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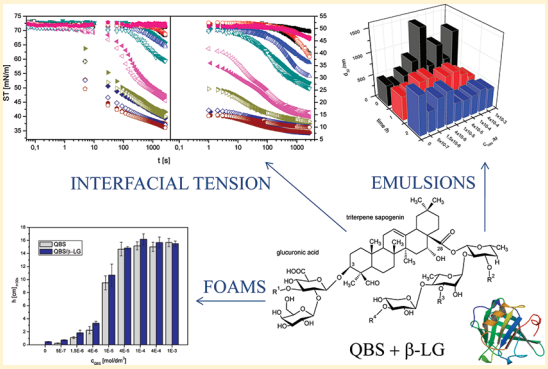
Biosurfactant–Protein Mixtures: *Quillaja* Bark Saponin at Water/Air and Water/Oil Interfaces in Presence of β-Lactoglobulin

Marek Piotrowski, Joanna Lewandowska, and Kamil Wojciechowski*

Department of Microbioanalytics, Faculty of Chemistry, Warsaw University of Technology, Warsaw, Poland

ABSTRACT: The adsorption kinetics of mixtures of a biosurfactant *Quillaja* Bark Saponin (QBS) with a globular protein, β-lactoglobulin (β-LG) at the water/air and water/tetradecane interfaces was investigated by measuring dynamic interfacial tension with axisymmetric drop shape analysis (ADSA) and maximum bubble pressure (MBP) techniques. With bulk concentration of β-LG fixed at 10^{-7} M, the most pronounced synergistic effects in the rate of the QBS adsorption at both interfaces were observed at low biosurfactant concentrations (5×10^{-7}–1×10^{-5} M). The synergistic effect due to a protein-biosurfactant complex formation is clearly noticeable, yet less pronounced than, e.g., previously studied QBS/lysozyme mixtures. The surface pressures attained at water/oil interface are higher than in the water/air system, although, at high biosurfactant/protein ratios, the presence of β-LG decelerates adsorption of the QBS/β-LG complex onto the water/tetradecane interface. In analogy to mixtures of synthetic surfactants with proteins, the adsorbed layer gets dominated by QBS at higher biosurfactant concentrations, although the presence of β-LG affects the surface pressures attained even at QBS/β-LG ratios as high as 10^4. The synergistic effects are much less noticeable in foamability and emulsion formation/stability, as probed by the modified Bikerman's and dynamic light scattering (DLS) techniques, respectively.



INTRODUCTION

Mixed protein–surfactant systems are ubiquitous in numerous aspects of everyday life. They ensure, e.g., the adequate sponginess of bread[1] or proper consistency of mayonnaise.[2] They are also present in most cosmetics formulations for hair and body, such as shampoos and creams.[3] Lipases/bile acids and a so-called pulmonary surfactant are just two examples of mixed protein/surfactant systems essential for living organisms. From a physical point of view, both surfactants and proteins adsorb on interfaces, reducing the respective interfacial tensions. While low molecular weight surfactants in most formulations are responsible for foam/emulsion formation thanks to a quick adsorption on the bubble/drop surface, the high-molecular weight proteins stabilize them on longer time scales, forming highly elastic and often electrically charged interfacial networks.[4] Interactions of proteins with ionic surfactants such as anionic sodium dodecyl sulfate (SDS) or cationic cetyltrimethylammonium bromide (CTAB) have been described in detail in the literature,[5] but reports on protein–biosurfactant interfacial properties are rather scarce. Interfacial interactions between proteins and (bio)surfactants are often studied using interfacial tension techniques, mainly based on analysis of the shape of bubbles or drops. The ultimate goal of these studies is to provide an understanding of relationships between the structure and dynamics of mixed interfacial layers[6] with foam/emulsion formation and stability.[7]

Milk proteins are key ingredients in a number of food formulations, but they find applications also in several other industries (pharmacy, cosmetic, etc.). β-lactoglobulin (β-LG) is one of the most widely studied mammalian milk proteins, together with β-casein,[8] and is a major milk whey protein. Interfacial properties of β-LG are similar to those of other globular proteins. At liquid/liquid and solid/liquid interfaces, interfacial unfolding has been evidenced in a number of studies[9−13] and is believed to be accompanied by exposition of reactive sulphydryl groups, which undergo slow surface polymerization.[9] However, for air/water interface, the interfacial unfolding is less obvious,[14,15] and some authors even suggest that only β-LG dimer dissociation (at neutral pH, β-LG exists as a dimer) takes place upon adsorption, without the subsequent unfolding.[16] In an alternative "colloidal approach", globular proteins are pictured as colloidal particles that do not unfold upon adsorption, and their slow adsorption kinetics is explained in terms of low probability of adsorbing a newly arriving protein molecule onto a limited number of available adsorption sites.[17] Whatever the molecular origin of these lengthy interfacial processes (with time scales of several hours), they lead to a highly elastic gel-like network formation at the interfaces, which seems to be a major long-term stabilizing factor for β-LG stabilized foams and emulsions.[18]

Despite applications in many branches of industry, such as food, beverage, personal care, and cosmetics,[19] where plant-derived saponins in mixtures with various proteins act as emulsifying agents, interfacial properties of such systems are

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still not sufficiently well characterized. More and more papers and patents are being devoted to applications of saponins, especially the soapbark tree (*Quillaja Saponaria* Molina) saponin: *Quillaja* Bark Saponin (QBS). However, only few publications report on QBS/protein[20] interfacial properties.[20−22] In our previous study,[21] we investigated short- and midterm adsorption kinetics of QBS at water/air interface. The results pointed to a nondiffusional control of QBS adsorption at water/air interface, the feature also observed recently by Stanimirova et al.[23] The presence of a positively charged globular protein-hen egg lysozyme (LYS) improves the kinetics of QBS adsorption at the water/air interface, probably due to the QBS/LYS interfacial complex formation. At low biosurfactant-to-protein ratios, the complexes showed a higher rate of surface tension decay than bare QBS or LYS, while at higher ratios, the interface was dominated by adsorption of uncomplexed QBS, in analogy to classical surfactant/protein mixtures. In contrast to LYS, β-LG used in the present study is negatively charged at employed pH, and its electrostatic interactions with QBS are thus expected to be less favorable. Nevertheless, binding of an anionic surfactant, SDS, to β-LG has been reported,[24] although as shown by dilational surface rheological study,[25] the secondary structure of the protein upon adsorption in the presence of anionic surfactants is much less disrupted than in the case of cationic ones. Therefore, solely on the basis of electrostatic considerations, much weaker attraction is expected for β-LG/QBS than for LYS/QBS.

In this work, the effect of β-LG on kinetics of adsorption of QBS at fluid/fluid interfaces will be described. In contrast to the previously described QBS/LYS mixtures,[21] in the present system, both protein and biosurfactant are negatively charged at the employed pH. The drop shape analysis was employed for studying interfacial tension of the mixtures at the water/air and water/tetradecane interfaces on medium time scale (5 s−45 min), and maximum bubble pressure method, for measurements on short time scales (50 ms−10 s) at the water/air interface. The predictions from dynamic interfacial tension data will be compared with foaming and emulsifying properties of the mixtures by measuring their foamability and emulsion drop size.

EXPERIMENTAL SECTION

Materials. *Quillaja* Bark Saponin (QBS, Figure 1), a mixture of triterpene-glycosides extracted from the bark of the tree *Quillaja saponaria* Molina was purchased from Sigma (84510). It consists of a triterpene backbone substituted at C-3 and C-28 with glucuronic acid and saccharides, respectively. Dissociated carboxylic acid group of the glucuronic acid residue determines the anionic nature of the molecule. Recently, Stanimirova et al.[23] found evidence of a nonionic character of QBS, as opposed to our previous report,[21] but it should be borne in mind that the two QBSs were obtained from different sources and may differ significantly in composition. There are over 60 known *Quillaja* saponins,[26] and for the purpose of this work, the QBS average molecular weight of M_w = 1680 Da was estimated on the basis of mass spectrometry data.[27]

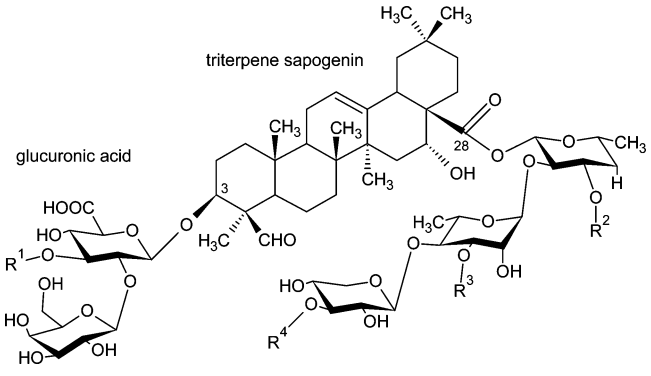

Figure 1. Chemical structure of QBS, R^1−R^4 represent either hydrogen or carbohydrate group.

β-lactoglobulin (β-LG) from bovine milk was purchased from Sigma (L0130). β-LG is a globular protein of a molecular weight M_w = 18 400 Da containing 162 amino acid residues with two disulfide bridges and one free cysteine group in its structure. Under conditions used in this study (pH = 7), β-LG forms dimers.[28] Because of its isoelectric point of 5.03 at neutral pH employed in this study, the net electrical charge of β-LG equals −5.[29] β-LG was stored at 4 °C and used as received, without further purification. Buffered solutions of β-LG and their mixtures with QBS were prepared in phosphate buffer (pH 7, ionic strength 0.1 M) using phosphate buffer powder purchased from Fluka (P7994) and ultrapure water (Millipore Synergy UV). The surface purity of phosphate buffer solution was checked before each preparation by performing a dynamic surface tension measurement for 45 min, the maximum time of all subsequent experiments. All solutions were prepared fresh before experiment. The oil phase used was tetradecane purchased from Sigma (172456). It was used without further purification because of the absence of surface active impurities confirmed by constant water/oil interfacial tension of 52 mN m^{-1} during 45 min. All the glassware was cleaned with Hellmanex II solution from Hellma, Inc. and rinsed with copious amounts of ultrapure water.

Methods. Dynamic surface tension on the short time scale (<10 s) was measured using a maximum bubble pressure tensiometer BP-1 (Kruess, Germany). A Teflon (ϕ = 1.2 mm) capillary was employed because the phosphate buffer components irreversibly adsorbed on the surface of glass capillaries (both bare and silanized), making it completely wettable for water, and thus preventing any reliable measurements. Axisymmetric drop shape analysis (ADSA) tensiometers: CAM-200 (KSV, Finland) and PAT-1 (SINTERFACE, Germany) were used for midterm (<45 min) dynamic interfacial tension measurements at water/air and water/oil interfaces, respectively. The stainless steel capillary of CAM-200 was fitted with a Teflon tube to avoid wetting of the steel capillary tip by the phosphate buffer solution in water/air experiments. On the contrary, we did not observe any problems related to the capillary wetting in water/tetradecane measurements.

Foamability was assessed by a modified Bikerman's method using a homemade device described in ref 30 (Figure 2). In brief, nitrogen gas was introduced at constant flow (20 dm^3 h^{-1}) into a column filled with the test QBS or QBS/β-LG solution through a porous sintered glass. After 30 s of bubbling, the foam height was read.

Emulsions were prepared by adding tetradecane (0.2% v/v) to a test QBS or QBS/β-LG solution, followed by sonication with the power of 34 W at 20 kHz using Sonopuls HD 2070 (BANDELIN, Germany) for 5 min. Initially, homogenization using Ultra-Turrax T25D (IKA, Germany) at 4000−19 000 rpm prior or instead of sonication was employed, but this step always resulted in increased foaming of emulsion and has been

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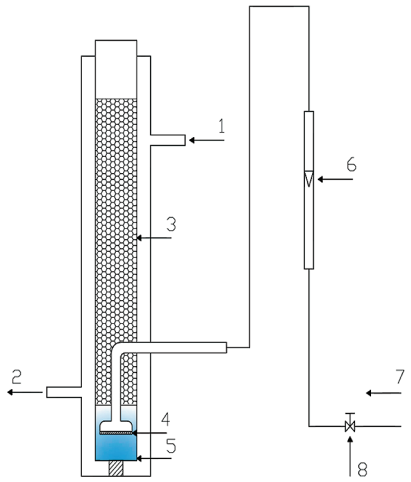

Figure 2. Scheme of an apparatus employed for foam formation measurements: 1, 2, thermostatted water inlet and outlet; 3, foam column; 4, sintered glass; 5, QBS/β-LG aqueous solution; 6, rotameter; 7, nitrogen gas inlet; 8, valve.

abandoned. Particle size of emulsions were measured with a Zetasizer 3000HS (MALVERN, UK) at 25 °C. All the experiments (dynamic surface tension, emulsion, and foam formation) were performed at 21 °C.

RESULTS

QBS and β-LG at Water/Air Interface. The short- and midterm adsorption kinetics of QBS/β-LG were probed by measuring dynamic surface tension using the maximum bubble pressure and axisymmetric drop shape analysis tensiometers, respectively. The β-LG concentration was fixed at 10^{-7} M, where the protein alone did not produce any appreciable change of surface tension on the time scale of our experiments (45 min). QBS concentration was varied in the range 5×10^{-7}–1×10^{-3} M. For the short-term measurements, the necessity of using a large diameter (ϕ = 1.2 mm) Teflon capillary prevented any reliable measurements for QBS concentrations above 1×10^{-4} M (for highly concentrated surfactant solutions, the use of such a wide capillary can introduce an error in the surface tension of more than 10%[31]). The combined short- and midterm results for QBS and QBS/β-LG at water/air interface are shown in Figure 3.

The effect of QBS on β-LG dynamic surface tension can be seen already at the lowest concentrations employed ($C_{QBS} = 5 \times 10^{-7}$ M and $C_{\beta\text{-LG}} = 10^{-7}$ M). When the biosurfactant and the protein are present alone at the respective bulk concentrations, the dynamic surface tension remains unchanged on the probed time scale. For bare QBS, no changes in dynamic surface tension were observed even until the concentration of 4×10^{-6} M.

With increasing QBS/β-LG ratio, the transient differences between the $\gamma(t)$ curves for QBS and QBS/β-LG become more pronounced, and their maxima shift to shorter times (see inset of Figure 3). At low QBS concentrations, the dynamic surface tension curves for the mixtures start to diverge significantly from those for pure QBS only in the final stages of adsorption. With increasing QBS concentrations, the beginning of this divergence shifts systematically to shorter times, and above the QBS concentration of 4×10^{-5} M is located in the short-term part of the dynamic surface tension curves, unavailable experimentally for high concentrations using the current

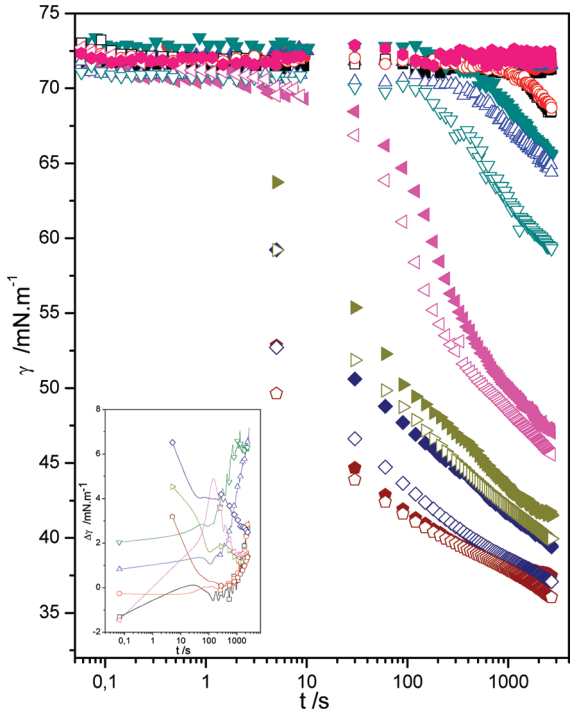

Figure 3. Water/air short- and midterm dynamic interfacial tension of QBS (closed symbols) and QBS/β-LG (open symbols) solutions. The concentration of β-LG was kept constant (1×10^{-7} M), while the QBS concentration was varied: 5×10^{-7} M (square); 1.5×10^{-6} M (circle); 4×10^{-6} M (rectangle); 1×10^{-5} M (downward facing triangle); 4×10^{-5} M (left facing triangle); 1×10^{-4} M (right facing triangle); 4×10^{-4} M (diamond); 1×10^{-3} M (pentagon). Pure β-LG (1×10^{-7} M) (hexagon).

setup. In general, the higher the QBS/β-LG ratio, the shorter the time required for the two dynamic curves (with and without β-LG) to diverge. In all cases, the presence of the protein seems to enhance the rate of adsorption of the mixtures, as it has been observed previously for QBS/LYS mixtures,[21] and several mixtures of synthetic surfactants with proteins.[32] In order to quantify this effect, an apparent diffusion coefficient for QBS/β-LG complex was estimated from the short-term dynamic surface tension data using a $t \to 0$ approximation of the Ward–Tordai equation combined with the Henry equation of state[33]

$$\left(\frac{d\gamma}{dt^{1/2}}\right)_{t\to 0} = -\frac{2nRTc}{\pi^{1/2}}(D^{1/2}) \quad (1)$$

where t is time, c is the bulk surfactant concentration, D is the diffusion coefficient, n = 1 for nonionic surfactants and n = 2 for ionic surfactants, R is the gas constant, and T is temperature.

The best-fit values of apparent diffusion coefficient, D_{app}, for the QBS/β-LG complex together with those obtained previously for QBS and QBS/LYS are shown in Table 1. Since QBS is not a strong electrolyte (a weakly acidic carboxylic group is responsible for its ionic character), in the presence of additional electrolyte (phosphate buffer) n should take some intermediate values between 1 (assuming that QBS behaves as nonionic surfactant) and 2 (assuming that QBS behaves as ionic surfactant). For this reason, two values of D_{app} are given in Table 1, for n = 1 and n = 2. A slightly higher value of diffusion coefficient for QBS/β-LG than for bare QBS corresponds well to the picture from Figure 3.

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Table 1. Apparent Diffusion Coefficients, D_{app}, for the QBS/β-LG Obtained from the Short-Term Dynamic Surface Tension Measurements, Together with the Corresponding Values for Pure QBS and QBS Mixtures with Hen Egg Lysozyme (QBS/LYS).[21]

	D_{app} (m^2 s^{-1})	
surfactant/protein	$n = 1$	$n = 2$
QBS	4.8×10^{-11}	1.2×10^{-11}
QBS/β-LG	5.6×10^{-11}	1.4×10^{-11}
QBS/LYS	1.3×10^{-10}	3.2×10^{-11}

Whatever is the molecular origin of this effect, adsorption of both QBS and QBS/β-LG is not controlled by diffusion.[21,23] In our case, the short-term approximation for the $\gamma(t)$ at water/air would produce unrealistically small values of apparent diffusion coefficient for bare β-LG, most likely due to the typical for some proteins induction time, when the surface pressure does not change, despite increasing surface concentration. However, the presence of even low quantities of β-LG affects the rate of surface pressure increase in QBS solutions, but mostly at intermediate and later stages of adsorption, for $t \rightarrow \infty$. For that reason, this effect is not fully reflected in the moderately higher value of the apparent diffusion coefficient of QBS/β-LG at the water/air interface, as determined from the short-term data (using an asymptotic solution to the Ward−Tordai equation valid for $t \rightarrow 0$).

Since the major driving force for studying the protein/biosurfactant interactions is a potential for practical applications, a foam formation ability of saponin and saponin/protein mixtures was also assessed. For this purpose, a modified Bikerman's method, as described in the experimental section, was employed, and the foam heights obtained after passing equal amounts of gas through the QBS and QBS/β-LG solutions are compared in Figure 4.

Adsorption of Saponin in the Presence of β-Lactoglobulin at Water/Tetradecane Interface. Water/oil interfacial tension dynamic decays for QBS and QBS/β-LG solutions were studied using an axisymmetric drop shape analysis technique. As a representative oil phase, tetradecane was chosen because its alkyl chain length is comparable to that of typical phospholipids constituting biological membranes. The results (Figure 5) show similar, but not identical trends as

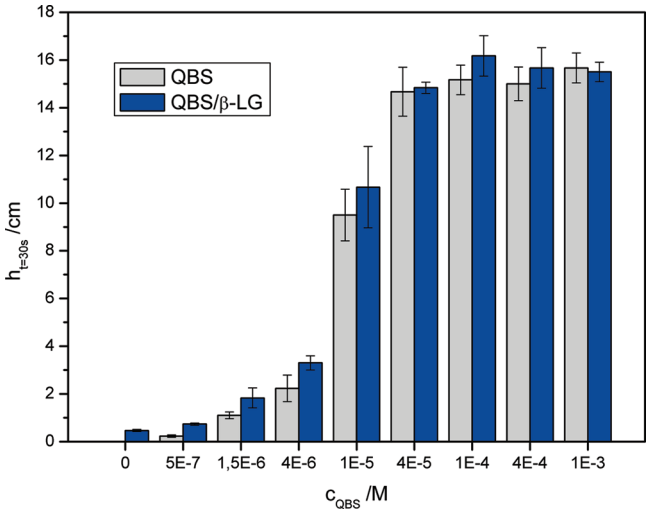

Figure 4. Foam heights obtained using a modified Bikerman's method after 30 s of bubbling nitrogen gas through the aqueous solutions of QBS and mixtures of QBS with β-LG. The concentration of β-LG in mixtures was constant (1×10^{-7} M), while the QBS concentration was varied in the range of surface tension measurements: 5×10^{-7}−1×10^{-3} M. The first bar ($C_{QBS} = 0$) represents pure β-LG solution.

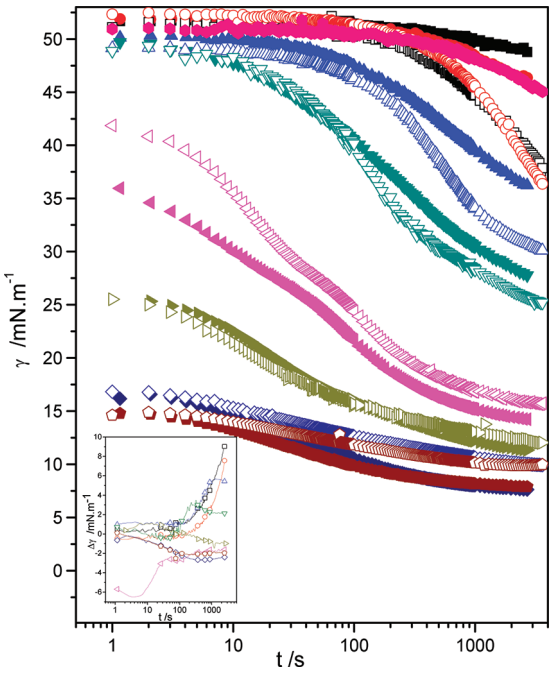

Figure 5. Water/tetradecane midterm dynamic interfacial tension of QBS (closed symbols) and QBS/β-LG (open symbols) solutions. The concentration of β-LG was constant (1×10^{-7} M), while the QBS concentration was varied: 5×10^{-7} M (square); 1.5×10^{-6} M (circle); 4×10^{-6} M (rectangle); 1×10^{-5} M (downward facing triangle); 4×10^{-5} M (left facing triangle); 1×10^{-4} M (right facing triangle); 4×10^{-4} M (diamond); 1×10^{-3} M (pentagon) [M]. Pure β-LG (1×10^{-7} M) (hexagon).

those for the water/air interface: at low biosurfactant concentrations, β-LG increases the adsorption rate of QBS mostly at later stages of the adsorption process ($t \rightarrow 45$ min). However, at intermediate QBS concentration, surprisingly, the protein has a decelerating effect on the QBS/β-LG mixture's adsorption kinetics at all times. At higher QBS concentrations, the effect of the protein is negligible at initial stages of adsorption and slightly decelerating at its later stages. This behavior is highlighted in the inset of Figure 5, where the differences between $\gamma(t)$ of the respective QBS and QBS/β-LG solutions are shown. Overall, a significantly higher decrease of interfacial tension (i.e., higher surface pressures) is observed, in comparison to the water/air interface.

In line with foam stabilization at the water/air interface, the ability to stabilize oil-in-water emulsion is another important prerequisite for any biosurfactant to become a potential replacement for its synthetic counterparts. The interfacial tension studies described above proved that QBS adsorbs to an even higher extent at the water/tetradecane than at the water/air interface. Even though no simple relationship exists between the interfacial tension and the ability to stabilize emulsions,[34] the lower interfacial tension certainly helps to overcome the energy barrier related to an increase of surface area associated with emulsion formation.

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In order to verify the ability to stabilize emulsions by both QBS and its mixtures with β-LG, the diluted tetradecane-in-water emulsions (0.2% w/w) were prepared by sonication of the respective aqueous solutions with tetradecane. The size distribution was measured by dynamic light scattering of emulsions freshly after their preparation, and after one and two hours of storage at room temperature. The results are presented as the Sauter mean diameter, d_{32}, in Figure 6. In full agreement with the interfacial tension results, QBS alone is able to stabilize tetradecane emulsion droplets even at very low bulk concentrations (5×10^{-7} M, Figure 6, top). The size of the droplets initially increases with an increase of the QBS concentration, to reach a maximum around 4×10^{-5} M, i.e., close to the maximum surface coverage, just below the cmc. Further increase of concentration causes a slight reduction of the droplet size in freshly prepared emulsions. In the whole range of QBS concentrations, the average size of emulsion droplets is not changed drastically within two hours of storage. In the presence of constant concentration of β-LG (Figure 6, bottom), freshly prepared emulsions behave differently: at low QBS concentrations, the droplets are smaller than without the protein, and at high concentrations, especially above cmc, the droplets are generally larger (although some scattering of the data can be observed). At low QBS concentrations, the droplets seem to grow with time, while at high QBS, they shrink.

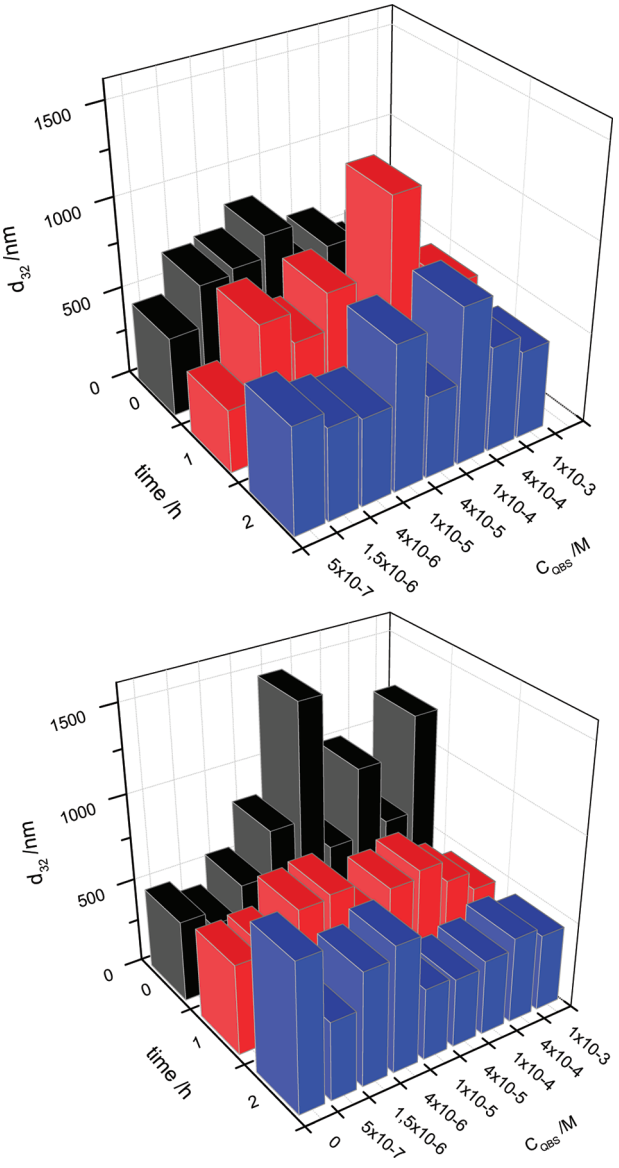

Figure 6. Average size (Sauter mean diameter, d_{32}) of tetradecane-in-water emulsion droplets stabilized by QBS alone (upper graph) and a mixture of β-LG ($C_{\beta\text{-LG}} = 10^{-7}$ M) with QBS as a function of QBS concentration for freshly prepared emulsion ($t = 0$), and after 1 and 2 h storage at room temperature.

DISCUSSION

In order to facilitate the comparison between interfacial behavior of QBS/β-LG mixtures at water/air and water/oil interfaces, the dynamic interfacial tension data from Figures 3 and 5 are redrawn as dynamic surface pressures in Figure 7. For all QBS concentrations, the surface pressures are higher at the water/oil interface, although this feature seems to be inherited mostly from QBS adsorption, which will be discussed in more detail in a separate paper. While at the water/air interface, β-LG at a concentration of 1×10^{-7} M exerts no surface pressure on the time scale of our measurements, replacement of air by the nonpolar oil phase significantly enhances the β-LG's surface affinity. This effect has been observed previously for β-LG[35,36] and many other proteins and is related to a good solvation of hydrophobic parts of β-LG by nonpolar oils.[10,37] Provided that the oil phase is sufficiently hydrophobic[38] (e.g., tetradecane, as used in our study), stabilization of the hydrophobic parts of the protein molecule by the oil phase favors its unfolding, which results in an increase of lateral (surface) pressure in the adsorbed layer. The faster and more pronounced increase of surface pressure at low QBS/β-LG ratios observed at water/tetradecane interface suggests that the biosurfactant/protein complexation facilitates the protein dimer dissociation and unfolding. However, at high QBS/β-LG ratios (>100), the opposite is observed, even a tiny amount of protein adsorbed at the water/oil interface decelerates adsorption of biosurfactant, probably by blocking its access to the interface. This was not observed at the water/air interface, where the β-LG unfolding is less pronounced on the time scale of our experiments, and the adsorbed protein layer is not inhibiting further adsorption of QBS. This observation is in good agreement with some suggestions that the globular structure is largely preserved at the water/air interface.[14] Perriman et al.[16] on the basis of their X-ray and neutron reflectivity results even suggested that only the β-LG dimer dissociation is possible at the water/air interface, with no consequent unfolding, unless a strong chemical denaturant is present in the solution. The question of blocking the interface by a protein requires some comment at this stage. In most of the previous studies, where small synthetic surfactants have been used in combination with proteins, the diffusion time scales of surfactant and protein had been well separated. Since most of the small amphiphiles adsorb to fluid/fluid interfaces with no or little barrier, the surfactants arrived first to (a fresh) interface, followed by proteins. Because of the large adsorption barrier observed for QBS ($1.2 \times 10^{-11} < D_{app}^{QBS} < 4.8 \times 10^{-11}$ m^2 s^{-1}, see Table 1), much higher than for typical small surfactants (e.g., $D_{app}^{SDS} = 3 \times 10^{-10}$ m^2 s^{-1}, $D_{app}^{Triton\ X\text{-}100} = 4 \times 10^{-10}$ m^2 s^{-1}[39]), in the present setup, the situation is different: there is little or no delay of the protein with respect to the biosurfactant. Hence, the situation corresponds more to that encountered in experiments with surfactant/protein systems in which the surfactant is added to a preformed protein layer.[40]

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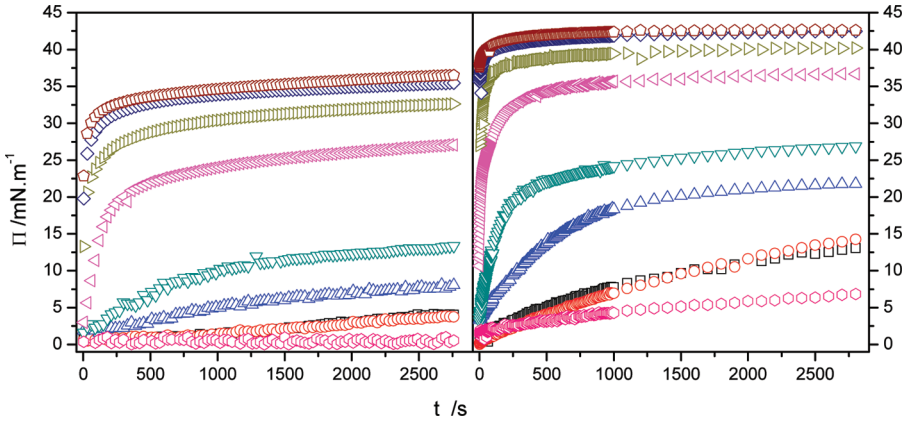

Figure 7. Midterm water/air (A) and water/tetradecane (B) surface pressure of β-LG (1×10^{-7} M) (hexagon) and QBS/β-LG solutions. The concentration of β-LG in mixtures was constant (1×10^{-7} M), while the QBS concentration was varied: 5×10^{7} (square); 1.5×10^{-6} (circle); 4×10^{-6} (rectangle); 1×10^{-5} (downward facing triangle); 4×10^{-5} (left facing triangle); 1×10^{-4} (right facing triangle); 4×10^{-4} (diamond); 1×10^{-3}(pentagon) [M].

At neutral pH, β-LG exists in solution predominantly as a dimer, which dissociates upon denaturation.[41] Binding small hydrophobic ligands (retinol, retinoic, and fatty acids, and even cholesterol) inside its central hydrophobic pocket (the calyx) strengthens even more this dimeric structure.[42] Noiseux et al. reported on the binding of peptides by β-LG and suggested that even the large ones (with molecular masses exceeding those of QBS) can bind inside the cavity, provided they are sufficiently hydrophobic.[43] However, the existence of a second binding site, situated on the surface of the β-LG's β-barrel has been postulated by several authors.[42,44,45] The latter can host small molecules (like SDS[24]), but also larger ones, which cannot be accommodated in the central hydrophobic pocket. Some authors suggest that the availability of this external binding site is higher in dissociated (monomeric) β-LG.[46] Upon denaturation, the β-LG molecule exposes some of the hydrophobic parts[47] (which normally constitute the calyx) and becomes more surface active.[48] It is thus expected that binding of large molecules on the surface of β-LG might enhance the protein's surface activity.

In view of the above, the mode of saponin binding to β-LG seems to be crucial to the understanding of the dynamic interfacial tension data for QBS/β-LG mixtures. The saponin used in the present study is very well soluble in water and is probably not sufficiently hydrophobic to be accommodated inside the calyx. This supposition is supported by the studies of enzymatic hydrolysis of β-LG in the presence of retinol and soybean saponin.[49] In retinol/β-LG mixtures, where retinol is known to locate in the calyx and stabilize the β-LG dimer, the protein's resistance to trypsin hydrolysis, as well as to heating and light irradiation, increases. In the case of saponin/β-LG mixtures, however, the opposite is observed: the protein's rigid β-barrel structure loosens, facilitating the attack of proteases, an effect assigned by the authors to binding of saponin onto the surface site of β-LG. The saponin-enhanced passage of β-LG through the small intestine in rats observed by Gee et al.[50] also seem to support the hypothesis of saponin binding to β-LG.

The apparent diffusion coefficient of β-LG found from the short-term approximation of the diffusion-based Ward−Tordai equation unexpectedly gave values 2−3 orders of magnitude higher than the bulk ones.[8] This is in clear contrast to our present results, where the short-term approximation would produce too low a value of D_{eff} for pure β-LG. The difference most likely stems from the fact that, in our system, the data on a time scale between 50 ms and 10 s were used, while in ref 8, a much longer-term data were employed (10−1000 s), which in our view do not necessarily fulfill the $t \rightarrow 0$ condition. The short-term data employed in this work lie largely within the induction period, where surface tension does not decay appreciably. Consequently, D_{eff} for pure β-LG would be largely underestimated, so we do not report these values here. However, adsorption of β-LG, as many other globular proteins, is not diffusion controlled at longer times. In the bulk, typically, the radius of gyration of 2.3−2.7 nm is found for β-LG dimer. This corresponds to the values of diffusion coefficient, D, between 7.48×10^{-11} m^2 s^{-1} and 8.65×10^{-11} m^2 s^{-1}.[35,51−53] The fluorescence correlation spectroscopy study of β-LG apparent diffusion coefficient at aqueous/oil interface produced, however, the values about 20 times lower than the bulk value. This suggests that the slow interfacial tension decays do not stem from a slow diffusion to the interface but from some adsorption barrier during the actual adsorption process, e.g., slow dimer dissociation and/or unfolding. The increased rate of interfacial tension decays described above is in full agreement with partial disruption of the ternary structure of β-LG by QBS. Loosening the rigid β-barrel structure speeds up the subsequent unfolding of β-sheets, which always accompanies β-LG adsorption at water/air and water/oil interfaces.[9,11−13] Binding of QBS to the surface of β-LG would thus have a 2-fold effect on the kinetics of β-LG adsorption: promoting dissociation of the protein dimer and loosening its rigid structure, both effects facilitating further unfolding upon reaching the interface and increasing the rate of surface pressure increase.

More enigmatic remains the question of an enhanced (at water/air interface) and reduced (at water/oil interface) rate of surface pressure increase at high QBS/β-LG ratios, where adsorption is dominated by QBS. Although acceleration of the surfactant adsorption has been observed even for non-interacting (hence adsorbing competitively) protein−surfactant pairs, e.g., β-LG/Tween 20 at low ($\leq$10) surfactant/protein ratios,[32] in our system, the effect can be observed at QBS/β-LG ratios as high as 10^4. The most likely reason of the reduction of the rate of interfacial tension decay at the water/oil interface is an electrostatic repulsion between the negatively charged QBS and β-LG domains. At neutral pH employed for this study, each β-LG molecule possesses on average five uncompensated

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negative charges, which may be differently exposed to the surrounding. The fact that the repulsion is more pronounced at water/oil interface points to different orientation and/or degree of unfolding, as compared to the water/air interface. The markedly different effect of β-LG on the rate of surface pressure increase at both interfaces in combination with the fact that in both cases the diffusion toward interface takes place in the same aqueous phase, points to the interface as the main source of the adsorption barrier. If any QBS aggregates existed in the aqueous phase, their dissociation by β-LG would be independent of the contacting nonaqueous phase. Therefore, β-LG most likely influences the actual adsorption of QBS, not its transport to the interface. Apparently, when the protein is adsorbed at the water/air interface, the weakly unfolded structure facilitates adsorption of QBS. The same seems to be true for water/oil structures but only at low QBS concentrations. The high extent of unfolding of the protein at water/tetradecane interface allows for only limited number of QBS to be adsorbed, and above certain concentration ($\sim 1 \times 10^{-5}$ M), any further adsorption is decelerated; hence, the rate of surface pressure increase drops.

No clear correlation exists between the dynamics of interfacial tension decays and the foam and emulsion formation ability. The differences in foam heights between the QBS and QBS/β-LG solutions are rather small, but the presence of the protein seems to enhance slightly the foaming ability of QBS, especially at low biosurfactant concentrations (5×10^{-7}–1×10^{-5} M). Similar to the pure QBS solutions, the foaming ability increases drastically around the concentration of 10^{-5} M, which corresponds to the onset of the steep descent of the QBS adsorption isotherm.[21] Around this concentration an appreciable decrease of dynamic surface tension curves for QBS and QBS/β-LG (Figure 3) appears within the time scale of the foaming experiment (30 s). It is worth noting that, in analogous experiments with QBS/LYS mixtures,[21] similar effects have been observed, although the effect of protein was much more pronounced than for β-LG.

The emulsion formation ability and short-term stability are probably linked to some transient processes in adsorption of QBS/BLG mixtures, which are also visible (although less pronounced) in interfacial tension measurements. At low QBS concentration, the synergistic effects in dynamic interfacial tension at the water/tetradecane interface are most pronounced at longer adsorption times (Figure 5). Apparently, the surface complex that brings additional reduction of the interfacial tension at later stages of adsorption is less capable of stabilizing the emulsion droplets, hence their growth with time at low QBS concentrations. These low-QBS emulsions behave similarly to that stabilized solely by the protein (C_{QBS} = 0, Figure 6), where the emulsion droplet distributions broaden with time.

CONCLUSIONS

Mixtures of *Quillaja* Bark Saponin (QBS) with β-lactoglobulin (β-LG) display both synergistic and antagonistic effects at water/air and water/oil interfaces due to protein/biosurfactant interactions. Depending on the biosurfactant/protein molar ratio, the rate of interfacial tension decay is affected by the presence of β-LG, mostly at later stages of adsorption. At the water/tetradecane interface, the surface pressures attained are higher than at the water/air one, although at high QBS concentrations, the presence of the protein reduces the surface pressures. This effect is probably related to blocking the water/oil interface by the unfolded protein, which can electrostatically repel the negatively charged biosurfactant molecules. QBS probably binds to a ligand binding site on the surface of β-LG, thus facilitating its dissociation (β-LG at neutral pH exists in a dimeric form) and unfolding. However, QBS adsorbs with a relatively large adsorption barrier, which is partially removed in the presence of β-LG. In contrast to most of the typical systems, in the present protein/(bio)surfactant combination, both components have comparable time scales for adsorption. Consequently, even tiny amounts of the protein (10^{-7} M) may have an effect on the kinetics of adsorption of the biosurfactant at QBS/β-LG ratios even as high as 10^4. This effect is probably not related to diffusion of the QBS/β-LG complexes in the bulk phase but to the actual adsorption process. The addition of a small amount (10^{-7} M) of β-LG has only a minor effect on the foamability as well as the emulsion formation and short-term stability of QBS solutions.

AUTHOR INFORMATION

Corresponding Author

*E-mail: kamil.wojciechowski@ch.pw.edu.pl.

Notes

The authors declare no competing financial interest.

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