Self-Assembly of Monoglycerides in β-Lactoglobulin Adsorbed Films at the Air−Water Interface. Structural, Topographical, and Rheological Consequences

Juan M. Rodríguez Patino,* Marta Cejudo Fernández, Mª. Rosario Rodríguez Niño, and Cecilio Carrera Sánchez

Departamento de Ingeniería Química, Facultad de Química, Universidad de Sevilla, C/Professor García González 1, 41012-Seville, Spain

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In this work, we have analyzed the structural, topographical, and surface dilatational characteristics of pure β-lactoglobulin adsorbed films and the effect of the self-assembly of monoglycerides (monopalmitin or monoolein) in β-lactoglobulin films at the air−water interface. Measurements were performed in a single device that incorporates a Wilhelmy-type film balance, Brewster angle microscopy, and interfacial dilatational rheology. The structural and topographical characteristics of β-lactoglobulin adsorbed and spread films are similar. However, the surface dilatational modulus of β-lactoglobulin films shows a complex behavior depending on film formation. The self-assembly of monoglyceride in a β-lactoglobulin adsorbed film has an effect on the structural, topographical, and dilatational properties of the mixed films, depending on the interfacial composition and the surface pressure (π). At low π, a mixed film of monoglyceride and β-lactoglobulin may exist. At high π (after the collapse of β-lactoglobulin), the mixed films are dominated by monoglyceride molecules. However, the small amounts of collapsed β-lactoglobulin have a significant effect on the surface dilatational properties of the mixed films. Protein displacement by monoglyceride is higher for monopalmitin than for monoolein. However, some degree of interaction exists between proteins and monoglycerides, and these interactions are more evident in adsorbed films than in spread films.

Introduction

Many food formulations are emulsions or foams (food colloids) containing droplets or bubbles, respectively.[1] These are microstructural entities stabilized by the formation of an interfacial emulsifier layer around the particles.[2−4] The properties of this interfacial layer are governed by the composition and structure of the adsorbed material. For this reason, biopolymers (proteins, polypeptides, and polysaccharides) and low-molecular-weight emulsifiers (LMWEs) have been used for many decades as emulsifiers in the production of food colloids.[1,5,6] On the molecular level, these ingredients and their chemical properties, including specific modifications, can be used to add new uses to these emulsifiers.[7] Real food dispersions usually contain a mixture of different emulsifiers (biopolymers and LMWEs); thus the way emulsifiers interact with each other at the interface (structure formation) influences the formation and stability of individual emulsion droplets or foam bubbles and the interaction between groups of droplets or bubbles.[2,8] This means that the macroscopic characteristics of dispersions, such as their rheological or textural properties, can be improved by controlling nanostructure formation at the interface.[9,10] This represents one way in which nanoscience approaches may lead to new possibilities in the fabrication of complex food colloids.[7]

Our knowledge of the structure of emulsifier assemblies on surfaces has undergone some revolutionary changes since 1995, when atomic force microscopy (AFM), which allowed direct imaging of surfactants at fluid interfaces, was first used.[3,9,11,12] Although self-assembly is a widely recognized phenomenon in nature, it has only recently been recognized in the area of fabrication and production.[7] So far, the potential of self-assembly has not been intentionally used in the manufacturing of food formulations. Thus, a better understanding of the supramolecular structuring principles[2,13] will bring new phenomena to light and lead to new manufacturing processes for high-added-value food products and emulsifiers. A prerequisite for this approach is a proper understanding of the fundamental principles of molecular interactions (noncovalent interactions, such as hydrogen bonding, electrostatic and van der Waals interactions, etc.), leading to supramolecular structures, and their influence on the generation and stabilization of higher hierarchical structures (droplets or bubbles) present in most food colloids. The need to control the processes involved in this molecular engineering has led to the dramatic development of advanced equipment[9,14−17] suitable for thin-layer characterization (AFM, Brewster angle microscopy (BAM), imaging ellipsometry, IR−UV reflection−absorption spectrometry, transmission fluorescence spectrometry, two-dimensional X-ray reflectometry, surface plasmon resonance, etc.). Besides these entirely new techniques, traditional instruments (Langmuir and Langmuir−Blodgett troughs) have also been equipped with new facilities and developed to produce constant and dynamic shearing and/or dilatation, in situ observation, molecular deposition, and so forth.[9,18−20]

The aim of this work was to analyze the effect of monoglycerides (monopalmitin and monoolein) on the interfacial behavior of a milk protein (β-lactoglobulin) previously adsorbed at the air−water interface by using complementary techniques (Wilhelmy-type film balance, BAM, and surface dilatational rheology). It involves (i) the effect of monoglyceride on the interfacial structure, morphology, and topography (self-assembly) of an

* To whom all correspondence should be addressed. Tel: +34 95 4556446. Fax: +34 95 4556447. E-mail: jmrodri@us.es.

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adsorbed β-lactoglobulin film, (ii) the compatibility (incompatibility) between monoglyceride and β-lactoglobulin at a fluid interface, (iii) the surface dilatational characteristics of monoglyceride + β-lactoglobulin adsorbed mixed films at fluid interfaces, and, finally, (iv) a discussion of the effect of film formation (by adsorption or spreading) and the type of protein on the formation and properties of emulsifier-mixed films at a fluid interface. Although monolayer technique has been used successfully for studying the properties of mixed emulsifiers spread at the air−water interface,[18] adsorbed films of mixed emulsifiers are more interesting from a technological point of view. However, there exists little information about these systems so far.[21,22] This paper complements previous works on pure proteins[23,24] and protein−monoglyceride mixed films adsorbed and spread at the air−water interface.[21,22]

Experimental Section

Chemicals. Synthetic 1-monohexadecanoyl-*rac*-glycerol (monopalmitin, DIMODAN PA 90) and 1-mono (*cis*-9-octadecanoyl) glycerol (monoolein, RYLO MG 19) were supplied by Danisco Ingredients (Brabran, Denmark) with over 95−98% purity. Whey protein isolate (WPI), a native protein with a very high content of β-lactoglobulin (protein 92 ± 2%, β-lactoglobulin > 95%, α-lactalbumin < 5%) obtained by fractionation, was supplied by Danisco Ingredients. Samples for the interfacial characteristics of WPI adsorbed films were prepared using Milli-Q ultrapure water and were buffered at pH 7. To form the mixed surface film on a previously adsorbed β-lactoglobulin monolayer, monoglyceride was spread in the form of a solution, using hexane/ethanol (9:1, v/v) as a spreading solvent. Analytical grade hexane (Merck, 99%) and ethanol (Merck, >99.8%) were used. The water used as subphase was purified by means of a Millipore filtration device (Milli-Q). A commercial buffer solution called trizma ($(CH_2OH)_3CNH_2$/$(CH_2OH)_3CNH_3Cl$; Sigma, >99.5%) was used to achieve pH 7. The ionic strength of the subphase was maintained constant at 0.05 M in all the experiments.

Surface Film Balance. Measurements of surface pressure (π)−area (*A*) isotherms of adsorbed β-lactoglobulin films and β-lactoglobulin−monoglyceride mixed films at the air−water interface were performed on a fully automated Wilhelmy-type film balance (KSV 3000, Finland) as described previously.[25,26] The maximum area of the trough between the two barriers is 51.5 × 15 cm^2. Before each measurement, the film balance was calibrated at 20 °C. For β-lactoglobulin adsorbed films from water protein solutions at $1\cdot10^{-5}$−$1\cdot10^{-4}$ wt % were left in the trough, and time was allowed for protein adsorption at the interface. These protein concentrations were selected from previous data of the adsorption isotherm.[27] At these protein concentrations in solution, the surface pressure after 24 h at the maximum area of the trough was practically zero. At this point, the monoglyceride (monopalmitin or monoolein) in the hexane/ethanol solution (at $2.5\cdot10^{14}$−$3.5\cdot10^{14}$ molecule·μl^{-1}) was spread by means of a micrometric syringe at different points on the β-lactoglobulin adsorbed film. During the spreading of the monoglyceride, the surface pressure of the previously adsorbed β-lactoglobulin film was zero, at the maximum area of the trough. Further details about operational conditions have been described elsewhere.[21,22] Mixtures of particular mass fractions—expressed as the mass fraction of monopalmitin, X_{MP}, or monoolein, X_{MO}, in the mixture—were studied. The compression rate was 3.3 cm·min^{-1}, which is the highest value for which isotherms were found to be reproducible in preliminary experiments. The π−*A* isotherm was measured four times. The reproducibility of the results was better than ±0.5 mN/m for surface pressure and ±0.05 m^2/mg for area.

Brewster Angle Microscopy (BAM). A commercial Brewster angle microscope, BAM2, manufactured by NFT (Göttingen, Germany), was used to study the topography of the film. The BAM was positioned over the film balance. Further characteristics of the device and operational conditions have been described elsewhere.[28,29] The surface pressure measurements, area, and gray level as a function of time were carried out simultaneously by means of a device connected between the film balance and BAM. To measure the relative reflectivity (I) of the film, a previous camera calibration is necessary.[28,29] The imaging conditions were adjusted to optimize both image quality and quantitative measurement of reflectivity. Thus, generally as the surface pressure or the protein content increased the shutter speed was also increased.

Surface Dilatational Rheology. To obtain surface rheological parameters, such as surface dilatational modulus (*E*), elastic (Ed) and viscous (Ev) components, and loss angle tangent (tan θ), the same modified Wilhelmy-type film balance (KSV 3000) was used as described elsewhere.[25,26] In this method, the surface is subjected to small periodic sinusoidal compressions and expansions by means of two oscillating barriers at a given frequency (ω) and amplitude (Δ*A*/*A*), and the response of the surface pressure is monitored. Surface pressure was directly measured by means of two roughened platinum plates situated on the surface between the two barriers. The dilatational modulus is a complex quantity and is composed of real and imaginary parts ($E = Ed + iEv$). The real part of the dilatational modulus or storage component is the dilatational elasticity, $Ed = |E|\cdot\cos\theta$. The imaginary part of the dilatational modulus or loss component is the surface dilatational viscosity, $Ev = |E|\cdot\sin\theta$. The loss angle tangent can be defined as the ratio between the viscous and elastic components of the modulus ($\tan\theta = Ev/Ed$). If the film is purely elastic, the loss angle tangent is zero. The amplitude of deformation was maintained constant at 5%. This percentage area change was determined to be in the linear region. The reproducibility of these results for two measurements was better than 5%.

Results

Structural Characteristics of β-Lactoglobulin Adsorbed Films. Figure 1A shows the π−trough area isotherms for an adsorbed film of β-lactoglobulin after successive compressions, formed from adsorption in solution at $1\cdot10^{-5}$ wt %. In these experiments, π−trough area isotherms at different times after the protein solution was left in the trough have been recorded, starting at 30 min and continuing for 36 h. However, to add clarity, only three π−trough area isotherms are included in Figure 1A. There was a difference in the π−trough area isotherms as a function of time after protein addition to the aqueous bulk phase. It can be seen that there was a shift of the π−trough area isotherms toward higher areas as the protein adsorption time increased to 30 h. This phenomenon may be attributed to adsorption of β-lactoglobulin at the interface, which increased with the adsorption time. The π−trough area isotherm for a first compression at 30 min of adsorption time (data not shown) indicates that a small amount of protein was adsorbed at the interface because the surface pressure at the minimum area was lower than the equilibrium spreading pressure for β-lactoglobulin ($\pi_e^{\beta-\text{lactoglobulin}} \cong 25.9$ mN/m).[27] Moreover, the π−trough area isotherms were parallel after successive compressions (Figure 1A). These data reveal that a long time interval of adsorption allows more β-lactoglobulin to adsorb at the surface, especially from low protein concentrations in solution such as those used in this work. After 30 h of adsorption time, the π−trough area isotherms after successive compressions show a shift toward lower areas, which may be explained by a condensation of the protein at the interface after repetitive compression−expansion cycles.

Since the surface concentration is actually unknown for the adsorbed film, the values were derived from plots in Figure 1A by assuming that the *A* values for adsorbed and spread films were equal at the collapse point of the mixed film (Figure

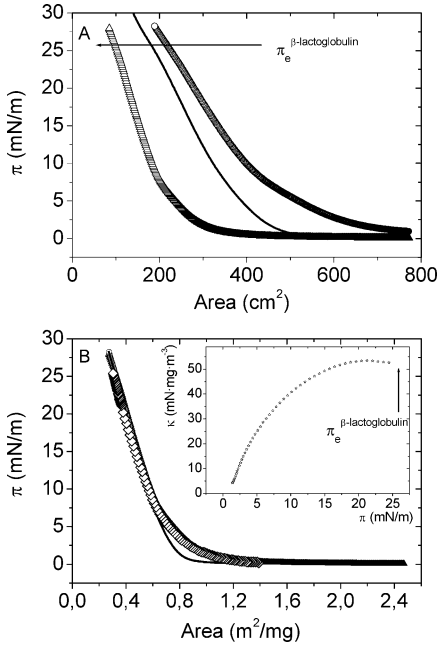

Figure 1. (A) $\pi-$trough area isotherms (compression curves) for adsorbed monolayers of β-lactoglobulin at an aging time of (△) 7 h, (○) 30, and (—) 36 h. (B) $\pi-A$ isotherms for adsorbed β-lactoglobulin monolayers at an aging time of (△) 7 h, (○) 30, and (—) 36 h, and (◇) spread β-lactoglobulin monolayer (compression curves).[24,26,31] The inset shows the surface pressure dependence of the compressional coefficient (κ) for an adsorbed β-lactoglobulin monolayer at an aging time of 30 h. Aqueous subphase at pH 7. Temperature, 20 °C. Concentration of β-lactoglobulin in the aqueous phase, $1\cdot10^{-5}$ wt %. The π_e of β-lactoglobulin ($\pi_e^{\beta-\text{lactoglobulin}}$) is indicated by means of an arrow.

1B).[21,22,30] This assumption can be supported by the fact that for β-lactoglobulin films, the equilibrium spreading pressure ($\pi_e^{\beta-\text{lactoglobulin}}$) and the surface pressure at the plateau for a saturated β-lactoglobulin adsorbed film[27] are the same. The $\pi-A$ isotherms deduced for adsorbed β-lactoglobulin films in the Wilhelmy-type film balance are in good agreement with those observed[21] in a Langmuir-type film balance. On the other hand, the $\pi-A$ isotherm deduced for adsorbed β-lactoglobulin film (Figure 1B) is similar to that obtained previously by spreading in the Wilhelmy-[26] and Langmuir-type[24,31] film balances. Thus, the structures of β-lactoglobulin films formed in the two different ways must be identical, at least for adsorption from low bulk protein concentrations. In this regard, β-lactoglobulin (a globular protein) and β-casein (a disordered protein)[32] behaved in a different way.

The results of the $\pi-A$ isotherms (Figure 1B) with the help of the compressional coefficient (insert in Figure 1B) deduced from the slope of the $\pi-A$ isotherm ($\kappa = -\mathrm{d}\pi/\mathrm{d}A$), confirm[21,26,31,33] that adsorbed β-lactoglobulin films at the air−water interface adopt a liquid expanded-like structure and the collapse phase. However, according to Graham and Phillips,[33] β-lactoglobulin retains elements of the native structure, not fully unfolded at the interface. Thus, most amino acid residues in β-lactoglobulin adopt loop conformation at the air−water interface. But the loop conformation is more condensed at higher surface pressures and is displaced toward the bulk phase at the collapse point (at $\pi \geq \pi_e^{\beta-\text{lactoglobulin}}$). The film collapses at a surface pressure of about 30 mN/m (Figure 1B), a value that is a little higher than the surface pressure at the plateau for a saturated adsorbed film and the equilibrium spreading pressure,[27] which is indicated in Figure 1A by means of an arrow. The morphology (Figure 2A-a) and, especially, the reflectivity (Figure 2B) as a function of surface pressure obtained with β-lactoglobulin adsorbed films reproduce those observed previously[21] and show that, for β-lactoglobulin adsorbed monolayers,[26,31] film thickness increases with the surface pressure and is a maximum at the collapse, at the highest surface pressure. These data corroborate the idea that a condensation in the monolayer structure takes place during the compression of the monolayer. BAM images also prove that β-lactoglobulin spread[26,31] and adsorbed films (Figure 2A-a) adopt a homogeneous morphology during the film compression.

Dilatational Characteristics of β-Lactoglobulin Adsorbed Films. A common trend of the surface pressure dependence of the dilatational modulus (E) for β-lactoglobulin adsorbed films (Figure 3A) is that E increases with increasing surface pressure up to the collapse point. This increase is a result of an increase in the interactions between the monolayer molecules (that is, of its structure), as deduced from $\pi-A$ isotherms (Figure 1B), and monolayer thickness (Figure 2B). That is, more condensed monolayer structures (at higher surface pressures) may lead to an increase in the forces of interaction between amino acid residues at the interface, which is consistent with the observed increase in E. Upon further increase of the surface pressure, E remains constant. That is, the molecular structure (self-assembly of amino acid residues) is important to understand the dynamic interfacial behavior of adsorbed protein at fluid interfaces,[26,31] as has been pointed out by means of simulations of dilatational rheology of adsorbed protein films.[34−36] These data also support the hypothesis that the compression of β-lactoglobulin films does not produce any change in loop conformation adopted by amino acid residues because E increases monotonically with π, but that a condensation of this conformation takes place. These results are in good agreement with those observed for β-lactoglobulin monolayers spread on the air−water interface[24,26,31] and for globular proteins in general.[24,37−43]

However, in contrast with the general behavior discussed previously, the surface dilatational modulus of β-lactoglobulin films shows a complex behavior depending on the experimental conditions for film formation and processing. From the data included in Figure 3A it can be deduced that

(i) The surface pressure dependence on E, during the compression of β-lactoglobulin adsorbed films from aqueous solutions at $1\cdot10^{-5}$ and $1\cdot10^{-4}$ wt % (this work), depends on the β-lactoglobulin concentration in solution. It can be seen that E values are higher for adsorbed films from higher β-lactoglobulin concentrations in solution.

(ii) The results for β-lactoglobulin adsorbed films under dynamic conditions, during the adsorption of the protein at the air−water interface, from different protein concentrations in solution and at different adsorption times,[24] are aligned in different $E-\pi$ lines for different protein concentrations. On the other hand, the E values during the adsorption of β-lactoglobulin from aqueous solutions under dynamic conditions[24] and at different concentrations are halfway between those during the compression of the adsorbed films from low protein concentrations in solution (this work). These results support the hypothesis that this protein is adsorbed at the air−water interface with different degrees of association (aggregation) at different concentrations in the bulk phase,[44] a phenomenon that can be described by means of theoretical models[35,36] and which depends on the history of film formation.

(iii) Over the range of surface pressures studied, the values of E for β-lactoglobulin spread films were higher than those for adsorbed films. This result was unexpected because the $\pi-A$ isotherms deduced for adsorbed and spread β-lactoglobulin films

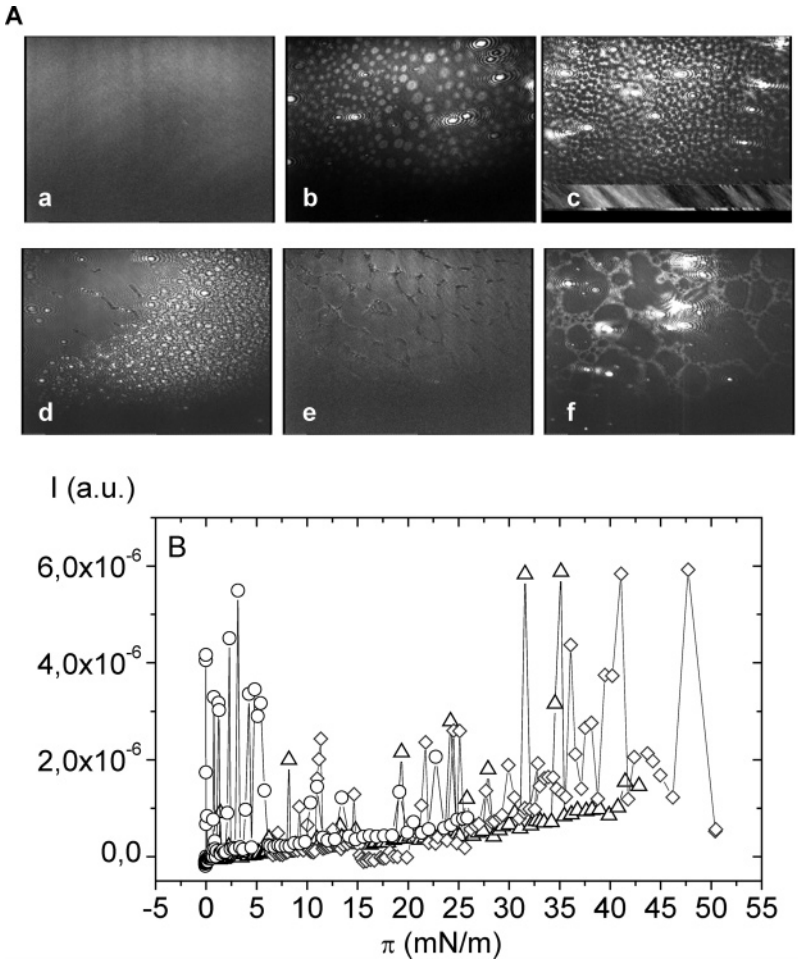

Figure 2. (A) Visualization of β-lactoglobulin and β-lactoglobulin-monopalmitin adsorbed mixed monolayers by BAM: (a) compression of β-lactoglobulin at $\pi < 30$ mN/m, (b) compression of β-lactoglobulin adsorbed film + monopalmitin at 15 mN/m, (c) compression of β-lactoglobulin adsorbed film + monopalmitin at 30 mN/m, (d) compression of β-lactoglobulin adsorbed film + monopalmitin at 45 mN/m, (e) compression of β-lactoglobulin adsorbed film + monopalmitin at 48 mN/m, and (f) expansion of β-lactoglobulin adsorbed film + monopalmitin at $\pi \approx 0-2$ mN/m. Shutter speed, 1/50 s. Mass fraction of monopalmitin in the mixture, $X_{MP} = 0.4$. The horizontal direction of the image corresponds to 630 μm, and the vertical direction corresponds to 470 μm. (B) Reflectivity (arbitrary units) during the compression for (○) an adsorbed β-lactoglobulin film, (△) β-lactoglobulin adsorbed film + monopalmitin at $X_{MP} = 0.25$, and (◇) β-lactoglobulin adsorbed film + monopalmitin at $X_{MP} = 0.4$. Shutter speed, 1/250 s. Temperature 20 °C, pH 7.

are practically the same (Figure 1B). One possibility is that the spreading process of a β-lactoglobulin monolayer[26] can be the cause of an interfacial unfolding of the protein, leading to a loss of its tertiary structure at the air−water interface. Under these conditions, the interactions between amino acid residues in spread films with loop conformation are stronger, especially at higher surface pressures (at the collapse point), as the formation of protein multilayers takes place.[26]

(iv) The limiting Gibbs' elasticity obtained from the $\pi-A$ isotherm of a spread β-lactoglobulin monolayer, defined as the E value measured at a frequency where no relaxation processes affect the surface pressure in the time scale of the area oscillation, $E_o = -A\cdot(\delta\pi/\delta A)_T$, shows the same surface pressure dependence as those of E for adsorbed and spread films. However, the E_o values are lower than those for adsorbed and spread films. The theoretical values of E_o for globular proteins are lower than those for adsorbed molecules since configurations with lower molecular areas are favored at the expense of the higher-area configurations, especially at high π.[36] This fact may also be explained by the viscoelastic behavior of β-lactoglobulin films, as will be analyzed later.

(v) Finally, the values of E for adsorbed and, especially, for spread β-lactoglobulin films are higher (with few exceptions) than those for an $E-\pi$ slope of 1 (dashed line), which implies an important nonideal behavior, [45] with higher molecular interactions as the surface pressure increases.

These findings support the hypothesis that the surface dilatational modulus of β-lactoglobulin films is not only determined by the interactions between protein molecules (which depend on the surface pressure), but also by others factors, such as the state of condensation of amino acid residues, which must be different for adsorbed than for spread films, or the film formation from low or high protein concentrations in solution, or even the dynamics of film formation and collapse.

The evolution of tan θ with π (Figure 3B) corroborates the idea that β-lactoglobulin adsorbed films from low protein concentrations in solution present a viscoelastic behavior at every surface pressure, in contrast with β-lactoglobulin adsorbed films from higher protein concentrations in solution, which present a viscoelastic behavior that changes to elastic at higher surface pressures. However, β-lactoglobulin spread films present a complex behavior. For spread β-lactoglobulin films, tan θ is not only lower at $\pi < \pi_e^{\beta-\text{lactoglobulin}}$ than for adsorbed films, but it also passes through a minimum at $\pi > \pi_e^{\beta-\text{lactoglobulin}}$, followed by an increases in tan θ at higher π as the β-lactoglobulin collapse and multilayer formation take place. The lower values of tan θ for spread β-lactoglobulin films as compared

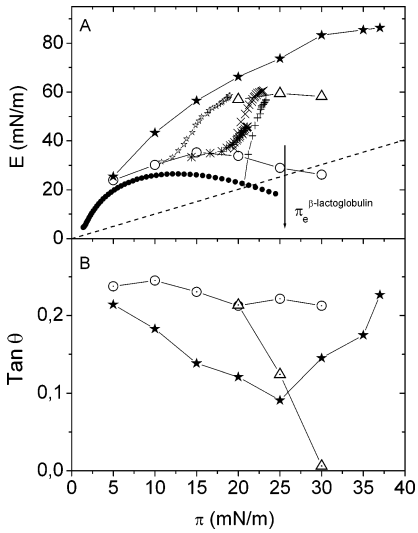

Figure 3. Surface pressure dependence of (A) surface dilatational modulus (E) and (B) loss angle tangent (tan θ) for pure β-lactoglobulin monolayers at pH 7 and at 20 °C. Open symbols (○, △) are for β-lactoglobulin films adsorbed from the aqueous phase at protein concentrations of $1\cdot10^{-5}$ wt % (○) and $1\cdot10^{-4}$ wt % (△); (★) is for a β-lactoglobulin spread monolayer, and (●) is the limiting Gibbs' elasticity (E_0) deduced from the $\pi-A$ isotherm for a spread monolayer. The surface dilatational modulus under dynamic conditions for β-lactoglobulin films adsorbed from aqueous solutions at protein concentrations (wt %) in the bulk phase of (+) 1, (×) 0.1, (∗) 0.01, and (☆) 0.001 from ref 24 are included for comparison. The dashed line with an $E-\pi$ plot slope of 1 represents an ideal behavior with low protein interactions.[45] The π_e of β-lactoglobulin ($\pi_e^{\beta-\text{lactoglobulin}}$) is indicated by means of an arrow.

with adsorbed films support the hypothesis that the spreading process facilitates the unfolding of the protein at the air−water interface and favors the formation of an interfacial gel with high elastic characteristics (i.e., with low values of tan θ). These results also confirm the idea that the surface viscoelastic behavior is sensitive to the structure, interactions, and collapse in β-lactoglobulin spread and adsorbed films at the air−water interface.

Self-Assembly of Monopalmitin in β-Lactoglobulin Adsorbed Films at the Air−Water Interface: Structural, Topographical, and Rheological Consequences. For analysis of the effect of the self-assembly of monopalmitin molecules in β-lactoglobulin films previously adsorbed at the air−water interface, mixtures of particular β-lactoglobulin/monopalmitin mass fractions of monopalmitin in the mixture (at X_{MP} of 0, 0.25, 0.4, and 1.0) were studied. The amount of spread monoglyceride was calculated on the basis of the mass of previously adsorbed β-lactoglobulin (which was deduced from the adsorbed $\pi-A$ isotherm). Thus, as opposed to spread monolayers,[31] for adsorbed films, the mixtures with mass fractions higher than $X_{MP} = 0.4$ saturate the interface under the experimental conditions used in this work.

The surface pressure as a function of trough area for β-lactoglobulin + monopalmitin adsorbed mixed films (compression curves) is shown in Figure 4A. As for pure β-lactoglobulin adsorbed films, the actual $\pi-A$ isotherms for β-lactoglobulin + monopalmitin adsorbed mixed films were derived by assuming[21,22] that the A values for adsorbed and spread films were equal at the collapse point. This assumption can be supported by the fact that the surface pressure at the collapse point for adsorbed (Figure 4B) and spread[31] mixed films is practically equal to that for the pure monoglyceride. Results derived from $\pi-A$ isotherms for β-lactoglobulin + monopalmitin adsorbed mixed films in the Wilhelmy-type trough (Figure 4B) are in good agreement with those obtained[21] in the Langmuir-type trough with the same β-lactoglobulin + monopalmitin adsorbed mixed films.

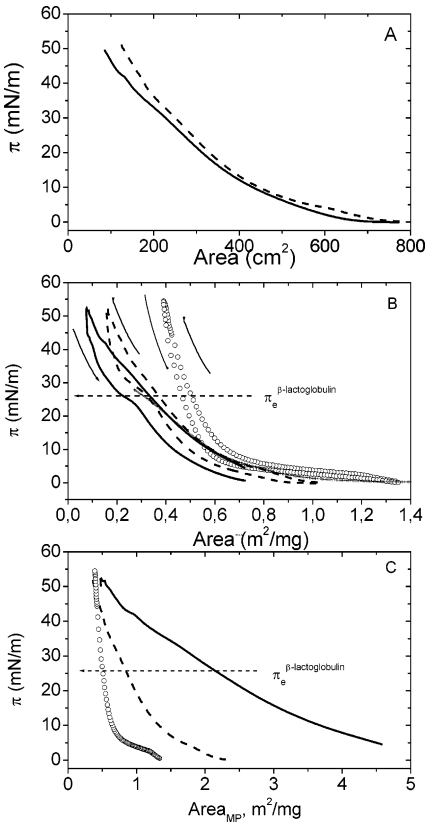

Figure 4. (A) Surface pressure−trough area isotherms (compression curves) and (B) surface pressure−area isotherms (compression−expansion curves) for mixed monolayers of monopalmitin spread on a β-lactoglobulin adsorbed film from buffered water. (C) Surface pressure−area isotherms (compression curves) for mixed monolayers of monopalmitin spread on a β-lactoglobulin adsorbed film on the basis that only monopalmitin is present at the air−water interface. Temperature 20 °C and pH 7. Concentration of β-lactoglobulin in the aqueous phase, $1\cdot10^{-5}$ wt %. Mass fraction of monopalmitin in the mixture, X_{MP}: (+) 0, (−) 0.25, (- - -) 0.4 and (○) 1.0. The direction of the expansion/compression curves is indicated by means of solid arrows. The π_e of β-lactoglobulin is indicated by means of a dash arrow.

Briefly, (i) there was a film expansion as the monopalmitin concentration in the mixture was increased, especially at higher surface pressures (Figure 4B). That is, the $\pi-A$ isotherm is displaced toward higher A as the concentration of monopalmitin in the mixture increases.

(ii) At surface pressures higher than that for β-lactoglobulin collapse (at $\pi > \pi_e^{\beta-\text{lactoglobulin}}$), the $\pi-A$ isotherm for mixed films was parallel to that of monopalmitin (Figure 4B). These data are also in agreement with those deduced for spread β-lactoglobulin + monopalmitin mixed films.[31] At the highest surface pressures, at the collapse point of the mixed film, the immiscibility between film-forming components is deduced because the collapse pressure of mixed films is similar to that of a pure monoglyceride monolayer (Figure 4B).

(iii) The hypothetical $\pi-A$ isotherms for β-lactoglobulin + monopalmitin adsorbed mixed films, calculated on the basis that only monopalmitin is present at the air−water interface (Figure 4C), at $\pi > \pi_e^{\beta-\text{lactoglobulin}}$, tend to that of a pure monopalmitin spread monolayer, especially at higher π and X_{MP} in the mixture. These results suggest that, at $\pi > \pi_e^{\beta-\text{lactoglobulin}}$, protein

displacement by the monoglyceride from the air−water interface takes place. At $\pi < \pi_e^{\beta-\text{lactoglobulin}}$, both β-lactoglobulin and monopalmitin coexist at the interface.

(iv) The protein displaced by monopalmitin from the interface during compression remains underneath the monoglyceride film, either through hydrophobic interactions between protein and lipid or by local anchoring through the monoglyceride layer,[21,22] and reenters the mixed film during the expansion. This statement is supported by the fact that the $\pi-A$ isotherms were repetitive after continuous compression−expansion cycles (data not shown).

(v) For adsorbed β-lactoglobulin + monopalmitin mixed films, a first-order-like phase transition was observed upon film expansion (Figure 4B) at $\pi \approx \pi_e^{\beta-\text{lactoglobulin}}$, with a degenerated plateau in the $\pi-$A isotherm. This result suggests[21,22] that the readsorption of previously displaced β-lactoglobulin has a kinetic character, which was not evident for spread mixed films.[31]

The evolution with the surface pressure of BAM images (Figure 2A) gives complementary information, at a microscopic level, on the structural characteristics and interactions of adsorbed β-lactoglobulin + monopalmitin mixed films, as deduced from $\pi-A$ isotherms (Figure 4). At $\pi < \pi_e^{\beta-\text{lactoglobulin}}$, a mixed film of monopalmitin and β-lactoglobulin may exist (Figure 2A-b) with small circular domains of monopalmitin uniformly distributed on the homogeneous β-lactoglobulin layer. The circular domains of monopalmitin, with a liquid condensed structure,[28] were more numerous as the surface pressure increased (Figure 2A-c), just as for a pure monopalmitin monolayer.[28] At $\pi > \pi_e^{\beta-\text{lactoglobulin}}$, a characteristic squeezing out phenomena of β-lactoglobulin by monopalmitin was observed (Figure 2A-c,d), and the mixed films were practically dominated by monopalmitin domains. That is, at higher surface pressures, collapsed β-lactoglobulin residues (bright region) may be displaced from the interface by monopalmitin molecules (circular dark regions). A topographical characteristic of the adsorbed film (not observed in spread mixed film[31]) was the presence of short fractures in the film at higher surface pressures, near to the collapse point of the mixed film (Figure 2A-e), which are characteristic of protein−monoglyceride adsorbed films.[21,22] Finally, after the expansion, the film undergoes breakup of the collapse structure to a three-dimensional foam structure (Figure 2A-f), which consists of a collapse protein phase in the plateau borders with foam cells containing a liquid-like protein and monopalmitin.

The segregation observed in BAM images (Figure 2A) for β-lactoglobulin + monopalmitin mixed adsorbed films is confirmed by the evolution of the reflectivity (I) with surface pressure (Figure 2B). The I peaks observed over the overall range of existence of the mixed film are an indication of the film heterogeneity because domains of monopalmitin (with low I) and β-lactoglobulin residues (with high I) are present during the monolayer compression−expansion cycle. The reflectivity of some spots of the mixed film is higher than that for pure β-lactoglobulin (Figure 2B) or pure monopalmitin (data not shown), especially at higher surface pressures. These results strengthen the hypothesis that, for adsorbed β-lactoglobulin + monopalmitin mixed films, some degree of attractive interactions between film-forming components exists at a microscopic level, giving a mixed film with greater thickness compared with those of pure components.

The surface viscoelastic properties of β-lactoglobulin + monopalmitin adsorbed mixed films on the air−water interface at a representative monopalmitin concentration in the mixture of $X_{PM} = 0.25$ are shown in Figure 5. In the same figure are included as a reference the viscoelastic properties of a pure monopalmitin spread monolayer and β-lactoglobulin + monopalmitin mixed monolayers spread at the air−water interface at $X_{PM} = 0.20$.[46] It can be seen that the $E-\pi$ plot for an adsorbed film showed an irregular shape. At $\pi < \pi_e^{\beta-\text{lactoglobulin}}$, the values of E increase with π and tend to a maximum plateau at $\pi \approx \pi_e^{\beta-\text{lactoglobulin}}$, just as E of a pure β-lactoglobulin adsorbed film or the E_o values for a spread film (Figure 3A). At these π values, the E values of the mixed film are higher than those for a pure β-lactoglobulin adsorbed film, but are much lower than those for a pure monopalmitin spread monolayer. The values of E tend to a maximum plateau at $\pi \approx \pi_e^{\beta-\text{lactoglobulin}}$. These results corroborate the idea that, at $\pi < \pi_e^{\beta-\text{lactoglobulin}}$, β-lactoglobulin and monopalmitin coexist in adsorbed and spread mixed films at the air−water interface.

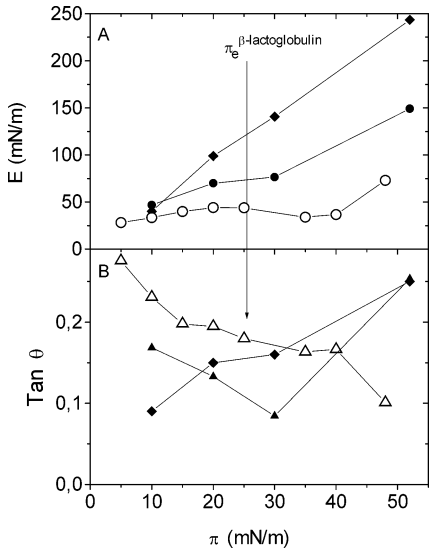

Figure 5. Surface pressure dependence of (A) the surface dilatational modulus (E) and (B) the loss angle tangent (tan θ) for mixed monolayers of monopalmitin and β-lactoglobulin in buffered water at pH 7 and at 20 °C. Symbols: (○, △) mixed monolayers of monopalmitin spread on a β-lactoglobulin adsorbed film at a mass fraction of monopalmitin in the mixture $X_{MP} = 0.25$, (●,▲) monopalmitin and β-lactoglobulin spread mixed films at a mass fraction of monopalmitin in the mixture $X_{MP} = 0.20$, and (◆) pure monopalmitin spread monolayer. Concentration of β-lactoglobulin in the aqueous phase, $1 \cdot 10^{-5}$ wt %. Open symbols are for adsorbed monolayers and closed symbols are for spread monolayers. The π_e of β-lactoglobulin ($\pi_e^{\beta-\text{lactoglobulin}}$) is indicated by means of an arrow.

At $\pi > \pi_e^{\beta-\text{lactoglobulin}}$, the values of E increase with π up to a maximum value at the collapse point of the mixed film, just as for a pure monopalmitin monolayer and for β-lactoglobulin + monopalmitin mixed spread monolayers. In addition, the $E-\pi$ plots for mixed films (either adsorbed or spread) were parallel to those of monopalmitin, which demonstrated the effect of monopalmitin on E at higher surface pressures. However, the data in Figure 5A also demonstrate that minor amounts of β-lactoglobulin collapsed residues at the interface, as deduced at a microscopic level from BAM images (Figure 2A), have an effect on the surface dilatational properties of the mixed films. In fact, the values of E for mixed films are lower than those for a pure monopalmitin monolayer, even at the collapse point of the mixed films. In addition, at higher π values, the E values for β-lactoglobulin + monopalmitin are lower for adsorbed films than for spread mixed films at the same surface pressures, as observed for pure β-lactoglobulin films (Figure 3A). Thus, the mechanical properties of the mixed films also demonstrated that,

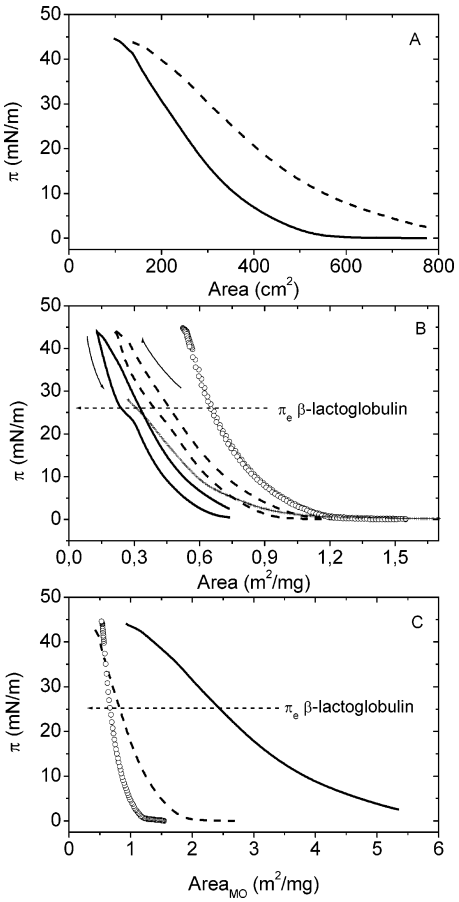

Figure 6. (A) Surface pressure−trough area isotherms (compression curves) and (B) surface pressure−area isotherms (compression−expansion curves) for mixed monolayers of monoolein spread on a β-lactoglobulin adsorbed film from buffered water at pH 7 and at 20 °C. (C) Surface pressure−area isotherms (compression curves) for mixed monolayers of monoolein spread on a β-lactoglobulin adsorbed film on the basis that only monoolein is present at the air−water interface. Concentration of β-lactoglobulin in the aqueous phase, $1 \cdot 10^{-5}$ wt %. Mass fraction of monoolein in the mixture (X_{MP}): (+) 0, (—) 0.2, (- - -) 0.4, and (○) 1.0. The direction of the expansion/compression curves is indicated by means of solid arrows. The π_e of β-lactoglobulin ($\pi_e^{\beta-\text{lactoglobulin}}$) is indicated by means of a dash arrow.

at higher π values, monopalmitin and β-lactoglobulin molecules coexist at the air−water interface.

From the values of the loss angle tangent (tan $\theta-\pi$ curves) for β-lactoglobulin + monopalmitin adsorbed mixed films, it can be concluded that these films behaved as viscoelastic at low π, but their behavior changes to elastic at higher π (Figure 5B). In this regard, adsorbed and spread films behave in a different way, especially at higher π (at $\pi > \pi_e^{\beta-\text{lactoglobulin}}$). The viscoelastic behavior of β-lactoglobulin + monopalmitin adsorbed mixed films also confirms the effect of β-lactoglobulin on the surface dilatational properties of the mixed films.

Self-Assembly of Monoolein In β-Lactoglobulin Adsorbed Films at the Air−Water Interface: Structural, Topographical, and Rheological Consequences. The effect of the self-assembly of monoolein molecules in β-lactoglobulin films previously adsorbed at the air−water interface on the structural characteristics of mixed films of β-lactoglobulin + monoolein, as deduced from $\pi-A$ isotherms, is illustrated in Figure 6. As in the preceding section, the actual $\pi-A$ isotherms for β-lactoglobulin + monoolein adsorbed mixed films (Figure 6B) were derived from the π−trough area plots (Figure 6A). As expected,[22,31] β-lactoglobulin + monoolein mixed films (Figure 6B) at $\pi < \pi_e^{\beta-\text{lactoglobulin}}$ adopt a liquid-like structure, just as for pure components. There was a film expansion due to the presence of monoolein in the mixture. At $\pi > \pi_e^{\beta-\text{lactoglobulin}}$, the $\pi-A$ isotherms for mixed films were practically parallel to those of monoolein. Under these experimental conditions, the hypothetical $\pi-A$ isotherms for mixed films, calculated on the basis that only monoolein is present at the air−water interface, tend to that of a pure monoolein monolayer, especially at higher X_{MO} (Figure 6C). These results prove that β-lactoglobulin is displaced from the air−water interface by monoolein. At the highest π, at the collapse point of the mixed film, the immiscibility between film-forming components is deduced because the collapse pressure of mixed films is similar to that of a pure monoolein monolayer (Figure 6B).

BAM images are characteristics for adsorbed β-lactoglobulin + monoolein mixed films (Figure 7A). At π lower than that of the collapse of the mixed film, the topographies of the pure components and the mixed film are practically identical (Figure 7A-a) because, in this region, both components and the mixed film form an isotropic (homogeneous) film without any difference in the domain topography. At π higher than that of the collapse of the mixed film, BAM images (Figure 7A-b) demonstrated that β-lactoglobulin and monoolein molecules adopted an isotropic structure in the mixed film with some white regions, which correspond to the presence of a thicker β-lactoglobulin collapsed film. BAM images and $I-\pi$ plots (Figure 7) prove that, at a microscopic level, (i) there exists a high degree of segregation between β-lactoglobulin and monoolein in adsorbed mixed films; however, the presence of monoolein in the mixed film attenuates the *I* peaks of collapsed β-lactoglobulin at low π; (ii) β-lactoglobulin and monoolein coexist at the air−water interface, even at the highest surface pressure (at the collapse point of the mixed film); and (iii) some degree of attractive interactions exists between film-forming components because the film thickness of the mixed film is higher than those of pure components, especially at higher surface pressures.

The surface viscoelastic properties of β-lactoglobulin + monoolein adsorbed mixed films on the air−water interface at a representative monoolein concentration in the mixture of $X_{MO} = 0.25$ are shown in Figure 8. In the same figure are included as a reference the viscoelastic properties of monoolein and spread mixed monolayers at the interface at $X_{MO} = 0.20$.[31] It can be seen that *E* increases with increasing π up to the collapse point of β-lactoglobulin. Upon further increase of the surface pressure, *E* decreases to a minimum value at the collapse point of the mixed film. Thus, the $E-\pi$ evolution of β-lactoglobulin + monoolein adsorbed mixed films (Figure 8A) is the same as that for a pure β-lactoglobulin adsorbed film (Figure 3A), but with *E* values a bit higher for the former. The differences between the *E* values for a pure monoolein monolayer or spread mixed films and β-lactoglobulin + monoolein adsorbed mixed films reach a maximum at the highest surface pressure, at the collapse point of the mixed films. Finally, at $\pi > \pi_e^{\beta-\text{lactoglobulin}}$, the $E-\pi$ plots for mixed films were not parallel to those of monoolein (Figure 8A). These results demonstrate that *E* values of the mixed films are dominated by the presence of β-lactoglobulin in the mixture, even at higher surface pressures ($\pi > \pi_e^{\beta-\text{lactoglobulin}}$).

From the values of the loss angle tangent (tan $\theta-\pi$ curves) for monoolein and β-lactoglobulin + monoolein adsorbed mixed films, it can be concluded that these films behaved as viscoelastic films at every surface pressure (Figure 8B). For the mixed adsorbed films, tan $\theta-\pi$ curves follow the same evolution as a

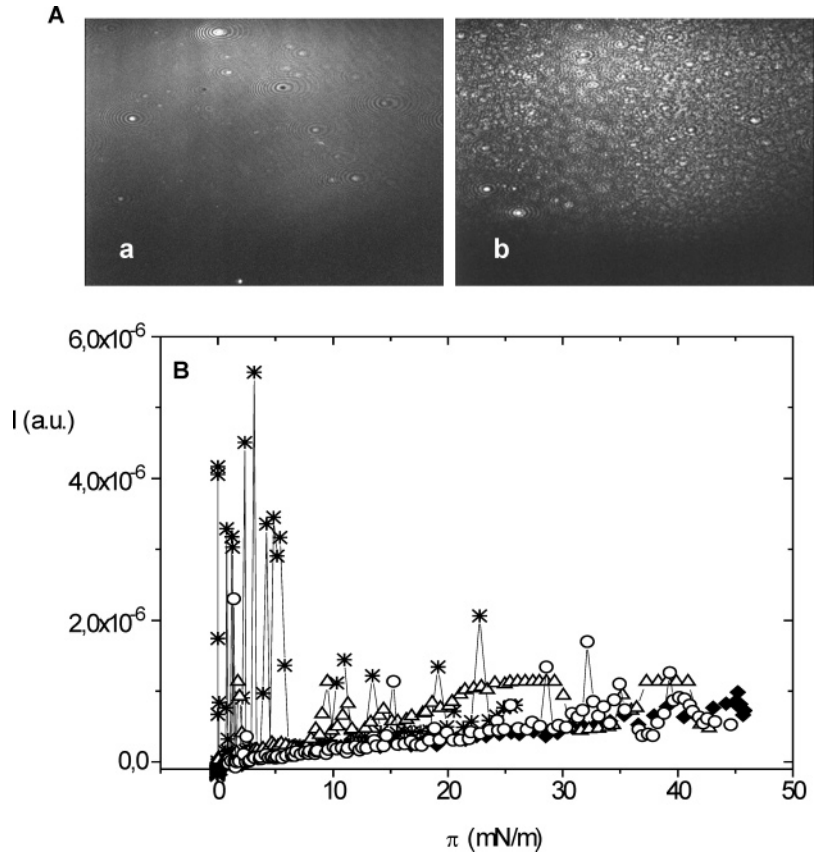

Figure 7. (A) Visualization of β-lactoglobulin−monoolein adsorbed mixed monolayers by BAM: (a) compression of β-lactoglobulin + monoolein adsorbed film at $\pi < 40$ mN/m and (b) compression of β-lactoglobulin + monoolein adsorbed film at $\pi = 44$ mN/m. Shutter speed, 1/50 s. Mass fraction of monoolein in the mixture, $X_{MO} = 0.4$. The horizontal direction of the image corresponds to 630 μm, and the vertical direction corresponds to 470 μm. (B) Reflectivity (arbitrary units) during the compression for (○) β-lactoglobulin adsorbed film + monoolein at $X_{MO} = 0.20$ and (△) β-lactoglobulin adsorbed film + monopalmitin at $X_{MO} = 0.4$. The reflectivity of pure (∗) adsorbed β-lactoglobulin and (◆) spread monoolein films are included as a reference. Shutter speed, 1/250 s. Temperature 20 °C, pH 7.

pure monoolein monolayer, with a more elastic character for the latter. β-Lactoglobulin + monoolein spread mixed films also show a more elastic character than adsorbed mixed films, except at the collapse point.

Discussion

Knowledge of the interfacial structure of adsorbed emulsifiers (proteins and lipids) on a micro/nanoscale and the interfacial properties derived from this structure (i.e., the surface dilatational properties) will have an important role in innovations in food dispersion formulations (emulsions and foams). In fact, the correlation between a specific product property and the micro/nanostructure (*property function*) can be obtained by the choice of suitable process conditions (*process function*), such as surface pressure or surface density, surface composition, film formation (spreading, adsorption, or both), and so forth. *Product engineering* (or *formulation engineering*), which is concerned with physical or physicochemical principles, may improve the quality and performance of products with added value due to an adequate correlation between property function and process function.[47,48] Thus, the results of this work have direct relevance to food engineering (specially for foam formation and stabilization[49]) and serve as a model study for extrapolation to more complex real food systems (foams and emulsions).

In this work, a single device that incorporates different interfacial techniques, such as Wilhelmy-type film balance, BAM, and interfacial dilatational rheology, has been used to analyze the static (structure, morphology, and interactions) and dynamic characteristics (surface dilatational properties) of β-lactoglobulin + monoglyceride mixed films adsorbed on the air−water interface.

The structural (Figure 1) and topographical (Figure 2) characteristics of β-lactoglobulin adsorbed and spread films are similar. However, the surface dilatational modulus of β-lactoglobulin films shows a complex behavior depending on film formation and the experimental conditions (Figure 3). The surface dilatational modulus and viscoelasticity of β-lactoglobulin films is not only determined by the interactions between protein molecules (which depend on the surface pressure), but also by other factors such as the state of condensation of amino acid residues, which, for adsorbed films, must be different from that for spread films, or the film formation from low or high protein concentrations in solution, or even the dynamics of film formation and collapse. That is, surface rheology is very sensitive to the structural characteristics and dynamic phenomena of proteins at fluid interfaces,[19,20] including the effect of film formation (Figure 3).

The structural, topographical and dilatational properties of monoglyceride (monopalmitin and monoolein) spread films have been analyzed in detail in the literature.[23] From the $\pi-A$ isotherm and BAM, different structures can be deduced for monoglyceride monolayers as a function of surface pressure. Monopalmitin monolayers show that liquid-expanded, liquid-condensed, and solid structures and, finally, the collapse at a surface pressure higher than the equilibrium spreading pressure ($\pi_e^{monopalmitin} \approx 45.2$ mN/m) take place as the surface pressure increases. In contrast with monopalmitin, a monoolein mono-

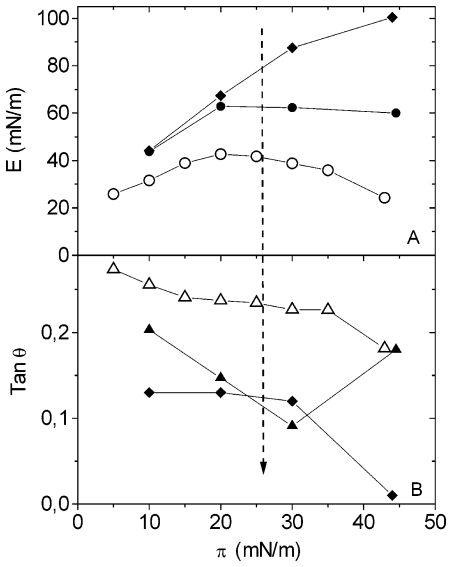

Figure 8. Surface pressure dependence of (A) the surface dilatational modulus (E) and (B) the loss angle tangent (tan θ) for mixed monolayers of monoolein and β-lactoglobulin in buffered water at pH 7 and at 20 °C. Symbols: (○, △) mixed monolayers of monoolein spread on a β-lactoglobulin adsorbed film at a mass fraction of monoolein in the mixture $X_{MO} = 0.20$, (●, ▲) monoolein and β-lactoglobulin spread mixed films at a mass fraction of monoolein in the mixture $X_{MO} = 0.20$, and (◆) pure monoolein spread monolayer. Concentration of β-lactoglobulin in the aqueous phase, $1 \cdot 10^{-5}$ wt %. Open symbols are for adsorbed monolayers and closed symbols are for spread monolayers. The π_e of β-lactoglobulin ($\pi_e^{\beta-\text{lactoglobulin}}$) is indicated by means of an arrow.

layer presents only the liquid expanded structure and the collapse at the equilibrium spreading pressure ($\pi_e^{\text{monoolein}} \approx 45.7$ mN/m). The evolution of reflectivity (film thickness) for monoglyceride films increases monotonically with monolayer compression, but, in contrast to monopalmitin monolayers, (i) no discontinuity was observed, which corroborates that, during the monolayer compression, a denser film is formed but without any change in its structure, and (ii) the reflectivity during monoolein monolayer compression, and especially at the collapse point, is lower than that for monopalmitin. A common trend of the surface pressure dependence of the dilatational modulus for monopalmitin and monoolein monolayers is that E increases with increasing π up to the collapse point. This increase is a result of an increase in the interactions between the monolayer molecules (that is, of its structure), as deduced from $\pi-A$ isotherms, BAM images, and film thickness.[20] However, for the more condensed monopalmitin film, this increase is higher than that for the more expanded monoolein film. This indicates that not only is E determined by the interactions between spread monoglyceride molecules (which depend on the surface pressure or surface density), but that the structure of the film molecules also plays an important role.

The self-assembly of monoglyceride in a β-lactoglobulin adsorbed film has an effect on the structural (Figures 4 and 6), topographical (Figures 2 and 7) and dilatational properties (Figures 5 and 8) of the mixed films, depending on the interfacial composition and the surface pressure. At $\pi < \pi_e^{\beta-\text{lactoglobulin}}$, a mixed film of monoglyceride and β-lactoglobulin may exist. At $\pi > \pi_e^{\beta-\text{lactoglobulin}}$, the mixed films were dominated by monoglyceride molecules. That is, at higher surface pressures, collapsed β-lactoglobulin residues may be partially displaced from the interface by monoglycerides. However, β-lactoglobulin displacement by monoglycerides is not quantitative at the monoglyceride concentrations studied in this work. The protein displacement by monoglyceride is higher for monopalmitin than for monoolein. From a rheological point of view, β-lactoglobulin + monoglyceride adsorbed films have a viscoelastic character (Figures 5 and 8). At $\pi < \pi_e^{\beta-\text{lactoglobulin}}$ the surface dilatational characteristics of the mixed films were dominated by the presence of β-lactoglobulin in the mixture. Even at higher surface pressures (at $\pi > \pi_e^{\beta-\text{lactoglobulin}}$) the small amounts of β-lactoglobulin collapsed residues with a variable extension and a random distribution at the interface have a significant effect on the surface dilatational properties of the mixed films. The results also corroborate the presence of significant differences in the viscoelastic character of β-lactoglobulin + monoglyceride (monopalmitin or monoolein) adsorbed mixed films, especially at higher π, with a more elastic character for β-lactoglobulin + monopalmitin adsorbed mixed films.

From the comparison between viscoelastic characteristics of adsorbed (Figures 5 and 8) and spread[31] β-lactoglobulin + monoglyceride mixed films, it can be concluded that (i) E values of adsorbed and spread mixed films are different, the lower E values being shown by the former, (ii) the elastic character is higher for spread films, except at highest π (at the collapse point of mixed films), and (iii) the viscoelastic characteristics of the mixed films corroborate the idea that protein displacement for monoglycerides is easier for adsorbed than for spread mixed films, especially for the β-lactoglobulin + monopalmitin system.

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