Influence of Competitive Adsorption of a Lysopalmitoylphosphatidylcholine on the Functional Properties of Puroindoline, a Lipid-Binding Protein Isolated from Wheat Flour

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The functional properties of a low molecular weight lipid-binding protein, puroindoline from wheat, were studied as a function of lysophosphatidylcholine (LPC) concentration. Puroindoline bound approximately five LPC molecules in a positively cooperative manner with a dissociation constant of 54.3 μM. The foaming properties of puroindoline were enhanced in mixtures containing LPC, and maximum stability was observed in the range of LPC to puroindoline molar ratios (R) of 1–10. The properties of isolated foam lamellae (thin films) were investigated to explain this observation. Film drainage, equilibrium thickness, surface diffusion in the adsorbed layer by fluorescence recovery after photobleaching, and surface displacement measurements were made. We conclude that the foaming properties of this highly foam active protein are enhanced by addition of LPC, probably by formation of a complex, which reduces puroindoline aggregation at the interface. However, foam stability begins to decrease once there is enough LPC present in the system to allow the protein to diffuse laterally in the adsorbed layer.

INTRODUCTION

Amphiphilic natural or synthetic lipids are low molecular weight surfactants and are widely used in the food industry to achieve the desired texture and appearance of products. As components of membranes, these molecules are also naturally present in most raw materials and contribute to the processing and quality of foods. For example, the endogenous phospholipids and glycolipids of wheat flour are known to exert important effects in baking. These lipids improve bread loaf volume and crumb texture by stabilizing air–water interfaces during gas expansion, occurring at the proofing and early stages of oven baking (MacRitchie, 1983). The surface properties of these polar lipids are not sufficient to explain their effect in dough. We have suggested (Marion, 1992) that their interaction with proteins, and especially with lipid-binding proteins, plays an important role as has been described for the pulmonary surfactant system (Notter *et al.*, 1987; Hagwood *et al.*, 1987). Different lipid-binding proteins were recently purified from wheat flour (Blochet *et al.*, 1991; Désormeaux *et al.*, 1992). Some of these proteins are tightly bound to amphiphilic lipids, and nonionic detergent is necessary for their extraction (Blochet *et al.*, 1991). Using Triton X-114 phase partitioning, a basic and cysteine-rich protein, named puroindoline, which has a unique tryptophan-rich domain, was recently purified and sequenced (Blochet *et al.*, 1993). Here we report a study in which we have probed the putative functional properties of puroindoline. Specifically, we have investigated the interaction between puroindoline and a phospholipid analogue, lysopalmitoylphosphatidylcholine (LPC). This lipid was chosen for its solubility in water and also because it is an important biosurfactant (Stafford and Dennis, 1988). Our study comprised an investigation of the binding of LPC to puroindoline, some foam stabiliity measurements, and drainage, thickness, surface diffusion, and protein displacement measurements on air-suspended thin liquid films of puroindoline, containing different concentrations of LPC.

MATERIALS AND METHODS

Materials. LPC (L-α-lysophosphatidylcholine, palmitoyl) was purchased from Sigma Chemical Co. (Product L-5254) along with fluorescein isothiocyanate (FITC; Product F-7250). All other chemicals were of AnalaR grade from BDH Chemicals and were used without further purification. All experiments were carried out in 10 mM sodium phosphate buffer, pH 7.0. The majority of experiments were performed at a constant solution concentration of puroindoline of 0.1 mg/mL (8.3 μM) unless stated otherwise. During the course of this study we refer to R, which is the molar ratio of LPC to puroindoline.

Methods. 1. Purification of Puroindoline. Puroindoline was purified according to a modification of the procedure described by Blochet *et al.* (1991). Soluble proteins were extracted for 2 h at 4 °C by gentle stirring of 2 kg of wheat flour (*Triticum aestivum* var. Camp Rémy) with 10 L of 100 mM Tris-HCl, pH 7.8, buffer (Tris buffer) containing 5 mM EDTA and 0.1 M KCl. After centrifugation at 5000*g* for 30 min, the sediment was extracted for 2 h at 4 °C with 5 L of the Tris buffer containing 4% Triton X-114. After centrifugation at 5000*g*, the supernatant was heated to 30 °C for 1 h and centrifuged for 15 min at 5000*g*. The upper detergent-poor phase was discarded, and the same volume of fresh Tris buffer containing 0.06% Triton X-114 was added. The solution was stirred for 1 h at 4 °C, and the phase-partitioning procedure was repeated. Finally, the upper phase was discarded, the lower detergent-rich phase was removed, and the proteins were precipitated overnight with 5 L of peroxide-free diethyl ether–ethanol (1:3 v/v) at −20 °C. After centrifugation at 2000*g*, the protein sediment was reextracted at −20 °C three times with 1 L of diethyl ether–ethanol and finally with 1 L of diethyl ether. After centrifugation at 2000*g*, the protein sediment was dried overnight under reduced pressure.

The dry white protein powder was solubilized in 50 mM acetic acid and fractionated on a column (10 cm × 100 cm) packed with Sephadex G-75 (Pharmacia). Each fraction was analyzed by SDS–PAGE according to the procedure of Laemmli (1970), and fractions containing polypeptides with apparent molecular weight below 20 000 were pooled and freeze-dried. This freeze-dried protein fraction was solubilized in 0.05 M ammonium acetate

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and fractionated on a cation-exchange column (Neobar) using a 0.05–0.7 M gradient of ammonium acetate buffer, pH 5.5, containing 20% acetonitrile. The puroindoline fraction was dialyzed overnight against deionized water and freeze-dried. Puroindoline gave a single band in SDS–PAGE and a single peak in reversed-phase HPLC under conditions described previously for the separation of Triton X-114 proteins (Blochet *et al.*, 1991). Finally, a single N-terminal sequence was obtained by automated sequencing of the reduced and alkylated protein, according to the procedures described by Désormeaux *et al.* (1992). An absorption coefficient of 1.94 mL/(mg cm) at 281 nm was determined for puroindoline and was used to determine solution concentration.

2. Binding. The interaction of LPC with puroindoline caused an enhancement of the intrinsic fluorescence of puroindoline, which could be used to study the binding process (Clark *et al.*, 1992). In a typical experiment, aliquots (5–10 μL) of a solution containing LPC (3 mM) and puroindoline (0.1 mg/mL) in 10 mM sodium phosphate buffer, pH 7.0, were added to 1 mL of puroindoline (0.1 mg/mL) in the same buffer, contained in a semimicro fluorescence cell. (Dilution of the protein was avoided during the titration by ensuring that both solutions contained equivalent concentrations of puroindoline.) After thorough mixing, the fluorescence emissiion resulting from excitation at 295 nm (slit 2.5 nm) was measured at 337 nm (slit 2.5 nm) on a Perkin-Elmer LS-5 luminescence spectrometer. The data were recorded at 20 °C. The titration was stopped when the fluorescence showed no further increase with added LPC. The background fluorescence from the LPC alone was small but was measured in a reference titration performed in the absence of protein and subtracted from the data. The fraction of occupied sites was calculated, as a function of added LPC, from the observed fluorescence by assuming that the plateau represented total saturation of the available binding sites.

In addition, some equilibrium dialysis measurements were made (Clark *et al.*, 1992). The dialysis cells that were used in this study contained five sample chambers per unit (Scienceware). Each chamber was divided into two half-chambers (1-mL volume) by a dialysis membrane formed from regenerated cellulose. The membranes had a nominal molecular weight cutoff of 6000 and a thickness of 0.073 nm. Small magnetic stirring bars were introduced into the chamber to accelerate equilibrium.

For any given chamber, both half-chambers were initially loaded with equal concentrations of LPC but puroindoline (0.5 mg/mL) was introduced into only one half-chamber. The chambers were sealed with screws and the cells placed on a magnetic stirrer for 20 h, to ensure that equilibrium was reached. A 0.5-mL sample was then removed from the half-chambers that contained LPC alone and assayed using the method of Raheja *et al.* (1973). The assay detected the amount of lipid phosphorus present in the sample. A chromogenic solution was carefully prepared, which contained 0.7% (v/v) mercury, 1% (w/v) ammonium molybdate, 2.7% (v/v) HCl, 13.5% (v/v) H_2SO_4, 28% (v/v) H_2O, 45% (v/v) methanol, and 5% (v/v) chloroform. This reagent (1 mL) together with 0.5 mL of sample and a further 1 mL of chloroform were mixed together in a test tube and held at 100 °C for 3 min in an oil bath. The solution was then cooled on ice, thoroughly mixed with another 1 mL of chloroform, and centrifuged to separate the phases. The chloroform (lower) phase was removed, and the absorption at 716 nm was measured on a Perkin-Elmer Lambda 9 spectrophotometer, against a chloroform reference. A calibration was performed with solutions containing known concentrations of LPC which had been through the equilibrium dialysis cell procedure in the absence of puroindoline. This compensated for any loss of LPC by adsorption to the walls of the dialysis cell.

The concentration of LPC in the half-chambers that contained LPC alone was equivalent to the concentration of free (unbound) LPC in the chamber containing both LPC and protein. The reduction in the concentration of LPC observed in this chamber at equilibrium was related to the fraction of sites occupied on the protein.

In these binding experiments, we have followed the analytical pathway shown below (Clark *et al.*, 1992). First, a plot of the raw data provided an indication of the type of binding present. Typically, we plotted fractional occupation of the protein binding sites (υ) as a function of total ligand concentration. This allowed identification of the binding plateau where saturation of the binding sites occurred. A Scatchard plot (Scatchard, 1949) was then generated (plotting υ against υ/[L], where [L] is the free ligand concentration). This was used to distinguish between independent and cooperative binding. If the Scatchard plot suggested some form of cooperation between sites (e.g., a humped curve indicates positive cooperativity), then the Hill equation (Hill, 1910) was used to determine the dissociation coefficient, K_d, and cooperativity coefficients. The Hill equation is

$$\upsilon = n([L]/K_d)^{Nh}/(1 + ([L]/K_d)^{Nh}) \quad (1)$$

where υ is the fraction of the protein with n occupied binding sites and Nh is the coefficient of cooperativity. If Nh < 1, binding is negatively cooperative, and if Nh > 1, binding is positively cooperative. Positive cooperativity enhances the binding of additional ligands to the protein.

3. Foaming. Foam stability was measured using a microconductivity method as described in detail previously (Clark *et al.*, 1991b). During the course of these experiments, the concentration of puroindoline was kept constant at 0.1 mg/mL, and measurements were performed as a function of LPC concentration in the range 0–500 μM. This corresponds to an R (molar ratio of [LPC]:[puroindoline]) of 0–60. The foaming properties of solutions of LPC alone were also investigated.

4. Thin Films. Air-suspended liquid thin films (approximately 100-μm diameter) were formed in a ground glass annulus (approximately 3-mm diameter) as described previously (Clark *et al.*, 1990a). The drainage of the thin films was observed under a Nikon Diaphot inverted microscope. The thickness of the films was measured by laser interferometry. Experiments were performed using solutions containing 0.1 mg/mL puroindoline, and the LPC concentration ranged between 0 and 33 μM (R = 0–4).

5. FRAP. The surface diffusion coefficient of FITC-labeled puroindoline was determined by fluorescence recovery after photobleaching (FRAP) as described previously (Clark *et al.*, 1990a,b). Fluorescent-labeled protein was prepared by incubating 2 mg/mL puroindoline with 0.1 mg of FITC for 2 h in 10 mM sodium phosphate buffer, pH 8.0. The labeled protein was separated from unreacted FITC by FPLC using a fast desalting column equilibrated in 10 mM sodium phosphate buffer, pH 7.0. FRAP experiments were performed on thin films (prepared as described above) that had reached equilibrium thickness. Fluorescence labeling of puroindoline did not alter the drainage or thickness properties of the thin films. Typically, labeled puroindoline preparations contained 0.2 mol of FITC/mol of protein. FRAP measurements were performed on thin films containing a constant concentration of FITC-labeled puroindoline (0.1 mg/mL) and LPC concentrations in the range 0–66 μM (R = 0–8).

6. Displacement. A simple semiquantitative method for monitoring the displacement of fluorescent-labeled molecules from the interfaces of thin films has been developed (Wilde and Clark, 1993) and was used to investigate the displacement of FITC-labeled puroindoline by LPC. A thin film was formed in the normal way, and a low-power beam from an argon-ion laser (λ = 488 nm) was focused on the film. The fluorescence emitted from the surface-adsorbed labeled protein was measured using a photon counting detector (Thorn-EMI). The magnitude of the fluorescence signal gave a qualitative measurement of the amount of puroindoline at the interface, provided that the thin film was irradiated with a fixed laser intensity. The progressive displacement of the protein was studied as a function of increasing LPC concentration. Each measurement was performed on a fresh, thin film 30 min after formation, to eliminate errors arising from time-dependent increases in protein–protein interactions at the air–water interface.

RESULTS

1. Binding Data. The Scatchard plot for the fluorescence titration data obtained from puroindoline and LPC in 10 mM sodium phosphate buffer, pH 7.0, is shown in Figure 1. The curve shown has a positive (humped)

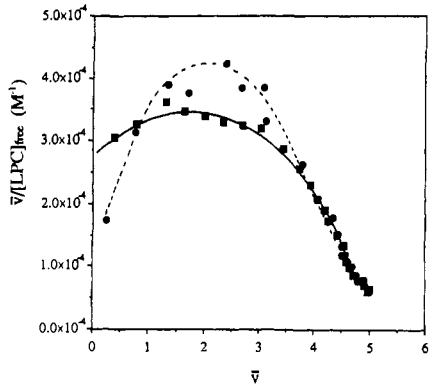

Figure 1. Scatchard plots of the fluorescence titration data obtained from the titration of puroindoline with LPC: (■) 10 mM Tris-HCl, pH 7.5; (●) 10 mM sodium phosphate, pH 7.0. υ is the fraction of occupied sites on the protein.

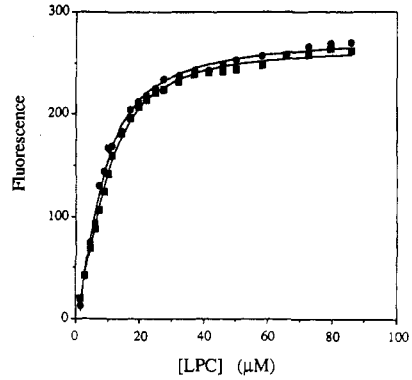

Figure 2. Fluorescence titration data obtained from the titration of puroindoline with LPC (symbols) and the computed fit to the Hill equation constrained to five binding sites: (■) 10 mM Tris-HCl, pH 7.5; (●) 10 mM sodium phosphate, pH 7.0.

shape which indicates positive cooperation between multiple LPC binding sites on the protein. An equilibrium dialysis experiment was performed to determine the number of LPC binding sites present on each molecule of puroindoline. Unfortunately, to obtain accurate equilibrium dialysis data, it is necessary to use much higher protein concentrations than those required for the fluorescence titration. Due to the limited availability of purified puroindoline, it was only practical to perform equilibrium dialysis measurements at three different LPC concentrations. LPC concentrations that provided half-saturation binding, saturation binding, and a 2-fold excess of saturation were chosen using the fluorescence titration data. These corresponded to LPC concentrations of 200, 400, and 800 μM, respectively. The concentration of LPC in the half-chamber that contained LPC alone was assayed after 20 h. The resulting concentrations were 135.6, 333.0, and 654.5 μM. Since LPC was applied to both sides of the dialysis membrane, the concentration of bound LPC was twice the concentration depleted from the nonprotein side. So the fractional occupation of puroindoline was 2.1 for the half-saturated sample, 5.4 for the saturated sample, and 4.7 for the sample with a 2-fold excess of LPC. Therefore, above the saturation concentration, each molecule of puroindoline bound approximately five molecules of LPC. Using the equilibrium dialysis results, it was possible to fit the Hill equation, constrained to five binding sites, to the fluorescence titration data. The calculated curve obtained is plotted in Figure 2 along with the fluorescence titration data. There is good agreement between the titration data and the computed fit. The fit returned a value for K_d of 54.3 μM with an Nh of 1.23.

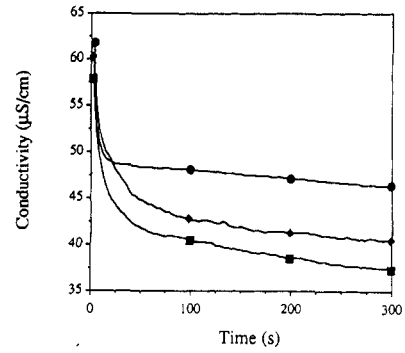

Figure 3. Foam conductivity decay curves for a sample containing (■) puroindoline alone and puroindoline in the presence of (♦) 1.3 μM (R = 0.16) and (●) 13.2 μM (R = 1.6) LPC.

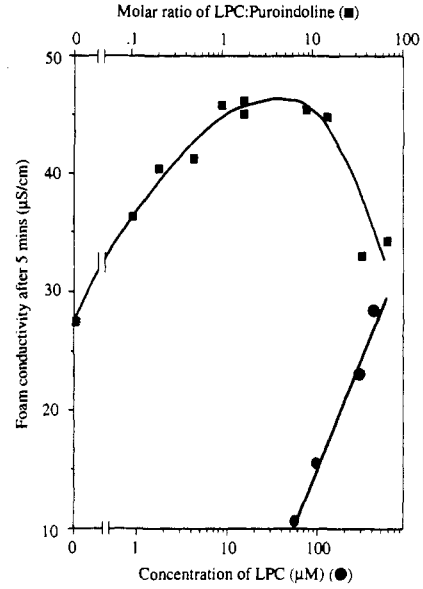

Figure 4. Stability of puroindoline foams as a function of LPC concentration as determined by conductivity remaining after 5 min of drainage. The data (■) are plotted in terms of the molar ratio of LPC:puroindoline and the total concentration of added LPC. Stability data are also shown for LPC alone (●).

2. Foaming. The conductivity decay curves for puroindoline alone and in the presence of 1.3 (R = 0.16) and 13.2 μM (R = 1.6) LPC are shown in Figure 3. The foam stability, as determined by the residual conductivity of the foam after 5 min of drainage, is shown as a function of LPC concentration in Figure 4. Puroindoline alone at 0.1 mg/mL formed a stable foam. Addition of LPC steadily increased the stability of the foam to a maximum at a molar ratio of R = 5. Above this ratio, the foam stability reduced to below the value obtained for puroindoline alone. Conductivity data for foams formed from equivalent concentrations of LPC alone (i.e., R = 10 is equivalent to 83 μM LPC) are also shown in Figure 4. A solution containing 83 μM LPC was the lowest concentration of LPC that was able to form a foam in the absence of puroindoline. The conductivity signal from LPC foams was lower than that observed in the equivalent mixed-component sample. The conductivity properties merge at concentrations above 100 μM (data not shown), suggesting that the interfaces of the foams formed from both LPC alone and mixed-component samples are dominated by LPC at these concentrations.

3. Thin Films. The drainage properties of the thin films were observed as a function of LPC concentration. The films drained in a manner typical for protein-stabilized films at values of R between 0 and 1 (Clark *et al.*, 1990a,

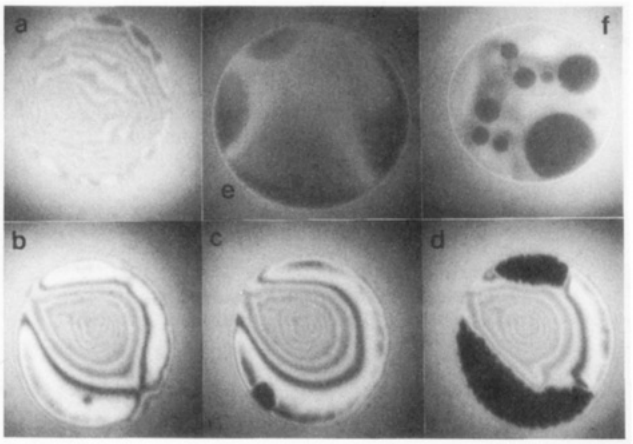

Figure 5. Photographs of stages in the drainage of air-suspended thin liquid films containing mixtures of puroindoline and LPC: (a) film containing aggregated protein typical of those observed at R values between 0 and 1; (b–d) different stages in the drainage of films showing transitional drainage behavior observed at R values between 1 and 2; (e, f) different stages in the drainage of films showing the surfactant-like drainage observed at R values greater than 2.

1991a). Although the solutions showed no evidence of protein aggregation, many aggregates were observed in the thin films even after filtration (Figure 5a). Between $R = 1$ and 2, an unusual phenomena was observed (Figure 5b–d). After formation, the thin films showed drainage behavior characteristic of films in which the protein–protein interactions at the interfaces were partially disrupted by competitive adsorption of low molecular weight surfactant. This has been observed previously with films containing mixtures of β-lactoglobulin and Tween 20 (Clark *et al.*, 1991a). However, this type of drainage was interrupted after approximately 1 min by the appearance of a thin black region which spread rapidly across the film in a few seconds (Figure 5c,d). Usually there is no difficulty in introducing interlamellar liquid into thin films by forcing liquid back into the film ring, even after equilibrium thickness has been attained. However, it was difficult to force liquid into the black region in these puroindoline/LPC films due to strong interactions present between the two surfaces of the film. This phenomenon was gradually reduced at LPC concentrations above $R > 2$ and was replaced by typical mobile surfactant-like drainage as shown in Figure 5e,f (Clark *et al.*, 1991a). Under these conditions, the initial drainage was much faster and more chaotic. The final transition to a black film occurred with very mobile, thin black "spots" moving around and gradually filling the film. As R was increased to greater than $R = 8$, the mobile black spots disappeared and the films thinned steadily to black. This latter behavior was also observed in thin films formed from LPC only.

Measurements of the equilibrium film thickness were made as a function of LPC concentration using an interferometric method (Clark *et al.*, 1990a). The results are shown in Figure 6. The broken line in Figure 6 indicates that the films did not reach equilibrium thickness between $R = 0$ and 1, due to high levels of protein aggregation in the films. The first thickness measurements were possible at $R = 1$, where the rapid transition to a black film was first observed (Figure 5b–d). This phenomenon ceased at approximately $R = 2$, which corresponded to the minimum observed equilibrium film thickness of 14 nm. A similar equilibrium thickness was observed for films containing LPC alone. In further experiments, lower concentrations of protein (0.05 mg/mL) were used and significantly less aggregation was observed. These films exhibited protein-

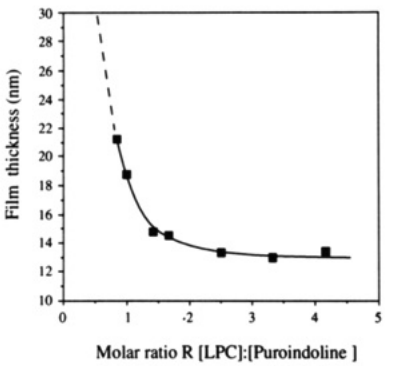

Figure 6. Equilibrium thickness of air-suspended thin liquid films of puroindoline as a function of LPC concentration determined by interferometry. The broken line indicates that the films did not reach a uniform equilibrium thickness at $R < 1$ due to the presence of protein aggregates.

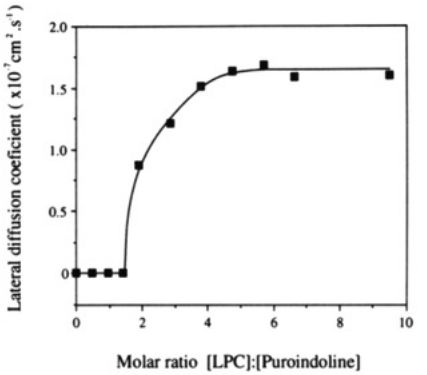

Figure 7. Lateral diffusion coefficient of adsorbed FITC-puroindoline as determined by FRAP measurements plotted as a function of LPC concentration.

like drainage behavior for solutions of R values between 0 and 1 and gradually drained to an equilibrium thickness of approximately 30 nm. We conclude that the unusual and rapid transition to a black film observed between $R = 1$ and 2 is due to the presence of LPC.

4. FRAP. Measurements of surface diffusion of FITC-labeled puroindoline in air-suspended thin films were made as a function of LPC concentration using FRAP. The lateral diffusion coefficient, D, for surface adsorbed FITC-puroindoline is shown as a function of R in Figure 7. A value for D of 0 indicated that there was no surface diffusion of the labeled protein (i.e., the protein was totally immobilized at the surface by protein–protein interactions). Surface diffusion of the FITC-puroindoline was first observed at $R = 2$. A rapid increase in D was observed between $R = 2$ and 3, which was followed by a more gradual increase in D up to $R = 5$, where a plateau value of 1.7×10^{-7} cm^2/s was attained. A typical FRAP data curve for a solution with an R value of 5 is shown in Figure 8.

5. Displacement. The results of a study of the displacement of puroindoline from the air–water interfaces of thin films by competitive adsorption of LPC are shown in Figure 9. It was not possible to make accurate measurements for solutions with $R < 1$, as the films did not reach equilibrium due to the presence of aggregation and contributions from soluble fluorescent-labeled protein in the interlamellar liquid. The amount of adsorbed FITC-puroindoline was gradually reduced between R values of 1 and 1.8. There was a sharp drop in the fluorescence and hence surface concentration of FITC-puroindoline between $R = 1.8$ and 3. At $R = 3$, the fluorescence signal was an order of magnitude less than its value at $R = 1.8$. There

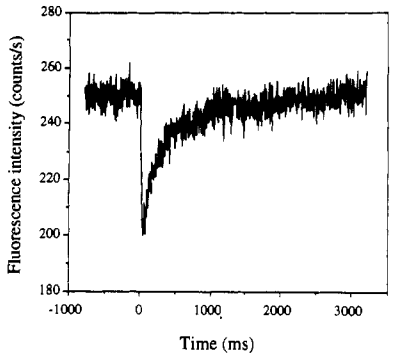

Figure 8. Typical FRAP data curve obtained from the summation of 10 experimental data curves measuring the lateral diffusion of adsorbed FITC-puroindoline in an air-suspended thin liquid film formed from a solution containing a 5-fold molar excess of LPC (R = 5).

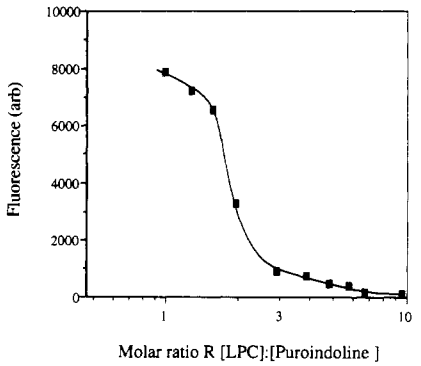

Figure 9. Reduction in the surface concentration of adsorbed FITC-puroindoline due to displacement by added LPC.

was a further steady decline in fluorescence at $R > 3$ as less and less FITC-puroindoline remained adsorbed at the interface.

DISCUSSION

A summary of the observed changes in the foaming and thin film properties of puroindoline as a function of added LPC is given in Table I. Puroindoline alone had extremely good foamability and foam stability properties and exceeded the foaming performance of recognized good foaming proteins, such as bovine serum albumin and β-lactoglobulin. The good foaming properties of this protein may be related to its tryptophan-rich domain. A tryptophan-rich synthetic peptide with excellent foaming properties has been described previously (Enser *et al.*, 1990).

The presence of relatively low concentrations of LPC enhanced puroindoline foam stability considerably. These low concentrations of LPC were less than the minimum concentration needed to form a foam from LPC alone. Therefore, the enhancement of foam stability is not explained by simple addition of the foaming properties of each of the individual components (puroindoline and LPC). This suggests that LPC and puroindoline may act synergistically, possibly via interaction to generate enhanced foam stability. Circumstantial evidence in support of this conclusion comes from foaming experiments with β-casein (data not shown), which demonstrated that LPC has very little effect on foam stability in this system. This is of interest because there is no evidence for interaction of LPC with β-casein. It is known that β-casein does not bind surfactants significantly (Clark *et al.*, 1992). We conclude that interaction of LPC with puroindoline results in the formation of a complex that is responsible for the change in foaming behavior of the mixtures.

Table I. Summary of Observed Changes in the Functional Properties of Puroindoline as a Function of Added LPC

R value	change in functional property
0–1	puroindoline foam stability significantly enhanced thin films contain aggregates and drain in a protein-like manner
1–1.5	smaller enhancement in foam stability thin films drain in a semimobile manner unusual transition to the common black film occurs displacement of puroindoline by LPC begins puroindoline is immobile at the air–water interface 9–15% of the total protein in solution is complexed in solution at R = 1.5 puroindoline starts to diffuse in the adsorbed layer
2–3	maximal foam stability film drainage becomes more chaotic puroindoline diffusion rate increases at the interface film thickness reaches minimum value most puroindoline has been displaced from the interface 15–25% of total protein in solution is complexed in solution
>3	foam stability decreases film thickness is same as for LPC alone film drainage becomes surfactant-like remaining puroindoline is gradually displaced puroindoline surface diffusion coefficient reaches a maximum

We have shown that LPC and puroindoline interact in solution. However, calculations based on the cooperative binding and dissociation coefficient reported in this paper suggest that at R = 1 only 6% of the puroindoline is in the form of LPC/puroindoline complex. This could be an underestimate of the true level of complex formed. The strong adsorption of puroindoline at the air–water interface will result in a high local concentration near the interface, which could lead to formation of a higher proportion of the LPC/protein complex.

Observations of thin films show that at R = 1 the drainage characteristics change from an aggregated protein-like drainage to semimobile drainage. This is indicative of a reduction in the strong protein–protein interactions usually formed at the surface. In addition, once the black film is formed, it is difficult to separate the two interfaces, which strongly suggests that an interaction that bridges across the two interfaces of the film is present. At this R value, FRAP shows that the adsorbed FITC-puroindoline is still immobile at the interface. These observations are evidence that strong protein–protein interactions exist at the interface throughout this range of R values, and this could account for the enhanced foaming properties of this lipid/protein complex. Paradoxically, the presence of adsorbed LPC or LPC/puroindoline complex at the interface also reduces the extent of protein aggregation in the thin films and the onset of semimobile drainage behavior. Our hypothesis is that these two separate effects are properties of the complex.

The films reduce in thickness more freely in the absence of protein aggregates and reach an equilibrium common black film relatively rapidly. This lack of aggregation and improved film thinning probably account for the unusual transition to a black film. It is possible that the protein adsorbs at the upper and lower interfaces of the film in a preferred orientation which may expose mutually attractive protein surfaces leading to bridging across the interlamellar region.

The unusual drainage behavior, which causes transition to a common black film, is more rapid and fluid at higher R values. Instead of a black area spreading across the

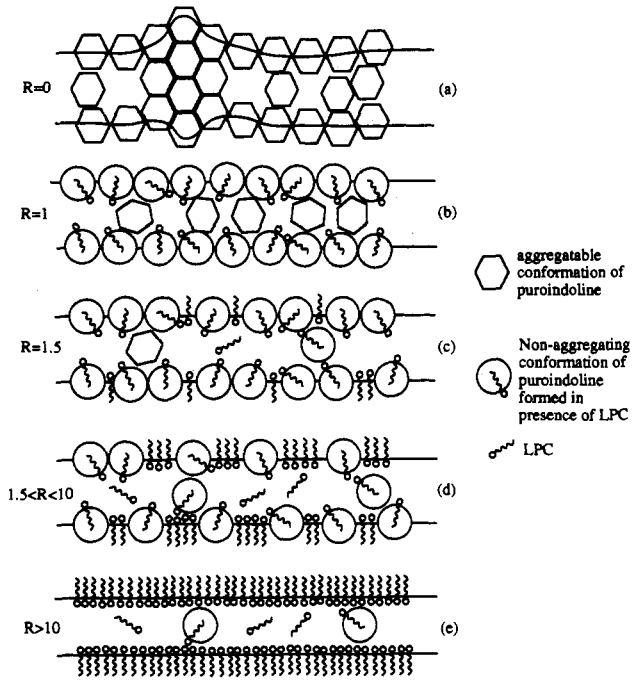

Figure 10. Speculative representation of changes in the structure of the adsorbed layer of puroindoline as a function of added LPC.

film, several black spots form and move around in the film. This change in drainage behavior is coincident with the onset and increase in lateral diffusion of FITC-puroindoline at the interface. In addition, a significant proportion of the protein of displaced from the film surface. The onset of protein surface diffusion correlates with a reduction in foam stability as was seen with mixtures of β-lactoglobulin and Tween 20 previously (Coke *et al.*, 1990). Thus, the stiff, immobile, viscoelastic protein film at the interface is disrupted by adsorbed LPC which reduces film and foam stability. Restabilization of the films and foam by the Marangoni mechanism, which relies on rapid surface diffusion of adsorbed species, will only occur when the relatively large, slowly diffusing protein molecules are completely displaced from the adsorbed layer by LPC. It would appear from our experiments that pure LPC foams are not as stable as puroindoline foams.

Above $R = 3$, the films behave more like the pure LPC-stabilized films. The black-spot stage of film drainage disappears, and the equilibrium thickness becomes similar to that of pure LPC films. Foam stability drops to that of LPC alone, and the displacement data show that more and more of the puroindoline is expelled from the surface by LPC. Therefore, LPC becomes the dominant species at the interface and the functional properties of the system revert to those of LPC alone.

A speculative representation of changes in the molecular composition of thin films of puroindoline as a function of LPC concentration is presented in Figure 10. High levels of aggregation are present in the films in the absence of LPC (i.e., $R = 0$) (Figure 10a). At $R = 1$, there is sufficient LPC present to either interact with the protein or adsorb at the interface and prevent aggregate formation (Figure 10b). This is represented in the scheme by a change in the conformation of puroindoline. At $R = 1.5$, the level of adsorbed LPC at the interface is sufficient to break up protein–protein interactions in the adsorbed layer with the resultant onset of surface diffusion (Figure 10c). Protein is progressively displaced from the interface with each further addition of LPC ($1.5 < R < 10$) (Figure 10d). The protein is effectively completely displaced from the interface above $R = 10$ (Figure 10e). The positive charges associated with the adsorbed layer of LPC probably repel puroindoline, which carries a net positive charge at neutral pH.

This protein could have important technological applications in the food industry. It is widely known that protein foams are very susceptible to destabilization by lipid. For example, egg white foams are sensitive to lipid contamination by egg yolk lipids. This protein may provide the means of complexing lipid in solution, preventing it from reaching the interface. Alternatively, the disruptive effect of lipid adsorption in protein-stabilized foams may be eliminated by interaction with puroindoline at the interface. Preliminary experiments with beer foam have demonstrated that the presence of low concentrations of puroindoline completely overcomes the foam destabilization by a selection of lipids (Clark *et al.*, 1993).

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