# Incompatibility of Mixing of Proteins in Adsorbed Binary Protein Films at the Air−Water Interface

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Competitive adsorption of proteins from several binary protein solutions to the air−water interface has been studied. With a few exceptions, the equilibrium composition of the saturated monolayer of mixed protein films at various bulk concentration ratios did not follow a Langmuir-type competitive adsorption model. The deviation from ideal behavior results from incompatibility of mixing of proteins in the film at the air−water interface. This immiscibility alters the ratio of the binding affinity of the proteins in a protein 1/protein 2/water ternary film compared to that in a protein 1/water and protein 2/water binary film. A method to determine the extent of incompatibility between two proteins in a mixed protein film has been developed. It is shown that the incompatibility index derived for 19 protein 1/water and protein 2/water systems studied show a linear relationship with the absolute difference between Flory−Huggins protein−solvent interaction parameters, that is, $|\chi_{1s} - \chi_2|$, of the constituent proteins. On the basis of the evidence, it is theorized that, because of incompatibility, proteins in a mixed protein film at interfaces may undergo two-dimensional phase separation.

**Keywords:** *Air−water interface; protein adsorption; thermodynamic incompatibility; phase separation at interfaces*

## INTRODUCTION

Proteins exhibit a high propensity to migrate and bind to air−water and oil−water interfaces and decrease the interfacial tension. In addition to lowering the interfacial tension, the adsorbed protein can form a strong viscoelastic film via intermolecular interactions, which can withstand thermal and mechanical perturbations (*1*, *2*). The latter property makes proteins more desirable than low molecular weight amphiphiles, such as lecithin and monoglycerides, as surfactants in emulsion and foam-type food products. In addition, conformational changes in proteins at interfaces allow them to form loops that protrude from the interface into the bulk phase. The steric repulsion caused by overlapping of the layer of protruding chains when they approach each other is considered to be the most important force stabilizing emulsions (*2*−*6*). However, proteins do differ significantly in their surface activity and in their ability to stabilize colloidal systems. This is attributed to differences in their structural properties and their ability to undergo conformational reorientation at interfaces (*7*, *8*).

Notwithstanding such structure-dependent innate differences among proteins, another important factor that might influence the stability of food emulsions and foams is the thermodynamic incompatibility among proteins in the adsorbed protein film at interfaces. Typical food proteins, notably protein blends used in food industries, are mixtures of several proteins. As a result, the protein film formed at air−water and oil−water interfaces usually consists of a mixture of proteins. The stability of the protein film, and consequently the stability of protein-stabilized emulsions and foams, will be dependent on the nature and intensity of protein−protein interactions in the film. Generally, interactions between two dissimilar polymers are thermodynamically incompatible. This is true of proteins as well. In concentrated aqueous solutions (10−20% w/v), mixtures of two proteins exhibit thermodynamic incompatibility of mixing and, as a result, undergo phase separation (*9*−*12*). The saturated monolayer coverage at the air−water interface for most proteins is in the range of 1−2 mg $m^{-2}$ (*8*). If the thickness of the protein film at the air−water interface is ∼50 Å (*13*), this surface concentration range is equivalent to a local concentration of ∼20−40% (w/v). At this high local concentration, conditions for incompatibility of mixing might exist and, as a consequence, two-dimensional phase separation of proteins also might occur in the film. This might affect the storage stability of food colloids.

Experimental verification of thermodynamic incompatibility between proteins in mixed protein films at interfaces is not simple. This situation is partly due to the lack of a theoretical framework and experimental approach to study phase behavior at interfaces. Recently, we have developed an empirical approach to study this phenomenon (*14*). The essential features of this approach are as follows: Adsorption of proteins at interfaces is generally assumed to follow a Langmuir adsorption model (*15*). The Langmuir model for reversible adsorption of proteins from a protein 1/protein 2/water ternary system to an interface is (*15*)

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

and

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$$\Gamma_2 = \frac{K_2 C_2}{1 + K_1 a_1 C_1 + K_2 a_2 C_2} \quad (2)$$

where $K_1$ and $K_2$ are the equilibrium binding constants of proteins 1 and 2, respectively, to the interface determined from protein 1/solvent and protein 2/solvent adsorption systems, $C_1$ and $C_2$ are their concentrations in the bulk mixture at equilibrium, $\Gamma_1$ and $\Gamma_2$ are concentrations in the mixed film at the interface at equilibrium, and $a_1$ and $a_2$ are the surface areas occupied by proteins 1 and 2, respectively, at saturated monolayer coverage in protein 1/solvent and protein 2/solvent adsorption systems. This Langmuir model for competitive adsorption in a protein 1/protein 2/water ternary system assumes that the surface concentrations of proteins 1 and 2 in the mixed protein film are affected only by their relative binding affinities to the interface, their concentration in the bulk phase, and the availability of vacant sites at the interface. It assumes a priori that the adsorbed protein species do not interact with each other. For a non-interacting protein 1/protein 2/water ternary system, from eqs 1 and 2, the Langmuir adsorption model predicts that

$$\frac{\Gamma_1}{\Gamma_{tot}} = \frac{K_1 C_1}{K_1 C_1 + K_2 C_2} \quad \text{and} \quad \frac{\Gamma_2}{\Gamma_{tot}} = \frac{K_2 C_2}{K_1 C_1 + K_2 C_2} \quad (3)$$

Knowing $K_1$ and $K_2$ from single-protein adsorption experiments, eq 3 can predict the $\Gamma_i/\Gamma_{tot}$ versus $C_i/C_{tot}$ profile of a protein 1/protein 2/water ternary system that obeys the Langmuir competitive adsorption model.

If the proteins of a protein 1/protein 2/water ternary system exhibit incompatibility of mixing at an interface, then the first impact of that would be on the binding affinities of the proteins to the interface. The ratio of binding affinities of the proteins in the ternary adsorption system under nonideal conditions would not be the same as the ratio determined from single-protein adsorption systems. Thus, if incompatibility exists between two proteins at an interface, then the experimental $\Gamma_i/\Gamma_{tot}$ versus $C_i/C_{tot}$ profile would not be the same as that predicted by the Langmuir competitive adsorption model (eq 3). The extent of deviation will be a direct measure of the extent of incompatibility between the proteins.

The thermodynamic incompatibility between proteins at an interface can be determined using another approach as follows. From eqs 1 and 2

$$\ln(\Gamma_1/\Gamma_2) = \ln(K'_1/K'_2) + \ln(C_1/C_2) \quad (4)$$

The value of $\ln(K'_1/K'_2)$ for a nonideal ternary system can be determined from the intercept of a plot of $\ln(\Gamma_1/\Gamma_2)$ versus $\ln(C_1/C_2)$. If $K_1$ and $K_2$ are the binding constants of two proteins in protein 1/water and protein 2/water binary adsorption systems, then the absolute difference between $\ln(K_1/K_2)$ and $\ln(K'_1/K'_2)$, that is, $|\Delta \ln K|$, can be regarded as a measure of thermodynamic incompatibility between the proteins.

In this paper we provide experimental evidence of incompatibility in several mixed protein films 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 (*16*). Acidic subunits of soy 11S globulin (AS11S) were prepared as described elsewhere (*17*, *18*). The purity of this preparation was >95% as judged from SDS-PAGE gel. All other proteins used in this study were purchased from 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). 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.

**Radiolabeling.** The proteins were radiolabeled with $^{14}C$ nuclide by reductive methylation of protein amino groups with [$^{14}C$]formaldehyde at pH 7.0 as described elsewhere (*19*, *20*). The specific radioactivity of the labeled proteins ranged from 1 to 3 μCi/mg. This typically amounted to labeling of one to two lysine residues in proteins. Because reductive methylation does not change the net charge of a protein, no conformational change in proteins is likely to occur as a result of labeling. The protein concentration was determined using the extinction coefficient values of the proteins reported in the literature.

**Adsorption Measurements.** Equilibrium adsorption of radiolabeled proteins at the air−water (20 mM phosphate-buffered saline solution, pH 7.0, $I = 0.1$) interface from dilute solutions was studied using a surface radiotracer technique as described elsewhere (*19*−*21*). In most cases, equilibrium adsorption occurred within 24−30 h at 25 °C. Coadsorption of proteins from binary protein solution mixtures was studied as follows: To monitor adsorption of protein 1 from a binary mixture, stock solutions of [$^{14}C$]protein 1 and unlabeled protein 2 were mixed with the buffer to the required final bulk concentration. The solution was poured into a Teflon trough (19 × 5.2 × 1.27 cm), and the surface of the solution was swept using a capillary tube attached to an aspirator. The proteins were then allowed to adsorb from the bulk phase to the air−water interface. Although both proteins 1 and 2 are adsorbed simultaneously to the air−water interface, the measured surface radioactivity would correspond only to the amount of $^{14}C$-labeled protein 1 at the interface. To determine the amount of protein 2 adsorbed at the interface, the experiment was repeated by mixing stock solutions of [$^{14}C$]protein 2 and unlabeled protein 1 with the buffer. Equilibrium adsorption values were obtained from surface radioactivity values after 24−30 h of adsorption.

Adsorption isotherms for each protein was constructed by determining $\Gamma_{eq}$ at various bulk concentrations, $C_b$, in single-protein systems. These isotherms were used to determine the binding affinity of proteins to the air−water interface using the Langmuir equation for a single-component system

$$\Gamma = KC/(1 + KaC) \quad (5)$$

which on rearrangement gives

$$\Gamma/(1 - a\Gamma) = CK \quad (6)$$

## RESULTS AND DISCUSSION

A basic assumption in the empirical approach described above to study thermodynamic incompatibility in mixed protein films at an interface is that protein adsorption is reversible under dynamic conditions. To ascertain the validity of this assumption, the ability of one protein to displace another protein from the air−water interface was investigated using the soy 7S/β-casein binary system. In this case, $^{14}C$-labeled soy 7S was first allowed to adsorb to the air−water interface. Then unlabeled β-casein was injected into the bulk phase to see if adsorption of β-casein would displace the $^{14}C$-labeled soy 7S from the interface. The results are shown in Figure 1. At 4.6 μg/mL bulk concentration, soy 7S reached an equilibrium surface concentration of ∼2.2 mg $m^{-2}$. The adsorption isotherm of soy 7S showed that this value was close to the saturated monolayer film of soy 7S (i.e., 2.8 mg $m^{-2}$) at the air−water interface. When β-casein was injected into the bulk phase (6.0 μg/

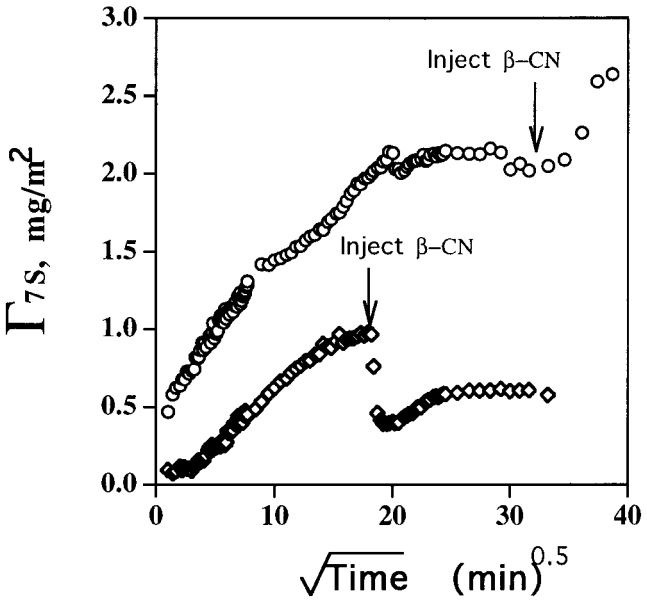


**Figure 1.** Displacement of $^{14}$C-labeled soy 7S by bulk phase unlabeled $\beta$-casein: (○) $^{14}$C-labeled soy 7S adsorbed to the air−water interface from a bulk phase containing 4.6 mg/mL soy 7S [after 900 min of adsorption (shown by the arrow), 6.0 mg/mL unlabeled $\beta$-casein was injected into the bulk phase]; (◇) $^{14}$C-labeled soy 7S adsorbed to the air−water interface from a bulk phase containing 0.5 mg/mL soy 7S [after 360 min of adsorption (shown by the arrow), 6.0 mg/mL unlabeled $\beta$-casein was injected into the bulk phase]. See the text for more details.

mL final concentration) after about 900 min of adsorption of soy 7S, that is, after soy 7S had formed a close-to-saturated monolayer film, there was no reduction in surface cpm, indicating that $\beta$-casein could not displace soy 7S from the interface. To elucidate whether the inability of $\beta$-casein (which is more surface active than soy 7S) to displace soy 7S is due to the film state of soy 7S at the interface, displacement experiments were carried out under adsorption conditions that allowed only a dilute unsaturated monolayer formation of soy 7S at the interface. In this case, $^{14}$C-labeled soy 7S was first allowed to adsorb to the air−water interface from a bulk solution containing 0.5 $\mu$g/mL of soy 7S. Under these conditions, the surface concentration of soy 7S approached an equilibrium value at ∼360 min of adsorption (Figure 1). This equilibrium value, which was ∼1 mg m$^{-2}$, was much lower than the saturated monolayer coverage, which was ∼2.8 mg m$^{-2}$. When $\beta$-casein was injected into the bulk phase (6.0 $\mu$g/mL final concentration) after about 360 min of adsorption, initially there was a marked reduction in surface concentration of soy 7S, followed by a slow increase and finally an equilibrium value (Figure 1). The data in Figure 1 suggest that so long as the surface concentration of soy 7S did not reach a saturated monolayer value and form a cohesive film at the interface, $\beta$-casein was able to displace it from the interface. It is often assumed that irreversibility of protein adsorption at an interface is related to conformational changes in proteins at the interface, which, owing to an increase in the number of contacts between the protein and the interface, increases the activation energy to detach it from the interface. However, this alone does not seem to explain the ability of $\beta$-casein to displace soy 7S at low surface coverage. It should be noted that 360 min is enough time for most of the protein molecules to undergo a significant extent of conformational change at the air−water interface. Thus, the data indicate that *reversibility* or *irreversibility* of protein adsorption is not solely dependent on the conformational state of the protein at the interface but might also be dependent on concentration-dependent intermolecular interactions at the interface. The inability of $\beta$-casein to displace soy 7S at saturated monolayer coverage might be due to surface aggregation and formation of a film via intermolecular cohesive interactions. In the saturated monolayer, the intermolecular cohesive interactions prevent displacement of soy 7S from the interface, whereas at low surface coverage the absence of such interactions makes it possible for $\beta$-casein to displace it from the interface.

**Table 1. Adsorption Isotherm Parameters of Various Proteins at the Air−Water Interface**

| protein | $K_L$[a] (cm) | $G_{sat}$ (mg m$^{-2}$) |
|---|---|---|
| soy 11S | 0.54 | 1.25 |
| soy 7S | 0.22 | 2.8 |
| soy AS11S[b] | 0.47 | 2.15 |
| BSA | 0.60 | 1.0 |
| α-casein (α-CN) | 1.07 | 1.69 |
| $\beta$-casein ($\beta$-CN) | 1.25 | 1.84 |
| egg lysozyme (EL) | 0.41 | 0.87 |
| α-lactalbumin (α-La) | 0.46 | 0.77 |
| $\beta$-lactoglobulin ($\beta$-Lg) | 1.08 | 1.10 |
| ovalbumin (OA) | 0.42 | 0.95 |

[a] Binding affinity determined using eq 6. [b] Acidic subunits of soy 11S globulin.

It should be noted that after $\beta$-casein is injected at 900 min, the increase of surface concentration of soy 7S continues with time. This suggests that $\beta$-casein apparently does not incorporate itself into the soy 7S film but allows adsorption of soy 7S to reach its saturated monolayer coverage.

The data in Figure 1 clearly indicate that during coadsorption of two proteins from the bulk phase to the air−water interface, the proteins may displace each other during the initial stages of adsorption and up until aggregation and film formation occurs. Thus, protein adsorption is essentially a reversible process, and therefore the formulation developed earlier for studying the thermodynamic incompatibility of mixing of proteins at the interface using the Langmuir model must be valid.

Table 1 shows the binding affinity ($K$), determined from adsorption isotherms, of various proteins to the air−water interface in single-protein systems. For all of the proteins listed in Table 1, the adsorption isotherm data up to 80−90% of monolayer coverage obeyed the Langmuir model (eq 6). Figure 2 shows fitting of the isotherm data of bovine serum albumin (BSA) and AS11S to the Langmuir model as examples. It should be pointed out that the Langmuir model is valid only for a reversibly adsorbing system below the monolayer coverage. The linear fit of the adsorption isotherm data to the Langmuir equation further confirms our argument that, up until aggregation and film formation occurs, protein adsorption at the air−water interface is essentially a reversible process.

Previously (*14*), a modified form of the Langmuir equation, which assumed cooperativity in binding of proteins to an interface (*15*), had been used to determine the binding affinity of proteins to the air−water interface. However, such a complicated method is unnecessary because only the ratio of binding affinities, not their absolute values, is required to elucidate incompatibility between proteins at the interface. In fact, the relative

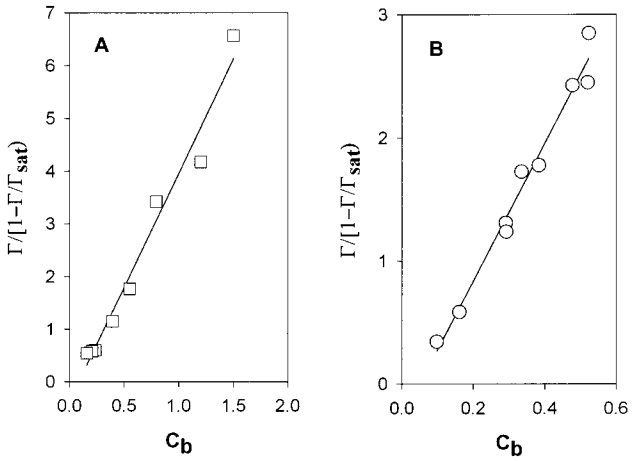


**Figure 2.** Adsorption isotherm data of BSA (A) and AS11S (B) plotted according to the Langmuir equation (eq 6).

**Table 2. Thermodynamic Incompatibility Parameters for Various Protein 1/Protein 2/Water Ternary Systems at the Air−Water Interface**

| protein 1/ protein 2 | $\ln(K_1/K_2)$ | $\ln(K'_1/K'_2)$ | $\lvert\Delta \ln K\rvert$ | $X_{12}$ | $\lvert\chi_{1s} - \chi_{2s}\rvert$ |
|---|---|---|---|---|---|
| α-La/β-CN | −1.00 | −0.78 | 0.22 | 0.06 | 0.007 |
| α-CN/α-La | 0.84 | 1.15 | 0.26 | 0.13 | 0.037 |
| BSA/β-CN | −0.73 | −1.56 | 0.83 | 0.20 | 0.103 |
| EL/β-CN | −1.11 | −2.02 | 0.91 | 0.30 | 0.219 |
| soy 11S/β-CN | −0.84 | −1.90 | 1.06 | 0.41 | 0.315 |
| α-CN/β-CN | −0.16 | −0.58 | 0.42 | 0.14 | 0.044 |
| EL/BSA | −0.38 | −2.37 | 1.99 | 0.68 | 0.278 |
| β-Lg/α-La | 0.85 | 0.56 | 0.29 | 0.05 | 0.205 |
| β-Lg/β-CN | −0.15 | 0.58 | 0.73 | 0.26 | 0.198 |
| AS11S/β-CN | −0.98 | 0.49 | 1.47 | 0.39 | 0.322 |
| AS11S/BSA | −0.24 | 0.89 | 1.13 | 0.35 | 0.381 |
| OA/BSA | −0.36 | −0.81 | 0.45 | 0.18 | 0.073 |
| α-La/BSA | −0.26 | 0.24 | 0.50 | 0.13 | 0.066 |
| β-Lg/BSA | 0.59 | 0.75 | 0.16 | 0.10 | 0.139 |
| α-CN/BSA | 0.58 | 0.98 | 0.40 | 0.20 | 0.103 |
| OA/β-CN | −1.09 | −1.33 | 0.24 | 0.11 | 0.073 |
| AS11S/β-Lg | −0.83 | 0.42 | 1.25 | 0.36 | 0.520 |
| soy 7S/BSA | −1.00 | 0.064 | 1.06 | 0.243 | 0.154 |
| soy 7S/β-CN | −1.74 | −0.507 | 1.23 | 0.217 | 0.095 |

values of binding affinities of proteins remain unchanged irrespective of the method used. For all proteins listed in Table 1, the adsorption isotherms, that is, plots of $\Gamma_{eq}$ versus $C_b$, showed a well-defined plateau in the bulk concentration range 1.5−5.0 $\mu$g mL$^{-1}$. The saturated monolayer coverage values, $\Gamma_{sat}$, for various proteins also are given in Table 1.

To determine the thermodynamic incompatibility between proteins in a mixed protein film at the air−water interface, the protein composition of the mixed protein film at equilibrium was determined at various bulk concentration ratios. The protein 1/protein 2/water ternary systems investigated are listed in Table 2. Figure 3 shows experimental $\Gamma_i/\Gamma_{tot}$ versus $C_i/C_{tot}$ curves for some of the ternary systems listed in Table 2. It should be pointed out that the corresponding plots for the second protein component of the system would appear as inverse of the curves shown. The theoretical curve, predicted by eq 3, based on the $K$ values obtained from single-component systems (Table 1), for each of the ternary systems is also shown in Figure 3 (dotted lines). It should be noted that, except for the β-lactoglobulin/BSA and ovalbumin/β-casein systems, the experimental $\Gamma_i/\Gamma_{tot}$ versus $C_i/C_{tot}$ curves deviated significantly from that of the predicted curves. Only the β-lactoglobulin/BSA and ovalbumin/β-casein systems exhibited close to the ideal competitive adsorption behavior at the air−water interface. The behavior of the ovalbumin/β-casein system is particularly interesting, because, in terms of their physicochemical characteristics, these two proteins are very dissimilar. Whereas ovalbumin is a hydrophilic albumin-type protein, β-casein is a hydrophobic disordered-type protein. Polyakov et al. (*11*) reported that a concentrated solution of a mixture of ovalbumin and β-casein separated into two phases with a phase separation threshold of 19.6. Thus, the phase behavior of a ovalbumin/β-casein/water ternary system at the air−water interface is at variance with its phase behavior in bulk solution. It is likely that this might be related to partial or extensive denaturation of ovalbumin at the air−water interface. Exposure of the interior hydrophobic groups of the protein may render it more hydrophobic in the denatured state at the air−water interface, and this might enable it to become more compatible with the hydrophobic β-casein.

Polyakov et al. (*11*) reported that BSA is highly compatible with ovalbumin in solution. This is presumably because both proteins are albumin-type proteins. Although no solution studies have been reported for compatibility between BSA and α-lactalbumin, it is likely that these two albumin-type proteins also are compatible in solution. The data in Figure 3, however, show that BSA is not compatible with either α-lactalbumin or ovalbumin at the air−water interface. On the other hand, BSA seems to be more compatible with β-lactoglobulin, which is a globulin-type protein. These contradictions clearly suggest that proteins that are known to be very compatible in solution need not be compatible at the air−water interface and vice versa.

As discussed earlier, the deviation of the experimental $\Gamma_i/\Gamma_{tot}$ versus $C_i/C_{tot}$ curves from the ideal behavior predicted by the Langmuir equation (Figure 3) is due primarily to incompatibility of mixing of the proteins in the mixed protein film. This assertion is confirmed by the fact that the total protein concentration at the interface in a mixed film is invariably less than that predicted by the Langmuir equation. For instance, at 2.0 $\mu$g/mL each of β-casein and soy 7S in the bulk phase, the Langmuir eqs 1 and 2 predict that the surface concentration of β-casein and soy 7S should be 0.92 and 0.815 mg m$^{-2}$, respectively. However, experimentally these concentrations were only 0.732 and 0.386 mg m$^{-2}$, respectively. Thus, the total concentration of protein in the mixed protein film was far less than that predicted by the Langmuir equation. This can occur only when there is incompatibility of mixing of the proteins in the film.

The extent of deviation of the experimental $\Gamma_i/\Gamma_{tot}$ versus $C_i/C_{tot}$ curves from the ideal behavior predicted by the Langmuir equation can be regarded as a measure of the degree of incompatibility between the two proteins at the interface. If two proteins are totally incompatible with each other, then they cannot coexist at the interface. That is, even at a minute concentration of one protein at the interface, the other protein cannot adsorb at all. For this situation, the areas highlighted in Figure 3 (as examples) can be regarded as total incompatibility. Then, the ratio of the area between the experimental and predicted curves to the area representing total incompatibility can be defined as the degree of incompatibility, $X_{12}$, between the proteins. The $X_{12}$ values for various ternary systems are shown in Table 2. The data show that the degree of incompatibility of β-casein,

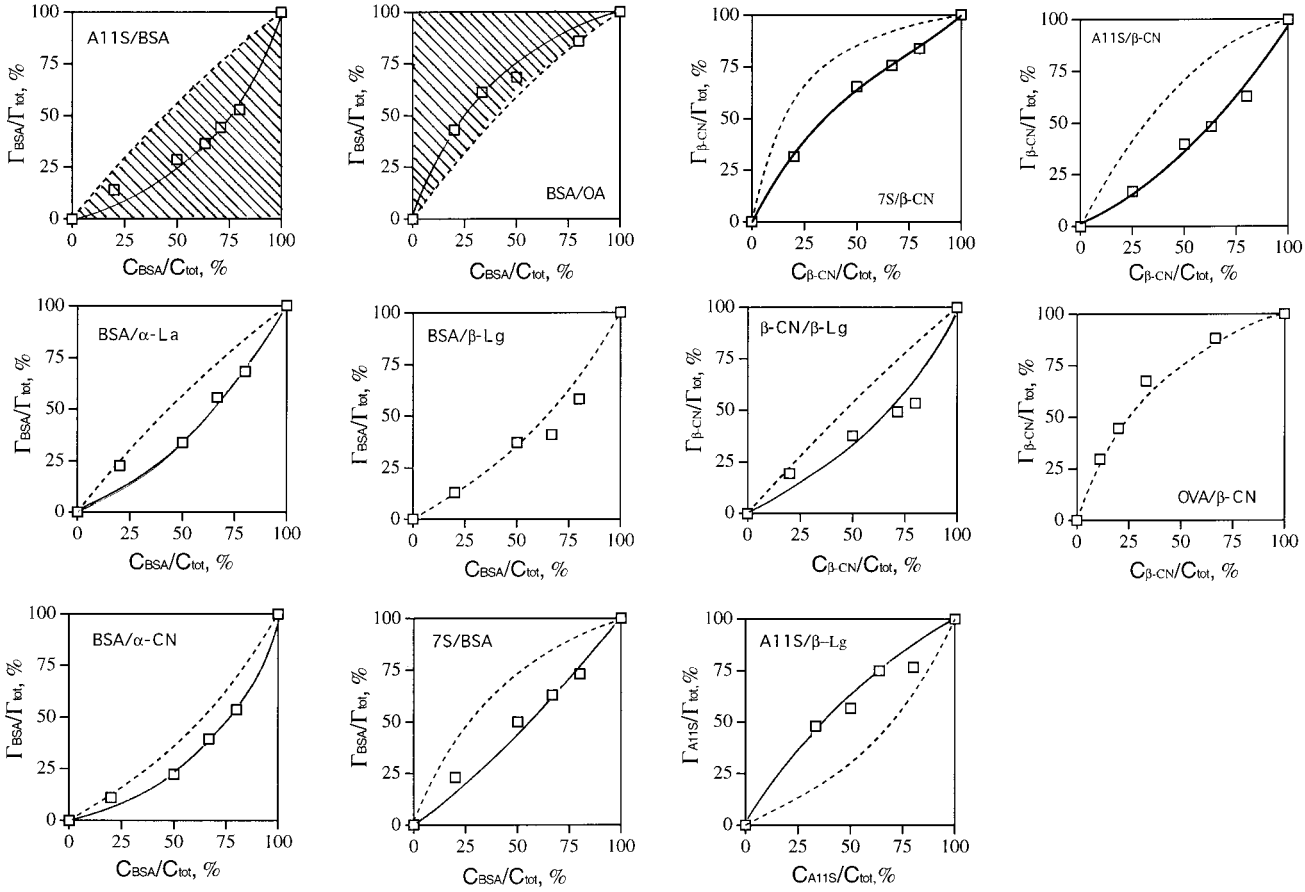


**Figure 3.** Plots of $\Gamma_1/\Gamma_{tot}$ versus $C_1/C_{tot}$ for various protein 1/protein 2/water ternary systems. The dotted lines represent the ideal curves predicted by eq 3.

which is a hydrophobic disordered-type protein, with other proteins at the air−water interface increases in the order α-lactalbumin < ovalbumin < α-casein < BSA < soy 7S < *β*-lactoglobulin < egg lysozyme < soy A11S < soy11S. On the other hand, the degree of incompatibility of BSA, which is a hydrophilic and albumin-type protein, with other proteins at the air−water interface increases in the order *β*-lactoglobulin < α-lactalbumin < ovalbumin < α-casein = *β*-casein < soy 7S < soy A11S < egg lysozyme. It should be noted that even though α-lactalbumin and *β*-casein are dissimilar proteins, this pair has the lowest $X_{12}$ value, suggesting that they are highly compatible at the air−water interface.

The $X_{12}$ values determined by the graphical method are empirical in nature. They provide relative differences in incompatibility of protein pairs. As discussed earlier, fundamentally, the deviation of the experimental $\Gamma_i/\Gamma_{tot}$ versus $C_i/C_{tot}$ curve from the predicted one is due to a change in the binding affinity of a protein to the interface in the presence of the other protein at the interface as a result of incompatibility. The relative changes in the binding affinities of proteins in a ternary system as compared to that in a protein/solvent binary system can be determined from eq 4 by plotting $\ln(\Gamma_1/\Gamma_2)$ versus $\ln(C_1/C_2)$. The values of $\ln(K'_1/K'_2)$ obtained from the intercept of such plots for the ternary systems at the air−water interface are given in Table 2. The values of $\ln(K_1/K_2)$, determined from protein/solvent binary systems at the air−water interface, and the absolute difference between $\ln(K_1/K_2)$ and $\ln(K'_1/K'_2)$, that is, $|\Delta \ln K|$, are also shown in Table 2. Because $|\Delta \ln K|$ represents a net change in the ratio of the binding constants between the ternary and binary systems at the interface, it represents the intensity of incompatible interactions between proteins at the interface. A linear relationship between $X_{12}$ and $|\Delta \ln K|$ (Figure 4) clearly demonstrates that these two parameters are reflections of each other.

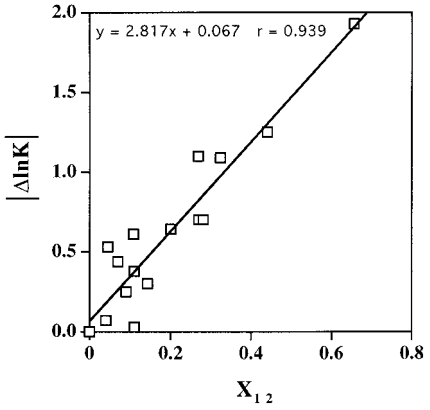


**Figure 4.** Relationship between the interaction parameter $|\Delta \ln K|$ and the empirical incompatibility parameter $X_{12}$.

Compatibility between two proteins in a ternary solution system is dictated by a delicate interplay of the protein 1/protein 2 interaction parameter ($\chi_{12}$) and the protein 1/solvent and protein 2/solvent interaction parameters ($\chi_{1s}$ and $\chi_{2s}$). From the Flory−Huggins theory, the critical interaction parameter, $\chi_c$, for a binary polymer−solvent system is 0.5, and for a binary mixture of polymers it is 0. The significance of the critical interaction parameter is that two components will be miscible if their interaction parameter is below $\chi_c$ but

will separate into two phases if its value is $>\chi_c$. Although, intuitively, two proteins in a protein 1/protein 2/water ternary system should be totally compatible when the interaction parameter $\chi_{12} = 0$, in reality they may still exhibit incompatibility in solution due to a difference in the protein−solvent interaction parameters, $|\chi_{1s} - \chi_{2s}|$, for as little as 0.03 (*22*). The magnitudes of the polymer−solvent interaction parameters, that is, $\chi_{1s}$ and $\chi_{2s}$, are not important. It is the difference, that is, $|\chi_{1s} - \chi_{2s}|$, that impacts polymer−polymer incompatibility in solution (*22*). The larger the difference in hydrophilicity (i.e., $\chi_{1s} - \chi_{2s}$) of two proteins, the greater would be the incompatibility and the lower would be the threshold for phase separation in a ternary system. On the other hand, if the solvent is equally good for each polymer (i.e., $\chi_{1s} \approx \chi_{2s}$), two polymers may yield a totally miscible solution despite a small positive value of $\chi_{12}$ (*23*). It should be pointed out that because the thermodynamic state of water at the interface is different from that in bulk solution, the $|\chi_{1s} - \chi_{2s}|$ value for a ternary film at the air−water interface may not be the same as that calculated for polymer solutions. Specifically, because the density of water in the interfacial region at the air−water interface is lower than its bulk density (*24*), the $|\chi_{1s} - \chi_{2s}|$ value for the ternary film will be different from that in bulk solution. Furthermore, because the conformational state of a protein at the air−water interface is invariably different from that in solution, it would also impact the absolute value of $|\chi_{1s} - \chi_{2s}|$ at the air−water interface. Thus, both the conformational state of proteins and the state of water at the interface would influence the thermodynamic compatibility between proteins at the interface.

If $X_{12}$ and $|\Delta \ln K|$ indeed are related to the thermodynamic incompatibility of mixing of proteins at the interface, then it is logical to expect a relationship between $|\chi_{1s} - \chi_{2s}|$ and these parameters. The protein−solvent interaction parameters $\chi_{1s}$ and $\chi_{2s}$ can be calculated from the Hildebrand solubility parameter ($\delta$) of proteins (*25*). The solubility parameter of a protein can be calculated from contributions of dispersion, polar, and hydrogen bonding interactions of each chemical group in the protein to its cohesive energy ($E_{coh}$) using the method of van Krevelan (*26*). The protein−solvent interaction parameter, $\chi_{ps}$, is given by

$$\chi_{ps} = (V_s/RT)(\Delta\delta_{ps})^2 \quad (6)$$

where $V_s$ is the molar volume of the solvent, $R$ is the gas constant, $T$ is the temperature, and $\Delta\delta_{ps}{}^2$ is

$$\Delta\delta_{ps}{}^2 = (\delta_p - \delta_s)^2 = (\delta_p - \delta_s)^2_{di} + (\delta_p - \delta_s)^2_{pi} + (\delta_p - \delta_s)^2_{h} \quad (7)$$

The subscripts di, pi, and h refer to the dispersion, polar, and hydrogen bonding contributions, respectively, to the solubility parameters of protein ($\delta_p$) and solvent ($\delta_s$). The $|\chi_{1s} - \chi_{2s}|$ values (where subscripts 1 and 2 refer to proteins 1 and 2, respectively) calculated in this manner for various protein pairs are given in Table 2. Figure 5 shows the relationship between $|\chi_{1s} - \chi_{2s}|$ and the parameter $X_{12}$ . These two parameters show a linear correlation with a correlation coefficient of 0.91. It should be pointed out that the data in Figure 5 do not include the data points for AS11S/$\beta$-lactoglobulin, lysozyme/BSA, and $\alpha$-lactalbumin/$\beta$-lactoglobulin systems. When these data are included, the correlation coefficient decreases to 0.746. The departure of the above three binary mixtures from the behavior of the other systems might be related to some specific interactions between the proteins involved. For instance, a mixed film of $\alpha$-lactalbumin/$\beta$-lactoglobulin at the oil−water interface is known to undergo sulfhydryl−disulfide interchange reaction, resulting in intermolecular cross-linking of the proteins (*27*). Similar cross-linking reactions also might occur in AS11S/$\beta$-lactoglobulin and lysozyme/BSA systems, which also contain free sulfhydryl and disulfide groups. Such a chemical reaction between the protein components of the mixed film might distort their incompatibility behavior, which is a physical phenomenon. Nevertheless, the data in Figure 5 strongly suggest that the deviation of competitive adsorption of proteins at the air−water interface from that of the Langmuir model is in fact due to thermodynamic incompatibility of mixing of the proteins in the adsorbed film.

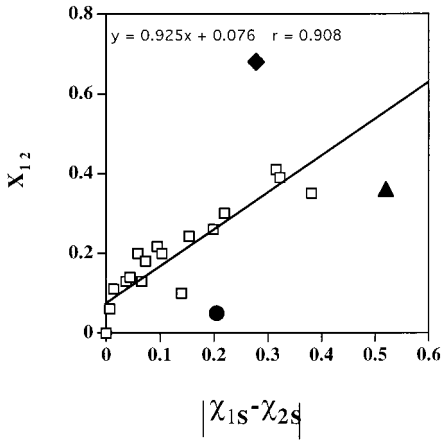


**Figure 5.** Relationship between the empirical incompatibility parameter $X_{12}$ and the net difference in protein−solvent interaction parameters, $|\chi_{1s} - \chi_2|$, for 16 protein binary pairs: (●) $\alpha$-La/$\beta$-Lg system; (◆) Lys/BSA system; (▲) AS11S/$\beta$-Lg system. The data points in bold symbols are not included in the regression.

The Flory−Huggins theory predicts that polymer systems that exhibit thermodynamic incompatibility of mixing would tend to separate into two phases under appropriate conditions. From a technological standpoint, incompatibility of mixing of proteins in a mixed protein film and consequent two-dimensional phase separation of the proteins in the film would have undesirable consequences on the stability of protein-stabilized dispersed systems such as foam and emulsion. The high energy *interface* between the phase-separated regions of the protein film may act as zones of instability in emulsions and foams prepared with typical food proteins, which are mixture of various proteins. Microscopic evidence for two-dimensional phase separation in several binary protein films at the air−water interface is presented in the next paper.

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