Heat-Induced Gelation of β-Lactoglobulin/α-Lactalbumin Blends at pH 3 and pH 7

Gaynor M. Kavanagh,[†,§] Allan H. Clark,[‡] Walraj S. Gosal,[†] and Simon B. Ross-Murphy*[,†]

Biopolymers Group, Division of Life Sciences, King's College London, Franklin-Wilkins Building, 150 Stamford Street, London SE1 8WA, U.K., and Unilever Research, Colworth House, Sharnbrook, Bedford MK44 1lQ, UK

Received March 28, 2000; Revised Manuscript Received July 14, 2000

ABSTRACT: Mixed globular protein gels, or gel composites, based on heating solutions of proteins from milk have been investigated for many years, although the great majority of these studies have used rather crude whey isolates containing a fixed ratio of the principal components β-lactoglobulin and α-lactalbumin and some denatured material. Moreover, most of the previous work has concentrated on examining structural and rheological properties of fully cured gels. In the present paper, attention is focused on well-defined mixtures composed of much purer samples of the principal components, these being present in systematically varying proportions. Protein−protein co-gels are formed at pH's 3 and 7, by heating the mixtures at 80 °C. Gelation is monitored by cure curve measurement and both gel times and long-time limiting moduli established. A considerable difference in behavior is observed at the two pH's and modeling based on segregated phase-separated structures gives only a partial explanation of the results. It seems probable that coupled networks of some kind form in both cases. The pH 3 α-lactalbumin systems show a transition from reversible gelation at low temperatures to irreversible network formation above the first gel melting temperature. Conclusions from this work have generic implications for other mixed gel composite systems.

1. Introduction

Heat-induced aggregation of globular proteins and the formation of single-component globular protein gels are the subject of several earlier papers from our group[1−5] and others.[6−16] When heated to temperatures above the protein denaturation temperature, typically ∼60 °C, and at concentrations ≳10% w/w protein solutions form either particulate or fibrillar irreversible networks. In the biomedical area there is currently considerable interest in this transition to fibrillar protein gels, in the present case induced by low pHs, because of the structurally similar β-amyloid material formed in certain diseases such as Alzheimer's.[17]

Much of the structural and rheological work on mixed biopolymer gels follows a paper of two of the present authors on a phase-separated protein−polysaccharide system,[18] which introduced what is now, sometimes, called the (effective concentration) modified Takayanagi (MT) model. There is also a considerable body of work on polysaccharide−polysaccharide gels, although most publications have been more concerned with defining and understanding the structure in terms of molecular features,[19−22] but rheological studies using the MT approach have also appeared.[23,24] Nevertheless there is an, albeit smaller, body of work on mixed protein−protein gels. These have attracted both academic and industrial interest, for example in the dairying sector. Consequently, quite a few of these papers address the heat-set gelation of mixtures (blends) of whey proteins. The great majority of these have used commercial whey concentrate samples, while relatively few workers have performed more fundamental studies in which purified samples of the original component proteins are mixed and the gelation properties of the resultant system investigated. Recent studies have, however, included work by Hines and Foegeding,[25] who studied mixed gel systems of α-lactalbumin (α-La), bovine serum albumin (BSA), and β-lactoglobulin (β-Lg) using dynamic shear oscillatory rheometry and size exclusion chromatography. Here, the amount of monomer consumed by the reaction was examined to establish the level of interaction among these proteins, and (apparent) reaction rates were calculated. BSA had the fastest reaction rate, followed by β-Lg and α-La. β-Lg and BSA were found to contribute significantly to the G' of the mixed gels, while α-La contributed less.

More recently, Tobitani and Ross-Murphy[4,5] have used rheological methods to investigate the incipient gelling behavior of single-component and mixed binary systems of BSA, α-La, and β-Lg at pH 6.6. Reproducible results were only obtained for BSA/β-Lg mixtures, the results obtained for mixtures of β-Lg and α-La being somewhat unreliable. Large deviations were observed when the amount of α-La exceeded that of the other protein present because of the high temperatures required for formation of α-La gels. Changes in gel times and temperatures in relation to concentration and composition were described using new models.

Since problems were encountered in this previous work when the α-La content was higher than that of the other protein constituent, some further investigation has been performed here in an attempt to clarify the matter. The objective of the present work was mainly to determine the gel modulus at long time for mixed systems of β-Lg and α-La induced to gel at 80 °C. Additional kinetic information (such as the gel time) was a bonus, however, as it provided further evidence about

† King's College London.
‡ Unilever Research.
§ Present address: DERA, Fort Halstead Laboratory, Sevenoaks, Kent, UK.
* Corresponding author: Telephone +44 (0) 20 78484081; Fax +44(0) 20 78484500; e-mail simon.ross-murphy@kcl.ac.uk.

10.1021/ma0005522 CCC: $19.00
Published on Web 09/01/2000

the formation of these composite gels. Measurements were carried out both at pH 7, where β-Lg is known to form highly branched and rather dense aggregate structures, and at pH 3, where more extended linear fibrillar structures are seen.

The structural differences between β-lactoglobulin networks formed under different conditions are now quite well characterized. Langton and Hermansson,[6] for example, have reported some detailed results using electron and optical microscopy. They have demonstrated the formation of particulate networks (based on concentrated gelled protein droplets) at pHs in a range surrounding the isoelectric point, i.e., 4−6, and more fibrillar ("fine-stranded") networks outside this range.[7,8] Under near isoelectric conditions the aggregates are almost spherical, and their size depends on the exact pH, while fine-stranded networks below pH 4 show stiff, short strands, which increase in width and length as the pH falls. Above pH 6 the chains are longer and more flexible, and it is suggested that increasing the pH increases the chain diameter.[7] Light and neutron scattering studies by Aymard and co-workers[12,13,26] of β-Lg aggregates formed at pHs 6 and 3 tend to confirm the microscope findings, fractal dimension arguments being used to identify the presence of extended rodlike structures at low pH, and to indicate more compact structures at pH 6.

Consequently, while the gelation of β-Lg has been studied over the past few years by both structural and mechanical approaches, relatively few similar measurements have been performed for α-La or its mixtures with β-Lg. We believe none of these contrast the behavior of such mixtures at pH 3 and 6, as in the present paper. One reason might be the relatively high cost of purified α-lactalbumin, but another must surely be the commercial importance of the whey mixture. This has attracted most attention, despite the fact that it contains lactalbumin and lactoglobulin in fixed proportions, as well as other impurities including partially denatured protein material. In the present work, better characterized mixtures of the two main whey protein constituents are examined, these being studied systematically over a wide compositional range.

In the present work small strain oscillatory shear measurements were employed to examine gelation times and long-time moduli (obtained by extrapolation) for the mixtures, some theoretical models being invoked to interpret findings. These models have been developed in an attempt to examine the generic dependence of properties of mixed gels on their composition.[4,5,18,27] In subsequent sections the theoretical bases of these approaches are examined only briefly, since they have been discussed in much more detail elsewhere.[28−30]

2. Experiments

In the work described here, β-Lg was initially chosen as the predominant protein, and α-La was the minor component, as it is in nature.[31] However, work was also carried out where α-La was the major species in the protein mixture. In most cases the total protein concentration was 15%, so the major component (85%) on a w/w basis was still the supporting aqueous electrolyte at pH's 3 and 7. In our earlier work[5] the total protein concentration was kept at 10% w/w, and the ratio of one protein to the other was varied. Difficulties encountered then in reproducing results were thought to be due to the long periods of time taken to form gels. It was felt that these difficulties could be reduced by increasing the cure temperature (from 80 °C) to shorten gel times. Accordingly, in the present work, preliminary experiments were performed for α-La using an RFSII rheometer to investigate such increases, but from the findings obtained it was clear that increasing the temperature to 85 °C, for example, had an insufficient effect on the gelation parameters. Moreover, significant additional problems were experienced because of sample drying in the parallel plate geometry. It was concluded that if reliable measurements were to be achieved using this experimental configuration, 80 °C was the highest useful temperature.

Table 1. Concentrations (% w/w) of β-Lg and α-La for Mixed Systems

β-Lg	15.0	14.0	13.0	12.0	11.0	10.0	6.0	4.0	2.0	0.0
α-La	0.0	1.0	2.0	3.0	4.0	5.0	9.0	11.0	13.0	15.0

In the present work the binary protein mixtures were induced to gel at 80 °C and measured using established rheological techniques. A total protein concentration of 15% w/w was maintained, as this decreased the gel times relative to those of the earlier work and enabled better estimates of the final gel modulus to be obtained.

2.1. Materials. The β-Lg (L-0130, lot no. 91H7005, Sigma Chemicals Co.) used here had the same product and lot numbers as that employed in previous gelation experiments for single systems.[28] According to the suppliers, it contains ca. 0.8% Na^+. The net charge on the polyanion β-Lg at pH 7 is −8, so the stated content of sodium ions is very close to that required to counter this contribution (8*23/18400 = 1%). This confirms that the sample is already extensively dialyzed and that at pHs around 7 there is very little additional supporting electrolyte (NaCl).

The α-La (L-6010, lot no. 76H7125) type III (from bovine milk, approximately 85% polyacrylamide gel electrophoresis) was also purchased from Sigma; they state it contains only trace amounts of Na^+ (as phosphate) and ammonium ions (as sulfate). This sample, which we and others have previously employed, has less Ca^{2+} than the "native" form, depletion appearing to be associated with the purification regime adopted in its preparation.

2.2. Sample Preparation. Each protein was weighed out separately, and the weighed amounts were mixed. The required amount of deionized water was then added to give a total protein concentration of 15% w/w, employing correction factors for the predetermined water contents of the proteins (90.0% for α-La and 90.1% for β-Lg). The mixture was then stirred with a magnetic stirrer to ensure all protein had dissolved. The pH was adjusted to either 3 or 7 using 0.1 M HCl (Analar, BDH). Table 1 shows the amounts of β-Lg and α-La present in selected mixtures. The natural pH values of the mixed β-Lg and α-La systems ranged from 7.4 to 7.2, with higher values observed when β-Lg was the major protein.

2.3. Rheological Measurements. Rheological measurements were performed with a strain-controlled Rheometrics fluids spectrometer II (RFSII, Rheometric Scientific Inc., USA). Small parallel plates were used with a diameter of 25 mm and a nominal gap of 1 mm. The gelation process was monitored isothermally at 80 °C, with frequency and strain set at 1 rad/s and 1%, respectively. The solutions were loaded quickly between preheated plates stabilized at 80 °C. A thin layer of paraffin oil (BDH, 29436) was pipetted onto the sample edge. Geometry covers, i.e., two semicircular pieces of equipment used to cover the plates without touching them, were placed around these to help minimize sample evaporation. The sample was held at 80 °C, and the storage modulus, G', and the loss modulus, G'', were monitored as functions of time. These modulus components were recorded over several hours. At the end of each time sweep, a frequency sweep (0.1−100 rad/s) and a strain sweep (0−25%) were performed. Since little *extra* information was obtained from these sweeps,[30] other than (a) a demonstration that prior measurements were within the linear viscoelastic limit and (b) confirmation that strong gel spectra were obtained, they will not be discussed further.[32] In the studies of the cold set α-La systems, some measurements were made using a Rheometrics Scientific DSR200 stress-controlled rheometer. Procedures were effectively the same as

described above, although this instrument was equipped with a Peltier heating stage, which is an advantage for some of the experiments performed (see later).

As in previous work,[30] the gelation time was determined by finding the time at which G' was greater than a threshold value (typically ∼1−10 Pa), and the "infinite time" value of gel modulus was obtained by extrapolation using the empirical equation

$$G' \approx G'_\infty \exp(-B/t) \quad (1)$$

as proposed by Kavanagh.[28] Here t is the time in seconds, B is an empirical parameter, and G'_∞ is the required value of G' at infinite time. This form can reproduce both the asymptotic limit as $t \rightarrow \infty$ and the logarithmic singularity ($\log G' \rightarrow 0$) as $t \rightarrow t_c$ from above. Both of these are required limits in a percolation-based kinetic gelation model but are not always addressed by other approaches.

As we have shown previously,[33−35] this apparently arbitrary approach to gelation time determination is well justified. For example, since the increase in gel modulus from its pre-gel threshold value is extremely rapid, the concentration dependence of gel time is almost always independent of the precise method employed, except when the kinetics of gelation are extremely slow (say gel times $\gtrsim 10^4$ s). In the latter case there are other problems at least as serious, for example, long time instrument drift. Lowering the pH did not affect the success of the extrapolation method of eq 1, as it could be applied equally well at pH ≳2 and pH 7. The curve fits involved were intrinsically weighted toward the final modulus values, although modification of the fit conditions could be employed to obtain gel time estimates as well. (Results not reported here.) The use of such an extrapolation procedure is a valuable step in "final modulus" determination. The importance of using G'_∞ rather than a time-truncated value of G', as employed by almost all previous workers, has already been discussed in some detail.[29,36]

3. Results and Discussion

3.1. Cure Curves at pH 7. Just as was observed for *β*-Lg and α-La as single components,[28] G' was found to be higher than G'' for all the pH 7 mixtures in the initial stages of cure, gelation occurring subsequently at the appropriate gel time. In practice, however, determination of the end point, i.e., when the log G' versus time "cure curve" appeared to level off, was more difficult for the mixed systems than for *β*-Lg alone. Measurements on samples containing a high ratio of α-La had to be stopped quickly after the gels had formed since small maxima in G' were observed, which suggested drying effects. All of the G' cure curve data for the mixed systems at pH 7 are shown in Figure 1. When the sample contained only *β*-Lg, it gelled within 4 min, whereas for 15% α-La, gelation took 50 min. Despite such slow kinetics, however, the long-time value of G' for the lactalbumin was approximately a decade higher than that for the corresponding pure *β*-Lg.

A transition in gelation behavior occurring around 6% *β*-Lg/9% α-La composition is suggested by Figure 1 and, interestingly, is also reflected in G'' data (not shown) which closely followed the growth shapes seen for G' but with absolute values approximately a decade lower, i.e., $\tan\delta \approx 0.1$. Significantly, for single-component *β*-Lg pH 7 systems, gels did not form below 12.2% w/w, while for the mixed systems at pH 7, gels formed at all weight ratios, demonstrating that α-La effectively enhances the gelation properties and final modulus of the *β*-Lg component.

3.2. Cure Curves at pH 3. Prolonged heating of systems at pH 3 caused gelation to occur within 10^4 s for all blends, except for the 15% pure α-La system. This

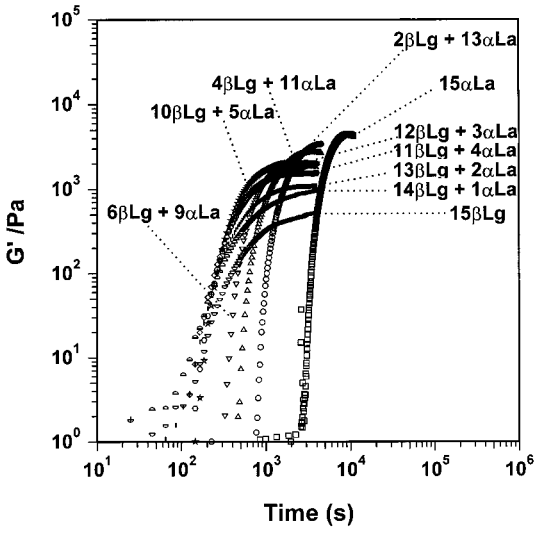

Figure 1. Combined time sweeps for different concentration of mixed samples at pH 7, showing G'.

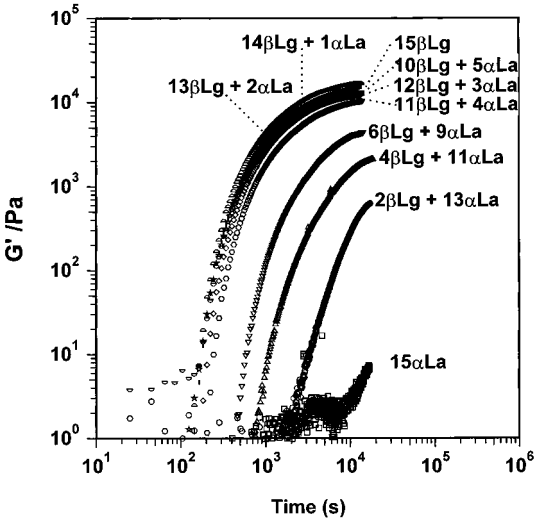

Figure 2. Combined time sweeps for different concentration of mixed samples at pH 3, showing G'.

did not appear to form a gel on heating within the time constraints of the experiment. More sophisticated methods for detection of the rheological gel point[37] are largely inappropriate, since the lack of torque signal precludes any reliable measurement of frequency dependence (say over the range 0.1−100 rad s^{-1}) prior to, and even just past, the gelation point.[4]

All the G' cure curve data for *β*-Lg and α-La mixtures at pH 3 appear in Figure 2. In general, at high concentrations of *β*-Lg and low concentrations of α-La, a gel formed within 4−5 min. When the amount of α-La was larger than the amount of *β*-Lg, however, the gel time increased. In addition and in contrast to the behavior at pH 7, the final modulus was reduced as the α-La composition increased. When *β*-Lg was the dominant protein, α-La appeared to contribute to the overall gelation behavior since the cure curve response was greater than that expected for *β*-Lg alone at its nominal concentration and was even greater than expected for *β*-Lg at the total system concentration (15% w/w). When the α-La became the major protein species by weight (e.g., 6% *β*-Lg and 9% α-La), the mixture still gelled, even though at pH 3, gels were not expected to form for pure *β*-Lg at concentrations less than 6.9%. For these

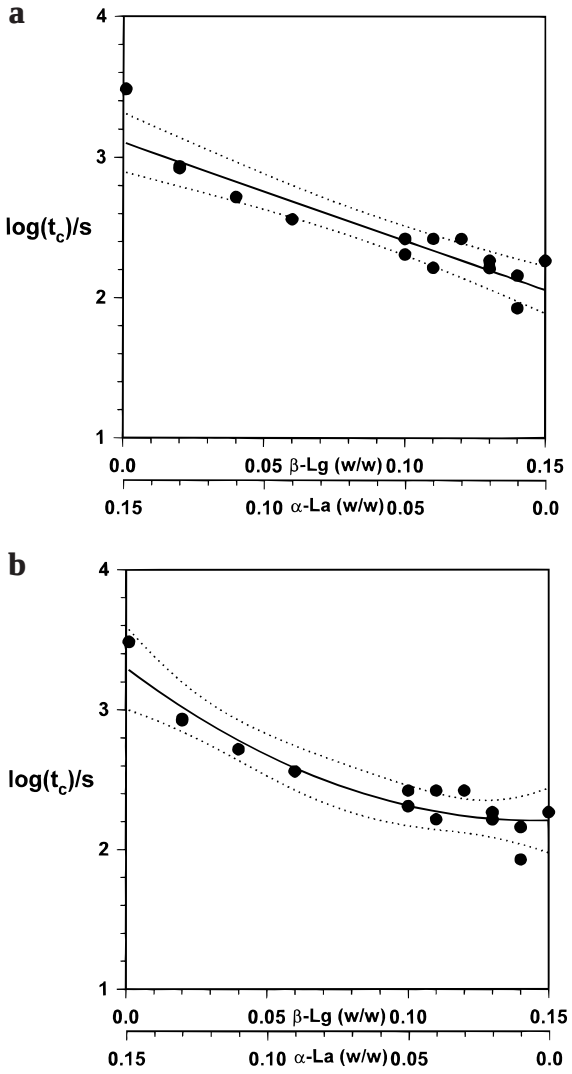

Figure 3. (a) Estimated gel times for mixtures of α-Lg/*β*-La at pH 7. The solid line shows the fit obtained with eq 2, with $a_3 = 0$. The dotted lines show the 95% confidence limits. (b) As in (a), but with $a_3 \neq 0$.

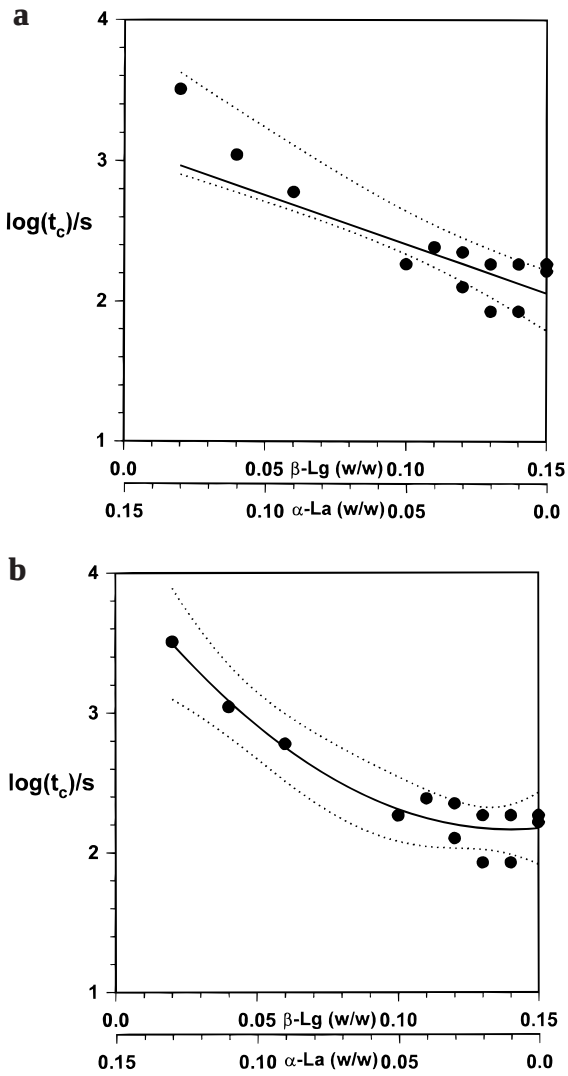

Figure 4. (a) As in Figure 3a at pH 3. (b) As in Figure 3b at pH 3.

mixed systems, gels actually formed at 2% *β*-Lg in the presence of 13% α-La, and indeed it may even be possible to form gels at almost 15% α-La, if a trace of *β*-Lg is added. Again these results at pH 3 are quite different from the findings at pH 7, since at that pH, 15% α-La took a long time to gel but, once gelled, reached the highest modulus value for the 15% mixture series. The same α-La concentration at pH 3 appeared not to form a gel on heating even after several hours heating (see section 4).

3.3. Gelation Times. Estimating gel times for the mixed systems at pH 7 and pH 3 when they contained high concentrations of *β*-Lg was difficult because, as soon as the sample had been loaded onto the measurement plate, the gelation process started. As soon as the concentration of α-La was greater than that of *β*-Lg, however, this initial gel time error was reduced, due to the significantly increased time required to form a gel. However, since the main objective of the present work was to establish final moduli for the systems studied, reducing the time of gelation was considered important.

In practice, gels always took longer to form when the amount of α-La was increased, and as discussed above, no gel appeared to form at all at pH 3 when the system contained only α-La. In some cases enough sample solution was prepared for two successive sample loadings, and the second loading was used as a comparison. When doing this, difficulties were experienced for the pH 3 mixtures when trying to load second (high-concentration) samples of α-La on the RFSII spectrometer. On standing at room temperature these samples appeared to become increasingly viscous. These initial findings of increasing viscosity at room temperature in an unheated sample led to further investigation of the pH 3 systems, which will be described later.

The estimated gel time values found for each ratio of *β*-Lg/α-La are plotted against concentration in Figures 3 and 4 for pH's 7 and 3, respectively. Results are also shown of fitting exercises conducted using the following (semiempirical) equation for the composition dependence of the gelation time:[5]

$$\ln(t_c) = a_1 r + a_2(1 - r) + a_3 r(1 - r) \qquad (2)$$

Here the *a*'s are coefficients (actually the log gel times of the pure components at the total protein concentration of 15% w/w), and *r* represents the ratio of concentration of the first species to the total protein concentration. Clearly if the cross-term (containing a_3) is zero, $\ln(t_c)$ is linearly related to the logarithms of the indi-

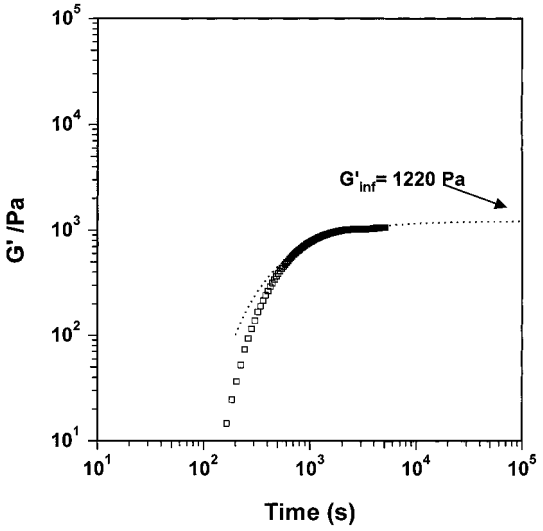

Figure 5. Example of data extrapolation at pH 7 for a high concentration of *β*-Lg (13% w/w) and a low concentration of α-La (2% w/w).

vidual component gel times (at total solids concentrations). The gel time data at pH 7 (Figure 3a,b) and pH 3 (Figure 4a,b) have been plotted (a) without and (b) with the interaction cross-term, together with 95% confidence limits. The description achieved is good but is essentially empirical. In no sense do the gel times of the two components reflect their nominal concentrations in the mixtures, as both individual values for these, and their averages, would show large maxima at intermediate concentrations. Nor do the gel times reflect a simple average of the pure 15% values, nor a transition from one pure 15% result to the other, something which might have been expected for a simple segregated system undergoing phase inversion (see below). The success of eq 2 probably reflects some more complex synergistic coupling between the two components (for example, the lactalbumin acting to catalyze and alter the mode of lactoglobulin gelation or even becoming involved in the formation of a mixed lactalbumin−lactoglobulin network).

3.4. Determination of G'_∞. At pH 7, and in the presence of high concentrations of *β*-Lg, the extrapolation method of eq 1 works well (Figure 5). Unfortunately, on increasing the amount of α-La, the quality of the fit decreased. The slopes of the cure traces, i.e., dG'/dt, became much steeper, and maxima in the G' data were observed at high concentrations of α-La. On removing the samples from the plates after completing experiments, the resulting gels were found to be transparent, although a large number of air bubbles were observed in samples which contained a high α-La content. These maxima were assumed to be due to artifacts in the experiment, probably due to long time sample drying, and in order to fully utilize the extrapolation method, data collected after the maxima were removed. Consequently, values obtained in the high α-La region may represent overestimates. No such problems were experienced when the extrapolation method was applied to the mixed pH 3 data, however, irrespective of the amount of α-La present.

3.5. Composition Dependence of G'_∞. G'_∞ values are plotted against composition at pH 7 (Figure 6) and pH 3 (Figure 7). This should be considered in light of the considerable literature that has appeared on the

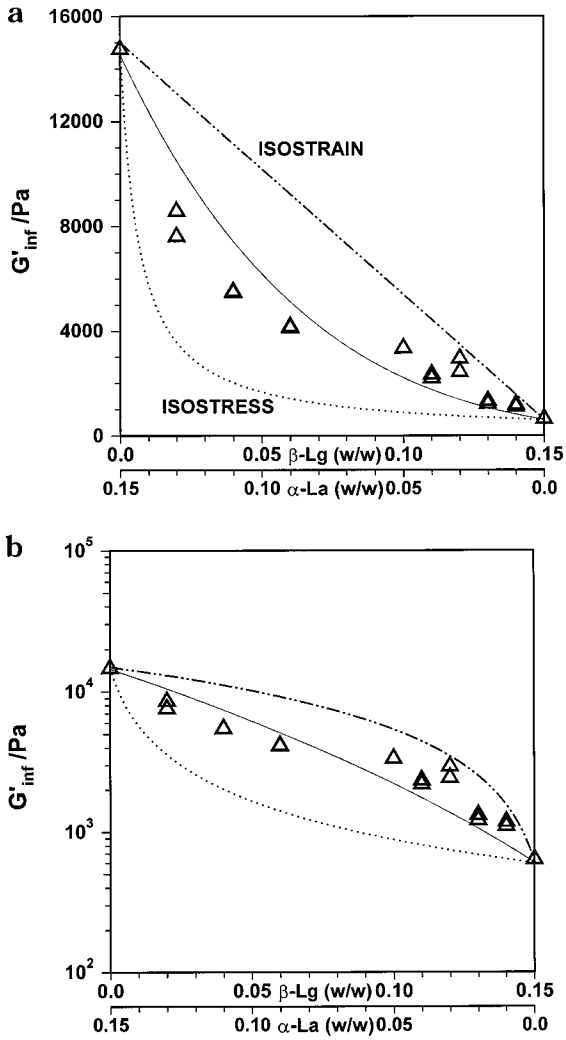

Figure 6. (a) Extrapolated modulus values for pH 7 mixed systems plotted against concentration. The data have been fitted using the isostrain (dot−dash), isostress (dotted), and bicontinuous (solid line) models. (b) As in (a), but plotted logarithmically.

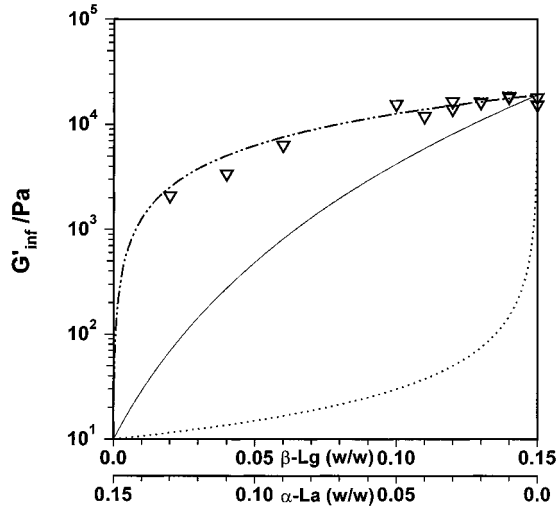

Figure 7. As in Figure 6b but for pH 3 mixed systems; here the isostrain model fits best.

measurement and prediction of the moduli of biopolymer gel mixtures since the work of Clark et al.;[18] developments in this field are discussed in a recent conference proceedings.[38]

For bulk mixed networks or melts, the modulus of a composite can be related to pure component behavior by use of so-called blending laws. If a high-modulus (filler) phase is dispersed within a low-modulus supporting phase, it is possible, by exact analogy with equations for the viscosity of filled fluid phases, to write

$$G_c = G_x(1 + f(\phi)) \tag{3}$$

in which ϕ is the volume fraction of the filler phase, G_c and G_x are respectively the composite modulus and the modulus of the supporting X-phase material, and $f(\phi)$ is an increasing function of ϕ. Here it is assumed that G_y, the modulus of the Y or filler phase, is effectively infinite. When G_y is of the same order as G_x, which is often the case when dealing with aqueous mixed gel composites, the approach pioneered by Takayanagi[39] for synthetic polymer blends is often useful. This is to apply series and parallel models to the problem of modulus addition. For $G_y > G_x$ this implies

$$\frac{1}{G_c} = \frac{1-\phi}{G_x} + \frac{\phi}{G_y} \tag{4}$$

and for $G_x > G_y$

$$G_c = (1-\phi)G_x + \phi G_y \tag{5}$$

The justification for these two so-called lower (4) and upper (5) bound models is that, in the first case, that of a hard filler in a soft matrix, the filler is deformed proportionately less than the matrix, so both components experience the same stress. Conversely, when the matrix is the stronger phase, it controls the total deformation. Consequently, (4) is sometimes called the isostress limit and (5) the isostrain limit.

The problem with applying even a simple bounds model of the above type to predicting the properties of most mixed aqueous gel systems is that the nominal concentrations of polymer appropriate to the initial solutions rarely represent their final local concentrations in phases generated by subsequent liquid−liquid demixing. Thus, while synthetic polymer blends are assembled from the pure components as separate phases which subsequently retain their identity, aqueous biopolymer mixtures generate their constituent phases kinetically by solution demixing driven by thermodynamic forces.[40] In other words, in the common case of fairly substantial segregation of the two polymer components, the real phase volume occupied by either one of these is determined by how the (aqueous) solvent is partitioned. To address this complication, the previously mentioned MT model of Clark and co-workers[18,27] introduced a so-called "avidity" parameter p to quantify such partition, this parameter now being recognized as related to the two polymer−solvent Flory−Huggins χ parameters for the solution.[41] The parameter p is defined in terms of the nominal mass fractions (x, y) of the polymers X and Y by

$$p = \frac{\alpha y}{x(1-\alpha)} \tag{6}$$

where α is a fraction such that αw of the solvent ($w = 1 - x - y$) is associated with the X-rich phase and $(1-\alpha)w$ with that containing Y. Under these circumstances, the effective concentrations (mass fractions) of X and Y in their respective compartments are

$$C_x = \frac{x}{x + \alpha w}$$

$$C_y = \frac{y}{y + (1-\alpha)w} \tag{7}$$

and are completely determined by p. When the density of the system is close to unity, mass fractions can be equated to volume fractions for the phases, these being given as

$$\phi_x = \frac{x(p + \{1-p\}y)}{px + y}, \quad \phi_y = 1 - \phi_x \tag{8}$$

Provided some relationship can be established between modulus and concentration for each of the components as single gelling systems, bounds for the modulus of the mixed gel can be evaluated using (4) and (5). In the original work, branching (cascade) theory was employed to interpolate (and extrapolate) pure system modulus data, but some subsequent workers[42] have employed an empirical single power law approach. Unfortunately, the latter approach is physically unsound, since it cannot reproduce the correct (singular) behavior as the critical concentration is approached.

The modified Takayanagi model (eqs 4−8) has been very widely employed over the years to describe mixed aqueous biopolymer gels, despite appeals for caution.[43] It is in fact subject to several limitations, not least those imposed by assuming total component segregation (rarely complete in practice), and the assumption that upper and lower bounds are valid descriptors of real behavior (i.e., are more than just bounds).

3.6. Treatment of *β*-Lactoglobulin/α-Lactalbumin Mixtures. In the present work, as a starting point in modeling, the conventional segregative approach was adopted first, and the MT model was used to describe the modulus composition data. A tendency toward compatibility in mixed protein systems (discussed more fully below) was recognized, to the extent that the simple $p = 1$ version of the model was assumed (proteins equally effective at attracting solvent). This implied that local protein concentrations would be fixed at the total solids content (i.e., 15% w/w), and phase volume fractions would coincide with the mass fractions of the components present (see eqs 6−8 with $p = 1$).

In Figures 6 and 7, isostrain and isostress bounds have been calculated in this way for the pH 7 and pH 3 data, using values of G'_∞ found for the 15% single component gels. For the pH 3 α-La system, an arbitrary value (=10 Pa, which corresponds to the gelation limit value) was assigned. Both linear G'_∞ (Figure 6a) and logarithmic G'_∞ plots (Figures 6b and 7) are presented. From Figures 6, the pH 7 data lie closer to the isostress model which (if the systems really are phase-separated) would suggest that β-Lg (assumed to be the supporting phase) was weaker than α-La (the discontinuous phase). However, on changing the pH to 3, the reverse is found since (see Figure 7) the isostrain model lies closer to the data. These results seem not unreasonable in terms of the relative moduli of the pure systems at the two pHs and 15% w/w concentration, but perhaps some form of phase inversion would have been expected as the lactoglobulin content fell, leading to a transition from one bound to another. The gel times might also have been expected to undergo an interchange from values appropriate to one 15% system to the other, with cure curves sometimes containing evidence of two phases of

gelling behavior. None of these characteristics of an explanation based on simple phase separation are observed, suggesting that the gels are more likely to be based on a mixed (coupled) protein network quite different from that assumed in the segregative approach.

Recently, Picullell and co-workers[44] have reintroduced a phenomenological model originally derived by Davies[45] for a granulated composite essentially analogous to that for random conductive percolation. They and others[24] have taken the bold step of applying it to bicontinuous polymer networks. This approach, given below, moderates the upper bound, but at the expense of another parameter. (Because it seems to us that there is nothing in the model that makes the exponent 0.2 specific, almost any other value in the appropriate range 0−1 could also be employed.)

$$G_c^{1/5} = (1 - \phi)G_x^{1/5} + \phi G_y^{1/5} \qquad (9)$$

Nevertheless, we have included this function fit in the above plots (solid lines), and it (although arguably since it introduces an extra parameter) furnishes a slightly better fit to the pH 7 data. However, we feel that segregative bicontinuous network formation is extremely unlikely in the present case.

Coupled networks are not improbable for mixed globular protein gels, since for mixed protein systems, it is not at all clear that even partial phase separation occurs, let alone the total separation implied by the models just described. Phase separation in conventional polymer mixtures is promoted by an unfavorable interaction energy between the polymer types, a difference in their thermodynamic interaction with solvent, and a low increase of entropy on mixing. However, the compact natures of globular proteins in native (intrinsic viscosities [η] typically ∼ 0.04 dL/g), denatured (volume change *per particle* usually no more than ∼30%), and aggregated states ("string of beads" model) appear to reduce the entropy argument. The energy case is weakened too by the chemical similarity of different unfolded proteins (provided that there are no large differences in charged states or degrees of hydrophobicity/hydrophilicity). In such situations virial coefficients may reflect reasonably favorable cross interactions between the components and not too much difference in interaction with solvent ($p = 1$), both factors promoting compatibility.

Compatibility in mixed globular protein gels should therefore be considered as an option in modeling exercises as well as the more conventional phase-separated description. When attempting this option, it should be remembered, however, that protein networks based on compatibility can take more than one form. These can range from types corresponding to the simple interpenetration of two distinct networks based on the separate species gelling essentially independently and for which, as discussed above, the Davies form was employed by more recent workers, to various forms of "coupled" network based on direct association of the polymers (e.g., mixed filaments, interaction between segregated filaments, etc.). There is also the problem that models are less available for these situations. At the simplest level one might argue that, for a mixed network, the modulus as given by the sum of the moduli expected for the individual species assumed to gel at their nominal concentrations (simple interpenetrating network model); gel times might also reflect such individual behavior. However, in the present case, the gel time data seem immediately to exclude this description, and at pH 3, at least, the model clearly fails from a modulus point of view, too. Here the lactalbumin contribution is always zero, and given the quite high critical concentration for pure lactoglobulin, modulus values would not be expected for the composite below about 8% β-Lg. Some form of more complex synergistic coupling is indicated, but before attempting a model, further work will be required involving, in particular, structural approaches (e.g., immuno-labeling and microscopy) to establish the exact nature of this.

We consider the effect seen at pH 7 is a consequence of mutual protein gelation rather than other factors such as the change in ionic strength induced by adding a salt containing protein. Such effects are significant when adding small amounts of salt to essentially salt-free systems. Here it is very important to distinguish between intrinsic ionic strength (essentially from essential counterions to the charge on the protein) and extrinsic ionic strength, contributed by deliberately adding salt. As discussed above, the net charge on the polyanion β-Lg at pH 7 and the cation concentration is very close to that required to just counter this. Since the α-La contains only trace cations, the overall effect of adding α-La to β-Lg systems modifies the gelation behavior, as reported previously.[25]

At pH 3 the situation is different, because β-Lg has an overall +22 charge. Acidification would protonate carboxyl groups and generate extrinsic Na ions. Here there will be an inevitable ionic strength variation, but this is unavoidable, since it a consequence of the neutralization. Adjusting toward acid pH will generate extrinsic sodium ions, but the concentration is not so great. For example, even at 15% β-Lg it will be ∼50 mM. An alternative approach would be to dialyze all mixtures against buffers of appropriate pH. However, in directly related work on single-component systems, we wanted to study the fibrillar gels, not the gross particulate structures found at higher salt levels, so we could compare our electron microscope images[29] with those of say Stading and co-workers.[9]

Most interestingly, the results suggest that the two protein species, when partially unfolded into the molten globule state, give results consistent with nonspecific mixed network formation between protein species, rather than a synergic interaction between the individual protein gel systems which may be regarded as the current "default" model. Whether this mixed network formation is by cross-interaction of fibrils of segregated types, i.e., β-lactoglobulin and α-lactalbumin, or by formation of mixed fibrils from the two unfolded species in the molten globule state, the techniques employed here cannot establish. However, our view is that what is most significant is the total concentration of protein (=15% w/w) in the molten globule state and not the individual protein species. We consider the smoothly varying behavior exhibited by Figures 3, 4, 6, and 7 supports this approach. In this respect, as in others, the results are of general applicability.

4. Further Experiments on pH 3 Systems

While performing heat-induced gelation experiments on mixed systems of β-Lg and α-La at pH 3, one sample did not gel on heating (15% α-La). The remains of this *unheated* sample were left at room temperature and

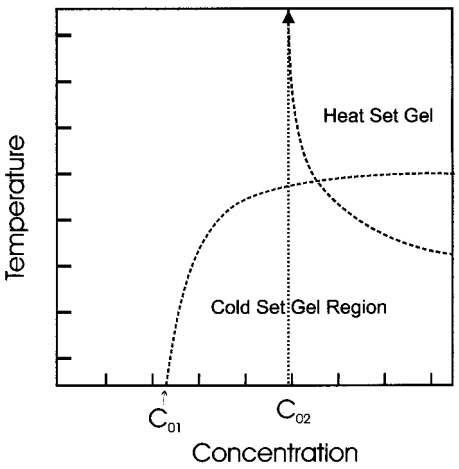

Figure 8. Apparent behavior of pH 3 α-La gels, arbitrary units, illustrating the two gelation modes (cold and heat set) and the corresponding critical concentrations.

after 3 days were observed to have apparently gelled. On heating this gel in a water bath at 80 °C for 5 min it became viscous, while on recooling a gel re-formed. In view of these findings it was decided to perform a few additional experiments to investigate the behavior of mixed pH 3 systems on cooling. A combination of measurements was performed using a Dynamic Stress rheometer with Peltier heating stage (DSR200, Rheometrics Scientific Inc., USA), a programmable glycerol/water bath, to apply a controlled temperature ramp, and some simple "test tube" experiments.[28] Most of these experiments were routine and will not be recounted here. However, what could be deduced was that when a series of α-La samples (10, 12.5, 15.0% w/w) were prepared at pH 3 and then left at room temperature for 3 days, the 12.5 and 15% samples appeared to have gelled, while the 10% had not, despite a notable increase in viscosity. On standing for a week, this sample also became more gel-like. The first two gels (12.5 and 15.0% systems after 3 days) were then heated to 80 °C and were found to melt at around 45 °C. Corresponding experiments were performed using *β*-Lg (2%):α-La (13%) systems, and similar effects were seen at room temperature; but of course, this latter system can also form a stable heat set gel.

Figure 8 shows diagrammatically what is believed to happen for these mixed and single systems at pH 3. We surmise that two modes of gelation are in operation. In acid conditions, and above a critical gelation concentration, C_{01}, gelation can occur at room temperature (cold-set gelation). The absolute critical concentration for mixed and single systems will probably vary slightly depending on the salt content. The gelation mechanism occurring at room temperature appears to be reversible, since on heating, the sample transforms back to a sol. On increasing the temperature further (and when the concentration was above a second critical concentration, C_{02}), gelation occurred through the more usual heat-setting mechanism. Once this heat-set gel formed, reducing the temperature had no melting/cold set gelation effect, since then the heat denaturation is essentially irreversible.

At the time we considered these results to be novel, since nothing similar appears to have been reported in the recent literature, but a more extensive search established that related results were reported in the 1960s by Kronman and co-workers[46,47] and subsequently discussed in the classical review by Mackenzie.[48] This work using the now largely neglected technique of analytical ultracentrifugation studied the aggregation of α-lactalbumin using sedimentation velocity, over the range 0−7% w/w and measured its time dependence.

After this work it was established that α-lactalbumin forms a so-called "A" state at acidic pH, particularly between pH 2 and pH 4, and this "A" state conformation corresponds to a "molten globule", with a hydrodynamic volume around 15% larger, as determined by light scattering and intrinsic viscosity measurements, compared to the native state at pH 7.4 as described in the recent review by Ptitsyn.[49] It has well-defined secondary structure, including intact disulfide linkage, but a fluidlike tertiary structure. The Kronman work shows that α-lactalbumin in its "A" state is prone to aggregation, and polymerization, compared to the native conformation, and that this acidic polymerization behavior is heavily time dependent. Indeed, polymerization occurred to such an extent that what they refer to as gels (but some of which we suspect, in the light of a more precise appreciation of gelation may actually be viscous pre-gel aggregates) were formed after 24 h at room temperature, at appropriate protein concentrations. The polymerization behavior was also shown to be dependent on temperature and solvent ionic concentration and could be reversed by changing conditions.

Since the acid-induced cold-set polymerization appears completely reversible, this appears to rule out any role for disulfide bonding in the mechanism and final network architecture. Because the molten globule conformer state of α-lactalbumin at low pH is documented to be structurally similar to the conformer formed at 80 °C, but essentially irreversible, it may be that the high-temperature irreversible gel involves some S−S cross-linking after all. More detailed experiments to investigate this behavior using both Ca^{2+} depleted α-La, and a corresponding sample with Ca^{2+} present, are currently planned.

5. Conclusions

At pH 7 all the *β*-Lg/α-La mixtures gelled. Although gelation took longer in the presence of high concentrations of α-La, the final modulus values were higher, the more α-La the sample contained. The final modulus values for 15% α-La samples were approximately a decade higher than for corresponding 15% *β*-Lg gels. On heating at pH 3, only the 15% α-La sample failed to gel. The gel times obtained for pH 3 mixed systems showed a similar trend to those obtained at pH 7. Increasing the amount of α-La in the mixture increased the gelation time, until eventually the system contained only α-La and no gel was formed. A clear distinction was observed between these different mixed pH systems, however. At pH 7, increasing the amount of α-La increased the final modulus, while at pH 3, it brought about a decrease.

The gel time and modulus data obtained for mixed *β*-Lg/α-La systems at pH 7 and pH 3 could only be explained partly in terms of a simple phase separation hypothesis. In fact it seems quite probable that, in this case, rather than conventional phase-separated systems being formed, so-called "mixed or coupled gel networks" have arisen, based on a low thermodynamic driving force for demixing, even during unfolding and aggregation of the components. While there is no direct experimental evidence to support this view, it seems reason-

able to assume that the molten globule structures usually associated with unfolded globular proteins could show a level of nonspecificity in their interaction or even an increased propensity for cross-interaction. Some form of catalytic activity of one on the aggregation of the other is also possible. Alternatively, it may be that the filamentous structures formed during segregated interaction, cross-link at the network-building level, and form a coupled network in that way. These several possibilities (and no doubt there are others) are likely to be difficult to distinguish on the basis of rheological data alone, particularly in the absence of theoretical models, so it seems that independent structural evidence is now required at each pH. This is difficult to obtain using conventional electron microscopy as contrasting the protein components using simple chemical stains is impossible, but immuno-labeling is available and will be attempted in the near future. Spectroscopic approaches based on secondary structure determination (IR/Raman) may also prove useful.

Mixed samples containing a high α-La ratio, at pH 3, formed gels when left at room temperature. The results from a series of experiments suggest that two possible gelation mechanisms exist for such systems. In the first instance, above a certain critical concentration, they can form thermoreversible cold-set gels. On further heating above the melting temperature, and above a different critical concentration, such blends can form more conventional, irreversible heat-set gels. These observations, which are consistent with the properties of the low-temperature acid-induced "A" conformation of α-La, suggest further structural investigations of the products of the two gelation modes would be worthwhile and provide a very useful addition to current understanding of globular protein gelation.

Acknowledgment. G.M.K. and W.S.G. thank the Biotechnology and Biological Sciences Research Council (BBSRC) and Unilever plc for the award of CASE Studentships. The Rheometric Scientific RFSII and DSR was purchased with financial support from the BBSRC under Grants F01033 and F06419, respectively.

References and Notes

(1) Richardson, R. K.; Ross-Murphy, S. B. *Int. J. Biol. Macromol.* **1981**, *3*, 315.
(2) Clark, A. H.; Judge, F. J.; Richards, J. B.; Stubbs, J. M.; Suggett, A. *Int. J. Pept. Protein Res.* **1981**, *17*, 380.
(3) Clark, A. H.; Saunderson, D. H. P.; Suggett, A. *Int. J. Pept. Protein Res.* **1981**, *17*, 353.
(4) Tobitani, A.; Ross-Murphy, S. B. *Macromolecules* **1997**, *30*, 4845.
(5) Tobitani, A.; Ross-Murphy, S. B. *Macromolecules* **1997**, *30*, 4855.
(6) Langton, M.; Hermansson, A. M. *Food Hydrocolloids* **1992**, *5*, 523.
(7) Stading, M.; Hermansson, A. M. *Food Hydrocolloids* **1990**, *4*, 121.
(8) Stading, M.; Hermansson, A. M. *Food Hydrocolloids* **1991**, *5*, 339.
(9) Stading, M.; Langton, M.; Hermansson, A. M. *Food Hydrocolloids* **1992**, *6*, 455.
(10) Foegeding, E. A.; Lowe, R.; Boland, M. J.; Hill, J. P. *Macromol. Symp.* **1999**, *140*, 137.
(11) Ikeda, S.; Foegeding, E. A.; Hagiwara, T. *Langmuir* **1999**, *15*, 8584.
(12) Aymard, P.; Durand, D.; Nicolai, T. *Int. J. Biol. Macromol.* **1996**, *19*, 213.
(13) Aymard, P.; Gimel, J. C.; Nicolai, T.; Durand, D. *J. Chim. Phys. Phys.-Chim. Biol.* **1996**, *93*, 987.
(14) Aymard, P.; Nicolai, T.; Durand, D.; Clark, A. H. *Macromolecules* **1999**, *32*, 2542.
(15) Gimel, J. C.; Durand, D.; Nicolai, T. *Macromolecules* **1994**, *27*, 583.
(16) Le Bon, C.; Nicolai, T.; Durand, D. *Macromolecules* **1999**, *32*, 6120.
(17) Sunde, M.; Blake, C. C. F. *Q. Rev. Biophys.* **1998**, *31*, 1.
(18) Clark, A. H.; Richardson, R. K.; Ross-Murphy, S. B.; Stubbs, J. M. *Macromolecules* **1983**, *16*, 1367.
(19) Miyoshi, E.; Nishinari, K. *Kobunshi Ronbunshu* **1998**, *55*, 567.
(20) Goycoolea, F. M.; Richardson, R. K.; Morris, E. R.; Gidley, M. J. *Macromolecules* **1995**, *28*, 8308.
(21) Goycoolea, F. M.; Morris, E. R.; Gidley, M. J. *Carbohydr. Polym.* **1995**, *28*, 351.
(22) Ridout, M. J.; Brownsey, G. J.; Morris, V. J. *Macromolecules* **1998**, *31*, 2539.
(23) Mohammed, Z. H.; Hember, M. W. N.; Richardson, R. K.; Morris, E. R. *Carbohydr. Polym.* **1998**, *36*, 27.
(24) Mohammed, Z. H.; Hember, M. W. N.; Richardson, R. K.; Morris, E. R. *Carbohydr. Polym.* **1998**, *36*, 37.
(25) Hines, M. E.; Foegeding, E. A. *J. Agric. Food Chem.* **1993**, *41*, 341.
(26) Aymard, P.; Durand, D.; Nicolai, T. *Int. J. Polym. Anal. Char.* **1996**, *2*, 115.
(27) Clark, A. H.; Ross-Murphy, S. B. *Adv. Polym. Sci.* **1987**, *83*, 57.
(28) Kavanagh, G. M. Rheological and structural studies of denatured globular protein gels and sols. PhD, University of London, 1998.
(29) Kavanagh, G. M.; Clark, A. H.; Ross-Murphy, S. B. *Int. J. Biol. Macromol.*, in press.
(30) Kavanagh, G. M.; Clark, A. H.; Ross-Murphy, S. B. *Langmuir*, in press.
(31) Swaisgood, H. E. In *Developments in Dairy Chemistry-1: Proteins*; Fox, P. F., Ed.; Applied Sciences: London, 1982; p 1.
(32) Ross-Murphy, S. B. In *Physical Techniques for the Study of Food Biopolymers*; Ross-Murphy, S. B., Ed.; Blackie Academic and Professional: Glasgow, 1994; p 342.
(33) Ross-Murphy, S. B. *Carbohydr. Polym.* **1990**, *14*, 281.
(34) Ross-Murphy, S. B. *Rheol. Acta* **1991**, *30*, 401.
(35) Kavanagh, G. M.; Ross-Murphy, S. B. *Prog. Polym. Sci.* **1998**, *23*, 533.
(36) Clark, A. H. *Polym. Gels Networks* **1993**, *1*, 139.
(37) Winter, H. H.; Chambon, F. *J. Rheol.* **1986**, *30*, 367.
(38) Hill S. E., Mitchell, J. R., Harding, S. E., Eds. *Biopolymer Mixtures*; Nottingham University Press: Nottingham, England, 1996.
(39) Manson, J. A.; Sperling, L. H. *Polymer Blends and Composites*; Heyden: London, 1976.
(40) Clark, A. H. In *Biopolymer Mixtures*; Hill, S. E., Mitchell, J. R., Harding, S. E., Eds.; Nottingham University Press: Nottingham, England, 1996; p 37.
(41) Durrani, C. M.; Prystupa, D. A.; Donald, A. M.; Clark, A. H. *Macromolecules* **1993**, *26*, 981.
(42) Morris, E. R. *Carbohydr. Polym.* **1992**, *17*, 56.
(43) Ross-Murphy, S. B. In *Biopolymer Mixtures*; Hill S. E., Mitchell, J. R., Harding, S. E., Eds.; Nottingham University Press: Nottingham, England, 1996; p 85.
(44) Piculell, L.; Nilsson, S.; Muhrbeck, P. *Carbohydr. Polym.* **1992**, *18*, 199.
(45) Davies, W. E. A. *J. Phys. D* **1971**, *4*, 1325.
(46) Kronman, M. J.; Andreotti, R. E. *Biochemistry* **1964**, *3*, 1145.
(47) Kronman, M. J.; Andreotti, R. E.; Vitols, R. *Biochemistry* **1964**, *8*, 1152.
(48) Mackenzie, H. A. *Adv. Protein Chem.* **1967**, *22*, 56.
(49) Ptitsyn, O. B. *Adv. Protein Chem.* **1995**, *47*, 83.

MA0005522