Cross-Linking of Milk Proteins with Transglutaminase at the Oil−Water Interface

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The influence of covalent cross-linking with transglutaminase on the time-dependent surface shear viscosity of adsorbed milk protein films at the *n*-tetradecane−water interface has been investigated for sodium caseinate, α_{s1}-casein, β-casein, and β-lactoglobulin. Proteins were adsorbed from 10^{-3} wt % aqueous solutions at pH 7, and apparent surface viscosities were recorded at 40 °C in the presence of various enzyme concentrations. Results for casein systems showed a rapid enhancement in surface viscoelasticity due to enzymatic cross-linking, with a substantially slower development of surface shear viscosity for α_{s1}-casein than for β-casein. While adsorbed β-lactoglobulin showed less relative increase in surface viscosity than the caseins, the results for β-lactoglobulin showed the presence of a substantial rate of cross-linking of the globular protein in the adsorbed state, whereas in bulk solution β-lactoglobulin was cross-linked only after partial unfolding in the presence of dithiothreitol. A maximum in shear viscosity at relatively short times following addition of a moderately high dose of enzyme was attributed to formation of a highly cross-linked protein film followed by its brittle fracture. Enzymatic cross-linking of protein before exposure to the oil−water interface was found to produce a slower increase in surface viscosity than enzyme addition immediately after interface formation or to the aged protein film.

Keywords: *Surface shear viscosity; transglutaminase; casein(ate); β-lactoglobulin; protein film; oil−water interface; surface rheology; enzymatic cross-linking*

INTRODUCTION

An adsorbed layer of protein at the oil−water or air−water interface provides the primary stabilization of oil droplets or air bubbles in most food colloids. The viscoelastic properties of the interfacial protein film are particularly important in relation to the stability of emulsions and foams toward coalescence (Dickinson, 1992). Surface shear viscometry at the oil−water interface is a technique that is very sensitive to the structure and composition of an adsorbed protein layer and to the nature of intermolecular interactions in the film (Dickinson *et al.*, 1985a, 1988a).

The different milk proteins vary enormously in their surface shear rheological behavior. Adsorbed films of the flexible disordered caseins have surface shear viscosities approximately 2 orders of magnitude lower than those of the globular whey proteins (Castle *et al.*, 1986; Dickinson *et al.*, 1988a). Under the same solution conditions, the surface shear viscosity of sodium caseinate at the oil−water interface is rather higher than that for either of the pure major proteins, α_{s1}-casein or β-casein. This is probably mainly attributable to the presence of κ-casein in sodium caseinate (Dickinson, 1989).

Measurements of the time-dependent surface shear rheology of β-lactoglobulin at the oil−water interface have shown that the surface viscosity of this globular whey protein continues to rise even after monolayer adsorption is apparently complete (Dickinson *et al.*, 1990). This is explained, at least in part, by the slow polymerization of β-lactoglobulin molecules at the interface via disulfide linkages (Dickinson and Matsumura, 1991). An apparent relationship between the surface shear rheology of β-lactoglobulin films at the oil−water interface and the orthokinetic stability of oil-in-water emulsions has been observed (Dickinson *et al.*, 1993; Chen *et al.*, 1993) in destabilization studies involving the addition of small amounts of nonionic surfactant to systems containing adsorbed protein films and protein-stabilized emulsions. The reduction in surface shear viscosity at very low surfactant/protein ratios correlates well with the observed rate of shear-induced coalescence. There are also other examples in the literature of an apparent relationship between surface shear viscosity and emulsion stability. For instance, it has been separately reported that heating β-lactoglobulin (to 70 °C) at the oil−water interface significantly enhances adsorbed layer surface viscoelasticity (Dickinson and Hong, 1994) and that the coalescence rate of β-lactoglobulin-stabilized emulsions decreases with heating time at 70 °C (Das and Kinsella, 1990).

One way of substantially changing the internal structure and aggregation state of protein molecules, with a view to influencing the surface rheology and colloid stabilizing properties, is through the introduction of new covalent bonds. Chemical modification is undesirable for food use because of the possibility of unknown side reactions and toxicity effects. Enzymatic cross-linking is, however, generally acceptable. One such enzyme that has potential application for protein cross-linking is transglutaminase (EC 2.3.2.13). This is a transferase that catalyzes an acyl transfer reaction between peptide-bound glutamines and a number of primary amines. When the ϵ-amino group of peptide-bound lysine acts as an acceptor in the reaction, the ϵ-(γ-glutamyl)lysine cross-link is formed (Folk and Finlayson, 1977); this bond can be intramolecular or intermolecular, depend-

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ing on whether the glutamine and lysine residues are situated on the same protein molecule or on two separate ones.

There have already been various reports of transglutaminase cross-linking applied to a range of food proteins, *e.g.*, caseins (Ikura *et al.*, 1980a; Traoré and Meunier, 1991; Sakamoto *et al.*, 1994), soybean proteins (Ikura *et al.*, 1980b; Kang *et al.*, 1994) and whey proteins (Aboumahmoud and Savello, 1990; Færgemand *et al.*, 1997), under various experimental conditions. Cross-linking of milk proteins in aqueous solution results in enhanced viscosity and/or gelation as a direct result of increased protein aggregation and network formation (Nio *et al.*, 1986a; Sakamoto *et al.*, 1994; Færgemand *et al.*, 1997). Transglutaminase-catalyzed cross-linking can also be used to convert liquid-like oil-in-water emulsions into elastic emulsion gels (Nio *et al.*, 1986b; Matsumura *et al.*, 1993; Dickinson and Yamamoto, 1996). It seems likely that the introduction of additional intermolecular covalent cross-links into adsorbed protein films by treatment with transglutaminase could influence the stability and rheology of protein-containing emulsions and foams. The aim of this present study is to investigate the effect of transglutaminase cross-linking on the surface shear viscosity of milk proteins at the oil−water interface.

MATERIALS AND METHODS

Materials. The microbial Ca^{2+}-independent transglutaminase (TGase) derived from *Streptoverticillium mobaraense* was obtained from Ajinomoto Co. (Tokyo, Japan). Before use, it was purified to remove salts from the enzyme preparation. A 25 g sample of the commercial product (with enzyme content of *ca.* 1%) was suspended in 200 mL of 20 mM sodium acetate (pH 6.0) and dialyzed extensively against the same buffer. After passing through a 0.45 μm filter, the solution was applied onto a Hiload SP-Sepharose column (16/10) (Pharmacia Biotech, Uppsala, Sweden) and equilibrated with 20 mM sodium acetate (pH 6.0), and the TGase was eluted in 30 column volumes with a linear gradient of 0−0.5 M NaCl at a flow rate of 4 mL min^{-1}. Pools from the Hiload SP-Sepharose column were concentrated in an Amicon cell equipped with a 10 kDa Diaflo membrane (Amico Inc.) followed by a buffer change to 20 mM sodium phosphate (pH 6.5). This solution was further purified on a Pharmacia Blue-Sepharose column (16/10) equilibrated with 20 mM sodium phosphate (pH 6.5). Elution of TGase was performed at a flow rate of 4 mL min^{-1} with a linear gradient of 0−1.0 M NaCl in 10 column volumes. The 3 mL fractions from the Blue-Sepharose column were pooled and concentrated in an Amicon cell as described above and dialyzed extensively against 20 mM sodium acetate (pH 6.0). This enzyme solution was stored at −18 °C, and its activity was determined at the beginning of the surface viscosity experiments. The specific activity of the purified enzyme preparation (composed of a single protein species) was 7.4 units per milligram (U/mg), as determined using the hydroxamate procedure described previously (Færgemand and Qvist, 1997), one unit of transglutaminase causing the formation of 1 μM of hydroxamic acid/min at pH 7.0 at 37 °C.

β-Lactoglobulin was provided by Danmark Protein (Videbaek, Denmark). Sodium caseinate was from DMV (Veghel, Netherlands). Purified individual caseins, α_{s1}-casein or β-casein, were obtained from the Hannah Research Institute (Ayr, U.K.). *n*-Tetradecane (99% pure) and all other reagents were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.).

Surface Shear Viscometry. Steady-state surface shear viscosity measurements were carried out in Leeds using the Couette-type surface rheometer described previously (Dickinson *et al.*, 1985a). Experiments were performed at 40.0 ± 0.2 °C at an intermittent steady shear rate of 1.3×10^{-3} rad s^{-1}. To ensure steady-state conditions, the plane interface was sheared continuously for 10 min prior to each set of readings being taken. Quoted values at particular adsorption times are averages of sets of five readings taken at 1 min intervals over a period of 5 min.

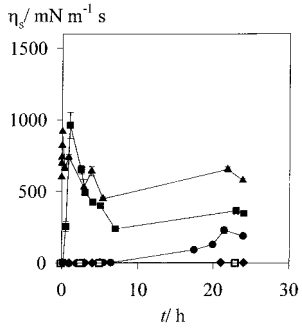

Figure 1. Influence of transglutaminase added immediately after formation of the oil−water interface on the surface shear viscosity of an adsorbed film of sodium caseinate (bulk protein concentration 10^{-3} wt %, pH 7, 40 °C). Apparent surface shear viscosity η_s is plotted against time t for various enzyme doses: (□) no TGase; (◆) 0.2 U/g; (●) 2 U/g; (■) 20 U/g; (▲) 200 U/g. Error bars indicate standard deviations based on sets of five consecutive readings.

A solution of 10^{-3} wt % protein in 20 mM imidazole buffer adjusted to pH 7.0 with HCl was made up freshly before each experiment. The solution was treated with TGase before, during (*i.e.*, immediately following the layering of *n*-tetradecane on top of the protein solution), or after formation of the interfacial film in the rheometer, as required for the particular experiment. When the protein solution was treated with TGase during or after the formation of the adsorbed protein film, the enzyme solution was injected through the hydrocarbon oil phase into the aqueous subphase, which at the same time was gently stirred to distribute the enzyme uniformly within the bulk solution. In the procedure involving treatment of the protein solution with TGase before forming the surface film, the protein solution was incubated with TGase for a specified time, and thereafter the enzyme activity was product-inhibited by increasing the ammonia content through addition of 10 mM NH_4Cl. It is well-known (Folk and Chung, 1969) that transglutaminase is inhibited by NH_4^+, and previously it was found (Færgemand, 1997) that a NH_4Cl concentration of 10 mM is sufficient to inhibit the activity of the enzyme in a concentrated solution of sodium caseinate, as indicated by the constancy of the bulk viscosity. In a few experiments, denatured TGase (inactivated by heating at 100 °C for 5 min) was added to the protein solution in order to check for (nonenzymatic) effects of the TGase protein preparation on the surface activity.

Surface Tensiometry. Interfacial tensions were measured at the hydrocarbon−water interface using the Wilhelmy plate method. Having been prepared as described above, the protein solution (180 mL) was poured into a temperature-controlled glass cell and *n*-tetradecane (45 mL) was gently poured on top of the aqueous solution. Immediately after the oil addition, a roughened mica plate was immersed through the oil layer down to a level approximately 7 mm into the aqueous phase. The pull on the plate was measured with a Sartorius balance. The interfacial tension at 40 °C was monitored continuously with time, and a pseudoequilibrium value was recorded after 10 h.

RESULTS AND DISCUSSION

Figure 1 shows the time-dependent apparent surface shear viscosity, $\eta_s(t)$, of an adsorbed film of sodium caseinate at the oil−water interface incubated with different concentrations of the microbial transglutaminase. In the absence of enzyme, the caseinate surface viscosity at 40 °C and pH 7 is *ca.* 4 mN m^{-1} s. In the presence of a moderately high TGase concentration (20 U/g), the surface viscosity of the caseinate film was found to increase very rapidly to a value of *ca.* 1000 mN m^{-1} s. This initial rapid increase in $\eta_s(t)$ was then

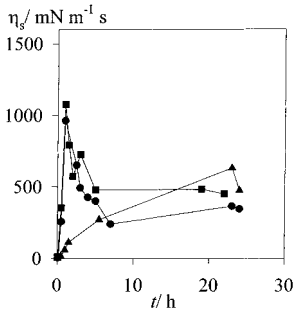

Figure 2. Comparison of time-dependent surface viscosities, $\eta_s(t)$, of films of α_{s1}-casein, β-casein, and sodium caseinate (10^{-3} wt %, pH 7, 40 °C) in the presence of 20 U/g TGase added immediately after forming the oil−water interface: (▲) α_{s1}-casein; (●) β-casein; (■) caseinate (containing α_{s1}-, α_{s2}-, β-, and κ-caseins).

Table 1. Initial Rate of Increase of Surface Shear Viscosity, $d\eta_s/dt$, for Caseins (10^{-3} wt %) at the *n*-Tetradecane−Water Interface (pH 7, Ionic Strength 20 mM, 20 U/g TGase, 40 °C)

protein	$d\eta_s/dt$ (mN m^{-1} s h^{-1})
sodium caseinate	970 ± 250
β-casein	930 ± 300
α_{s1}-casein	120 ± 70

followed by a somewhat slower decrease, reaching an apparently limiting constant value after 5−6 h at high doses. The rate of increase in apparent surface viscosity was much lower at the low enzyme doses, and at a dose of 0.2 U/g no discernible surface viscosity increase was observed over 24 h. So it seems that both the final limiting viscosity and its initial rate of increase are dependent on enzyme concentration. The initial peak in the $\eta_s(t)$ plot, which is observed at high but not low doses, can be attributed to the rapid formation and subsequent fracture of a highly cross-linked and brittle protein network, followed by some re-formation of a different and less uniformly cross-linked interfacial structure, which never reaches such a high surface viscosity as the enzyme-cross-linked film before fracture. (With significance probably just beyond the estimated experimental precision, a further minor peak is sometimes discernible after the primary fracture peak, *e.g.*, in the 200 U/g data set in Figure 1, and in data in some of the subsequent figures. This may be indicative of secondary fracture of the re-formed film.)

Sodium caseinate contains a mixture of α_{s1}-, α_{s2}-, β-, and κ-caseins in the approximate ratio 4:1:4:1. These individual caseins have different surface properties (Dickinson, 1989), and β-casein has a particularly low surface viscosity—approximately 15 times lower that that of sodium caseinate. It is therefore of interest to establish whether the very high surface viscosities reached by cross-linking sodium caseinate can also be achieved with β-casein. Figure 2 compares $\eta_s(t)$ of β-casein when incubated with 20 U/g TGase with that of sodium caseinate, and also with α_{s1}-casein under similar conditions. We can see that there is a considerable difference in behavior of the individual caseins: whereas the surface viscosity of adsorbed β-casein increases as fast as that for adsorbed caseinate, the surface viscosity of adsorbed α_{s1}-casein increases much more slowly. The rates of viscosity increase, and the estimated errors therein are given in Table 1. The much slower structural buildup with α_{s1}-casein than with β-casein is probably due to at least two factors. First, the rate of accumulation of protein at the oil−water interface is slower for α_{s1}-casein, as demonstrated by

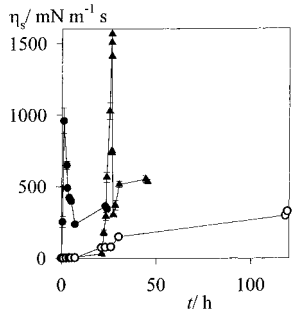

Figure 3. Effect on the developing surface viscoelasticity of the timing of the addition of 20 U/g TGase with respect to formation of the oil−water interface. Apparent surface shear viscosity η_s of the adsorbed film of sodium caseinate (10^{-3} wt %, pH 7, 40 °C) is plotted against time t: (a) (●) enzyme added immediately after surface formation ($t \approx 0$); (b) (○) protein solution incubated with enzyme for 60 min and then enzyme inhibited prior to surface formation; (c) (▲) enzyme added to subphase below film aged for 21 h. Error bars indicate standard deviations based on sets of five consecutive readings.

Table 2. Interfacial Tension γ of Sodium Caseinate (10^{-3} wt %) at the *n*-Tetradecane−Water Interface (pH 7, Ionic Strength 20 mM, 40 °C) after 10 h

aqueous phase	γ (mN m^{-1})[a]
buffer only	41.2
caseinate	12.8
caseinate with 20 U/g TGase added at $t = 0$	15.3
cross-linked caseinate (20 U/g TGase, 1 h)	16.1

[a] Estimated experimental error ±0.05 mN m^{-1}.

the rate of increase in surface pressure (Dickinson *et al.*, 1985b). Secondly, even though both proteins are highly disordered, it appears that there is a lower accessibility of available reactive residues in α_{s1}-casein; a higher specificity toward β-casein than α_{s1}-casein has been reported for human FXIIIa transglutaminase (Traoré and Meunier, 1991). The similar kinetic behavior for sodium caseinate and β-casein is consistent with the preferential adsorption of β-casein over α_{s1}-casein (Dickinson *et al.*, 1988b) and the faster cross-linking of β-casein in bulk solution and at the interface.

All the results mentioned so far are for experiments where the TGase was added to the protein phase immediately after forming the oil−water interface. Figure 3 compares this situation (experiment a) with cases where the enzyme was added either before forming the surface (experiment b) or after a substantial time following establishment of the adsorbed protein layer (experiment c). For the case when the enzyme was added (at a dose of 20 U/g) after 21 h following adsorption of sodium caseinate from a 10^{-3} wt % protein solution at pH 7, Figure 3 shows that the resulting rate of increase in η_s is very large, but still only *ca.* 25% of that determined when the enzyme was added immediately after forming the oil−water interface. It is also clear from Figure 3 that the development of the surface viscosity of sodium caseinate proceeded very much more slowly when the protein was cross-linked before adsorption (experiment b) than when it was cross-linked during adsorption (experiment a) or after adsorption (experiment c). This is probably due to the greatly reduced rate of diffusion-controlled adsorption of aggregates of cross-linked protein. Table 2 shows that the interfacial tension reached after 10 h for a 10^{-3} wt % solution of enzymatically cross-linked caseinate is slightly higher than that of the untreated protein. This may be attributed to a lower flexibility of the cross-linked casein molecules, which leads to a poorer accessibility of apolar residues for the interface, and also to the fact

that the "equilibrium" tension may still not have been reached even after this extended period. In this context, it is noteworthy in Figure 3 (experiment b) that, while the value of η_s of the adsorbed film made from the caseinate cross-linked before forming the surface starts off very low, after several days it is of comparable magnitude to that reached when TGase was added during the formation of the film (experiment a).

To summarize the results on the casein systems, we can say that TGase cross-linking does generally produce a very substantial increase in surface shear viscosity at the oil−water interface for both commercial sodium caseinate and the pure major individual caseins, although the rate of development of surface viscoelasticity is dependent on whether the protein is cross-linked before or after adsorption. This behavior at the oil−water interface is broadly consistent with bulk viscosity measurements on concentrated protein solutions and is further confirmation that the flexible caseins are indeed good substrates for the enzyme (Nio *et al.*, 1985, 1986a).

At the same time as the casein molecules are cross-linked by TGase at the interface, it is possible that some casein molecules in solution also become cross-linked to previously adsorbed molecules in the original film, especially in the later stages of the developing surface viscoelasticity. Such additional cross-linking would lead to the formation of a secondary layer of adsorbed casein covalently attached to the primary casein monolayer. While a change in layer thickness without any change in uniform density of cross-links should in theory have no effect on surface rheology, the undoubtedly nonuniform character of the protein segment distribution and the cross-link distribution within the adsorbed layer means that such secondary cross-linking will probably make some contribution to the developing surface rheology of the enzyme-treated casein film. In the corresponding emulsion system, the increase in effective adsorbed layer thickness caused by the secondary cross-linking would be expected to have a positive influence on steric stabilization of casein-coated oil droplets.

We now consider the surface shear viscosity of the cross-linked globular protein β-lactoglobulin. Previous work has shown that in normal bulk solution this protein is not particularly susceptible to TGase cross-linking and that some unfolding is generally required to facilitate the process (Aboumahmoud and Savello, 1990; Traoré and Meunier, 1992; Færgemand *et al.*, 1997). On this basis, we might not necessarily expect a large effect with β-lactoglobulin incubated with TGase in the absence of a reductant, although it seems likely that unfolding upon adsorption should increase the accessibility to the enzyme (Dickinson and Yamamoto, 1996).

Figure 4 shows the effect of changing enzyme concentration on $\eta_s(t)$ for a 10^{-3} wt % β-lactoglobulin solution at pH 7 and 40 °C. The first point to note is that the untreated protein itself yields a substantial surface viscosity of *ca.* 200 mN m^{-1} s. The second point is that treatment at a very low TGase dose (0.2 U/g), or with thermally inactivated TGase, does actually seem to produce a slight reduction in η_s. The reason for this is not clear; it could be due to the presence of a small amount of surface-active impurity in the enzyme preparation. Thirdly, and more importantly, however, at moderate or high doses, the enzyme treatment does lead to a substantial positive effect. The presence of TGase at a moderately high dose (20 U/g) leads to an increase by nearly 1 order of magnitude in the surface viscosity reached after 10−20 h. While the rate of increase is clearly much lower for β-lactoglobulin than for the caseins, the final value reached is higher. This substantial effect of TGase treatment on the β-lactoglobulin surface viscosity suggests that sufficient unfolding of the globular protein takes place on adsorption to expose the reactive glutamines and lysines to the enzyme. This is consistent with recent work on α-lactalbumin (Matsumura *et al.*, 1996) that has demonstrated that this protein is more susceptible to TGase-catalyzed cross-linking when the molecule is in the molten globule state; analogies between the adsorbed and molten globule states of α-lactalbumin have been discussed (Dickinson and Matsumura, 1994).

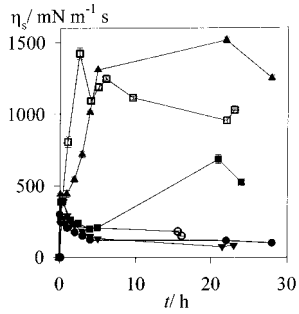

Figure 4. Influence of TGase added immediately after forming the oil−water interface on the surface shear viscosity of an adsorbed film of β-lactoglobulin (10^{-3} wt %, pH 7, 40 °C). Apparent surface shear viscosity η_s is plotted against time t for various enzyme doses: (○) no TGase; (▼) 0.2 U/g; (■) 2 U/g; (▲) 20 U/g; (□) 200 U/g; (●) inactivated TGase (20 U/g).

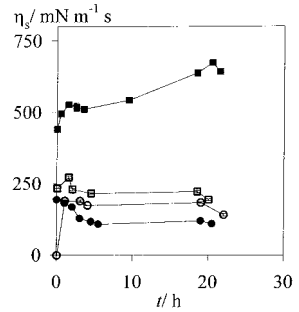

Figure 5. Influence of dithiothreitol (DTT) on the time-dependent surface shear viscosity, $\eta_s(t)$, of an adsorbed film of β-lactoglobulin (10^{-3} wt %, pH 7, 40 °C) at the oil−water interface: (○) no TGase or DTT present; (●) protein incubated with 20 U/g TGase for 4 h (without DTT) and then enzyme inhibited prior to surface formation; (■) protein incubated with 20 U/g TGase for 4 h with 10 mM DTT and then enzyme inhibited prior to surface formation; (□) protein incubated with 10 mM DTT but without TGase for 4 h.

To attempt to confirm the enhanced rate of TGase cross-linking in the unfolded globular protein adsorbed state, further experiments were carried out as recorded in Figure 5. When β-lactoglobulin was incubated with TGase in bulk solution, and then the enzyme inactivated before forming the oil−water interface, the resulting $\eta_s(t)$ was found to be the same (within experimental error) as for the untreated system. This implies that there is no significant cross-linking of the native protein in bulk solution. However, when the incubation was carried out with TGase in bulk solution in the presence of dithiothreitol (DTT), the resulting surface viscosity was increased by about a factor of 3, which agrees with separate experiments (Færgemand *et al.*, 1997) showing that cross-linking of β-lactoglobulin is only possible after partial unfolding of the protein. Adsorption of pre-cross-linked protein did not lead to such a high surface viscosity as when the TGase was added during or after film formation (*cf.* Figures 4 and 5). This could be associated with effects of the aggregation of the β-lactoglobulin on its surface activity (see Table 3) and its

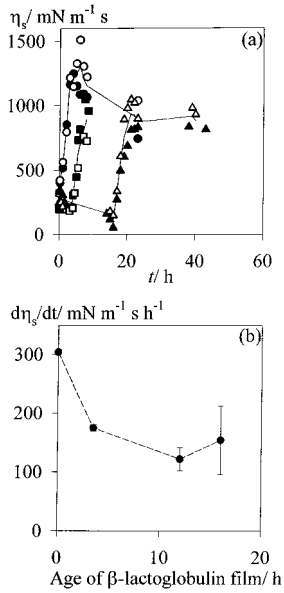

Figure 6. Effect of film aging on the developing surface shear viscosity η_s of β-lactoglobulin (10^{-3} wt %, pH 7, 40 °C) at the oil−water interface following enzyme addition after various times t following surface formation. (a) Time-dependent apparent surface shear viscosity: (●, ○) $t = 0$; (■, □) $t = 3.5$ h; (▲, △) $t = 18$ h. In each case, the filled and open symbols represent duplicate experiments, and the lines represent averages of the two experiments. (b) Rate of initial increase in surface viscosity after adding TGase. The rate was estimated by linear regression of the first five measurement points following TGase addition (taken over equal intervals for all samples).

Table 3. Interfacial Tension γ of β-Lactoglobulin (10^{-3} wt %) at the *n*-Tetradecane−Water Interface (pH 7, Ionic Strength 20 mM, 40 °C) after 10 h

aqueous phase	γ (mN m^{-1})[a]
buffer only	41.2
β-lactoglobulin	13.6
β-lactoglobulin with 20 U/g TGase added at $t = 0$	14.6
cross-linked β-lactoglobulin (20 U/g TGase, 4 h) with 10 mM DTT	13.8
β-lactoglobulin with 10 mM DTT	11.0

[a] Estimated experimental error ±0.05 mN m^{-1}.

ability to pack into a compact monolayer at the oil−water interface.

The effect of aging of the β-lactoglobulin layer at the interface prior to addition of enzyme is indicated in Figure 6a. It would seem that, while there is little effect of aging on the ultimate surface viscosity reached, there is a reduction in the rate of increase of the surface viscosity, as shown in Figure 6b. Previous work in the absence of TGase has demonstrated (Dickinson and Matsumura, 1991) slow interfacial cross-linking of β-lactoglobulin through intermolecular disulfide bridges. It seems likely that the loss of polypeptide flexibility and the masking of reactive glutamine and lysine residues by disulfide bond formation could lead to a reduction in the rate of TGase-catalyzed cross-linking and hence in the rate of development of the surface viscoelasticity.

The occurrence of a high maximum value of η_s at short times, which was apparent with TGase added to sodium caseinate (Figures 1−3), is also seen to some extent with β-lactoglobulin, *e.g.*, in Figures 4 (at 200 U/g) and 6. For the caseinate case already discussed, we attributed this behavior to the rapid formation of a highly cross-linked network structure at low strain values, followed by brittle fracture and the reduction in mechanical strength on further prolonged deformation. (Bulk gels formed by enzyme-induced cross-linking of milk proteins in solution or dispersion also exhibit this kind of brittle fracture at large strains.) The change in film structure is complicated, however, by the fact that, although some network bonds may be broken by the shear deformation, others can later be formed due to further TGase-catalyzed cross-linking.

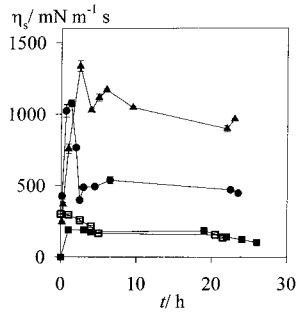

Figure 7. Effect of inhibition of TGase (added immediately after surface formation) during protein cross-linking at the oil−water interface. Apparent surface shear viscosity η_s of β-lactoglobulin adsorbed film (10^{-3} wt %, pH 7, 40 °C) is plotted against time t: (■) no TGase and no inhibitor; (□) inhibitor (10 mM NH_4Cl) present throughout (no TGase); (▲) 200 U/g TGase (no inhibitor); (●) 200 U/g TGase, inhibitor (10 mM NH_4Cl) added after 1 h. Error bars indicate standard deviations based on sets of five consecutive readings.

Figure 7 shows the result of an experiment in which enzyme-induced cross-linking of β-lactoglobulin was deliberately switched off by addition of inhibitor (10 mM NH_4Cl) after a time (1 h) corresponding approximately to the maximum in $\eta_s(t)$. We can see that, following inhibitor addition to the enzyme cross-linked layer, there is a sudden fall in surface viscosity (by about 50%) followed by little further change. (A control experiment showed that fall was not simply due to the inhibitor acting by itself.) This result suggests that, when TGase remains active, the fractured network can be partially reconstructed through further interfacial cross-linking of the already adsorbed β-lactoglobulin molecules and possibly also new ones arriving at the surface. In contrast, once the TGase is inactivated, there is no mechanism for "healing" the fractured network structure, and $\eta_s(t)$ falls to a steady value approximately midway between the values for the fully cross-linked β-lactoglobulin and the untreated β-lactoglobulin.

Comparing the β-lactoglobulin results with those for the casein(s) suggests that the β-lactoglobulin film is more effectively repaired by a process of continuing interfacial cross-linking following fracture than is the casein film. Due to the greater molecular flexibility and greater accessibility of reactive groups on the adsorbed protein during the early stages of treatment with TGase, we can assume that the casein film is more extensively cross-linked at the fracture stress. Hence, once fractured, there is less scope for structural rearrangements to take place in the resulting casein film. Also, at the time of fracture, the casein is already likely to be extensively cross-linked in bulk solution, and so the effectiveness of casein molecules in solution being able to link up with the freshly fractured film and heal breaks in the interfacial structure is presumably much less than that for β-lactoglobulin that is not cross-linked by TGase in bulk solution.

In conclusion, these experiments have demonstrated that enzymatic cross-linking of milk proteins at the oil−water interface leads to a substantial increase in the surface viscoelasticity of the adsorbed films, especially for the caseins. The experiments also show that there may be an optimum level of cross-linking to produce a

film with the desirable combined qualities of flexibility and mechanical strength. If the adsorbed milk proteins become excessively cross-linked, however, the fracture of an initially formed brittle network structure leads to a film of lower surface shear viscosity.

On the basis that increased film surface viscoelasticity may correlate with improved stability of protein-coated droplets and bubbles, these results suggest that enzymatic cross-linking is a potential way of controlling the stability of protein-containing emulsions and foams. The main practical problem in nondilute emulsions is likely to be how to cause cross-linking within the adsorbed layer without also inducing some flocculation of droplets by generating cross-links between proteins on different droplets (Dickinson and Yamamoto, 1996). Of considerable importance in relation to foam and emulsion stability are the surface dilational properties of adsorbed films. Related experiments on the effect of TGase cross-linking on the surface dilational modulus and viscosity of adsorbed milk protein films are currently in progress in this laboratory.

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