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Competitive adsorption behavior of β-lactoglobulin, α-lactalbumin, bovin serum albumin in presence of hydroxypropylmethylcellulose. Influence of pH

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ABSTRACT

The interfacial properties at the air–water (A/W) of each individual whey proteins (β-lactoglobulin, β-lg; α-lactalbumin, α-la; bovin serum albumin, BSA), and their mixtures with a surface-active polysaccharide, hydroxypropylmethylcellulose (HPMC) were studied at pH 3 or 6. The interfacial films were studied by measurement surface pressure (π) isotherms and dynamics of adsorption. At equilibrium proteins surface activity was affected by pH only at low concentrations (below $1{\cdot}10^{-2}$ % wt/wt), due to their pH-dependent conformational changes. HPMC resulted less surface active at pH 3 (below $1{\cdot}10^{-4}$ % wt/wt concentration) that at pH 6. On kinetic studies (π–t), the behavior of β-lg, HPMC and BSA did not change with pH but α-la presented a higher surface activity at pH 3 than 6, even on saturating bulk concentrations. Mixtures of β-lg or BSA with HPMC showed a behavior in between that of single components revealing a net competence for the interface but the mixture α-la and HPMC at pH 6 showed an enhance adsorption. Rheological studies (surface dilatational elastic, E_d, over time) presented the major differences for pHs evaluated. The α-la formed extremely viscoelastic films at pH 6.0, while at pH 3 has the lowest E_d value. β-lg and HPMC films were more viscoelastic at pH 6, being E_d protein film higher. Finally, BSA presented the lowest viscoelastic films without differences between both pHs. For mixtures: i) at pH 6 β-lg/HPMC mixture E_d was dominated by HPMC; at pH 3.0, E_d begins dominated by HPMC, reaching an intermediate value; ii) α-la/HPMC mixture formed more viscoelastic films at pH 6.0 with an intermediate E_d value, while at pH 3.0 the E_d is dominated by protein; iii) BSA/HPMC mixture presented a similar trend in E_d behavior at both pHs.

1. Introduction

Proteins and polysaccharides are usually present together in food products, contributing to stability, texture and shelf life of those products. The formation and stability of food dispersions (foams and emulsions) are quality parameters in a wide range of food products and are determined by the interfacial properties of their components (proteins, polysaccharides and other surfactants), as well as by the interactions between them. Proteins contribute principally to texture and stability by their surfactant character and gelling properties (Dickinson, 2003). On the other hand, polysaccharides are mainly used by their thickening and/or gelling characteristics, derived from their hydrophilic character and their ability to establish ionic interactions (Baeza, Carrera Sánchez, Pilosof, & Rodríguez Patino, 2004).

The surfactant properties of proteins lie in its amphiphilic nature: they are able to constitute an interfacial monolayer since they can orientate their hydrophobic segments to the hydrophobic phase (air or oil), while their hydrophilic regions will be orientated to the aqueous phase (Lankfeld & Lyklema, 1972). Moreover, the number of segments that are distributed in the interface will depend on the molecular flexibility and on the protein affinity for the sub-phase (Dickinson, 1992, pp. 140–173) as well as their interaction to form a viscoelastic film.

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In general, high MW polysaccharides with mainly hydrophilic nature do not have a great tendency to adsorb at an air–water interface. However, those polysaccharides derived from cellulose constitute an exception since the introduction of hydrophobic groups allows them to behave as surfactants. In the particular case of hydroxypropylmethylcellulose (HPMC), those groups are the hydroxypropyl and methyl substitutes. Thus, this macromolecule is capable of adsorbing at the air and oil–water interfaces (Ochoa-Machiste & Buckton, 1996; Wollenweber, Makievski, & Daniels, 2000). Pérez, Carrera Sanchez, Rodríguez Patino, and Pilosof (2006) determined the surfactant properties and structural characteristics of the surface films formed by three commercial HPMCs (denominated E4M, E50LV and F4M). These authors found that HPMC molecules are capable of diffusing and saturate the air–water interface at very low bulk concentrations. Additionally, the three HPMC types formed very elastic films (Pérez, Carrera Sanchez, et al., 2006; Pérez, Carrera Sánchez, Rodríguez Patino, & Pilosof, 2007; Pérez, Carrera Sánchez, Rodríguez Patino, & Pilosof, 2008). Thus, due to its surfactant character, HPMC could be adsorbing in a competition with proteins. In particular, the adsorption process was deeply studied for mixtures of WPC with three commercial HPMCs (E4M, E50LV, F4M) (Pérez, Carrera Sánchez, Pilosof, & Rodríguez Patino, 2009). In presence of E4M a net competence for interface can be observed at short adsorption time, while an enhance adsorption was observed for celluloses E50LV and F4M as surface pressure was higher that each single component. Such differences were attributed to differences in the molecular weight and degree and molar substitution among the HPMCs (Pérez et al., 2007).

Although these mixed systems are quiet known, the properties of protein/polysaccharide mixtures interfacial films are not fully characterized. In particular, is important to study the relationship between interfacial film properties and the aqueous phase characteristics, since the interfacial properties of mixed films are affected by the interaction between biopolymers: the understanding of the relationship between the phase behavior of a protein/polysaccharide aqueous mixture and the interfacial mixed films properties results of crucial interest for the technological and functional development of food dispersions (Dickinson, 2003; Murray, 2002).

Thus, the current research aims to assess the relationship between aqueous protein/polysaccharide mixtures phase behavior and the interfacial properties, adjusting the mixtures pH to promote the biopolymers thermodynamic compatibility or incompatibility. The proteins selected were β-lactoglobulin (β-lg), α-lactalbumin (α-la), and bovine serum albumin (BSA), whereas the polysaccharide selected was E50LV, which is a hydroxypropylmethylcellulose widely used.

2. Materials and methods

2.1. Materials

BioPURE β-lactoglobulin was supplied by DAVISCO Foods International, Inc. (Le Sueur, MN). Its composition was: protein (dry basis) 97.8% being β-lactoglobulin 93.6% of total proteins, fat 0.3%, ash 1.8% and moisture 5.0%.

Lacprodan® Alpha-10, provided by Arla Foods Ingredients (Viby, Denmark), presented α-lactalbumin protein (dry mass) 88 ± 4.5%, lactose max. 10%, fat max. 2%, ash max. 5%, moisture max. 5.5%.

Bovine serum albumin (BSA) was purchased from Sigma–Aldrich (product number, A6003; batch 048K7400, Saint Louis, MO). As stated by the manufacturer, the composition of this lyophilized powder was: ≥96% BSA, ≤5.0% water and 0.01% free fatty acid.

Commercial hydroxypropylmethylcellulose (HPMC) Methocell® E50LV (Dow Chemical Company®) was gently donated by Colorcon-Argentina. This is a food quality HPMC and was used without further purification.

The whole glass material used to prepare any solution was perfectly washed with detergent, rinsed with distilled water and treated with a chromo-sulfuric blend (concentrated sulfuric acid and ammoniac per-sulfate, 8 g/L). Finally, it was rinsed again with distilled water. This procedure eliminates grease residues and avoids any contamination by any surface-active substance.

2.2. Stock solutions and mixed systems

Proteins stock solutions (4% wt/wt) were prepared in phosphate buffer (pH 6.0, 50 mM) and in acetate buffer (pH 3.0, 50 mM). The appropriate mass powder was weighted and dispersed in the buffer at 25 °C, with continues agitation for 30 min to allow complete dissolution. A few drops of NaN_3 (0.2N) were added as an antimicrobial agent and pH was adjusted with HCl (1N) or NaOH (1N) if necessary.

E50LV stock solutions (1% wt/wt) were prepared by dispersing the appropriate mass powder in phosphate or acetate buffers previously heated to 85 °C, with agitation to allow complete dissolution of the powder. Then, the solutions were cooled at ambient temperature and stored at 4 °C over night to allow the polysaccharide to reach its maximum hydration.

2.3. Surface pressure isotherm

Surface tension measurements were registered by the Wilhelmy plate method, using a rectangular platinum plate with an exactly known geometry, vertically suspended and attached to a Sigma 701 digital tensiometer (KSV, Finland). The lower edge of the plate is put into contact with the sample liquid and the surface tension is measure as the force (F) that experiments the plate toward the liquid. The surface tension (γ) is then defined as $\gamma = F/(L_b \cdot \cos\theta)$, where L_b is the wet length and θ is the contact angle (between the plate surface and the tangent to the wet line). The temperature of the system was maintained constant at 25 °C within ±0.5 °C by a circulating Heto thermostat. A device connected to the tensiometer recorded the reduction in surface tension, γ, continuously. Equilibrium was assumed to be reached when the surface pressure did not change by more than 0.1 mN/m in 45 min.

Surface activity was expressed by the surface pressure, $\pi_{eq} = \gamma_o - \gamma_{eq}$, where γ_o and γ_{eq} are the aqueous sub-phase surface tension (72.0 ± 0.5 mN/m) and the surface tension of the biopolymer solutions at equilibrium, respectively. With the obtained surface tension data at the equilibrium, the isotherms of surface pressure were constructed by plotting the surface pressure against the logarithmic biopolymer bulk concentration.

In this experiment biopolymers were studied individually in a concentration range from $1 \cdot 10^{-1}$ to $1 \cdot 10^{-8}$ % wt/wt at pH 3.0 or 6.0. Before measurements these solutions were stored at 4 °C for 24 h to achieve the biopolymer adsorption. Measurements were replicated for at least 3 times.

2.4. Dynamic interfacial properties

Time-dependent surface pressure and surface viscoelastic parameters of β-lg, α-la, BSA, HPMC, and protein/HPMC mixed systems films at the air–water interface were determined with an automatic drop tensiometer (Tracker, IT Concept, Longessaigne, Francia) as described elsewhere (Rodríguez Niño & Rodríguez Patino, 2002).

Protein/HPMC mixed solutions at the desired pH were prepared by mixing the appropriate volume of each stock solution in order to obtain the systems protein (2% wt/wt)/HPMC (0.5% wt/wt)

(saturating interface concentrations). The mixture was agitated for 30 min to achieve a homogeneous distribution of both components and pH was controlled again. Then, each mixed solution was placed in a 500 μl glass Hamilton syringe equipped with a stainless steel needle and then in a rectangular glass cuvette (5 ml) covered by a compartment, which was maintained at constant temperature (20 ± 0.2 °C) by circulating water from a thermostat, and was allowed to stand for 30 min to reach constant temperature and humidity in the compartment. Then a drop of sample solution (5–8 μl) was delivered and allowed to stand at the tip of the needle for about 180 min to achieve biopolymer adsorption at the air–water interface. The image of the drop was continuously taken from a CCD camera and digitized. The surface tension (γ) was calculated by analyzing the profile of the drop (Rodríguez Niño & Rodríguez Patino, 2002). The surface pressure is $\pi = \gamma_o - \gamma$, where γ_o is the surface tension of pure water in the absence of any surface-active component and γ the time-dependent surface tension of biopolymer solutions.

The surface viscoelastic parameters (surface dilatational modulus, E, and its elastic, E_d, and viscous, E_v, components) were measured with the same drop tensiometer (Rodríguez Patino, Rodríguez Niño, & Carrera Sánchez, 1999) as a function of time, θ, at 10% deformation amplitude ($\Delta A/A$) and 100 mHz of angular frequency (ω). The method involved a periodic automated controlled, sinusoidal interfacial compression and expansion performed by decreasing and increasing the drop volume, at the desired amplitude ($\Delta A/A$). The sinusoidal oscillation for surface dilatational measurement was made with five oscillation cycles followed by a time of 50 cycles without any oscillation up to the time required to complete adsorption. The average standard accuracy of the surface pressure is roughly 0.1 mN/m. However, the reproducibility of the results (for at least two measurements) was better than 0.5%. The surface dilatational modulus derived from the change in interfacial tension (dilatational stress), σ (Eq. (1)), resulting from a small change in surface area (dilatational strain), A (Eq. (2)), may be described by Eq. (3) (Lucassen & Van Den Tempel, 1972):

$$\sigma = \sigma_0 \sin(\omega\theta + \delta) \tag{1}$$

$$A = A_0 \sin(\omega\theta) \tag{2}$$

$$E = \frac{d\sigma}{dA/A} = \frac{d\pi}{d \ln A} \tag{3}$$

where σ_0 and A_0 are the stress and strain amplitudes, respectively, and δ is the phase angle between stress and strain.

The dilatational modulus is a complex quantity and is composed of real and imaginary parts (Eq. (4)). The real part of the dilatational modulus or storage component is the dilatational elasticity, $E_d = |E| \cos \delta$. The imaginary part of the dilatational modulus or loss component is the surface dilatational viscosity, $E_v = |E| \sin \delta$. The absolute modulus (E), a measure of the total unit material dilatational resistance to deformation (elastic + viscous) and δ is the phase angle between stress and strain. For a perfect elastic material the stress and strain are in phase ($\delta = 0$) and the imaginary term is zero. In the case of a perfectly viscous material, $\delta = 90°$ and the real part is zero. The loss angle tangent, tan δ, can be defined by Eq. (5). If the film is purely elastic, the loss angle tangent is zero.

$$E = E_d + iE_v \tag{4}$$

$$tg\delta = \frac{E_v}{E_d} \tag{5}$$

3. Results and discussion

3.1. Surface pressure isotherms of pure components

First, the equilibrium surface pressure of individual whey proteins and E50LV at pH 6.0 and 3.0 was determined as a function of biopolymer sub-phase concentration by the Wilhelmy tensiometer. The adsorption isotherms (π vs. concentration) for β-lg, α-la, BSA and E50LV are shown in Fig. 1a–d, respectively. The surface pressure isotherms for all the pure components showed a sigmoid behavior, typical of surfactants biopolymers (Álvarez Gómez & Rodríguez Patino, 2006; Pérez, Carrera Sanchez, et al., 2006; Pérez et al., 2007; Rodríguez Patino, Carrera Sánchez, & Rodríguez Niño, 2008).

In general, all biopolymers showed a surface pressure close to zero at low concentrations independently of pH: although the hydrophobic residues are located in the air–water interface but their number is too small to cause a noticeable decrease in the surface tension (Fig. 1a–d). At higher concentrations, a monolayer of adsorbed molecules with an "expanded" conformation is constituted, which then suffer a transition to a "condensed" structure as the interface biopolymer concentration increase. The pressure at which occurs this transition is the "critical pressure", π_{cr}. The pseudo-equilibrium is reached when the monolayer is saturated of molecules irreversible adsorbed. A pseudo-equilibrium is more appropriate to define this condition because is not possible to observed a real adsorption equilibrium. Thus, the pseudo-equilibrium is assumed to achieve when the surface pressure does not change after 24 h by more than 0.1 mN/m as compared with the values at 48 h (Benjamins, Lyklema, & Lucassen-Reynders, 2006; Rodríguez Niño & Rodríguez Patino, 1998). At even more concentrated conditions, biomolecules form multi-layers underneath the original monolayer (Graham & Philips, 1979; Pérez, Carrera Sanchez, et al., 2006).

As can be observed in Fig. 1a, β-lg shows a surface activity significantly higher at pH 6 than at pH 3 in the concentration range $5 \cdot 10^{-7}$–$1 \cdot 10^{-2}$ % (wt/wt). In this range, the surface pressure increased from 2.5 ± 0.5 to 17.3 ± 0.3 mN/m. Wüstneck, Moser, & Muschiolik (1999) found a similar behavior working with this protein at pH 7.0. Then, a gradual increase in the surface pressure was observed up to achieve the final pseudo-equilibrium state. At pH 6.0, β-lg undergoes the transition from "expanded" to "condensed" molecular structure at a π_{cr} of 13.5 ± 0.2 mN/m corresponding to a $5 \cdot 10^{-5}$ % wt/wt. Similar π_{cr} values were reported in literature for β-lg at pH 7 (Baeza et al., 2004; Wüstneck et al., 1999).

At pH 3.0 it was required a concentration higher than $1 \cdot 10^{-5}$ wt/wt to observe an increase in the surface pressure. Then, protein transition from "expanded" to "condensed" molecular structure occurred at a $\pi_{cr} = 11.5 \pm 0.2$ mN/m.

The reduced surface activity of β-lg at pH 3.0 below monolayer saturation may be attributed to pH-dependent conformational changes (Taulier & Chalikian, 2001). Among those changes, Tanford transition is the most important; moreover it is believed to be related with the biological function of the protein (mainly, fatty acids transportation). Tanford, Bunville, and Nozaki (1959) found that this conformational change is accompanied by a reversible proton lost in the carboxylic group of the glutamic acid located at position 89 (Glu89) at pH 7.5. This amino acid is on a loop, which forms one of the central cavity extremes. When Glu89 is protonated, it hides inside that cavity taking the β-lg protein to a "closed" structure, however if this residues is exposed and de-protonated, β-lg adopts the "open" form. This conformational change seams to control the β-lg ligand binding because this loop would regulate the access to central cavity (Qin et al., 1998). One of the main β-lg characteristics is its capacity to bind diverse hydrophobic ligands,

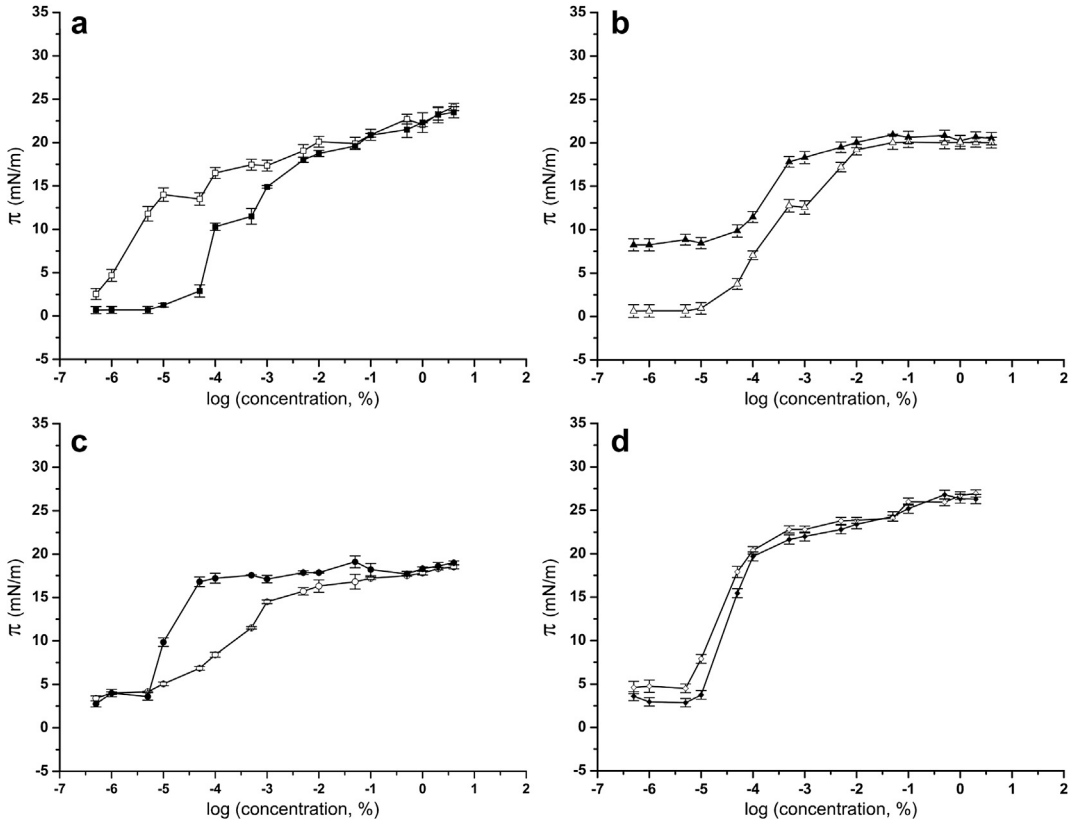

Fig. 1. Pure biopolymers surface pressure isotherms at the A/W interface at pH 6 (empty symbols) or 3 (full symbols): (a) β-lg (□, ■); (b) α-la (△, ▲); (c) BSA (○, ●); (d) E50LV (◇, ◆). Temperature 25 °C, $I = 5$ mM. Error bars indicate standard deviation.

like fatty acids, being the central cavity the principal binding site (Kontopidis, Holt, & Sawyer, 2004).

Sakurai, Konuma, Yagi, and Goto (2009) studied the β-lg folding as a function of pH, determining that at pH 3.0 most of protein molecules are in their "close" state, while at pH 6.0 exists a higher proportion of molecules that are in the "open" state. Thus at pH 6.0 β-lg, in its "open" state, exposes more hydrophobic groups which would allow the protein to adsorb at the air–water interface easier than at pH 3.0, where it would be in its "closed" form.

Finally, above $1 \cdot 10^{-2}$ % wt/wt differences in protein surface pressure with pH were not longer observed. This could be due to the high β-lg concentration, which would be enough to saturate the interface independently of the protein conformational state.

Fig. 1b shows that α-lactalbumin presented surface activity from bulk concentrations of $1 \cdot 10^{-5}$ % wt/wt at pH 6.0. On the other hand, at pH 3.0, α-la solutions exhibited a surface pressure of 8.4 ± 0.6 mN/m even at very low concentrations as $5 \cdot 10^{-7}$ % wt/wt and saturation was achieved when surface pressure reached its maximum value (20.5 ± 0.6 mN/m) without significant differences between pH values.

The differences in surface activity of α-la at pH 6 or 3 observed at low concentrations can be explained by environment-dependent conformations of the protein. α-Lactalbumin is a globular, calcium metalloprotein with a MW of 14.2 kDa, 4 disulfide bonds, and no free thiol groups (Swaisgood, 1982). This protein exists in a number of conformations, including the holo (native, calcium-bound) form, which is the major form under the physiological conditions and the molten globule states which is intermediate in the unfolding pathway of a globular protein and have native-like secondary structure but have completely lost tertiary structure. Thus, it presents a higher surface hydrophobicity than the native state since the molecules posses substantial conformational flexibility (Cornec, Dennis, & Narsimhan, 2001). The molten globule state of α-la is induced at acidic pH. Thus, the rigid compact conformation of native α-la at pH 6.0 allows relatively limited unfolding after initial adsorption in comparison with the flexible molten globule state of α-la at pH 3.0 (Kronman, 1989).

Razumovsky and Damodaran (1999) showed that the surface activity of globular proteins is directly related to their molecular flexibility and their susceptibility to conformational changes at the interface. The structure of α-la is stabilized by four disulfide bonds compared to two for β-lg, thus pseudo-equilibrium surface pressure was found to be higher for β-lg. This suggests that the adsorbed molecules of β-lg exert more effect on the surface pressure than the adsorbed α-la. Denaturation of α-la upon adsorption at the air–water interface was reversible suggesting that no breakdown in disulfide bonds occurred upon adsorption. On the other hand, β-lg showed the highest degree of denaturation upon adsorption and the conformational changes were irreversible.

The surface pressure isotherms of BSA at pH 3.0 or 6.0 are shown in Fig. 1c. BSA presented surface activity from bulk concentration of $1 \cdot 10^{-5}$ % wt/wt at pH 3.0, and from this point a fast increase in surface pressure was observed, reaching the pseudo-equilibrium (18.2 ± 0.4 N/m) at $5 \cdot 10^{-5}$ % wt/wt. On the other hand, at pH 6.0 the increment was much more gradual and always below the pH 3.0 curve. BSA at pH 6.0 showed a continuous increase in the surface pressure in the concentration range $1 \cdot 10^{-5}$–$1 \cdot 10^{-1}$ % wt/wt, at this point it reached the pseudo-equilibrium with no significant differences with pH 3.0 due to the high concentration level. The differences between the two curves can be explained by the different conformational behavior of BSA at acid or neutral pH values. BSA has a MW of 66 kDa and three homologous domains, which are predominantly helical. Fatty-acid-free BSA has a

triangular or heart like shape but undergoes several well-recognized conformational changes by varying pH (Foster, 1977). The normal "N" BSA form is found close to the isoelectric point and up to neutral pH, and has a 55% of helix motifs. When lowering pH to 4.3, the acid-induced structural changes of BSA are characterized by changes in secondary as well as tertiary structure. At this stage, the faster "F" BSA form (from the "faster" migrating species in the PAGE) is adopted, which is characterized by a longer, less compact (11% volume increase) and increasingly asymmetric molecule and a decrease of the percentage of helical motifs to 45%. Although all structural transitions are reversible, the result is an abrupt opening of the molecule involving breakup of salt bridges and hydrophobic interactions thereby increasing its availability for surface adsorption. At pH values below 3, BSA undergoes another transition to the expanded "E" form, with another decrease in helical content up to 35% (Cascão Pereira, Théodoly, Blanch, & Radke, 2003). These conformational changes may promote BSA affinity for the interface at pH 3.0 increasing its surface activity as seen in Fig. 1c.

Fig. 1d shows surface pressure isotherm for E50LV at pH 3.0 or 6.0. As in the case of other HPMCs (Pérez, Carrera Sanchez, et al., 2006), the equilibrium surface pressure was considered to be reached after 24 h. The curves exhibited a sigmoid behavior with the increment of E50LV concentration in the whole range studied being observed surface activity from a concentration of $5 \cdot 10^{-7}$ % wt/wt. Slight significant differences at concentrations because of pH were observed below $1 \cdot 10^{-4}$ % wt/wt, where a higher π value was obtained at pH 6.0. At higher concentrations no significant differences were observed between pH 6.0. Camino, Carrera Sánchez, Rodríguez Patino, and Pilosof (2011) demonstrated that the E50LV surface activity is strongly influenced by pH. Moreover, these authors showed that the interfacial properties of the films formed by this polysaccharide decreased at pH 3.0 due to electrostatic repulsion of the negative charged molecules (Camino, Pérez, & Pilosof, 2009). Thus, E50LV molecules that diffuse to the interface from the sub-phase are impaired to adsorb because of the negative charge of previously adsorbed molecules.

3.2. Dynamics of adsorption and viscoelastic properties of mixed whey proteins and HPMC films as affected by pH

3.2.1. β-Lactoglobulin/HPMC mixed systems

The time-dependent surface pressure (π) of the β-lg/E50LV mixed system at pH 6.0 is shown in Fig. 2a. The concentration of β-lg (2% wt/wt) and E50LV (0.5% wt/wt) allowed both biopolymers to saturate the air–water interface (see previous section). For comparison, in the same Fig. 2a is also shown the surface pressure evolution for individual components (β-lg and E50LV). The surface pressure increased immediately after the drop formation, which is associated with the protein (Baeza, Carrera Sánchez, Pilosof, & Rodríguez Patino, 2005; Damodaran & Song, 1988; Graham & Phillips, 1979) and/or polysaccharide adsorption (Pérez et al., 2008; Pérez, Wargon & Pilosof, 2006).

It can be seen that E50LV had a higher surface activity than β-lg (Fig. 2a). The protein reached a π value of 25.5 mN/m at long adsorption times, which agrees with previous reported values for β-lg. For example, Baeza et al. (2005) reported a π value of 23 mN/m at 5000 s working with β-lg (2% w/w, pH 7) and coincides with the one found by Waniska and Kinsella (1985) for β-lg (2% wt/wt, pH 6.3) after 6000 s of adsorption. For E50LV (1% wt/wt, pH 7), a π value of 28.7 mN/m after 10,800 s of adsorption has been reported (Pérez et al., 2008). For such an adsorption time (10,800 s), the surface pressure found in this work for E50LV (0.5% wt/wt and pH 6.0) resulted within the same order with a π value of 30 mN/m.

There is a competitive adsorption between β-lg and E50LV, which could be deduced from the π–time curves comparison of individual and mixed biopolymers. Since β-lg is less surface active than E50LV at these concentrations, the replacement of the polysaccharide by the protein in the mixed systems decreased the surface pressure value of the mixture, mainly at low adsorption time. The competitive adsorption between biopolymers may affect directly the surface pressure by displacement of the more surface active component by other with less surfactant activity. At pH 6.0, it can be observed that E50LV dominated the surface pressure of the mixture (Fig. 2a) at longer adsorption times (more than 8000 s). Similar results were reported for WPC/E50LV mixtures at pH 7 (Pérez et al., 2007).

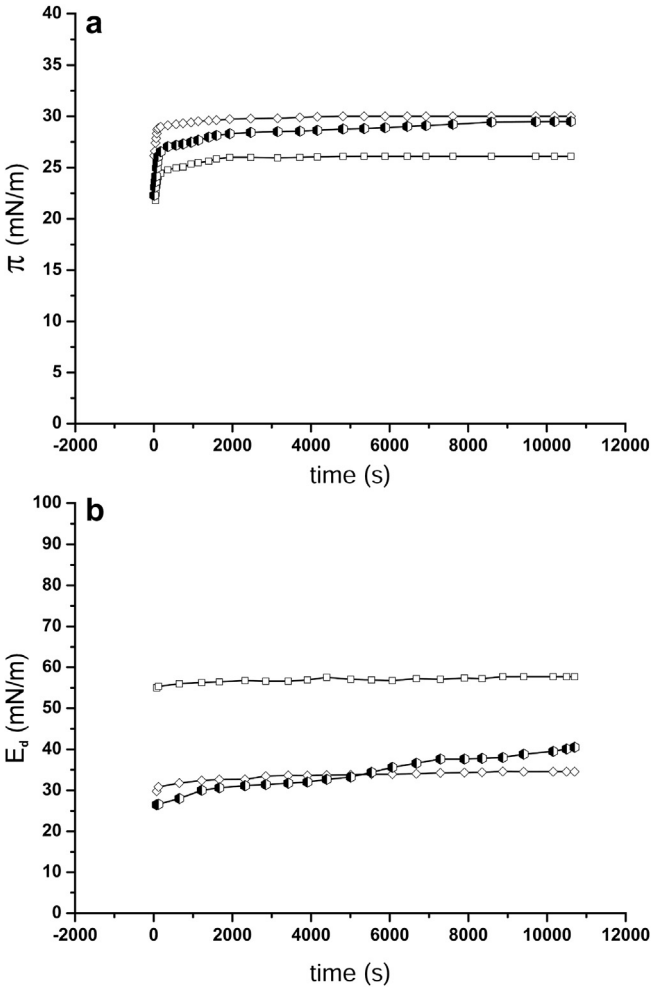

Fig. 2. Interfacial pressure (π) or dilatational elasticity (E_d) time-adsorption dependence, (a) or (b) respectively, for β-lg 2% wt/wt/E50LV 0.5% wt/wt mixture at the A/W interface at pH 6 compared with the biopolymers alone. β-lg (□), E50LV (◇), mixture (◐). Temperature 25 °C, $I = 5$ mM, Oscillation frequency 100 mHz.

Fig. 2b shows the time-evolution of the elastic component (E_d) of the dilatational modulus (E) for the β-lg/E50LV mixed film at pH 6.0 and for individual components. The time-dependent increment of E_d is due to the protein and/or polysaccharide adsorption at the interface, indicating that E_d depends on the interfacial film formation, which increases with time (Martínez, Carrera Sánchez, Pizones Ruiz-Henestrosa, Rodríguez Patino, & Pilosof, 2007).

Unlike the adsorption kinetics, E_d presented a different behavior for the protein and the polysaccharide at pH 6.0 (Fig. 2b). E50LV E_d slightly increased with time reaching an equilibrium value of 34.2 mN/m, after an adsorption time of 5000 s (Fig. 2b). On the contrary, β-lg film reached an equilibrium E_d (constant) of 56.5 mN/m at short adsorption times of approximately of 2000 s (Fig. 2b), indicating that the protein film is more viscoelastic than the HPMC

film. Martínez, Carrera Sanchez, Rodríguez Patino, and Pilosof (2009) found a similar behavior working with β-lg (4% wt/wt, pH 7). The E_d for β-lg/E50LV mixed film at pH 6.0 was similar to that of E50LV, indicating that the polysaccharide dominates the film elasticity (Fig. 2b). This is in agreement with the kinetic results obtained, where it was shown that E50LV also dominated the surface pressure at long adsorption times.

When analyzing the π–time curves at pH 3.0 (Fig. 3a), it can be noticed that the adsorption behavior of single components did not change with pH as shown in the pressure isotherm (Fig. 1a and d) at concentrations above interface saturation. However, the mixture behavior was slightly different from pH 6 (Fig. 2a): at shorter adsorption times (less than 1000 s) surface pressure was dominated by E50LV, while at longer adsorption times (more than 8000 s) surface pressure reaches an intermediate π value between those of individual components (Fig. 3a).

Unlike the adsorption kinetics, E_d did change with pH for the individual biopolymers (Fig. 3b). The β-lg films at pH 3.0 presented an E_d lower than at pH 6.0 which increased gradually with time without reaching an equilibrium value, even at long adsorption times (9000 s). As explained in the previous section, at pH 6.0 β-lg molecules are mostly in their "open" form (exposing hydrophobic amino acids), while at pH 3.0 most of the β-lg population are in their "close" form (Sakurai et al., 2009) which would decrease its ability to form an elastic interfacial film (Damodaran & Song, 1988). Besides this fact, it can be considered that pH also affects the monomer – associated states (dimmers, tetramers) equilibrium of β-lg (Gottschalk, Nilsson, Roos, & Halle, 2003; Sakurai & Goto, 2007; Uhrínová et al., 2000) which impacts on protein adsorption dynamic as well as its E_d behavior. Moreover, at neutral pH β-lg forms dimers that dissociate under acidic conditions, being its surface activity and emulsifying capacity lower at acidic pH than at neutral pH (Touhani & Dutcher, 2009).

On the contrary, E50LV at pH 3.0 exhibited a lower film elasticity (25.6 mN/m) than at pH 6.0 (Fig. 3b). Camino et al. (2011) have demonstrated that at pH 3.0 there is a decrease in the hydrophobic interactions between HPMC molecules due to their small negative net charge, decreasing their ability to associate by their hydrophobic groups once adsorbed at the interface (Pérez et al., 2008).

The E_d of the mixed film at pH 3.0 showed that at longer times (more than 5000 s), the protein started to dominate the viscoelastic character of the interface (Fig. 3b). This behavior also correlates with the kinetic adsorption results (Fig. 3a).

3.2.2. α-Lactalbumin/HPMC mixed systems

Fig. 4a shows the surface pressure (π) evolution upon time for α-lactalbumin, E50LV and their mixture at pH 6.0 at bulk concentrations high enough to saturate the interface. The surface pressure

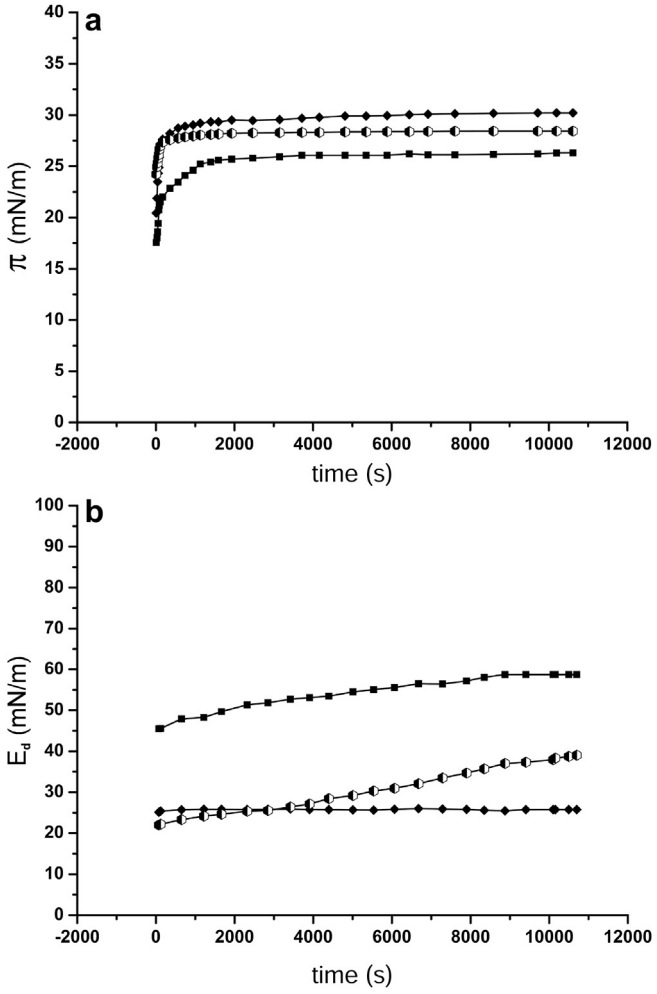

Fig. 3. Interfacial pressure (π) or dilatational elasticity (E_d) time-adsorption dependence, (a) or (b) respectively, for β-lg 2% wt/wt/E50LV 0.5% wt/wt mixture at the A/W interface at pH 3 compared with the biopolymers alone. β-lg (■), E50LV (◆), mixture (◐). Temperature 25 °C, $I = 5$ mM, Oscillation frequency 100 mHz.

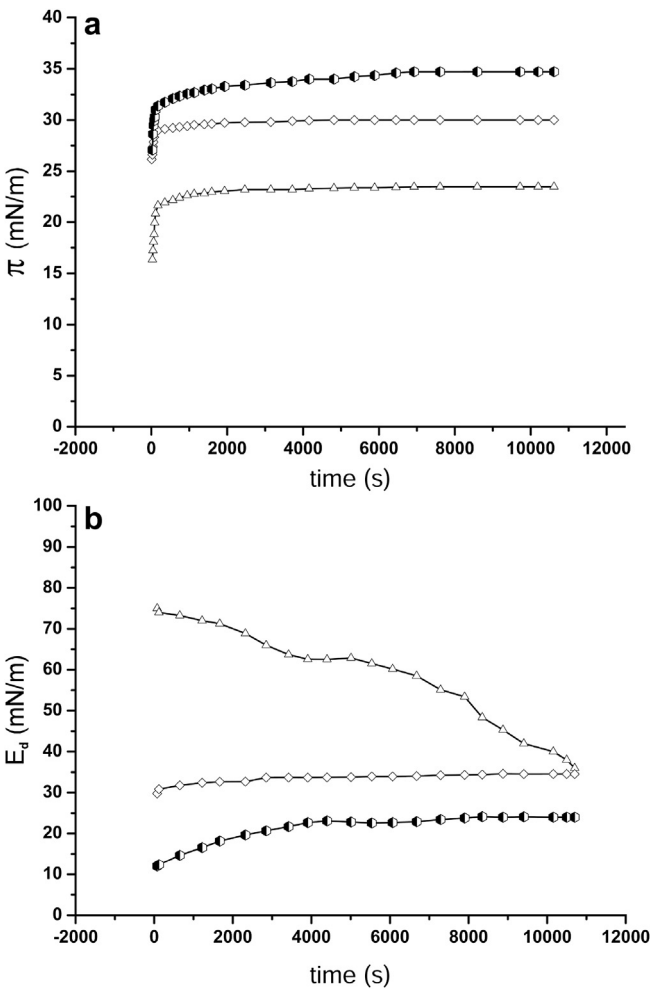

Fig. 4. Interfacial pressure (π) or dilatational elasticity (E_d) time-adsorption dependence, (a) or (b) respectively, for α-lac 2% wt/wt/E50LV 0.5% wt/wt mixture at the A/W interface at pH 6 compared with the biopolymers alone. α-la (△), E50LV (◇), mixture (◐). Temperature 25 °C, $I = 5$ mM, Oscillation frequency 100 mHz.

values for α-la were lower (23 mN/m) than for E50LV (30 mN/m). In the mixture a synergism is observed since the surface pressure was higher than that of E50LV (33 mN/m). Contrarily, at pH 3.0 it has been observed an inverse behavior (Fig. 5b), where α-la reached surface pressure values higher than at pH 6.0 (32.6 mN/m), even exceeding to E50LV values (30 mN/m). Regarding to the mixture, in this case there is a competitive adsorption between protein and polysaccharides.

These facts indicate that at pH 6.0 the adsorption, penetration and unfolding at the air–water interface is quiet difficult for native α-la. This trend keeps good correspondence with that obtained from the π–C isotherms, where α-la presented a higher affinity for the interface in its molten globule state, pH 3.0, than in its native state, pH 6.0 (Figs. 5a and 4a, respectively) (Kronman, 1989).

Fig. 4b shows the surface dilatational elasticity (E_d) for the pure components and the mixture at pH 6.0. Initially, the α-la film showed a higher elastic component value (75 mN/m) but it decreased over time until reaching a value similar to E50LV (35 mN/m); however, the mixed film was dominated by E50LV and exhibited even lower values indicating an antagonistic interaction. These results highlight the dominant role of E50LV in determining the rheological behavior of mixed films due to the incapacity of the protein to displace the HPMC at this pH.

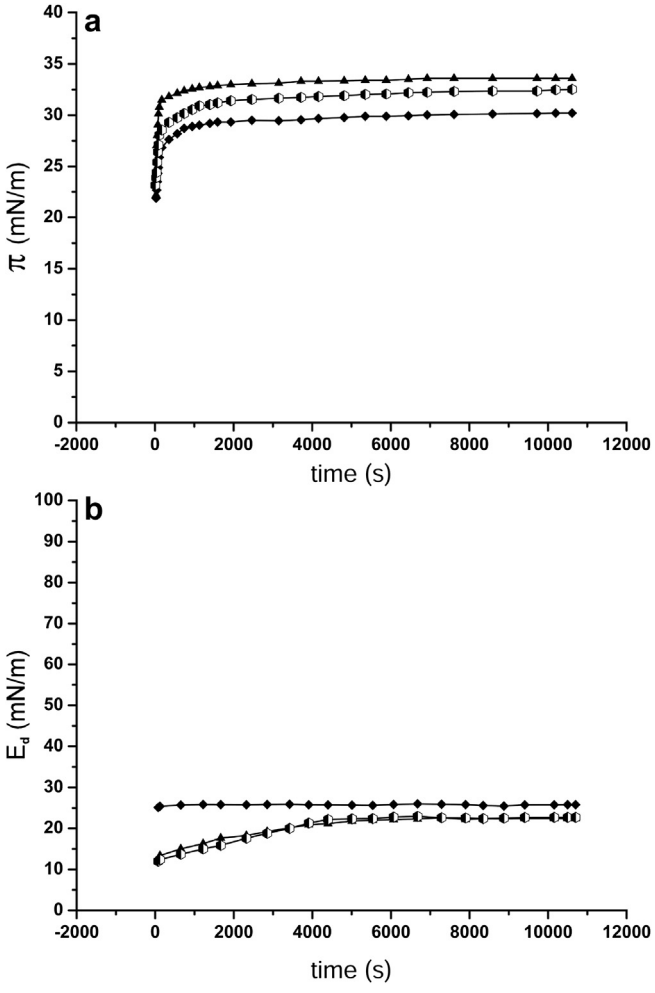

Fig. 5. Interfacial pressure (π) or dilatational elasticity (E_d) time-adsorption dependence, (a) or (b) respectively, for α-lac 2% wt/wt/E50LV 0.5% wt/wt mixture at the A/W interface at pH 3 compared with the biopolymers alone. α-la (▲), E50LV (◆), mixture (◐). Temperature 25 °C, $I = 5$ mM, Oscillation frequency 100 mHz.

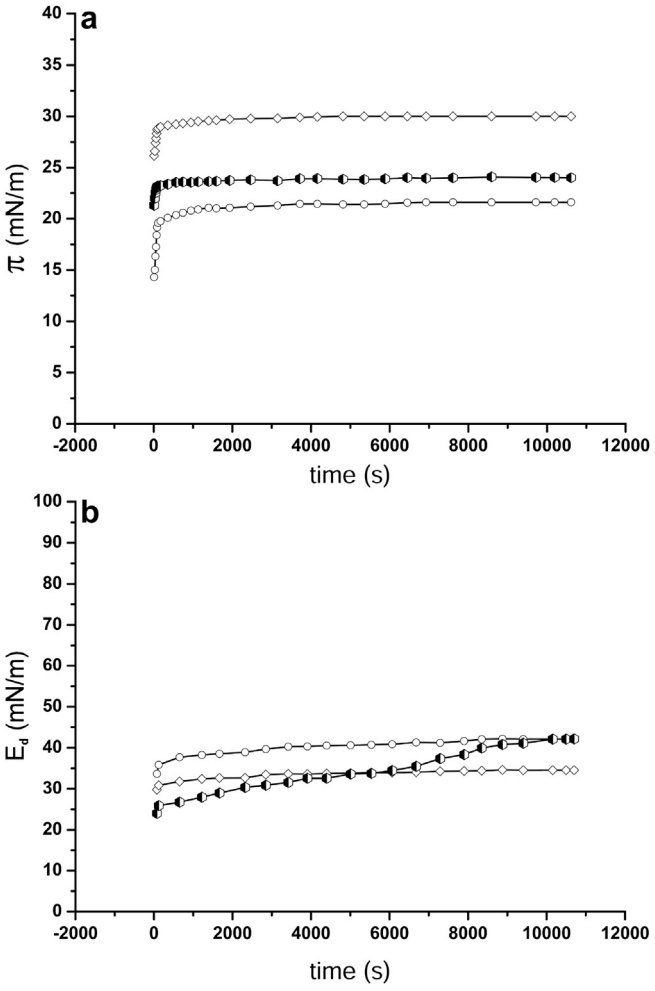

Fig. 6. Interfacial pressure (π) or dilatational elasticity (E_d) time-adsorption dependence, (a) or (b) respectively, for BSA 2% wt/wt/E50LV 0.5% wt/wt mixture at the A/W interface at pH 6 compared with the biopolymers alone. BSA (○), E50LV (◇), mixture (◐). Temperature 25 °C, $I = 5$ mM, Oscillation frequency 100 mHz.

The results at pH 3.0 showed a reverse scenario since α-la presented equilibrium π values (34 mN/m) higher than that of E50LV (30 mN/m), while the mixed system appears to have an intermediate behavior (32 mN/m) with comparable contributions of both biopolymers (Fig. 5a) as the molten globule state of α-la facilitates the adsorption at interface because of increase in the surface hydrophobicity due to the tertiary conformational changes and partially unfolding of the molecule at this pH (Cornec et al., 2001). However, in spite of more α-la adsorption at the interface due to the more surface active molten globule conformation at pH 3.0, the E_d values (Fig. 5b) were considerably lower than those for native α-la conformation at pH 6.0 (Fig. 4b). The fact that the time dependence of the surface dilatational elasticity did not follow the same trend as the surface pressure (Figs. 4a and 5a) indicates that E_d does not depend exclusively on the surface coverage, which increases with time, but also on the intermolecular interactions between the adsorbed proteins to form a viscoelastic film. The dilatational elasticity of the mixed film was dominated by α-la in spite of the small differences between the single components.

3.2.3. BSA/HPMC mixed systems

At pH 6.0 or 3.0 BSA was less surface active than E50LV (Figs. 6a and 7a) and the mixture exhibited an intermediate behavior reflecting the adsorption of both biopolymers. The BSA film

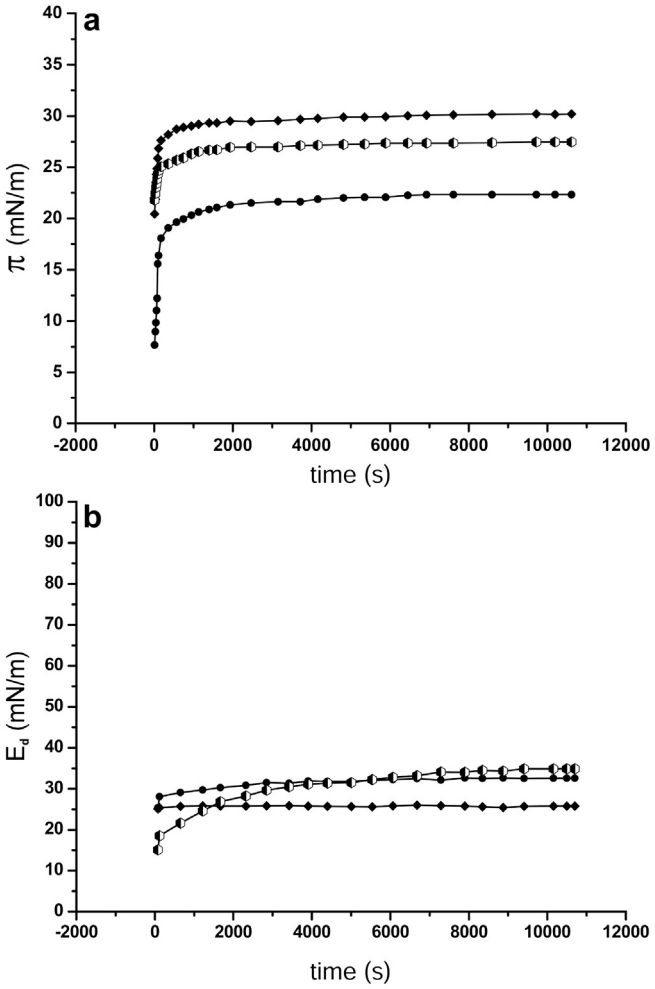

Fig. 7. Interfacial pressure (π) or dilatational elasticity (E_d) time-adsorption dependence, (a) or (b) respectively, for BSA 2% wt/wt/E50LV 0.5% wt/wt mixture at the A/W interface at pH 3 compared with the biopolymers alone. BSA (●), E50LV (◆), mixture (◐). Temperature 25 °C, $I = 5$ mM, Oscillation frequency 100 mHz.

showed however a higher E_d than E50LV film and dominated the rheological behavior of the mixed film at long adsorption time (Figs. 6b and 7b).

Nevertheless, some differences due to pH may be observed. The film elasticity for BSA at pH 6.0 resulted much higher (from 33 to 42.5 mN/m) than at pH 3.0 (from 25 to 32.5 mN/m), indicating that BSA in its "N" structure forms a more viscoelastic film than it its open "F" structure (Figs. 6b and 7b) (Cascão Pereira et al., 2003). Regarding the E_d of mixed films, at pH 6.0, an antagonist behavior at adsorption time below 4000 s is observed; then E_d increased reaching the value of single BSA (42.5 mN/m) (Fig. 6b). At pH 3.0 E_d presented a similar trend, but an antagonist behavior was apparent below 2000 s. Then E_d reached values similar to BSA (35.0 mN/m), and even higher at long adsorption times ($t > 7000$ s) (Fig. 7b). Thus, the evolution of E_d upon time points out rearrangements of adsorbed HPMC and BSA at the interface, where BSA interactions increase over time.

4. Conclusions

WPC and HPMC mixtures at neutral pH are generally unstable and upon a critical biopolymer concentration separate in a protein rich-phase and a polysaccharide rich-phase (Pérez et al., 2006).

At pH 6.0, under conditions of limited thermodynamic compatibility between each protein and the HPMC, at the bulk biopolymers concentration using in this work allowing monolayer saturation, no macroscopic phase separation was observed (Jara & Pilosof, 2009). There will be a competition for the interface being the final composition of the interface and its rheological properties dependent on the surface activity, rate of adsorption and film forming ability of the protein and polysaccharide (Rodríguez Patino & Pilosof, 2011). Thus, at pH 6.0 the HPMC was more surface active than each one of the proteins, but formed surface film of lower dilatational elasticity. Moreover, at this pH, β-lg exhibited the higher surface activity and dilatational elasticity among the three proteins; nevertheless when it co-adsorbed with the HPMC, both the surface pressure and film elasticity, were dominated by polysaccharide, revealing that β-lg can be easily displaced from the interface by HPMC. For BSA, that exhibited the lowest surface activity among proteins and intermediate values of film dilatational elasticity, when it co-adsorbed with HPMC, could compete for the interface as indicated by surface pressure values of mixture which were in between that of single biopolymer but closer to BSA; moreover BSA also dominated the film elasticity. Finally, α-la that exhibited an intermediate surface pressure among proteins and the lowest film elasticity, performed in a synergistic way when co-adsorbed with HPMC, but an antagonism on dilatational elasticity of mixed film was apparent, suggesting some kind of molecular interactions between both adsorbed biomolecules.

At pH 3.0, it has been reported that WPC/HPMC mixtures do not show any macroscopic phase separation, but a partial compatibility corresponding to a microscopic phase separation has been determined from glass transition measurements (Jara & Pilosof, 2009). At this pH proteins carry a positive charge while HPMC has a small negative net charge (Camino et al., 2011) which can explain their increased compatibility. Under these conditions, the three proteins could compete with the HPMC for the interface as the surface pressure reached by the mixture was in between the values for single biopolymers. The surface dilatational elasticity was dominated by the proteins, except for β-lg/HPMC mixture.

It can be concluded that in compatibility conditions (pH 3.0) the three proteins performed similarly on co-adsorption with the surface active HPMC, but in conditions of incompatibility (pH 6.0), strong differences arise between proteins that may be attributed to molecular features of each protein that would induce different interactions with HPMC. β-lg, in spite of being the more surface active and the better film forming protein cannot compete with HPMC for the air–water interface. Contrarily, BSA could compete with the HPMC for the interfaces, determining also the elasticity of mixed film. Regarding α-la, thermodynamic incompatibility gives some interesting benefits as the synergistic increase of surface pressure on co-adsorption with HPMC that suggest a strong incompatibility in this mixture.

Finally, it has been proposed that interactions between proteins and polysaccharides at fluid interfaces would occur by a complexation mechanism or indirectly by exclusion volume effects (Rodríguez Patino & Pilososf, 2011). Because of the existence of a limited thermodynamic compatibility each adsorbed biopolymer would concentrate in different domains, leading to a synergistic increase of surface pressure. These segregative interactions at the interfaces could hinder the association of each biopolymer leading to an antagonistic behavior in film elasticity, mainly if both components have poor film forming abilities, i.e. BSA/HPMC mixture.

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