for the first few hours (lag phase), then increased rapidly (growth phase) and finally plateaued (stationary phase).

Eq. (2) fitted fluorescence data extremely well, and kinetic parameters given by Eq. (3) – (5) are shown in Table 1. This modified logistic function was shown by Morris et al. (2008) to fit a wide range of protein aggregation data extremely well. They attributed mechanistic meaning to its parameters based on a two-step reaction scheme comprising first order nucleation and autocatalytic growth. However, conversion of β-lactoglobulin from monomers into fibrils appears to require a hydrolysis step prior to nucleation (Akkermans et al. 2008), so the two-step autocatalytic mechanism is not suitable. Here we use Eq. (2) as a fitting function with arbitrary parameters because it provides a convenient and unbiased calculation of t_{lag}, $t_{0.5\,max}$ and $(df/dt)_{max}$.

There was a consistent decrease in t_{lag} from 5.5 h at pH 2.4 to 3.4 h at pH 1.8. The rate at which fluorescence increased during the growth phase, $(df/dt)_{max}$, was dramatically higher at pH 1.6 than at pH 1.8 - 2.4, resulting in much lower $t_{0.5\,max}$. The maximum fluorescence was significantly higher at pH 1.6 and 1.8 than pH 2.4.

Hydrolysis is expected to occur faster at lower pH due to the excess of H_3O^+ driving the reaction forward, and this explains the decrease in t_{lag} between pH 2.4 and pH 1.8. However there is little change in t_{lag} between pH 1.6 and pH 1.8, which implies that the rate of hydrolysis does not control the length of the lag phase here. Formation of fibril nuclei may instead be limiting, and once sufficient nucleation has occurred, the abundance of hydrolysis products would facilitate very rapid growth of fibrils, hence the large $(df/dt)_{max}$ at pH 1.6.

These results show that it is possible to increase both the rate of fibril formation and the yield of fibrils by reducing pH below 2.0.

Effect of NaCl and $CaCl_2$. The effect of NaCl and $CaCl_2$ on the rate of fibril formation was also measured with the ThT fluorescence assay. Samples of 1% w/v β-lactoglobulin at pH 2.0 were heated at 80°C for 8 h in the presence of 0 - 100 mM NaCl or $CaCl_2$.

ThT fluorescence data in the growth phase were fitted with a straight line (Figure 2), as there were insufficient data for a more complex model. The slope of the straight line is referred to hereafter as the 'growth rate'. The t_{lag} was calculated as the intercept of the fitted straight line with the time axis.

With 100 mM $CaCl_2$, the ThT fluorescence decreased between 4 h and 8 h (Figure 2B). This coincided with the appearance of transparent gel particles in the sample, which are thought to be composed of fibrils. Fibrils in the interior of gel particles are invisible to the ThT assay, and the decrease in fluorescence reflects the local gelling of part of the sample.

Both NaCl and $CaCl_2$ increased the growth rate to an approximately equal extent on an equimolar basis, but $CaCl_2$ also decreased t_{lag} substantially, whereas NaCl had little effect on t_{lag} (Figure 3). Arnaudov and de Vries (2007) reported a more dramatic effect of NaCl concentration on β-lactoglobulin fibril formation kinetics, but this may reflect differences in

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experimental and theoretical approaches. Aymard et al. (1999) found that NaCl increased both the rate of aggregation and the yield of aggregates.

$CaCl_2$ produces higher ionic strength than NaCl, and would therefore reduce electrostatic repulsion and enhance aggregation more effectively at the same molar concentration. However, comparing lag times in the presence of 10 mM $CaCl_2$ (ionic strength 30 mM, t_{lag} = 2.4 h) or 50 mM NaCl (ionic strength 50 mM, t_{lag} = 3.1 h) shows that increasing ionic strength alone does not speed up fibril formation. If NaCl and $CaCl_2$ data are compared on the basis of equal Cl^- concentration, e.g. 100 mM NaCl vs. 50 mM $CaCl_2$, NaCl increased the fibril growth rate slightly more than $CaCl_2$, but $CaCl_2$ decreased t_{lag} much more than NaCl.

Increasing the ionic strength or concentration of Cl^- may accelerate aggregation of charged peptides by screening positive charges and thereby reducing intermolecular repulsion (Schokker et al. 2000), but cation-specific effects are larger.

At pH 2 there will be no negative charges on peptides for cations to interact with. However, cations could interact with carboxyl groups on Asp and Glu side chains, C-terminal carboxyl groups or peptide carbonyls, providing the spatial configuration and cation size permit. Bridging of two such groups by a Ca^{2+} ion may facilitate the formation of a nucleus, thereby accelerating self-assembly (Graveland-Bikker et al. 2004).

Cations can also interact with delocalised π electrons around the aromatic amino acids phenylalanine (Phe), tryptophan (Trp) and tyrosine (Tyr), a phenomenon named 'cation-π interactions' (Dougherty, 1996). The aromatic amino acids appear to play an important role in the early stages of heat-induced β-lactoglobulin fibril formation, particularly where well-separated from positively charged amino acids, which would repel cations.

Akkermans et al. (2008) identified seven peptides thought to be present in fibrils, five of which contained Trp_{19} and Tyr_{20}, which are some distance from Lys_{14}. The remaining two peptides contained Phe_{105} or Phe_{151}, whose nearest charge-bearing neighbours were Lys_{101} and Arg_{148}, respectively. Molecular dynamics simulations (de la Paz et al., 2005) indicated that Phe promoted β-sheet formation at any position within an amyloidogenic hexapeptide.

Hamada et al. (2009) showed that designed peptides based on β-strands A, G, H and I in the β-lactoglobulin molecule could form fibrillar aggregates in 5M urea, with fibril-forming ability following the order βG >> βA > βI >> βH > βF. Their βG peptide contained Phe_{105} but also Cys_{106}, which may have facilitated nucleation by forming disulphide bonds (Euston et al. 2007; Hamada et al. 2009). The βA peptide contained Trp_{19} and Tyr_{20}, and βI contained Phe_{151}. The βH peptide was designed with an extra Trp at the N-terminal end to facilitate detection by UV absorption, but otherwise contained no aromatic residues. The βF peptide contained Tyr_{99}, but this was immediately adjacent to Lys_{100} and Lys_{101}. The βB region contains Tyr_{42}, and may have the potential to form fibrils.

Both Na^+ and Ca^{2+} can take part in cation-π interactions, but *ab initio* calculations (Bhattacharjee 2000; Reddy and Sastry 2005) indicate that Ca^{2+} binds to aromatic amino acid side chains with 2 - 4 times the affinity of Na^+. We note that amino acids arginine, histidine and lysine could also participate in cation-π interactions at acidic pH.

On the basis of current evidence, it is not possible to identify the type of the interactions between Na^+ or Ca^{2+} and fibril-forming peptides. However the large differences between Ca^{2+} and Na^+ effects reported here indicate that the nature of the cation has an important influence on the strength and/or duration of interactions.

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Fibril morphology

Effect of pH. The effect of pH on the morphology of β-lactoglobulin fibrils is shown in Figure 4. At pH 2.0, fibrils were semi-flexible, 5 - 10 nm in diameter and up to 5 μm long. Long semi-flexible fibrils were entangled with others in loose networks approximately 5 - 10 μm wide. Fibrils did not appear to be branched, although it was often difficult to follow a single fibril along its entire length because of overlapping with other fibrils. In addition to long semi-flexible fibrils, a small number of spherical aggregates with diameter 15 - 25 nm was seen.

At pH 2.2, fibrils were also long, semiflexible and unbranched, and in many cases had a 'wavy' appearance (see Figure 4 inset). Waves had a period of 63.9 ± 1.5 nm (n = 5) and amplitude of approximately 8 nm. A few spherical aggregates were again present, but fibrillar aggregates predominated. At pH 2.4, fibrils were more closely entangled in networks, but they appeared to be long, unbranched and somewhat wavy.

At pH 1.8 and 1.6, fibril morphology was very similar to pH 2.0 fibrils, except that at pH 1.6 a small number of tightly-curled fibrils were present (see Figure 4 inset).

Kavanagh et al. (2000b) found highly flexible fibrils up to approximately 150 nm long when β-lactoglobulin was heated at pH 2.5. Mudgal et al. (2009) produced fibrils at pH 3.35 with contour length of approximately 130 nm. We have found no other reports of the effect of pH on fibril morphology.

Effect of NaCl. β-Lactoglobulin fibrils formed in the presence of NaCl are shown in Figure 5. Morphology was virtually unchanged with up to 50 mM NaCl, although at 50 mM fibrils were often 'wavy', as seen at pH 2.2. At 60 mM NaCl, long semi-flexible fibrils were formed but they were slightly shorter than with less NaCl. There were also short, tightly curled fibrils similar to the 'worm-like' fibrils produced when β-lactoglobulin is incubated with solvents (Gosal et al., 2004). At higher NaCl concentrations the two types of fibrils again coexisted, and the proportion of worm-like fibrils increased with increasing NaCl concentration.

The worm-like fibrils seen here look similar to those seen by others (Arnaudov and de Vries, 2006; Durand et al., 2002; Kavanagh et al., 2000b). The progressive increase in the proportion of worm-like fibrils with increasing NaCl concentration is in agreement with the findings of Arnaudov and de Vries (2006).

Effect of $CaCl_2$. $CaCl_2$ had a similar effect of on fibril morphology to that of NaCl (Figure 6). With <33 mM $CaCl_2$, fibrils were indistinguishable from those formed in the absence of $CaCl_2$. With ≥33 mM $CaCl_2$, the long semi-flexible fibrils became slightly shorter, and worm-like fibrils were also present. Increasing $CaCl_2$ concentration produced shorter semi-flexible fibrils and a higher proportion of worm-like fibrils. With 80 mM or 100 mM $CaCl_2$, only worm-like fibrils were formed.

Multi-fibril helices. Under all conditions tested there was a small proportion of fibrils twisted together into multi-fibril helices, examples of which are shown in Figure 7. Jung and Mezzenga (2010) found similar 'helical bundles' of β-lactoglobulin fibrils, which had a pitch of 60 - 100 nm. They noted that the number of helices increased significantly when the pH was raised from 2 to 4.

Kad et al. (2001; 2003) showed AFM and TEM images of β_2-microglobulin fibrils (termed protofilaments by them) twisting into three different types of multi-fibril helical structures. They termed them Type I: two protofilaments twisted into a helix; Type II: four protofilaments

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helically twisted together; and Type III: fibrils with a 'twisted ribbon' appearance, which was thought to involve lateral association of four protofilaments.

Multi-fibril β-lactoglobulin structures were putatively labelled 'Type I' and 'Type II' in Figure 7 based on a similar appearance to the corresponding type of β_2-microglobulin fibrils and, where possible the number of single fibrils twisting together. No Type III β-lactoglobulin fibrils were seen.

Figures 7C and 7E each show two fibrils twisting together, and the resulting helices were labelled Type I for that reason. However in both cases the pitch is quite different to Type I fibrils in Figure 7A and 7F, so these may be a different variety of Type I fibrils. Adding 30 to 60 mM NaCl or 10 to 40 mM $CaCl_2$ gave fibrils a wavy appearance, and in a small number of cases several adjacent fibrils were lined up parallel with interlocking waves, such as Figure 7D.

Persistence length. The persistence length (L_p) of a polymer is a measure of its flexibility, and represents the typical length at which thermal fluctuations begin to bend the polymer in different directions (Mackintosh, 1998). Cifra (2004) compared three approaches suitable for calculating L_p from micrographs. At least two of these approaches have been applied to food proteins: Akkermans et al. (2007) used an equation based on bond orientation correlation to calculate L_p of soy protein fibrils, and Mudgal et al. (2009) calculated L_p of β-lactoglobulin fibrils from the average cosine of the bond angle. The former approach assumes an exponential decay of the correlation between the orientation of an end segment of fibril and the orientation of subsequent segments along the fibril's length. This calculation was tried here, but correlation decay was not exponential. The bond orientation correlation approach was rejected in favour of the average cosine method, which gave L_p values comparable to those of Mudgal et al. (2009).

It was often difficult to follow the entire length of a fibril, either because it extended outside a field of view or because its course could not be unequivocally traced beyond an overlap with other fibrils. For that reason, most L_p measurements were derived from 1 - 5 µm sections of long semi-flexible fibrils or 175 - 684 nm sections of worm-like fibrils. Short semi-flexible fibrils showed little curvature over their length, and it was thought that they would give artificially low L_p, so longer fibrils were chosen for measurements. Diluting fibril dispersions more extensively before TEM would make it easier to visualise entire fibrils, but harder to find fibrils on the TEM grid.

Our L_p measurements are summarised in Table 2. It was not possible to measure L_p from TEM images of pH 2.4 fibrils because they were too closely entangled. L_p of fibrils formed at pH 1.6, 1.8 or 2.2 was not significantly different from the control at pH 2.0. The L_p values of worm-like fibrils formed in the presence of 100 mM NaCl and 33 or 100 mM $CaCl_2$ were statistically indistinguishable, and were an order of magnitude lower than L_p values of long semi-flexible fibrils formed under those and other conditions.

The L_ps of long semi-flexible fibrils formed in the presence of 50 mM NaCl or 33 mM $CaCl_2$ were not significantly different from the control, but at 100 mM NaCl long semi-flexible fibrils had L_p higher than control fibrils. Cifra (2004) noted a tendency for the average cosine calculation to overestimate L_p for stiff rods, though it is difficult to know whether that is occurring here.

Table 3 summarises L_p measurements of protein fibrils reported by others, as well as contour length (L_c) data, i.e. the length of fibrils at maximum extension. Our results are in agreement with those of Aymard et al. (1999), who also found a large decrease in L_p of β-lactoglobulin fibrils with increasing ionic strength. The L_ps of β-lactoglobulin fibrils at pH 3.35 (Mudgal et al., 2009) are the same order of magnitude as our worm-like fibrils, and pH 3.35 fibrils look

somewhat similar to our worm-like fibrils. A comparison of L_p data for different proteins suggests that β-lactoglobulin fibrils are stiffer than BSA, ovalbumin or soy glycinin fibrils.

Conclusions

β-Lactoglobulin fibrils have the potential to enhance food functionality in a very efficient way. Heat-induced transformation of β-lactoglobulin into fibrils occurs only at low pH and low ionic strength, but there is still relatively little understanding of the effects of pH and salts on the process, and only a limited range of conditions has been explored. Here we have shown that the transformation occurs faster at pH below 2.0 and in the presence of salts.

Lowering pH below 2.0 accelerates fibril formation during the growth phase and increases the yield of fibrils, but has little effect on the duration of the lag phase. NaCl also accelerates fibril formation during the growth phase without shortening the lag phase, but $CaCl_2$ both accelerates growth and shortens the lag phase. We hypothesise that Ca^{2+} accelerates nucleation by 'bridging' between two peptides *via* nucleophilic moieties. NaCl and $CaCl_2$ alter fibril morphology dramatically, which may also alter the functionality of β-lactoglobulin fibrils as food ingredients.

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Table 1. Kinetic parameters describing the effect of pH on the rate of β-lactoglobulin fibril formation during heating at 80°C, measured via ThT fluorescence of a 1% w/v β-lactoglobulin solution (Fig. 1).

pH	t_{lag}	$\left(\frac{df}{dt}\right)_{max}$	$t_{0.5\ max}$	max fluorescence[a]	adjusted r^2	$S_{y\|x}$[b]
	[h]	[FU.h^{-1}]	[h]	[FU]		
1.6	3.7	94.4	4.8	204.9 (12.7) ab	0.985	10.4
1.8	3.4	29.4	7.2	227.2 (16.8) a	0.964	14.5
2.0	3.9	24.4	7.4	169.5 (16.1) bc	0.967	10.3
2.2	4.7	32.5	7.4	174.0 (9.0) bc	0.976	9.5
2.4	5.5	29.2	7.8	136.2 (8.6) c	0.977	7.1

a: the average fluorescence intensity measured after heating for 8 h (pH 1.6) or 24 h (pH 1.8 - 2.2). Figures in brackets are standard errors of triplicates. Different lower case letters indicate significant differences in Fisher pairwise comparisons with 95% confidence.

b: estimated standard error of the regression.

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Table 2. Persistence lengths (L_p) of β-lactoglobulin fibrils created by heating a 1% w/v solution for 6 h at 80°C, pH 1.6 to 2.2 with 0 – 100 mM added NaCl or 0 - 100 mM added $CaCl_2$. L_p values were calculated from TEM images using Eq (1).

description	pH	NaCl [mM]	CaCl2 [mM]	type[a]	L_p [nm]	N	group[c]
control	2.0	0	0	LS	2607 (511)[b]	9	b
pH	1.6	0	0	LS	3191 (786)	6	ab
	1.8	0	0	LS	2150 (221)	5	b
	2.2	0	0	LS	2924 (835)	6	ab
NaCl	2.0	50	0	LS	3162 (500)	8	ab
	2.0	100	0	WL	81 (7)	5	c
	2.0	100	0	LS	4307 (747)	5	a
$CaCl_2$	2.0	0	33	WL	84 (20)	6	c
	2.0	0	33	LS	1846 (140)	5	b
	2.0	0	100	WL	67 (4)	6	c

a: LS, long semi-flexible; WL, worm-like

b: figures in brackets are standard errors

c: groupings are based on Fisher pairwise comparisons; groups with different letters are significantly different at 95% confidence.

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Table 3. Persistence length (L_p) and contour length (L_c) measurements of food protein fibrils reported by others.

study and method for measuring L_p	Protein	concentration [% w/v]	ionic strength	pH	heating temperature [°C]	heating time [h]	L_c [nm]	L_p [nm]
Aymard et al. 1999	β-lactoglobulin	4	0.013	2	80	?[a]	?	600
x-ray and neutron scattering		4	0.03	2	80	?	?	300
		4	0.1	2	80	?	?	38
Mudgal et al. 2009	β-lactoglobulin	1.5	<0.05	2	80	10	2500	788
TEM image analysis		4	<0.05	3.35	85	3	130	36
		8	<0.05	3.35	85	3	300	34
Akkermans et al. 2007 TEM image analysis	soy glycinin	2	?	2	85	20	1100	2300 ± 1400
Pouzot et al. 2005	ovalbumin	5.5	0.003	7	78-80°C	24	>757[b]	130
x-ray scattering		0.4	0.100	7	78-80°C	24	?	60
Sagis et al. 2004	β-lactoglobulin	?	?	2	80	?	4500 ± 2500	1600 ± 400
adjusted random contact rheological model	BSA[c]	?	?	2	60	10	255 ± 76	16 ± 4
	ovalbumin	?	?	2	80	1	51 – 190[d]	300 ± 75

a: not specified

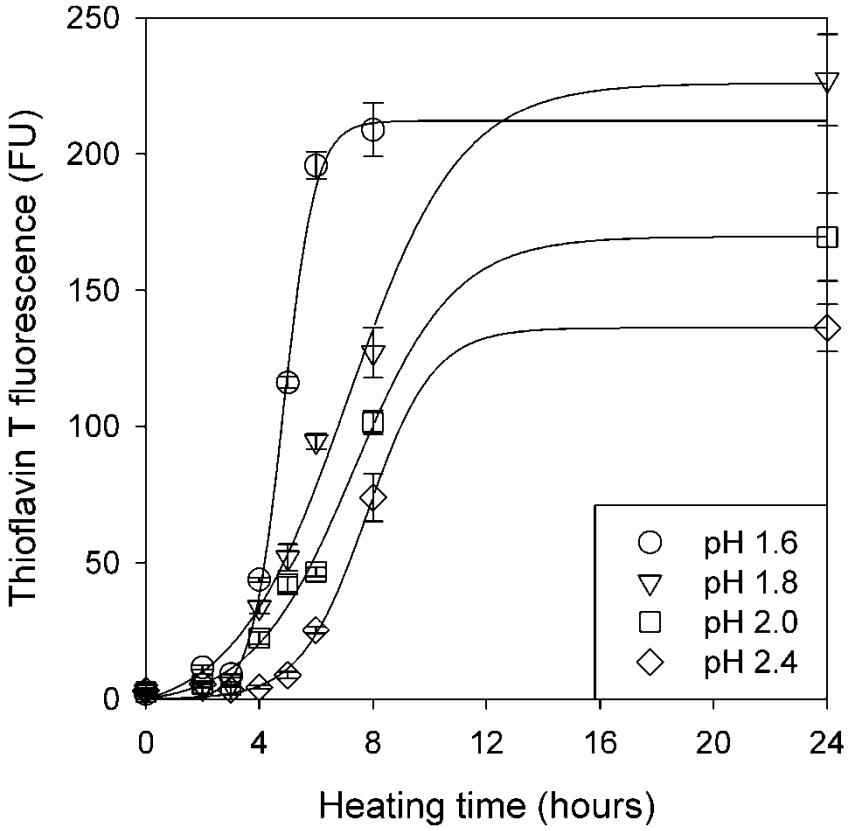
b: calculated by the present authors using image analysis with Figure 4 of Pouzot et al. (2005).

c: bovine serum albumin

d: contour length depended on ionic strength and protein concentration

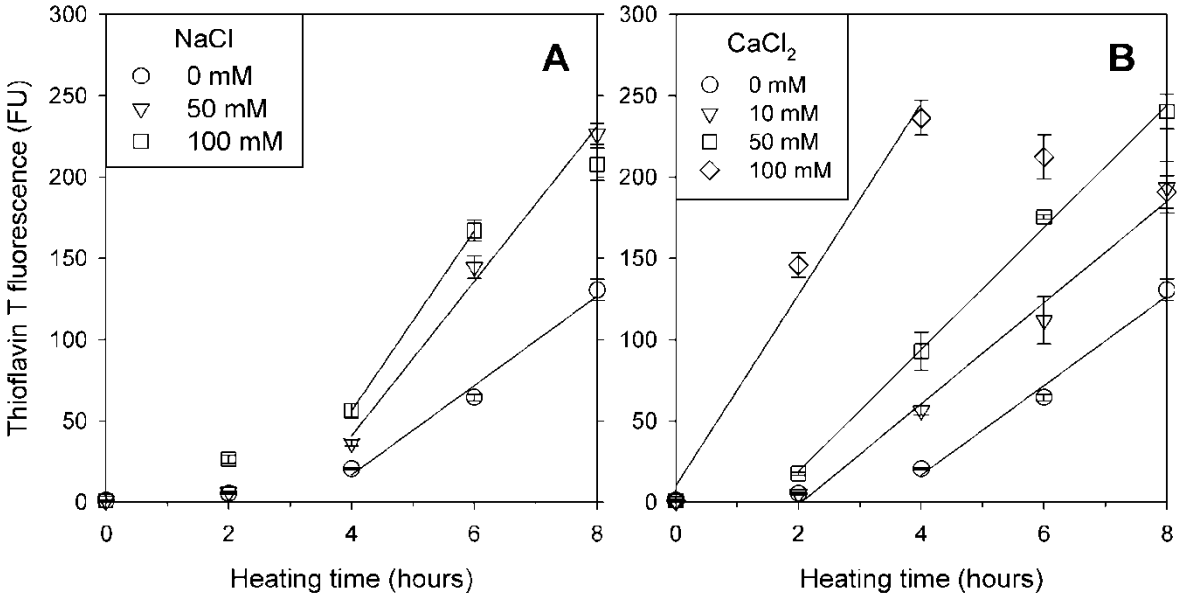
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Figure 1. Effect of pH on the kinetics of β-lactoglobulin fibril formation, measured *via* Thioflavin T fluorescence of 1% w/v β-lactoglobulin solutions heated at 80°C. Fluorescence data at pH 2.2 were almost identical to pH 2.0 data, and are not shown for that reason. Lines show the fit with empirical Eq. (2), and vertical bars are standard errors of triplicate assays.



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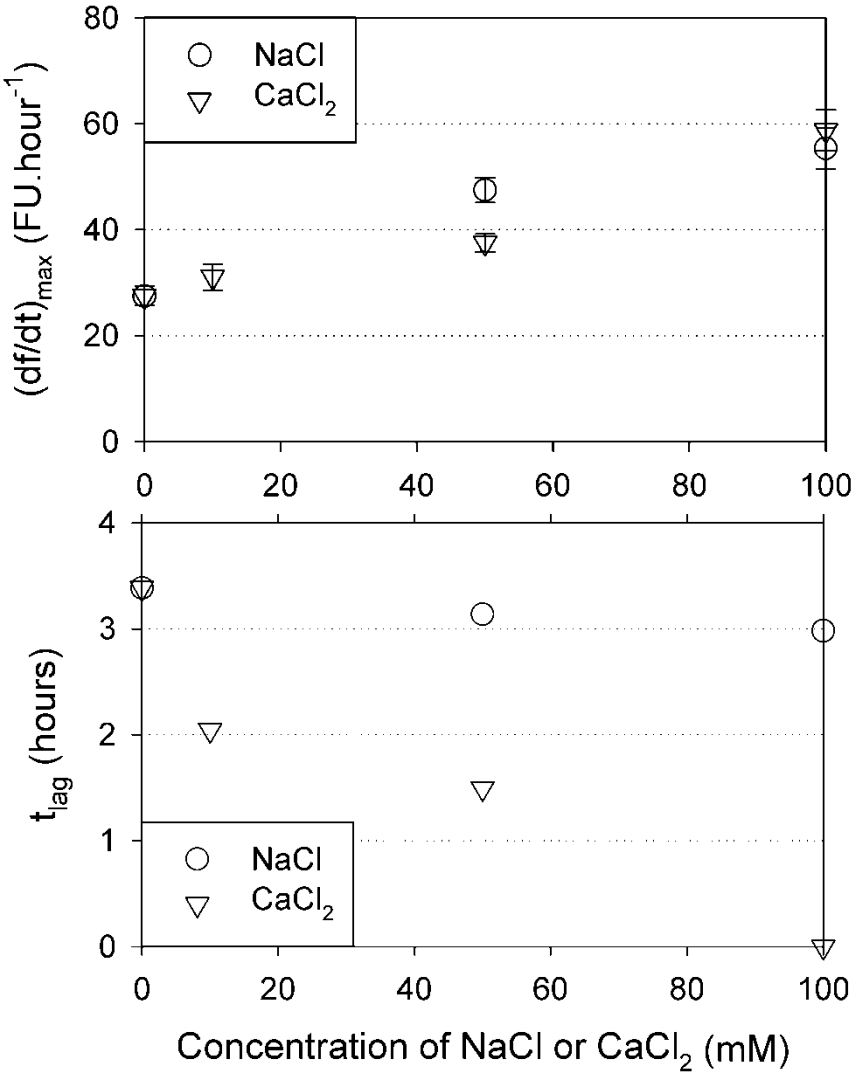
Figure 2. Effect of NaCl (A) and $CaCl_2$ (B) on the kinetics of β-lactoglobulin fibril formation, measured *via* Thioflavin T fluorescence of 1% w/v β-lactoglobulin solutions at pH 2.0, heated at 80°C. Solid lines show linear regression fits of data in the growth phase. Vertical bars are standard errors of triplicate assay results.



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Figure 3. Effect of NaCl and $CaCl_2$ on fibril growth rate, $(df/dt)_{max}$, and lag time, t_{lag}, measured *via* Thioflavin T fluorescence of 1% w/v β-lactoglobulin solutions at pH 2.0, heated at 80°C. Growth rates were calculated by linear regression of Thioflavin T fluorescence (Figure 2) and t_{lag} is the intersection of the fit line with the time axis.



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Figure 4. TEM images of β-lactoglobulin fibrils prepared at pH 1.6 - 2.4 by heating 1% w/v β-lactoglobulin solutions at 80°C for 6 h.

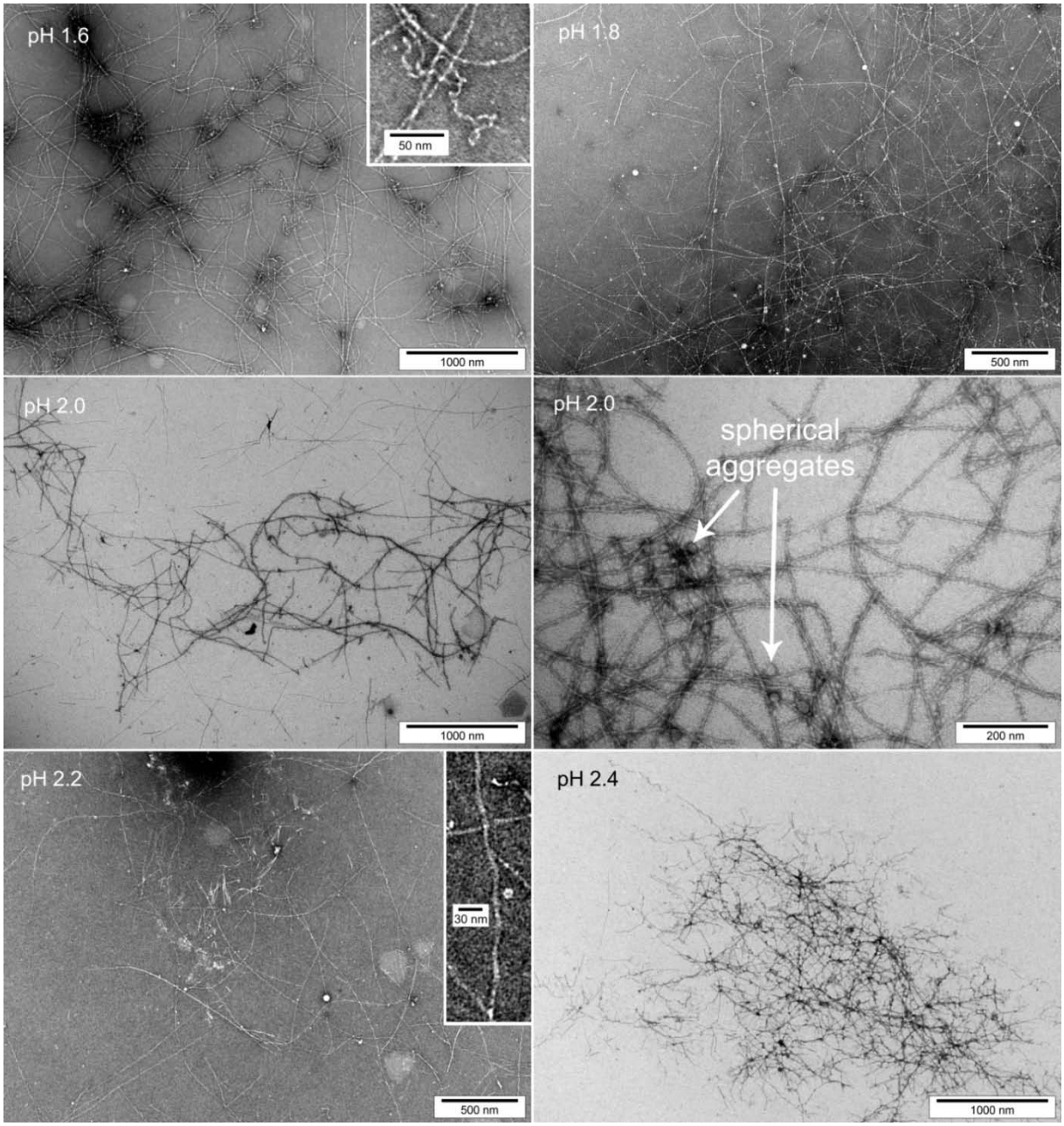
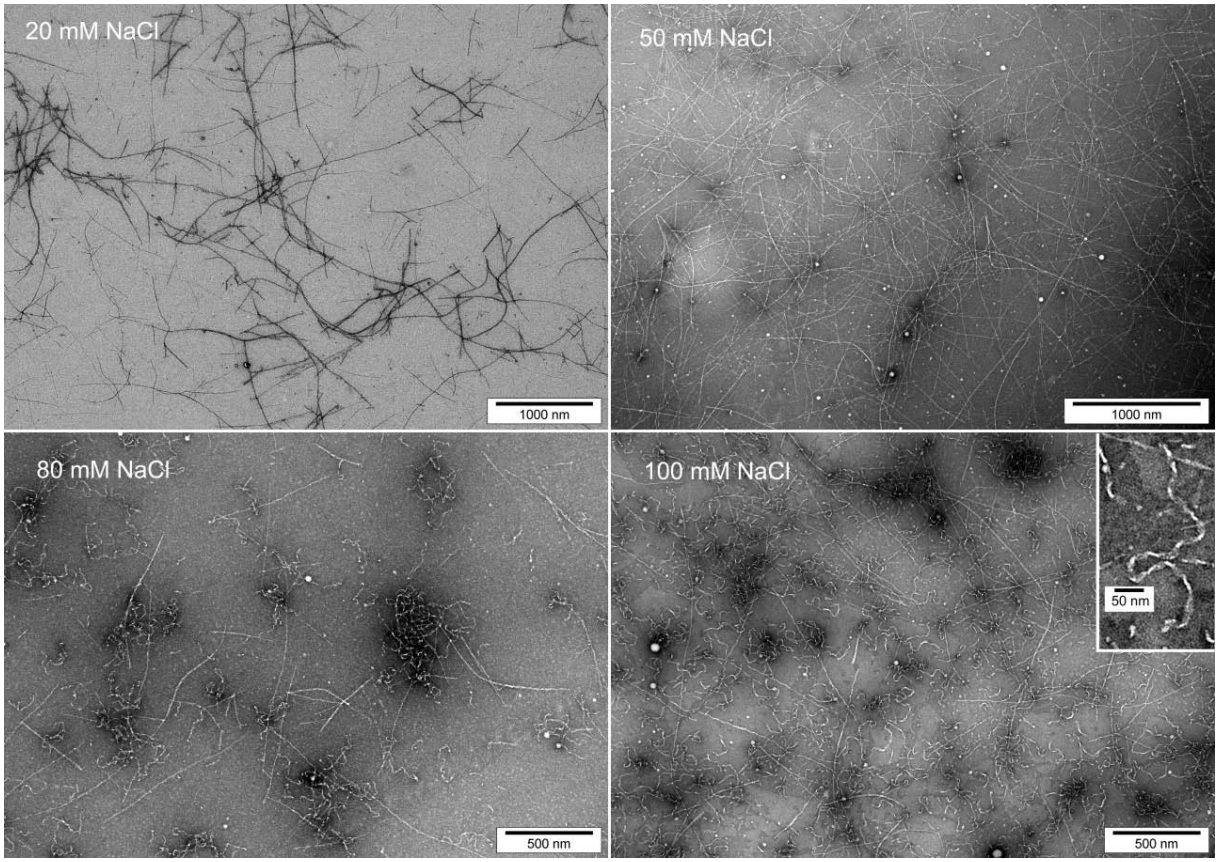
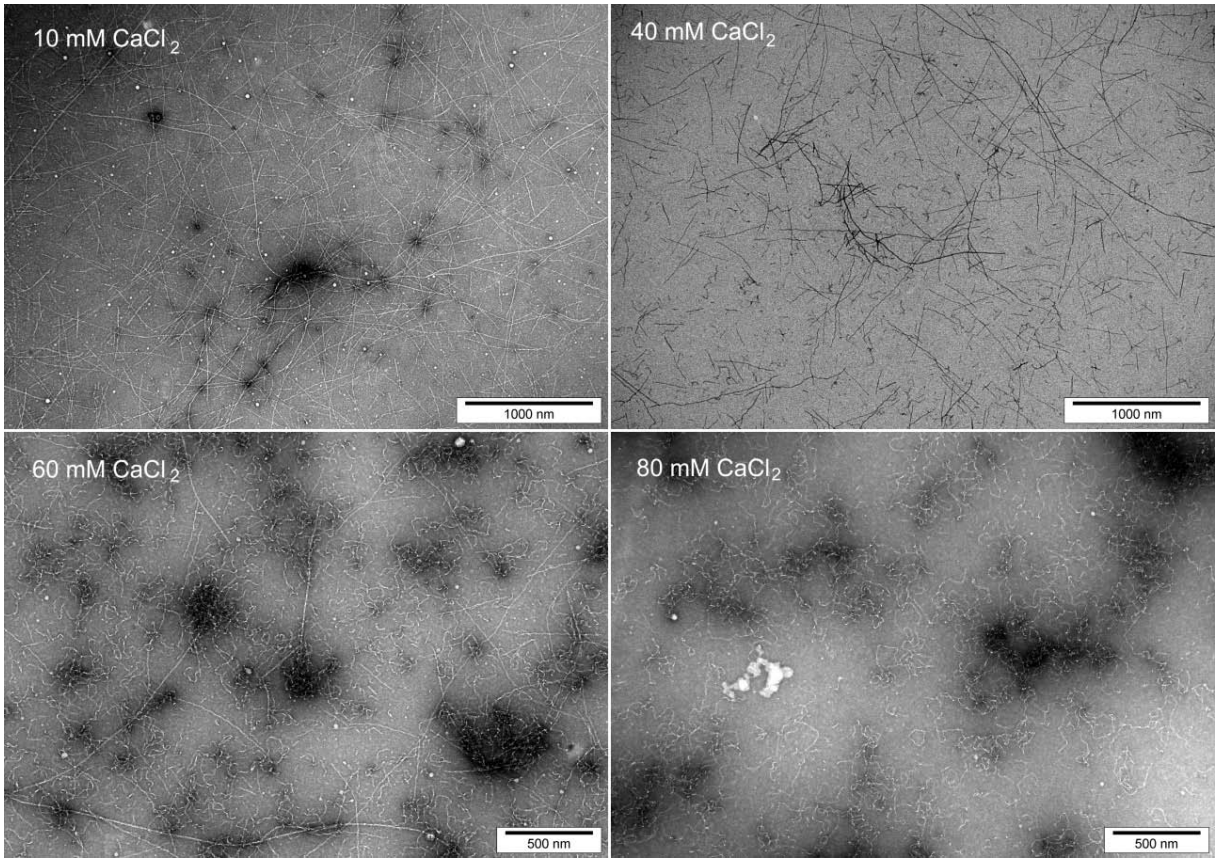


Figure 5. TEM images of β-lactoglobulin fibrils prepared at pH 2.0 with 20 – 100 mM NaCl by heating 1% w/v β-lactoglobulin solutions at 80°C for 6 h.



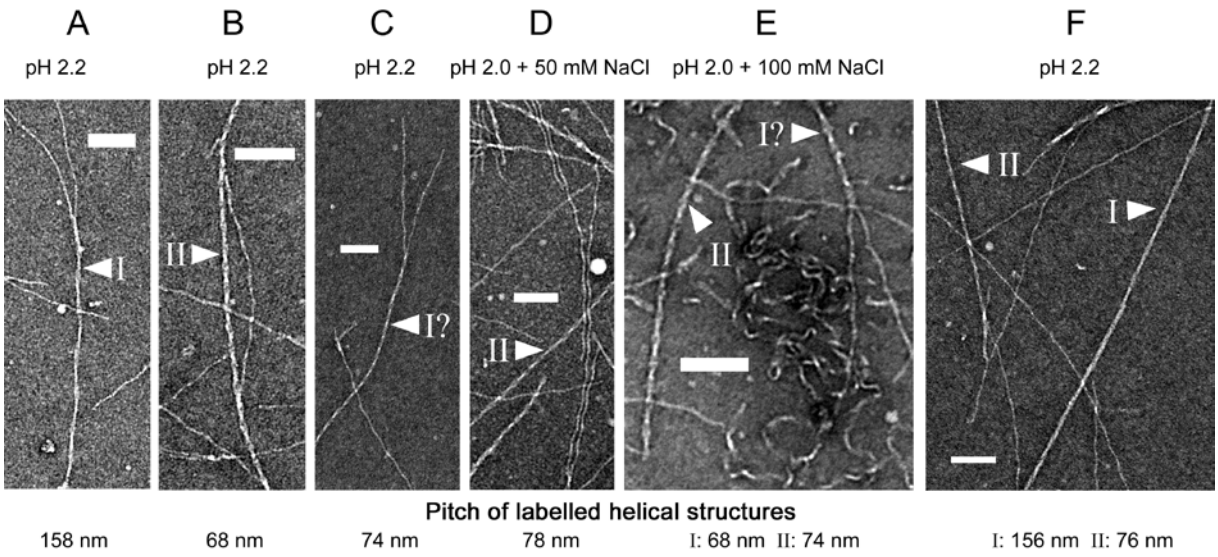
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Figure 6. TEM images of β-lactoglobulin fibrils prepared at pH 2.0 with 10 – 80 mM $CaCl_2$ by heating 1% w/v β-lactoglobulin solutions at 80°C for 6 h.



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Figure 7. TEM images of β-lactoglobulin fibrils prepared at pH 2.0 or 2.2 with 0 - 100 mM NaCl. 1% w/v β-lactoglobulin solutions were heated at 80°C for 4 h (A) or 6 h (B to F). Arrows indicate putative types of multi-fibril structures according to the types defined by Kad et al. (2001; 2003), and lengths below each frame are the pitch of labelled helical structures. All scale bars are 100 nm.



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