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Rheology of Mixed β-Casein/β-Lactoglobulin Films at the Air−Water Interface

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The adsorption of dilute mixtures of β-casein/β-lactoglobulin to the air−water interface was investigated using surface dilatation and surface shear rheology. The data were fitted to simple rheological models to try to gain further information regarding the composition and nature of the interface. The dilatational measurements suggested that the composition of the interface could be determined using these models and that the surface concentration was dominated by the β-casein in the early stages of adsorption but that high levels of β-lactoglobulin were present in the final stages. Surface shear rheological measurements showed a similar trend. However, the shear measurements appeared to be more sensitive to the strength of the network than to the composition of the interface. Fluorescence microscopy supported the findings and demonstrated that any "phase separation" capable of affecting the surface rheological measurements occurred at the sub-micrometer scale. The results also demonstrated that the heterogeneity of the interface, once formed, is kinetically trapped, and no further phase separation occurs over the time span of the experiments.

KEYWORDS: Protein; adsorption; surface rheology; phase separation

INTRODUCTION

The majority of food foams and emulsions are created and stabilized by proteins. They are abundant, relatively inexpensive, and perceived to be natural and wholesome ingredients. The most widely used proteins are derived from milk. They are produced in large quantities in a form that is easily accessible. In fact, many of the proteins used in milk are there to stabilize the milk fat; therefore, they tend to be highly functional proteins, which have been well characterized and have a range of applications (*1*, *2*). Milk protein based ingredients tend to be complex mixtures of protein having compositions and functionalities largely dependent on the extraction, purification, and subsequent processing procedures (*3*). The functional behavior of these mixtures can therefore be complex and often unpredictable. The molecular basis of the functionality of many of the individual milk proteins has been well characterized (*2*, *4*−*12*). It is vital, therefore, if we are to be able to control or predict the functionality of milk protein products, that we understand, in as much detail as possible, the mechanisms underlying the functionality of milk protein mixtures.

Surface rheological techniques have been used extensively in the area of food colloids, as proteins tend to form viscoelastic interfaces, with rheological properties similar to those of three-dimensional protein gels (*6*, *12*−*22*). The technique is very useful for studying interactions at interfaces, such as between proteins and other surface-active ingredients such as low molecular weight emulsifiers and lipids (*7*, *14*, *21*, *23*−*27*), which possess very little in the way of surface viscoelasticity. Other interactions such as with enzymes or cross-linking agents can be detected through an increase in the surface rheology (*16*, *28*−*30*). Although many proteins exhibit similar surface tension behavior, their surface rheological characteristics can be very different (*14*, *27*). This can be due to gross structural differences such as in the case of globular proteins versus aperiodic proteins (*12*, *14*). Even very small structural changes such as the differences between genetic variants of the same protein can cause a measurable difference in surface rheological behavior (*11*) and indeed functionality (*9*).

The surface viscoelastic fingerprints of different proteins have been used in the past to differentiate between two different proteins at the interface (*7*, *15*, *16*, *18*, *19*, *24*); this was used primarily to study the way that one protein may disrupt or displace another adsorbed protein. For example, it has been shown that β-casein will disrupt α_{s1}-casein and α-lactalbumin adsorbed layers more easily than the other way around (*18*, *19*, *31*) and that β-lactoglobulin is also effective at disrupting other adsorbed protein layers (*31*). There has been a recent resurgence in protein surface rheology, due to the availability of commercial surface rheometers; hence, there is likely to be an increase in the volume of surface rheological data in the literature. The limitation is that interpretation and analysis of these data is a long way behind that developed for three-dimensional rheology (e.g., refs *32* and *33*); therefore, if surface rheology is to become a useful tool in the future, sound analytical methods will be required.

This present study is a preliminary investigation into quantitative analysis of the surface rheology of mixed proteins. We have

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studied the adsorption of binary mixtures of the milk proteins β-lactoglobulin and β-casein. These proteins were chosen because they have very different adsorption and surface rheological behaviors (*12*, *14*, *27*). Briefly, the globular β-lactoglobulin forms strong, elastic adsorbed films, whereas β-casein is a flexible, disordered molecule capable of adsorbing very quickly, but the interfacial film is very weak. We chose some very simple models that have been applied to the bulk rheology of composite systems (*34*, *35*). This allowed us to estimate the dynamic surface coverage of the two proteins at different ratios and then interpret these data in terms of the surface properties of the individual components. In light of some recent work by the group of Damodaran (*36*−*38*) who claim to have observed dynamic phase separation effects between adsorbed proteins, these data, together with fluorescent microscopy of the mixed protein interface, should allow the development of a better understanding of the interfacial behavior of simple protein mixtures. This will naturally be extended to more complex "real" protein ingredients.

EXPERIMENTAL PROCEDURES

Solutions of β-casein (BCAS) (Sigma Chemical Co., lot 12H9550) and β-lactoglobulin (BLG) (Sigma Chemical Co., lot 91H7005) were prepared at a concentration of ∼1 mg mL^{-1} in ultrapure water (Elga, Maxima L.S), allowed to equilibrate for 30 min, and used without further purification. These solutions were then diluted further with ultrapure water to give a final total protein concentration of 200 nM, containing differing ratios of β-casein and β-lactoglobulin. This solution was again allowed to equilibrate before adsorption and testing under dilation or shear rheology. The adsorption of the proteins was monitored over 3 h.

Surface shear rheology utilized a Camtel CIR100 (Camtel Instruments, Royston, U.K.) surface rheometer, in normalized resonance mode (*39*) and at an applied stress of 3000 μrad, using a 13 mm diameter De Nouy ring. Calculation of the surface modulus is by comparison with a reference sample (ultrapure water). Measurements were carried out at 20 °C. The instrument is controlled by a feedback loop system. This forces the ring to oscillate in a condition of "phase-resonance" at a frequency above the resonant frequency of the measuring system. Surface dilatation measurements used the ring-trough method (*40*), where a Wilhelmy plate is suspended within a 100 mm diameter glass ring (ring-trough). The ring-trough was oscillated sinusoidally in the vertical plane at low frequency (0.82 rad s^{-1}), imposing a calculated change in surface area of ∼4%, these values allowing measurements to be within the elastic response envelope of the sample.

The surface rheology data were analyzed to estimate the surface composition and possibly derive some structural information (i.e., degree of mixing). Two models, applied to the bulk rheology of non-interacting composites and known as the series and parallel models (*34*, *35*), were applied. These represent two extremes of composite modulus for (i) equal stress distribution or (ii) equal strain distribution by the composite, respectively

$$G_{mix} = \phi_1 G_1 + \phi_2 G_2 \quad (1)$$

and

$$1/G_{mix} = (\phi_1/G_1) + (\phi_2/G_2) \quad (2)$$

where G_{mix}, G_1, and G_2 are the moduli of the mixture and individual components, respectively, and ϕ_1 and ϕ_2 are the volume fractions of the individual components in the mixture, respectively. In eq 1 the stronger phase is assumed to make up the continuous network and therefore dominates the behavior, whereas in eq 2 the weaker phase makes up the continuous network and dominates the behavior. In our experiments ϕ_1 and ϕ_2 were taken as the surface area (rather than volume) fractions of BBAC and BLG, respectively. G values were taken as the measured surface shear elastic modulus (G') or surface dilatational modulus (E'), depending on the experiment.

Fluorescence microscopy was undertaken on mixed, fluorescently labeled BCAS/BLG adsorbed films. BCAS was labeled with fluorescein isothiocyanate (FITC, Sigma Aldrich Ltd., Poole, U.K.) and BLG with Rhodamine 6G (Molecular Probes Inc., Eugene OR) as described elsewhere (*41*). The proteins were mixed together thoroughly at known ratios and then allowed to adsorb. Adsorbed films were transferred to glass microscope cover slides by Langmuir−Blodgett (LB) dipping, prior to imaging (*42*). Films were allowed to stabilize over periods of up to 4 days prior to transfer and imaging (*36*), although no further changes were observed in the films after the first few hours of adsorption.

RESULTS AND DISCUSSION

Pure Proteins. The individual adsorption of either BLG or BCAS to an air−water interface has been studied previously under a variety of concentrations and conditions (*14*, *17*, *19*, *27*). **Figure 1** compares surface tension−time (**Figure 1a**), surface dilatational modulus−time (**Figure 1b**), and surface dilatational modulus−surface pressure (**Figure 1c**) for the individual proteins at a concentration of 200 nM. BCAS adsorbs to the surface more quickly than BLG, as shown in **Figure 1a**, with the surface tension dropping more rapidly over the first hour. This is due to its random, flexible structure in solution (*10*). In contrast, BLG possesses a greater degree of secondary structure in the bulk (*2*), which tends to slow the surface unfolding (*12*). This was in part demonstrated in **Figure 1a**, with BCAS having lower surface tension values than BLG, and also in **Figure 1b**, where the dilatational elastic modulus for BCAS was higher than that of BLG at short times (<12 min). The BCAS adsorbed layer appeared to undergo a (relatively) rapid reorganization to a more condensed, albeit weaker, layer, at intermediate times (15−30 min) and surface pressures (8−15 mN/m). This is thought to be caused by the collapse of the charged "tail" region (residues 1−50) of the molecule into the subphase and is indicative of the flexible BCAS molecule rapidly rearranging to a more stable conformation (*43*). A subsequent rearrangement to a stronger, nonequilibrium surface occurred after 80 min (surface pressure > 18mN/m). This is thought to be due to the formation of a more compact interface as the remaining part of the BCAS molecule approaches the close packed limit leading to stronger intermolecular interactions. Similar behavior has been observed for other systems; for example, recently Cornec et al. (*5*) inferred that α-lactalbumin was able to change orientation at high surface concentrations. BLG almost reached an equilibrium state over the course of the experiments (**Figure 1b**). Initially the rise in modulus is rapid, and then it plateaus at very high dilatational modulus values compared to BCAS. A comparison of the surface pressure−modulus data (**Figure 1c**) shows that at low surface pressures the two proteins behaved very similarly, but diverged markedly as surface pressure increased. The increase in modulus for BCAS observed after 80 min occurred over a very short surface pressure increase (**Figure 1b**) and, as indicated, is probably due to increased surface packing and interactions. In contrast, the modulus for BLG steadily increased with surface pressure. This behavior has been described previously and is due in part to van der Waals forces, hydrogen bonding, and possibly intermolecular disulfide bonding (*8*) and a relatively slow conformational change at the interface (*12*).

The surface shear rheology results for the individually adsorbing proteins are shown in **Figure 2**. The differences between the two proteins were even more marked, as observed previously (*6*, *19*, *25*, *27*). After ∼5 min of adsorption, BLG began to rapidly develop the surface shear elasticity. This continued to increase over the time course of the experiment,

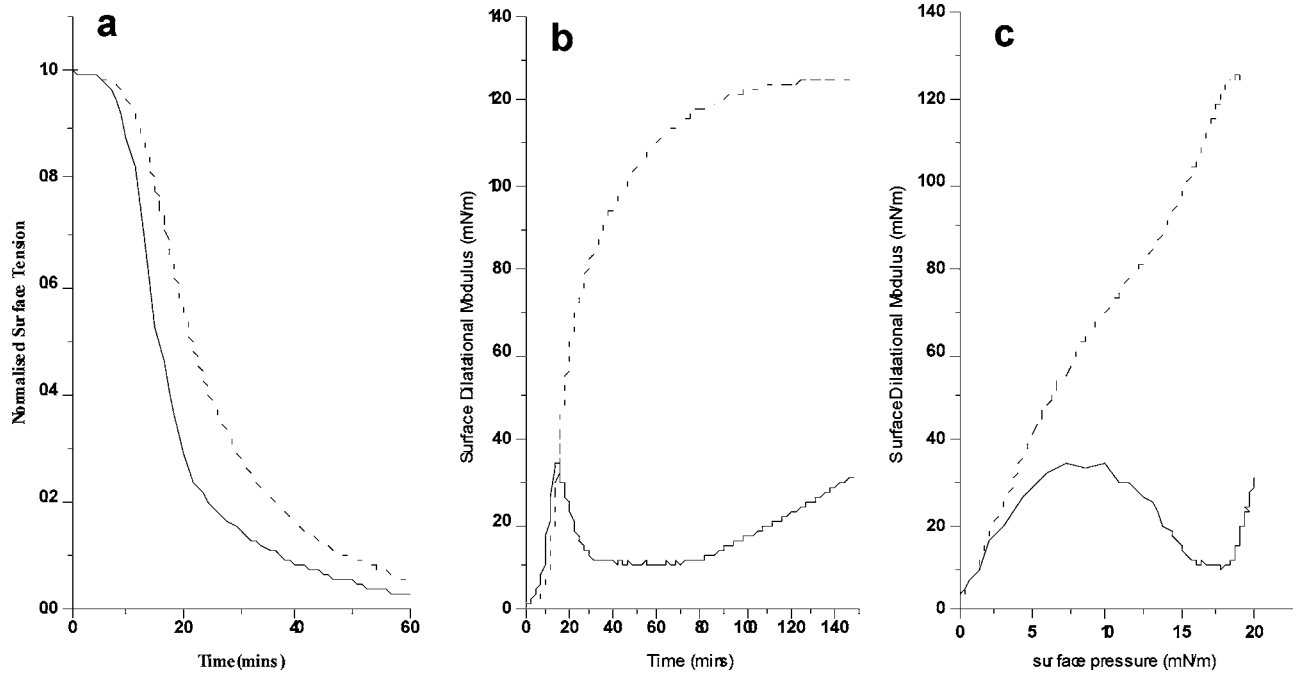

Figure 1. Surface tension and surface dilatational moduli of 200 nM β-casein (solid lines) and 200 nM β-lactoglobulin (dashed line): (**a**, **b**) surface tension and surface dilational modulus, respectively, as a function of adsorption time; (**c**) surface dilational modulus as a function of the surface pressure.

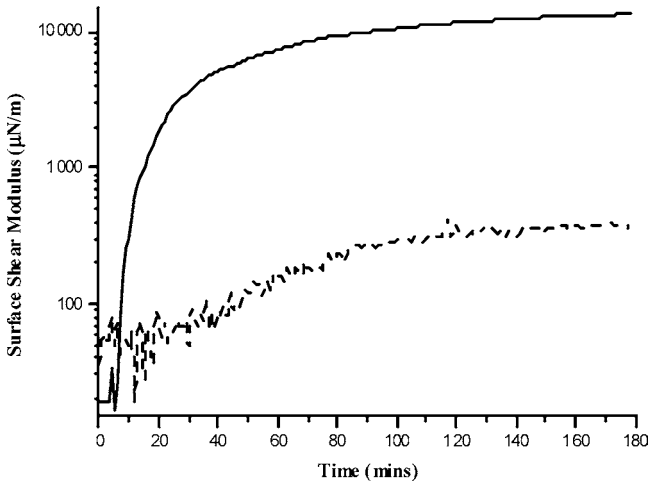

Figure 2. Surface shear elastic moduli as a function of adsorption time for 200 nM β-casein (dashed line) and 200 nM β-lactoglobulin (solid line). The modulus is presented on a logarithmic scale to highlight the small changes in the values for β-casein.

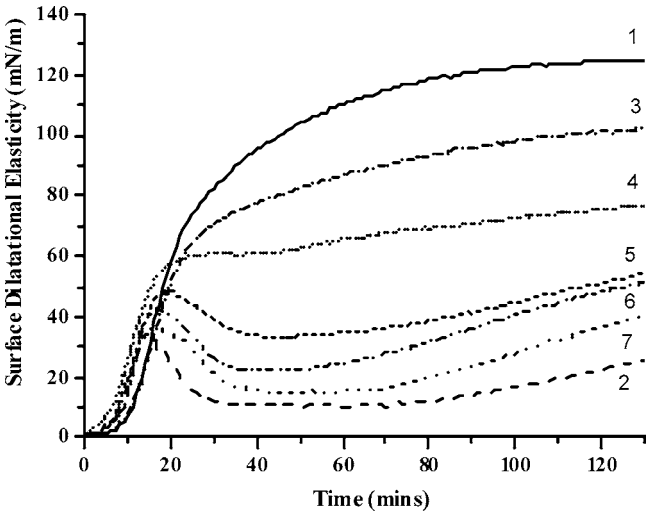

Figure 3. Surface dilatational elastic modulus as a function of adsorption time for BCAS/BLG mixtures. Total protein concentration is 200 nM. Molar ratios of BCAS/BLG are as follows: curve 1, 0:1, curve 2, 1:0; curve 3, 1:9; curve 4, 1:4; curve 5, 1:2; curve 6, 1:1; curve 7, 3:1.

as seen with the dilatational measurements, eventually forming a strong surface layer. BCAS, in contrast, showed considerably weaker surface structure by shear measurements. This can be attributed to the very weak nature of the BCAS network, as seen by the dilatation results (**Figure 1**). After ~20 min, BCAS began to slowly develop measurable surface shear elasticity. The reason for this appears to be the high surface density as the molecules reach the packing limit. The surface shear rheology is generally thought to be very sensitive to any interactions that may occur in the interfacial network (*14*, *19*). At short times, the adsorbed BCAS network probably has a small number of very weak interactions not least because of the molecular rearrangement that takes place as the charged "tail" region collapses. As adsorption time increases (higher surface pressures) the number or strength of the interactions increases (as discussed above) to give rise to a sufficiently strong film that can be monitored by surface shear techniques.

Mixed BCAS/BLG Films. The time-dependent adsorption behavior of BCAS and BLG mixtures, measured by surface dilatational rheology, was studied, and the results are shown in **Figure 3**. The concentrations were chosen so that the time scale of the adsorption process was sufficiently long to allow accurate measurement by these techniques. The first observation is that all of the data for the mixed proteins fall between the values for the individual components; therefore, no synergistic interaction was observed. Interestingly, for nearly all of the datasets, the initial behavior of BCAS (t < 30 min) largely dominated the behavior of the mixed interface. This is further evidence of the importance of the faster adsorption of BCAS shown earlier (**Figure 1a**). At longer times the behavior of the films appeared to be more intermediate between the behaviors of the individual proteins. However, considering the molecular ratios of the mixtures, the influence of BCAS is much greater than expected

from the solution composition. Only at very low levels of BCAS (10%) could the behavior be described as BLG-like, possibly implying some form of phase transition behavior in the mixtures. Similar results have also been found with binary mixtures of BCAS and α-lactalbumin or gelatin (*15*). Here, BCAS was found to dominate the surface shear viscosity at a relative concentration of only 25%. Presumably, the protein that adsorbs more quickly (in this case BCAS, **Figure 1a**) will have a greater chance of forming a continuous network at the interface and dominating the surface rheological properties. The protein that adsorbs more slowly either fills in the available space or, if the bulk concentration is great enough, will continue to adsorb and perhaps even disrupt or displace the first protein (*19*, *31*). Significant displacement is unlikely to occur here, as the surface pressures reached were not great enough to induce significant displacement as shown in previous displacement studies with surfactants (*42*). It is likely that any interactions which develop over time may also lead to changes in the observed behavior, making it difficult to fit the simple models used here.

Surface rheology can impart information regarding the surface composition of mixed protein interfaces that tensiometry cannot. This has been demonstrated previously when it was shown how one protein can disrupt an interface formed by another (*15*, *24*, *18*, *19*, *31*). Careful analysis of the data may also be able to give information about the nature of the mixed interface, in the same way that bulk rheological analysis imparts information about the bulk (*34*, *35*). Heterogeneous structures have already been observed in mixed protein/surfactant interfaces using probe microscopy (*42*, *44*) and Brewster angle microscopy (*45*, *46*), but to date it has not been possible for these techniques to differentiate between two adsorbed proteins. As indicated earlier, it is thought that proteins adsorbed to an interface provide a two-dimensional representation of the three-dimensional network behavior observed in the bulk. This is supported by the fact that bulk protein gels and adsorbed protein films both have non-Newtonian viscoelastic properties that have analogous concentration, frequency, and stress dependencies. Models of this bulk rheological behavior vary in complexity (*35*), but can be very simple (*34*). Initially, we have fitted the simplest models to our observed moduli data (both shear and dilatation) to see if it is possible to predict the nature of the interface.

Using the dilatational data for BCAS and BLG adsorption (**Figure 1c**) we generated "predicted moduli" for the mixed systems at various BCAS/BLG ratios using eqs 1 and 2. In **Figure 4a** BLG was seen as forming the continuous network and exerting the most influence over the moduli, whereas in **Figure 4b** BCAS was deemed to form the continuous network and therefore dominate the properties. The similarities between **Figures 3** and **4b** are quite marked. The observed data strongly suggest that BCAS dominates the behavior of the mixed system at all but the lowest BCAS concentrations. This can be seen in the peak in elasticity at ~18 min of adsorption time (**Figure 3**), which is characteristic of BCAS. It is also interesting to note that at the higher levels of BLG present in the bulk, the surface properties were more accurately predicted by eq 1, the model that assumes that the stronger component is in the continuous phase. This might possibly be expected because (a) there may not be sufficient BCAS present to form a continuous network and (b) the dilatational modulus of BLG is considerably higher than that of BCAS.

Figure 5 further demonstrates the dependency of the final surface properties on the bulk composition. Here, surface dilatational viscosity is plotted against surface pressure and there are clearly two distinct behavioral regimes, one dominated by the BCAS, even when bulk contains as little as 30% BCAS, and the other at lower BCAS levels, where the viscous component appears to be dominated entirely by the BLG and no evidence of the weaker BCAS is seen. This is probably a consequence of the typical behavior of the protein forming the continuous network, BLG producing a highly viscoelastic

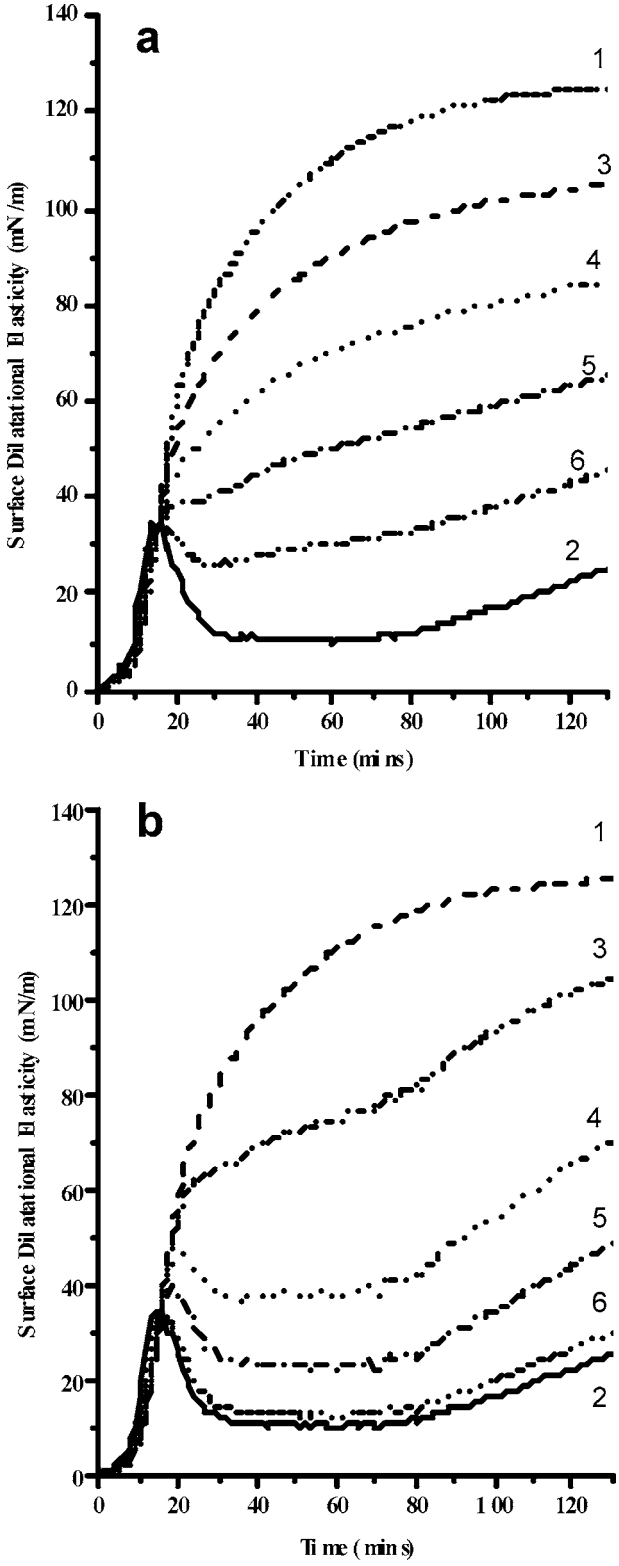

Figure 4. Calculated surface dilatational moduli of BCAS/BLG mixtures, using (**a**) eq 1 and (**b**) eq 2. Molar ratios of BCAS/BLG are as follows: curve 1, 0:1; curve 2, 1:0; curve 3, 1:4; curve 4, 2:3; curve 5, 3:2; curve 6, 4:1.

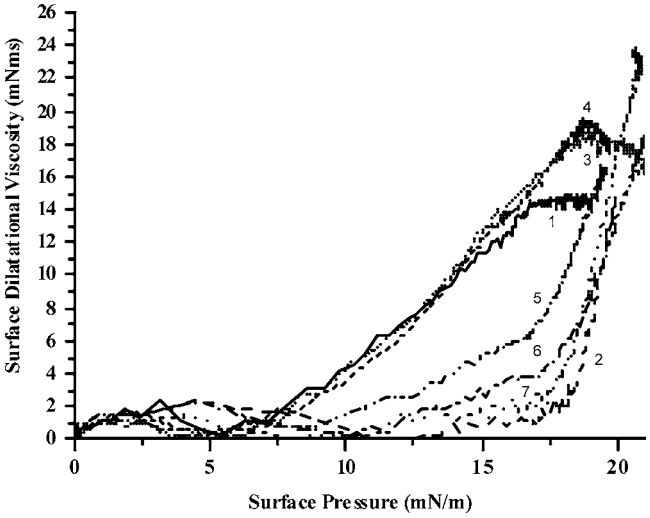

Figure 5. Surface dilatational viscosity as a function of adsorption time for BCAS/BLG mixtures. Total protein concentration is 200 nM. Molar ratios of BCAS/BLG are as follows: curve 1, 0:1; curve 2, 1:0; curve 3, 1:9; curve 4, 1:4; curve 5, 1:2; curve 6, 1:1; curve 7, 3:1.

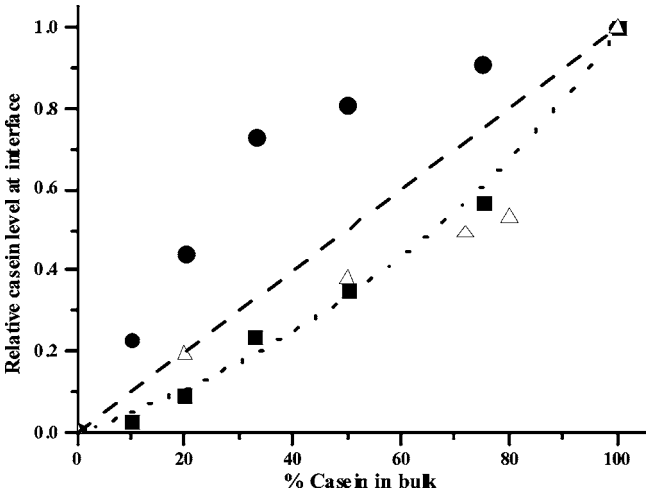

Figure 6. Relative surface concentration of BCAS as a function of relative proportion of BCAS in solution calculated using either eq 1(●) or eq 2 (■). Measured values of surface concentrations of BCAS (△) from Razumovsky and Damodaran (*48*) are also shown.

surface, whereas BCAS demonstrates a very small surface dilatational elasticity.

Another interesting phenomenon was the time-dependent changes in apparent surface composition. Fitting the data over small time scales, for example, in 15 min blocks, gave differing ratios of BCAS to BLG at the surface up to an adsorption time of 30−40 min (data not shown). This indicated that although BCAS adsorbed more quickly initially, the relative surface proportion of BCAS appeared to decrease at longer times. Thereafter, the relative concentrations of the two proteins at the surface remained unchanged over the duration of the experiments. These data are in agreement with the recently published studies of Cornec et al. (*5*), who argued that adsorption was diffusion controlled at short times.

Analysis of the experimental data from dilatational measurements using the two models was undertaken to see if the relative surface concentrations of the two proteins could be determined. The results shown in **Figure 6** are for values calculated over the whole data range. Equation 1 always predicted high BCAS levels at the interface and demonstrated two differing behavior

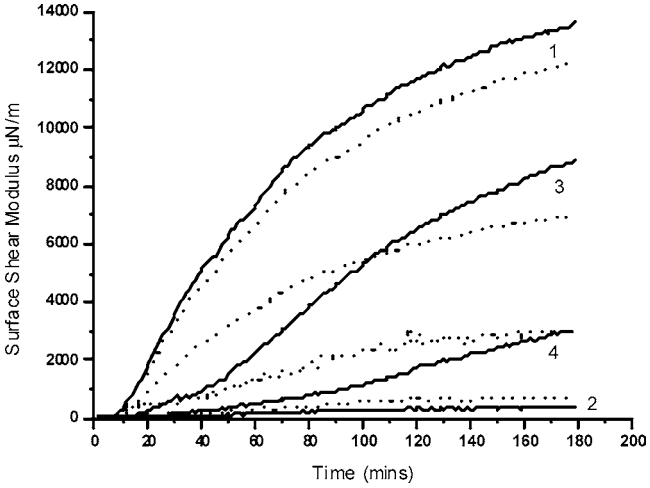

Figure 7. Experimental and calculated values of the surface shear modulus of BCAS/BLG mixtures. Total protein concentration is 200 nM. Molar ratios of BCAS/BLG are as follows: curve 1, 0:1; curve 2, 1:0; curve 3, 1:1; curve 4, 9:1.

regimes (**Figure 5**); we note that this model was a very poor fit at times up to 60+ min (as could be inferred from **Figure 4**), whereas the other model (eq 2) fits the majority of the data more accurately. Hence, the data (eq 1) were biased toward the longer adsorption time. Whereas eq 2 appeared to be a better fit, the calculated BCAS surface concentrations always appeared lower than would be expected from the relative adsorption rates. This suggested that adsorbing BLG began to dominate the network at longer times. Included in **Figure 6** are the surface-radio-counting data from Razumovsky and Damodaran (*48*), which were measured under similar experimental conditions. The values they have measured are very close to those predicted by the calculations from eq 2.

The surface shear data are somewhat revealing and on inspection appeared not to fit either model. The results are shown in **Figure 7** and appear on first observations to agree with the analysis of the surface dilatational data. That is, the surface moduli of the mixed protein solutions appeared to be dominated by BCAS at short times, again emphasizing the importance of the relative adsorption rates, but at longer times the behavior gradually becomes dominated by the much stronger BLG network. When the models were applied to the data, the strong BLG network completely dominated the predicted adsorption behavior, and the calculated surface concentrations showed no relationship to those from the surface dilatational measurements or from the experiments of Razumovsky and Damodaran (*48*); in fact, eq 2 only resembled the data at short adsorption times. It is thought that surface shear rheology is more sensitive to intermolecular interactions formed in the network than the dilatational measurements, and, as already stated, the two models assume that there is no interaction between the competing species. Analysis of the surface dilatational measurements suggested that the surface composition equilibrated within the first hour of adsorption, whereas the surface shear elastic moduli (**Figure 7**) continued to increase long after this. This suggests that complex interactions continued to develop, probably as a result of molecular rearrangement between the same, or different, protein species. Either way, this highlights the deficiencies in these simple models as molecular interactions are not accounted for in contributing to the surface rheological properties. Therefore, further work is required to develop a model with two separate dynamic processes, namely, adsorption (surface composition) and molecular interactions.

It was hoped that the surface rheological data could be used to reveal information about possible structures at the interface. As indicated earlier, interfacial protein films can be considered in a similar way as bulk systems, but restricted to two dimensions. The rheology has demonstrated that the interface is heterogeneous, with the dilatation results fitting well to simple models describing composite systems. The degrees of mixing at the interface must be restricted to mixed (which can allow interaction), demixed (phase separated), which may allow interaction at the BLG/BCAS junction, or a combination of both (which would also allow some interaction). Recent work by Damodaran and co-workers (*36*−*38*) has demonstrated large-scale phase separation between BLG and BCAS at the interface. Their studies have also suggested that phase separation due to thermodynamic incompatibility should be the norm. Interestingly, despite stating that the interfacial protein concentrations remain constant once the film has developed (some 24−30 h) (from radiolabeling methods) (*48*), phase separation on the scale they publish does not occur until nearly 96 h have passed (*36*−*38*). The scales of the phase-separated regions are truly impressive; it might be expected that phase separation would occur relatively evenly across the complete interface, but their results indicated regions of hundreds of micrometers where one protein dominates over another. One has to question the apparent delay time and sudden onset of such massive regions of phase separation, as one would expect a driving force leading to this behavior that would be observed at shorter times. In recent studies Mackie et al. (*47*) have observed the displacement of mixed protein films from an interface using surfactants. Here it was shown that the displacement occurs not in large regions, as would be predicted from the studies of Sengupta et al. (*36*−*38*), but rather at discrete sub-micrometer levels, indicative of either an intimately mixed films or films where phase separation occurs at scales significantly smaller than those demonstrated by Sengupta et al. (*36*−*38*).

To assist interpretation of the surface rheological measurements and to study the development of interfacial phase separation of these systems, fluorescent microscopy of the mixtures was undertaken. BCAS and BLG were fluorescently labeled separately, and adsorbed films were prepared as in the surface rheological experiments. A fluorescent image of a 1:1 mixture (4 days old, although images at earlier times were almost identical) is shown in **Figure 8**. Here, as expected, the image is dominated by BCAS, due to its relatively faster adsorption. The image does show several different regions: BCAS dominated (green), BLG dominated (red), and regions where the two proteins are intimately mixed. These data show that there is some small-scale heterogeneity of the mixed interface. No large-scale phase separation was detected at any time during these experiments. It also agrees with the surface composition as determined by the surface rheology insofar that it did not alter over long time periods. It should also be noted that surface pressures sufficient to lead to displacement from the interface were not reached (*42*). The image shown also differs from the results presented by Sengupta et al. (*36*−*38*); the phase separation they observe occurs catastrophically. The results they present are, like ours, at equilibrium surface concentration and surface pressure and have been for many hours previously. The differences in the preparation are quite significant, however. In our experiments the glass slides were thoroughly cleaned prior to LB dipping, whereas in their work the slide is exhaustively prepared and coated using 3-aminopropyl triethoxysilane (APTES) prior to Langmuir−Schaefer (LS) transfer. In previous experiments on interfacial protein displacement by SDS, imaged by atomic force microscopy, Mackie and co-workers demonstrated that the results from LB dipping and LS dipping can be markedly different (*44*).

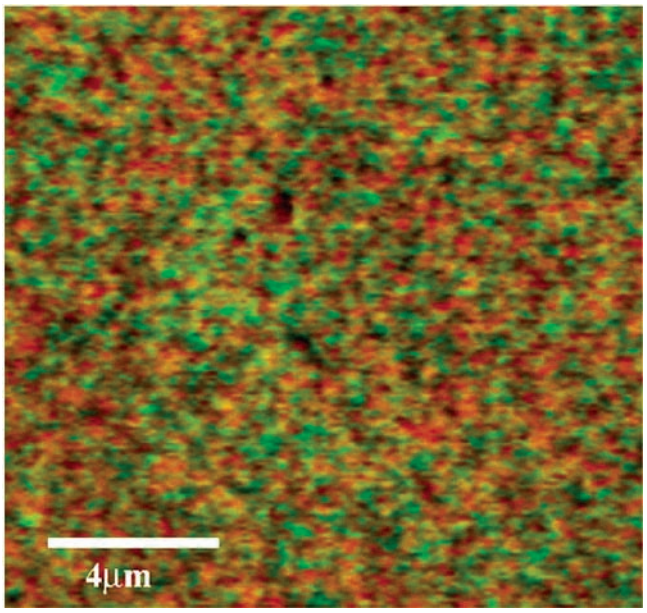

Figure 8. Fluorescence micrograph of LB film transferred from the surface of a 1:1 BCAS/BLG mixture (total protein concentration = 200 nM). BCAS signal is shown in green, and BLG signal is shown in red.

By applying a simple rheological model, we have shown that the surface dilatational moduli could be used to estimate the surface composition. This is probably because this technique compresses the interface, and so the response is sensitive to the molecular structure of the individual components rather than any interactions between the molecules. The surface shear moduli are more sensitive to the interactions forming within the network, which control the integrity of the film. The models used here assumed no interactions between the components and so were of limited value for analyzing the surface shear results. However, the data suggest that although the surface composition equilibrated within the first 40 min, interactions continue to develop and strengthen the network. The source of these interactions is not yet fully understood and requires further investigation. Also, if the surface pressure does not increase beyond a critical value where displacement occurs, over the time scale of the experiments, the surface composition remains constant. Considering the spatial distribution of the components within the interface and that the surface diffusion of pure protein interfaces was close to zero compared to surfactants (*4*), it seems unlikely that the extensive phase separation in these mixed protein systems reported recently (*36*−*38*) will occur over the time scale of these experiments. The fluorescence microscopy data appeared to confirm this. Small-scale heterogeneity was observed, probably as a result of the differing adsorption rates and interactions between BCAS and BLG. Further rearrangements or demixing waswere not observed. To further investigate this, we are using atomic force microscopy and scanning near field optical microscopy to look at mixed films at even higher magnification. The studies are also being moved to higher concentration regimes, where the surface pressures reached may be sufficient to induce displacement of one (or both) protein(s).

Conclusions. The relative surface concentrations of mixed BCAS/BLG adsorbed films were estimated using surface dilatational rheology in conjunction with a simple rheological model. Surface shear rheology was sensitive to component

interactions at the interface, making it difficult to apply simple models to these measurements. As expected, these models are limited and require modification to account for dynamic changes in component interactions. The relative adsorption rates and surface activities of the "competing" proteins were important factors for determining the composition of the adsorbed layer. The interfacial composition and structure reached equilibrium within the first hour of adsorption; thereafter, as long as the experimental conditions remained constant, no further rearrangement or phase separation was observed.

ABBREVIATIONS USED

BCAS, β-casein; BLG, β-lactoglobulin; LB, Langmuir−Blodgett; LS, Langmuir−Schaeffer.

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