Competitive Adsorption of Egg White Proteins at the Air–Water Interface: Direct Evidence for Electrostatic Complex Formation between Lysozyme and Other Egg Proteins at the Interface

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The competitive adsorption of five major egg white proteins, viz., ovalbumin, ovotransferrin, ovoglobulins, ovomucoid, and lysozyme, from a bulk solution having relative protein concentration ratios similar to those in egg white to the air–water interface has been studied at low and high ionic strengths. At 0.1 ionic strength, only ovalbumin and ovoglobulins adsorbed to the interface, and ovotransferrin, ovomucoid, and lysozyme were essentially excluded from the interface. Notably, the surface concentration of lysozyme was essentially zero, indicating that at 0.1 ionic strength it was not electrostatically associated with the other egg white proteins at the interface. However, at 0.002 ionic strength, a significant amount of lysozyme adsorbed to the interface along with the other egg white proteins. Evidence is provided which suggests that at low ionic strength lysozyme forms binary or ternary electrostatic complexes with the other proteins and thus its adsorption to the interface is facilitated by the adsorption of other proteins.

Keywords: *Egg proteins; competitive adsorption; air–water interface; electrostatic interactions; lysozyme; foaming properties; functional properties*

INTRODUCTION

Egg albumen is extensively used as a functional protein ingredient in many processed foods because of its excellent foaming properties. Several studies on the foaming and emulsifying properties of egg white proteins have been reported in an attempt to understand the role of various constituent proteins of egg albumen in the expression of its surface active properties (Li-Chan and Nakai, 1989; Acton et al., 1990; Johnson and Zabik, 1981). Comparison of the stabilities of ovalbumin-, ovotransferrin-, and lysozyme-stabilized emulsions showed that emulsions stabilized by ovotransferrin and ovalbumin were more stable than that stabilized by lysozyme (Acton et al., 1990). This has been attributed to less molecular flexibility of lysozyme as compared to ovotransferrin and ovalbumin. Johnson and Zabik (1981) reported that the foaming properties of egg white proteins followed the relative order globulins > ovalbumin > ovotransferrin > lysozyme > ovomucoid > ovomucin. Although these studies described the relative surface activities of the constituent proteins of egg white, it is not known what role, if any, interactions among these proteins play in the expression of surface activity of egg white.

It has been suggested that the excellent foaming properties of egg albumen are attributable to different charge characteristics of the constituent proteins (Poole et al., 1984). Specifically, it has been suggested that the positively charged lysozyme, which has an isoelectric point of 10.7, plays a vital role in the formation and stability of egg albumen foams (Poole et al., 1984). It is believed that during foaming, both the positively charged lysozyme and other negatively charged egg white proteins migrate to the air–liquid interface. At the interface, the positively charged lysozyme interacts electrostatically with the other negatively charged proteins, and this effectively reduces electrostatic repulsive interactions in the protein film and thus stabilizes the foam (Poole et al., 1984; Clark et al., 1988). Studies on the foaming properties of bovine serum albumin (BSA) showed that both foam expansion and foam stability improved dramatically in the presence of lysozyme (Poole et al., 1984; Clark et al., 1988). On the basis of these findings, Poole (1989) suggested that, as a general rule, the foaming properties of acidic proteins can be enhanced by adding a basic protein that has a molecular weight of at least 4000 and a p*I* of >9. However, careful examination of the foaming properties of several food proteins in the presence of lysozyme indicates that this may not be a universal effect (Poole et al., 1984). For instance, while lysozyme enhanced both foam expansion and foam stability of BSA, bovine β-globulin, and β-lactoglobulin, it had no effect on soy protein isolate, sodium caseinate, fibrinogen, and several individual egg white proteins, particularly ovalbumin, ovotransferrin, and ovoglobulin (Poole et al., 1984).

Although the positive effect of lysozyme on the foaming properties of several acidic proteins has been experimentally observed, there is no direct experimental evidence showing electrostatic complex formation between lysozyme and acidic proteins *in* the adsorbed protein film at the air–water interface. Circumstantial evidence based on the detection of lysozyme in drained foam by electrophoresis is unreliable because of the possibility of entrapment of lysozyme in the plateau borders of the foam during drainage (Poole et al., 1984). Furthermore, several acidic proteins form insoluble

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electrostatic complexes with lysozyme (Damodaran and Kinsella, 1986; Poole et al., 1984), and the insoluble complexes are generally not good foaming agents.

In this paper, competitive adsorption of five egg white proteins, namely, lysozyme, ovalbumin, ovotransferrin, ovomucoid, and ovoglobulins, from a bulk mixture (in which the concentration ratios of the above five proteins was the same as in egg white) to the air−water interface was studied by quantitatively determining the concentration of each constituent protein of egg white in the mixed-protein film at the air−water interface. It is shown that, of the five major egg white protein constituents, only ovalbumin and globulins adsorbed to the air−water interface at 0.1 ionic strength and the other three proteins were essentially excluded from the interface. However, at 0.002 ionic strength a significant amount of lysozyme was incorporated into the interfacial protein film along with the other four egg white proteins.

MATERIALS AND METHODS

Chicken egg white proteins, i.e., lysozyme, ovalbumin, ovotransferrin, ovomucoid, and ovoglobulins, were purchased from Sigma Chemical Co. (St. Louis, MO). Except for ovoglobulins, which is a mixture of different globulins (Nakamura et al., 1980), all other proteins were at least 99% pure as indicated by a single band in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE). Ultrapure NaH_2PO_4 and Na_2HPO_4 were purchased from Aldrich Chemical Co. (Milwaukee, WI). [^{14}C]Formaldehyde was from New England Nuclear Co. (Boston, MA). The specific radioactivity of the [^{14}C]formaldehyde was 58 mCi/mmol. All other reagents in this study were of analytical grade.

Extreme care was taken in purifying water for adsorption studies. A Milli-Q ultrapure water purification system (Millipore Corp., Bedford, MA) with a Qpak-1 cartridge package capable of removing inorganic and organic impurities was used to purify water. The resistivity of water was usually 18.2 mΩ cm. To check water quality, the surface tension of water was measured at 20 °C. If the surface tension of water was not 72.9 ± 0.1 mN/m and did not remain constant during 24 h of aging, it was discarded.

Radiolabeling. The proteins were radiolabeled with ^{14}C nuclide by reductive methylation of amino groups with [^{14}C]-formaldehyde at pH 7.5 as described previously (Xu and Damodaran, 1992, 1993, 1994). At the reaction pH 7.5, only the N-terminal α-amino group, not the ε-amino group of lysyl residues, will be predominantly methylated. Since the methylated amine groups retain their charge (Jentoft and Dearborn, 1979), the electrostatic properties of the proteins will not be affected. The protein concentration was determined using $E^{1\%}$ values of 26.3 at 281 nm for lysozyme, 7.5 at 280 nm for ovalbumin, 4.55 at 280 nm for ovomucoid, and 11.6 at 280 nm for ovotransferrin. The concentration of ovoglobulin was determined according to the Lowry et al. (1951) method. The specific radioactivities of the labeled proteins were 1.38, 0.78, 0.59, 0.957, and 1.49 μCi/mg, respectively, for lysozyme, ovalbumin, ovomucoid, ovotransferrin, and globulins. These values correspond to incorporation of less than 1 mol of $^{14}CH_3$/mol of protein. At this low level of reductive methylation, neither the conformation of the proteins nor their net charge would be significantly altered.

Adsorption Studies. The kinetics of adsorption of radiolabeled proteins at the air−solution interface was studied as described elsewhere (Xu and Damodaran, 1992, 1993). Briefly, the rate of change of protein concentration at the air−water interface of radiolabeled protein solutions (120 mL) in a Teflon trough (21 × 5.56 × 1.27 cm) was monitored by measuring surface radioactivity using a rectangular gas proportional counter (8 × 4 cm) (Ludlum Measurements, Inc., Sweetwater, TX). The entire experimental setup was housed in a refrigerated incubator maintained at 25 ± 0.2 °C. A carrier gas composed of 98% argon and 2.0% propane was passed continuously through the gas proportional counter at a rate of 20 mL/min. A calibration curve relating cpm versus surface radioactivity (μCi/m^2), constructed by spreading ^{14}C-labeled β-casein on the air−water interface, was used to convert surface cpm versus adsorption time to μCi/m^2 versus time. The surface concentration (mg/m^2) as a function of adsorption time was then calculated by multiplying μCi/m^2 with specific radioactivity (μCi/mg) of the protein. The rationale for using ^{14}C-labeled β-casein to construct the cpm versus surface radioactivity (μCi/m^2) has been discussed elsewhere (Xu and Damodaran, 1993). The contribution of bulk radioactivity to cpm was corrected using a standard curve relating cpm versus specific radioactivity of $CH_3{}^{14}COONa$ solutions. The rate of change of surface pressure was monitored according to the Wilhelmy plate method using a thin sand-blasted platinum plate (1 cm width) hanging from an electrobalance (Cahn Instruments, Co., Escondido, CA). Both surface concentration and surface pressure were monitored simultaneously for each protein solution.

In competitive adsorption experiments involving mixtures of ovalbumin, ovotransferrin, ovomucoid, ovoglobulins, and lysozyme, the following approach was used to monitor adsorption of each protein component from the bulk mixture to the air−solution interface (Anand and Damodaran, 1995; Xu and Damodaran, 1994): To determine the kinetics of adsorption of ovalbumin from the bulk mixture, an aliquot of ^{14}C-labeled ovalbumin stock solution was mixed with unlabeled stock solutions of the other proteins to the required final concentration ratios. The protein mixture solution was poured into the Langmuir trough, and the rate of increase of surface radioactivity was monitored. Although all five proteins would simultaneously adsorb to the air−solution interface, the measured rate of increase of surface radioactivity would correspond only to the rate of increase of the concentration of ovalbumin at the interface. Following this procedure, the rate of adsorption and the equilibrium concentration of each protein component at the interface was studied by including radiolabeled proteins one at a time in the bulk mixture. In all cases, the relative concentration ratios of various proteins and the total protein concentration in the bulk mixture were the same.

Table 1. Composition and Some Physicochemical Properties of Major Proteins of Egg White

protein	% of total protein	mol wt[a]	isoelectric point
ovalbumin	54	45000	4.5
ovotransferrin	12−13	77700	6.0
ovomucoid	11	28000	4.1
lysozyme	3.4−3.5	14300	10.7
ovoglobulins	8	49000	5.5−5.8

[a] From Li-Chan and Nakai (1989).

RESULTS AND DISCUSSION

Table 1 shows the composition and some physicochemical characteristics of the major egg white proteins (Li-Chan and Nakai, 1989). To simulate competitive adsorption of the major egg proteins at the air−water interface of foams, it is essential to keep the relative concentration ratios of the five major proteins in the bulk phase of the model system the same as those found in egg white. Thus, the bulk concentrations used in this study were 5.4, 1.2, 1.1, 0.8, and 0.35 μg/mL, respectively, for ovalbumin, ovotransferrin, ovomucoid, ovoglobulin, and lysozyme. The rationale for selecting these concentrations is as follows: If the concentrations are lower than the above, then the extent of adsorption of some of the protein components may be so low that it may not be possible to monitor their kinetics and equilibrium adsorption. On the other hand, if the concentrations are much higher than the above concentrations, then some of the components may adsorb so

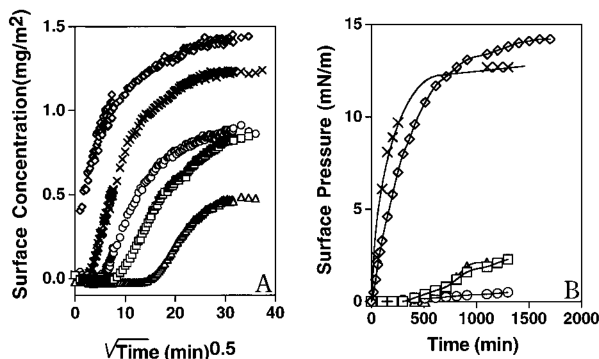

Figure 1. Time-dependent increase of surface concentration (A) and surface pressure (B) during adsorption of egg white proteins at the air−water interface in single-protein systems at 25 °C. The initial protein concentrations of the bulk phase (20 mM phosphate-buffered saline, pH 7.0, $I = 0.1$) were 5.4, 1.2, 1.1, 0.8, and 0.35 μg/mL, respectively, for ovalbumin (◇), ovotransferrin (□), ovomucoid (○), ovoglobulins (×), and lysozyme (△).

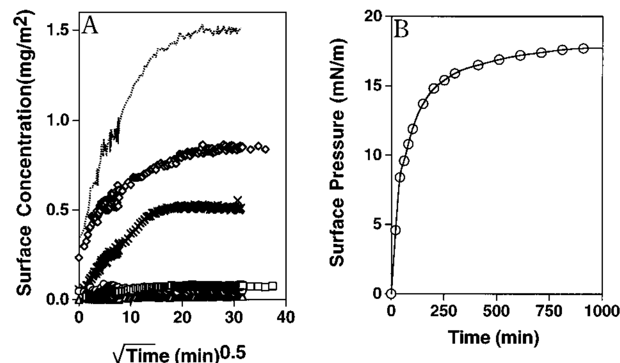

Figure 2. Time-dependent increase of surface concentration (A) and surface pressure (B) during adsorption of egg white proteins at the air−water interface in a five-component protein system at 25 °C. The initial protein concentrations of the bulk phase (20 mM phosphate-buffered saline, pH 7.0, $I = 0.1$) were 5.4, 1.2, 1.1, 0.8, and 0.35 μg/mL, respectively, for ovalbumin (◇), ovotransferrin (□), ovomucoid (○), ovoglobulins (×), and lysozyme (△). The solid line in (A) represents the sum total of all curves.

rapidly that it may not be possible to study the kinetics of adsorption. Preliminary experiments showed that the above concentrations were adequate for monitoring both the kinetics and the equilibrium adsorption of all five proteins both in single-component and multicomponent systems.

Figure 1A shows the rate of change of surface concentration of ovalbumin, ovotransferrin, ovomucoid, ovoglobulin, and lysozyme in single-protein systems from a 20 mM phosphate-buffered saline (PBS) solution (pH 7.0, $I = 0.1$). The adsorption data are plotted as square-root-of-time kinetics because the kinetics of arrival of proteins from a quiescent bulk phase to the air−water interface is believed to follow a diffusion-controlled adsorption process according to the expression

$$\Gamma_t = 2C_0(D/3.1416)^{1/2}t^{1/2} \quad (1)$$

where Γ_t is surface concentration at time t, C_0 is bulk protein concentration, and D is diffusion coefficient. All five egg white proteins apparently reached equilibrium adsorption after ∼1000 min. At the bulk concentration levels used, the rate and extent of adsorption of ovalbumin were greater than those of the other egg white proteins. This was partly because the bulk concentration of ovalbumin was much higher than those of the other proteins. It should be noted, however, that the adsorption curves of ovotransferrin, ovomucoid, ovoglobulins, and lysozyme showed a lag phase. This indicates that adsorption did not begin soon after a fresh air−water interface was created and that these proteins experienced an initial energy barrier for adsorption at the air−water interface (Xu and Damodaran, 1992). The lag times for adsorption of ovoglobulins, ovomucoid, ovotransferrin, and lysozyme were 7, 25, 80, and 220 min, respectively. It should be noted that the lag time, the rate of adsorption, and the extent of equilibrium adsorption of the egg white proteins were not related to their bulk phase concentration (Figure 1A). For example, although the bulk concentration of ovoglobulins (0.8 μg/mL) was lower than that of ovotransferrin (1.2 μg/mL), the rate and equilibrium adsorption of ovoglobulins were much greater than those of ovotransferrin. Similarly, although the bulk concentration of ovoglobulin was ∼7-fold lower than that of ovalbumin, the surface concentration of ovoglobulin at equilibrium adsorption was only slightly lower than that of ovalbumin. Conversely, at identical bulk concentrations, the rate and extent of adsorption of ovoglobulins at the air−water interface would have been much greater than that of the other egg white proteins. This clearly suggests that differences in physicochemical characteristics, such as conformational flexibility and hydrophobicity/hydrophilicity characteristics of the proteins' surface, were the major determinants of surface activities of egg white proteins (Damodaran, 1989).

Figure 1B shows the time-dependent increase of surface pressure during adsorption of egg white proteins from a 0.1 ionic strength PBS solution to the air−water interface in single-component systems. The rate of increase of surface pressure (i.e., the net reduction in surface tension) of ovoglobulins was faster than that of ovalbumin, even though its rate of increase of surface concentration was slower than that of ovalbumin (Figure 1A). At equilibrium, however, the surface pressure of ovalbumin was higher than that of the other egg white proteins. Taken together, the data in parts A and B of Figure 1 provide several interesting facts. First, there seems to be no correlation between equilibrium surface concentration and equilibrium surface pressure. For example, although the equilibrium surface concentration of ovomucoid was ∼0.9 mg/m^2 compared to ∼1.25 mg/m^2 for ovoglobulins, the equilibrium surface pressure of ovomucoid was <1 mN/m, whereas that of ovoglobulins was ∼13 mN/m. Similarly, even though the equilibrium surface concentrations of ovomucoid and ovotransferrin were very similar, the surface pressure of ovotransferrin was ∼2.5 mN/m compared to <1 mN/m for ovomucoid. These results indicate that although ovomucoid and ovotransferrin adsorb to a significant extent to the air−water interface, they do not reduce the free energy of the air−water interface as much as ovoglobulins do. This may be related to differences in the abilities of these globular proteins to unfold at the air−water interface and occupy a greater area at the interface (Damodaran, 1997).

Figure 2A shows kinetics of competitive adsorption of each of the five egg white proteins from a bulk mixture to the air−water interface at 0.1 ionic strength. The concentration of each protein in the bulk mixture was exactly the same as that in single-component

experiments shown in Figure 1A. The ratio of the proteins in the bulk mixture was the same as that in egg white. The adsorption behavior of these proteins was quite different from that in single-component systems. Only ovalbumin and ovoglobulins adsorbed to the interface, and ovotransferrin, ovomucoid, and lysozyme were essentially excluded from the interface. Notably, the surface concentration of lysozyme was essentially zero.

The exclusion of ovomucoid, ovotransferrin, and lysozyme from the air–solution ($I = 0.1$) interface in the multiprotein system might be related to differences in the rates of arrival of ovalbumin, ovotransferrin, ovoglobulins, ovomucoid, and lysozyme at the interface. It should be pointed out that, in the single-component systems, lysozyme, ovomucoid, and ovotransferrin exhibited long lag times for the initiation of adsorption at the interface, whereas ovalbumin readily adsorbed and ovoglobulins displayed only ~7 min of lag time (Figure 1A). If these lag periods for adsorption also existed in the multiprotein system, then by the time ovomucoid began to adsorb to the interface, the interface would have been covered with ~0.3 mg/m^2 of ovoglobulins and ~0.9 mg/m^2 of ovalbumin. The extent of adsorption of ovomucoid to the interface would then depend on the unoccupied area available between the adsorbed ovalbumin and ovoglobulin molecules at the interface. If this area was smaller than the minimum area required for ovomucoid to adsorb, then adsorption of ovomucoid might not take place. Since the lag times for ovotransferrin and lysozyme were much longer (i.e., 80 and 220 min, respectively) than that for ovomucoid, adsorption of these also might not be possible if the area available at the interface at the time of their arrival at the interface was smaller than the minimum area required for these proteins to anchor to the interface. In other words, exclusion of ovomucoid, ovotransferrin, and lysozyme from the air–water interface in the multiprotein system was related to the relative rate of arrival of the proteins at the interface. The proteins (ovalbumin and ovoglobulins) that arrived first at the interface adsorbed to the interface, and the late-arriving proteins (ovotransferrin, ovomucoid, and lysozyme) could not displace the adsorbed proteins.

It is notable that among the egg white proteins lysozyme was almost totally excluded from the interface. This suggests that, at 0.1 ionic strength, lysozyme was not electrostatically associated with any of the other egg white proteins and that in the multicomponent system it followed an adsorption kinetics similar to that in the single-component system. Furthermore, even when it arrived at the interface, its collision with the already adsorbed ovalbumin and ovoglobulin molecules at the interface did not lead to electrostatic complex formation.

The development of surface pressure during adsorption of egg white proteins in the five-component system at 0.1 ionic strength PBS is shown in Figure 2B. The maximum surface pressure reached ~18 mN/m. The rate of increase of surface pressure in the five-component system was faster than that of any of the five proteins in the single-component systems (Figure 1B), although the surface concentration at equilibrium in the five-component system was similar to that of ovalbumin in the single-component system. This suggests that protein–protein interactions, mainly between ovalbumin and ovoglobulins, in the mixed-protein film might reduce the interfacial free energy much more effectively

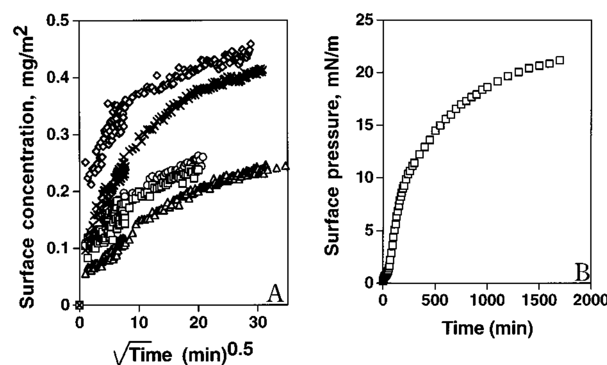

Figure 3. Time-dependent increase of surface concentration (A) and surface pressure (B) during adsorption of egg white proteins at the air–water interface in a five-component protein system at 25 °C at low ionic strength. The initial protein concentrations of the bulk phase (1.0 mM phosphate buffer, pH 7.0) were 5.4, 1.2, 1.1, 0.8, and 0.35 μg/mL, respectively, for ovalbumin (◇), ovotransferrin (□), ovomucoid (○), ovoglobulins (×), and lysozyme (△).

than either ovalbumin or ovoglobulins alone. To determine if coadsorption of lysozyme at the air–solution interface occurs at low ionic strength, the kinetics of competitive adsorption of each of the five egg white proteins from a 1.0 mM phosphate buffer (pH 7.0, $I = 0.002$) bulk phase to the air–water interface was studied. The concentration of each protein and the concentration ratios of the proteins in the bulk mixture were exactly the same as in Figure 2A. As shown in Figure 3A, the competitive adsorption behavior of these proteins at 0.002 ionic strength was quite different from that at 0.1 ionic strength. Lysozyme, ovomucoid, and ovotransferrin, which were excluded from the air–solution interface at 0.1 ionic strength (Figure 2A), were able to adsorb to the air–solution interface at 0.002 ionic strength. However, since the total amounts of protein adsorbed at the interface at equilibrium were almost the same in both systems, adsorption of these three proteins seems to have occurred mainly at the expense of a decrease in the extent of adsorption of ovalbumin and ovoglobulin. The extent of adsorption of ovalbumin decreased from 0.9 to ~0.45 mg/m^2, whereas that of ovoglobulins decreased only from 0.5 to ~0.4 mg/m^2 (Figures 2A and 3A).

The rate of increase and the equilibrium surface pressure of the protein film formed at 0.002 ionic strength (Figure 3B) were significantly different from those of the protein film formed at 0.1 ionic strength (Figure 2B). The rate of increase of surface pressure was slower, but the equilibrium surface pressure was higher at ionic strength 0.002 than at 0.1. The apparent equilibrium surface pressure was ~21 mN/m at 0.002 ionic strength compared to ~16.5 mN/m at 0.1 ionic strength. The slower rate of increase of surface pressure at low ionic strength might be related to a slower rate of adsorption of the high molecular weight electrostatic complexes formed between lysozyme and the other proteins. However, once adsorbed, these complexes formed a cohesive film at the interface and thus decreased the interfacial tension much more efficiently than at 0.1 ionic strength.

Adsorption of lysozyme to the air–water interface at 0.002 ionic strength might occur via two different mechanisms. First, at 0.002 ionic strength, lysozyme might form electrostatic complexes with the other proteins in the bulk phase and then adsorb to the air–solution interface. Second, lysozyme may remain un-

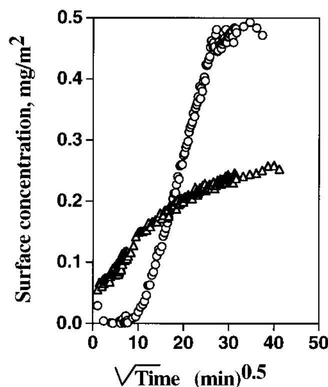

Figure 4. Rate of increase of surface concentration of radiolabeled lysozyme in single-component (○) and in five-component (△) systems in 1.0 mM phosphate buffer, pH 7.0, at 25 °C. Lysozyme concentration in the bulk phase in both cases was 0.35 μg/mL.

associated with the other proteins in the bulk phase. After adsorbing to the interface, it may form complexes with the other proteins in the interface. To determine which of these two pathways is dominant, the kinetics of adsorption of lysozyme alone in 1.0 mM phosphate buffer containing 0.35 μg/mL lysozyme was studied and compared with its kinetics in the five-component system. Figure 4 shows differences between the kinetics of adsorption of lysozyme at 0.002 ionic strength in single-component and five-component systems. In the single-component system, lysozyme exhibited a lag time for adsorption, whereas it was absent in the five-component system. This clearly indicates that at 0.002 ionic strength, lysozyme predominantly existed as an electrostatic complex with other proteins, and its adsorption to the interface was facilitated by the adsorption of other proteins.

At equilibrium, the ratio of surface concentrations of ovalbumin/ovotransferrin/ovomucoid/ovoglobulins/lysozyme in the adsorbed film was about 9:5:5:8:4. This ratio was much higher than the concentration ratios of the proteins in the bulk phase. This indicated that the minor protein components adsorbed more at the expense of the major component, viz., ovalbumin. Since the concentration ratios of egg proteins used in this study were similar to that found in egg white, it is reasonable to assume that concentration ratios of various proteins in the protein films of a real egg white foam also might be similar to the above ratio.

The results of this study provide direct evidence for the involvement of lysozyme in the formation of egg white protein films at the air–water interface at low ionic strength. The data also show that incorporation of all five major egg white proteins in the protein film at the air–water interface occurs only at very low ionic strength. At 0.1 ionic strength, adsorption of only ovalbumin and ovoglobulins occurs and the other three proteins are totally excluded from the interface. However, it should be recognized that although the ratio of egg white proteins used in this study was similar to that which occurs in egg white, the protein concentrations were much lower than that normally present in liquid egg white. Kato et al. (1975) showed that a considerable amount of lysozyme exists in the form of electrostatic complexes with ovotransferrin, ovoalbumin, and ovomucin in liquid egg white. Thus, it is possible that, depending on the magnitude of the association constant of lysozyme with other egg white proteins, at higher protein concentrations a fraction of lysozyme may still exist in the form of an electrostatic complex with other egg white proteins even at 0.1 ionic strength. If this is the case, then, even at high ionic strength, lysozyme may be involved in electrostatic stabilization of egg white foams.

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