Rheokinetic Analysis of Protein Films at the Air−Aqueous Phase Interface. 1. Bovine Serum Albumin Adsorption on Ethanol Aqueous Solutions

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The dilational rheological properties and surface tension of bovine serum albumin (BSA) adsorbed at the air−aqueous phase interface were measured as a function of time, protein concentration (1×10^{-1}−3×10^{-3}% w/w), and subphase composition (aqueous ethanol solutions from 0 to 2 M). The temperature was maintained at 20 °C. Adsorbed BSA films on water and aqueous ethanol solutions exhibited rheological properties that were mainly elastic and not very frequency dependent. The time dependence of surface tension and surface rheological properties was related with the rate of protein adsorption and the influence of ethanol on competitive adsorption. This phenomenon as well as protein−ethanol interactions could be supported by a significant reduction of the surface dilational modulus as either ethanol concentration increased (at constant BSA content) or BSA concentration decreased (at constant ethanol content). Circular dichroism measurements showed no significant change in the secondary structure of BSA in the presence of ethanol, at the concentrations used in these experiments.

Keywords: *Superficial dilational rheology; adsorption; surface properties; bovine serum albumin; air−water interface*

INTRODUCTION

The adsorption of proteins at liquid interfaces and their behavior in the adsorbed state play an important role in many technological processes. In formulated foods, especially in foam- and emulsion-based products, proteins are often used as functional ingredients to perform the role of a surface active agent (Halling, 1981; Dickinson, 1989; Damodaran, 1990). The important initial step involved in the formation and stabilization of protein-based foams and emulsions is the adsorption and spreading of the protein at the interface. It would appear that the rate of formation of an emulsion stabilized by proteins is limited by the rate of adsorption of protein at the interface (Dickinson, 1992), whereas information exists which relates the stability of a foam with the rate of protein adsorption (Kato and Nakai, 1980). Moreover, in a great variety of model food systems, the highly viscoelastic characteristics of adsorbed protein films correlates with increased stability (Chen and Dickinson, 1995a−c; Clark et al., 1990a,b; Kim and Kinsella, 1985; Djabbarah and Wasan, 1985). For further information concerning the interfacial rheological characteristics of adsorbed films of proteins and emulsifiers in the food context, the reader is referred to the reviews of Lucassen-Reynders (1993) and Murray and Dickinson (1996).

Protein adsorption can be considered to occur in three main stages (MacRitchie, 1978; Graham and Phillips, 1979a; Tornberg, 1978): (i) diffusion of the native protein molecules to the interface, (ii) adsorption in the globular form by penetration into the interface, and (iii) structural rearrangements or surface denaturation, involving spreading or unfolding of adsorbed molecules. In connection with the study of the foaming and emulsifying properties of proteins, their interfacial behavior, as reflected in surface/interfacial tension (σ) decay, is an important property. It is reasonable to assume that any process responsible for the time dependence of the reduction in σ by a protein molecule must involve an increase in the number of adsorbed segments per unit area with time (Damodaran, 1990; Xu and Damodaran, 1992, 1994). In the case of protein molecules adsorbing at interfaces, Graham and Phillips (1979a) have shown that the primary layer of molecules is largely responsible for the value of σ.

Proteins, in addition to lowering the interfacial tension, can form a continuous film at the interface via complex intermolecular interactions and thus impart structural rigidity to the interface (Bos et al., 1996; Castle et al., 1987; Sarker et al., 1995). The orientation of the adsorbed molecules, molecular interactions and packing, formation of complex, or structural transformations at the interface can result in a peculiar rheological behavior, which can depend on shear rate or age (Malhotra and Wasan, 1988). The intermolecular interactions at the interface involve hydrogen bonding and electrostatic and hydrophobic interactions. The extent of these interactions at the interface is dependent on the conformation of the adsorbed protein molecules. An optimum balance of these interactions leading to the formation of a cohesive, viscous film is required to stabilize the emulsions or foams. In addition to the physicochemical properties of the protein, several factors, such as protein concentration and the presence of other food components (ethanol, sugars, lipids, etc.), can

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affect the properties of adsorbed protein films and hence their foaming and emulsifying properties.

The aim of this series is to systematically study the transient dilational rheology of a protein (BSA) adsorbed from aqueous solutions of ethanol (Part 1) and sucrose (Part 2). We have determined surface viscoelastic parameters as a function of adsorption time and at a characteristic time once the surface had reach a quasi-equilibrium. In previous work we have observed that both the rate of BSA adsorption at the interface and the surface tension at equilibrium (Rodríguez Patino and Rodríguez Niño, 1995) depend on the aqueous phase composition. A lag period was observed with the presence of ethanol in the subphase; this could reflect the existence of BSA−solute interactions in the aqueous phase and at the interface. However, the rate of BSA adsorption increased when sucrose was present in the bulk phase. The influence of ethanol in the subphase on surface properties of BSA has been also studied recently (Dussaud et al., 1994; Dussaud and Vignes-Adler, 1994a,b).

The role of interfacial rheology in real food products is very complicated (Murray and Dickinson, 1996); therefore, in these papers we will focus on the interfacial rheology of model systems. Investigating the effect of ethanol and/or sucrose on the adsorption behavior and interfacial rheology of protein films may further our knowledge of the behavior of food colloids (emulsions or foams). Moreover, by incorporating typical food solutes in the aqueous phase, we hope to advance our knowledge from the behavior of simple well-defined model to complex, real food formulations—such as ice cream, imitation dairy products, mayonnaise, desserts, bakery products, whipped cream, soft drinks, and cream liqueurs, to name only a few (Als and Krog, 1991; Krog et al., 1985).

EXPERIMENTAL PROCEDURES

Materials. BSA (Fluka, >96% pure), and analytical grade ethanol (Merck, >99.8%), potassium dihydrogen phosphate (Merck, 99.5%), and dipotassium hydrogen phosphate (Merck, 99%) were used without further purification. All samples were prepared using double-distilled surface chemically pure water.

Method. The surface rheological parameters—such as surface dilational modulus and elastic and viscous components—and the surface tension were measured according to the method of Kokelaar et al. (1991) as a function of time and radial frequency. The method involves a periodic, sinusoidal interfacial expansion and compression performed in a special Langmuir trough using a circular barrier which is oscillated vertically through the interface. The surface tension is simultaneously measured by a Wilhelmy glass plate in permanent contact with the liquid surface. The surface dilational modulus, E, derived from the change in surface tension, resulting from a small change in surface area, A, may be described by the equation (Lucassen and van den Temple, 1972)

$$E = \frac{\mathrm{d}\sigma}{\mathrm{d}A/A} = -\frac{\mathrm{d}\pi}{\mathrm{d}\ln A}$$

where $\pi = \sigma_o - \sigma$ is the surface pressure and σ_o is the subphase surface tension.

The dilational modulus is a complex quantity and is composed of real and imaginary parts, $E = |E|(\cos\theta + i\sin\theta)$. The real part of the dilational modulus or storage component is the dilational elasticity, $E_d = |E|\cos\theta$. The imaginary part of the dilational modulus or loss component is the surface dilational viscosity, $\eta_d\omega = |E|\sin\theta$, where θ is the loss angle of the modulus and ω is the frequency of the oscillation. The loss angle tangent can be defined as $\tan\theta = \eta_d\omega/E_d$. If the film is purely elastic, the loss angle tangent is zero.

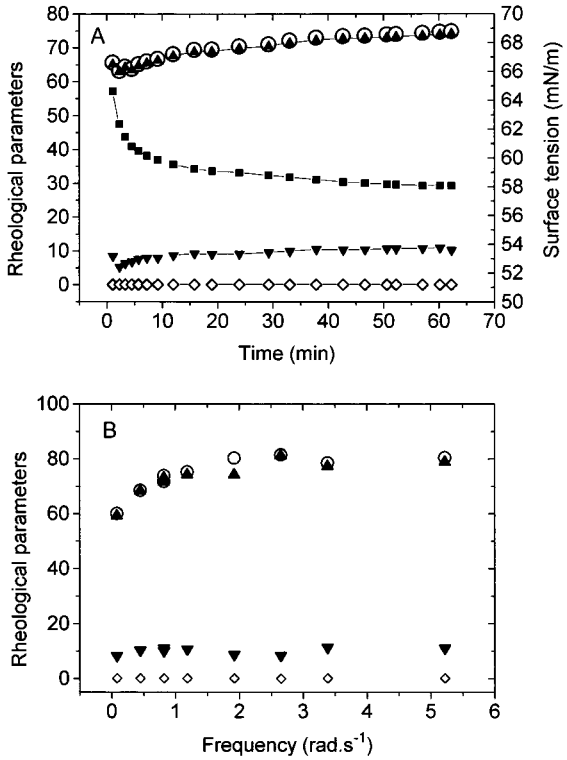

Figure 1. (A) Time dependence of surface tension (σ, ■, mN/m), surface dilational modulus (E, ○, mN/m), surface dilational elasticity (E_d, ▲, mN/m), surface dilational viscosity ($\eta_d\omega$, ▼, mN/m), and loss angle tangent (tan θ, ◇) for BSA films adsorbed on water, angular frequency = 0.81 rad s^{-1}. (B) Angular frequency dependence of rheological parameters. T = 20 °C. BSA concentration = 0.1% w/w.

The measurements of surface tension and surface dilational properties as a function of time were carried out at 20 °C. All of the aqueous subphases were prepared in 50 mM phosphate buffer and adjusted to pH 7.0. To study the rate of protein adsorption, recording began at the moment the BSA solution (200 mL, pre-equilibrated at 20 °C) was placed in the trough. In experiments with ethanol in the subphase (ethanol concentrations of 0.1, 0.5, 1.0, and 2.0 M), protein solutions were prepared at 20 °C by stirring for 30 min and then placed in the trough. Surface measurements are very sensitive to the presence of impurities, so extreme care was taken to ensure that all materials and equipment used in this study were clean. The absence of surface active contaminants in the aqueous subphase was checked. Measurements were performed at least twice. The reproducibility of the results was better than 6%.

The secondary structure of BSA was determined using circular dichroism spectroscopy. The near-UV (340−250 nm) and far-UV (260−180 nm) spectra of 1.0 mg/mL BSA in 0.1 mm path length quartz cells were obtained using a Jasco J-710 spectropolarimeter (Jasco Corp., Tokyo). The far-UV spectra were analyzed using the selcon method (Sreerama and Woody, 1993), fitting to three structural parameters—α-helix, β-sheet, and aperiodic.

RESULTS

The experimental transient surface dynamic properties—such as surface tension and surface dilational properties (surface dilational modulus, surface dilational elasticity, surface dilational viscosity, and loss angle tangent)—for BSA solutions at several ethanol concentrations (0, 0.1, 0.5, 1.0, and 2.0 M) are plotted in Figures 1−5. In the absence of ethanol, the time-dependent surface dynamic properties are shown in Figure 1. The data for BSA adsorbed from aqueous ethanol solutions at 0.1, 0.5, 1.0, and 2.0 M are shown in Figures 2−5, respectively.

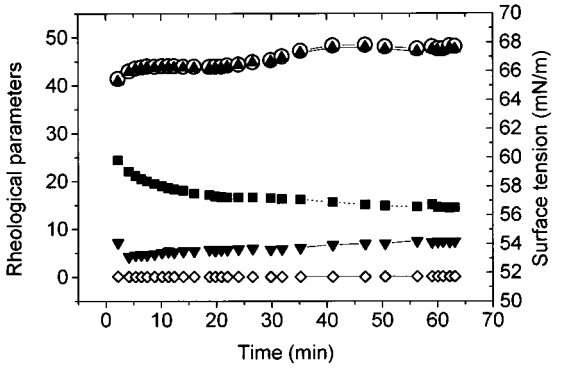

Figure 2. Time dependence of surface tension (σ, ■, mN/m), surface dilational modulus (E, ○, mN/m), surface dilational elasticity (E_d, ▲, mN/m), surface dilational viscosity ($\eta_d\omega$, ▼, mN/m), and loss angle tangent (tan θ, ◇) for BSA films adsorbed on a 0.1 M ethanol aqueous solution, angular frequency = 0.81 rad s^{-1}. T = 20 °C. BSA concentration = 0.1% w/w.

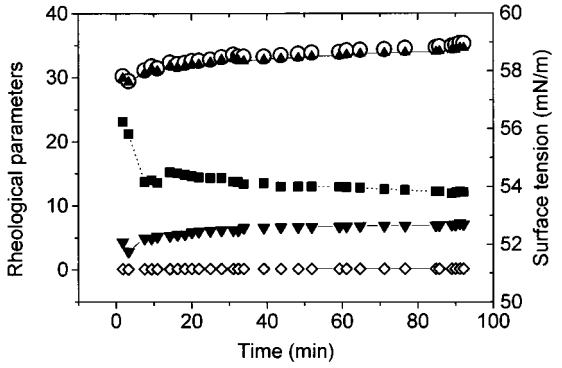

Figure 3. Time dependence of surface tension (σ, ■, mN/m), surface dilational modulus (E, ○, mN/m), surface dilational elasticity (E_d, ▲, mN/m), surface dilational viscosity ($\eta_d\omega$, ▼, mN/m), and loss angle tangent (tan θ, ◇) for BSA films adsorbed on a 0.5 M ethanol aqueous solution, angular frequency = 0.81 rad s^{-1}. T = 20 °C. BSA concentration = 0.1% w/w.

The results show some interesting features from a rheological point of view: (i) the values of surface dilational modulus (E) were similar to that of surface dilational elasticity (E_d); (ii) the values of surface dilational viscosity ($\eta_d\omega$) were low and became practically zero at the highest ethanol concentration; (iii) the loss angle tangent (tan θ) was practically zero, especially as the ethanol content increased (at an ethanol concentration of 2 M the loss angle tangent was <0.1); and (iv) the frequency dependence of E and E_d for BSA on water and aqueous ethanol solutions characterizes a viscoelastic behavior of the surface that is practically elastic over the range of frequencies studied. Figures 1 and 4 show this dependence for BSA on water and on a 1 M ethanol solution (as an example), respectively. E varied from 60.2 and 12.5 mN/m (ω = 0.45 rad s^{-1}) to plateau values of 81.6 and 20 mN/m (ω = 5.2 rad s^{-1}) for BSA on water and a 1 M ethanol solution, respectively. Different ethanol concentrations in the aqueous phase (data not shown) behave in a similar way.

From a kinetic point of view, the time dependence of the surface tension and surface dilational properties depended on the ethanol concentration. At ethanol concentration <0.5 M, the surface tension decreased and surface dilational modulus increased monotonically to a plateau value with time (Figures 1−3). At ethanol concentrations >0.5 M, the time dependence of the surface dynamic properties was more complex (Figures 3−5). It can be seen that a lag period exists during BSA adsorption. The induction period increased with ethanol concentration.

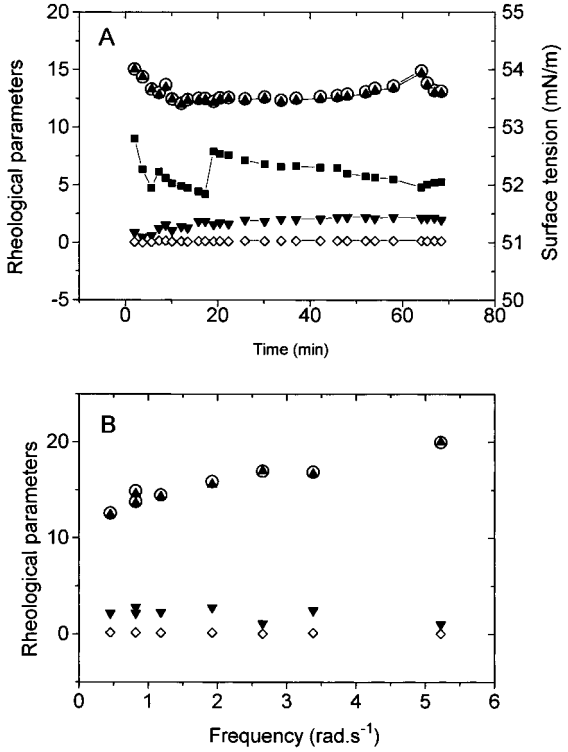

Figure 4. (A) Time dependence of surface tension (σ, ■, mN/m), surface dilational modulus (E, ○, mN/m), surface dilational elasticity (E_d, ▲, mN/m), surface dilational viscosity ($\eta_d\omega$, ▼, mN/m), and loss angle tangent (tan θ, ◇) for BSA films adsorbed on a 1.0 M ethanol aqueous solution, angular frequency = 0.81 rad s^{-1}. (B) Angular frequency dependence of rheological parameters. T = 20 °C. BSA concentration = 0.1% w/w.

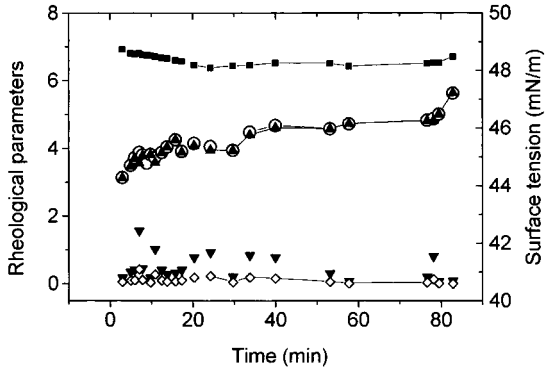

Figure 5. Time dependence of surface tension (σ, ■, mN/m), surface dilational modulus (E, ○, mN/m), surface dilational elasticity (E_d, ▲, mN/m), surface dilational viscosity ($\eta_d\omega$, ▼, mN/m), and loss angle tangent (tan θ, ◇) for BSA films adsorbed on a 2.0 M ethanol aqueous solution, angular frequency = 0.81 rad s^{-1}. T = 20 °C. BSA concentration = 0.1%% w/w.

Transient surface dynamic properties also depend on BSA concentration (Figures 4 and 6). In these experiments different BSA concentrations (1×10^{-1}, 2×10^{-2}, and 3×10^{-3}% w/w) on a solution of 1 M ethanol were studied. The surface rheological characteristics were practically independent of BSA concentration. That is, the film displayed an elastic behavior—it can be seen that E and E_d values are similar and the loss angle tangent is practically zero (Figures 4 and 6). The adsorption rate of BSA—as deduced from the slope of σ−time or E−time plots—increased as the BSA concentration increased. At the lower BSA concentrations a

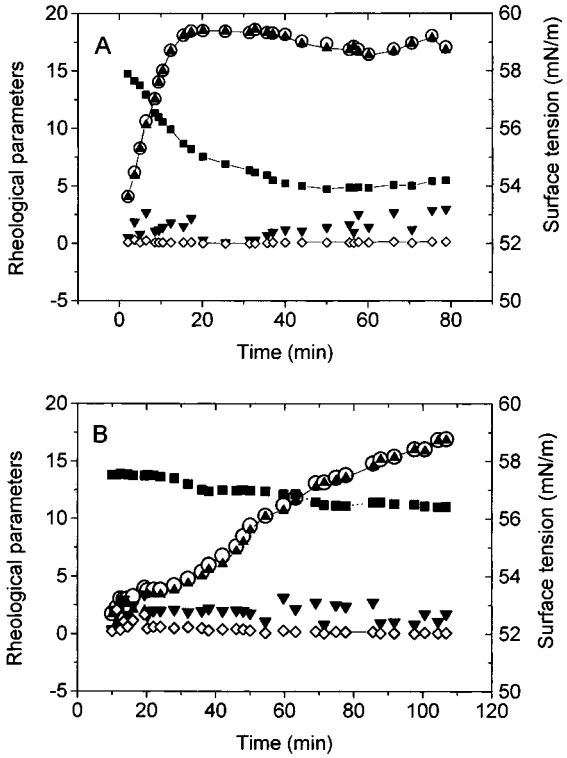

Figure 6. Time dependence of surface tension (σ, ■, mN/m), surface dilational modulus (E, ○, mN/m), surface dilational elasticity (E_d, ▲, mN/m), surface dilational viscosity ($\eta_d\omega$, ▼, mN/m), and loss angle tangent (tan θ, ◇) for BSA films adsorbed on a 1.0 M ethanol aqueous, angular frequency = 0.81 rad s^{-1}. T = 20 °C. BSA concentration in bulk phase: (A) 2×10^{-2}% w/w; (B) 3×10^{-3}% w/w.

Table 1. Secondary Structure Composition of BSA in the Presence and Absence of 2 M Ethanol, As Determined by Far-UV Circular Dichroism

sample	α-helix (%)	β-sheet (%)	aperiodic (%)
BSA	55.7	7.9	36.8
BSA + 2 M ethanol	55.8	7.6	36.0

quasi-equilibrium state was not reached after an adsorption time of about 110 min (Figure 6B).

No secondary structural changes to BSA were observed in the presence of 2 M ethanol, as determined using circular dichroism. Table 1 shows the results of the analysis of the far-UV spectra and shows no significant change in secondary structure. Tertiary structure was also not affected as the near-UV spectra of BSA were identical in the presence and absence of 2 M ethanol.

DISCUSSION

Transient Surface Dynamic Properties. The surface dynamic properties of BSA on water and aqueous ethanol solutions over the concentration range 0−2 M has been measured as a function of time (Figures 1−6). The time dependence of surface tension for alcoholic solutions of BSA is typical, as compared with previous data obtained with the same systems by surface tensiometry (Rodríguez Patino and Rodríguez Niño, 1995; Dussaud et al., 1994a). The main difference occurs with the time required to reach a quasi-equilibrium state. The rate of change of the surface tension is higher in the ring trough than in a tensiometer using either the de Nouy ring (Dussaud et al., 1994a) or the Wilhelmy plate (Rodríguez Patino and Rodríguez Niño, 1995) method. This is due to the existence of convection at the interface and/or the bulk phase, originated by the oscillatory sinusoidal movement of the glass. The same reasoning could be used to explain the smaller lag period during BSA adsorption from alcoholic solutions in the ring trough as compared with previous data in a surface tensiometer (Rodríguez Patino and Rodríguez Niño, 1995). That is, the convection in the aqueous bulk phase and at the interface could improve the BSA diffusion to the interface and the protein−ethanol interaction at the interface and in the aqueous phase.

The decrease in surface tension and an increase in the surface rheological properties with time, especially the surface dilational modulus and surface dilational elasticity, are associated with BSA adsorption at the interface (MacRitchie and Alexander, 1963; Damodaran and Song, 1988; Graham and Phillips, 1979b). The transient surface dynamic properties of BSA films on water and alcoholic solutions can be correlated with the competitive adsorption of BSA and ethanol at the interface.

A detailed analysis of the BSA adsorption on water (Rodríguez Patino and Rodríguez Niño, 1995) has shown that after a rapid diffusion of protein to the interface, according the Ward and Torday equation (Ward and Torday, 1946), the rate of BSA adsorption is controlled by the spreading, unfolding, and rearrangement of adsorbed molecules. The existence of a lag period can be associated with the competitive adsorption of BSA and ethanol at the interface. The effect of ethanol in reducing the surface tension of water (Rodríguez Patino and Martín, 1994) reflects the fact that ethanol molecules adsorb at the interface, but only weakly. The surface excess concentration of ethanol can be calculated by using the classical Gibbs equation (Adamson, 1990). From this calculation (data not shown) it can be concluded that the surface coverage by ethanol at equilibrium is near saturation at ethanol bulk concentrations >5 M. However, proteins can compete with ethanol due to their higher affinity for the interface, as a consequence of their greater hydrophobicity.

Finally, the frequency dependence of the surface dilational modulus is an indication that a reorientation of the molecules at the interface or an exchange of molecules between the interface and the subsurface during the compression−expansion cycle could exist.

Effect of Ethanol Concentration. The effect of ethanol concentration on surface dynamic properties at 30 and 60 min of adsorption time is shown in Figure 7. The surface tension and surface rheological parameters, especially surface dilational modulus and elasticity, both decrease with increased ethanol concentration. These results are of practical importance because the film viscoelasticity decreases to a low value at the highest ethanol concentration (2 M). Similar surface dilational data were obtained by Dussaud and Vignes-Adler (1994a) for BSA and by Brierley et al. (1996) for beer proteins, at the air−ethanol solution interface. These data are in agreement with those reported by Dickinson and Woskett (1988) on shear viscosity of caseinate solutions at the oil−water interface. In these experiments a sudden drop in surface viscosity was observed when 1 wt % of ethanol was added to the aqueous phase.

The phenomena reported here must be associated with the protein−ethanol interactions, either at the interface or in the bulk phase. The interactions of BSA−ethanol at the interface could reduce the amount of adsorbed protein (Dussaud et al., 1994a) or disrupt the protein−protein interactions by forming a mixed BSA−ethanol adsorbed film. Moreover, the denaturing effect of ethanol (Tanford, 1962) could weaken hydrophobic bonds, decrease protein solubility, and promote

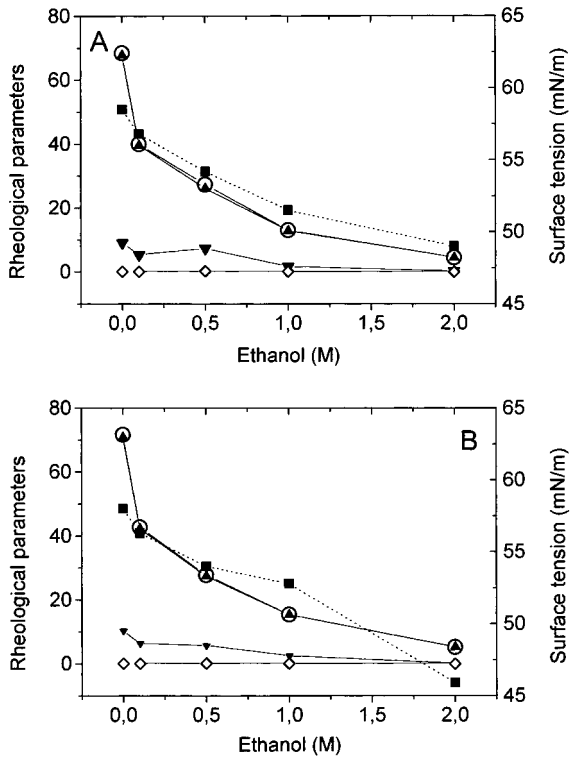

Figure 7. Ethanol concentration dependence of surface tension (σ, ■, mN/m), surface dilational modulus (E, ○, mN/m), surface dilational elasticity (E_d, ▲, mN/m), surface dilational viscosity ($\eta_d\omega$, ▼, mN/m), and loss angle tangent (tan θ, ◇) for BSA films adsorbed on ethanol aqueous solutions, angular frequency = 0.81 rad s^{-1}. T = 20 °C. Adsorption time: (A) 30 min; (B) 60 min. BSA concentration = 0.1% w/w.

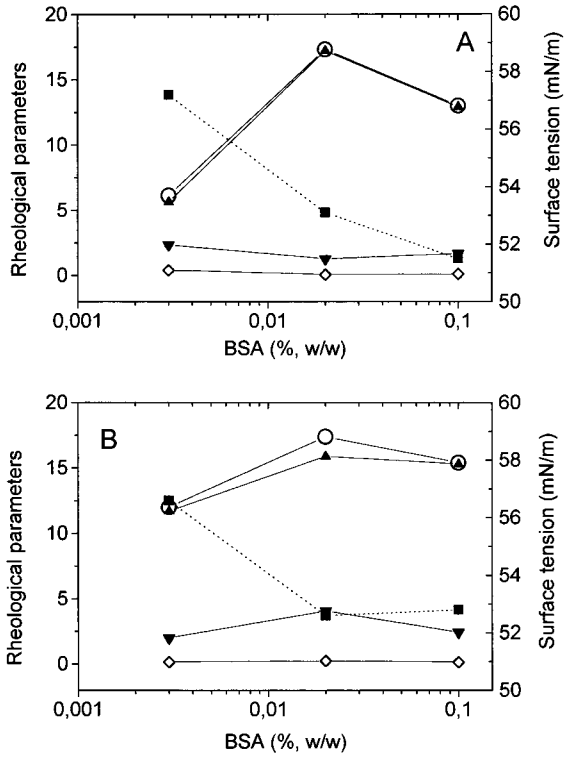

Figure 8. BSA concentration dependence of surface tension (σ, ■, mN/m), surface dilational modulus (E, ○, mN/m), surface dilational elasticity (E_d, ▲, mN/m), surface dilational viscosity ($\eta_d\omega$, ▼, mN/m), and loss angle tangent (tan θ, ◇) for BSA films adsorbed on a 1.0 M ethanol aqueous solution, angular frequency = 0.81 rad s^{-1}. T = 20 °C. Adsorption time: (A) 30 min; (B) 60 min.

aggregation (Aoki et al., 1981). It is also possible that ethanol may destabilize native protein by hydrophobic or electrostatic interactions as can be deduced from circular dichroism data (Clark and Smith, 1989); however, as presented here in Table 1, there appear to be no significant changes in secondary or tertiary structure of BSA in the presence of 2 M ethanol. As a consequence of these interactions between ethanol and BSA, a complex between both components may be formed, resulting in an adsorbed layer with weaker interfacial structure, which agrees with the data presented in Figure 7.

Effect of BSA Concentration. The effect of BSA concentration on surface dynamic properties at 30 and 60 min of adsorption time, in a 1 M aqueous ethanol solution, is shown in Figure 8. It can be seen that the surface dilational modulus and elasticity increase with protein until a plateau value is attained, once the surface is occupied by a monolayer of BSA (Phillips, 1981; Tornberg, 1978). The shape of the σ−BSA concentration plot is similar to that previously obtained by other authors (Damodaran and Song, 1988; Graham and Phillips, 1979a; Phillips, 1981; Suttiprasit et al., 1992). It is apparent that the surface dilational properties increase and σ decreases with increasing protein bulk concentration until a plateau is reached. This plateau begins at the point where σ reaches its minimum value. The similarity between surface tension and superficial density versus concentration plots—obtained by ellipsometry (Graham and Phillips, 1979a) and radioactive marking (Damodaran and Song, 1988; Graham and Phillips, 1979b)—provides supporting evidence for relating the surface dilational properties and surface tension value to the presence of protein at interface (Graham and Phillips, 1979a,b). At BSA concentrations of 10^{-2}–10^{-3}%, steady-state BSA adsorption forms a film that consists of irreversibly adsorbed molecules in a monolayer. As the surface dilational properties increase, the protein is packed more efficiently in the monolayer, leading to higher surface density and a thicker film. At surface densities higher than that of monolayer coverage, protein molecules may form further layers which stack beneath the primary monolayer but do not contribute significantly to σ (Graham and Phillips, 1979a) and surface dilational properties.

The differences observed in parts A and B of Figure 8 as a consequence of the adsorption time could be associated with the effect of ethanol on the rate of BSA adsorption, as previously analyzed. In fact, at the lowest BSA concentration (3×10^{-3}% w/w) the surface dynamic properties are time dependent. It can be seen that rheological parameters are lower at an adsorption time of 30 min than at 60 min. So, it can be concluded that the effect of ethanol on surface dynamic properties for BSA films depends on the BSA/ethanol ratio. At higher ethanol content or at lower BSA content decreases both the rate of BSA adsorption and the magnitude of the surface rheological properties. That is, at lower ethanol content the effect of BSA concentration at the interface is predominant, but at high ethanol concentrations, the ethanol dominates the interfacial behavior and limits the extent of BSA adsorption, independent of the concentration of BSA.

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