Interfacial Dilatational Properties of Milk Proteins Cross-Linked by Transglutaminase

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Milk proteins (β-lactoglobulin and sodium caseinate) were adsorbed at air−water (A−W) and oil−water (O−W) interfaces and cross-linked with a microbial Ca^{2+}-independent transglutaminase. Interfacial dilatational moduli were measured by interfacial tension relaxation. Moduli were generally higher at the O−W interface than at the A−W interface for native β-lactoglobulin, but lower at the O−W interface for native sodium caseinate. Cross-linked films generally had higher dilatational moduli than non-cross-linked films at both the A−W and O−W interfaces. Especially for β-lactoglobulin, it was found that 2 h of cross-linking had a more marked effect at the O−W interface than at the A−W interface. This could be explained by greater unfolding of β-lactoglobulin at the O−W interface, as confirmed by measurement of surface pressure−area (π−A) isotherms for β-lactoglobulin spread at both types of interface. For sodium caseinate enhanced unfolding and adsorption at the O−W interface may have inhibited enzyme action at the interface.

Keywords: *β-Lactoglobulin; caseinate; protein(s); transglutaminase; dilatational rheology*

INTRODUCTION

Surface properties of proteins are important for the formation and stabilization of emulsions and foams. Milk proteins (especially the caseins) are highly surface active and this makes them excellent emulsifiers (Dickinson, 1989). However, the lowering of the interfacial free energy due to their adsorption is not the main role of the protein as an emulsifier. The main function of the protein is to form a macromolecular layer at the interface to protect emulsion droplets or air bubbles from recoalescence just after formation (Walstra, 1988). The rheological properties of the protein interfacial layer are important with respect to its stability to rupture and coalescence (Dickinson, 1992) and are therefore significant for both the initial emulsion/foam formation and the long-term storage stability of emulsions and foams.

A distinction can be made between interfacial shear rheology and interfacial dilatational rheology. Interfacial shear viscosity measurements have been shown to be highly sensitive to interactions between proteins at the air−water (A−W) or oil−water (O−W) interface (Dickinson et al., 1985, 1990), and recently we have shown that enzymatic cross-linking increases the surface shear viscosity of milk proteins significantly (Færgemand et al., 1997a). Though interfacial shear viscosity may be related to the resistance of interfacial protein films to rupture, it is clear that shear is not the most dominant sort of deformation during the formation and handling of emulsions. Most of the processes involved in emulsion/foam formation and stability are connected with changes in interfacial area, so that dilatational rheology is often considered to be of more direct significance to these processes (Murray and Dickinson, 1996). Dilatational rheology of some food proteins has been studied recently. Benjamins and van Voorst Vader (1992) found that the compact globular proteins ovalbumin and bovine serum albumin (BSA) at the A−W interface formed films with high elastic moduli, whereas the more flexible sodium caseinate formed films with lower elastic moduli. The same relative order of magnitude was found with respect to the surface shear modulus of the proteins. The dilatational properties of ovalbumin, BSA, and caseinate were also studied at the O−W interface (Benjamins et al., 1996). Here the same pattern was found; the globular proteins had higher elastic moduli than caseinate; furthermore, it was observed that the moduli of the protein films were all lower at the O−W than at the A−W interface. The oil used was a purified triglyceride. Williams and Prins (1996) investigated β-lactoglobulin and β-casein at the A−W and O−W interfaces. They found that the two proteins gave similar moduli at low concentrations (below 0.1 wt % protein), whereas β-lactoglobulin gave higher moduli than β-casein at higher concentrations. It was suggested that at low concentrations, the adsorbed proteins are equally mobile, probably because the β-lactoglobulin is highly unfolded and its structure more closely approaches that of the more random coil of β-casein. Williams and Prins (1996) also observed that at concentrations above 0.1 wt % β-lactoglobulin the elastic modulus was higher at the O−W interface than at the A−W interface, but at concentrations below 0.1 wt % the difference was negligible. The oil used was a purified paraffin oil. Murray (1997a) and Murray et al. (1998) have recently also observed significantly higher dilatational moduli for β-lactoglobulin at an oil (tetradecane)−water interface.

Thus far studies of different proteins have shown that dilatational rheological properties differ with protein type and the type of interface, particularly the type of oil, and it seems that the more structured proteins have

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higher elastic moduli than less structured proteins (Graham and Phillips, 1980). It is therefore of interest to investigate how interfacial dilatational properties change when further structuring is introduced, for example, by enzymatic cross-linking. Transglutaminase (EC 2.3.2.13) produces cross-links between peptide-bound glutamine and primary amines, such as lysine (Folk and Finlayson, 1977), and has been shown to cross-link both caseins (Sakamoto et al., 1994) and β-lactoglobulin (Færgemand et al., 1997b) in the bulk, as well as at the O−W interface (Færgemand et al., 1997a). In this study we have incubated β-lactoglobulin and sodium caseinate with transglutaminase to investigate the effect of introducing new covalent bonds on the interfacial dilatational rheology, to compare with the previous study of the interfacial shear viscosity (Færgemand et al., 1997a).

MATERIALS AND METHODS

Microbial Ca^{2+}-independent transglutaminase (TGase) derived from *Streptoverticillium mobaraense* was obtained from Ajinomoto Co. (Japan). The enzyme was purified from the commercial enzyme preparation as described previously (Færgemand et al., 1997a). The specific activity of the enzyme preparation was 7.4 units/mg determined using the hydroxamate procedure as described previously (Færgemand and Qvist, 1997), with 1 unit of transglutaminase causing the formation of 1 μmol of hydroxamic acid/min at pH 7.0 and 37 °C. The β-lactoglobulin was a native sample prepared from commercial whey protein, provided by Danmark Protein (Videbaek, Denmark). Sodium caseinate was from DMV (Veghel, The Netherlands). *n*-Tetradecane (99%) was from Sigma Chemical Co. (St. Louis, MO). All other chemicals were AnalR grade reagents from BDH Merck (Poole, Dorset, U.K.). All water used was from a Millipore alpha-Q purification system with a surface tension of 72.0 mN m^{-1} at 25 °C.

Interfacial Rheological Measurements. Surface dilatational moduli were measured using a Langmuir trough method developed recently (Murray and Nelson, 1996). Measurements were made on adsorbed or spread protein films at the A−W or O−W interface. All measurements were performed at 40 °C for consistency with the previous measurements on the interfacial shear rheology. (At this temperature the action of the enzyme is conveniently a little more rapid.)

Adsorbed Films. After solubilization, 10^{-3} wt % protein solutions in phosphate buffer (pH 7.0, $I = 0.05$ M) were kept at 40 °C for 30 min prior to film formation to equilibrate to the measuring temperature. Approximately 600 mL of the protein solution was then poured into the trough. The surface tension was then measured using a Wilhelmy plate (of roughened mica) dipped into the protein solution. Immediately after the protein solution was added to the trough, the interface was rapidly reduced to a very small area (smaller than used in the subsequent measurements) and the surface was sucked clean with a vacuum line. The interface was then re-expanded to 40% of the maximum obtainable area and the trough was covered with a thermostated box. This procedure was to avoid contamination from the air as far possible during the setting up of the film. At the A−W interface, the adsorption of protein to the interface was followed from this point by measuring the surface tension over time. For the O−W interface *n*-tetradecane—which had been kept at 40 °C for 30 min—was gently poured on top of the protein solution at this stage and the interfacial tension was followed using a hydrophobic Wilhelmy plate, made from carbon black coated mica, completely submerged in the oil layer and suspended at the O−W interface. It was ensured that the plate made zero contact angle with the oil phase throughout the experiment. [see Murray and Nelson (1996) and Murray (1997a) for details]. When the equilibrium interfacial tension had been reached, the interface was subjected to a sudden area increase of 10% (from 0.4 to 0.44 of the maximum obtainable interfacial area) within 1 s, and the resultant behavior of the interfacial tension (γ) with time was monitored. Each experiment was repeated a least two times, and the results were reproducible to within 0.3 mN m^{-1}. The errors on the corresponding dilatational moduli (see below) were therefore of the order of 3 mN m^{-1}.

Spread Films. Full details of the procedures used for preparing and examining protein films on the Langmuir trough are given by Murray and Nelson (1996) and Murray (1997a). Phosphate buffer (at 40 °C) was poured into the trough and the surface sucked clean as described above. After the surface had been cleaned, the surface was re-expanded to 70% of the maximum obtainable area. For the A−W interface spreading was then performed. The concentration of the aqueous protein spreading solution was 0.2 mg/mL and typically 50 μL was spread. At the O−W interface, *n*-tetradecane was added on top of the clean buffer surface before spreading of the protein. Spreading took ≈10 min, and measurements were then started 20−30 min later, when the temperature had re-equilibrated at 40 °C. (The temperature dropped during spreading because the trough was open to the atmosphere.) The interface was compressed at a constant slow speed to obtain a surface pressure−area ($\pi-A$) isotherm. The speed of compression was $dA_r/dt \approx 1.5 \times 10^{-4}$ s^{-1}, where A_r is the area relative to the maximum trough area and t is time. This compression speed was previously found to be slow enough that compression at half this speed resulted in no noticeable change in the isotherm (Murray and Nelson, 1996). The isotherms were therefore considered to be "equilibrium" isotherms. Isotherm experiments were repeated at least two times, and the interfacial pressure readings were reproducible to within 0.2−0.3 mN m^{-1}.

Cross-Linking of Protein Films. After experiments on the films of native protein had been performed, protein films were cross-linked by adding microbial transglutaminase. A few milliliters of the enzyme solution were injected into the aqueous phase via a syringe. This was followed by gentle stirring via a glass rod, which swept over the base of the trough to ensure that the enzyme was evenly dispersed throughout the aqueous phase within ≈1 min. Enzyme was dosed in at 20 units of transglutaminase/g of substrate protein in the trough, and cross-linking was allowed for usually 2 h before the properties of the cross-linked film were measured. Occasionally, films were left overnight and measurements made after 20 h of cross-linking. Measurements were performed as described for the native protein films. The injectate itself was shown previously to have negligible surface activity at the low concentrations of added enzyme used (Færgemand et al., 1997a).

RESULTS AND DISCUSSION

When the area, A, of the protein films is expanded, there is a rise in interfacial tension, γ, due to a lower concentration of protein at the interface. The rate of change of interfacial stress (i.e., γ) with respect to the increase in interfacial strain (i.e., the relative change in interfacial area) defines the dilatational elasticity, ϵ

$$\epsilon = d\gamma/d \ln A \quad (1)$$

Similarly, the interfacial dilatational viscosity, κ, is defined by

$$\kappa = d\gamma/[(d \ln A)/dt] \quad (2)$$

where t is time. The higher the dilatational elasticity and viscosity, then the greater the resistance to expansion of the film. Dilatational moduli were obtained by applying a Fourier transform to the decay of the interfacial tension back to the equilibrium value. For a step increase in area, ΔA, which proceeds more rapidly than the time scale (frequency) of the recovery consid-

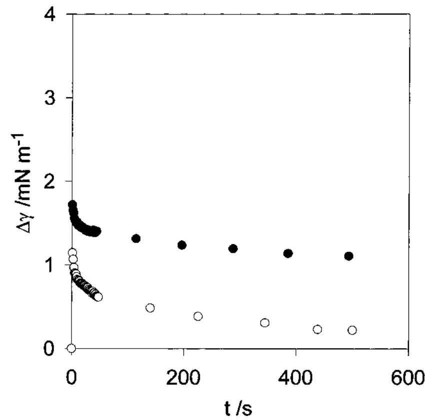

Figure 1. Relaxation of interfacial tension (γ) in adsorbed β-lactoglobulin films at the A−W interface after expansion of the interfacial area by 10%: (open symbols) native β-lactoglobulin; (solid symbols) β-lactoglobulin cross-linked for 2 h.

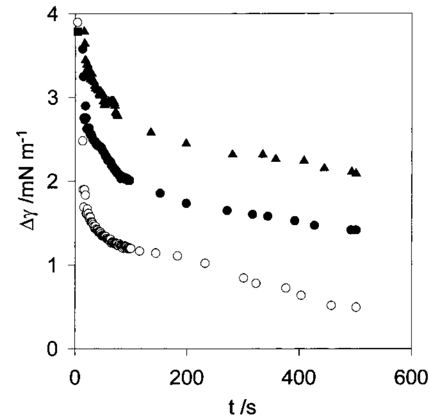

Figure 2. Relaxation of interfacial tension (γ) in adsorbed β-lactoglobulin films at the O−W interface after expansion of the interfacial area by 10%: (○) native β-lactoglobulin; (●) β-lactoglobulin cross-linked for 2 h; (▲) β-lactoglobulin cross-linked for 20 h.

ered, the equations are (Loglio et al., 1984; Cárdenas-Valera and Bailey, 1993)

$$\epsilon' = \epsilon = \omega/(\Delta A/A)\int_0^\infty \Delta\gamma(t)\sin(\omega t)\,\mathrm{d}t \qquad (3)$$

and

$$\epsilon'' = \omega\kappa = \omega/(\Delta A/A)\int_0^\infty \Delta\gamma(t)\cos(\omega t)\,\mathrm{d}t \qquad (4)$$

where ϵ' and ϵ'' are the real (elastic or "storage") and imaginary (viscous or "loss") parts of the complex modulus, $\omega = 2\pi f$, f is the frequency, and $\pi = 3.141$. The requirement of integration between zero time and infinite time in practice means meaningful results are obtained only for frequencies no higher than ($1/2\tau$), where τ is the shortest time step between measurements. In this case τ is limited to 1 s, the time of expansion of the film. Similarly, the decay curve should be integrated until the displacement of the interfacial tension $\Delta\gamma$ has decayed to zero, which in practice depends upon the experimental error in measuring the tension, and this determines the practical low-frequency limit of the measurements. Numerous other methods have of course been employed to measure dilatational rheology of protein films [see Murray and Dickinson (1996), Warburton (1996), and Miller et al. (1996) for recent reviews]. For adsorbed films two contributions to the relaxation of γ back to the equilibrium value may be distinguished: (a) the adsorption of further protein from the bulk aqueous solution and (b) the redistribution and reorientation of protein already adsorbed at the interface. In the case of spread protein monolayers, only the latter contribution exists.

***β*-Lactoglobulin.** Figure 1 shows the relaxation behavior of adsorbed β-lactoglobulin films at the A−W interface for the first 600 s after the expansion. The figure shows results for a native β-lactoglobulin film and for a film that was cross-linked for 2 h with transglutaminase prior to expansion. It is clear that for the cross-linked film there was a larger initial rise in interfacial tension, followed by a much slower relaxation compared with the behavior of the native film. Since transglutaminase is not capable of cross-linking β-lactoglobulin in the bulk under these conditions, the different relaxation behavior must be ascribed to the interfacial protein. At the O−W interface it has been shown that β-lactoglobulin is accessible to enzymatic cross-linking because it becomes unfolded (Færgemand et al., 1997a) so that the different behavior of the film at the A−W interface when exposed to the enzyme is also probably due to transglutaminase-induced cross-links between the unfolded molecules. A higher density of covalent cross-links is expected to slow the rate of rearrangement of molecules in the film and the contribution that this makes to the relaxation back to the equilibrium interfacial tension. Evidently, adsorption of protein from the bulk is not able to compensate for the inhibition of the interfacial cross-linked protein to rearrange—highlighting the significant contribution of the latter to the dilatational behavior (Murray, 1997a,b).

Figure 2 shows relaxation of adsorbed β-lactoglobulin films at the O−W interface before and after cross-linking with transglutaminase. For the native protein at the O−W interface there was a larger initial change in interfacial tension compared with the native protein at the A−W interface (Figure 1). The relaxation of the film that had been cross-linked for 2 h was much slower than the relaxation of the native film, as expected from the above results at the A−W interface. In a another experiment the film was incubated with transglutaminase for 20 h and the relaxation was slower still. This agrees well with the formation of additional bonds by letting the enzyme cross-link for a longer time. It is difficult to compare the relaxation of the films at the O−W and A−W interfaces because the magnitude of the initial increase in interfacial tension varies in each case. A better way is to compare the dilatational moduli obtained via Fourier transformation of the decay curves as described earlier. After the first 600 s of the decay, the interfacial tension decreased slowly back to the equilibrium tension value within 1−2 h for all of the experiments. These full decay curves were Fourier transformed to obtain the dilatational moduli: Figure 3 shows the results of Fourier transforming the data for β-lactoglobulin film at the A−W interface (Figure 1).

The native β-lactoglobulin film at the A−W interface had a (maximum) dilatational elastic modulus of ~6 mN m^{-1} at a frequency of 10^{-3} Hz. This value (obtained at 40 °C) is a little lower than measurements made elsewhere at 30 °C (Murray et al., 1998). The adsorbed β-lactoglobulin film could be described as a viscoelastic material, characterized by dominantly viscous behavior in the low-frequency range and predominantly elastic behavior at higher frequencies, with a crossover point

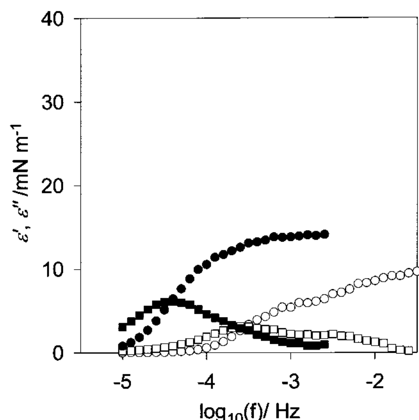

Figure 3. Frequency dependence of interfacial dilatational moduli of adsorbed β-lactoglobulin films at the A−W interface: (○) ϵ' for native β-lactoglobulin; (□) ϵ'' for native β-lactoglobulin; (●) ϵ' for β-lactoglobulin cross-linked for 2 h; (■) ϵ'' for β-lactoglobulin cross-linked for 2 h.

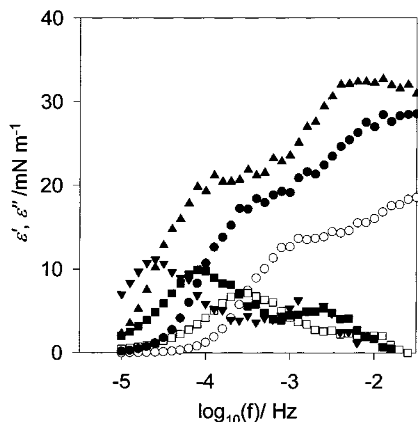

Figure 4. Frequency dependence of interfacial dilatational moduli of adsorbed β-lactoglobulin films at the O−W interface: (○) ϵ' for native β-lactoglobulin; (□) ϵ'' for native β-lactoglobulin; (●) ϵ' for β-lactoglobulin cross-linked for 2 h; (■) ϵ' for β-lactoglobulin cross-linked for 2 h; (▲) ϵ' for β-lactoglobulin cross-linked for 20 h; (▼) ϵ'' for β-lactoglobulin cross-linked for 20 h.

between the storage and loss modulus at 3×10^{-4} Hz. Generally, the moduli for the cross-linked β-lactoglobulin film at the A−W interface were higher than for the native film. At 10^{-3} Hz the elastic dilatational modulus of the cross-linked film was ~14 mN m^{-1}. Furthermore, the cross-linked film was characterized by a lower crossover frequency, ~3×10^{-5} Hz. One can calculate the loss tangent (θ), which is, by analogy to bulk rheology, defined as

$$\tan \theta = \epsilon''/\epsilon' \qquad (5)$$

For the native β-lactoglobulin film $\theta = 0.41$ and for the cross-linked film $\theta = 0.08$ at 10^{-3} Hz, so that although the elastic modulus increased by “only” a factor 2−3, the mechanical properties of the interfacial film changed significantly. Fourier analysis was also applied to the data obtained at the O−W interface (Figure 2), and the results are shown in Figure 4.

It is evident that the properties of the adsorbed β-lactoglobulin film at the O−W interface differed from the properties of the film at the A−W interface. The moduli for β-lactoglobulin films were higher at the O−W interface than at the A−W interface over most of the frequency range. This may reflect the fact that the

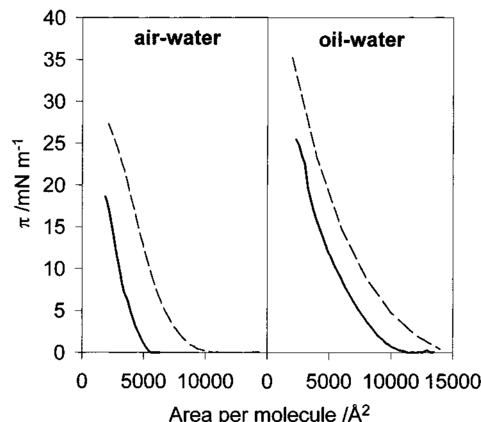

Figure 5. π−A isotherms for spread β-lactoglobulin monolayers at the A−W (left) and O−W (right) interfaces: (solid lines) native β-lactoglobulin; (broken lines) β-lactoglobulin monolayers cross-linked for 2 h. Areas per molecule were calculated using the molecular mass for a β-lactoglobulin dimer (36.8 kDa).

protein is more unfolded at the O−W interface, possibly due to better solubilization of the hydrophobic side chains of the protein. As noted earlier, Williams and Prins (1996) also found that β-lactoglobulin films were slightly more viscous at the O−W interface compared to the A−W interface over a narrow range of concentrations, whereas Benjamins et al. (1996) found the opposite. It is quite clear that cross-linking significantly increased the film elasticity at both interfaces. The higher dilatational moduli obtained for the cross-linked film at the O−W interface compared to the A−W interface are also possibly due to greater unfolding at the O−W interface (see below), which aids cross-linking of the film by the enzyme. Cross-linking the O−W film for 20 h rather than 2 h gave a modest further increase in the moduli. This is in agreement with the earlier study of the interfacial shear viscosity, which increased rapidly in the first few hours after cross-linking but which exhibited only slow further increase after about 4 h of exposure to the same enzyme concentration.

It should be noted that the dilatational moduli were measured at a single value of strain (10%). Dilatational measurements on protein films are frequently made at strains of this order of magnitude and the rheological behavior claimed to be in the linear regime, while shear moduli are often highly nonlinear at even small strains/rates of strain (Murray and Dickinson, 1996). More recent measurements on non-cross-linked β-lactoglobulin films, measured via the same technique (Murray et al., 1998), also suggest that if a linear regime exists, this is below 10% strain. The highly complex, cross-linked nature of the enzyme-treated films probably means that they behave similarly. Thus, in this respect the moduli might be more correctly viewed as *apparent* moduli, dependent on the strain applied.

To investigate further the properties of the interfacial films, π−A isotherms of spread β-lactoglobulin were measured. Figure 5 shows the π−A isotherms of native and enzymatically cross-linked β-lactoglobulin monolayers at the A−W and O−W interfaces, assuming the molecular mass of the β-lactoglobulin dimer (36.8 kDa). Although the dimer may in fact dissociate at the interface and it is clear that the molecular mass must change on cross-linking, this serves as a method of comparing the behavior of the films before and after cross-linking. It is clear that the β-lactoglobulin was

more expanded at the O−W interface compared to the A−W interface, and this agrees with earlier studies on β-lactoglobulin and bovine serum albumin films (Murray and Nelson, 1996; Murray, 1997a). This supports the hypothesis that β-lactoglobulin was more extensively cross-linked at the O−W interface, resulting in higher dilatational moduli, because of a greater degree of unfolding at the O−W interface. When the spread β-lactoglobulin monolayers were cross-linked, the $\pi-A$ isotherms were shifted toward higher areas per molecule for the same π, showing that the cross-linked films were more resistant to compression.

The existence of more expanded films at the O−W interface is opposite to the effect noted by Graham and Phillips (1979), who studied radiolabeled lysozyme, bovine serum albumin (BSA), and β-casein at the A−W and paraffin−water interfaces. As noted in the Introduction, it appears that protein film behavior is dependent on the type of oil. This is not surprising, since the solvency of a triglyceride oil for the nonpolar segments of the protein might be expected to be different from that for a paraffin oil, for example. It should also be noted that the radiolabeled proteins such as those used by Graham and Phillips had different surface activities compared to the native proteins (Adams et al., 1971) and the more condensed state at the O−W interface was explained in terms of placing a higher density of hydrophobic polypeptide "loops" into the oil phase (Graham and Phillips, 1979). To our knowledge, it has never been demonstrated that polypeptide chains of any amino acid are soluble in oil, which is probably prevented by the polar nature of the peptide bond. It seems more likely that a greater attraction of an oil phase for the hydrophobic segments leads to greater protein unfolding and therefore a higher area per molecule, as observed here.

To summarize, the results for adsorbed and spread β-lactoglobulin films show that cross-linking took place at both the A−W and O−W interfaces, resulting in higher dilatational moduli. Cross-linking was most efficient at the O−W interface, possibly due to the more unfolded state of β-lactoglobulin at this interface.

Sodium Caseinate. From a technological point of view, one of the most important proteinaceous emulsifiers, besides whey protein, is sodium caseinate. It is therefore useful to compare the effects of transglutaminase cross-linking on the interfacial rheological properties of caseinate with those of β-lactoglobulin—the main component of whey protein. This is also of interest because the structural properties of the caseins and whey proteins are quite different—the major casein components being relatively unstructured proteins while the whey proteins are predominantly highly folded globular proteins.

Figure 6 shows the surface dilatational moduli of adsorbed sodium caseinate films, obtained from relaxation experiments performed at the A−W interface in the same way as for the β-lactoglobulin films.

If we compare the curves in Figure 6 with those in Figure 3 (for β-lactoglobulin), it is seen that the moduli of the caseinate films are slightly lower than the moduli of the β-lactoglobulin films. The interfacial shear viscosity of native caseinate is much lower than for β-lactoglobulin [e.g., see Færgemand et al. (1997a)]. From Figure 6 it is evident that for caseinate films also, enzymatic cross-linking increases the dilatational elasticity. The rheological properties of the cross-linked and

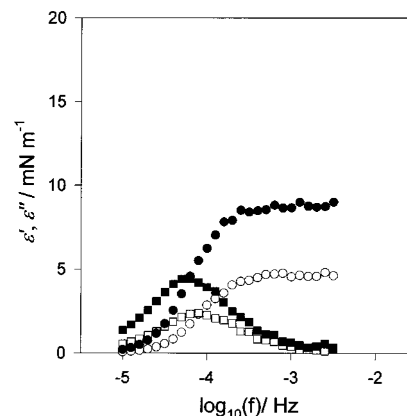

Figure 6. Frequency dependence of interfacial dilatational moduli of adsorbed sodium caseinate films at the A−W interface: (○) ϵ' for native caseinate; (□) ϵ'' for native caseinate; (●) ϵ' for caseinate cross-linked for 2 h; (■) ϵ'' for caseinate cross-linked for 2 h.

native caseinate may be compared with the properties of the β-lactoglobulin films by calculating the moduli and loss tangents at 10^{-3} Hz. The native caseinate film had an elastic modulus of 4.5 mN m^{-1} and a loss tangent of $\approx$0.08 at 10^{-3} Hz; the corresponding values for a film that had been cross-linked for 2 h were 8 mN m^{-1} and 0.07. For the native β-lactoglobulin film the corresponding values were 6 mN m^{-1} and 0.41 and for the cross-liked film, 14 mN m^{-1} and 0.08. Thus, the cross-linked caseinate at the A−W interface forms a film that is slightly more elastic than native β-lactoglobulin but somewhat less elastic than cross-linked β-lactoglobulin film. This is probably due to the more flexible nature of the casein molecules compared to β-lactoglobulin. However, interpretation of the effects of transglutaminase action in the caseinate system is a little more difficult, since transglutaminase will also cross-link the caseins in the bulk, because their structure is already considerably unfolded. Cross-linking in the bulk will lead to an increase in the molecular weight of the adsorbing species and will inhibit relaxation of the interfacial tension back the equilibrium value. Clearly, however, this effect is not strong enough to increase the elasticity of the film above that of the cross-linked β-lactoglobulin system, in which cross-linking is only possible in the interface. The lower overall moduli for the caseinate systems may therefore be attributed to greater flexibility and rapidity of rearrangement and adsorption of the caseinate proteins. An additional possibility is that cross-linking occurs between protein in the adsorbed film and protein in the bulk. This would further inhibit adsorption of casein from the bulk (and relaxation back to equilibrium) by forming a layer that is more difficult for free protein to diffuse through, as well as inhibiting the access of the enzyme molecules to the interface to perform further cross-linking. However, we have no evidence that such cross-linking does occur or has a significant effect.

Figure 7 shows the interfacial dilatational moduli of caseinate at the O−W interface. The results clearly differ from those obtained at the A−W interface. Although both the elastic and viscous moduli increased after 2 h of cross-linking, there was no significant shift in the ϵ'' peak or the attainment of the ϵ' plateau value to lower f. Thus, recovery of the interfacial tension took place over a similar time scale as for the native film.

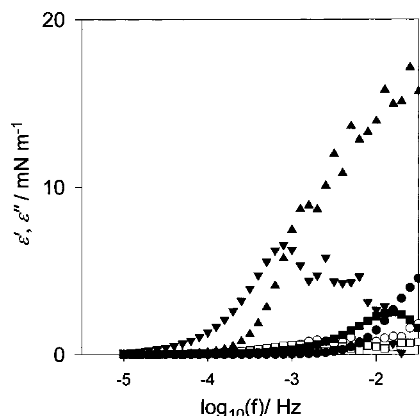

Figure 7. Frequency dependence of interfacial dilatational moduli of adsorbed sodium caseinate films at the O−W interface: (○) ϵ' for native caseinate; (□) ϵ'' for native caseinate; (●) ϵ' for caseinate cross-linked for 2 h; (■) ϵ'' for caseinate cross-linked for 2 h; (▲) ϵ' for caseinate cross-linked for 20 h; (▼) ϵ'' for caseinate cross-linked for 20 h.

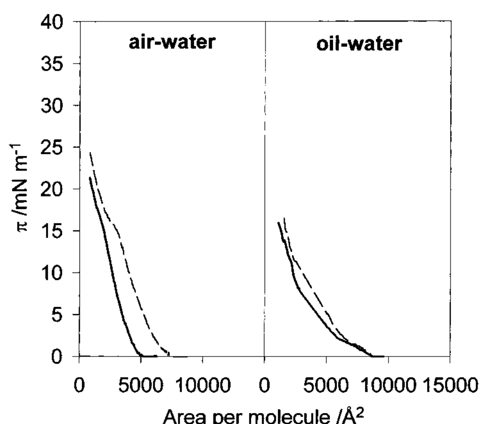

Figure 8. $\pi-A$ isotherms for spread sodium caseinate monolayers at the A−W (left) and O−W (right) interfaces at 40 °C and pH 7.0: (solid lines) native sodium caseinate; (broken lines) sodium caseinate cross-linked for 2 h. Areas per molecule were calculated using an average molecular mass for sodium caseinate of 23.5 kDa.

This was surprising, since initially it was expected that the caseinate film at the O−W interface might possess the most unfolded protein and therefore form the most cross-linked film, with the least capacity for rearrangement to establish the equilibrium tension. One explanation for this behavior may be that if protein becomes very unfolded and too strongly adsorbed at the O−W interface, then it is difficult for the enzyme to access the substrate groups and perform the cross-linking. (After 20 h of cross-linking, the behavior approached more closely the behavior of the cross-linked A−W film, with further increase in the moduli and a shift in peak values to lower *f*.) Figure 8 indeed shows that, similar to β-lactoglobulin, the native caseinate monolayers were more expanded at the O−W interface compared to the A−W interface, but there was not so much difference on cross-linking caseinate at the O−W interface. Areas per molecule were calculated using an average molecular mass for sodium caseinate of 23.5 kDa, which is an average of the molecular masses of the principal components of caseinate: α_{s1}-, α_{s2}-, β-, and κ-casein. This was for the purpose of comparing the behavior before and after cross-linking, since the exact state of aggregation of the different caseins in the film, before or after cross-linking, is unknown.

Conclusion. In conclusion, this study shows that the surface dilatational moduli of milk proteins at the O−W and A−W interfaces increase significantly after enzymatic cross-linking with transglutaminase. These results agree qualitatively with previous measurements of interfacial shear viscosity—although the magnitude of the effect on the dilatational moduli is not as large as on shear viscosity. The results indicate that enzymatic cross-linking may be a valuable tool for controlling interfacial rheological properties, which may be of importance for the stability of emulsions or gas bubbles toward coalescence.

LITERATURE CITED

Adams, D. J.; Evans, M. T. A.; Mitchell, J. R.; Phillips, M. C.; Rees, P. M. Adsorption of lysozyme and some acetyl derivatives at the air−water interface. *J. Polym. Sci.*: *Part C* **1971**, *34*, 167−179.

Benjamins, J.; van Voorst Vader, F. The determination of the surface shear properties of adsorbed protein layers. *Colloids Surf.* **1992**, *65*, 161−174.

Benjamins, J.; Cagna, A.; Lucassen-Reynders, E. H. Viscoelastic properties of triacylglycerol/water interfaces covered by proteins. *Colloids Surf. A: Physicochem. Eng. Aspects* **1996**, *114*, 245−254.

Cárdenas-Valera, A. E.; Bailey, A. I. The interfacial rheological behaviour of monolayers of PEO/PMMA graft copolymers spread at the air/water and oil/water interfaces. *Colloids Surf. A: Physicochem. Eng. Aspects* **1993**, *79*, 115−127.

Dickinson, E. Surface and emulsifying properties of caseins. *J. Dairy Res.* **1989**, *56*, 471−477.

Dickinson, E. *An Introduction to Food Colloids*; Oxford Science Publishers: Oxford, U.K., 1992.

Dickinson, E.; Murray, B. S.; Stainsby, G. Time-dependent surface viscosity of adsorbed films of casein + gelatin at the oil−water interface. *J. Colloid Interface. Sci.* **1985**, *106*, 259−262.

Dickinson, E.; Rolfe, S. E.; Dalgleish, D. G. Surface shear viscometry as a probe of protein−protein interactions in mixed milk protein films adsorbed at the oil−water interface. *Int. J. Biol. Macromol.* **1990**, *12*, 189−194.

Færgemand, M.; Qvist, K. B. Transglutaminase: Effect on rheological properties, microstructure and permeability of set style acid milk gel. *Food Hydrocolloids* **1997**, *11*, 287−292.

Færgemand, M.; Murray, B. S.; Dickinson, E. Cross-linking of milk proteins with transglutaminase at the oil−water interface. *J. Agric. Food Chem.* **1997a**, *45*, 2514−2519.

Færgemand, M.; Otte, J.; Qvist, K. B. Enzymatic crosslinking of whey proteins by a Ca^{2+} independent microbial transglutaminase from *Streptomyces lydicus*. *Food Hydrocolloids* **1997b**, *11*, 19−25.

Folk, J. E.; Finlayson, J. S. The ϵ-(γ-Glutamyl)lysine Crosslink and the Catalytic Role of Transglutaminases. *Adv. Protein Chem.* **1977**, *31*, 2−120.

Graham, D. E.; Phillips, M. C. Proteins at liquid interfaces. *J. Colloid Interface Sci.* **1979**, *70*, 403−414, 415−426, 427−439.

Graham, D. E.; Phillips, M. C. Proteins at liquid interfaces. *J. Colloid Interface Sci.* **1980**, *76*, 227−239.

Loglio, G.; Tesei, U.; Cini, R. Dilational properties of monolayers at the oil-air interface. *J. Colloid Interface Sci.* **1984**, *100*, 393−396.

Miller, R.; Wüstneck, R.; Kragel, J.; Kretzschmar, G. Dilational and shear rheology of adsorption layers at liquid interfaces. *Colloids Surf. A* **1996**, *111*, 75−118.

Murray, B. S. Equilibrium and dynamic surface pressure−area measurements on protein films at the air−water and oil−water interfaces. *Colloids Surf. A* **1997a**, *125*, 73−83.

Murray, B. S. Dynamics of proteins at air−water and oil−water interfaces using novel Langmuir trough methods. *Prog. Colloid Polym. Sci.* **1997b**, *103*, 41−50.

Murray, B. S.; Dickinson, E. Interfacial rheology and the dynamic properties of adsorbed films of food proteins and surfactants. *Food Sci. Technol. Int.* **1996**, *2*, 131−145.

Murray, B. S.; Nelson, P. V. A novel Langmuir trough for equilibrium and dynamic measurements on air−water and oil−water monolayers. *Langmuir* **1996**, *12*, 5973−5976.

Murray, B. S.; Ventura, A.; Lallemant, C. Dilational rheology of protein + nonionic surfactant films at air−water and oil−water interfaces. *Colloids Surf. A* **1998**, in press.

Sakamoto, H.; Kumazawa, Y.; Motoki, M. Strength of protein gels prepared with microbial transglutaminase as related to reaction conditions. *J. Food Sci.* **1994**, *59*, 866−871.

Walstra, P. The role of proteins in the stabilisation of emulsions. In *Gums and Stabilisers for the Food Industry*; Phillips, G. O., Williams, P. A., Wedlock, D. J., Eds; IRL Press: Oxford, U.K., 1988.

Warburton, B. Interfacial rheology. *Curr. Opin. Colloid Interface Sci.* **1996**, *1*, 481−486.

Williams, A.; Prins, A. Comparison of the dilational behaviour of adsorbed milk proteins at the air−water and oil−water interface. *Colloids Surf. A* **1996**, *114*, 267−275.

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