Lateral Phase Separation in Adsorbed Binary Protein Films at the Air−Water Interface

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Lateral phase separation in two-dimensional mixed films of soy 11S/β-casein, acidic subunits of soy 11 (AS11S)/β-casein, and α-lactalbumin/β-casein adsorbed at the air−water interface has been studied using an epifluorescence microscopy method. No distinct lateral phase separation was observed in the mixed protein films when they were examined after 24 h of adsorption from the bulk phase. However, when the soy 11S/β-casein and AS11S/β-casein films were aged at the air−water interface for 96 h, phase-separated regions of the constituent proteins were evident, indicating that the phase separation process was kinetically limited by a viscosity barrier against lateral diffusion. In these films, β-casein always formed the continuous phase and the other globular protein the dispersed phase. The morphology of the dispersed patches was affected by the protein composition in the film. In contrast with soy 11S/β-casein and AS11S/β-casein films, no lateral phase separation was observed in the α-lactalbumin/β-casein film at both low and high concentration ratios in the film. The results of these studies proved that proteins in adsorbed binary films exhibit limited miscibility, and the deviation of competitive adsorption behavior of proteins at the air−water interface from that predicted by the ideal Langmuir model (Razumovsky, L.; Damodaran, S. *J. Agric. Food Chem*. **2001**, *49*, 3080−3086) is in fact due to thermodynamic incompatibility of mixing of the proteins in the binary film. It is hypothesized that phase separation in adsorbed mixed protein films at the air−water and possibly oil−water interfaces of foams and emulsions might be a source of instability in these dispersed systems.

Keywords: *Phase separation; protein films; air−water interface; thermodynamic incompatibility; protein adsorption; epifluorescence microscopy*

INTRODUCTION

The stability of protein-stabilized food colloids, such as food foams and food emulsions, is inherently dependent on the structure and rheological properties of the adsorbed protein film at the interface. Whereas intermolecular interactions between the adsorbed protein molecules create a viscoelastic film that can withstand thermal and mechanical perturbations (*1*, *2*), loop and tail configurations of the protein chain at the interface minimize interparticle interactions and thus provide steric stabilization against coalescence of the oil droplets during storage (*2*−*5*). These attributes are, in turn, dependent on the physicochemical properties, such as molecular flexibility, hydrophilicity/hydrophobicity, and charge characteristics, of proteins. Another important factor, which has not been considered in the past, that might impact the stability of food colloid dispersions is the composition of the protein film. Typical food proteins, such as milk proteins, legume proteins, and egg proteins, are mixtures of several component proteins, so it is conceivable that the composition, rheological properties, and integrity of the protein film formed at interfaces might be affected by the relative affinity of the component proteins to the interface and thermodynamics of interactions among them in the interfacial film.

Generally, polymer mixtures are thermodynamically incompatible. This has been shown to be true of proteins as well. In concentrated aqueous solutions, mixtures of two proteins exhibit thermodynamic incompatibility of mixing and, as a result, undergo phase separation (*6*−*8*). Because the local concentration of proteins in an adsorbed protein film at the air−water interface is equivalent to 15−30%, it is likely that two-dimensional phase separation of proteins might occur in the film (*9*). In the preceding paper and elsewhere (*10*), we have shown that thermodynamic incompatibility of mixing exists in mixed protein films formed at the air−water interface for several binary protein systems. Because, in solution, thermodynamic incompatibility between proteins inevitably leads to phase separation (*8*), it is only logical to expect that similar two-dimensional phase separation should occur in mixed protein films formed at air−water and oil−water interfaces, where the local concentration of protein is very high. In this paper, using epifluorescence microscopy, we present experimental evidence of two-dimensional phase separation in several binary protein films formed at the air−water interface.

MATERIALS AND METHODS

Materials: Soy 11S was isolated from defatted soy flour (Central Soya Co., Chicago, IL) as described by Thanh and Shibasaki (*11*). Acidic subunits of soy 11S globulin (AS11S) were prepared as described elsewhere (*12*, *13*). The purity of this preparation was >95% as judged from SDS-PAGE gel. All other proteins used in this study were purchased from

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Sigma Chemical Co. (St. Louis, MO). Ultrapure Na_2CNBH_3, NaH_2PO_4, Na_2HPO_4, and NaCl were from Aldrich Chemical Co. (Milwaukee, WI). Fluorescin-5-EX succinimidyl ester was purchased from Molecular Probes (Eugene, OR), and Sulforhodamine 101 acid chloride (also known as Texas Red), 3-aminopropyltriethoxysilane (APTES), and Sigmacote (chlorinated organopolysiloxane in heptane) were purchased from Sigma Chemical Co. (St. Louis, MO). A Slowfade Light Antifade reagent in glycerol−water, which prevents photobleaching of fluorescent-labeled proteins, was obtained from Molecular Probes. 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 experiments.

Fluorescent Labeling of Proteins. Phase separation in protein 1/protein 2 mixed protein films at the air−water interface was examined using an epifluorescence microscopy technique. In this approach, protein 1 was labeled with Fluorescin-5-EX succinimidyl ester, and protein 2 was labeled with Texas Red. In both cases, the fluorescence labeling was at the lysyl residues of the proteins. Labeling with Fluorescin-5-EX succinimidyl ester was carried out as follows: To 5 mL of protein solution in 0.1 M carbonate buffer (pH 8.5), containing ∼1 μmol of protein was added 0.5 mL of anhydrous ethanol containing ∼10 μmol of the dye. The mixture was stirred for 3.5 h. After the reaction, the mixture was applied to a Sephadex G-25-50 gel filtration column and eluted with the carbonate buffer. The fractions corresponding to the first elution peak that contained the protein, as judged from absorption at 280 nm, were pooled and dialyzed against phosphate-buffered saline solution (pH 7.0 and ionic strength 0.1 M) and lyophilized. Under these reaction conditions, ∼0.85 mol of Fluorescin-5-EX succinimidyl ester was incorporated per mole of the protein.

Labeling of proteins with Texas Red was carried out according to the method outlined by Sigma Chemical Co. Briefly, the protein (40 mg) was dissolved in 8 mL of ice-cold 0.1 M carbonate buffer (pH 8.5) and stirred continuously. A stock solution of Texas Red (4 mg/mL) was prepared in ethanol. To start the reaction, 0.2 mL of the Texas Red−ethanol solution was added to the protein solution, and the mixture was stirred under ice-cold conditions for 10 min. This procedure was repeated every 10 min by adding 0.2 mL of the dye−ethanol solution to the protein−buffer solution until all of the dye solution was consumed. After the last cycle, the reaction mixture was stirred under ice-cold conditions for an additional hour. The labeled protein was then purified as described above. Under these reaction conditions, the level of labeling was typically ∼0.132 mol of Texas Red/mol of the protein.

Epifluorescence Microscopy. For epifluorescence microscopy, first the fluorescent-labeled proteins were allowed to adsorb to the air−water interface from a solution containing a mixture of the proteins in a Teflon trough for a period of 4 days. The mixed protein film formed at the air−water interface was transferred to a clean microscope slide that was precoated with APTES. Coating of the microscope slide with APTES was carried out as described elsewhere (*14*). Transfer of the film was done using a horizontal lifting method in which the microscope slide held horizontally by a vacuum tube was gently lowered to make contact with the aqueous surface for ∼10 s. After the film had been lifted, excess water was allowed to drain off for 1 min. The slide was then dipped in water to rinse off loosely bound proteins on the glass slide. The slide was then air-dried. A drop of SlowFade Light Antifade reagent in glycerol−water was added to the glass slide and covered thereafter with a clean cover glass. This reagent prevented photobleaching of the fluorophores during observation under the microscope. The slides, thus prepared, were immediately observed under a computerized Olympus epifluorescence microscope. The microscope was equipped with a wide excitation and band-pass emission Oregon Green optical filter cube assembly selective for green (excitation, 495 ± 15 nm; emission, 545 ± 25 nm), a narrow excitation and a long-pass emission Texas Red filter cube assembly selective for red (excitation, 545−550 nm; emission, 610 nm and above), and a wide excitation and a long-pass emission fluorescin filter cube assembly (excitation, 460−490 nm; emission, 515 nm and above) for simultaneous detection of both dyes. The fluorescence images were digitized and analyzed with Olympus Image-Pro Analysis software.

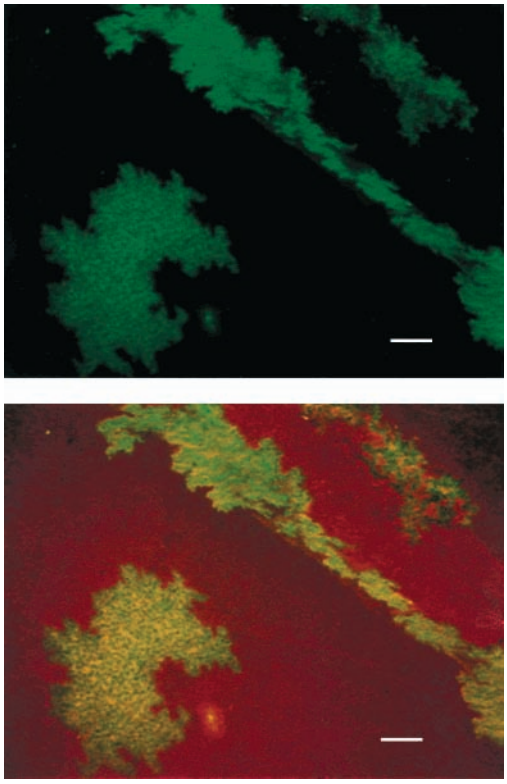

Figure 1. Fluorescence microscope image of a soy 11S/β-casein mixed protein film at the air−water interface: (A, top) image taken with a green filter; (B, bottom) image taken with a mutual filter that allowed both red and green fluorescence. The green region is soy 11S and the red region is β-casein. The surface concentrations of soy 11S and β-casein in the mixed film were about 0.054 and 1.42 mg m^{-2}, respectively. Scale bar = 100 μm.

RESULTS AND DISCUSSION

Preliminary investigations showed that protein films from the air−water interface can be lifted intact using a clean microscope glass slide precoated with APTES. Figures 1−5 show epifluorescence microscope images of soy 11S/β-casein, AS11S/β-casein, and α-lactalbumin/β-casein mixed protein films transferred from the air−water interface. In initial experiments, when the protein films were transferred soon after they formed a saturated monolayer at the interface, that is,, after 24 h of adsorption, they did not show well-defined phase-separated regions under the epifluorescence microscope. However, when the films were aged at the air−water interface for 4 days and then transferred to the glass slide, distinct phase-separated regions could be observed. Apparently, during the initial stages of adsorption, two proteins are adsorbed randomly at the interface, forming a homogeneous film. The proteins then undergo phase separation due to mutual incompatibility. Because the local concentration of protein in the film is very high, phase separation appears to be kinetically limited by the viscosity barrier to lateral diffusion of the protein molecules. Figure 1 shows two-dimensional phase separations in a soy 11S/β-casein binary film. The red and green regions in these fluorescence images correspond to β-casein and soy 11S,

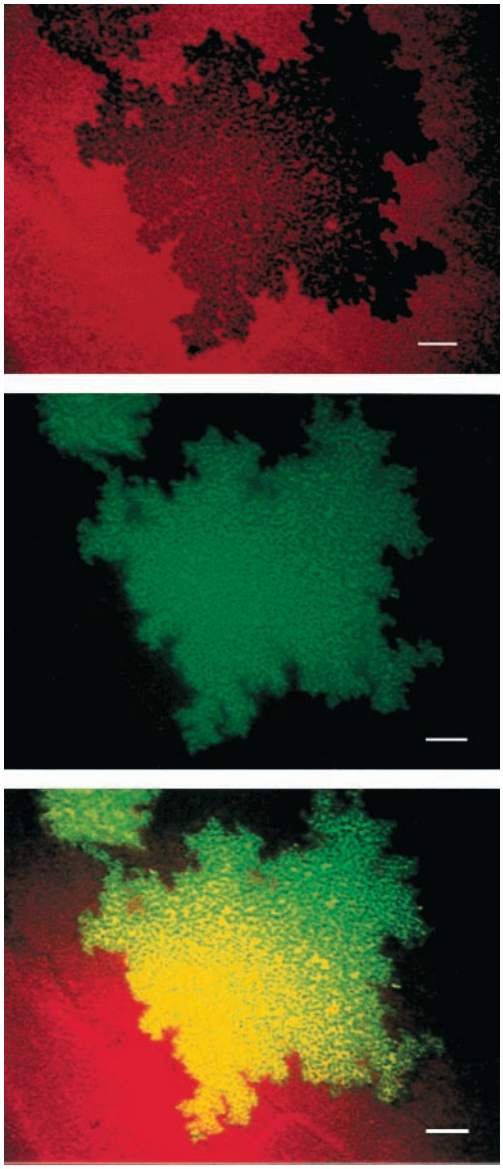

Figure 2. Fluorescence microscope image of an AS11S/β-casein mixed protein film at the air−water interface: (A, top) image taken with a red filter; (B, middle) image taken with a green filter; (C, bottom) image taken with a mutual filter. The concentrations of AS11S and β-casein in the bulk phase were 0.25 and 1.5 μg/mL, respectively. The red and green regions are β-casein and AS11S, respectively. The yellow region is a mixture of red and green as seen by the mutual filter. Scale bar = 100 μm.

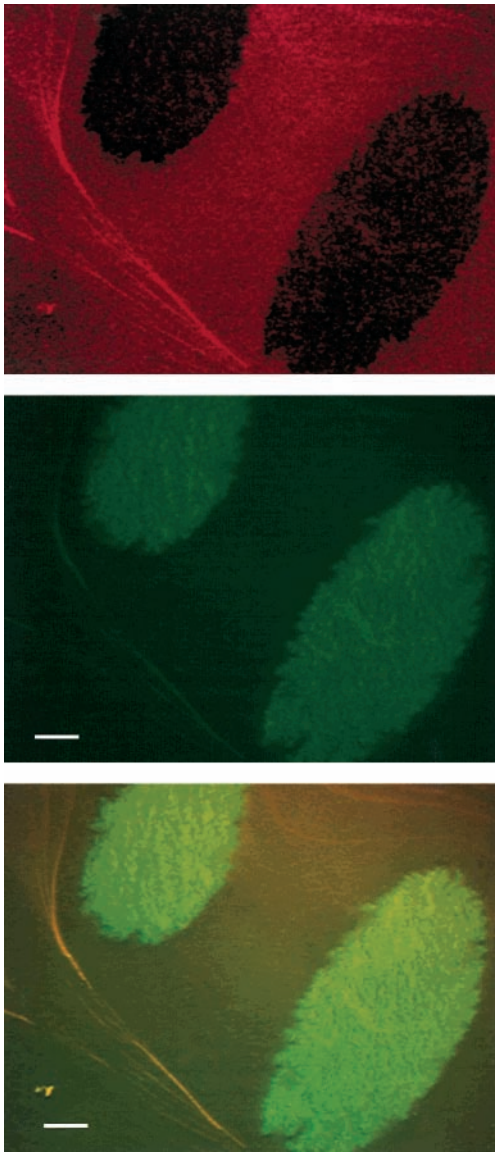

Figure 3. Fluorescence microscope image of an AS11S/β-casein mixed protein film at the air−water interface: (A, top) image taken with a red filter; (B, middle) image taken with a green filter; (C, bottom) image taken with a mutual filter. The surface concentrations of AS11S and β-casein in the mixed film were about 0.43 and 0.74 mg m^{-2}, respectively, corresponding to initial bulk concentrations of 0.5 and 1.5 μg/mL, respectively. The color schemes are as in Figure 2. Scale bar = 100 μm.

respectively. Figure 1A shows the fluorescence image of the binary film formed from a bulk mixture containing 0.25 μg/mL soy 11S and 1.5 μg/mL β-casein. At this bulk concentration ratio, the surface concentrations of soy 11S and β-casein were 0.54 and 1.4 mg m^{-2}, respectively. The image taken with the green filter (Figure 1A) shows irregular green patches (soy 11S) surrounded by a dark continuous phase. When the same image is taken with the mutual filter that allowed both red and green emissions, it shows green-yellow regions surrounded by a continuous red (β-casein) region. At high soy 11S/β-casein bulk concentration ratios, the patches of soy 11S in the film became denser and larger in size (data not shown). In all cases soy 11S tended to form the dispersed phase and β-casein the continuous phase in the binary film at the air−water interface.

Figures 2 and 3 show epifluorescence images of AS11S/β-casein mixed films at the air−water interface at two different bulk concentration ratios. Figure 2 shows fluorescence microscope images of a section of the mixed film taken using the three different filters. The bulk concentrations of AS11S and β-casein were 0.25 and 1.5 μg/mL, respectively. In the image taken with the Texas Red filter (Figure 2A), there is a large black patch with specks of red surrounded by a continuous red region (β-casein). This black patch appears as an intense green patch and the red region appears as black region when the image is taken with the green filter (Figure 2B). This clearly shows that, whereas the continuous phase contains only β-casein, the dispersed phase contains mainly AS11S. When the image is taken with the mutual filter, the black patch with specks of red appears as green and yellow and the continuous phase appears as predominantly red (Figure 2C). The yellow region in Figure 2C is a mixture of AS11S (green)

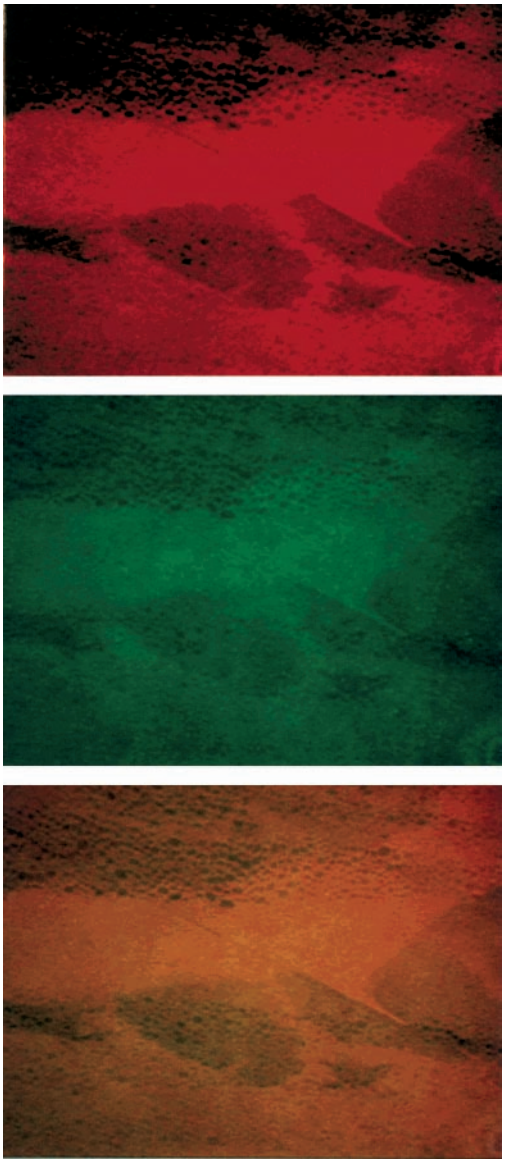

Figure 4. Fluorescence microscope image of an α-lactalbumin/β-casein mixed protein film at the air−water interface: (A, top) image taken with a red filter; (B, middle) image taken with a green filter; (C, bottom) image taken with a mutual filter. The surface concentrations of α-lactalbumin and β-casein in the mixed film were about 0.12 and 1.1 mg m^{-2}, respectively, corresponding to initial bulk concentrations of 0.15 and 1.5 μg/mL, respectively. The color schemes and scale are as in Figure 2.

and β-casein (red) as seen by the mutual filter. It should be noted that, at this concentration ratio, the AS11S patches appear irregular in geometry and fractal-like in appearance.

As the concentration ratio of AS11S to β-casein in the mixed film is increased, the patches of AS11S in the film become denser and smaller in size, as shown in Figure 3. Figure 3 shows images of a mixed film formed from a bulk solution containing 0.5 μg/mL AS11S and 1.5 μg/mL β-casein, which corresponds to 0.4 mg m^{-2} of AS11S and 0.74 mg m^{-2} of β-casein in the film. It should be noted that the AS11S regions are oval in shape, and in the image taken with the mutual filter (Figure 3C), the continuous phase is olive green in color and the dispersed phase is larger and irregular in shape. This is in contrast with the image in Figure 2C, in which the continuous phase is red in color. The olive green color

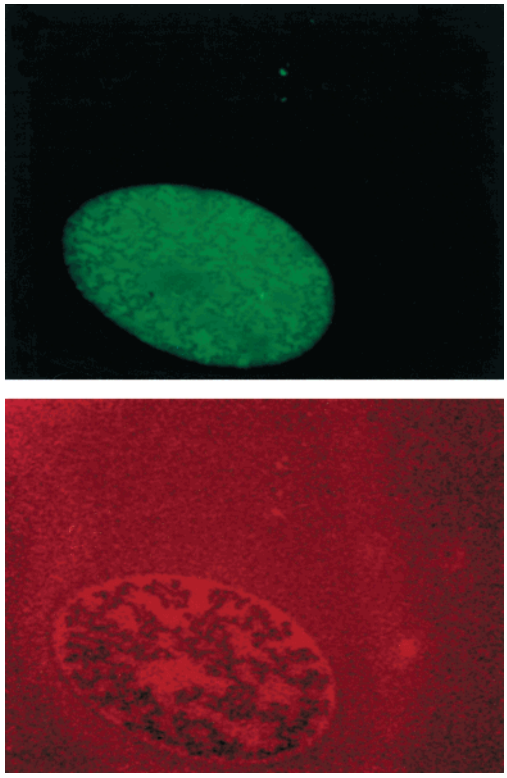

Figure 5. Fluorescence microscope image of an α-lactalbumin/β-casein mixed protein film at the air−water interface: (A, top) image taken with a red filter; (B, bottom) image taken with a green filter. The surface concentrations of α-lactalbumin and β-casein in the mixed film were about 0.32 and 0.92 mg m^{-2}, respectively, corresponding to initial bulk concentrations of 1.5 and 1.5 μg/mL, respectively. The color schemes and scale are as in Figure 2.

indicates coexistence of AS11S with β-casein in the continuous phase. It is also probable that at high ratios of AS11S to β-casein in the film, complete separation of AS11S into a dispersed phase might be kinetically limited and a longer aging time would have allowed more separation of AS11S from the continuous phase.

Figures 4 and 5 show epifluorescence images of α-lactalbumin/β-casein mixed film at the air−water interface at two different bulk concentration ratios. Figure 4 shows images of a film formed from a bulk mixture of 0.15 μg/mL α-lactalbumin and 1.5 μg/mL β-casein. This corresponds to 0.12 mg m^{-2} α-lactalbumin and 1.1 mg m^{-2} β-casein in the film. It is interesting to note that, unlike the other binary films, no phase separation between α-lactalbumin and β-casein occurs at this concentration ratio. The images taken with the red (Figure 4A) and green (Figure 4B) filters show essentially the same pattern, without any distinct phase-separated regions. This is further confirmed by the image taken with the mutual filter (Figure 4C), which shows a uniform orange color, indicating a homogeneous mixture of α-lactalbumin (green) and β-casein (red) in the film. Figure 5 shows images of α-lactalbumin/β-casein mixed film obtained from a bulk mixture of 1.5 μg/mL α-lactalbumin and 1.5 μg/mL β-casein. The actual concentrations of α-lactabumin and β-casein in the film were 0.32 and 0.92 mg m^{-2}, respectively. At this high concentration ratio, α-lactalbumin seems to form oval-shaped phase-separated regions (Figure 5A). However, when the image is taken with a red filter, a significant amount of β-casein (red) is also present in the oval-shaped region, suggesting

that these two proteins coexist in the form a complex in this region.

Among the three binary protein films at the air−water interface investigated in this study, the soy 11S/β-casein and AS11S/β-casein systems clearly exhibit incompatibility and two-dimensional phase separation, whereas the α-lactalbumin/β-casein system exhibits limited compatibility. These results agree rather very well with the magnitude of interaction parameter, $|\Delta \ln K|$, which is related to the intensity of thermodynamic incompatibility between two proteins, of these systems (*10*). The values of $|\Delta \ln K|$ for the soy 11S/β-casein and AS11S/β-casein systems are 1.06 and 1.47, respectively, whereas that for the α-lactalbumin/β-casein system is 0.22 (*10*). The greater the value of $|\Delta \ln K|$, the more incompatible the proteins are. Another parameter that influences the extent of incompatibility between two proteins in a protein 1/protein 2/solvent ternary system is the net difference in the extent of interaction of the proteins with the solvent. That is, if χ_{1s} and χ_{2s} are the interaction parameters of proteins 1 and 2, respectively, with the solvent, then the magnitude of $|\chi_{1s} - \chi_{2s}|$ will have an influence on the compatibility of mixing between the proteins. The larger the difference of $|\chi_{1s} - \chi_{2s}|$, the greater would be the incompatibility and the lower would be the threshold for phase separation in a ternary system. The values of $|\chi_{1s} - \chi_{2s}|$ for soy 11S/β-casein, AS11S/β-casein, and α-lactalbumin/β-casein systems are calculated to be 0.315, 0.322, and 0.007, respectively (*10*). According to these values, the α-lactalbumin/β-casein system should be more compatible than the soy 11S/β-casein and AS11S/β-casein systems, which in fact seems to be the case.

Taken together, the two-dimensional phase separation behaviors of the three binary protein films at the air−water interface investigated in this study as well as those reported recently elsewhere (*15*, *16*) unambiguously prove that the deviation of competitive adsorption of proteins at the air−water interface from that predicted by the ideal Langmuir model (*10*) is in fact due to thermodynamic incompatibility of mixing of the proteins in the binary film, and the $|\Delta \ln K|$ value can be used meaningfully as an indicator of the intensity of incompatibility between two proteins at the air−water interface.

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