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Dynamics of Competitive Adsorption of α_s-Casein and β-Casein at Planar Triolein–Water Interface: Evidence for Incompatibility of Mixing in the Interfacial Film

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Competitive adsorption of α_s-casein and β-casein from a bulk solution mixture to the triolein−water interface has been studied. Although the binding affinity of α_s-casein to the triolein−water interface was lower than that of β-casein in single-component systems, in a 1:1 mixture of α_s-casein and β-casein in the bulk solution the ratio of interfacial concentrations of α_s-casein to β-casein at equilibrium was about 2:1, indicating that α_s-casein was preferentially adsorbed to the triolein−water interface. Furthermore, the equilibrium composition of α_s-casein and β-casein in the interfacial film at various bulk concentration ratios did not follow a simple Langmuir adsorption model. This deviation from ideal behavior was mainly due to thermodynamic incompatibility of mixing of these caseins in the interfacial region. The value of the incompatibility parameter, X_{12}, for these caseins at the triolein−water interface was much greater than that at the air−water interface. Displacement experiments showed that while α_s-casein could dynamically displace β-casein when the latter was in an unsaturated monolayer state at the interface, it could not do so when β-casein was in a saturated monolayer film state. It is hypothesized that, because of thermodynamic incompatibility of mixing, the α_s-casein and β-casein mixed film at the oil−water interface may undergo two-dimensional phase separation.

KEYWORDS: Oil−water interface; kinetics of protein adsorption; competitive adsorption; thermodynamic incompatibility at interfaces; α_s-casein; β-casein

INTRODUCTION

Recently, several studies (*1*−*6*) have been reported on competitive adsorption of proteins at the air−water interface. These studies have revealed that, in the initial stages of adsorption, i.e., before the formation of a protein film at the interface, competitive adsorption of protein components to the air−water interface from a bulk mixture was mostly reversible and the proteins displaced each other from the interface up until surface aggregation and film formation occurred (*5*, *7*). It was also noted that the surface concentration of individual proteins in the mixed binary protein films at equilibrium did not follow the ideal Langmuir adsorption model, suggesting that the binding affinities of the protein components to the interface in a binary system were not the same as those in single-component systems (*6*, *7*). This was attributed to thermodynamic incompatibility of mixing of the protein components in the adsorbed film at the interface (*6*, *7*).

Recent studies on competitive adsorption of α_{S1}-casein and β-casein at the air−water interface have shown that these two proteins dynamically displaced each other as they adsorbed from a bulk mixture to the air−water interface (*5*). At a bulk concentration ratio of 1:1 (1.5×10^{-4}% w/v each), the α_{S1}-casein arrived at the interface first, but was easily displaced by the late arriving β-casein owing to its higher binding affinity for the interface. At equilibrium, the ratio of α_{S1}-casein to β-casein in the mixed film was 1:2. It was also demonstrated (*6*, *8*) that although the two caseins were apparently compatible in solution, they exhibited incompatibility of mixing in the film at the air−water interface and eventually separated into α_{S1}-casein and β-casein rich phases at the interface.

Although several systematic studies have been conducted on competitive adsorption of proteins at the air−water interface (*3*−*7*), no such studies have been reported for the oil−water interface. It has been shown that the kinetics and energetics of protein adsorption at the oil−water interface is quite different from that at the air−water interface (*9*). This has been attributed to stronger dispersion forces emanating from the oil phase than from the gas phase. In this respect, it is conceivable that the competitive adsorption behavior, incompatibility of mixing, and the dynamics of exchange/displacement of proteins at the oil−water interface also may be quite different from that observed at the air−water interface. These may affect emulsifying and emulsion stabilizing properties of protein mixtures. The objective of the present study was to investigate competitive adsorption of α_S-casein and β-casein at a planar triolein−water interface and compare the results with those from the air−water interface studies to obtain a better understanding of the influence of the oil phase on competitive adsorption behavior of proteins.

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MATERIALS AND METHODS

Triolein (99% pure), lyophilized and salt free bovine β-casein (min 90%), and lyophilized bovine α_s-casein (min 85%) were obtained from Sigma Chemical Co. (St. Louis, MO). These protein samples were used without further purification. Methyl oleate (99% pure), sodium cyanoborohydride (95% pure), ultrapure Na_2HPO_4 and NaH_2PO_4 and NaCl were purchased from Aldrich Chemical Co. (Milwaukee, WI). ^{14}C-Formaldehyde with a specific radioactivity of 10 mCi/mmol was purchased from New England Nuclear Co. (Boston, MA). Extreme care was taken in purifying water for adsorption studies. A Milli-Q Ultrapure water purification system (Millipore Corp., Bedford, MA) with a Qpak1cartridge package (composed of activated charcoal, reverse osmosis, ion-exchange, and ultrafiltration cartridges) capable of removing inorganic and organic impurities was used to purify the water. The resistivity of the water used was 18.2 mΩ·cm. The surface tension of this water at 25 °C was 71.9 $\pm$ 0.1 mN/m.

The purity of α_s-casein and β-casein was determined by alkaline urea polyacrylamide gel electrophoresis (urea−PAGE) as described previously (*10*, *11*).

Proteins were radiolabeled by reductive methylation of the amino groups using sodium cyanoborohydride and ^{14}C-formaldehyde at pH 7.5, as described elsewhere (*12*). The protein concentrations were determined using $E^{1\%}$ values of 4.6 and 10.5 at 280 nm for β-casein, and α_s-casein, respectively. The specific radioactivities of ^{14}C-radiolabeled β-casein and α_s-casein were 0.795 μCi/mg and 1.181 μCi mg^{-1}, respectively. These specific radioactivity values corresponded to incorporation of about1.9 and 1.8 mol of $^{14}CH_3$ per mole of protein, respectively.

Adsorption Studies at the Oil−Water Interface. The kinetics of protein adsorption at the triolein−water interface was studied as described previously (*13*). The method essentially involved spreading of a 1000 Å thick film on water surface and monitoring adsorption of ^{14}C-labeled proteins using a surface radiotracer probe. The surface tension was measured by the Wilhelmy plate technique using a ST 9000 surface tensiometer (Nima Technology Ltd., Coventry, England), interfaced with an IBM computer. A thin sand-blasted-platinum plate was used as the sensor.

The apparatus consisted of a Teflon trough having inner dimensions of 17.45 cm length, 5.5 cm width, and 4 cm depth. One side of the trough had a small hole (1 mm diameter) capped tightly with a septum for injecting protein solution into the bulk phase. The entire experimental set up was placed on a plexiglass platform, which was designed to damp any vibrations. In each experiment, 350 mL of solution consisting of 20 mM phosphate buffer (pH 7.0) adjusted to 0.1 M ionic strength with NaCl was used as the bulk phase. Prior to spreading the triolein film over the water (buffer) surface, a very thin (3 mm diameter, 12.7 mm length) Teflon-coated magnetic stir bar was placed at the center of the trough and the radiotracer probe ((Ludlum Measurements, Inc., Sweetwater, Texas) and the Wilhelmy plate were placed in position. The method for spreading a 1000 D thick triolein film has been described in detail elsewhere (*13*).

To initiate protein adsorption in single protein adsorption experiments, a known volume (0.5−2.0 mL) of protein stock solution (1% w/v) was injected through the hole in the side of the trough, without disturbing the oil film. The final bulk concentration of protein in the trough was in the range of 10^{-5}−10^{-2} % (w/v) in adsorption isotherm studies. The surface tension and surface radioactivity (cpm) measurements were initiated soon after injection of the protein solution. The bulk phase was gently stirred by using the stir bar, which had been preadjusted to about 60 rpm. The stir bar was very slowly moved along the length of the trough by moving the magnetic stirrer beneath the plexiglass platform for proper mixing of the injected protein solution. Proper care was taken to prevent ripples on the oil film. To the naked eye, the oil-covered water surface remained perfectly calm under this gentle stirring motion. The stirring was continued only for the first 15 min, after which it was stopped.

The surface tension and surface cpm were continuously monitored until they reached an equilibrium value, which usually took about 16−20 h depending on protein concentration in the bulk. The cpm were integrated using a rate meter (model 2200, Ludlum Measurements, Sweetwater, Texas) and printed out on a strip chart recorder interfaced with the rate meter. The cpm measurements were recorded for every minute for the first hour of the experiment, followed by cp-10 min measurements thereafter for 16−20 h. Calibration curves required for converting cpm readings at the oil−water interface into interfacial protein concentrations (mg m^{-2}) were constructed as described elsewhere (*13*). The cpm versus interfacial radioactivity (μCi m^{-2}) calibration curve was constructed by spreading ^{14}C-labeled β-casein at the oil−water interface as described earlier (*13*). The cpm arising from radioactivity of protein in the bulk solution was determined from a standard curve relating surface cpm versus bulk radioactivity of $Na_2{}^{14}CO_3$. The interfacial radioactivity (μCi m^{-2}) of the adsorbed protein was determined by dividing the background-corrected cpm with the slope of the cpm versus interfacial radioactivity calibration curve. The interfacial protein concentration (mg m^{-2}) was obtained by dividing the instantaneous interfacial radioactivity (μCi m^{-2}) with the specific radioactivity of the protein (μCi mg^{-1}).

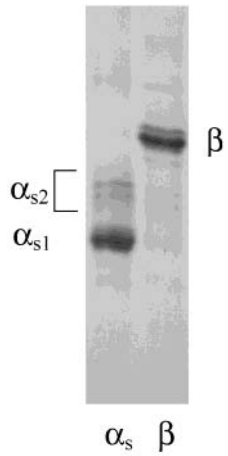

Figure 1. Alkaline urea–PAGE profile of α_s-casein and β-casein samples used in this study.

Competitive Adsorption. In competitive adsorption experiments involving α_s-casein and β-casein, the total bulk protein concentration was maintained constant at 4×10^{-4} % (w/v) and the concentration of each component was varied from 0.25×10^{-4}% to 3.75×10^{-4}% (w/v). Only in one experiment the total concentration of the proteins was maintained at 3×10^{-4} % (w/v) with each protein at 1.5×10^{-4} % (w/v) concentration. The binary adsorption experiments were carried out in sets. Each set consisted of a pair of experiments with identical bulk concentrations of α_s-casein and β-casein. In the first experiment, ^{14}C-labeled β-casein and unlabeled α_s-casein were used. This permitted monitoring of adsorption of β-casein only, even though both proteins adsorbed simultaneously. In the other experiment belonging to the same pair, ^{14}C-labeled α_s-casein and unlabeled β-casein were used to monitor adsorption of α_s-casein only. The sum of interfacial concentrations of both caseins at any instant yielded the dynamic total protein concentration at the triolein−water interface.

Displacement. The ability of α_s-casein to displace β-casein from an aged β-casein film at the oil−water interface and vice versa was investigated as follows: First, ^{14}C-labeled α_s-casein (or ^{14}C-labeled β-casein) was allowed to adsorb to the triolein-water interface from a 1.5×10^{-4}% (w/v) bulk solution for 24 h or until the interfacial concentration reached a constant value. Then, an aliquot of a stock solution (1%) of unlabeled β-casein (or unlabeled α_s-casein) was injected into the bulk phase so that the final bulk concentration of β-casein (or α_s-casein) was 1.5×10^{-4}% w/v. The change in the interfacial radioactivity (cpm) was monitored continuously for several hours to determine if the unlabeled β-casein (or unlabeled α_s-casein) from the bulk phase displaced the ^{14}C-labeled α_s-casein (or ^{14}C-labeled β-casein) at the interface.

RESULTS AND DISCUSSION

Figure 1 shows the alkaline urea−PAGE pattern of α_s-casein and β-casein samples used in this study. The α_s-casein contained

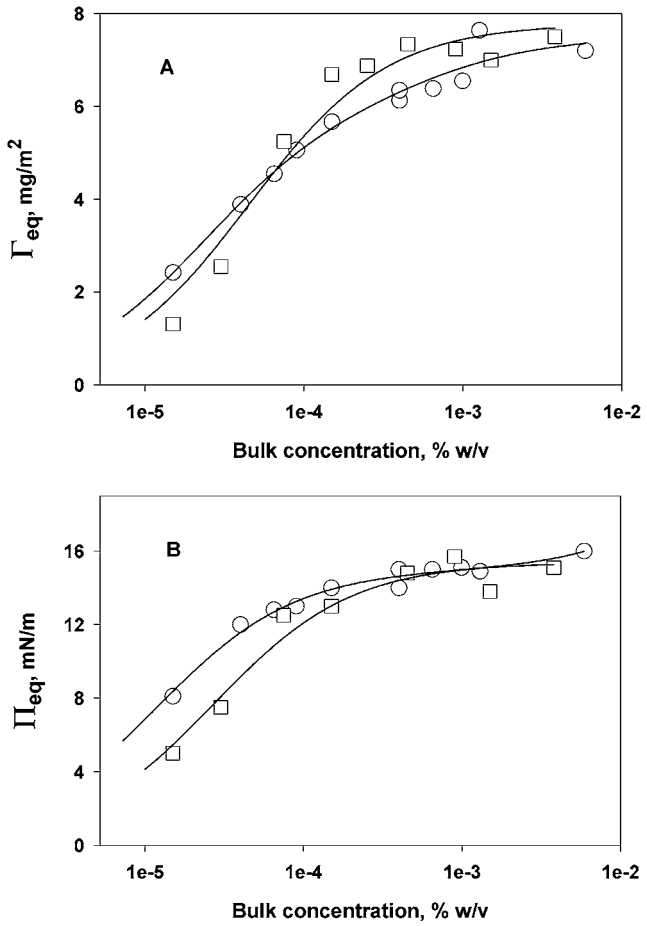

Figure 2. Adsorption (A) and interfacial pressure (B) isotherms of α_s-casein (○) and β-casein (□) at the triolein–water interface. The bulk phase was phosphate buffered saline solution, pH 7.0, $I = 0.1$.

only α_{s1} and α_{s2} fractions and was devoid of any β-casein contamination. Similarly the β-casein sample contained only the major β-casein fractions and was essentially devoid of α_s-casein contamination.

Figure 2a shows adsorption isotherms of ^{14}C-labeled α_s-casein and β-casein in single component systems. Both these proteins exhibited a plateau above 6×10^{-4}% (w/v) bulk protein concentration. The saturated monolayer coverage, Γ_{sat}, for β-casein and α_s-casein at the triolein−water interface was 7.6 and 7.1 mg m^{-2}, respectively. **Figure 2b** shows the equilibrium interfacial pressure (Π) of adsorbed α_s-casein and β-casein films in single component systems over the same bulk concentration range. The Π_{sat} for α_s-casein and β-casein at saturated monolayer coverage were very similar, about 15 mN m^{-1}. It should be noted that α_s-casein used in this study is a natural mixture of α_{s1}-casein and α_{s2}-casein with a composition as shown in **Figure 1**. Therefore, the adsorption isotherm and other kinetic data on α_s-casein should be regarded as an average behavior of this mixture.

Adsorption of proteins at an interface is generally assumed to follow a Langmuir adsorption model when the protein concentration in the bulk phase is below the critical concentration at which saturated monolayer formation occurs. The equilibrium interfacial concentration, Γ_{eq}, under these conditions is given by the relationship (*8*)

$$\Gamma_{eq} = \frac{KC_b}{1 + KaC_b} \text{ or } \frac{\Gamma_{eq}}{1 - (\Gamma_{eq}/\Gamma_{sat})} = KC_b \qquad (1)$$

where, K is the equilibrium constant, a is the average area occupied by the protein molecule at saturated monolayer coverage (i.e., $1/\Gamma_{sat}$), and C_b is the bulk concentration of protein at equilibrium. The adsorption isotherms of α_s-casein and β-casein were analyzed according to eq 1, and the results are shown in **Figure 3**. The equilibrium binding constants, calculated from the slope of the lines in **Figure 3**, are 1.75 and 3.63 cm, respectively, for α_s-casein and β-casein, suggesting that β-casein has greater affinity than α_s-casein for the oil−water interface. Previously, it has been reported that the binding affinities of β-casein and α_s-casein to the air−water interface were 1.25 and 1.07 cm, respectively (*7*). These values clearly show that β-casein inherently has greater affinity than α_s-casein for both air−water and oil−water interfaces, and that for both caseins the oil−water interface is more attractive than the air−water interface.

The time-dependent increases in Γ and Π during adsorption of α_s-casein and β-casein to the triolein−water interface in single-component systems at 1.5×10^{-4}% (w/v) bulk concentration are shown in **Figure 4**. For both these proteins, Γ did not reach a true equilibrium value even after 24 h of adsorption. This also was the case with the surface pressure values. Therefore, the adsorption isotherms shown in **Figure 2**, which were based on 24 h values, should be regarded as apparent adsorption isotherms. Both $\Gamma - t^{1/2}$ and $\Pi - t^{1/2}$ curves exhibited a biphasic behavior, suggesting existence of two molecular processes affecting adsorption of caseins to the interface. One may involve initial anchoring of the protein to the interface and the other may involve two-dimensional aggregation at the interface (*14*). The initial rate of adsorption of proteins at an interface from a dilute solution is generally regarded as diffusion-controlled (*15*) and follows the relationship

$$\Gamma_t = 2C_0\left(\frac{D}{\pi}\right)^{1/2} t^{1/2} \qquad (2)$$

where C_0 is the bulk concentration of protein, t is time, and D is the diffusion coefficient of the protein. The apparent diffusion coefficient of α_s-casein and β-casein, calculated from the initial slopes of $\Gamma_t - t^{1/2}$ curves in **Figure 4A**, is 148×10^{-7} and 124×10^{-7} cm^2/s, respectively. These values are about 2 orders of magnitude greater than those determined from adsorption studies at the air−water interface (*9*) and their diffusivity in bulk solution. It should be pointed out that these high values could not be attributed to convection since the bulk phase was not stirred during the time course of adsorption, except very gently for the first 15 min. Previously, it has been shown that dispersion interactions between proteins and the oil−water interface is attractive, whereas those between proteins and the air−water interface were generally repulsive (*9*). Thus, the faster rate of adsorption and higher Γ_{eq} values of caseins at the oil−water interface than at the air−water interface might be due to the dominant attractive dispersion interactions between the proteins and the oil phase.

Figure 5 shows competitive adsorption of α_s-casein and β-casein from a 1:1 bulk mixture to the triolein−water interface. The total protein concentration in the bulk was 3.0×10^{-4} % (w/v). Under these conditions, the interfacial concentration of α_s-casein and β-casein reached an equilibrium value of 3.69 and 1.92 mg m^{-2}, respectively. That is, the ratio of α_s-casein to β-casein concentration in the mixed film at the triolein−water interface was about 2:1. The total protein load at the triolein−water interface at equilibrium was 5.61 mg m^{-2}, which was lower than the value for either of the proteins in single component systems at 3×10^{-4}% bulk concentration (**Figure**

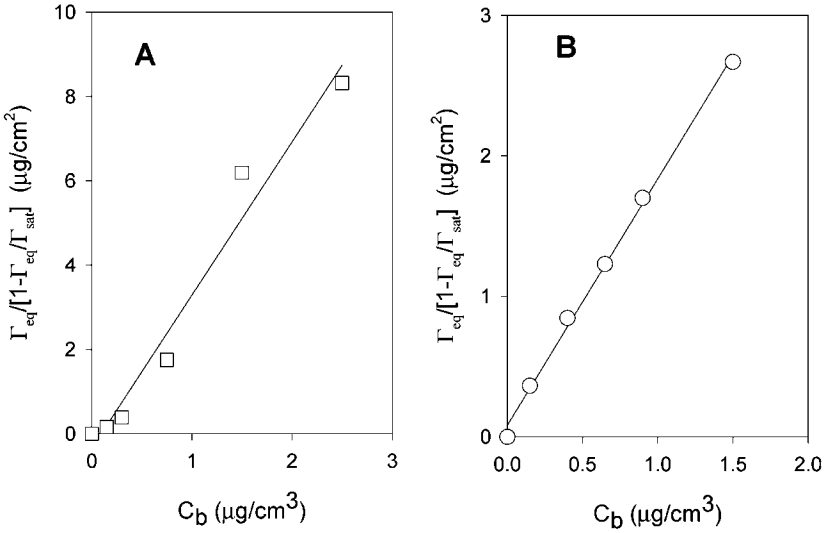

Figure 3. Adsorption isotherm data of (A) β-casein and (B) α_s-casein plotted according to the Langmuir equation (eq 1). The solid lines are linear regression.

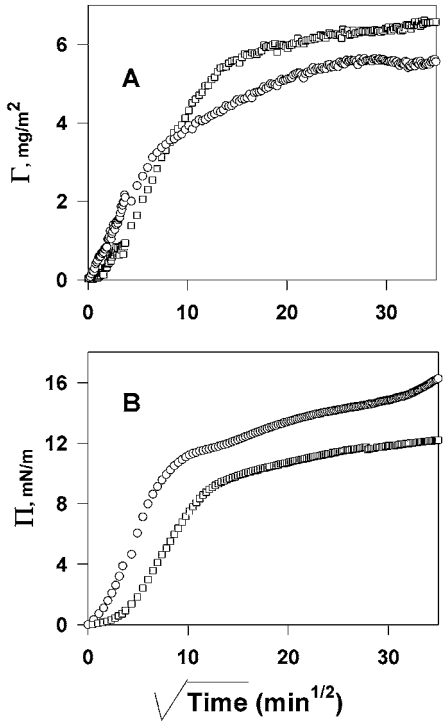

Figure 4. Evolution of interfacial concentration (A) and interfacial pressure (B) with time during adsorption of α_s-casein (○) and β-casein (□) in single-component systems at the triolein–water interface from a bulk solution containing 1.5×10^{-4}% (w/v) protein.

2). Previously, competitive adsorption of α_s-casein and β-casein under similar conditions at the air−water interface showed that the ratio of α_s-casein to β-casein at the air−water interface at equilibrium was about 1:2 (*5*), which was exactly the opposite of the behavior observed at the triolein−water interface. It should be noted that, in the binary system, the Γ values of both α_s-casein and β-casein reached stable equilibrium values after about 400 min of adsorption (**Figure 5A**), which was not the case in single component systems (Figure 4A). The surface pressure of the mixed protein film did not reach a true equilibrium value although it appeared to reach an apparent equilibrium value after about 1000 min of adsorption (**Figure 5b**).

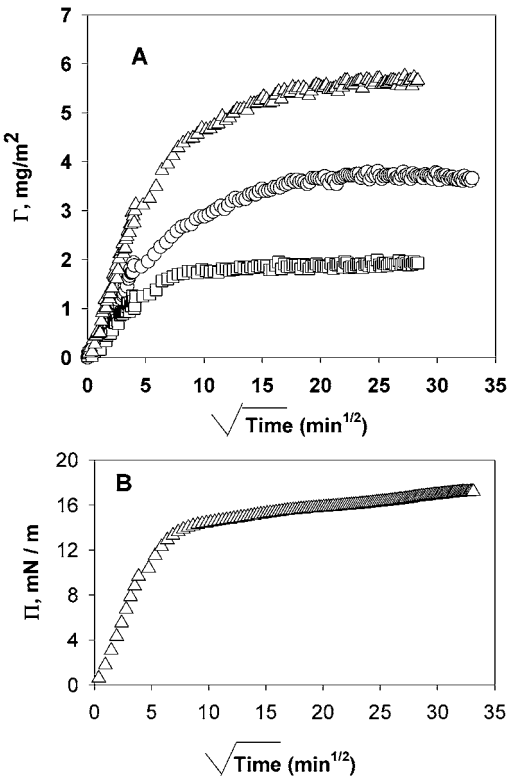

Figure 5. (A) Kinetics of competitive adsorption of α_s-casein (○) and β-casein (□) at the triolein–water interface from a 1:1 bulk solution mixture containing 1.5×10^{-4}% (w/v) of each protein. Δ represents total interfacial concentration obtained from the sum of ○ and □ curves. (B) Evolution of interfacial pressure during adsorption of α_s-casein and β-casein at the triolein–water interface in the binary protein system.

The most intriguing aspect of the data in **Figure 5A** is that even though the apparent binding affinity of α_s-casein to the triolein−water interface was less than that of β-casein, its Γ_{eq} was twice that of β-casein in the binary system containing 1.5×10^{-4}% (w/v) each of α_s-casein and β-casein in the bulk solution. This suggests that factors other than binding affinity per se seem to influence the final composition of these caseins in the binary film as opposed to in the single component film at the triolein-water interface. One possible explanation may be that these two caseins may exhibit incompatibility of mixing

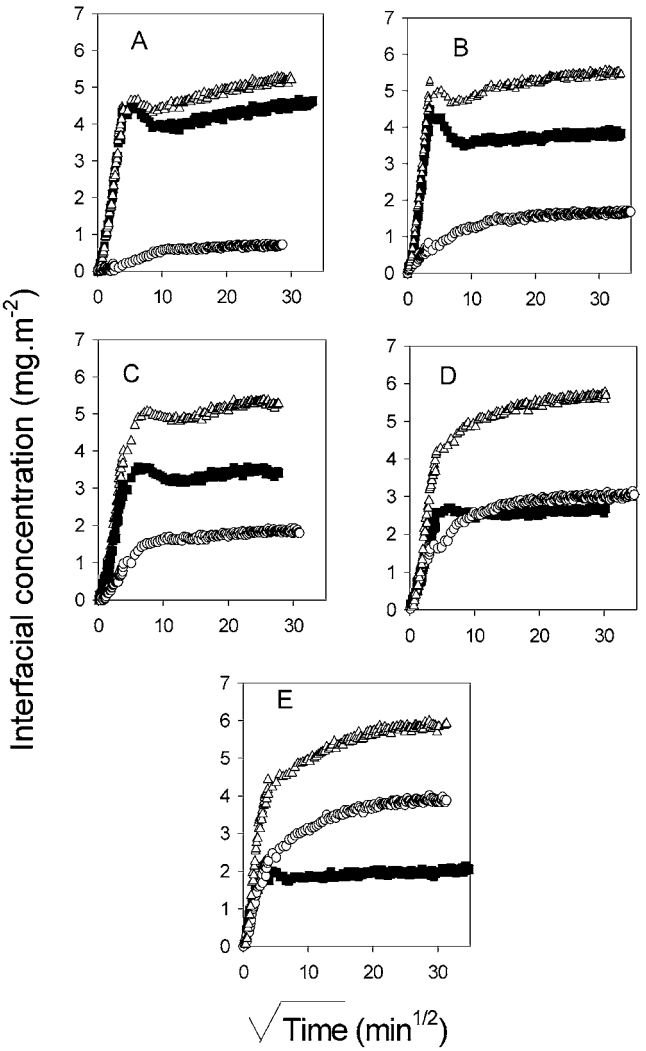

Figure 6. Kinetics of competitive adsorption of α_s-casein (○) and β-casein (■) at the triolein–water interface from bulk solutions containing various concentration ratios of α_s-casein and β-casein. The △ curve represents total interfacial concentration, obtained from the sum of ○ and ■ curves. The bulk concentration ratios of α_s-casein/β-casein were (A) 0.25/3.75, (B) 0.44/3.56, (C) 0.67/3.33, (D) 1.33/2.67, and (E) 2/2. The total protein concentration in all these binary solutions was 4×10^{-4}% w/v.

in the film at the interface. This may facilitate preferential adsorption of one protein at the expense of the other. Previously, using an epi-fluorescence microscopy technique, it has been demonstrated that α_{s1}-casein and β-casein exhibited two-dimensional phase separation in films formed at the air−water interface because of incompatibility of mixing (*16*). That such a phenomenon may also exist at the triolein−water interface is evident from the fact that the total amount of Γ_{eq} in the binary system (i.e., 5.61 mg m^{-2}) was less than that in single component systems at 3.0×10^{-4}% (w/v) bulk concentration (**Figure 2**). This could occur only when the presence of one protein at the interface alters the binding affinity of the other to the interface because of incompatibility of mixing (*6*, *7*).

To determine if α_s-casein and β-casein dynamically displaced each other during adsorption at the triolein−water interface, competitive adsorption at various bulk concentration ratios was studied. **Figures 6** and **7** show $\Gamma - t^{1/2}$ and $\Pi - t^{1/2}$ curves, respectively, for competitive adsorption of α_s-casein and β-casein at five different bulk concentration ratios. The total protein concentration in the bulk solution in all these experiments was 4.0×10^{-4} % (w/v). At low α_s-casein to β-casein

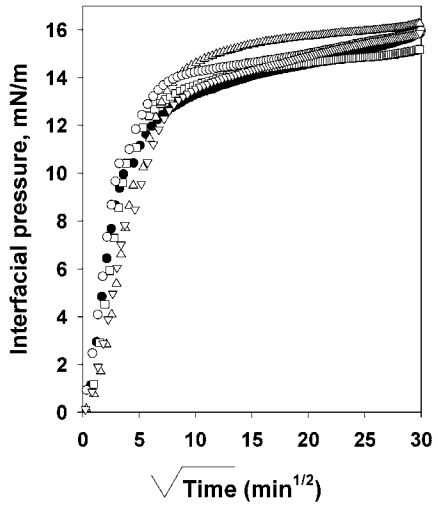

Figure 7. Evolution of interfacial pressure with time during adsorption of α_s-casein and β-casein at the triolein–water interface in various binary systems described in Figure 5. The symbols correspond to α_s-casein/β-casein bulk ratios, (▽) 0.25/3.75, (○) 0.44/3.56, (△) 0.67/3.33, (□) 1.33/2.67, and (●) 2/2.

ratio in the bulk, for example at α_s-casein to β-casein ratio of 0.25:3.75 (**Figure 6A**), the interfacial concentration of β-casein initially increased rapidly to reach a peak at about 25 min of adsorption, then gradually decreased with time up to about 100 min and then slowly increased with time. On the other hand, the interfacial concentration of α_s-casein increased continuously during the desorption phase of β-casein and reached an equilibrium value of about 0.8 mg m^{-2}. The data in **Figure 6A** suggest that after the initial rapid adsorption of β-casein to the interface, owing to its high bulk concentration, it was displaced from the interface by the late arriving α_s-casein and the system reached a new equilibrium value. As the α_s-casein to β-casein bulk concentration ratio was increased, keeping the total protein concentration in the bulk phase constant at 4×10^{-4}% (w/v), the Γ_{eq} of α_s-casein increased and that of β-casein decreased (**Figures 6B**−**E**). The desorption phase of β-casein (as a result of displacement by α_s-casein) also gradually decreased as the α_s-casein to β-casein bulk ratio was increased. However, while the shape of the $\Gamma - t^{1/2}$ curves of α_s-casein was typically hyperbolic at all bulk ratios, that of β-casein was nonhyperbolic, suggesting that adsorption of β-casein to the interface was abruptly stopped after a period of time, presumably by α_s-casein. Desorption of α_s-casein by β-casein did not take place even at high α_s-casein to β-casein bulk ratios, for example at 3:1 ratio (data not shown). This is surprising given the fact that the binding affinity of β-casein for the triolein−water interface was greater than that of α_s-casein. As discussed earlier, this might be related to thermodynamic incompatibility of mixing between α_s-casein and β-casein above a threshold concentration in the interfacial region, favoring preferential adsorption of α_s-casein.

The Γ_{eq} values of both caseins in each of the binary experiments are summarized in **Table 1** and the Γ_{eq} versus α_s-casein/β-casein bulk ratio profiles are shown in **Figure 8.** The Γ_{eq} of α_s-casein increased and that of β-casein decreased with increasing ratio of α_s-casein to β-casein in the bulk. The extent of increase of Γ_{eq} of α_s-casein was larger and the extent of decrease of Γ_{eq} of β-casein was smaller for a unit increase in α_s-casein to β-casein concentration ratio in the bulk solution. It should be noted that while the Γ_{eq} of α_s-casein and β-casein in single component systems at 4×10^{-4}% (w/v) bulk concentration was 6.13 and 7.15 mg m^{-2}, respectively, the Γ_{total}

Table 1. Compositions of the Mixed α_s-Casein/β-Casein Films at the Triolein–Water Interface at Various Bulk Ratios[a]

bulk protein conc, % (w/v)		interfacial protein conc at equilibrium (mg m^{-2})		
α_s-casein	β-casein	α_s-casein	β-casein	total
4.0×10^{-4}	0.0	6.13	0.0	6.13
2.0×10^{-4}	2.0×10^{-4}	3.86	2.09	5.95
1.33×10^{-4}	2.67×10^{-4}	3.01	2.64	5.65
1.0×10^{-4}	3.0×10^{-4}	2.37	3.25	5.62
0.67×10^{-4}	3.33×10^{-4}	1.83	3.46	5.29
0.44×10^{-4}	3.56×10^{-4}	1.65	3.82	5.47
0.25×10^{-4}	3.75×10^{-4}	0.69	4.45	5.14
0.0	4.0×10^{-4}	0.0	7.15	7.15

[a] The total bulk protein concentration was 4×10^{-4}% (w/v).

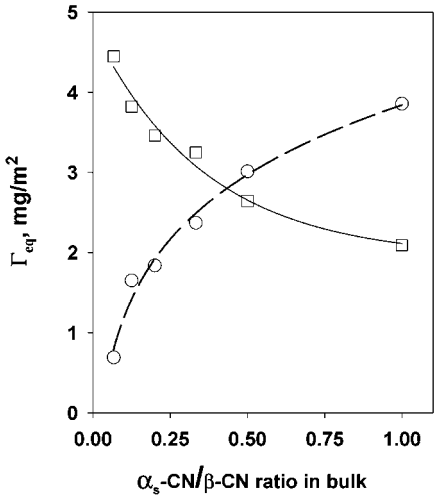

Figure 8. Relationship between equilibrium interfacial concentration of α_s-casein (○) and β-casein (□) and the bulk concentration ratio of α_s-casein to β-casein.

at equilibrium in all these binary experiments was in the range of 5.14−5.95 mg m^{-2} (**Table 1**), which was lower than the Γ_{eq} values in single component systems. For instance, in the case of 0.25:3.75 bulk ratio of α_s-casein to β-casein, the Γ_{total} was only about 5.14 mg m^{-2} whereas when α_s-casein was omitted from the system the Γ_{total} increased to 7.15 mg m^{-2}. This suggests that the presence of even a small concentration of α_s-casein in the bulk impedes adsorption of β-casein to the interface. This might be due to incompatibility of mixing of these two caseins at the triolein−water interface.

It has been suggested that reversibility/irreversibility of protein adsorption at an interface and exchange between protein molecules in the bulk and at the interface was dependent on the magnitude of concentration-dependent intermolecular interactions at the interface (*7*). According to this view, protein adsorption is intrinsically reversible and exchangeable so long as the adsorbed protein molecules are in a nonaggregated state at the interface. However, once film formation via intermolecular interactions occurs above a critical Γ, reversibility and exchangeability are lost. To determine if this is true in the case of α_s-casein/β-casein at the triolein−water interface, displacement experiments were carried out on 24 h aged monolayer films of α_s-casein and β-casein. The results are shown in **Figure 9**. When unlabeled β-casein was injected into the bulk phase of ^{14}C-labeled α_s-casein film aged for 24 h at the triolein−water interface, no perceptible change was observed in the interfacial cpm readings, implying that β-casein could not displace α_s-casein from the triolein−water interface. Similarly, α_s-casein

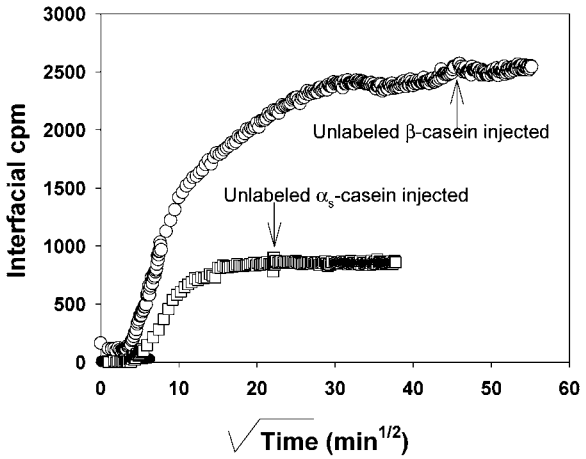

Figure 9. Displacement of ^{14}C-labeled α_s-casein by unlabeled β-casein (○) and ^{14}C-labeled β-casein by unlabeled α_s-casein (□) from the triolein-water interface. The arrows represent the times at which the unlabeled proteins were injected into the bulk phase. See text and Materials and Methods for further details. The specific radioactivities of α_s-casein and β-casein were 0.795 and 0.34 μCi/mg, respectively.

also could not displace β-casein from the triolein−water interface. It should be recalled that during simultaneous adsorption of α_s-casein and β-casein from the bulk phase, α_s-casein could dynamically displace β-casein from the triolein−water interface during initial phases of the adsorption process (**Figure 6**). Coupled with the data of **Figure 6**, the data in **Figure 9** clearly demonstrate that so long as the interfacial concentration of β-casein is below the threshold concentration to form a film, α_s-casein from the bulk phase can displace β-casein from the triolein−water interface.

The inability of either of the proteins to displace the other when the latter is in an aged film state at the triolein−water interface is in stark contrast with their behavior at the air−water interface (*5*). It has been shown that both α_{s1}-casein and β-casein could exchange and displace each other from the air−water interface even in an aged saturated monolayer film (*5*). This tentatively suggests that both α_s-casein and β-casein form a more cohesive film at the triolein-water interface than at the air−water interface.

Recently, an empirical approach has been developed to study thermodynamic incompatibility of mixing of proteins at interfaces (*6*, *7*). The Langmuir model for competitive adsorption of two proteins from a bulk phase to an interface is given by (*1*)

$$\Gamma_1 = \frac{K_1 C_1}{1 + K_1 a_1 C_1 + K_2 a_2 C_2} \quad (3)$$

$$\Gamma_2 = \frac{K_2 C_2}{1 + K_1 a_1 C_1 + K_2 a_2 C_2} \quad (4)$$

where Γ_1 and Γ_2 are interfacial concentrations at equilibrium, C_1 and C_2 are bulk concentrations at equilibrium, a_1 and a_2 are areas occupied per molecule at saturated monolayer coverage (i.e., $1/\Gamma_{sat}$), and K_1 and K_2 are equilibrium binding constants of protein 1 and protein 2, respectively, at the interface. This Langmuir model for competitive adsorption of proteins assumes that Γ_{eq} of proteins 1 and 2 in the mixed film are affected only by their concentrations in the bulk, the number of vacant sites available at the interface, and their relative binding affinities to the interface. It also implicitly assumes that the adsorbed proteins

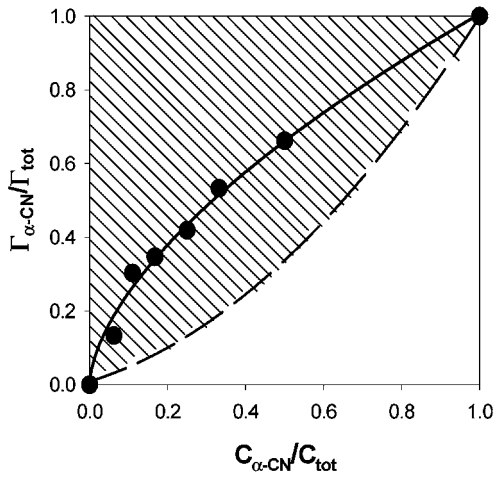

Figure 10. Plot of $\Gamma_{\alpha s-CN}/\Gamma_{tot}$ versus $C_{\alpha s-CN}/C_{tot}$ at equilibrium for adsorption of α_s-casein and β-casein at the triolein–water interface in the binary systems. The broken line represents the ideal Langmuir curve predicted by eq 5.

do not aggregate and/or interact with each other. For such a noninteracting ideal system, from eqs 3 and 4

$$\frac{\Gamma_1}{\Gamma_{tot}} = \frac{K_1C_1}{K_1C_1 + K_2C_2} \text{ and } \frac{\Gamma_2}{\Gamma_{tot}} = \frac{K_2C_2}{K_1C_1 + K_2C_2} \qquad (5)$$

Knowing K_1 and K_2 from single-component systems, the value of Γ_1/Γ_{tot} (or Γ_2/Γ_{tot}) can be calculated for any combination of C_1 and C_2. If the proteins in a binary film at an interface exhibit incompatibility of mixing, then the binding affinities K_1 and K_2 in the binary system would not be the same as those in single component systems (*6*). Thus, if incompatibility exists, the experimental Γ_1/Γ_{tot} versus C_1/C_{tot} profile would not be the same as that predicted by the Langmuir model. The extent of deviation will be a direct measure of the degree of nonideality of mixing between the proteins (*6*, *7*).

Figure 10 shows experimental $\Gamma_{\alpha s-CN}/\Gamma_{tot}$ versus $C_{\alpha s-CN}/C_{tot}$ curve along with that predicted by eq 5. A similar plot for β-casein will be similar in profile but inverted diagonally. It is evident that there is significant deviation of the experimental curve from the predicted one, indicating that the α_s-casein/β-casein binary film at the triolein−water interface exhibits a nonideal adsorption behavior, probably because of nonideal mixing at the interface. If these two proteins are completely incompatible, then they cannot coexist at all in the interface. That is, the presence of one protein at the interface will totally prevent adsorption of the other. For this condition, the shaded area in **Figure 10** can be regarded as a measure of 100% incompatibility or immiscibility. Then, the ratio of the area between the predicted and experimental curves and the total shaded area can be defined as the true degree of incompatibility, X_{12}, between α_s-casein and β-casein in the triolein−water interfacial region. The incompatibility parameter X_{12} calculated in this manner for α_s-casein/β-casein binary film at the triolein−water interface is 0.38. Previously, it has been shown that α_{s1}-casein and β-casein also exhibited incompatibility of mixing at the air−water interface and the value of incompatibility parameter in that system was 0.14 (*6*, *7*). This suggests that the degree of incompatibility between α_s-casein and β-casein at the triolein−water interface is much stronger than at the air−water interface.

Comparison of the results of competitive adsorption of α_s-casein and β-casein at the triolein−water interface and those of previous studies at the air−water interface (*5*−*7*) reveal several important differences. First, even though the apparent binding affinity of α_s-casein is lower than that of β-casein to both air−water and triolein−water interfaces, α_s-casein is preferentially adsorbed to the triolein−water interface, whereas this was not the case at the air−water interface. Although the exact molecular reasons for this behavior are not clear, it might be the net result of the energetics of protein−protein, protein−water, and protein−oil interactions in the interfacial region. It is likely that the magnitude of Flory-Higgins protein−solvent interaction parameters, χ_{ps}, of the two proteins in the triolein−water interfacial region may play a role in amplifying incompatibility of mixing of the casein in this interface (*7*). In a protein 1/protein 2/solvent ternary system, even if the interaction parameter $\chi_{12} = 0$, meaning that the polymers are compatible with each other, they can still exhibit incompatibility of mixing in a solvent medium if there is a difference in the protein−solvent interaction parameters, $|\chi_{1s} - \chi_{2s}|$ (*17*). In the case of oil−water interface, the situation is more complicated than the air−water interface because the solvent in the interfacial region is an inhomogeneous mixture of oil and water. Thus, χ_{1s} and χ_{2s} will have two components, viz., χ_{1w} and χ_{2w}, depicting interaction with water, and χ_{1o} and χ_{2o}, depicting interaction with oil. Therefore, for two proteins at the oil−water interface

$$|\chi_{1s} - \chi_{2s}| = |(\chi_{1w} + \chi_{1o}) - (\chi_{2w} + \chi_{2o})| = |(\chi_{1w} - \chi_{2w}) + (\chi_{1o} - \chi_{2o})| \qquad (6)$$

This expression basically relates to the sum of differences in hydrophilicities and lipophilicities of the two proteins. The larger the difference, the greater would be the incompatibility at the oil−water interface. Previously (*17*), it has been pointed out that incompatibility between two polymers can manifest even when the value of $|\chi_{1s} - \chi_{2s}|$ is as little as 0.03.

An alternative explanation for the preferential adsorption of α_s-casein may be related to its propensity to self-aggregate and form network structure. This property, which is lacking in β-casein, may enable α_s-casein to form a self-aggregated film at the triolein−water interface, thereby forcing β-casein to desorb from the interface. The absence of this phenomenon at the air−water interface might be related to the fact that the saturated monolayer coverage for α_s-casein at the air−water interface is only about 1.6 mg m^{-2}, which may not be high enough to form a strong network structure.

Previously (*18*) it has been reported that during aging of a tetradecane-in-water emulsion stabilized by a mixture of α_{s1}-casein and β-casein, β-casein from the bulk serum phase gradually displaced α_{s1}-casein from the emulsion droplet surface. In the emulsion formed with a 1:1 mixture of α_s-casein + β-casein, the surface load of β-casein was twice that of α_s-casein (*18*). The results of the present study disagree with those previous reports. It is quite likely that several washing and centrifugation steps used in the previous emulsion studies for determining protein composition and load at the interface might have resulted in experimental artifacts. It is also likely that the observed displacement of α_{s1}-casein by β-casein in those studies might not have been from the primary adsorbed layer, but from loosely held multilayers around oil droplets. The data presented here pertain only to dynamics of displacement at monolayer coverage since no multilayer formation was obvious from the adsorption isotherms in the bulk concentration range studied (**Figure 2**). Moreover, in the previous study (*18*), *n*-tetradecane was used as the oil phase in the preparation of oil-in-water emulsion. It is quite possible that the energetics of

interaction of α_s-casein by β-casein with the triolein−water interface might be very different from that with the *n*-tetradecane−water interface. It is quite possible that the pure hydrocarbon−water interface of the *n*-tetradecane−water systems may behave very similar to the air−water interface. The fact that the ratio of interfacial load of α_s-casein to β-casein in the *n*-tetradecane-in-water emulsion was 1:2 when the concentration ratio of the proteins in the bulk phase was 1:1 (*17*), which was exactly the same as that found at the air−water interface under similar conditions (*5*), indirectly supports the above supposition.

The results of this study clearly demonstrate that competitive adsorption of α_s-casein and β-casein from a bulk solution to the oil−water interface does not follow the simple Langmuir model, and this deviation might be related to incompatibility of mixing of these caseins in the oil−water interfacial region. Since thermodynamic incompatibility generally leads to phase separation, it is likely that α_s-casein and β-casein in the mixed film at the triolein−water interface may undergo time-dependent two-dimensional phase separation. Two-dimensional phase separation in α_s-casein/β-casein mixed films at the air−water interface has been reported (*16*). If phase separation does occur at the triolein−water interface, then the interface between phase-separated regions in the mixed protein film around oil droplets in emulsions may act as a source of instability in these emulsions.

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