Coadsorption of β-Casein and Bovine Serum Albumin at the Air–Water Interface from a Binary Mixture

Yunhong Cao and Srinivasan Damodaran*

Department of Food Science, University of Wisconsin–Madison, 1605 Linden Drive, Madison, Wisconsin 53706

Competitive adsorption of bovine serum albumin (BSA) and β-casein from binary solutions to the air–water interface have been studied. Adsorption of these proteins followed a kinetically controlled, instead of a Langmuirian-type thermodynamically controlled, competitive adsorption mechanism. Molecular area calculations indicated that the excluded area between protein molecules in the binary protein film at the air–water interface was greater than that in saturated monolayers of single protein systems, indicating thermodynamic incompatibility of mixing of these proteins. Adsorption of 3 molecules of BSA excluded adsorption of 16 molecules of β-casein from the interface and *vice versa*. Sequential adsorption experiments showed that β-casein can displace β-casein from the interface, whereas it cannot displace adsorbed BSA. In contrast, BSA can neither displace β-casein nor exchange with adsorbed BSA. The results showed that the protein component that arrives first to the interface adsorbs first, and the late-arriving protein component cannot displace the adsorbed component regardless of its surface active properties.

Keywords: *Air–water interface; competitive adsorption; β-casein; serum albumin; proteins; thermodynamic incompatibility*

INTRODUCTION

Food proteins are generally mixtures of several protein components. Thus, functional properties, such as foaming and emulsifying properties, of food proteins are innately dependent on the composition and relative surface activity of each protein component in the mixture. For example, the superior foaming and emulsifying properties of egg white arise from complex interactions among its protein constituents, namely ovalbumin, conalbumin, lysozyme, ovomucin, and other proteins. Recent studies have indicated that the foaming properties of acidic proteins can be dramatically improved by mixing them with basic proteins, such as lysozyme and clupine (Poole, 1989; Poole et al., 1984, 1987). This enhancing effect is thought to be related to formation of an electrostatic complex between acidic and basic proteins. The rheological properties of mixed protein films formed at the oil–water interface were also shown to be dependent on interfacial composition, type and concentration of protein constituents in the bulk phase, and interactions among proteins at the interface (Castle et al., 1987).

It is generally assumed that when a protein solution is bubbled or whipped to create a foam, the composition of the adsorbed protein layer is similar to that of the bulk phase. Implicite in this notion is that all proteins have equal affinity to the air–water interface, and thus the concentration of each protein in the adsorbed layer is only affected by its relative concentration in the bulk phase. However, since protein components in a protein mixture differ in size, shape, surface hydrophobicity, and conformational stability at interfaces, composition of an adsorbed protein layer must be a complex function of differences among the proteins in terms of their rates of adsorption, their affinities to an interface, their thermodynamic compatibility of mixing at an interface, and their concentration ratio in the bulk phase. The importance of these factors on competitive adsorption of proteins from bulk mixtures and the influence of competitive adsorption on properties of protein-stabilized foams are not well understood.

Studies on coadsorption of two structurally very different proteins, namely β-casein and lysozyme, showed that the extent of adsorption of these two proteins to the air–water interface from binary mixtures followed a kinetically controlled mechanism (Xu and Damodaran, 1994). The protein that arrived first at the interface adsorbed first, and it was not displaced by a late-arriving second protein, irrespective of the second protein's affinity to the interface. The β-casein/lysozyme binary system represents two extremely different proteins. Lysozyme is a highly ordered, hydrophilic, and basic protein, whereas β-casein is a flexible, hydrophobic, and negatively charged protein. To elucidate if the conclusions drawn from β-casein/lysozyme binary system are true for other binary protein systems, we studied the kinetics of coadsorption of proteins from a β-casein/bovine serum albumin binary system. We report that even in the case of a β-casein/BSA system, which represents a flexible/rigid but negatively/negatively charged binary system, coadsorption at the air–water interface did not follow a Langmuirian-type competitive adsorption mechanism but rather a kinetically controlled adsorption mechanism.

MATERIALS AND METHODS

Bovine serum albumin (BSA, crystallized and lyophilized) and β-casein were from Sigma Chemical Co. (St. Louis, MO). Ultrapure Na_2HPO_4 and NaH_2PO_4 were from Aldrich Chemical Co. (Milwaukee, WI). [^{14}C]Formaldehyde was from New England Nuclear Co. (Boston, MA). Purified water from a Milli-Q ultrapure water system (Millipore Corp., Bedford, MA) with a resistivity of 18.2 mΩ·cm was used in all adsorption experiments.

Radiolabeling and Protein Assay. Proteins were radiolabeled by reductive methylation of amino groups with [^{14}C]-formaldehyde at pH 7.0 as described elsewhere (Xu and

* Author to whom correspondence should be addresssed [telephone (608) 263-2012; fax (608) 262-6872; e-mail sdamodar@facstaff.wisc.edu].

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Damodaran, 1992). The protein concentration was determined using an $E^{1\%}$ value of 6.67 at 279 nm for BSA and 4.6 at 280 nm for β-casein. The specific radioactivities of the labeled proteins were 1.91 and 1.62 mCi/mg, respectively, for BSA and β-casein.

Measurements of the Rate and Degree of Adsorption. The kinetics of adsorption of proteins at the air–water (20 mM phosphate-buffered saline solution, pH 7.0, I = 0.1, 24 ± 0.2 °C) interface were studied as described in detail elsewhere (Xu and Damodaran, 1992, 1993a,b). 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 24 ± 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 counts per minute versus surface radioactivity (millicuries per square meter), constructed by spreading ^{14}C-labeled β-casein on the air–water interface, was used to convert counts per minute to millicuries per square meter. The surface concentration (milligrams per square meter) was then calculated by multiplying surface activity with specific radioactivity (millicuries per milligram) of the protein. The contribution of bulk radioactivity to counts per minute was corrected using a standard curve relating counts per minute versus specific radioactivity of $CH_3{}^{14}COONa$ solutions. The rate of change of surface pressure was monitored by the Wilhelmy plate method using a thin sand-blasted platinum plate (1 cm width) hanging from an electrobalance (Cahn Instruments, Co., Cerritos, CA). Both surface concentration and surface pressure were monitored simultaneously for each protein solution.

Adsorption isotherms of proteins were studied by incubating protein solutions at 24 ± 0.2 °C for 24 h and measuring equilibrium surface concentrations at various bulk concentrations.

Coadsorption Experiments. The following approach was employed to study coadsorption of BSA and β-casein from binary solution mixtures. To monitor adsorption of BSA, stock solutions of [^{14}C]BSA and unlabeled β-casein were mixed with buffer to the required bulk concentration ratio and poured gently into the Teflon trough; the surface was swept and the rate of change of surface radioactivity monitored. Similarly, to monitor the rate of adsorption of β-casein from binary mixtures, stock solutions of [^{14}C]-β-casein and unlabeled BSA were mixed with buffer to the required bulk concentration ratio, and the rate of change of surface radioactivity was measured. In all coadsorption experiments, the bulk concentration of BSA was fixed at 1.5 mg/mL and only β-casein concentration was varied.

Displacement Experiments. The ability of BSA and β-casein to displace each other from the interface was studied as follows. First, ^{14}C-labeled protein 1 was allowed to adsorb for a given time from a 1.5 mg/mL protein solution, and then an aliquot of a stock solution of unlabeled protein 2 was injected into the bulk phase of protein 1. The final bulk concentration of protein 2 also was 1.5 mg/mL. The surface radioactivity of adsorbed protein 1 was monitored for several hours. If protein 2 displaced protein 1 from the air–water interface, it should be reflected in a gradual decrease in surface radioactivity.

RESULTS AND DISCUSSION

Figure 1 shows adsorption isotherms of β-casein and BSA at the air–water interface in single component systems. The plot of Γ against bulk concentration for β-casein shows a well-defined plateau in the range of 1.5–4.0 mg/mL, indicating that a saturated monolayer is formed in this bulk concentration region (Graham and Phillips, 1979). The saturated monolayer coverage for β-casein is about 1.85 mg/m^2. This corresponds to an area of 2154 Å^2 per molecule. The plot of Γ against bulk concentration for BSA does not exhibit a well-defined plateau. However, the data suggest that a saturated monolayer film is apparently formed in the bulk concentration range 1.5–10 mg/mL. A Γ value of 1.0 mg/m^2, representing the midpoint of this range, is assumed as the saturated monolayer coverage for BSA at the air–water interface. This corresponds to an area of 10 958 Å^2 per molecule. Both β-casein and BSA exhibit a C_{crit} value, that is, the minimum bulk concentration above which formation of a saturated monolayer begins, of about 1.5 mg/mL.

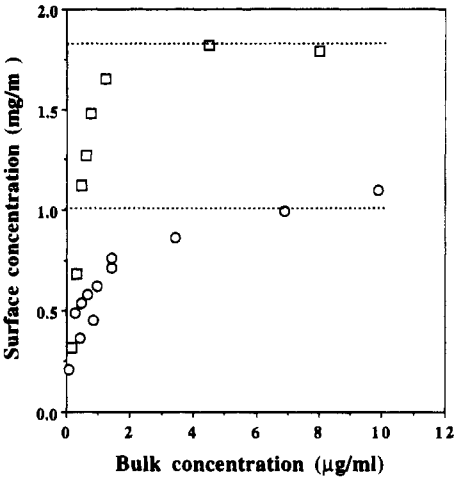

Figure 1. Adsorption isotherms of β-casein (□) and BSA (○) at the air–water interface at 24 °C. The subphase was 20 mM phosphate-buffered saline solution, pH 7.0, I = 0.1. Surface concentrations were measured after 24 h of adsorption. The dotted lines are the assumed saturated monolayer coverages.

The molecular weights of BSA and β-casein are 24 000 and 66 000, respectively (Swaisgood, 1986; Kragh-Hansen, 1981). The Γ_{eq} values of saturated monolayers of β-casein and BSA indicated that, even though the ratio of the molecular mass of BSA to that of β-casein is about 2.75 (i.e., 66 000/24 000), BSA occupies a molecular area equivalent to about 5 times the area occupied by β-casein. This might be due to differences in the structural properties of the two proteins. It appears that β-casein behaves like a stiff random coil polymer with a small radius of gyration and BSA behaves like a flexible globular protein with a large radius of gyration at the interface.

Adsorption of proteins at interfaces generally follows Langmuirian kinetics when the bulk concentration is below C_{crit}, (Hunter et al., 1990), and under these conditions Γ_{eq} is given by the relationship

$$\ln K - \lambda\Gamma_{eq}^{n} = \ln \frac{\Gamma_{eq}}{C(1 - a\Gamma_{eq})} \qquad (1)$$

where K is the equilibrium constant, a is the average area occupied per protein molecule at saturated monolayer coverage (i.e., $1/\Gamma_{eq}$ at saturated monolayer coverage), C is bulk concentration of protein, Γ_{eq} is surface concentration at equilibrium, n is an exponent related to cooperativity among adsorbing protein molecules, and λ is related to an activation energy barrier for adsorption. The adsorption isotherms of β-casein and BSA were analyzed according to eq 1. The right-hand side of eq 1 was plotted against Γ^n for n = 1, 2, 3, and 4. The lowest value of n that gave a straight line with the highest correlation coefficient was selected as its value. As shown in Figure 2, for both β-casein and BSA a best fit is obtained with n = 2. The value of λ (slope) is negative for β-casein, indicating that the activation

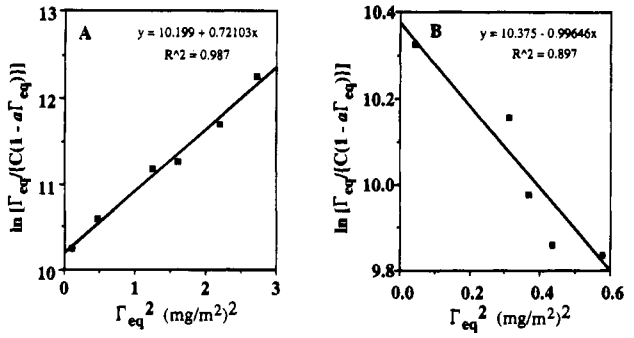

Figure 2. Plots of low bulk concentration adsorption isotherm data of β-casein (A) and BSA (B) according to eq 1.

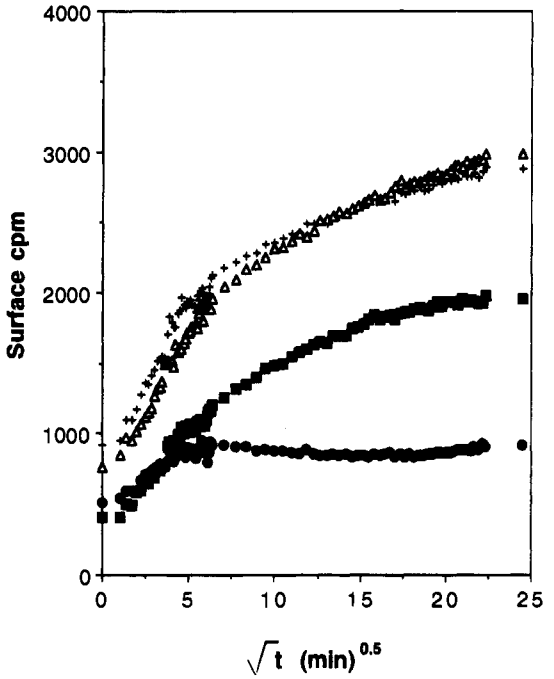

Figure 3. Changes in surface cpm with time during coadsorption of β-casein and BSA from bulk solutions containing 1.5 mg/mL each: ■, [^{14}C]-β-casein + unlabeled BSA; ●, [^{14}C]-BSA + unlabeled β-casein; △, [^{14}C]-β-casein + [^{14}C]BSA; +, arithmetic sum of the ■ and ● curves.

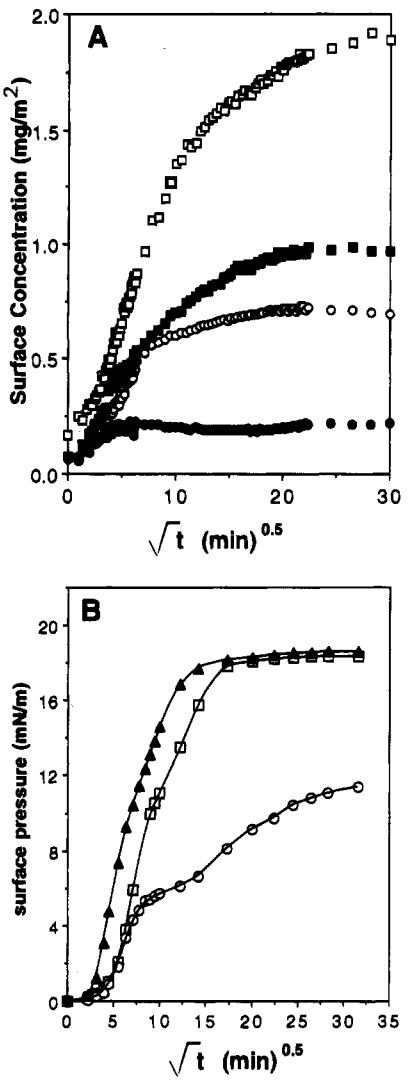

Figure 4. (A) Kinetics of adsorption of β-casein (squares) and BSA (circles) in single component (open symbols) and 1:1 mixture (solid symbols) systems. Concentration of each protein in both systems was 1.5 mg/mL. (B) Changes in surface pressure during adsorption of β-casein (□) and BSA (○) in single-component systems and in a binary protein system (▲) containing 1.5 mg/mL each.

energy barrier for adsorption decreases with increasing surface coverage, which is indicative of positive cooperativity among adsorbing β-casein molecules, whereas the value of λ is positive for BSA, indicating that the activation energy barrier increases with increasing surface coverage, which is indicative of negative cooperativity among adsorbing BSA molecules. The values of $K_{\beta C}$ and K_{BSA}, determined from the intercepts, are 2.7×10^4 and 3.2×10^4 mg (m^2 wt %)$^{-1}$, respectively.

Figure 3 shows the rates of change of surface counts per minute of dilute protein solutions containing [^{14}C]-BSA plus unlabeled β-casein (i.e., BSA* + βC), [^{14}C]-β-casein plus unlabeled BSA (BSA + βC*), and [^{14}C]BSA plus [^{14}C]-β-casein (BSA* + βC*). The bulk concentrations of BSA and β-casein in these 1:1 binary protein solutions were 1.5 mg/mL each. It should be noted that the arithmetic sum of BSA* + βC and BSA + βC* surface counts per minute curves is almost the same as the surface counts per minute of the BSA* + βC* curve, indicating that the methodology used in this study to monitor interfacial adsorption of proteins is highly reliable and reproducible.

Figure 4A shows the rates of change of surface concentration (Γ) of BSA and β-casein during adsorption from a 1:1 bulk mixture (1.5 mg/mL each) and also from single-protein solutions containing 1.5 mg/mL [^{14}C]BSA or [^{14}C]-β-casein. The changes in surface pressure with time of single-protein and binary protein solutions are presented in Figure 4B. In single-protein systems, positive adsorption of BSA and β-casein commences soon after a fresh air–water interface is created in the trough. β-Casein requires about 600 min to reach equilibrium adsorption with a Γ_{eq} of about 1.8 mg/m^2, whereas BSA attains a Γ_{eq} value of about 0.7 mg/m^2 within 300 min.

The rate and extent of adsorption of BSA and β-casein from the 1:1 binary mixture solution are significantly lower than those in single-component systems (Figure 4A). In the binary system, β-casein reaches a Γ_{eq} value of about 0.935 mg/m^2 after about 600 min of adsorption, whereas BSA reaches a steady state Γ_{eq} of only about 0.224 mg/m^2 within 30 min. The net decrease in Γ_{eq} of β-casein is about 0.865 mg/m^2, and that of BSA is about 0.5 mg/m^2 compared to the Γ_{eq} values in single-protein systems. The total protein concentration of the mixed film at equilibrium is only about 1.15 mg/m^2, which is much lower than Γ_{eq} of β-casein in the single-component system.

The surface pressure of a single-component BSA solution apparently does not reach a steady-state value

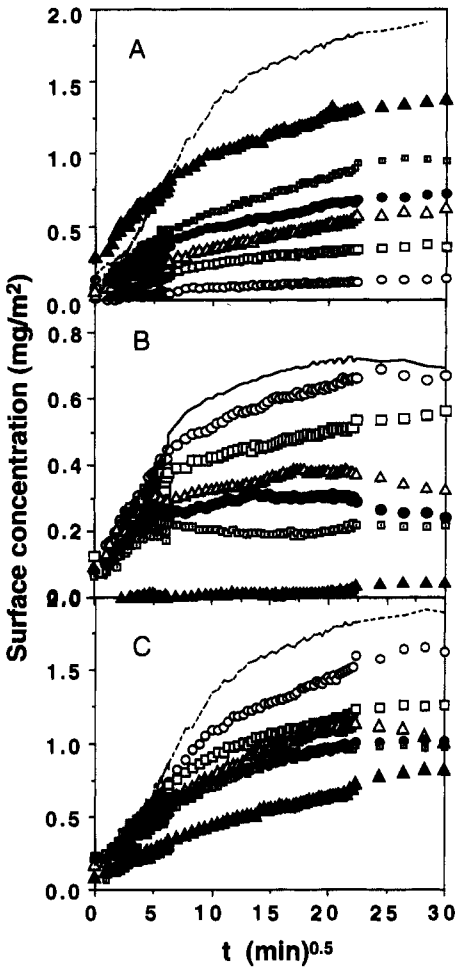

Figure 5. (A) Kinetics of adsorption of [^{14}C]-β-casein at the air–water interface from binary protein solutions containing 1.5 mg/mL unlabeled BSA and various concentrations of [^{14}C]-β-casein were as follows: ○, 0.15 mg/mL; □, 0.45 mg/mL; △, 0.75 mg/mL; ●, 1.05 mg/mL; ⊡, 1.5 mg/mL; ▲, 4.2 mg/mL. The dotted line represents adsorption of β-casein in a single-component system at 1.5 mg/mL bulk concentration. (B) Kinetics of adsorption of [^{14}C]BSA at the air–water interface from binary protein solutions containing 1.5 mg/mL [^{14}C]BSA and various concentrations of unlabeled β-casein. The symbols correspond to bulk concentration of β-casein shown for (A). The dotted line represents kinetics of adsorption of [^{14}C]BSA in a single-component system at 1.5 mg/mL bulk concentration. (C) Kinetics of adsorption of [^{14}C]-β-casein at the air–water interface from binary protein solutions containing 1.5 mg/mL [^{14}C]-β-casein and various concentrations of unlabeled BSA. The bulk concentrations of unlabeled BSA were as follows: - - - , no BSA; ○, 0.15 mg/mL; □, 0.45 mg/mL; △, 0.75 mg/mL; ●, 1.05 mg/mL; ⊡, 1.5 mg/mL; ▲, 4.2 mg/mL.

during the time interval studied but attains a value of about 11 mN/m after about 1000 min, whereas β-casein reaches a steady-state surface pressure of about 18 mN/m within about 600 min (Figure 4B). In the case of a 1:1 BSA/β-casein binary solution, the rate of change of surface pressure and the steady-state surface pressure are similar to those of the single-component β-casein system, indicating that even though the total protein concentration in the adsorbed mixed protein film is only 1.15 mg/m^2, the free energy (surface tension) of the interface decreases to a value similar to that of the single-component β-casein system.

The effects of the ratio of bulk concentrations of β-casein to BSA on the kinetics of adsorption of [^{14}C]-β-casein and [^{14}C]BSA at the air–water interface are shown in Figure 5. The bulk concentration ratio was altered by keeping the concentration of one of the proteins at 1.5 mg/mL and varying the concentration

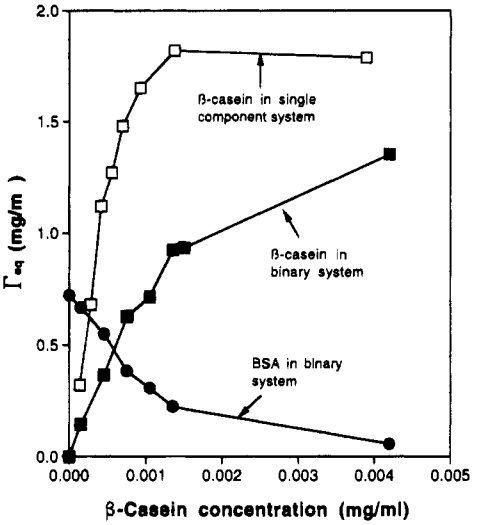

Figure 6. Plot of equilibrium surface concentrations of β-casein and BSA against bulk concentration of β-casein in single-component and binary protein systems. Data were extracted from Figure 5A,B.

of the other protein from 0.03 to 4.2 mg/mL. The rate and extent of adsorption of β-casein increase with increasing bulk ratio of [^{14}C]-β-casein to BSA (i.e., βC*/BSA) at a constant BSA concentration (Figure 5A). However, Γ_{eq} values of [^{14}C]-β-casein at various bulk concentrations of β-casein in the βC*/BSA binary system are consistently lower than its values in a single-component system at the same bulk concentrations (Figure 6). In contrast, the rate and extent of adsorption of [^{14}C]BSA decrease progressively with increasing β-casein to [^{14}C]BSA ratio (i.e., βC/BSA*) at a constant [^{14}C]BSA concentration (Figure 5B). At a βC/BSA* ratio of 2.8, no significant adsorption of BSA occurs for about 400 min and Γ reaches a value of only about 0.06 mg/m^2 after 600 min (Figure 5B). The results indicate that at high ratios of β-casein to BSA, the interfacial film is mainly composed of β-casein and BSA is completely excluded from the interface.

To elucidate if BSA can completely inhibit adsorption of β-casein at the interface, adsorption of [^{14}C]-β-casein from solutions containing 1.5 mg/mL [^{14}C]-β-casein and various concentrations of unlabeled BSA was studied (Figure 5C). Both rate and extent of adsorption of [^{14}C]-β-casein decrease with increasing BSA/βC* ratio at a constant [^{14}C]-β-casein concentration. Comparison of data in parts B and C of Figure 5, however, indicates that β-casein has a much greater inhibiting effect on BSA adsorption than does BSA on β-casein. For example, at a βC/BSA* bulk ratio of 4.2/1.5, β-casein completely inhibits adsorption of BSA (Figure 5B), whereas at a BSA/βC* bulk ratio of 4.2/1.5, BSA is unable to completely inhibit adsorption of β-casein at the interface (Figure 5C). In the latter case, the equilibrium surface concentration of β-casein is about 0.85 mg/m^2, which is about half of the value in the single-component system.

The rate of adsorption of proteins at an air–water interface from a dilute solution is generally regarded as diffusion controlled (MacRitchie and Alexander, 1963; Ward and Tordai, 1946) and follows the relationship

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

where Γ_t is surface concentration at time t, D is a diffusion coefficient, C_0 is bulk protein concentration, and t is the time. To determine if the presence of one

Table 1. Diffusion Coefficients of β-Casein and BSA in Single-Component and Binary Systems

βC*/BSA, BSA*/βC, or BSA/βC ratio[a,b]	diffusion coefficient × 10^7 cm²/s: of BSA in βC/BSA* binary system	of βC in BSA/βC* binary system	of βC in βC*/BSA binary system
0	3.64	7.64	
0.1	1.77	4.33	DM[c]
0.3	1.64	4.47	DM
0.5	0.88	3.85	DM
0.7	0.47	4.31	2.00
0.9			1.52
1.0	0.95	3.85	1.53
2.8	0.03	0.65	2.23

[a] Bulk concentration of the component in the denominator was 1.5 μg/mL in all cases. [b] The asterisk indicates the radiolabeled component. [c] DM, difficult to measure.

protein in the bulk phase influences diffusion of the second protein to the interface, apparent diffusion coefficient values of BSA and β-casein, calculated from the initial slopes of $\Gamma - t^{1/2}$ curves of Figure 5, were analyzed. The apparent diffusion coefficient of BSA in a single-component system is 3.63×10^{-7} cm²/s, which is slightly lower than its conventional diffusion coefficient in solution (5.9×10^{-7} cm²/s). Addition of increasing amounts of β-casein to BSA* solution (1.5 mg/mL) progressively decreases the apparent diffusion coefficient of BSA (Table 1, column 2). For example, the apparent diffusion coefficient of BSA decreases by 1 order of magnitude at a βC/BSA* bulk ratio of 1.0 and by 2 orders of magnitude at a ratio of 2.8 compared to that in a single-component BSA system. In contrast, a significant reduction in the apparent diffusion coefficient of β-casein only occurs at a BSA/βC* bulk ratio of 2.8 (Table 1, column 3). These results indicate that even at low bulk concentrations, β-casein successfully competes with BSA for adsorption and this decreases the number of adsorption sites available for BSA at the interface, causing a decrease in its apparent diffusion coefficient. It is noteworthy that in the βC*/BSA system (Table 1, column 4), in which the bulk concentration of BSA was kept constant, the apparent diffusion coefficient of β-casein is independent of its bulk concentration.

Several important pieces of information regarding the adsorption behaviors of these two proteins in the binary system can be derived from the data in Figure 5. As discussed earlier, the adsorption isotherms of β-casein and BSA show that the minimum areas per molecule at saturated monolayer coverage are 2154 and 10 958 Å² for β-casein and BSA, respectively. It is reasonable to assume that, in the absence of any cooperativity among these proteins during coadsorption from a binary solution, β-casein would require a minimum interfacial area of 2154 Å² or a unit cell of dimension 46 × 46 Å and BSA would require a minimum interfacial area of 10 958 Å² or a unit cell of dimension 105 × 105 Å for adsorption at the interface. If there is either positive or negative cooperativity among these proteins during or after coadsorption at the interface, it may alter the average unit cell dimensions of each of these proteins in the mixed film. For a noninteracting coadsorption process, the fraction of the total surface area occupied at saturated monolayer coverage can be expressed as

$$n_{BSA}a_{BSA} + n_{\beta C}a_{\beta C} = \phi_a \quad (3)$$

where n_{BSA} and $n_{\beta C}$ are the number of molecules per square meter, respectively, of BSA and β-casein at equilibrium adsorption in the mixed protein monolayer, a_{BSA} and $a_{\beta C}$ are molecular areas occupied by these proteins at saturated monolayer coverage in single component systems, and ϕ_a is the fraction of the total interfacial area occupied. If each β-casein and BSA molecule occupies 2154 Å² ($a_{\beta C}$) and 10 958 Å² (a_{BSA}), respectively, in a close-packed saturated monolayer, then $\phi_a = 1$. The values of n_{BSA} and $n_{\beta C}$ at various β-casein to BSA bulk ratios and the calculated ϕ_a are given in Table 2. It should be noted that at all bulk concentration ratios of β-casein to BSA, only about three-fourths fraction of the total interfacial area is found to be covered by the mixed protein film even though the bulk phase contained more than enough protein to form a saturated monolayer. For example, β-casein is able to form a saturated monolayer ($\phi_a = 1$) at 1.5 mg/mL bulk concentration in a single component system. However, in the presence of 1.5 mg/mL bulk concentration of BSA (i.e., in a 1:1 mixture), ϕ_a is only about 0.76, even though the total bulk phase protein concentration is 3 mg/mL, which should be more than sufficient to form a saturated monolayer of a mixed protein film. *A priori,* if one assumes that a saturated mixed protein monolayer forms at the air–water interface in a 1:1 binary solution, then it must be concluded that the molecular areas of BSA and β-casein in the mixed protein film are greater than their values in saturated monolayers of single-component systems. However, the increase in molecular areas might not be related to the area physically occupied by each molecule, but might be due to an increase in the excluded area between protein molecules. If this is the case, it would suggest that there is a negative cooperativity among adsorbing BSA and β-casein molecules. That is, adsorption of one protein decreases the probability of adsorption of the other protein. In other words, adsorption of BSA into a β-casein film, or *vice versa* (two-dimensional mixing), is thermodynamically unfavorable. The increase in excluded area might arise from electrostatic repulsion between BSA and β-casein molecules and/or from thermodynamic incompatibility of mixing of these proteins in the adsorbed film. The thermodynamic incompatibility of mixing might be attributable to dissimilarity among these proteins in terms of their hydrophobic and hydrophilic characteristics (Polyakov et al., 1985, 1986). Because of this thermodynamic incompatibility, phase separation might take place

Table 2. Composition of BSA/β-Casein Mixed Film Adsorbed at the Air–Water Interface from Bulk Solutions Containing Various Concentration Ratios of BSA and β-Casein[a]

βC/BSA ratio	Γ_{BSA} (mg/m²)	$n^{BSA} \times 10^{-15}$	$\Gamma_{\beta C}$ (mg/m²)	$n_{\beta C} \times 10^{-15}$	$n_{BSA}a_{BSA}$	$n_{\beta C}a_{\beta C}$	$n_{BSA}a_{SA} + n_{\beta C}a_{\beta C}$
0.1	0.667	6.0	0.144	3.6	0.687	0.076	0.763
0.3	0.549	4.9	0.364	9.1	0.561	0.191	0.752
0.5	0.384	3.5	0.627	15.7	0.400	0.329	0.729
0.7	0.306	2.8	0.715	17.9	0.320	0.375	0.695
1.0	0.224	2.0	0.935	23.5	0.229	0.493	0.722
2.8	0.057	0.5	1.353	34.0	0.057	0.713	0.760

[a] n is the number of molecules per square meter at equilibrium. a is molecular area occupied at saturated monolayer coverage in a single-component system. Bulk concentration of BSA in all cases was 1.5 μg/mL.

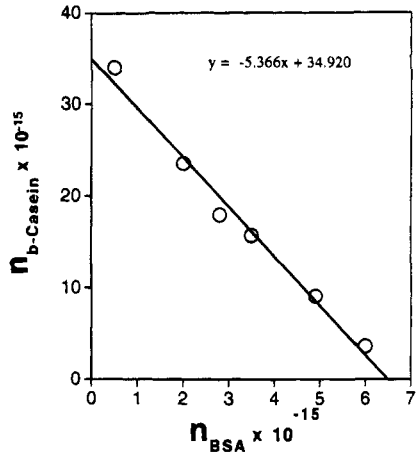

Figure 7. Correlation between the number of molecules per square meter of area of BSA and β-casein in mixed protein films formed at equilibrium at the air–water interface from solutions containing various β-casein to BSA bulk concentration ratios.

above a critical interfacial composition, resulting in selective exclusion of one of the proteins from the interface.

To elucidate if there was a correlation between concentrations of BSA and β-casein in mixed protein films, the values of n_{BSA} (from Table 2) were plotted against $n_{\beta C}$. The plot exhibits a straight line (Figure 7) with a slope of 5.3. This linear correlation indicates that adsorption of 3 molecules of BSA at the air–water interface excludes adsorption of 16 molecules of β-casein from the interface and *vice versa*. The y intercept of the plot indicates that if 34.94×10^{15} molecules/m^2 (or 1.39 mg/m^2) of β-casein were initially adsorbed to the interface, it would completely exclude adsorption of BSA to the interface. Conversely, the x intercept indicates that if 6.5×10^{15} molecules/m^2 (or 0.745 mg/m^2) of BSA were initially adsorbed to the interface, it would not allow adsorption of β-casein to the interface.

To elucidate if coadsorption of BSA and β-casein followed a Langmuirian-type thermodynamically controlled competitive adsorption mechanism, the data in Figure 5 and Table 2 were analyzed as follows. For a single-component Langmuirian-type adsorption process the equilibrium surface concentration is (Hunter et al., 1991)

$$\Gamma_{\beta C} = K_{\beta C} a_{\beta C} / (1 + K_{\beta C} a_{\beta C} C_{\beta C}) \quad (4)$$

and

$$\Gamma_{BSA} = K_{BSA} C_{BSA} / (1 + K_{BSA} a_{BSA} C_{BSA}) \quad (5)$$

where $\Gamma_{\beta C}$ and Γ_{BSA} are equilibrium surface concentrations of β-casein and BSA, respectively, $K_{\beta C}$ and K_{BSA} are equilibrium constants, and $a_{\beta C}$ and a_{BSA} are the average areas occupied per molecule of β-casein and BSA, respectively, at saturated monolayer coverage. If adsorption of β-casein and BSA from a binary mixture also follows a Langmuirian-type competitive mechanism, then interfacial concentrations of β-casein and BSA at any bulk protein ratio are given by the relationships (Hunter et al., 1991)

$$\Gamma_{\beta C} = K_{\beta C} C_{\beta C} / (1 + K_{\beta C} a_{\beta C} C_{\beta C} + K_{BSA} a_{BSA} C_{BSA}) \quad (6)$$

and

$$\Gamma_{BSA} = K_{BSA} C_{BSA} / (1 + K_{BSA} a_{BSA} C_{BSA} + K_{\beta C} a_{\beta C} C_{\beta C}) \quad (7)$$

Using the values of $K_{\beta C}$, K_{BSA}, $a_{\beta C}$, and a_{BSA} determined from adsorption isotherms of β-casein and BSA, the theoretical values of $\Gamma_{\beta C}$ and Γ_{BSA} were calculated from eqs 6 and 7 for various bulk ratios of β-casein and BSA. If the predicted values are the same as experimental values, then it can be concluded that adsorption follows a thermodynamically controlled competitive adsorption mechanism. The predicted and experimental $\Gamma_{\beta C}$ and Γ_{BSA} values are given in Table 3. The data show that the experimental Γ_{BSA} values are much lower than the predicted Γ_{BSA} values, whereas the experimental values of $\Gamma_{\beta C}$ are much higher than the predicted $\Gamma_{\beta C}$ values at all bulk concentration ratios. It seems that an increase in adsorption of β-casein takes place at the expense of a decrease in adsorption of BSA. These results indicate that equilibrium adsorptions of these proteins from binary solutions do not follow the Langmuirian-type thermodynamically controlled process. The differences in rates of adsorption, and not the relative affinities of these proteins to the interface, seem to be the primary factor influencing the composition of the adsorbed protein layer. That is, the interfacial protein composition is kinetically controlled. Because β-casein adsorbs at a much faster rate than does BSA, it occupies a greater fraction of the interfacial area, and the late-arriving BSA molecules do not seem to possess the ability to displace the adsorbed β-casein molecules.

To determine if an adsorbed protein molecule can be displaced by a molecule from the bulk phase, sequential adsorption experiments were carried out. Figure 8 shows that injection of unlabeled BSA (1.5 mg/mL final concentration) into a [^{14}C]-β-casein solution (1.5 mg/mL) after about 700 min of adsorption does not decrease the surface counts per minute for several hours, indicating that unlabeled bulk phase BSA molecules could not displace adsorbed [^{14}C]-β-casein molecules. Similarly, injection of unlabeled β-casein (1.5 mg/mL final concentration) into the [^{14}C]BSA solution after about 1100 min of adsorption does not decrease surface radioactivity, indicating that bulk phase β-casein was unable to displace adsorbed BSA molecules from the interface. These results indicate that, regardless of their surface active properties, bulk phase BSA molecules cannot displace adsorbed β-casein molecules and bulk phase β-casein molecules cannot displace adsorbed BSA molecules from the air–water interface. Conversely, in a heterologous system, the protein component that arrives at the interface first adsorbs first and cannot be displaced by the protein component that arrives at the interface later. Thus, the composition of the binary protein film is mainly determined by the rates of adsorption of the proteins, which may permit sequential desorption of polypeptide segments from the interface.

Figure 9 shows the ability of bulk phase molecules to displace adsorbed molecules in single-protein component systems. Injection of unlabeled BSA into [^{14}C]BSA solution after 1100 min of adsorption does not decrease the surface counts per minute, showing that, once adsorbed at the air–water interface, adsorbed BSA molecules cannot exchange with bulk phase BSA molecules. This must be due to conformational changes in adsorbed BSA molecules, which may increase the activation energy for displacement from the interface. In contrast, the results of [^{14}C]-β-casein/β-casein exchange experiment show that adsorbed β-casein molecules readily exchange with bulk phase β-casein molecules. This might be attributable to its random coil structure both in the adsorbed film and in bulk phase.

Table 3. Experimental and Predicted Surface Concentrations for Coadsorption of BSA and β-Casein[a]

$C_{b(BSA)}$ (μg/mL)	$C_{b(\beta C)}$ (μg/mL)	$\Gamma_{(BSA)}$ (expt) (mg/m²)	$\Gamma_{(BSA)}$ (predict) (mg/m²)	$\Gamma_{(\beta C)}$ (expt) (mg/m²)	$\Gamma_{(\beta C)}$ (predict) (mg/m²)
1.431	0.135	0.667	0.820	0.144	0.065
1.443	0.412	0.549	0.764	0.364	0.185
1.460	0.685	0.384	0.715	0.627	0.287
1.468	0.976	0.306	0.670	0.715	0.384
1.477	1.403	0.224	0.613	0.935	0.504
1.494	4.060	0.057	0.401	1.353	0.955

[a] C_b represents bulk concentration at equilibrium.

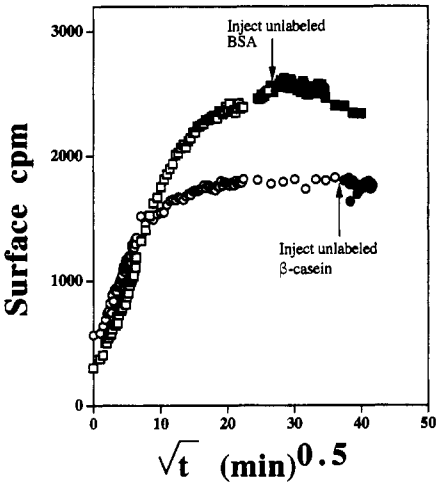

Figure 8. Displacement of adsorbed [^{14}C]-β-casein by bulk phase unlabeled BSA (□) and displacement of [^{14}C]BSA by bulk phase unlabeled β-casein (○). □ and ■ represent, respectively, surface cpm of [^{14}C]-β-casein before and after injection of unlabeled BSA into the bulk phase. ○ and ● represent, respectively, surface cpm of [^{14}C]BSA before and after injection of unlabeled β-casein. The final bulk concentrations of unlabeled proteins were 1.5 mg/mL. The arrows indicate the time of injection of unlabeled proteins.

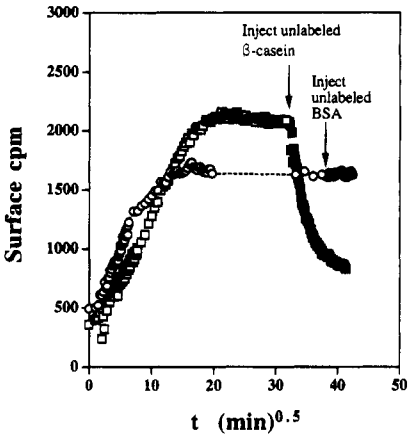

Figure 9. Exchange between adsorbed [^{14}C]BSA and bulk phase unlabeled BSA (○) and between bulk phase unlabeled β-casein and adsorbed [^{14}C]-β-casein (□). ○ and ● represent, respectively, surface cpm of [^{14}C]BSA before and after injection of unlabeled BSA into the bulk phase. □ and ■ represent, respectively, surface cpm of [^{14}C]-β-casein before and after injection of unlabeled β-casein. The final bulk concentrations of unlabeled proteins were 1.5 mg/mL. The arrows indicate the time of injection of unlabeled proteins.

The results of exchange and/or displacement experiments tentatively suggest that a globular protein can displace neither a globular nor a random coil protein from the air–water interface, whereas a random coil protein can displace another random coil protein but cannot displace a globular protein from the interface.

In summary, the results presented here show that competitive adsorption of β-casein and BSA from binary solutions to air–water interface follows a kinetically controlled mechanism. In this regard, the composition of the mixed protein film is mainly influenced by (1) the rates of arrival of proteins at the interface, (2) the ratio of concentrations of proteins in the bulk phase, and (3) the thermodynamic compatibility of mixing of the proteins in the adsorbed film.

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