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Soy Protein Hydrolysis and Polysaccharides Interactions: Concentration Effect On Kinetic Adsorption at Air-Water Interface

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ABSTRACT

The objective of the work was to study the influence of soy protein concentration, and the hydrolysis on kinetic adsorption to the air-water interface with the effect of polysaccharides addition. As starting material, a sample of commercial soy protein isolate was used (SP) and hydrolysate (H) at 2, 10^{-2} and 10^{-3}%wt/wt of concentrations, was produced by an enzymatic reaction. The degree of hydrolysis was 2%. The polysaccharides (PS) used at 0.25%wt/wt of final concentration were hydroxypropylmethylcellulose (E4M) and lambda carrageenan (□C). The dynamic surface pressure of films was evaluated with a drop tensiometer. We determined the kinetic parameters of adsorption to the air-water interface: the diffusion (Kd), penetration (Kp) and rearrangement (Kr) rates of SP, H and the mixed systems with PS. The parameters of adsorption depended on the protein size; concentration and PS used. Kd and Kp showed protein or PS effects depended the concentration of protein; however, when Kr was analyzed, the effect of hydrolysis and PS added showed to have a big importance in the films properties, which represents the behavior at long times of adsorption.

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1. Introduction

The use of soy proteins (SP) as functional ingredients in food manufacturing is increasing because of their role in human nutrition and health. The major globulins of SP are conglycinin (7S) and glycinin (11S). Native SP, because of its quaternary and compact tertiary structure has limited foaming (Kinsella, 1979; Utsumi, Matsumura and Mori, 1997; Yu and Damodaran, 1991) and emulsifying (Kinsella, 1979; Liu, Lee and Damodaran, 1999) properties. However, structural modifications by chemical methods such as deamidation,

succinilation, reduction or denaturation, allowing greater conformational flexibility of protein, may improve its surface behavior and functionality (Carp et al., 1997; Kim and Kinsella, 1987a, 1987b; Wagner and Gueguen, 1999).

In addition, at high degrees of hydrolysis of protein, the decrease in molecular size can be expected to decrease the ability of the polypeptides at the interface to interact so that less viscoelastic films will cause a decrease in systems stability. Therefore, because of the decreased dispersed systems stability of hydrolyzed proteins, polysaccharides addition would be essential for enhancing the properties.

In a previous work (Martinez et al., 2005) the effect of different polysaccharides on the foaming properties of intact and hydrolyzed sunflower protein isolate (SunP) (degree of hydrolysis of 1.5 and 9.8%) at pH where a limited incompatibility between macromolecules can occur has been studied. A limited enzymatic treatment substantially enhanced foaming properties of sunflower protein (Martínez et al., 2005). A small degree of hydrolysis (DH=1.5%) enhanced both foam overrun and foam stability against liquid drainage and collapse. However, an increase of DH to 9.8% did not further improve foaming properties. The overrun of foams was decreased in the presence of all the polysaccharides used but the performance of polysaccharides as stabilizers of foams depended on the protein hydrolysis, the structure of the polysaccharide and its concentration in the liquid used to make the foam.

In the present work, we analyzed the kinetic interfacial performance of mixed systems at different protein concentrations. In the basis of previous studies where a limited hydrolysis of this protein (2%) seemed to be the best strategy to improve penetration and rearrangements rates of stages of protein adsorption, with and without polysaccharides (Martinez, Carrera and Pilosof, 2016), it was selected to combine it at different concentrations in the current work.

2. Materials and Methods

2.1. Materials

A commercial soy protein isolate (SP) (90% protein) from Sambra, Brazil was used as substrate for the hydrolysis with fungal protease from *Aspergillus oryzae* with endopeptidase activity, provided by Quest International. The protein isolate was denatured as detected by differential scanning calorimetry. The polysaccharides (PS) used were: hydroxypropylmethylcellulose (HPMC) called Methocel E4M as surface active polysaccharide from Dow Chemical Co.; lambda carrageenan "λC") by Sanofi Bioindustries, Argentina, all used without further purification. The final concentration used of polysaccharides in mixed systems was 0.25% wt/wt, usual in the industrial food processing additives.

2.2. Enzymatic hydrolysis

SP isolate (72 g in 1200 ml of water) was hydrolyzed according to Zylberman and Pilosof (2002) batch-wise by treatment with fungal protease at pH 7, 50 ºC for 1 h, with enzyme/substrate (E/S) ratio: 0.5/100. Hydrolysis was stopped by heating at 80 °C for 10 min. The variation in pH was very small (maximum decrease

0.3 pH units) and was adjusted back to the original value with diluted NaOH. The hydrolysate was lyophilized. The degree of hydrolysis (DH), defined as the percentage of peptide bonds cleaved, was calculated from the determination of free amino groups by reaction with *o*-phthaldialdehyde (OPA) according to Church, Swaisgood, Porter & Catignani (1983).

Protein hydrolysate with 2% DH (H) was obtained.

2.3. Preparation of solutions

Solutions for interfacial studies were prepared by dissolving the proteins inMilli-Q ultrapure water. The pH and ionic strength were kept constant at 7 and 0.05M, respectively, by using a commercial buffer solution called Trizma $(CH_2OH)_3$ $CNH_2/(CH_2OH)_3$ CNH_3Cl (Sigma,499.5%). The mixed systems had a protein/ polysaccharide concentration of 2/0.25; 10^{-2}/0.25 and 10^{-3}/0.25%wt/wt.

2.4. Dynamic surface tension

Time-dependent surface pressure (π) of adsorbed mixed films at the air–water interface was performed by an automatic drop tensiometer as described elsewhere (Rodrıguez Niño and Rodrıguez Patino, 2002). Aqueous solutions of SP and their hydrolyzates, PS and their mixtures were placed in a 15 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 ºC) by circulating water from a thermostat, and were all lowed to stand for 30 min to reach constant temperature and humidity in the compartment. Then a drop of solutions (5–8 μl) was delivered and allowed to stand at the needle tip for about 180 min to achieve adsorption at the air–water interface. The image of the drop was continuously taken from a CCD camera and digitalized. The surface tension (σ) was calculated through the analysis of the drop profile (Labourdenne et al., 1994). The surface pressure is $\pi = \sigma_o - \sigma$, where σ_o is the surface tension of pure water in the absence of macromolecules. The average accuracy of the surface tension was roughly 0.1 mN/m. However, the reproducibility of the results (for at least two measurements) was better than 1%.

2.5. Kinetics of adsorption

The kinetics of protein adsorption at the air–water interface can be monitored by measuring changes in surface pressure. MacRitchie, (1990), has summarized the main features of the adsorption of proteins, which can be extended to surface-active polysaccharides (Pérez et al., 2007). The adsorption of these biopolymers at a fluid interface includes (*i*) the diffusion of the protein from the bulk onto the interface, (*ii*) adsorption (penetration) and interfacial unfolding, and (*iii*) aggregation (rearrangement) within the interfacial layer, multilayer formation and even interfacial gelation. During the first step, at relatively low surface pressures, when diffusion is the rate-determining step, a modified form of the Ward and Tordai equation (Ward and Tordai, 1946) can be used to correlate the change in surface pressure with time (Eq. (1)).

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

where "C_0" is the concentration in the bulk phase, "K" is the Boltzmann constant, "T" is the absolute temperature, and "D" is the diffusion coefficient. If the diffusion of the biopolymer at the air–water interface controls the adsorption process, a plot of π versus $t^{1/2}$ will then be linear (MacRitchie et al.,1978; Xu and Damodaran, 1994), and the slope of this plot will be the diffusion rate constant (Kd). At higher adsorption time, in the period after that affected by the diffusion, an energy barrier for mixtures adsorption exists, which can be attributed to adsorption, penetration, unfolding and rearrangements of the macromolecules at the interface (Rodrıguez Patino, Rodrıguez Niño and Carrera, 1999).

Because the interfacial concentration of adsorbed macromolecules is several times higher than that in the bulk phase, the molecular unfolding and rearrangement steps are magnified processes happening at interface, especially for high molecular weight macro-molecules. To monitor adsorption/penetration/unfolding of adsorbed molecules, the approach proposed by Graham and Phillips (Graham and Phillips, 1979) was used. Thus, the rate of these processes can be analyzed by a first order (Eq. (2)):

$$\ln (\pi_{180} - \pi_{\phi}) / (\pi_{180} - \pi_0) = -k_{i\phi} \quad (2)$$

where π_{180}, π_0 and π_t are the surface pressures at 180 min of adsorption time, at time t= 0, and at any time t, respectively, and k_i is the first-order rate constant.

In practice, a plot of Eq. (2) usually yields two or more linear regions. The initial slope is taken to correspond to a first-order rate constant of adsorption (Kp), while the second slope is taken to correspond to a first-order rate constant of rearrangement (Kr), occurring among a more or less constant number of adsorbed molecules.

All measures were made at least two times and errors less of 10% were obtained.

3. Results

3.1. Polysaccharides addition effect on kinetic adsorption of SP as function of concentrations

Surface pressure immediately increased after drop formation, a fact that should be associated with the adsorption of these biopolymers at the air–water interface (Graham and Phillips, 1979; Damodaran and Song, 1988).

In the Figure 1 it can be seen the surface pressure increase as a function of time for SP and the polysaccharides combinations (Martinez et al., 2007).

For adsorption of SP and its mixed systems from aqueous solutions it is known that diffusion at the interface controls the adsorption process at short adsorption time (Minones Jr. and Rodrıguez Patino, 2007).

Thus, from the slope of the plot of π against t ½ it was deduced the diffusion rate (Kd) of protein towards the interface. The π–$t^{1/2}$ plots showed that at 2%wt/wt concentration an aqueous phase diffusion step was too fast to be detected by the experimental technique used in this work (π > 10 mN/m). However, for these

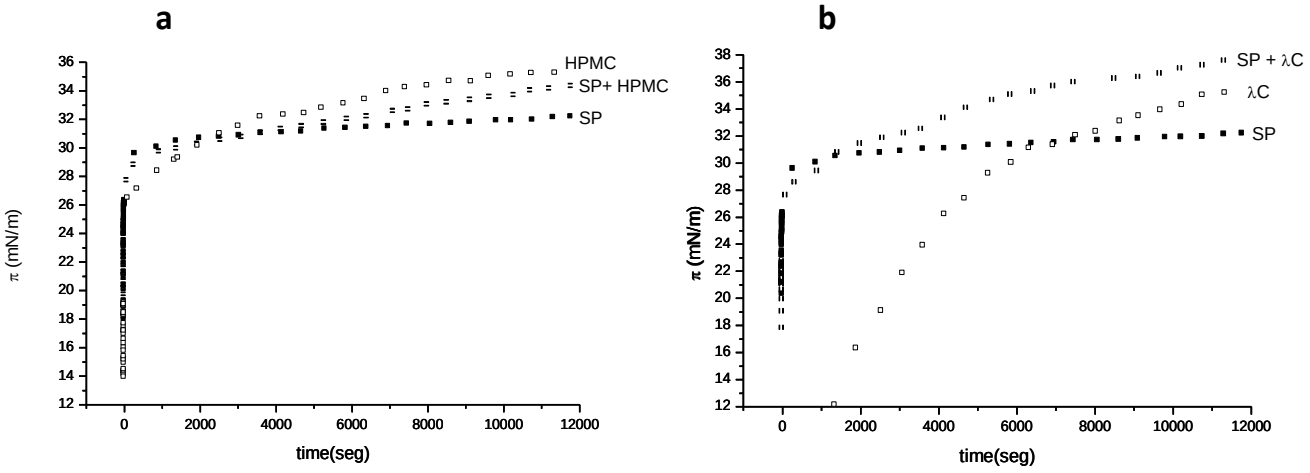

Figure 1: The transient surface pressure π for (a) soy protein (SP), HPMC (E4M) and SP + HPMC, (b) soy protein (SP), λC and SP + λC.

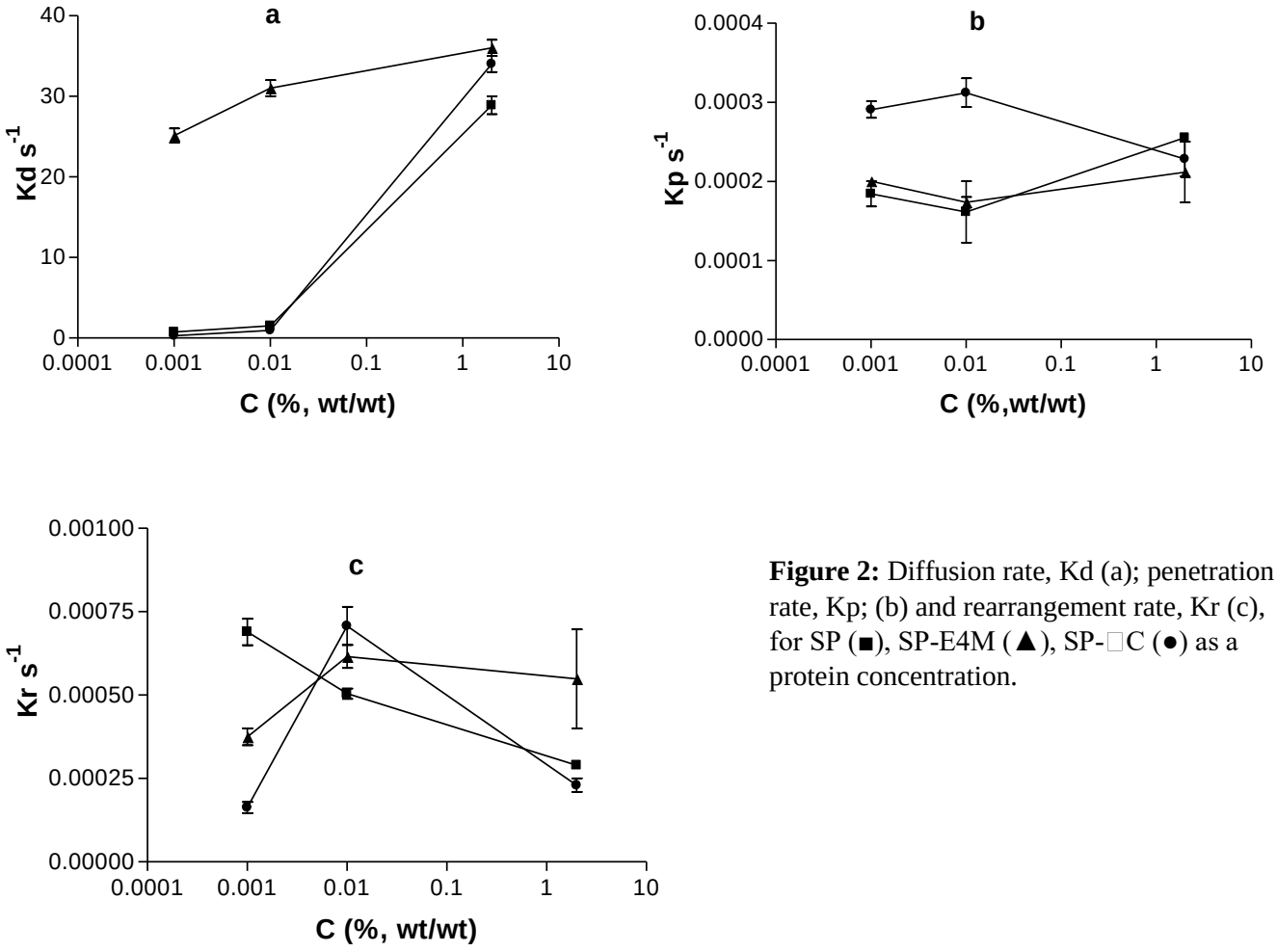

Figure 2: Diffusion rate, Kd (a); penetration rate, Kp; (b) and rearrangement rate, Kr (c), for SP (■), SP-E4M (▲), SP-□C (●) as a protein concentration.

systems the slope of the π–$t^{1/2}$ curve at the beginning of the adsorption (at 0.5 s) can be considered as a measure of the apparent rate of diffusion, Kd to compare with other concentrations.

In the adsorption at the air–water interface from protein solutions it was observed that the rate of surface pressure change over time increased when the protein concentration in the bulk phase increased (Minones Jr. et al., 2007). The fact that the time dependence of the surface pressure follows the same trend as the protein surface concentration (Rodrıguez Patino et al., 2003) indicates that π depends on the surface coverage, which is expected to increase with time and concentrations.

In the Figure 2 a-c it can be seen Kd, Kp and Kr as a function of concentrations (10^{-3}, 10^{-2} and 2%wt/wt) of SP with the PSs addition.

Kd of SP (Figure 2a) resulted in a great increase at 2%wt/wt. The addition of E4M showed an extraordinary diffusion rate increase at lower concentrations (10^{-3} %wt/wt and 10^{-2}%wt/wt) as consequence of surface active presence of PS and it higher proportion in the systems respect to SP. In these concentrations the PS was the responsible of the π change registered, giving a synergistic effect at the highest SP concentration (Figure 1a). In these conditions, Kd of E4M alone was higher than mixed systems (not shown), indicating that at higher adsorption times a marked increase of π in the mixed systems could be attributed to E4M adsorption (Martinez et al., 2007).

In other hand, λC only provoked a slight synergistic effect on Kd at the highest concentration. In this case, λC alone showed and intermediate value of Kd in between protein and mixed system (not shown) attributed to the presence of surface-active contaminant (Martinez et al., 2007).

In previous publications we showed that the presence of E4M and λC greatly increased the interfacial parameters on the basis of different mechanisms. E4M competed for the interface with SP, but due to its unusual strong surface activity it could dominate the surface pressure and improve film viscoelasticity, even at short times of adsorption. Whereas, the modification of surface pressure and rheological properties of adsorbed SP films in the presence of λC necessarily suggests the participation of λC contaminants at the interface. Pure λC could influence the interface by a complexation mechanism, or indirectly by a depletion mechanism in the vicinity of the interface in the present work, at the highest concentration, (Martinez et al.,2007).

In the Figure 2 b-c, the initial slope from eq, (2) to correspond to a first-order rate constant of adsorption (Kp), and the second slope (Kr) were taken to correspond to the penetration and rearrangement rate respectively of biopolymers as a function of protein concentration with the

PSs addition. Because the interfacial concentration of adsorbed protein is several times higher than that in the aqueous phase, molecular unfolding and rearrangement steps are magnified processes happening at interface, mainly for high-molecular weight biopolymers.

The higher π values at higher protein concentrations in solution correspond to the higher quantity of protein adsorbed (Martín, Bos and van Vliet, 2002). When an activation energy barrier to adsorption exists at this point as deduced from the π–time dependency at short adsorption time (Rodríguez Patino et. al., 2003), the ability of the protein molecules to create space in the existing film and penetrate and rearrange at the interface is rate-determining. This phenomenon is reflected in the evolution of the values of Kp and Kr with protein and systems concentrations. It is supposed that, penetration of protein at the interface is facilitated at higher protein concentrations in the bulk phase.

It can be clearly seen for the SP behavior (Figure 2b), where a huge Kp increase can be observed at 2%wt/wt for SP concentration. However, a different behavior was observed when PSs were added to SP isolate. When E4M was added, penetration reached almost at the same value as was obtained at the lowest SP concentration system (10^{-3}%wt/wt). In a previous submitted publication (Martinez, Carrera and Pilosof, 2016) E4M alone at 0.25%wt/wt showed the highest penetration rate, but it was decreased when proteins at 2% were present. It means that by comparing separately, the PS had a better ability to penetrate to the interface, but when both biopolymers were together, interactions between them would promote different performance on dynamics measurements. It was seen that in these conditions, in general, an increase of rates was observed due to a faster diffusion of proteins to the interface, phase separation (i.e aggregation of the protein induced by the polysaccharide) and increase of surface hydrophobicity by the unfolding of protein, (Baeza et al., 2004; Rodrıguez Patino and Rodrıguez Nino, 1999). However, in the present work a contrary effect was observed. Probably, the viscosity effect imparted by 0.25%wt/wt of E4M that reduces the penetration rate of the system respect to SP alone showed an unfavorable interaction between biopolymers. This behavior suggests that the presence of E4M at 0.25%wt/wt in the aqueous phase has a significant effect on proteins at low concentrations at the interfacial characteristics and on its ability to penetrate the air–water interface even though the proteins may control this phenomenon at the highest concentration.

In other hand, when λC was added to SP in an increasing concentration, a different

performance was observed. Definitely, the higher Kp values were obtained at lower SP concentrations. In spite of their non-surface active nature of λC, this polysaccharide can act as an active way. In a previous work, we studied the interfacial behavior of mixed SP and PS systems at same conditions to gain knowledge on the interactions between these biopolymers at the air–water interface under dynamic conditions at neutral pH where a limited incompatibility between macromolecules can occur, (Martínez, et al., 2007). It was observed that the adsorption of pure λC at the air–water interface is unlikely because its structure does not have any significant proportion of hydrophobic groups. However, the presence of surface-active contaminant in the λC produced a slow increase in the surface pressure. A review of literature evidence suggests that much of the reported surface activity of hydrophilic polysaccharides is explicable in terms of contamination of small amounts of surface-active protein, (Dickinson, 2003). In addition, surface active contaminant of λC if strongly bound to the polysaccharides and could bring some polysaccharides molecules at the interface. In other hand, pure λC could influence the interface by a complexation mechanism, or indirectly by a depletion mechanism in the vicinity of the interface Therefore, at the highest SP concentration, the penetration rate could be delayed by the protein-PS interaction that provoke to retard the penetration of mixed system to the air-water interface, whereas at lower SP concentrations, only λC contaminants led to an Kp of system increment together with the relative higher concentration and Kp value for λC alone (not shown).

When rearrangement rate for SP was analyzed as a function of concentration, (Figure 2c), a similar tendency as the penetration rate was obtained. However, when PSs were added at every SP concentration, rearrangement rate was kept in a low value at all SP concentration range for mixed systems. These results suggest a possible occlusion of the SP hydrophobic surface sites in the presence of PSs at all studied concentration, which could be associated with the existence of interactions between protein and PSs and/or with a protein aggregation in the presence of PS, delaying the Kr at long adsorption times.

3.2. Polysaccharides addition effect on kinetic adsorption to the air-water interface of soy protein hydrolysate as function of concentrations

The diffusion rate depends on the size, structure and protein concentration which is expected to increase with bulk concentrations as resulted in the SP kinetic adsorption.

In the Figure 3 a-c it can be seen Kd, Kp and

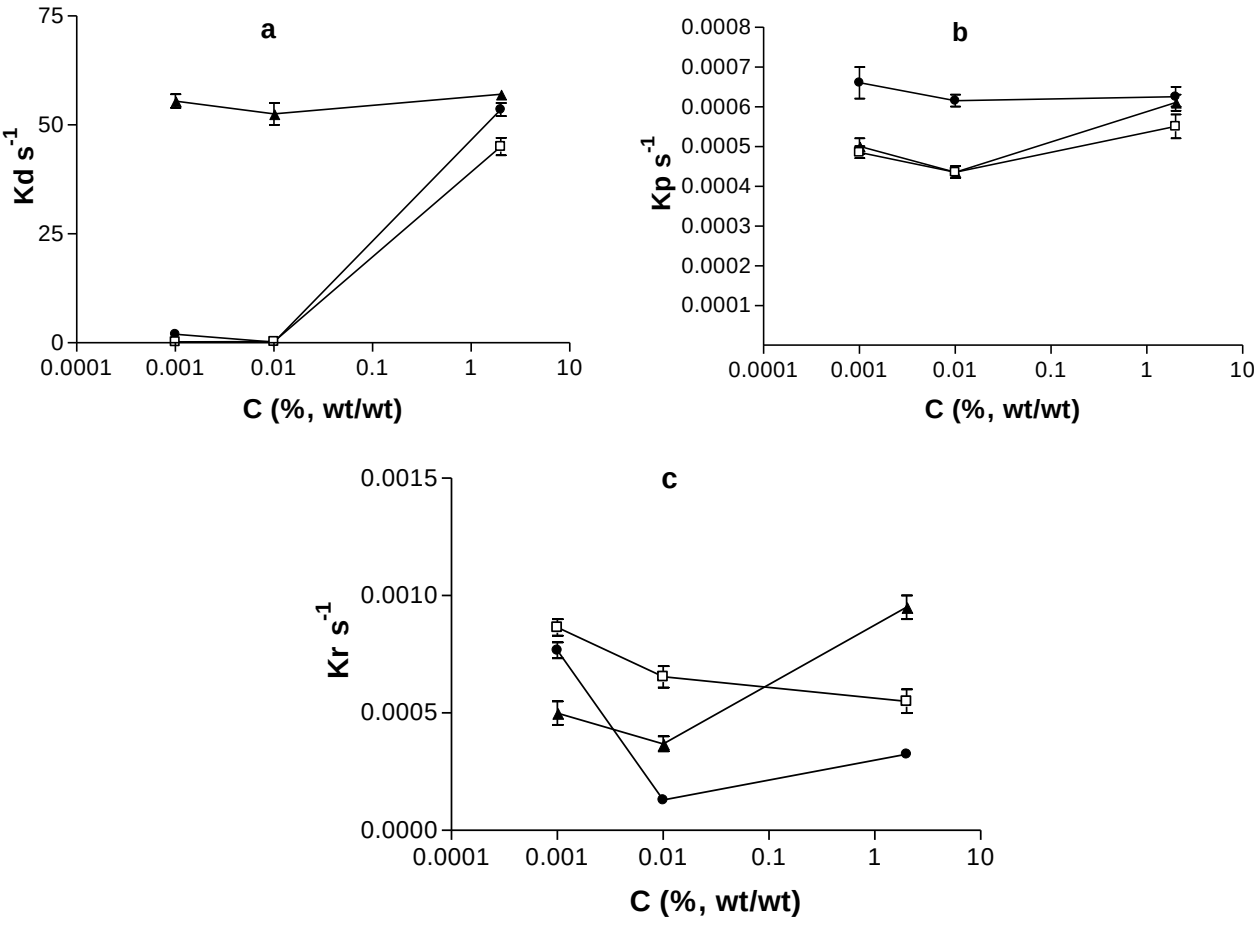

Figure 3: Diffusion rate, Kd (a); penetration rate, Kp; (b) and rearrangement rate, Kr (c), for H (□), H-E4M (▲), H-λC (●) as a protein concentration.

Kr as a function of H (10^{-3}, 10^{-2} and 2%wt/wt) with the PSs addition.

Kd of H (Figure 3a) resulted in a great increase at 2%wt/wt as resulted in the unhydrolyzed protein. The diffusion of the protein is driven by the concentration gradient, in agreement with previous results by other authors (Benjamins, 2000; Rodrıguez Nino and Rodrıguez Patino, 2002; Rodrıguez Patino et al., 2003).

The addition of E4M showed as same way an extraordinary Kd increase at low concentrations (10^{-3} and 10^{-2}%wt/wt) as consequence of their surface active presence and relative concentrations, but giving a less synergistic effect with H at high concentration by comparing with the corresponding mixed SP-E4M systems. λC provoked a slight synergistic effect of Kd at the highest concentration, following the same tendency as was observed for SP-λC system.

It can be observed that the strong surface activity of E4M, contaminants presence of λC and their higher PSs relative concentrations, which were stated as the responsible for the observed behavior with SP, performed as similar way with an increasing H concentration. Nevertheless, it can be seen that the diffusion rate values were higher for H-PS systems for all studied H concentrations. As was mentioned before, the Kd depends on the molecular size and shape, and the chemical nature of the protein surface (such as the hydrophobicity) among other factors. Since the diffusion coefficient is inversely proportional to the cube root of the molecular weight, in agreement with the penetration theory (Ward and Tordai, 1946), the hydrolysis may affect the diffusion rate to the interface. In fact, the reduction of molecular masses in soy hydrolysates might promote faster diffusion of molecules to fluid interfaces (air–water and oil–water, respectively) (Horne and Rodrıguez Patino, 2003; Rodrıguez Nino and Rodrıguez Patino, 2002; Rodrıguez Nino et al., 2003; Rodrıguez Patino et al., 1999).

In the Figure 3b the Kp as a function of H concentration with the PSs addition can be seen. As resulted as SP-PS systems, Kp rate for H alone greatly increased at the highest H concentration, whereas the other H-PS systems resulted in a lower Kp at this condition. Probably the viscosity effect by the PS addition was also very important on this molecular structure. As same way, the λC contaminants would diminish these values a high concentration. It can be also seen an increased Kp at low H concentrations for the mixed systems.

Thus, it can be concluded that PS effect was meanly the responsible for this tendency, controlling this velocity step independent on the molecular structure of protein. Nevertheless, by comparing with SP-PS systems it can be seen again that the rates of penetration were lower for SP than for H. That is, the reduction of the enzymatic treatment would facilitate the penetration and unfolding of the protein at the air–water interface in comparison with SP even of PSs presence.

When rearrangement rate for H was analyzed as a function of their concentration, (Figure 3c), a different tendency by comparing with SP-PS systems was obtained.

Firstly, a decreasing of Kr was observed as concentration increase for H. It can be also seen that the PSs addition promotes an increase of Kr at higher H concentration and a general decrease at low H concentrations by comparing with H alone. It means, only at high H concentration, the PSs addition helped to promote a faster film rearrangement at long times of adsorption.

Some authors have been demonstrated that during the adsorption from aqueous solutions

the kinetics depends on the protein. The slope of eq (2) during the rearrangement of the protein is different depending to the molecular structure. Rodriguez Patino et al., (2003), studied the surface dilatational properties of soy globulins (β-conglycinin, glycinin, and reduced glycinin with 10 mM o dithiothreitol (DTT)) adsorbed onto the air–water interface, as a function of adsorption time. The experiments were performed at constant temperature (20 ◦C), pH (8.0), and ionic strength (0.05 M). The surface rheological parameters were measured as a function of protein concentration (ranging from 1 to 1 × 10^{-3}% wt/wt). They found that the surface dilatational modulus, increases, and the phase angle, (inverse of film viscoelasticity) decreases with time, which may be associated with protein adsorption. These phenomena have been related to protein adsorption, unfolding, and/or protein–protein interactions (at long-term adsorption) as a function of protein concentration in solution.

The main conclusion was that the dilatational properties of the adsorbed films depended on the molecular structure of the protein. The rearrangement of the protein was different for β-conglycinin than for glycinin. For β-conglycinin the rearrangement of the aminoacid residues at the air–water interface produced a reduction in the value of film elastic module. This module time dependence was different for glycinin. Thus, the adsorption of β-conglycinin and glycinin follows different trends. These results strengthen the hypothesis that the structural characteristics of the protein (Ortiz et al., 2003) have a role in the structure and topography of films (Carrera et al, 2004) and in the kinetics of the adsorption and surface dilatational properties of the adsorbed film (Rodriguez Patino et al., 2003).

4. Conclusions

We have determined the kinetic parameters of adsorption to the air-water interface: the diffusion (Kd), penetration (Kp) and rearrangement (Kr) rates of soy protein isolate (SP) and a hydrolysate (H) of 2% degree of hydrolysis as a function of protein concentration (10^{-3}, $10^{-2}$and 2%wt/wt). It was studied also the interactions with polysaccharides, E4M and λC at 0.25%wt/wt of concentration.

In conclusion, molecular weight decrease and the chemical changes as a consequence of enzymatic hydrolysis would promote an increase of diffusion and penetration rates on kinetic adsorption at air-liquid interfaces, following the polysaccharides addition and concentration effect tendency. In other hand, it was observed a different behavior regarding the rearrangement rate. It was found an influence of hydrolysis, polysaccharides type addition and relative concentrations effect for this rate.

Thus, more detailed analysis of the last period of adsorption should be specially studied due to particular behavior presented in front of the other steps of the adsorption.

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