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Effect of enzymatic hydrolysis and polysaccharide addition on the β-lactoglobulin adsorption at the air–water interface

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ABSTRACT

The effect of enzymatic hydrolysis and polysaccharide addition on the interfacial adsorption of β-lactoglobulin (β-LG) was investigated in this work. The enzymatic treatment was performed in the hydrolysis degree (HD) range of 0.0–5.0% using bovine α-chymotrypsin II immobilized on agarose beads. Anionic non-surface active polysaccharides (PS), sodium alginate (SA) and λ-carrageenan (λ-C) were studied in the concentration range of 0.0–0.5 wt.%. The adsorption process at the air–water interface was evaluated by means of tensiometry and surface dilatational rheology. Biopolymer interactions in solution were analyzed by extrinsic fluorescence spectroscopy. The enzymatic hydrolysis improved β-LG interfacial properties. On the other hand, at low HD (1.0%), PS addition enhanced surface and elastic properties of β-LG hydrolysate films probably due to a higher repulsion between biopolymers in solution. However, at high HD (3.0–5.0%), SA addition caused a deterioration of surface and elastic properties of β-LG hydrolysate films probably due to the segregation and hydrolysate aggregation in solution, whereas λ-C addition could promote the formation of soluble complexes leading to a better control of elastic properties of β-LG hydrolysate films.

1. Introduction

Many foods are produced as colloidal dispersions such as foams and emulsions. Since these food systems are thermodynamically unstable due to their large interfacial area, the formation of foams and emulsions conveys the use of surfactant ingredients in order to minimize the energy input involved in foaming and emulsification processes (Walstra, 1993). This fact is based on the surfactant ability to decrease interfacial tension through an adsorption process, which leads to film formation (Halling, 1981; Damodaran, 1990; Dickinson, 1992). Moreover, the stability of colloidal dispersions depends on the composition, structure and rheological properties of surfactant adsorbed films at fluid interfaces (air–water and oil–water), as well as on the ingredient interactions, both in solution and at the interfacial vicinity (Rodríguez Patino et al., 2008).

Biopolymer ingredients most used in foam and emulsion production are proteins and polysaccharides (Dickinson, 1992, 2003). Proteins tend to be adsorbed at fluid interfaces due to their amphiphilic nature, whereas polysaccharides commonly control the aqueous subphase rheology because of their more hydrophilic nature. Since foods are multicomponent systems, protein–polysaccharide interactions have been extensively researched in order to find new and better applications for these biopolymers (Schmitt et al., 1998; Rodríguez Patino and Pilosof, 2011). Under different aqueous medium conditions (pH, ionic strength and relative concentration), biopolymer interactions could be handled in order to optimize and/or improve quality attributes (stability, texture, sensory perception, shelf life, etc.) of food products based on foam and emulsion (Dickinson, 2006; McClements, 2006; Rodríguez Patino and Pilosof, 2011). Furthermore, protein–polysaccharide interactions can be greatly affected by biopolymer structural modifications through thermal, enzymatic, and high pressure treatments (Galazka et al., 1999; Martínez et al., 2007; Santipanichwong et al., 2008). Therefore, fundamental studies about biopolymer interactions (both in solution and at fluid interfaces) are necessary to address new strategies for engineering and formulation of colloidal food dispersions.

In this work, the effect of enzymatic hydrolysis (as an engineering strategy) and PS addition (as a formulation strategy) on the β-LG adsorption at the air–water interface was studied. The main milk whey protein, β-LG, was selected in this study due to its susceptibility to enzymatic treatment (Caessens et al., 1999; Ipsen et al., 2001; Davis et al., 2005; Galvão et al., 2009) and its increased use in food colloid production (Purwanti et al., 2010). Enzymatic hydrolysis was employed as a tool for β-LG structural modification. It is well known that a limited treatment with proteases (at low

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hydrolysis degree) usually improves interfacial properties of globular proteins, mainly due to an increase in exposed hydrophobic areas (Damodaran and Paraf, 1997; Kilara and Panyam, 2003). Anionic non surface-active polysaccharides (PS), sodium alginate (SA) and λ-carrageenan (λ-C) were investigated on account of their wide utilization in food industry as stabilizer and thickener agents, and for their proven ability to interact with commercial milk whey protein, both in solution and at the vicinity of air–water interface (Perez et al., 2009a,b). Nevertheless, the effect of the addition of these PS on the bulk and interfacial behavior of β-LG hydrolysates has not been reported in literature.

2. Materials and methods

2.1. Biopolymer samples

β-Lactoglobulin (β-LG) was supplied by Danisco Ingredients (Brabrand, Denmark). Nitrogen solubility index (NSI) was determined by standard methods (AACC, 1983). For this, a 2 wt.% β-LG solution in Milli Q water was prepared. Solution pH was adjusted to 7 with 1 M NaOH and was shaken for 1 h at room temperature (25 °C). Then, it was centrifuged at 7720*g* for 15 min. The supernatant was collected, and analyzed for nitrogen content by the standard Kjeldahl method using conversion factor of 6.38. NSI value was expressed as the percentage nitrogen content of supernatant divided by the overall nitrogen content in the starting dispersion. The β-LG sample had an NSI = 99.70% at pH 7. The β-LG sample composition was: protein 92.00% (N × 6.38), moisture 6.00%, fat 0.20%, lactose 0.20%, ash 1.50% (Na^+ 0.50%, K^+ 1.30%, Ca^{2+} 0.10%, Mg^{2+} 0.10%). Samples of anionic non-surface active polysaccharides (PS), sodium alginate (SA) and λ-carrageenan (λ-C) were kindly supplied by Cargill (Buenos Aires, Argentina). The molecular weights of PS were 135 and 1000 kDa for SA and λ-C, respectively (data supplied by Cargill). The SA sample had the following composition: carbohydrate 63.00%, moisture 14.00%, and ash 23.0% (Na^+ 9.30%, K^+ 0.80%). The λ-C sample composition was: carbohydrate 68.00%, moisture 8.00%, and ash 24.00% (Na^+ 2.70%, K^+ 5.00%, Ca^{2+} 0.35%, Mg^{2+} 0.50%).

2.2. Enzymatic hydrolysis

β-LG enzymatic hydrolysis was carried out in a batch bioreactor using bovine α-chymotrypsin type II (EC 3.4.21.1) at pH 8 and 50 °C (optimal enzymatic conditions). Bovine α-chymotrypsin was purchased from Sigma Chemical Company (St. Louis, MO) and immobilized on agarose beads (Hispanagar S.A., Spain). The α-chymotrypsin derivatives were loaded with 40 mg of protein/g of support (enzyme activity: 60 U/mg support). The agarose beads were activated with 75 μequiv. of aldehyde groups/ml of support (6% glyoxyl-agarose). For this, glyceryl-supports were prepared by mixing agarose beads under stirring with an aqueous solution containing NaOH 1.7 M and $NaBH_4$ 0.75 M (glycidol) in ice bath. Then, 0.48 ml of glycidol/g of bead were added, kept under mechanical stirring for 18 h and washed until neutrality. Glyoxyl-supports (75 μequiv. of aldehyde groups/ml of support) were obtained by contacting beads with 2 ml of 0.1 M $NaIO_4$ solution per gram of gel for 2 h at room temperature (25 °C). Afterwards, they were washed with an excess of distilled water until neutrality. Subsequently, a mass of α-chymotrypsin (40 mg of enzyme/g of support) in bicarbonate buffer 100 mM, pH 10.05 was added to the activated support (ratio w/v of 1/10). The preparation was kept under mild stirring at 25 °C for 24 h. After that, enzyme derivatives were washed with distilled water and sodium phosphate buffer 0.1 M, pH 7.0. β-LG hydrolysates were obtained at different hydrolysis degree (HD): 1.0% (H1), 3.0% (H2) and 5.0% (H3). Enzymatic hydrolysis was controlled by the pH-stat method and the HD was calculated according to the procedure described by Spellman et al. (2003). At the desired HD, β-LG hydrolysate dispersions were filtered and subsequently lyophilized in Heto FD-25 equipment (Heto-Holten, Denmark). The composition of β-LG hydrolysates was: (i) H1: protein 88.09%, moisture 6.30%, and ash 4.20%, (ii) H2: protein 88.00%, moisture 7.30%, and ash 4.31%; and (iii) H3: protein 85.90%, moisture 7.43%, and ash 5.50%. SDS–PAGE analysis confirmed the reduction of β-LG molecular size as a consequence of the applied enzymatic treatment (data not shown).

2.3. Pure and mixed aqueous systems

β-LG, its hydrolysates and PS (SA and λ-C) powders were dissolved in Milli-Q ultrapure water at room temperature (25 °C), pH and ionic strength being adjusted to 7 and 0.05 M, respectively, with a commercial buffer solution called trizma ($(CH_2OH)_3$–C–NH_2/$(CH_2OH)_3$–C–NH_3Cl) (Sigma, USA). The absence of surface-active contaminants in the aqueous buffered solution was checked by interfacial tension measurement before the preparation of dispersions. No aqueous solutions with a surface tension other than that accepted in the literature (72–73 mN/m at 20 °C) were used. Stock PS dispersions (1.0 wt.%) were stirred for at least 30 min at 80 °C to ensure complete dispersion and they were subsequently left overnight at 4–5 °C to hydrate appropriately. The presence of surface active impurities in PS aqueous solutions was checked by surface tension measurement and removed by repetitive suctions. After five suctions (the last one after 24 h of preparation) the samples had a surface pressure of ~3 mN/m, which confirmed that most surface active impurities in PS aqueous solutions had been removed. These purified PS aqueous solutions were the ones used in this study. Mixed systems (Prot:PS) were obtained by mixing the appropriate volume of each double concentrated biopolymer solution up to the final required bulk concentration. It should be noticed that there was a very slight difference in the ionic strength of the aqueous systems due to ions contained in the biopolymer samples.

2.4. Protein surface hydrophobicity

Surface hydrophobicity (S_0) of β-LG and hydrolysates (in pure and mixed systems) was determined by extrinsic fluorescence spectroscopy using the fluorescence probe 1-anilino-8-naphthalene sulfonic acid (ANS, Fluka Chemie AG, Switzerland) (Kato and Nakai, 1980). Serial dilutions in trizma buffer were obtained from pure and mixed systems. Dilutions were prepared at pH 7 up to a final concentration of 0.01–0.50 mg/ml. Ten microliters of ANS (8 mM) were added to 2 ml of each dilution and the fluorescence intensity (FI) was measured at 350 nm (excitation) and 470 nm (emission). The initial slope of the FI (arbitrary unit, a.u.) versus protein concentration (mg/ml) plot was calculated by linear regression analysis, and was used as an index of S_0. Measurements were obtained in triplicate.

2.5. Surface pressure isotherms

Equilibrium surface tension (σ_{eq}, mN/m) for β-LG and hydrolysate adsorbed films at the air–water interface was determined by the Wilhelmy plate method, using a rectangular platinum plate attached to a Sigma 701 digital tensiometer (KSV, Finland) as described in Rodríguez Niño et al. (2001). Protein aqueous solutions in an increased range of concentration (1×10^{-6}–2.0 wt.%) were allowed to age for 24 h at 4–5 °C prior to each measurement to achieve the interfacial adsorption. Equilibrium condition was assumed when σ did not change by more than 0.1 mN/m in 30 min. Equilibrium surface pressure (π_{eq}) was calculated as $\pi_{eq} = \sigma_0 - \sigma_{eq}$,

where σ_0 is the trizma buffer surface tension and σ_{eq} is the surface tension at equilibrium. Finally, surface pressure isotherms were obtained graphically as π_{eq} versus log concentration plots.

2.6. Dynamic surface properties

Dynamic measurements of surface pressure (π, mN/m) and dilatational rheological properties were performed with an automatic pendant drop tensiometer (TRACKER, IT Concept, Longessaine, France) as it has been outlined in Rodríguez Patino et al. (2005). Aqueous solutions of pure and mixed systems were placed in a syringe and subsequently in a compartment, and they were allowed to stand for 30 min to reach 20 °C. Then a drop was delivered and allowed to stand for 10,800 s to achieve interfacial adsorption. For dilatational measurements, the applied method involved a sinusoidal interfacial compression and expansion performed by decreasing and increasing the drop volume at 10% of deformation amplitude ($\Delta A/A$) and at 0.1 Hz of angular frequency (ω). Surface dilatational modulus (E) derived from the change in interfacial tension (σ) resulting from a small change in the surface area may be described by Eq. (1) (Lucassen and van den Tempel, 1972):

$$E = d\sigma/(dA/A) = -d\pi/d\ln A = |E|e^{i\phi} = E_d + iE_v \quad (1)$$

where $|E| = (|E_d|^2 + |E_v|^2)^{1/2}$, E_d is dilatational elasticity, E_v is dilatational viscosity, and ϕ is the phase angle between stress and strain. For a perfectly elastic material, stress and strain are in phase ($\phi = 0$) and the imaginary term is zero. In the case of a perfectly viscous material, $\phi = 90°$ and the real part is zero. Measurements were made in triplicate.

2.7. Adsorption kinetics

Adsorption kinetics was monitored by measuring changes in surface pressure (π). In order to quantify the protein diffusion step toward the interface, a modified form of Ward and Tordai equation (Ward and Tordai, 1946) was used to correlate the change in π with adsorption time, θ (Eq. (2)):

$$\pi = 2C_0KT(D\theta/3.14)^{1/2} \quad (2)$$

where C_0 is the protein bulk concentration, K is the Boltzmann constant, T is the absolute temperature, and D is the protein diffusion coefficient. If the diffusion step controls the adsorption process, a plot of π against $\theta^{1/2}$ will then be linear, and its slope corresponds to diffusion rate constant (k_{diff}). Moreover, in order to quantify the penetration and rearrangement at air–water interface, the following first-order equation was applied (Graham and Phillips, 1979):

$$\ln(\pi_f - \pi_\theta)/(\pi_f - \pi_0) = -k_i\theta \quad (3)$$

where π_f, π_0, and π_θ are the surface pressures at final θ of each step, at initial θ, θ_0, and at any time θ, respectively, and k_i is a first-order rate constant. Usually, the initial slope corresponds to penetration rate constant (k_P), while the second slope corresponds to rearrangement rate constant (k_R).

2.8. Statistical analysis

One way analysis of variance (ANOVA) was carried out using StatGraphics Plus 3.0 software, and statistical differences among systems were determined using LSD test at 95% confidence level.

3. Results and discussion

3.1. Effect of HD on the β-LG surface properties

3.1.1. Surface pressure isotherms

Fig. 1A shows the effect of HD (0.0–5.0%) on the adsorption isotherm for β-LG adsorbed film at the air–water interface. It can be seen that β-LG adsorption isotherm has a sigmoid shape which is a characteristic of globular proteins (Carrera Sánchez et al., 2005). Moreover, the enzymatic treatment caused a slight change in the shape of β-LG adsorption isotherm. This behavior could be associated with a transition and/or modification of β-LG interfacial structuration as a result of enzymatic hydrolysis (Martínez et al., 2009). Furthermore, equilibrium surface pressure (π_{eq}) for β-LG and its hydrolysates reached a constant value or *plateau* at 1.0 wt.%. This concentration value corresponds to the protein bulk concentration which is able to saturate the air–water interface (Graham and Phillips, 1979). At this concentration, π_{eq} for β-LG adsorbed film increased with the increment in HD. This result would suggest an increase in β-LG surface activity as a consequence of the enzymatic treatment (Miñones Conde and Rodríguez Patino, 2007; Martínez et al., 2009). Bovine α-chymotrypsin is a serine protease that hydrolyzes peptide bonds with aromatic or large hydrophobic side chains (Tyr, Trp, Phe, Met, Leu) on the carboxy end of the bond (Galvão et al., 2009). Therefore, a limited enzymatic treatment with

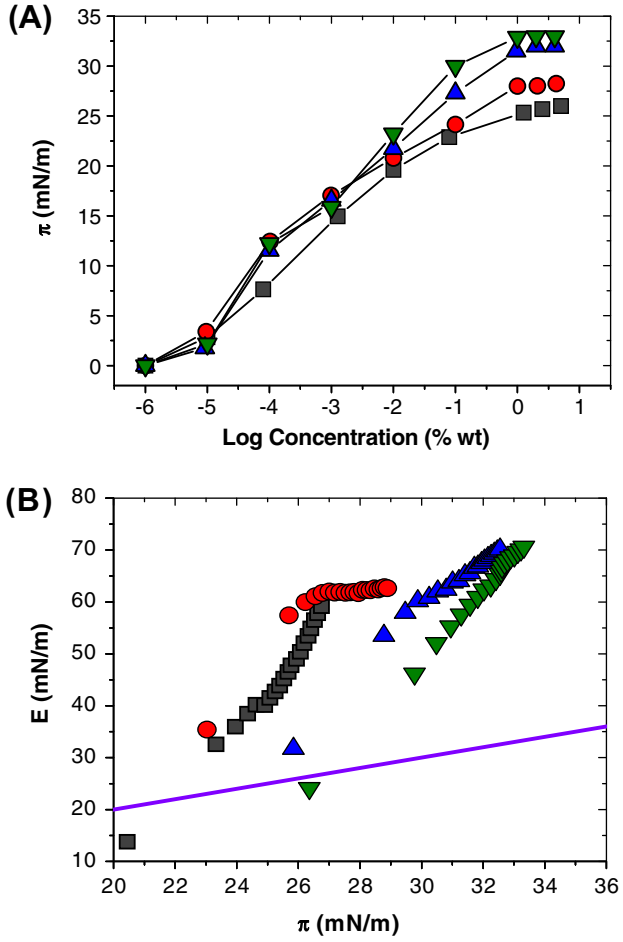

Fig. 1. Effect of HD on the surface pressure adsorption isotherm for β-LG (A) and on the molecular structuration (given by E_d–π plot) of β-LG adsorbed film at the air–water interface (B). Symbols HD: 0.0% (■), 1.0% (●), 3.0% (▲) and 5.0% (▼). Protein bulk concentration 1.0 wt.%, temperature 20 °C, pH 7 and I 0.05 M. Deformation amplitude ($\Delta A/A$) 10%, angular frequency (ω) 0.1 Hz.

α-chymotrypsin could substantially increase the exposure of hydrophobic areas, thus promoting an increment in β-LG surface activity. Based on these results, the protein (β-LG and hydrolysates) bulk concentration chosen to carry out dynamic interfacial experiments was 1.0 wt.%.

3.1.2. Surface pressure at long term adsorption

The effect of HD (0.0–5.0%) on the surface pressure (π_f) at long term adsorption (10,800 s) for β-LG film is shown in Table 1. It can be deduced that π_f value for β-LG film significantly increases with the increment in HD ($p < 0.05$). This result would suggest that the extent of the enzymatic hydrolysis could affect β-LG adsorption process. This hypothesis will be discussed from a kinetic point of view.

3.1.2.1. Diffusion. As described previously, the diffusion step of β-LG and its hydrolysates was determined from time evolution of π (Eq. (2)). At 1.0 wt.% protein bulk concentration, the diffusion proceeded too rapidly (with $\pi > 10$ mN/m) to be detected by the experimental method used. Therefore, at short θ, the interfacial adsorption of β-LG and its hydrolysates would not be controlled by a diffusive phenomenon. However, at the beginning of the adsorption process (at 0.5 s), the slope of the π–$\theta^{1/2}$ plot could be considered as a measure of apparent diffusion rate, k^a_{diff} (Perez et al., 2009b). As it can be observed in Table 2, k^a_{diff} value for β-LG significantly increased with the increment in HD ($p < 0.05$). This behavior could be related to: (i) reduction of β-LG molecular size, and/or (ii) increment in β-LG surface activity due to enzymatic hydrolysis (as it can be deduced from Fig. 1A) (Miñones Conde and Rodríguez Patino, 2007). This result is consistent with the surface hydrophobicity (S_0) analysis (also shown in Table 2) which could explain the increase in k^a_{diff} value with HD due to an increment in the number of hydrophobic areas exposed on β-LG.

3.1.2.2. Penetration and rearrangement. The effect of HD on the rate constants of penetration (k_P) and rearrangement (k_R) for β-LG is shown in Table 2. It was observed that k_P and k_R values for β-LG increased significantly with the increment in HD ($p < 0.05$). This result could be explained in terms of: (i) an increased number of exposed hydrophobic areas on β-LG which could make contact with the air–water interface, and (ii) an increased β-LG molecular flexibility which could facilitate protein unfolding and interactions among adsorbed segments (Graham and Phillips, 1979; Chobert et al., 1988; Miñones Conde and Rodríguez Patino, 2007). Moreover, k_P values were lower than those of k_R, suggesting that penetration would be the limiting step for the overall interfacial adsorption for both β-LG and its hydrolysates.

3.1.3. Dilatational rheological behavior at long term adsorption

Surface dilatational parameters (E_f, E_{df}, E_{vf} and ϕ_f) at long term adsorption (10,800 s) for β-LG and its hydrolysates are shown in Table 1. It was observed that: (i) E_f and E_{df} values were high, and (ii) E_{vf} and ϕ_f values were low. Therefore, it could be concluded that surface dilatational behavior for β-LG and its hydrolysates was essentially elastic at the angular frequency (ω) applied (0.1 Hz). Moreover, the increase in HD produced: (i) a significant increment in E_f and E_{df} values and (ii) a decrease in ϕ_f values ($p < 0.05$), suggesting an increased elastic character for β-LG film. This result could be explained in terms of a greater number of interactions among β-LG adsorbed segments (Graham and Phillips, 1979; Damodaran and Song, 1988) which could also be promoted by limited enzymatic hydrolysis (Ipsen et al., 2001).

3.1.4. Molecular structuration at the air–water interface

Normally, E increase with π values, which could suggest an increment in the number of interactions among protein adsorbed segments (Bos and van Vliet, 2001). Therefore, all E data would be normalized in a single E–π master curve which could give information about the protein interfacial structuration (Rodríguez Patino et al., 2003, 2005).

The effect of HD (0.0–5.0%) on the β-LG interfacial structuration (given by E–π plot) is shown in Fig. 1B. It can be seen that: (i) E–π slopes were greater than one (represented by the solid line) which would confirm a non-ideal behavior of films characterized by a great number of interactions among protein adsorbed segments (Lucassen Reynders et al., 1975), (ii) E–π plots are not normalized in a single master curve, indicating that enzymatic hydrolysis could promote different degrees of β-LG interfacial structuration according to HD (Miñones Conde and Rodríguez Patino, 2005), and (iii) the increment in HD caused a gradual displacement of

Table 1
Effect of PS (SA and λ-C) concentration (0.0–0.5 wt.%) on surface pressure (π_f) and dilatational rheological parameters (E_f, E_{df}, E_{vf} and ϕ_f) for β-LG and its hydrolysates at: 1.0% (H1), 3.0% (H2) and 5.0% (H3) at long-term adsorption (10,800 s) at the air–water interface.

Systems	Prot:PS (% wt)	π_f[A] (mN/m)	E_f[A] (mN/m)	E_{df}[A] (mN/m)	E_{vf}[A] (mN/m)	ϕ_f[A]
β-LG	1.0:0.0	26.7 ± 0.4^{a}	50.4 ± 0.3^{c}	47.5 ± 0.2^{c}	16.9 ± 0.2^{a,b}	19.6 ± 0.2^{j}
β-LG:0.1SA	1.0:0.1	26.5 ± 0.5^{a}	57.6 ± 0.4^{d}	55.6 ± 0.3^{d}	20.5 ± 0.3^{c,d,e}	18.6 ± 0.3^{i}
β-LG:0.5SA	1.0:0.5	27.5 ± 0.5^{b}	57.6 ± 0.3^{d}	55.6 ± 0.3^{d}	19.5 ± 0.2^{c,d,e}	17.7 ± 0.2^{g,h}
β-LG:0.1λ-C	1.0:0.1	27.1 ± 0.7^{a,b}	58.9 ± 0.5^{e}	56.6 ± 0.4^{e}	18.7 ± 0.4^{b,c}	18.4 ± 0.2^{i}
β-LG:0.5λ-C	1.0:0.5	27.4 ± 0.6^{b}	60.1 ± 0.4^{f}	57.0 ± 0.5^{e}	21.1 ± 0.3^{e}	17.4 ± 0.4^{g}
H1	1.0:0.0	28.9 ± 0.5^{c,d}	62.7 ± 0.2^{g}	59.7 ± 0.2^{f}	17.0 ± 0.2^{a,b}	17.8 ± 0.2^{g,h}
H1:0.1SA	1.0:0.1	28.5 ± 0.2^{c}	63.0 ± 0.2^{g,h}	60.5 ± 0.2^{g}	19.7 ± 0.2^{c,d,e}	18.2 ± 0.1^{h,i}
H1:0.5SA	1.0:0.5	30.0 ± 0.3^{e,f}	63.5 ± 0.3^{h}	61.9 ± 0.2^{h}	18.5 ± 0.3^{b,c}	16.3 ± 0.5^{f}
H1:0.1λ-C	1.0:0.1	28.7 ± 0.4^{c}	67.1 ± 0.5^{i}	65.2 ± 0.5^{j}	20.3 ± 0.4^{c,d,e}	17.3 ± 0.4^{g}
H1:0.5λ-C	1.0:0.5	28.4 ± 0.7^{c}	69.6 ± 0.4^{j}	67.4 ± 0.5^{k}	19.6 ± 0.5^{a,b}	17.5 ± 0.5^{g}
H2	1.0:0.0	32.5 ± 0.5^{h}	72.2 ± 0.4^{k}	70.1 ± 0.4^{l,m}	15.6 ± 0.3^{a}	13.6 ± 0.3^{d}
H2:0.1SA	1.0:0.1	31.3 ± 0.3^{g}	71.6 ± 0.3^{k}	69.6 ± 0.3^{l}	18.9 ± 0.2^{b,c}	13.6 ± 0.3^{d}
H2:0.5SA	1.0:0.5	31.3 ± 0.2^{g}	66.4 ± 0.3^{i}	64.5 ± 0.3^{i}	19.2 ± 0.1^{c,d}	14.5 ± 0.2^{e}
H2:0.1λ-C	1.0:0.1	31.4 ± 0.7^{g}	85.4 ± 0.5^{m}	83.3 ± 0.4^{n}	18.9 ± 0.4^{b,c}	12.8 ± 0.3^{c}
H2:0.5λ-C	1.0:0.5	30.8 ± 0.8^{f}	91.5 ± 0.5^{n}	89.3 ± 0.5^{o}	19.9 ± 0.3^{c,d,e}	11.3 ± 0.3^{b}
H3	1.0:0.0	33.3 ± 0.3^{h}	73.1 ± 0.4^{l}	70.6 ± 0.3^{m}	16.0 ± 0.4^{a}	15.0 ± 0.3^{e}
H3:0.1SA	1.0:0.1	31.4 ± 0.6^{g}	38.9 ± 0.6^{b}	36.4 ± 0.6^{b}	16.2 ± 0.5^{a}	18.2 ± 0.4^{h,i}
H3:0.5SA	1.0:0.5	29.6 ± 0.4^{d,e}	32.2 ± 0.5^{a}	30.6 ± 0.5^{a}	18.9 ± 0.3^{b,c}	20.5 ± 0.5^{k}
H3:0.1λ-C	1.0:0.1	30.6 ± 0.3^{f,g}	106.7 ± 0.7^{o}	104.5 ± 0.6^{p}	21.4 ± 0.5^{d,e}	11.6 ± 0.5^{b}
H3:0.5λ-C	1.0:0.5	29.0 ± 0.5^{c,d}	114.5 ± 0.5^{p}	113.0 ± 0.5^{q}	16.0 ± 0.5^{a}	8.5 ± 0.5^{a}

Protein bulk concentration 1.0 wt.%, temperature 20 °C, pH 7 and I 0.05 M. Deformation amplitude ($\Delta A/A$) 10%, angular frequency (ω) 0.1 Hz.
[A] Values are presented as mean ± SD. Different letters in each column indicate significant differences among systems ($p < 0.05$).

Table 2
Effect of PS (SA and λ-C) concentration (0.0–0.5 wt.%) on surface hydrophobicity (S_0), molecular apparent diffusion (k^a_{diff}), penetration (k_P) and configurational rearrangement (k_R) parameters for adsorption dynamics of β-LG and its hydrolysates at: 1.0% (H1), 3.0% (H2) and 5.0% (H3) at the air–water interface.

System	Prot:PS (% wt)	S_0 (u.a.)[A]	k^a_{diff} (mN/m $s^{-0.5}$)[A]	k_P (10^{-4} s^{-1})[A]	k_R (10^{-4} s^{-1})[A]
β-LG	1.0:0.0	181 ± 2^{a}	22.2 ± 0.6^{a}	2.23 ± 0.04^{a,b}	6.81 ± 0.16^{b}
β-LG:0.1SA	1.0:0.1	235 ± 7^{c}	23.0 ± 0.2^{b}	2.24 ± 0.06^{a,b}	7.01 ± 0.21^{b,c}
β-LG:0.5SA	1.0:0.5	350 ± 9^{g}	24.6 ± 0.4^{c,d}	2.27 ± 0.04^{b,c}	7.00 ± 0.10^{b,c}
β-LG:0.1λ-C	1.0:0.1	213 ± 9^{b}	22.5 ± 0.2^{a,b}	2.28 ± 0.03^{b,c}	6.80 ± 0.12^{b}
β-LG:0.5λ-C	1.0:0.5	333 ± 8^{f}	24.4 ± 0.3^{c}	2.31 ± 0.04^{c}	7.24 ± 0.16^{c}
H1	1.0:0.0	368 ± 5^{h}	24.6 ± 0.5^{c,d}	2.28 ± 0.01^{b,c}	7.84 ± 0.10^{d}
H1:0.1SA	1.0:0.1	390 ± 8^{k}	25.5 ± 0.3^{f,g}	2.38 ± 0.03^{d}	8.20 ± 0.12^{e}
H1:0.5SA	1.0:0.5	438 ± 18^{l}	26.8 ± 0.6^{i}	2.46 ± 0.02^{e}	8.53 ± 0.15^{f}
H1:0.1λ-C	1.0:0.1	373 ± 2^{h,i}	24.8 ± 0.2^{c,d,e}	2.38 ± 0.02^{d}	8.75 ± 0.14^{f,g}
H1:0.5λ-C	1.0:0.5	389 ± 7^{k}	25.0 ± 0.3^{d,e}	2.47 ± 0.02^{e}	9.05 ± 0.13^{h,i}
H2	1.0:0.0	397 ± 2^{k}	26.2 ± 0.3^{h}	2.79 ± 0.05^{g}	9.03 ± 0.19^{h,i}
H2:0.1SA	1.0:0.1	394 ± 6^{k}	26.0 ± 0.2^{g,h}	2.81 ± 0.02^{g}	8.97 ± 0.16^{g,h}
H2:0.5SA	1.0:0.5	389 ± 7^{k}	25.6 ± 0.2^{f,g}	2.69 ± 0.03^{f}	8.65 ± 0.11^{f}
H2:0.1λ-C	1.0:0.1	387 ± 10^{k,j}	26.0 ± 0.3^{g,h}	2.94 ± 0.05^{h}	9.31 ± 0.10^{j}
H2:0.5λ-C	1.0:0.5	375 ± 6^{h,i,j}	24.7 ± 0.2^{c,d}	3.46 ± 0.06^{i}	10.30 ± 0.11^{k}
H3	1.0:0.0	385 ± 2^{i,j,k}	27.3 ± 0.3^{i}	2.99 ± 0.02^{h}	9.23 ± 0.12^{i,j}
H3:0.1SA	1.0:0.1	290 ± 4^{e}	25.3 ± 0.3^{e,f}	2.20 ± 0.05^{a}	6.32 ± 0.18^{a}
H3:0.5SA	1.0:0.5	269 ± 4^{d}	24.6 ± 0.2^{c,d}	2.18 ± 0.02^{a}	6.15 ± 0.16^{a}
H3:0.1λ-C	1.0:0.1	321 ± 4^{a,f}	25.9 ± 0.2^{g,h}	3.52 ± 0.04^{i}	12.55 ± 0.19^{l}
H3:0.5λ-C	1.0:0.5	266 ± 15^{d}	24.4 ± 0.2^{c,d}	3.67 ± 0.06^{h}	15.47 ± 0.20^{m}

Protein bulk concentration 1.0 wt.%, temperature 20 °C, pH 7 and *I* 0.05 M.
[A] Values are presented as mean ± SD. Different letters in each column indicate significant differences among systems ($p < 0.05$).

E–π plots toward higher π values, which suggested an enhanced packaging and/or condensation of adsorbed β-LG segments.

3.2. Effect of PS on the surface properties of β-LG and its hydrolysates

3.2.1. Surface pressure at long term adsorption

The effect of PS concentration (0.0–0.5 wt.%) on the surface pressure (π_f) at long term adsorption (10,800 s) for β-LG and its hydrolysates is shown in Table 1. In general, it was observed that the PS (SA and λ-C) addition caused: (i) an increase in π_f values for β-LG and H1, suggesting a synergistic effect of PS on the interfacial adsorption of β-LG and H1, and (ii) a decrease in π_f values for H2 and H3, indicating that PS could promote a lower interfacial adsorption of these hydrolysates. From these results, it can be deduced that the PS addition would affect the interfacial adsorption process of β-LG and its hydrolysates, depending on different biopolymer interactions both in solution and at the air–water interface (Baeza et al., 2005; Martínez et al., 2007). This hypothesis will be discussed from a kinetic point of view.

3.2.1.1. Diffusion. In mixed systems, the diffusion step of β-LG and its hydrolysates was observed to be too fast ($\pi > 10$ mN/m), so that k_{diff} values could not be calculated according to Eq. (2). Therefore, k^a_{diff}, was calculated as previously described. Table 2 shows the effect of PS concentration on the k^a_{diff} value for β-LG and its hydrolysates. It was observed that:

(i) The increase in SA concentration caused a significant increment in k^a_{diff} values for β-LG and H1 ($p < 0.05$), reaching a maximum value for H1:0.5SA system (1.0:0.5 wt.%), whereas it caused a significant reduction in k^a_{diff} values for H2 and H3 ($p < 0.05$), with a minimum value for H3:0.5AS system (1.0:0.5 wt.%).
(ii) The addition of 0.1 wt.% λ-C had no significant effect on k^a_{diff} values for β-LG and H1, whereas it caused a significant reduction in k^a_{diff} values for H2 and H3 ($p < 0.05$). However, the addition of 0.5 wt.% λ-C produced: (a) a significant increase in k^a_{diff} value for β-LG ($p < 0.05$), (b) no significant effect on k^a_{diff} value for H1, and (c) a significant reduction in k^a_{diff} values for H2 and H3 ($p < 0.05$).

From these results, it can be deduced that the diffusion rate of mixed systems would be strongly affected by interaction dynamics between biopolymers in solution. Therefore, in order to evaluate the incidence of these interactions on the diffusion step toward the interface, the surface hydrophobicity (S_0) of mixed systems was evaluated. Table 1 shows the effect of PS concentration on the S_0 values for β-LG and its hydrolysates, as follows:

(i) The increment in PS concentration produced a significant increase in S_0 values for β-LG and H1 ($p < 0.05$). The SA addition caused a greater effect on S_0 as compared to λ-C. The increase in S_0 value could be due to a greater number of hydrophobic areas exposed on proteins as a consequence of modifications in their thermodynamic activity in the PS presence (Pavlovskaya et al., 1993). At neutral pH and low ionic strength, the high repulsion between proteins and PS in solution could increase the exposure of protein hydrophobic areas, resulting in increased interaction with ANS and, consequently, a higher S_0 value (Uruakpa and Arntfield, 2006). Thermodynamically, this situation would be unfavorable and the system could tend to minimize the contacts between protein exposed hydrophobic areas and the aqueous medium through hydrophobic effect, which could enhance the diffusion step toward the interface (Grinberg and Tolstoguzov, 1997).
(ii) The PS addition (in the evaluated concentration range) had no significant effect on S_0 value for H2.
(iii) The increment in PS concentration caused a significant decrease in S_0 value for H3 ($p < 0.05$). In mixed systems, the reduction in S_0 value could be related to a lower accessibility of the exposed hydrophobic areas on H3 for its association with ANS (Perez et al., 2009a, 2010, 2011). This behavior could explain the lower diffusion rate of H3/PS systems through two types of biopolymer interactions in solution:
    (I) Thermodynamic incompatibility between biopolymers, leading to a reduction of protein hydrophobic areas accessible to ANS through segregation phenomenon which could promote protein aggregation in solution (Uruakpa and Arntfield, 2006).

(II) Association between protein and PS, which could hinder sterically the access to ANS to interact with the surface hydrophobic areas on the protein. This association phenomenon could be explained in terms of the soluble complexes formation via electrostatic attraction between the positive *patches* on protein and negative charges on PS (Galazka et al., 1999).

In H3/PS systems, both types of biopolymer interaction would be feasible considering; (i) the protein structural modification subsequent to enzymatic hydrolysis (e.g. exposure of hydrophobic areas, possible alteration of the surface net charge, etc.) and, (ii) different chemical structures of PS. Under the aqueous medium conditions evaluated (neutral pH and low ionic strength), it can be argued that H3/PS systems containing carboxylic PS, such as SA, could be governed by segregation and H3 aggregation in solution (i.e. interaction behavior type I), whereas H3/PS systems containing sulphated PS, such as λ-C, could be characterized by soluble complexes formation (i.e. interaction behavior type II) (Grinberg and Tolstoguzov, 1997; Perez et al., 2009a). Thus, the interaction dynamics and, consequently, diffusion behavior in H3/PS systems could be mainly determined by the chemical nature of PS.

From these results, a close relationship was found between k^a_{diff} and S_0 values of mixed systems (Fig. 2A). From this relationship, it could be deduced that biopolymer interactions that promote lower S_0 values (e.g. soluble complexes formation and/or segregation with protein aggregation in solution) could produce lower diffusion rates of β-LG and hydrolysates. However, biopolymer interactions that promote higher S_0 values (e.g. high exposure of protein hydrophobic areas due to the high repulsion between biopolymers) could produce higher diffusion rates toward the air–water interface. Therefore, it can be concluded that different interactions in solution between proteins (β-LG and hydrolysates) and PS could exert a great influence on the diffusion rate and, consequently, on the interfacial film formation.

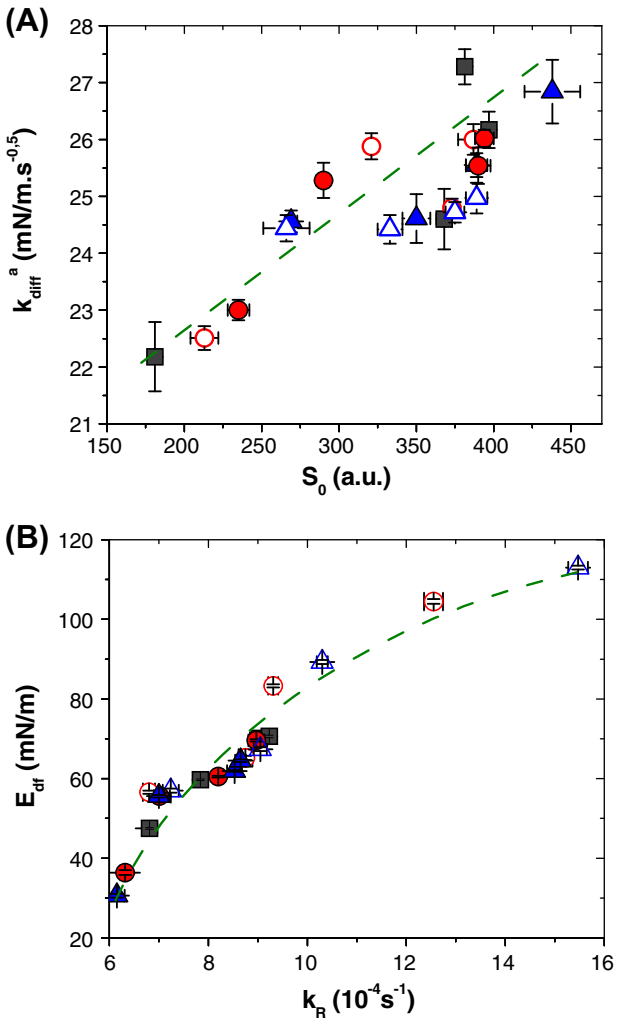

Fig. 2. Relationship between surface hydrophobicity (S_0) with apparent diffusion rate constant (k^a_{diff}) (A) and relationship between rearrangement rate constant (k_R) and dilatational elasticity (E_{df}) of films at long-term adsorption (10,800 s) (B) for Prot:PS systems: 1.0:0.0 wt.% (■), 1.0:0.1 wt.% (AS ●, λ-C ○), 1.0:0.5 wt.% (AS ▲, λ- C △). Protein (β-LG and its hydrolysates) bulk concentration 1.0 wt.%, temperature 20 °C, pH 7 and *I* 0.05 M. Deformation amplitude ($\Delta A/A$) 10%, angular frequency (ω) 0.1 Hz.

3.2.1.2. Penetration and rearrangement. The effect of PS concentration on the rate constants of penetration (k_P) and rearrangement (k_R) for β-LG and its hydrolysates is shown in Table 2. It was observed that:

(i) The SA addition did not significantly affect k_P and k_R values for β-LG, whereas k_P and k_R values for H1 significantly increased with increased SA concentration ($p < 0.05$). This behavior could be linked to the high diffusion rate in H1/SA systems (Baeza et al., 2005; Perez et al., 2009b). However, an antagonistic behavior was observed with the increment in HD. Under these conditions, the SA addition caused a significant reduction in k_P and k_R values ($p < 0.05$), mainly in H3/SA systems. This behavior could be associated with slower diffusion rate in H3/SA systems. Therefore, from these results it can be deduced that the penetration and rearrangement rates for β-LG and its hydrolysates would depend on the strength and nature of biopolymer interactions in solution according to the SA relative concentration and/or to protein structural modification subsequent to enzymatic hydrolysis.

(ii) The λ-C addition did not significantly affect the k_P and k_R values for β-LG. However, the increment in λ-C concentration produced a significant increase in k_P and k_R values for β-LG hydrolysates (H1, H2 and H3) ($p < 0.05$). Despite slower diffusion rates in H2/λ-C and H3/λ-C systems (as it can also be deduced from Table 2), the kinetics at long adsorption times could be favored by the increment in λ-C concentration (Perez et al., 2009b), and/or the greater β-LG structural change (as a consequence of enzymatic hydrolysis).

(iii) Finally, k_P values were lower than those of k_R, suggesting that the penetration step would be the limiting step of the interfacial adsorption process of mixed systems.

3.2.2. Dilatational rheological behavior at long term adsorption

The effect of PS concentration (0.0–0.5 wt.%) on surface dilatational parameters (E_f, E_{df}, E_{vf} and ϕ_f) for β-LG and its hydrolysate films at long term adsorption (10,800 s) is also shown in Table 1. It was observed that: (i) E_f and E_{df} values were high, and (ii) E_{vf} and ϕ_f values were low. Therefore, it could be concluded that surface dilatational behavior of mixed systems was essentially elastic at the angular frequency (ω) applied (0.1 Hz). Besides, Table 1 shows that:

(i) PS addition produced an increase in E_{df} value for β-LG ($p < 0.05$), the effect produced by λ-C being greater. However, increased PS concentration caused a significant decrease in ϕ_f value for β-LG (similar effect for both PS) ($p < 0.05$).

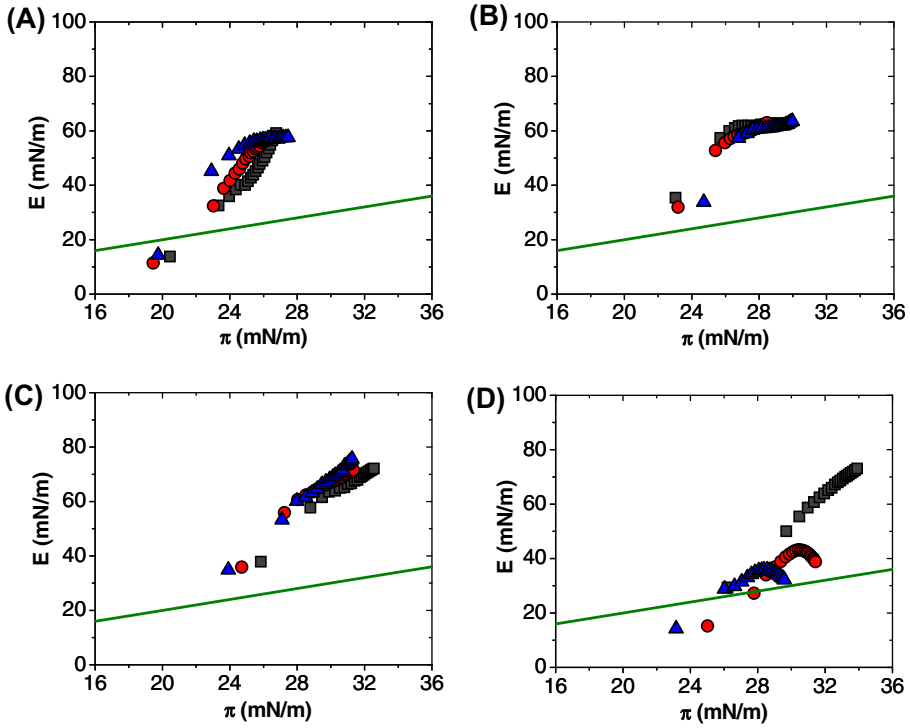

Fig. 3. Effect of AS concentration on the interfacial structuration (given by E–π plot) of β-LG (A) and its hydrolysates at: 1.0%, H1 (B), 3.0%, H2 (C) and 5.0%, H3 (D) adsorbed films at the air–water interface. Symbols Prot:SA systems: (■) 1.0:0.0 wt.%, (●) 1.0:0.1 wt.%, (▲) 1.0:0.5 wt.%. Protein (β-LG and its hydrolysates) bulk concentration 1.0 wt.%, temperature 20 °C, pH 7 and I 0.05 M. Deformation amplitude ($\Delta A/A$) 10%, angular frequency (ω) 0.1 Hz.

(ii) The increment in PS concentration produced a significant increase in E_{df} value for H1 ($p < 0.05$), the effect produced by λ-C being greater. However, increased SA concentration caused a significant decrease in ϕ_f value for H1 ($p < 0.05$), while the presence of λ-C did not affect ϕ_f value for H1.

(iii) The increment in SA concentration produced a significant decrease in E_{df} value and an increase in ϕ_f value for H2 and H3 ($p < 0.05$). However, increased λ-C concentration caused the opposite effect, i.e. an increase in E_{df} value and a decrease in ϕ_f value for H2 and H3. These effects were seen to be more pronounced in H3/PS systems.

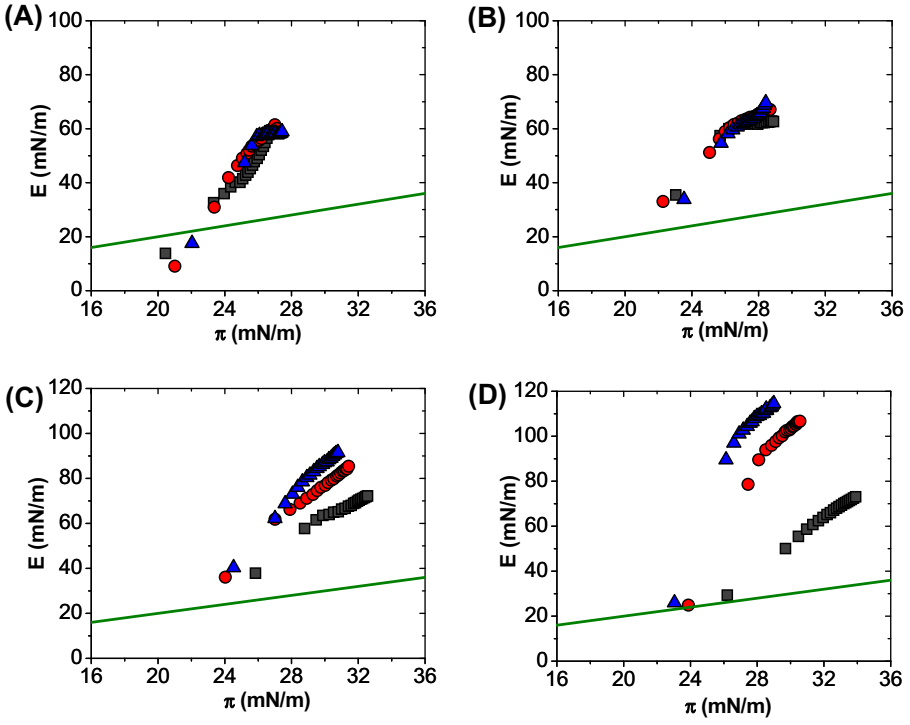

Fig. 4. Effect of λ-C concentration on the interfacial structuration (given by E_d–π plot) of β-LG (A) and its hydrolysates at: 1.0%, H1 (B), 3.0%, H2 (C) and 5.0%, H3 (D) adsorbed films at the air–water interface. Symbols Prot:λ-C systems: (■) 1.0:0.0 wt.%, (●) 1.0:0.1 wt.%, (▲) 1.0:0.5 wt.%. Protein (β-LG and its hydrolysates) bulk concentration 1.0 wt.%, temperature 20 °C, pH 7 and I 0.05 M. Deformation amplitude ($\Delta A/A$) 10%, angular frequency (ω) 0.1 Hz.

Dilatational rheological behavior (E_{df} and ϕ_f values) of mixed system adsorbed films could be explained in terms of different interactions between proteins (β-LG and hydrolysates) and PS both in solution and at the interfacial vicinity (Martínez et al., 2007; Perez et al., 2009b, 2010, 2011). Thus, higher E_{df} values and lower ϕ_f values could suggest the presence of a significant synergism on the elastic character of films (β-LG/PS, H1/PS, H2/λ-C and H3/λ-C) which could be promoted by a greater number of interactions among adsorbed protein segments. Nevertheless, the gradual decrease in E_{df} values and the progressive increase in ϕ_f values for H2/SA and H3/SA films could be attributable to a reduction in the number of interactions among adsorbed hydrolysate segments, and/or an increase in the film viscous character (Perez et al., 2009b, 2011).

On the other hand, a close relationship was found between the elastic character of films (E_{df}) and the rearrangement rate (k_R) in mixed systems. This finding is represented as an example in Fig. 2B. From this relationship, it could be deduced that biopolymer interactions that promote lower k_R values (e.g. segregation with protein aggregation in solution) could produce lower E_{df} values. However, biopolymer interactions that promote higher k_R values (e.g. high exposure of protein hydrophobic areas due to the high repulsion between biopolymers and/or soluble complexes formation) could produce higher E_{df} values. Therefore, it can be concluded that different interactions in solution between β-LG and its hydrolysates and PS could exert a great incidence on interfacial rearrangement rate and, consequently, on the elastic character of films. Martínez et al. (2005, 2007, 2009, 2011), using soy and sunflower protein isolates, have also shown that bulk and surface rheological properties of protein/polysaccharide systems are strongly affected by the enzymatic hydrolysis of proteins.

3.2.3. Molecular structuration at the air–water interface

Figs. 3 and 4 shows the effect of PS concentration (0.0–0.5 wt.%) on interfacial structuration (given by E–π plot) of β-LG and hydrolysates. In general, it was observed that:

(i) E–π curves were located above the line that denotes the film ideal behavior (solid lines).
(ii) E–π slopes were greater than one, suggesting a large number of interactions among adsorbed protein segments (Lucassen Reynders et al., 1975).
(iii) E–π curves were not normalized with master curves for pure proteins (β-LG and hydrolysates) indicating that protein hydrophobic segments could be adsorbed with different degrees of structure (packaging and/or condensation) at the air–water interface (Miñones Conde and Rodríguez Patino, 2005; Rodríguez Patino et al., 2005), depending on the PS type, their relative concentration and HD.

The effect of SA on the interfacial structuration of β-LG and hydrolysates is shown in Fig. 3. It was observed that the increase in SA concentration produced.

(i) A gradual displacement of E–π curve for β-LG toward high E values, especially at low π values (Fig. 3A), indicating a synergistic effect on interactions among adsorbed β-LG segments.
(ii) A slight effect on the E–π curve for H1 (Fig. 3B).
(iii) A gradual displacement of the E–π curve for H2 and H3 films towards low E values (Fig. 3C and D). In addition, E–π curves for H3:0.1SA system (1.0:0.1 wt.%) and H3:0.5SA system (1.0:0.5 wt.%) showed maximum peaks at $\pi \approx 28$ mN m and at $\pi \approx 30$ mN/m, respectively (Fig. 3D). These π values could be considered as critical surface pressures (π_c) associated with a transition in the viscoelastic behavior of H3 film (Bos and van Vliet, 2001). Moreover, these π values were lower than π_f value of H3 (33.4 mN/m) (as it can be seen in Table 1). Therefore, the collapse of H3 adsorbed film at the air–water interface could take place at π_c with the consequent deterioration of its elastic character (Perez et al., 2009b). This behavior could be due to a reduction in the number of interactions among adsorbed H3 segments promoted by increasing AS concentration and HD (as it can be deduced by lower k_P and k_R values from Table 2).

On the other hand, Fig. 4 shows the effect of λ-C on the interfacial structuration of β-LG and hydrolysates. It was observed that an increase in λ-C concentration caused:

(i) A displacement of E–π curve for β-LG towards high E values, mainly at low values of π (Fig. 4A), suggesting the presence of a synergistic effect of λ-C on interactions between adsorbed β-LG segments.
(ii) A slight effect on E–π curve of H1, especially at high π values (Fig. 4B).
(iii) A higher displacement of E–π curves of H2 and H3 toward high E values (Fig. 4C and D) indicating a greater number of interactions between adsorbed hydrolysate segments. These results confirm the presence of a synergistic effect of interactions between biopolymers (both in solution and at the vicinity of the interface) on the film interfacial structure promoted by higher adsorption kinetic parameters (k_P and k_R) and film elastic character (Perez et al., 2009b). This behavior could be favored by the increased λ-C relative concentration, the greater structural alteration of β-LG (due to enzymatic hydrolysis) and/or the combined effect of these factors.

4. Conclusions

The enzymatic treatment using bovine α-chymotrypsin II (in the range of HD = 0.0–5.0%) improved the surface and elastic properties of the β-LG adsorbed film due to an increased exposure of hydrophobic areas.

On the other hand, PS addition (0.0–0.5 wt.%) affected β-LG interfacial adsorption according to different biopolymer interactions in solution, which would mainly depend on the extent of enzymatic treatment. Low β-LG hydrolysis (HD, 1.0%) enhanced surface and elastic properties of adsorbed films possibly due to the higher repulsion between proteins and PS in solution. However, greater β-LG hydrolysis (HD = 3.0–5.0%) promoted: (i) a deterioration of surface and elastic properties of adsorbed films which could be due to the segregation and hydrolysate aggregation in the presence of SA or (ii) an enhancement of interfacial properties of films possibly due to the adsorption of soluble complexes formed in mixed systems with λ-C. These soluble complexes could exert a better control of elastic properties of β-LG hydrolysate films.

The results confirm the hypothesis that interfacial adsorption process of β-LG could be conveniently manipulated through structural modification by limited enzymatic hydrolysis (as an engineering strategy) and interactions with polysaccharides (as a formulation strategy). The information derived from this work could be used for the development and improvement of protein food foams.

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