

Published in final form as:

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(3), R47-R55.

doi: 10.1111/j.1750-3841.2009.01098.x

Factors Affecting Rheological Characteristics of Fibril Gels: The Case of β -Lactoglobulin and α -Lactalbumin

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Abstract

Some of the factors that affect the rheological characteristics of fibril gels are discussed. Fibrils with nanoscale diameters from β -lactoglobulin (β -lg) and α -lactalbumin (α -la) have been used to create gels with different rheological characteristics. Values of the gelation time, t_c , the critical gel concentration, c_0 , and the equilibrium value of the storage modulus, G , such as G'_{inf} at long gelation times, derived from experimental rheological data, are discussed.

Fibrils created from β -lg using solvent-incubation and heating result in gels with different rheological properties, probably because of different microstructures and fibril densities. Partial hydrolysis of α -la with a serine proteinase from *Bacillus licheniformis* results in fibrils that are tubes about 20 nm in diameter. Such a fibril gel from a 10.0% w/v α -la solution has a higher modulus than a heat-set gel from a 10% w/w β -lg, pH 2.5 solution; it is suggested that one reason for the higher modulus might be the greater stiffness of α -la fibrils. However, the gelation times of α -la fibrils are longer than those of β -lg fibrils.

Keywords: shear modulus, protein fibrils, persistence length, nanoscale diameter, gelation time

Introduction

β -Lactoglobulin (β -lg) and α -lactalbumin (α -la) are the two major globular proteins in milk. β -lg has two major forms, known as A and B (Farrell and others 2004), which are globular proteins with molecular masses of 18,363 and 18,277 Da, respectively, and each has a radius of about 2 nm (Aymard and others 1999); their isoelectric point is pH 5.13 (Farrell and others 2004). Loveday and others (2007) have shown that high-concentration β -lg dispersions, ϕ_s of about 0.58, are colloidal glasses. α -la has a molecular mass of 14,178 Da and an isoelectric point of 4.2–4.5, depending on the sample (Farrell and others 2004). It can bind Ca^{2+} and is involved in the regulation of lactose biosynthesis (Goers and others 2002).

Self-assembly of molecules occurs in many biological materials. One wide-spread example of biological self-assembly is the folding of proteins into their compact three-dimensional structures. However, a wide variety of pathological conditions arise when proteins fail to fold or to remain folded correctly (Dobson 2003). The deposition of protein aggregates with fibrillar structures has been implicated in amyloidosis, a generic term for a subset of protein misfolding diseases (e.g., Alzheimer's and Creutzfeldt-Jakob) and in amyloid-related disorders (e.g., Huntington's disease). Typically, the fibrils are long, unbranched and often twisted structures that are a few nanometers in diameter. Amyloid fibrils formed from proteins almost exclusively involve β -sheets whose strands run perpendicular to the fibril axis and an irreversible process (Dobson 2003).

It has been recognized that many peptides and proteins, including many globular food proteins that are used as gelling agents, foaming agents, or emulsifiers, can form amyloid structures in vitro, and that they possess useful mechanical properties desirable to create useful structures (Gosal and others 2004a, Higham 2007). In addition, actin and tubulin fibrils play a role in the properties of the cytoskeleton, and fibrin, an amyloid structure, is found in blood clots (Shah and Janmey 1997, Sagis and others 2004). Because of the extremely high ratio of length (micrometer) to width (nanometer), fibril solutions can form meso-phases such as nematic phases, providing opportunities for the development of new protein-based functional foods (Sagis and others 2004; van der Linden and Vennema 2007). The mechanism of self-assembly varies and appears to be specific for each protein (Dobson 2003, Sagis and others 2004).

Both β -lg and α -la have been used to create fibrils, which in turn have been used to create gels. The techniques for creating fibrils have been discussed in several studies: those for fibrils from β -lg can be found in Gosal and others (2004a) and Jung and others (2008); those for fibrils from α -la can be found in Ipsen and others (2001) and Graveland-Bikker and de Kruif (2006). Table 1 contains a summary of the procedures and the fibril structures. It should be noted that, whereas the fibrils from β -lg and α -la have generally accepted nanoscale diameters (1–100 nm), both α -la fibrils (Ipsen and Otte 2007) and β -lg fibrils (Gosal and others 2004a) are relatively long, from about 500 nm to over 1 μm . Recently, Jung and others (2008) obtained β -lg aggregates with different structures upon heating a 1 wt% protein solution at different values of pH: rod-like aggregates at pH 2.0, spherical aggregates at pH 5.8, and worm-like primary aggregates at pH 7.0.

Bolder and others (2007a) observed that no fibrils formed on heating either pure α -la or pure bovine serum albumin at pH 2. However, fibrils did form in pure β -lg and whey protein isolate (WPI) solutions (Bolder and others 2006). Experiments indicated that β -lg was the only whey protein involved in fibril formation; further, when the WPI fibrils formed at pH 2 were stored at pH 7 or pH 10, disulfide bonds were formed in the samples; however, it was suggested that the disulphide bonds were formed between "non-assembled proteins," i.e., the proteins and

peptides not incorporated into fibrils (Bolder and others 2007a). The conversion was found to vary from about 5% for 0.5 wt % WPI solutions to about 45% for 5 wt % WPI solutions (Bolder and others 2007b).

In this work, we review recent filament network models and point out the fibril-structure data that would permit better understanding of the rheology of their gels. We then review some of the characteristics of β -lg and α -la fibrils, and the rheological properties of their gels. We believe that characterization of the features of the fibrils is important for developing practical food applications. The applicability of various models, such as the cascade, percolation, and fractal models, to the rheological data is also covered.

Models for analyzing fibril gels

Like many other biopolymer gels, the gels created from β -lg and α -la fibrils are physical gels, as opposed to chemically cross-linked systems. For these and other physical gels, Ross-Murphy (2005) noted that the significant parameters that are derived from experimental data are the gelation time, t_c , the critical gel concentration, C_0 , and the equilibrium value of the shear modulus G , such as G'_{inf} at long gelation times. Useful information, particularly on the β -lg fibril gels, was also obtained by applying the cascade (Gordon and Ross-Murphy 1975), percolation theory (de Gennes 1979), and the fractal model (Shih and others 1990). It is emphasized that in most of the above efforts, characteristics of gels were derived from experimental rheological data. Reviews of the cascade, percolation, and fractal models can also be found elsewhere (Ikeda and others 1999, Clark 1994, Sagis and others 2004, Rao 2007, chapter 6).

Additional information on the microstructure would help understand the differences among the rheological properties of the gels of different fibrils: heat-induced β -lg, alcohol-induced β -lg, and α -la. Because insightful research work has been done on biological (e.g., F-actin) networks, we look for potential clues in the theoretical and experimental studies on them. For clarity and to be consistent with the terminology in the literature, we use the terms: filaments and networks for these systems. We hasten to add that we are not suggesting direct extrapolation of the results. We suggest to exercise considerable caution in such application because networks of filaments of actin, collagen, and fibrin have viscoelastic properties that differ from those of gels formed by flexible polymers; in particular, they show, after a linear region, a significant increase in the elastic modulus with increasing strain (strain-stiffening) (Shah and Janmey 1997, Storm and others 2005). In contrast, β -lg fibril gels showed strain-softening (Akkermans 2008, p. 130-131).

Biopolymer network models

We note that polymer theory deals with three types of filaments, characterized by two length scales: the persistence length, l_p , and the contour length L_c . The persistence length, defined as: $l_p = \kappa / (k_B T)$, is the typical length at which thermal fluctuations begin to bend the polymer in different directions; it is used to characterize the flexibility or rigidity of a filament (MacKintosh 1998). Increase in filament rigidity results in decrease in shear modulus of a gel because of reduced entanglement (MacKintosh 1998). The contour length, L_c , of a filament is its length at maximum extension. A filament is considered flexible when $l_p \ll L_c$, and rigid when the opposite holds ($l_p \gg L_c$); many biological filaments are in a third intermediate category: semiflexible filaments with l_p and L_c are of comparable magnitude (Storm and others 2005).

The persistence length has been obtained from many experimental techniques, including dynamic light scattering, microscopic observation of thermal fluctuations (Palmer and Boyce 2008), and transmission electron microscopy (TEM) data (Akkermans 2008, page 62). The persistence length of the β -lg fibrils, obtained by heating at pH 2, 80 °C, was approximately 1.6 μm , which was slightly lower than the 10 μm contour length of the fibrils (Sagis and others 2004).

To derive an expression for the gel modulus (Palmer and Boyce 2008), the force-extension behavior of the individual filaments is captured using an applicable constitutive relationship. That relationship is then used in conjunction with a network model to obtain the three-dimensional multiaxial stress-strain behavior of the network. In one constitutive model (Palmer and Boyce 2008), the magnitude of the network modulus is a function of the material properties: l_p , L_c , the filament density, n (filaments m^{-3}), and the initial filament end-to-end length, r_0 . In principle, r_0 and L_c are measurable from micrographs, l_p is measurable from single molecule bending or as noted above. The filament density, n (filaments m^{-3}), is defined as: $n = \rho_L / L_c$ where the polymer length density ($\mu\text{L m}^{-3}$), ρ_L , is estimated from the experimental monomer concentration, the linear polymer density ($Da \mu\text{m}^{-1}$) and the molecular mass of each monomer ($Da \text{ monomer}^{-1}$); both of the linear polymer density and the latter quantities are defined a priori (Palmer and Boyce 2008). One hopes that measurements based on quantities such as mass fraction of the fibrils and their density would provide a means of estimating fibril density in a gel.

The cellular solids and filler–matrix composite models

The cellular solids model, originally developed by Gibson and Ashby (1988), was extended to F-actin networks (Satcher and Dewey 1996). It was assumed that bending and twisting of actin filaments was the basic mode by which the actin network developed mechanical stress. The physical model used for the cytoskeletal network was a cubical frame and the network modulus was based on solid fraction (ϕ_s) and deformation of the cell edges. Further, for the limiting case of maximum cross-link density, the shear modulus of an elastic-filament network is related to the solid fraction as $G \sim \phi_s^{5/2}$ (MacKintosh 1998, Palmer and Boyce 2008).

Another model for gels, the filler–matrix composite model (Lewis and Nielsen 1970, Brownsey and others 1987, Carnali and Zhou 1996) of spherical particles embedded in a matrix, describes the modulus (rigidity) of a composite in terms of the rigidity of the matrix, the particle volume fraction, the rigidity of the filler particles, and the adhesion of the fibrils to the matrix.

For protein fibril gels, it seems that models based on their microstructure and the fibril density would be better suited, than the cellular solids and filler-matrix models, for quantitative estimation of their moduli and comparison of the values of different fibril gels.

Experimental Rheological Parameters

Dynamic rheological experiments

Dynamic rheological experiments, also called small-amplitude oscillatory tests, provide suitable means for monitoring the gelation process of many biopolymers (Rao 2007, chapter 6). In these experiments, a sinusoidal oscillating stress or strain with a frequency ω is applied to the material and the phase difference between the oscillating stress and strain and the amplitude ratio are measured. The following rheological parameters are obtained: the storage modulus G' (Pa), the loss modulus G'' (Pa), and the loss tangent, $\tan \delta = G''/G'$. For strain values within the linear range of deformation, G' and G'' are independent of strain. The loss tangent is the ratio of the energy dissipated to that stored per cycle of deformation. A complex modulus $G^*(\omega)$, as defined below, can also be measured:

$$|G^*| = \sqrt{(G')^2 + (G'')^2} \quad (1)$$

The continuous evolution of the viscoelastic properties throughout the gelation process can be followed using this method. Because the strain is kept small, modification of molecular structure caused by shear is minimized. In a gel-cure study, data on G' as a function of time are usually obtained (Kavanagh and others 2000a, Gosal and others 2004b, Rao 2007, chapter 6). However, sometimes, G^* is recorded as a function of time (Ipsen and others 2001).

In general, for a gelling system, G' (or G^*) continues to increase with time and, to a limited extent, is dependent on the measurement frequency. To handle the low pH samples at high temperatures, Gosal and others (2004b) made modifications to the CSL-100 rheometer using accessories specially designed and constructed. This included a ~ 2 mm stainless steel false bottom plate, and a stainless steel cover-slip made in two parts. They noted that together with a solvent-trap, these modifications greatly limited evaporation and damage to the genuine bottom plate, during measurements made over several hours. Additionally, they applied a thin layer of petroleum jelly over the cover-slip to further enhance its effectiveness. However, in long-duration experiments, one feature that is found in the G' vs. time (cure) data is a "dipdown" in the modulus at long times, which is probably due to the seepage of the silicone oil between the gel surface and the rheometer plates (Gosal and others 2004b).

Estimation of G'_{inf}

Limiting values of G' at long times, G'_{inf} , were determined by extrapolation from the gel-cure data using the relationship (Kavanagh and others 2000a):

$$G' \approx G'_{inf} \exp(-B/t) \quad (2)$$

where t is the time in seconds, B is an empirical parameter, and G'_{inf} is the value of G' at infinite time. Equation (2) reproduced satisfactorily both the asymptotic limit as $t \rightarrow \infty$ and the logarithmic singularity as $t \rightarrow t_c$ (Kavanagh and others 2000a). Both of these limits are required for subsequent testing of the data against physical models, such as a percolation-based kinetic gelation model. However, few workers have made such an extrapolation (Kavanagh and others 2000a, Gosal and others 2004b).

Linear extrapolation of a $\log G'$ vs. $(1/\text{time})$ plot also provides an estimate of G' at very long times, i.e., at G'_{inf} . However, it could lead to overestimation of the limiting modulus values, particularly for the data at the lowest concentrations, where the cure curves may simply level

off. For example, using the G^* vs. time data of Ipsen and others (2001) for a 10% w/v α -la gel, we obtained (not shown here) an estimated value of 2.38×10^5 Pa for G_{inf}^* using Equation (2), whereas extrapolation based on a plot of $\log G^*$ vs. (1/time) resulted in a value of 2.52×10^5 Pa.

Estimation of gelation time, t_c .

Experimental detection of the gel point is not always easy because the equilibrium shear modulus is technically zero at the gel point (Rao 2007, chapter 6). However, the small-amplitude oscillatory shear technique can be used to identify the gel point from the measured dynamic moduli during the gelation process. Different methods have been used to determine the gelation time.

Often, the gel point is taken to be the time at which G' and G'' cross each other at a given frequency; this technique is known as the classical technique. Thus, Ipsen and others (2001) determined the gelation time, t_c , to be the time at which the phase angle, δ , dropped below 45° .

However, often, either the rheometer signal is weak when gelation begins or the G' , G'' cross-over does not exist. In such instances, other criteria are used to determine the gel point. Kavanagh and others (2000a) estimated the gelation time, t_c , as the time at which G' was greater than a threshold value, approximately 5–10 Pa. They suggested that, because $\log G'$ increases very rapidly in this critical region, using alternative criteria makes little or no difference to the concentration dependence of t_c . Gosal and others (2004b) determined t_c as the time at which there was a logarithmic discontinuity in the gel-cure curve.

Gosal and others (2004c) found that the gelation times estimated using the G' , G'' cross-over method were slightly higher than those estimated using the method based on the discontinuity in the gel-cure curve. However, the slopes of the \log (gelation time) vs. concentration plots were nearly the same.

Estimation of critical concentration, c_0 .

The critical concentration for gelation has also been estimated using different techniques. From a consideration of the critical behavior near the gel point, a semi-empirical model that related gelation time and critical concentration was developed:

$$t_c^{-1} \propto \left\{ \left(\frac{c}{c_0} \right)^n - 1 \right\}^p \quad (3)$$

In Equation (3), n and p are arbitrary parameters. The critical concentration can also be estimated when the cascade model is applicable (Kavanagh and others 2000a).

Variation in G_{inf}' of β -lg fibril gels due to source variation and sample preparation

The rheological properties of β -lg fibril gels are quite sensitive to pH, ionic strength, and thermal history. Slight variations in buffer composition can therefore impact on the reproducibility of rheological measurements. For example, Gosal and others (2004b) fitted G_{inf}' vs. concentration data for a β -lg fibril gel with a power law model, and obtained exponents of 5.1 and 5.7 in two separate experiments using Sigma β -lg with the same lot number. Sagis and others (2002) reported more dramatic variation between four experiments

with β -lg from a given lot. The exponent for fitting the G' vs. concentration data varied between 3.0 and 11.5, whereas c_0 fell into two groups at approximately 2% and 9%. The authors suggested that pH and ionic strength differences could have affected the length, flexibility, and spatial organization of aggregates. This suggestion is borne out by the transmission electron micrographs of Kavanagh and others (2000b).

Soto and others (1995) found considerable variation in the secondary structure and the amyloidogenic potential of amyloid-beta peptides sourced from different companies, and also between batches and within batches from a given company. Similar differences may apply to commercial β -lg products.

The salt content in different lots of commercial β -lg can vary by 20%, and this markedly affects the gelation time at pH 7 (Kavanagh and others 2000a). Some laboratories routinely dialyze β -lg solutions against buffer to remove excess salt before preparing fibrils (Aymard and others 1999, Sagis and others 2002, Akkermans and others 2006). Dialysis makes t_c slightly more sensitive to protein concentration, but creates little difference in the final modulus (Kavanagh and others 2000a). In the case of 12% β -lg at pH 3, dialysis increased t_c from about 500 s to about 1000 s (Kavanagh and others 2000a), which suggests that impurities and/or salts removed by dialysis affect the kinetics of fibril nucleation in a significant and commercially relevant way (Nilsson 2004).

Effect of pH and concentration on the modulus of β -lg fibril gels

As shown in Figure 1, the pH of a β -lg fibril solution has a marked effect on the values of G'_{inf} (Kavanagh and others 2000a). The highest values of G'_{inf} were found at pH 3, whereas the lowest values were found at pH 7; the values of G'_{inf} at pH 2 and pH 2.5 were about the same magnitude as those at pH 3. Recently, Akkermans and others (2006) observed that pulsed and continuous shear treatments resulted in enhancement in the growth of fibrils. We also note that the highly acidic conditions at pH 2–3 would require suitable acid-resistant production equipment.

The very marked influence of the concentration of β -lg on G'_{inf} at each pH is also seen in Figure 1. The values of G'_{inf} increased at a greater rate at the lower β -lg concentrations, whereas they leveled off somewhat at higher concentrations. The cascade model (Gordon and Ross-Murphy 1975) was used to describe the modulus vs. concentration data at each pH value. The values of the critical concentration, c_0 (% w/w), for β -lg fibril gels at pH values of 2.0, 2.5, 3.0, and 7.0, determined using Equation (3), were 8.0, 5.10, 7.45, and 11.35, respectively (Kavanagh and others 2000a). Fibrils would be more highly charged at low pH, so electrostatic repulsion would strengthen the gel. Later, Gosal and others (2004a) reported that solutions of β -lg with concentrations of < 6% w/w and heated at pH 2 formed very weak gels; i.e., they did not percolate throughout the sample.

Results from Percolation Theory

Percolation describes the geometrical transition between disconnected and connected phases as the concentration of bonds in a lattice increases (Rao 2007, chapter 6). It is the foundation for the physical properties of many disordered systems and has been applied to gelation phenomena (de Gennes 1979, Stauffer et al. 1982). At just above gelation threshold, denoting the fraction of reacted bonds as p and $p = p_c + \Delta p$, p_c the critical concentration (infinite cluster), the scaling law (critical exponents) for the modulus (E) are:

$$E \cong (p - p_c)^t \quad (4)$$

A number of authors have used concentration of the polymer, c , in place of p . Sagis and others (2004) fitted G' data on β -lg fibrils heated at 80 °C for 10 hours using the scaling relation $G' \propto (c - c_c)^t$, where c is the protein concentration and t is an universal exponent. Using a regression procedure, the parameters c_c and t were determined at two values of ionic strength: 70 mM, $c_c = 1.2 \pm 0.1$, $t = 1.9 \pm 0.3$, and 10 mM, $c_c = 2.3 \pm 0.1$, $t = 2.1 \pm 0.2$. An adjusted random contact model for charged semiflexible fibrils was used to explain the decreasing percolation concentration with increasing ionic strength.

For amyloid fibrillar β -lg gels, prepared at pH 2, plotting values of G' extrapolated to infinite time, G'_{inf} , versus $(c/c_o) - 1$, Gosal and others (2004b) obtained values of the exponent, t , in the range 2.2-2.8, and a value of 3.1 for the data of Pouzot and others (2004). They suggested that for heat-set β -lactoglobulin gels, prepared at pH 2, the exponent appears to be between 2 and 3. The higher values could be consistent with the Bethe lattice value of 3 and the values smaller than 3 could also be explained by the classical approach if network wastage is taken into account.

Results from Fractal Analysis

A scaling relationship for the elastic properties of colloidal gels was developed by Shih and others (1990) considering the structure of the gel network to be a collection of close packed fractal flocs of colloidal particles. Two separate rheological regimes were defined depending on the strength of the interfloc links relative to that of the flocs themselves: (1) the strong-link regime is observed at low particle concentrations, allowing the flocs to grow to be very large, so that they can be considered weak springs. Thus, the links between flocs have a higher elastic constant than the flocs themselves, and (2) the weak-link regime is observed at high particle concentrations, where the small flocs are stronger springs, and the links between flocs have a lower elastic constant than the flocs themselves. The weak-link regime should be applicable to gels that are well above the gelation threshold (Shih and others 1990) where the elastic modulus, G' , is related to the particle (solids) volume fraction (ϕ_s) by the following relationship:

$$G' \sim \phi_s^{(d-2)/(d-D_f)} \quad (5)$$

where, D_f is the fractal dimension of the colloidal floc and d is the Euclidean dimension of the network –usually three.

The power relationship between the modulus and the volume fraction of solids implied in Equation 5 has been utilized to determine the fractal dimension of several food gels, including β -lg gels, and values of D_f between about 1.9 and 2.9 have been reported (Rao 2007, chapter 2). We note that Wu and Morbidelli (2001) extended the above model to include gels that are intermediate between the strong-link and the weak-link regimes.

Pouzot and others (2004) reported applicability of the fractal model to β -lactoglobulin gels prepared by heating at 80 °C and pH 7 and 0.1M Na Cl. They suggested that the gels may be considered as collections of randomly close packed “blobs” with a self-similar structure characterized by a fractal dimension $D_f = 2.0 \pm 0.1$.

One concern with applying the fractal model to gels is that the critical concentration, c_o , is assumed to be zero. In addition, for the amyloid fibrillar networks derived from β -lg, Gosal and others (2004c) suggested that the assumption of self-similar structures would not be

valid. They also pointed out that for conclusive comparison of the cascade and the fractal models, the measurements of G'_{inf} would have to be extended to cover a wider concentration range, i.e., to include measurements at both higher and lower concentrations. For globular protein systems, the limiting slopes of modulus-concentration plots are not reached until the concentration exceeds $10x_{c_0}$. For systems with c_0 about 5-7% w/w, such a region is almost impossible to access (Gosal and others 2004c).

Comparison of Heated vs. Solvent-Incubated Fibril Gels

Minimum β -lg concentration in solvent-incubated gels

Even though fibrils formed by incubating β -lg in water-alcohol mixtures (e.g., water-trifluoroethanol (TFE) and water-ethanol) may not be of major interest for creating new food products, they are of interest for studying the role of structure in the rheological properties of gels. Both the gelation time, t_c , and the critical concentration, c_0 , for gelation were influenced by different alcohols. For example, gels formed at concentrations of approximately 4% w/w in 50% v/v water-methanol, whereas the critical concentration for gelation was approximately 10% w/w for water-TFE.

We note here that solvent incubation times of about 16 h were used in TFE-induced pH 7 and ethanol-induced pH 2 gel-cure experiments; the alcohol content in each case was fixed at 50% v/v. However, incubation times as long as 40-90 days were used for obtaining transmission electron micrographs of the fibrils.

G'_{inf} of heated and solvent-incubated β -lg fibril gels

Figure 2 contains values of G'_{inf} for gels from fibrils obtained by heating and solvent incubating β -lg at various concentrations. It can be seen that the values of G'_{inf} for the gels from fibrils obtained by heating β -lg at pH 2 at either 75 °C or 80 °C were higher than those of the solvent-incubated β -lg fibril gels. Atomic force microscope images of fibrils obtained by heating and incubating in water-TFE are shown in Figures 3 and 4, respectively.

The differences in the values of G'_{inf} may be due to differences in the characteristics of the fibrils: l_p , L_c , the filament density, n (filaments m^{-3}), and the initial filament length, r_0 . It would be reasonable to assume that the fibrils obtained by either heating or solvent incubating β -lg would exhibit similar force-deformation behavior and the gels can be described by similar network models. Thus, the lower values of the modulus of the solvent-incubated, especially the ethanol-incubated, gels are probably due to the lower fibril densities in these gels. However, no data on either the fibril density or the mass fraction of the fibrils seem to exist.

In addition to the fibril density and modulus of the fibrils, the modulus of the gel matrix may also play an important role (Lewis and Nielsen 1970; Brownsey and others 1987). In this connection, we note that, for heat-set β -lg at pH 2, the starting solutions showed solid-like character ($G' > G''$) that was ascribed to "structuring" through protein repulsion (Gosal and others 2004c). In contrast to the heat-set systems, for the solvent-induced systems, the values of G'' were initially greater than the values of G' and the order was reversed at or just after the gel point; subsequently, G' became significantly greater than G'' . These observations suggest better structuring of the heat-set systems and, consequently, higher rigidity of the matrix. Nevertheless, the influence of fibril density would be significant.

Gelation time of β -lg fibril gels.

The gelation time provides practical information for the production of β -lg fibril gels. Such data reported by Gosal and others (2004b, 2004c) for both heat-induced and solvent-induced β -lg fibril gels are plotted in Figure 5. As expected, the gelation time decreased with an increase in the β -lg concentration. A power law may be used to describe the relationship between gelation time and β -lg concentration for both heat- and solvent-induced gels. The gelation times for ethanol-induced fibril gels were lower than those for heat-induced fibril gels and TFE-induced fibril gels. Interestingly, the slopes for the ethanol-induced fibril gels and the heat-induced fibril gels were nearly the same.

Mechanism of Gelation of β -lg Fibrils

Kavanagh and others (2000a) pointed out that it is difficult to develop realistic models to describe gelation mechanisms and the features of the gel-cure curves of β -lg fibril gels because the reactivity of polymerization sites is rarely uniform for biopolymers. Also, the sites themselves are difficult to define (physical cross-links), and the aggregation processes are often nonrandom and complex. Further, to include all these effects in a theoretical description would result in complex models; therefore, to simplify, several assumptions have to be made. In the final analysis, justification of these assumptions rests on their success in producing a satisfactory fit to the data. It was speculated that the marked concentration dependence of the gelation time suggests: (1) that models need to be modified to include nucleation and growth events; (2) that the results are not entirely independent of the finer details of molecular self-assembly (Gosal and others 2004b).

Bolder and others (2007b) proposed a model for fibril formation in whey protein isolate solutions, that included: activation, nucleation, growth, and termination steps. Akkermans (2008) found that, after heating 20 h at 85 °C at pH 2, β -Lactoglobulin was hydrolyzed into peptides with molecular masses between 2,000 and 8,000 Da, and that the fibrils were composed of specific peptides and not intact β -lactoglobulin. It was concluded that specific peptides were present in the fibrils because of the low charge, charge distribution along the sequence, hydrophobicity, and capacity to form β -sheets. Further, the part of the sequence that was completely absent in the peptides of the fibrils had a high charge and low capacity to form β -sheets.

Creation of α -la Fibril Gels

Minimum α -la concentration

Partial hydrolysis of α -la with a serine proteinase from *Bacillus licheniformis* was shown to form fibrils that were tubes about 20 nm in diameter and reaching several microns in length (Ipsen and Otte 2007). Ipsen and Otte (2007) reported that the minimum concentration of α -la necessary to form nanometer-diameter tubes (nanotubes) was 3.0% w/v (at 50 °C, 75 mM Tris buffer, pH 7.5, 2 mol Ca^{2+} /mol α -la); linear fibrils about 5 nm in diameter and/or random aggregates were obtained at α -la concentrations below 3% w/w. Earlier, Ipsen and Otte (2003) pointed out that gelation was not possible below pH 6.5 and that the gelation time decreased from 86 min at pH 6.5 to 58 min at pH 7.5, whereas Otte and others (2005) suggested that the minimum concentration of α -la was 2.0% w/v. The detailed structure of the nanotubes, presented in Graveland-Bikker (2005), suggests that they are built as a ten-start right-handed helix with an outer diameter of about 20 nm, an inner diameter of about 8 nm, and a pitch of about 110 nm.

Modulus and Gelation time of α -la Fibril Gels

Few published data were found on the rheological properties of α -la fibril gels at different concentrations. In Figure 6, the G^* vs. time (gel-cure) data of Ipsen and others (2001) for 10.0% w/v α -la fibrils heated for 4 h at 50 °C. We note that authors applied a thin layer of silicone oil to minimize evaporation at 50 °C. As stated earlier, using those data, we obtained (not shown here) an estimated value of 2.38×10^5 Pa for G_{inf}^* using Equation (2), whereas extrapolation based on a plot of $\log G^*$ vs. (1/time) resulted in a value of 2.52×10^5 Pa.

It is recognized that, for a given gel, G^* will be higher than G' because, as shown in Equation (1), the former includes the contribution of G'' . Also, for most gels, the values of G'' are less than those of G' . Assuming that the values of G' and G'' are equal, the values of G' for α -la fibril gels were estimated and are also shown in Figure 6.

The complex modulus and the gelation time of fibril gels from 10.0% w/v α -la with added Ca^{2+} , hydrolyzed with a serine proteinase from *B. licheniformis*, heated for 4 h at 50 °C, and after cooling to 25 °C for 30 min are shown in Figure 7. Interestingly, the values of the modulus after cooling to 25 °C were lower than those at 50 °C. This phenomenon was attributed to the decrease in the stability of hydrophobic bonds and salt-bridges in proteins with decreasing temperature. The gels, made from α -la, were almost transparent and somewhat whitish. Further, they were more than 20 times stiffer (measured as G^*) than equivalent gels, treated with *B. licheniformis*, made from β -lg of the same concentration (Ipsen and others 2001).

Even though α -la fibril gels were created by enzymatic hydrolysis while β -lg fibril gels were created by heat-induced denaturation at low pH, their gelation times are useful in practical applications. At the same concentration of 10%, from the data for β -lg fibril gels in Figure 5 and for α -la fibril gels in Figure 7, we note that the gelation time for α -la fibril gels is approximately 4680 s, whereas t_c for β -lg, pH 2.5 fibril gels is approximately 600 s.

Similar observations on the values of the gelation times and the modulus for pure (nonfibril) 15% β -lg and α -la solutions heated at 80 °C, pH 7 were reported (Kavanagh and others 2000c). The 15% β -lg solution gelled within 4 min, whereas the 15% α -la solution required 50 min. Further, the long-time value of G' , G_{∞}' , for the α -la gel was approximately a decade higher than that for the corresponding pure β -lg gel.

Possible reasons for the difference in the modulus values of the α -la gels and the β -lg gels are their different fibril densities and the fibril microstructures, such as the values of: l_p and L_c . The modulus of fibrils may play an important role through its influence on l_p because the latter characterizes the rigidity of a filament (MacKintosh 1998). In this connection, Graveland-Bikker and de Kruif (2006) reported that the Young's modulus of α -la fibrils, determined using an atomic force microscope, was of the order of 0.1 GPa. They also noted that the α -la nanotubes were stiffer than myofibrils and casein micelles, which have typical moduli of 10^{-1} MPa; they attributed the higher stiffness to the higher content of protein material in the α -la fibrils. However, values of the modulus of β -lg fibrils do not seem to have been published.

Mechanism of α -la Fibril Formation

Ipsen and Otte (2007) suggested a mechanism of self-assembly of α -la after partial hydrolysis using *B. licheniformis*. They pointed out that the outcome of the process depends on the initial concentration of α -la and the presence of calcium. Nanotubes are formed at α -la concentrations ≥ 30 g/L and a calcium/ α -la ratio > 1.5 . However, linear fibrils are formed at α -la concentrations < 30 g/L.

Conclusions

Food gels with different rheological properties can be created by using fibrils from β -lg obtained using either solvent-incubation or heating. The higher values of G'_{inf} for gels from fibrils obtained by heating β -lg at pH 2 at 75 °C and 80 °C compared with those of solvent-incubated β -lg fibril gels may be due to higher values of the density of the fibrils and different material properties: l_p and L_c . In this respect, the persistence and contour lengths of the β -lg fibrils, obtained by heating at pH 2, 80 °C, have been reported (Sagis and others 2004); the modulus of α -la fibrils that affects their persistence length was measured by Graveland-Bikker and de Kruif (2006). Partial hydrolysis of α -la with a serine proteinase from *B. licheniformis* results in fibrils that are hollow tubes about 20 nm in diameter. The gels created from the α -la nanotubes seem to be stronger than the gels from β -lg fibrils; again, the higher modulus is probably due to higher values of the density of the fibrils and different fibril properties: l_p and L_c .

More data on the rheological properties of the fibril gels created using a wide range of protein concentrations are needed, including gel-cure data similar to those summarized here. In addition, systematic studies should include: strain sweep data to determine whether strain-stiffening or strain-softening behavior is exhibited, and the persistence and contour lengths of fibrils, as well as fibril density (or fibril content) data. In addition, studies on the stress-strain behavior of the fibrils and fibril-network models to predict modulus-strain relationships would be desirable.

Notation

c_c the critical percolation concentration, % w/w

c_0 critical gelation concentration, % w/w

d Euclidean dimension of a network

D_f fractal dimension of a colloidal floc

E compression modulus, Pa

G shear modulus, Pa

G' storage modulus, Pa

G'' the loss modulus, Pa

G^* complex modulus, Pa

G'_{inf} storage modulus at long gelation time, Pa

κ bending stiffness of a filament or fibril, Nm

k_B Boltzmann's constant 1.38×10^{-23} , N m K⁻¹

L_c contour length, m

l_p persistence length, defined as $l_p = \kappa / (k_B T)$, m

n filament density, filaments m⁻³

r_0 end-to-end length, m

t universal percolation exponent

t_c gelation time, s

T absolute temperature, K

$\tan \delta = G'' / G'$ loss tangent, -

ϕ_s solids volume fraction, -

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Acknowledgments

We thank Fonterra Cooperative Ltd and the NZ Foundation for Research, Science and Technology for funding this work.

Table 1. Creation of fibrils from β -lactoglobulin and α -lactalbumin

<i>Protein</i>	<i>Process</i>	<i>General description of structures</i>	<i>Reference</i>
β -lactoglobulin	heating aqueous solutions, pH 2 and low ionic strength ~24 h; maximum between 10 and 34 h.	~5-7 nm in width, and >1 μ m in length	Gosal and others 2004a, Bolder and others 2007a.
β -lactoglobulin	incubation in water-ethanol at pH2 or water-trifluoroethanol at pH 7 for 10 h.	wormlike, ~7 nm width and <500 nm length	Gosal and others 2004a
α -lactalbumin	limited proteolysis by a protease from <i>Bacillus licheniformis</i> (BLP) 4 h at 50 C, pH=7.5; 3 ml a-LA + 33 μ l of enzyme solution: 100 mg BLP in 5 ml of water. Enzyme to substrate ratio of 0.02.	fibrillar and tubular, globular subunits ~20 nm in dia, fibril length >1 μ m in length	Ipsen and others 2001, Otte and others 2005

Figure 1 – Effect of β -lactoglobulin (β -lg) concentration and pH on values of G'_{inf} for heat-set fibril gels (from Kavanagh and others 2000a)

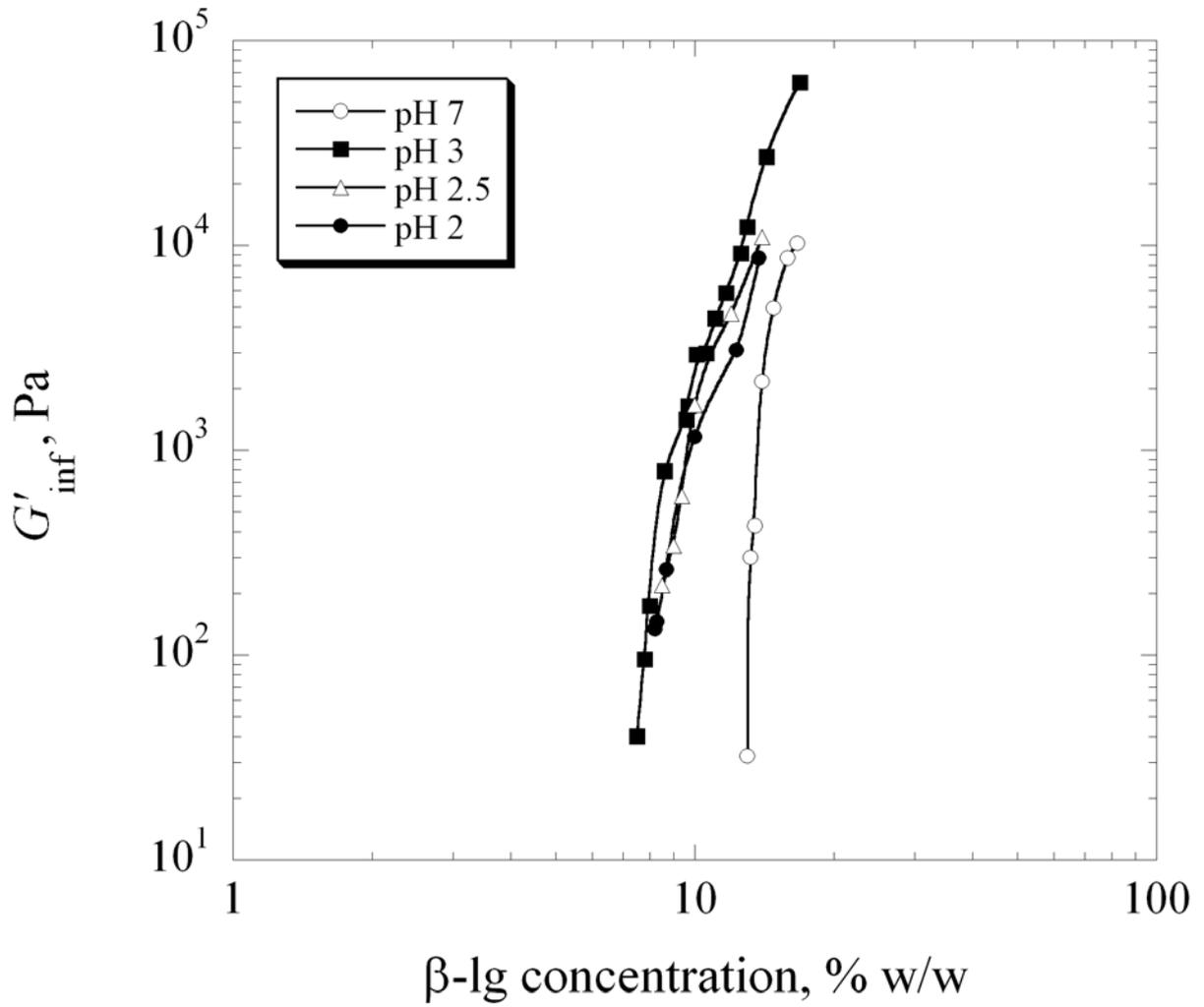


Figure 2 – Values of G'_{inf} for β -lactoglobulin (β -lg) fibril gels produced by incubating in water–ethanol (EtOH) and water–trifluoroethanol (TFE), and by heating at pH 2 at 75 °C and 80 °C (from Gosal and others 20004b, 2004c)

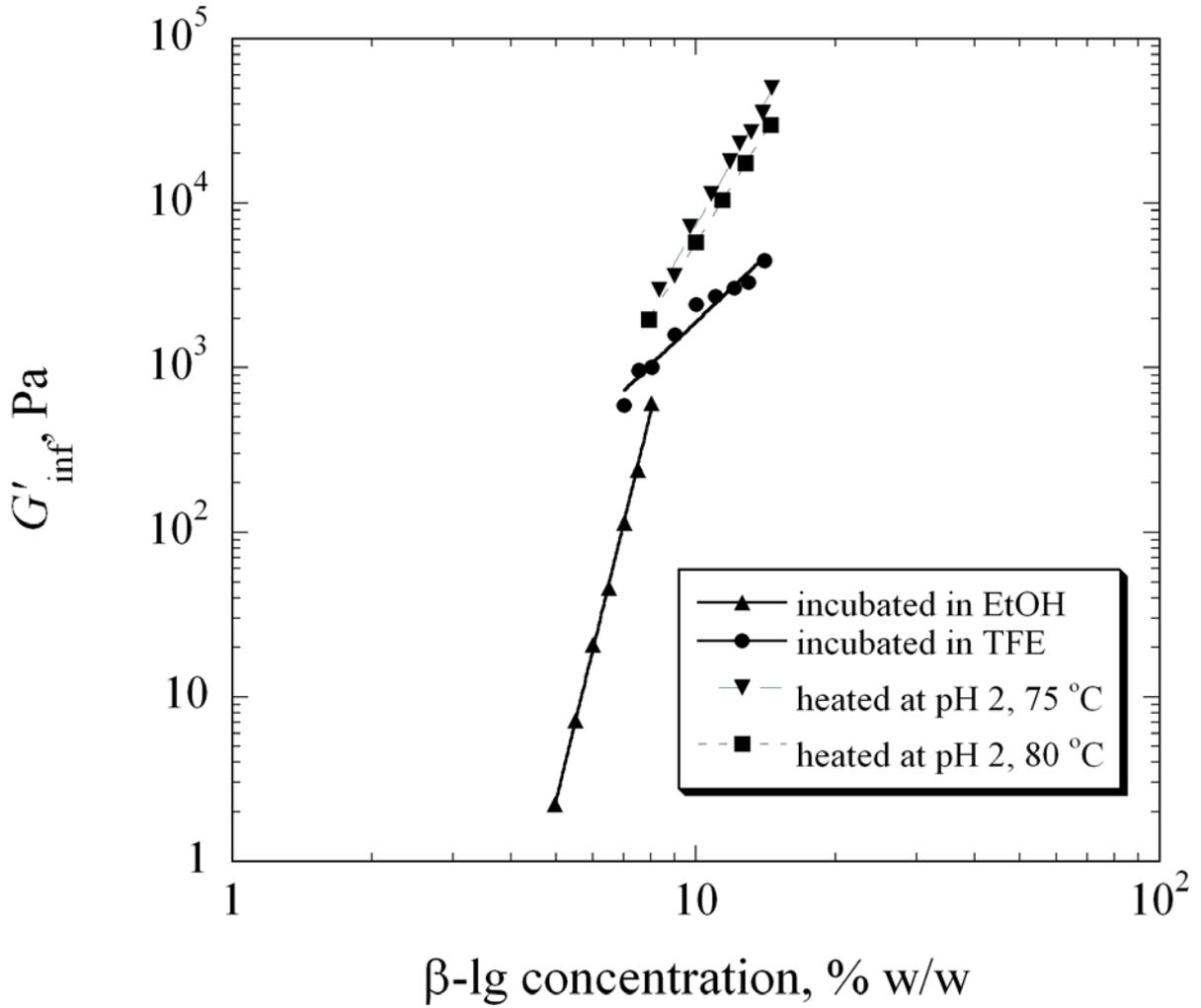


Figure 3 – Time-lapse atomic force microscope images of fibrils in 4% β -lactoglobulin (β -lg), pH 2, 80 °C (from Gosal and others 2004a)

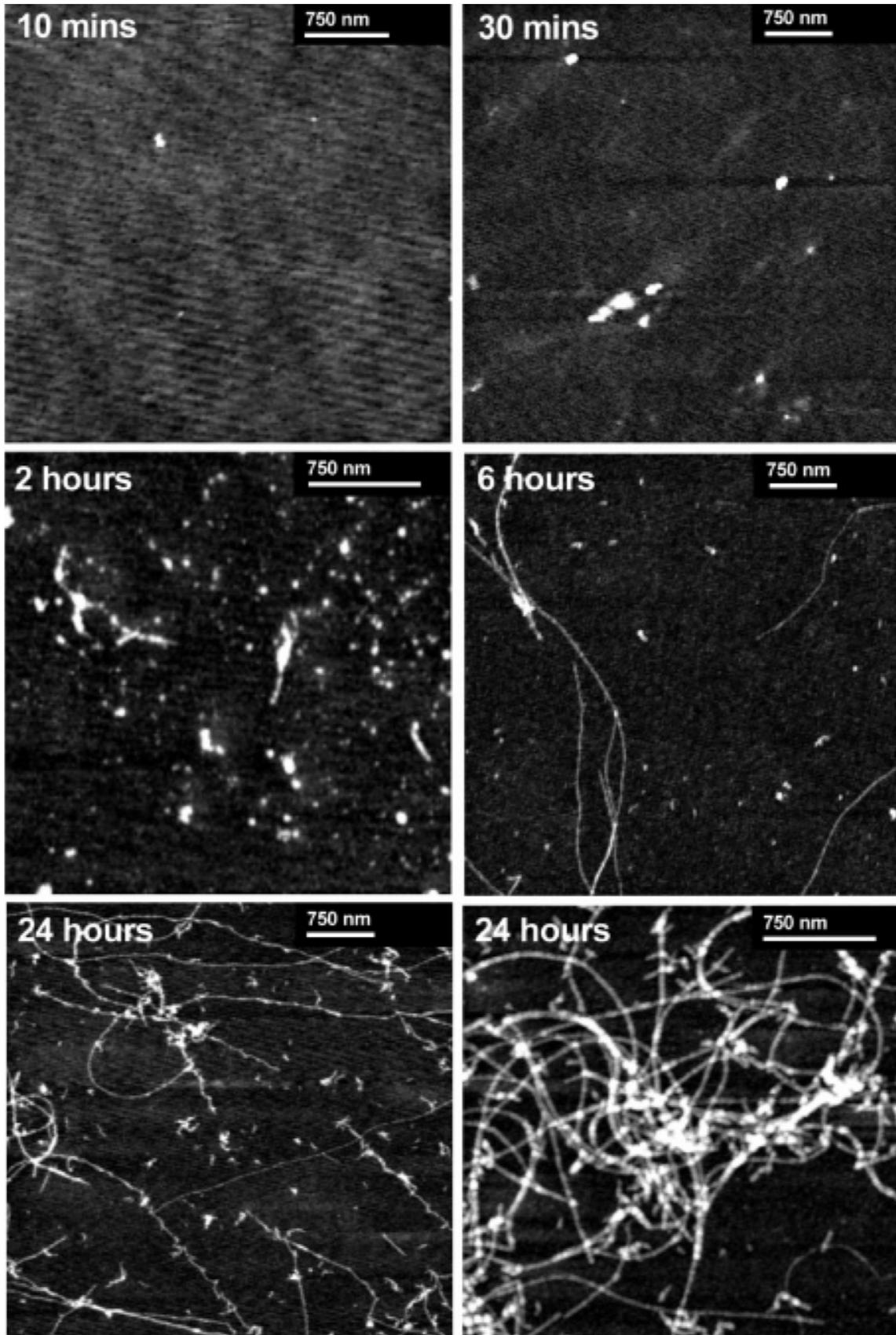


Figure 4 – Atomic force microscope images of fibrils obtained by incubating β -lactoglobulin (β -lg) in water–trifluoroethanol (TFE), pH 2, for 40 days (from Gosal and others 2004a)

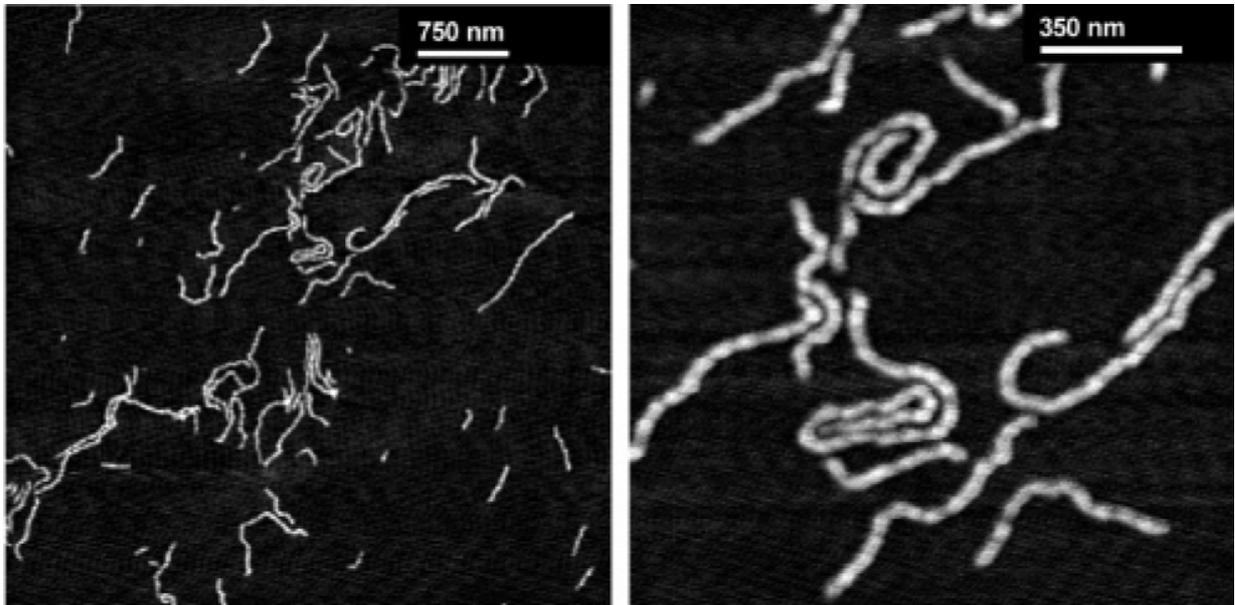


Figure 5 – Gelation time for heat- and solvent-induced β -lactoglobulin (β -lg) fibril gels (from Gosal and others 20004b, 2004c)

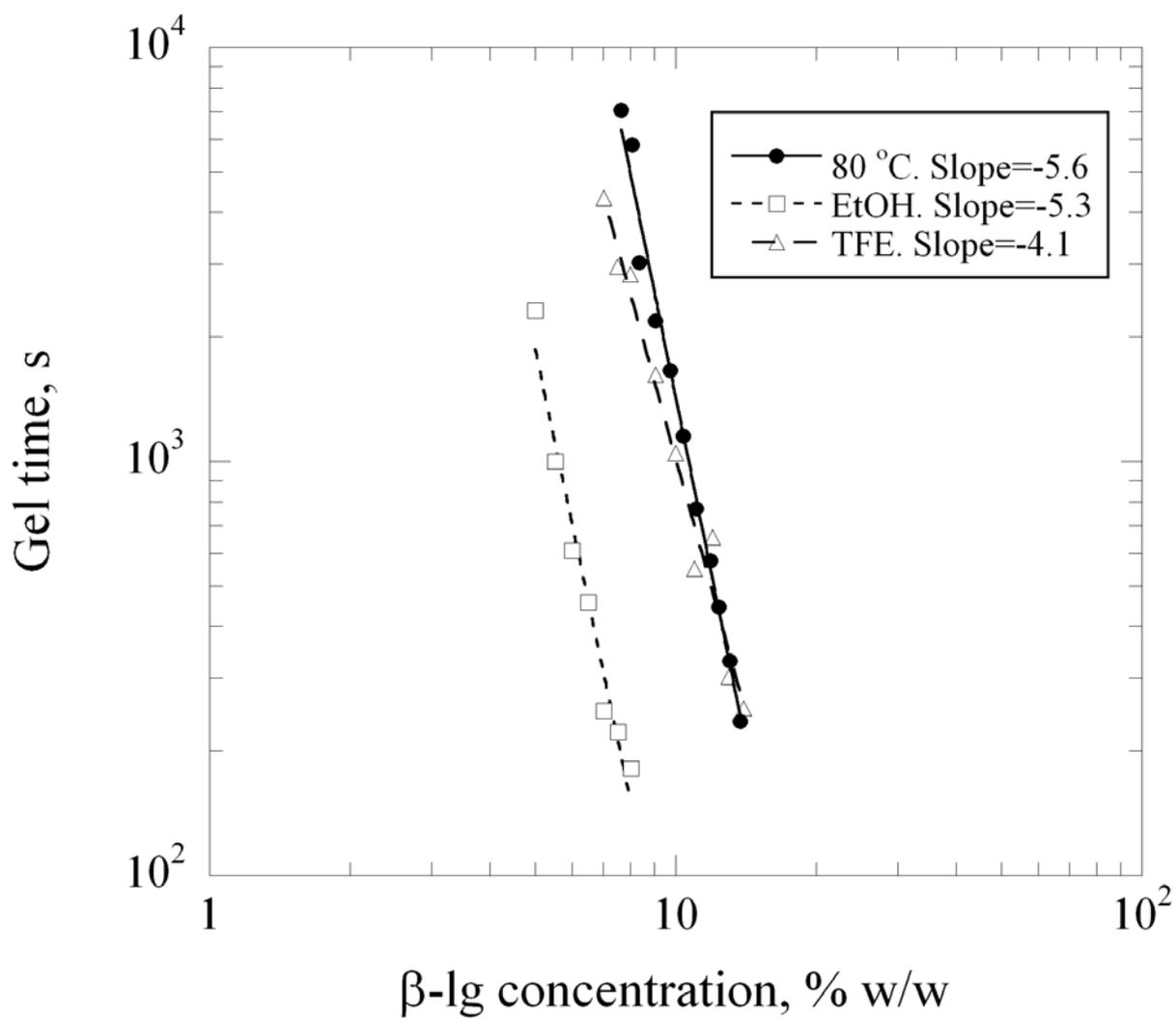


Figure 6 – The values of G^* vs. time data of Ipsen and others (2001) for 10.0% w/v α -lactalbumin (α -la), 10 mM Ca^{2+} , fibril gel heated for 4 h at 50 °C. Also shown are estimated values of G' for 10% α -la fibril gels.

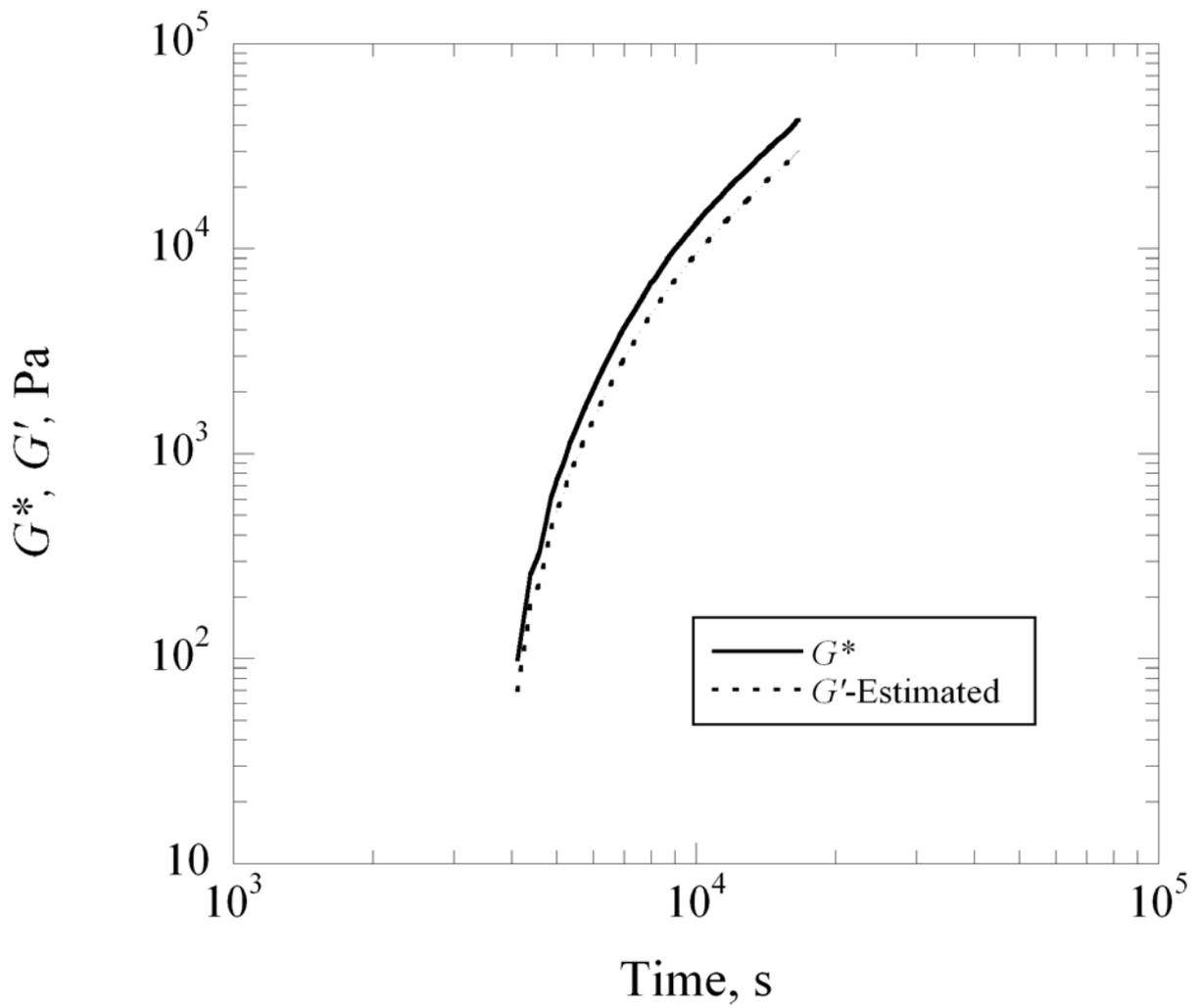


Figure 7 – Complex modulus and gelation time for fibril gels from 10.0% w/v α -lactalbumin (α -la), pH 7.5, with added Ca^{2+} , hydrolyzed with a serine proteinase from *Bacillus licheniformis*, heated for 4 h at 50 °C, and after cooling at 25 °C for 30 min (from Ipsen and others 2001).

