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INFLUENCE OF EMULSIFIER ON THE COMPETITIVE ADSORPTION OF WHEY PROTEINS IN EMULSIONS

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Abstract

The influence of Tween 20 (polyoxyethylene sorbitan monolaurate) on the composition of adsorbed protein films in hydrocarbon oil-in-water emulsions containing β-lactoglobulin and α-lactalbumin has been investigated at neutral pH. The kinetics of polymerization of adsorbed β-lactoglobulin via sulphhydryl-disulphide interchange is little affected by Tween 20. Adding emulsifier after homogenization leads to slightly greater competitive displacement of protein from the interface for emulsions made with pure β-lactoglobulin than for equivalent emulsions made with α-lactalbumin. For emulsions made with the two proteins in a 1:1 molar ratio, adding emulsifier after homogenization leads to more displacement of β-lactoglobulin than α-lactalbumin. In an emulsion containing both proteins and Tween 20 added before homogenization at an emulsifier-to-protein molar ratio of 2:1, there is three times as much α-lactalbumin adsorbed at the droplet surface as β-lactoglobulin. It appears that the kinetics of competitive protein exchange between the bulk phase and the oil-water interface is facilitated by the presence of water-soluble surfactant.

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Key words: Competitive adsorption, whey proteins, β-lactoglobulin, α-lactalbumin, displacement, emulsion, Tween 20, disulphide bonds, surface viscosity, surfactant

Introduction

The research reported in this paper forms part of an ongoing investigation into the competitive adsorption of milk proteins and small-molecule surfactants in oil-in-water emulsions. Previous publications have concerned emulsion systems containing various binary mixtures of individual milk proteins (Dickinson et al., 1988, 1989b; Dalgleish et al., 1991) and mixtures of nonionic surfactants with β-lactoglobulin or β-casein (Courthaudon et al., 1991a, 1991b). Also studies have been undertaken of surface activity and interfacial viscosity of complementary mixed films at the macroscopic oil-water interface (Dickinson et al., 1989a, 1990a, 1990b). These experiments have shown that the behaviour of protein + surfactant systems is qualitatively different from that of protein + protein systems. Over a time-scale of a few minutes, milk proteins can be completely removed from the emulsion droplet surface by concentrations of water-soluble nonionic surfactants high enough to give saturated monolayer coverage, in accordance with statistical theories (Cohen Stuart et al., 1984; de Feijter et al., 1987; Woskett, 1989; Dickinson and Woskett, 1989) and computer simulations (Dickinson, 1988; Dickinson and Rustom, 1989). On the other hand, with the notable exception of αs1-casein + β-casein, the ability of one milk protein to competitively displace another milk protein already adsorbed at the interface is both slow and limited in extent (Dickinson, 1991).

A more realistic model is provided by the ternary adsorbing system composed of surfactant + protein + protein. In this study we take as the surfactant the water-soluble emulsifier Tween 20 (polyoxyethylene sorbitan monolaurate); the two proteins are α-lactalbumin and β-lactoglobulin. Each of these components is found in a range of manufactured food colloids.

The purpose of the experimental study is to see how the presence of emulsifier affects the adsorption behaviour in the mixed globular protein system. It was shown earlier (Courthaudon et al., 1991b), in experiments with β-lactoglobulin alone, that the addition of Tween 20 up to an emulsifier-to-protein molar ratio of 1:1 leads to a dramatic lowering in the surface shear viscosity at the macroscopic oil-water interface, while there is...
no significant change in the amount of β-lactoglobulin adsorbed at the emulsion droplet surface. Addition of larger amounts of Tween 20, however, did lead to protein displacement from the emulsion droplet surface, and β-lactoglobulin displacement was found to be complete at an emulsifier-to-protein molar ratio of 8:1. In a separate study of emulsions made with α-lactalbumin and β-lactoglobulin but no small-molecule surfactant, it was found (Dalgleish et al., 1991) that the two whey proteins were adsorbed together at the interface during emulsification in proportion to their bulk concentrations, and that there was very little displacement of one protein by the other after emulsification. It seems plausible that the presence of a small-molecule surfactant, at a bulk concentration high enough to greatly reduce the surface viscosity, but still low enough not to be able to displace the protein completely, could have the effect of facilitating the preferential adsorption of one whey protein over the other by loosening up the structure of the adsorbed layer and hence increasing the rate of protein/protein exchange at the interface.

So, the question we are asking is this: though mixed globular protein adsorption appears to be effectively irreversible and kinetically controlled in the absence of emulsifier, could it become much more reversible and thermodynamically controlled in the presence of emulsifier? This question is of practical importance since small-molecule surfactant adsorption after homogenization, appropriate amounts of Tween 20 were mixed into aliquots of the freshly made protein-stabilized emulsion. (In one experiment, the protein-stabilized emulsion was left for 24 hours prior to adding Tween 20.) It was confirmed that there was no change in the droplet-size distribution following addition of surfactant.

One hour after preparation (and emulsifier addition), the emulsion samples were centrifuged at 1.5 × 10^6 g for 15 minutes to separate the oil droplets from the aqueous serum phase. After filtration through a 0.22 μm Millipore filter, the aqueous phase was analysed for protein by FPLC using a Pharmacia Mono-Q ion-exchange column as described previously (Dickinson et al., 1989b). Bulk phase concentrations of α-lactalbumin and β-lactoglobulin were calculated by reference to standard calibration graphs of peak area against protein content. Surface concentrations of α-lactalbumin and β-lactoglobulin were calculated from the known surface area per unit volume and the difference between the amount of protein(s) measured in the serum after centrifugation and the amount used to make the original emulsion.

Some emulsions made with β-lactoglobulin were examined with respect to time-dependent surface polymerization as described previously (Dickinson and Matsumura, 1991). These samples were treated with N-ethylmaleimide prior to centrifugation to stop the sulphhydryl/disulphide interchange reaction. Adsorbed protein in the cream phase after centrifugation was completely displaced from the droplet surface by 4 wt% sodium dodecyl sulphate (SDS) dissolved in an aqueous buffer solution (pH 6.8). Adsorbed and unadsorbed protein fractions were assayed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (without mercaptoethanol) with standard molecular weight markers and Coomassie brilliant blue as staining agent. Relative concentrations of β-lactoglobulin oligomers in the SDS-PAGE bands were determined densitometrically using a separately measured calibration graph of density against concentration.

Materials and Methods

Materials

The freeze-dried bovine β-lactoglobulin (1.84 X 10^9 daltons), prepared by the method of Aschaffenburg and Drewry (1957), was obtained from BDH Chemicals. The bovine α-lactalbumin (1.42 X 10^9 daltons), prepared by the method of Brodbeck et al. (1967), was obtained from Sigma Chemicals. The purity of the protein samples (> 99 wt%) was demonstrated by fast protein liquid chromatography (FPLC) as described in a previous paper (Dickinson et al., 1989b). High purity Tween 20 (Surfact-Amp 20, 1.24 X 10^5 daltons) was obtained from Pierce Chemicals. Analytical-grade n-tetradecane (> 99 wt%) was obtained from Sigma Chemicals. Buffer solutions were prepared from Analytical-grade reagents and double-distilled water.
Results and Discussion

We first present results for the emulsions containing β-lactoglobulin without α-lactalbumin. The volume–surface average diameter was found to be \( d_{32} = 0.60 \pm 0.03 \mu m \) for emulsions containing no emulsifier or emulsifier added after preparation. There is a slight reduction in \( d_{32} \) on adding Tween 20 before homogenization; values of average droplet size for all the β-lactoglobulin emulsions studied, with emulsifier-to-protein ratio up to \( R = 16 \), were in the range \( 0.50 \leq d_{32}/\mu m \leq 0.60 \). This reduction in average droplet size with emulsifier added before homogenization is broadly consistent with our previous studies of emulsions containing milk proteins + polyoxyethylene \((C_{2.8}E_n)\) surfactants (Dickinson et al., 1989a; Courthaudon et al., 1991a).

Figure 1 shows the effect on protein surface coverage of adding Tween 20 either before or after emulsification. The surface concentration \( \Gamma \) of protein is plotted against the emulsifier-to-protein molar ratio \( R \). We see that, while a low Tween 20 concentration \((R \leq 0.5)\) does not lead to any significant change in β-lactoglobulin surface coverage, a high Tween 20 concentration \((R = 10)\) leads to complete displacement of β-lactoglobulin from the droplet surface \( \Gamma = 0 \). It is worthy of note that the displacement behaviour is roughly the same irrespective of whether the emulsifier is present before or after homogenization, as was pointed out previously by de Pijter et al. (1987). Nevertheless, it is apparent in Figure 1 that there is a small but consistent trend towards slightly lower protein coverages with \( 1 \leq R \leq 4 \) in the case of systems with Tween 20 added before homogenization. Assuming all the available Tween 20 is bound to oil–water interface (or protein), this trend is consistent with the slightly lower mean droplet size and hence larger interfacial area as more surfactant is made available during homogenization (Dickinson et al., 1989a).

Analysis for protein oligomers by SDS-PAGE, after displacement of adsorbed β-lactoglobulin from the emulsion droplet surface by excess SDS, has indicated that the presence of Tween 20 up to \( R = 2 \) does not inhibit the polymerization of β-lactoglobulin at the oil–water interface. Table 1 lists the relative amounts of the various oligomers after 0, 2, 24 and 72 hours following homogenization, where the tetramer values also include the amounts of all \( n \)-mers with \( n > 4 \). The adsorbed protein immediately after emulsification (with or without Tween 20) consists only of the β-lactoglobulin monomer \((1.84 \times 10^4 \text{ daltons})\), as does the unadsorbed protein at all times after emulsification. After 2 hours at the oil–water interface, approximately 20% of the monomers are polymerized; this figure goes up to approximately 70% after 72 hours. There seems to be a slightly faster polymerization rate at \( R = 2 \) than at \( R = 0 \), though the difference is only just outside the estimated experimental uncertainty (Dickinson and

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Effect of Tween 20 at an emulsifier-to-protein molar ratio ( R = 2 ) on the relative amounts of different oligomers of β-lactoglobulin at the oil–water interface various times after emulsion formation. Numbers in brackets refer to relative amounts in the absence of emulsifier ((R = 0)).</th>
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<tr>
<td></td>
<td>0 hours</td>
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<tr>
<td>Monomer</td>
<td>100 (100)</td>
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<td>Dimer</td>
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<td>Trimer</td>
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<td>Tetramer</td>
<td>0 (0)</td>
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<tr>
<td>Total density b</td>
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\(^a\) Also includes all polymers larger than the tetramer.

\(^b\) Sum of peak areas for all oligomers (arbitrary units). The reduction in total protein surface concentration on addition of Tween 20 as determined by SDS-PAGE is consistent with that determined by FPLC (Figure 1), i.e., there is 25% protein displacement from the emulsion droplet surface for \( R = 2 \). [See Dickinson and Matsumura (1991) for further details of the SDS-PAGE experiments.]
The difference may be related to the much greater mobility in the adsorbed film in the presence of surfactant (Courthaudon et al., 1991b).

The main conclusion of the results in Table 1 is that, at concentrations much less than that which causes complete protein displacement, Tween 20 does not have much influence on sulphydryl-disulphide interchange at the emulsion droplet surface. This means that, although Tween 20 binds to β-lactoglobulin to form a 1:1 complex (Coke et al., 1990; Clark et al., 1991), the surfactant—protein binding appears not to affect the number of disulphide bonds available for reaction. Thus, whatever is the reason for the dramatic drop in surface viscosity on addition of surfactant up to $R = 1$ (Courthaudon et al., 1991b), the effect is not explicable in terms of an inhibition of the protein polymerization at the interface.

We now turn to emulsions containing the whey protein α-lactalbumin. The volume-surface average diameter was found to be $d_{32} = 0.52 \pm 0.02 \mu m$ for pure α-lactalbumin systems containing no Tween 20 or Tween 20 added after homogenization. Figure 2 shows the effect on the protein surface coverage $\Gamma$ of adding emulsifier after emulsion formation. The general trend of competitive displacement of protein by surfactant is similar to that found with pure β-lactoglobulin (see Figure 1), but we note that even at $R = 32$ the α-lactalbumin is not completely displaced from the interface. So it takes more Tween 20 to displace protein from a pure α-lactalbumin emulsion surface than from a pure β-lactoglobulin emulsion surface. Also shown in Figure 2 are surface coverages in α-lactalbumin emulsions with Tween 20 added before preparation ($R = 0.5, 1$ and 2). The values do not differ in any significant way from those for emulsifier added afterwards.

Figure 2. Effect of Tween 20 on surface coverage in pure α-lactalbumin emulsions. Protein surface concentration $\Gamma$ is plotted against molar ratio $R$: •, emulsifier added after homogenization; ○, emulsifier added before homogenization. Error bar on point at $R = 0$ denotes maximum estimated average experimental uncertainty.

Figure 3 shows the effect of Tween 20 added after homogenization on surface coverage in emulsions made with a 1:1 molar ratio of α-lactalbumin + β-lactoglobulin. Protein surface concentration $\Gamma$ is plotted against emulsifier-to-protein molar ratio $R$: ■, total protein; ○, α-lactalbumin; ●, β-lactoglobulin.

Thus far, we have considered emulsions made with just a single pure whey protein. We now turn to emulsions made with the same total amount of protein (0.5 wt%), but now of a composition which corresponds to a 1:1 molar ratio of the globular proteins (i.e., 0.216 wt% α-lactalbumin + 0.284 wt% β-lactoglobulin). The average droplet size was found to be intermediate between the values for pure α-lactalbumin and pure β-lactoglobulin. Figure 3 shows the effect of Tween 20 added after homogenization on the total protein surface concentration as well as on the surface coverages of the separate individual proteins. In the absence of emulsifier ($R = 0$), there is at least as much α-lactalbumin at the surface as there is β-lactoglobulin, even though the latter is present in larger amount (by weight) in the emulsion as a whole. At an emulsifier-to-protein molar ratio $R = 16$, the β-lactoglobulin is completely removed from the surface, whereas the α-lactalbumin is not. This is consistent with the earlier results for the emulsions containing pure proteins which suggested that the α-lactalbumin was less easy to displace from the interface by Tween 20 than was the β-lactoglobulin. Figure 3 shows that the competitive difference between the two whey proteins with respect to surfactant is most evident in the range $2 \leq R \leq 4$. Under conditions of partial protein displacement from the surface by surfactant, there is almost twice as much α-lactalbumin at the interface as β-lactoglobulin in the mixed protein emulsion system.

Figure 4 shows the total and individual protein surface concentrations for emulsions made with α-lactalbumin + β-lactoglobulin and Tween 20 added before homogenization up to an emulsifier-to-protein molar ratio $R = 2$. In the absence of emulsifier, the two whey proteins occur at the
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![Graph showing competitive adsorption](image)

Figure 4. Effect of Tween 20 present before homogenization on surface coverage in emulsions made with a 1:1 molar ratio of α-lactalbumin + β-lactoglobulin. Protein surface concentration $\Gamma$ is plotted against emulsifier-to-protein molar ratio $R$: $\bullet$, total protein; $\square$, α-lactalbumin; $\triangle$, β-lactoglobulin.

The surfactant (Tween 20) can displace protein from the emulsion droplet surface in roughly equal proportions as found previously (Balgleish et al., 1991). On the other hand, with increasing additions of Tween 20, while the surface coverage of α-lactalbumin stays essentially constant, there is a reduction in the surface concentration of β-lactoglobulin, and hence in the total surface coverage of protein. Indeed, at $R = 2$, there is three times as much α-lactalbumin at the emulsion droplet surface as β-lactoglobulin. So the presence of surfactant during emulsification allows preferential α-lactalbumin adsorption from the mixture of the whey proteins, whereas there is no such competitive effect with the two proteins in the absence of surfactant (Balgleish et al., 1991).

Comparison of the data in Figures 3 and 4 is suggestive of the fact that competitive removal of β-lactoglobulin by α-lactalbumin occurs to a greater degree the earlier the oil–water surface is exposed to the surfactant. That is, the longer the whey proteins have been adsorbed at the droplet surface, the more difficult it is for any interchange of α-lactalbumin and β-lactoglobulin molecules to occur upon addition of Tween 20 to the system, or for either protein species to be displaced by surfactant. This much increased difficulty of displacing aged protein films from the emulsion droplet surface has been observed previously in casein + gelatin systems (Dickinson et al., 1987).

To test for protein film aging effects, some emulsions made with α-lactalbumin or a mixture of α-lactalbumin + β-lactoglobulin were left for 24 hours prior to addition of Tween 20 ($R = 5.6$). In each case, it was found that less protein was displaced from the interface than in the previous experiments with Tween 20 added immediately after emulsion formation (Figures 2 and 3). The protein surface concentration was determined as before from the known surface area of the emulsion and the serum phase protein concentrations measured by FPLC. With the pure α-lactalbumin emulsion, the surface coverages are 0.4 mg m$^{-2}$ and 0.8 mg m$^{-2}$ for emulsifier added immediately and after 24 h, respectively. With the mixed protein emulsion, the surface coverages (total protein) are 0.45 mg m$^{-2}$ and 0.7 mg m$^{-2}$ ($\alpha$-lactalbumin 0.42 mg m$^{-2}$, β-lactoglobulin 0.28 mg m$^{-2}$), respectively. These results do indeed show that competitive adsorption in the α-lactalbumin + β-lactoglobulin + Tween 20 system is facilitated by the simultaneous exposure of all three components to the interface at the time of emulsification. The preponderance of α-lactalbumin over β-lactoglobulin in the adsorbed layer is consistent with the lower interfacial tension of α-lactalbumin as compared with β-lactoglobulin (Jackson and Pallansch, 1961). This preferential adsorption of the more surface-active component during emulsification with a binary mixture of milk proteins occurs in the absence of surfactant for α$_{3}$-casein + β-casein (Dickinson et al., 1988).]

It is clear from this study that the presence of a water-soluble food surfactant has considerable influence on the distribution of globular proteins between the bulk aqueous phase and the oil–water interface in a protein-stabilized emulsion. When addition of surfactant is delayed, the competitive adsorption is less pronounced, though displacement of protein by surfactant still readily takes place. These conclusions appear to be relevant to interfacial composition and structure in a wide range of dairy emulsion products.

Acknowledgement

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References


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Discussion with Reviewers

E.H. Lucassen-Reynders: Interestingly, you find that Tween 20 is more efficient in the displacement of $\beta$-lactoglobulin than in that of $\alpha_1$-lactalbumin. Could part of the explanation lie in the reported complexation between Tween 20 and $\beta$-lactoglobulin? Presumably, such complex formation makes the surfactant more hydrophobic, which is likely to lead to desorption. This possibility could be verified by measuring the concentration of $\beta$-lactoglobulin and $\alpha_1$-lactalbumin in the presence of oil-soluble surfactant.

Authors: By reducing the overall molecular hydrophobicity of $\beta$-lactoglobulin and hence lowering its surface activity, the formation of a complex with Tween 20 could be a factor in making it easier to displace from the interface. But, since only one surfactant molecule binds to each protein monomer, the effect on the hydrophilic-lipophilic balance might be expected to be rather small. Certainly we do not notice any reduction in emulsifying capacity of the l:1 mixture of $\beta$-lactoglobulin + Tween 20 as compared with the protein alone; in fact, if anything, the opposite appears to be the case (see Figure 1). Of more significance, perhaps, is the observation that $\alpha_1$-lactalbumin alone is especially difficult to completely displace from the droplet surface. In separate experiments on Tween 20 added to $\beta$-casein containing emulsions (Courthaudon et al. 1991a), we found that the $\beta$-casein is completely removed from the interface at R = 16. It would indeed be interesting to extend these experiments to oil-soluble surfactants.

E.H. Lucassen-Reynders: You rightly state that intermolecular interactions in protein films are still poorly understood. Even less is known about the link between such interactions and surface shear viscosity. Therefore, the suitability of this particular rheological parameter as a means of assessing such interactions seems doubtful. In principle, one would expect more information on interactions from methods measuring the interfacial tension of the mixed monolayers, both at equilibrium and under compression/expansion. The latter experiment would yield the surface dilational viscosity, which is generally much larger than the shear viscosity. Would you agree that such methods are likely to provide a more sensitive criterion for surface interactions?

Authors: Interfacial tension measurement can certainly be a useful indicator of composition of films adsorbed from binary protein mixtures, as shown, for instance, in a recent study of mixed systems of milk proteins and the egg-yolk protein phosvitin [Dickinson E, Hunt JA, Dalgleish DG (1991) Food Hydrocolloids 4, 403-414]. However,
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steady-state tension measurements are insensitive to protein-protein interactions, whereas surface viscometry is very sensitive to such interactions when they occur in the interfacial layer. This is because tension measurements are dependent on free energy changes occurring in the immediate vicinity of the Gibbs dividing surface (train segments of a flexible linear polymer, mainly), whereas surface rheology also probes connected regions further away from the surface (loops and tails of flexible polymers, as well as interacting secondary layers, possibly composed of a different polymer species). We have used surface shear viscosity as a probe of the strength of attractive protein-protein film interactions (Dickinson et al., 1987; 1990b) as well as of protein-polysaccharide interactions [Dickinson E, Euston SR (1991) In: Food Polymers, Gels and Colloids, Dickinson E (ed), Royal Society of Chemistry, Cambridge, 132-146]. I am not aware of any published work which reports equivalent information derived from surface dilational viscosity measurements, and, while such dilational measurements would certainly be of interest and value, I am not convinced that they provide a more sensitive probe of surface interactions than do shear measurements.

N. Krog: It is known that the type of oil phase influences emulsion stability due to variations in interfacial film properties. Results obtained with n-tetradecane may not be comparable with those obtained with triglycerides. Your results are of interest for whipable emulsions. Have you studied systems based on triglyceride oils and weakly polar emulsifiers such as monoglycerides or propylene glycol esters, which we use in food emulsions? Commercial dairy emulsions are homogenized at high temperature (e.g. 80°C) and stored at low temperature (e.g. 5°C). What temperature was used in your experiments, and what effect will temperature changes have on your results?

Authors: I agree that there may be considerable differences between hydrocarbon oils and triglyceride oils in terms of interfacial film properties, especially in systems containing oil-soluble surfactants. However, with nonionic water-soluble surfactants (e.g. Tween 20), we find that replacement of n-tetradecane by pure triglyceride oil makes little difference to the amount of surfactant necessary to displace the protein from the emulsion droplet surface, though the amount of protein adsorbed at the triglyceride interface is significantly lower at surfactant concentrations below the displacement point. So far, all our emulsions have been prepared and investigated at ambient temperature (15-20°C). It would be interesting to study effects of temperature changes as you suggest.

K. Chen: How do these adsorption phenomena affect the texture and stability of emulsions?

Authors: Competitive adsorption during emulsion formation may lead to bridging flocculation of droplets with substantial implications for bulk rheology and texture [Dickinson E, Flint FO, Hunt JA (1989) Food Hydrocolloids 3, 389-397; Dickinson E, Galazka VB (1991) J. Chem. Soc. Faraday Trans. 87, 963-969]. Displacement of protein from the surface of emulsion droplets by small-molecule surfactants makes them more susceptible to shear-induced aggregation. Such considerations are important in relation to the stability of aerated foods such as whipped desserts or ice-cream.