Food Chemistry 116 (2009) 104–113

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Interactions between milk whey protein and polysaccharide in solution

Adrián A. Perez [a], Carlos R. Carrara [a], Cecilio Carrera Sánchez [b], Juan M. Rodríguez Patino [b], Liliana G. Santiago [a,*]

[a] Instituto de Tecnología de Alimentos, Facultad de Ingeniería Química, Universidad Nacional del Litoral, Santa Fe, Argentina
[b] Departamento de Ingeniería Química, Facultad de Química, Universidad de Sevilla, Sevilla, España

ARTICLE INFO

Article history:
Received 9 September 2008
Received in revised form 4 January 2009
Accepted 9 February 2009

Keywords:
Hydrocolloid
Milk protein
Whey protein concentrate
Sodium alginate
λ-Carrageenan
Protein–polysaccharide interactions
Biopolymer in solution

ABSTRACT

In this work we have studied the interactions between a commercial whey protein concentrate (WPC) and two anionic polysaccharides (sodium alginate, SA, and λ-carrageenan, λ-C) in the aqueous phase. The concentration of WPC at 1.0% and the pH 7.0 of the aqueous phase were maintained constant, while polysaccharides (PS) were evaluated within a 0.0–1.0% concentration range. Interactions between WPC and PS in the aqueous phase were analysed by fluorescence spectroscopy, absorption spectroscopy in the presence of methylene blue (MB), and confocal laser scanning microscopy. The results from these methodologies revealed differences in the molecular dynamics of mixed systems. The nature of the interactions between WPC and PS depended on the PS type, its relative concentration in the aqueous phase and also on the two WPC fractions. Whey protein concentrate/sodium alginate (WPC/SA) mixed systems were distinguished by a tendency to protein aggregation in the aqueous phase and their segregation into separated microdomains. On the other hand, WPC/λ-carrageenan (WPC/λ-C) mixed systems showed a high degree of attractive interactions over the whole range of concentrations. The ultrastructure revealed the existence of hybrid macromolecular entities (biopolymer network). Interaction of WPC and polysaccharide in the aqueous phase has an effect on the adsorption of mixed systems at the air–water interface and on their foaming characteristics.

1. Introduction

Protein and polysaccharide interactions are of interest in a variety of scientific areas. Protein–polysaccharide interactions find many applications at the biological level (Tolstoguzov, 2003a) and in the food, cosmetic, and pharmacological industries. Individual biopolymer functionality can be improved by synergistic interactions between different biopolymers (i.e. proteins and polysaccharides), with repercussions for the stability, texture, and shelf-life of many food products (Dickinson, 2003; Tolstoguzov, 2003b). In this context, different methods have been applied, in order to understand the nature, strength, and molecular dynamics of these interactions (Benichou, Aserin, Lutz, & Garti, 2007; Girard, Turgeon, & Gauthier, 2002).

Depending on the aqueous environmental conditions – such as pH, ionic strength, and relative concentration of both biopolymers – four different behaviours have been described for the interaction between milk proteins and anionic polysaccharides in the aqueous phase (Weinbreck, de Vries, Schroogen, & de Kruif, 2003): (i) at neutral pH and low ionic strength, both proteins and polysaccharides have negative charges and, although attractive electrostatic interactions could exist between positive protein patches and negative polysaccharide charges, these biopolymers are co-soluble at low concentrations, (ii) at pH near the protein isoelectric point (p*I*) or at relatively low values, electrostatic protein–polysaccharide complexes are formed, (iii) a higher reduction of the aqueous phase pH leads to aggregation of the soluble complex and to complex coacervation, and (iv) at pH below 2.5, electrostatic biopolymer complexes are generally suppressed, due to the protonation of acid functional groups of the polysaccharide.

On the other hand, because most food dispersions are foams and/or emulsions, protein–polysaccharide interactions, both in the aqueous phase and at fluid interfaces, have an effect on protein interfacial adsorption (Baeza, Carrera Sánchez, Pilosof, & Rodríguez Patino, 2004, 2005; Baeza, Carrera, Rodríguez Patino, & Pilosof, 2006; Martinez, Baeza, Millán, & Pilosof, 2005; Martinez, Carrera, Pizones, Rodríguez Patino, & Pilosof, 2007b) and, consequently, on formation and stability of dispersed colloidal systems (Ganzevles, Cohen Stuart, van Vliet, & de Jongh, 2006; Rodríguez Patino, Rodríguez Niño, & Carrera, 2007, 2008).

In order to understand the functionality of a commercial milk whey protein concentrate (WPC) and two anionic polysaccharides (sodium alginate, SA, and λ-carrageenan, λ-C), the interactions between these biopolymers in the aqueous phase, at neutral pH and low ionic strength, have been studied by various complementary techniques. This contribution complements previous studies on intermolecular interactions between WPC and anionic polysaccha-

* Corresponding author. Tel.: +54 342 4571150; fax: +54 342 4571148.
E-mail address: lsanti@fiqus.unl.edu.ar (L.G. Santiago).

0308-8146/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2009.02.017

rides (PS) at the air–water interface (Perez, Carrara, Carrera, Santiago, & Rodríguez Patino, 2008).

These polysaccharides are used in the food industry as thickening and stabilising agents, being commonly considered as non surface-active polysaccharides (Stephen, 1995). Sodium alginate (SA) is a salt of alginic acid, extracted from brown algae, and formed by 1,4 copolymerisation of D-mannuronic acid and L-guluronic acid units (Moe, Graget, Skjak-Braek, & Smidsrod, 1995; Sharma, 1981). At neutral pH, SA has negative charge, due to the ionisation of carboxylic groups (Harnsilawat, Pongsawatmanit, & McClements, 2006). λ-Carrageenan (λ-C) is a polysaccharide extracted from red algae, consisting of galactose sulphate esters with three sulphate groups per disaccharide unit (Piculell, 1995; Sharma, 1981). λ-Carrageenan is negatively charged at neutral pH, due to the ionisation of the sulphate group.

2. Materials and method

2.1. Materials

Commercial whey protein concentrate (WPC) was kindly provided by Arla Food (Porteña, Cordoba, Argentina). The product is a WPC produced from sweet whey, after rennet casein precipitation by low-temperature ultrafiltration, followed by spray-drying. Its composition was: protein 76.81% ($N \times 6.38$), moisture 4.52%, lactose (max.) 9.0%, fat 2.01%, ash 2.05%, and 5.61% other components. Ions present in WPC powder were quantified by atomic-flame emission spectroscopy of an ash sample and were: Ca^{2+} 0.31%, Na^{+} 0.2%, Mg^{2+} 0.1%, Cl^{-} 0.05%, K^{+} 0.6%, and phosphorus 0.3%. Commercial samples of polysaccharides (PS) were kindly supplied by Degussa (Buenos Aires, Argentina) and used without purification. The nitrogen solubility index (NSI) was determined by the standard methods (AACC, 1983) with a factor ($N \times 6.38$). For the WPC sample at pH 7 NSI = 94.26%. The polysaccharides used were sodium alginate (SA) and λ-carrageenan (λ-C). The molecular weights of PS were 135 kDa and 1000 kDa for SA and λ-C, respectively (data supplied by Degussa). The SA sample has the following composition: carbohydrate 63%, moisture 14%, and ash 23% (Na^{+} 9300 mg/100 g and K^{+} 800 mg/100 g). The λ-C has the following composition: carbohydrate 68%, moisture 8%, and ash 24% (Na^{+} 2700 mg/100 g, K^{+} 5000 mg/100 g, Ca^{2+} 350 mg/100 g, and Mg^{+2} 500 mg/100 g). For an aqueous solution of λ-C at 1% weight and at 25 °C, the viscosity, measured in a Brookfield RVT viscometer, N° 2 spindle, and at 20 rpm, was 330–400 cps. For an aqueous solution of SA at 1% weight and at 25 °C, the viscosity measured in a Brookfield RVT viscometer, N° 1 spindle, and at 20 rpm, was 30–60 cps.

2.1.1. Size-exclusion HPLC

The size-exclusion (SEC) HPLC for commercial WPC at pH 6.8 and 0.05 M ionic strength (I) was determined in a model 9012 chromatograph equipped with a model 9050 UV/vis detector (Varian, Palo Alto, CA) and using a Biosep SEC S2000 column (hydrophilic bonded silica, exclusion range 5 μm). The mobile phase was monitored at 214 or 280 nm. A calibration curve of five standard proteins was used. The molecular weights of protein standards were bovine thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), chicken ovalbumin (44 kDa), horse myoglobin (17 kDa), and vitamin B_{12} (1.35 kDa). Protein samples were dispersed in the mobile phase at 0.05% (w/w protein) and filtered through a cellulose acetate membrane with pore size of 0.45 μm. The chromatogram of the commercial WPC sample showed numerous small peaks at low elution time, which could be ascribed to WPC aggregates with a molecular weight within the range 178–523 kDa. At higher elution time a peak of high intensity, presumably corresponding to the dimeric β-lactoglobulin form (≈36 kDa), was also observed.

2.1.2. Differential scanning calorimetry

Differential scanning calorimetry (DSC) was used to determine the peak temperature (T_p) and the enthalpy (ΔH) involved in WPC thermal denaturation. A DSC821e Mettler Toledo differential scanning calorimeter (Schwerzenbach, Switzerland) was used. The instrument was calibrated with indium (156.6 °C), lead (327.5 °C) and zinc (419.6 °C). The thermal parameters were determined by heating 60 μl of the sample in 160 μl capacity pans from 5 to 100 °C at 10 °C/min. An empty pan was used as reference. The average value of two replicates is reported. DSC for the commercial WPC sample reveals the presence of an endothermic peak ($\Delta H = 6.60 \pm 0.15$ J/g) at 75.2 °C (denaturation temperature), and onset temperature (T_{onset}) = 61.7 ± 0.05 °C. These data were similar to those reported by de Wit, Hontelez-Backx, and Adamse (1988), Spahn, Baeza, Santiago, and Pilosof (2007) for another commercial WPC. These results suggest the presence of a significant amount of native protein in the sample.

2.1.3. Determination of denatured protein

The percentage of denatured protein in the WPC sample was determined according to the method by de Wit et al. (1988) with some modifications. An aliquot of WPC (10.0%) was taken and pH value adjusted to 4.6 ± 0.1 with HCl (2 M) for precipitation of the proteins. The sample was centrifuged at 20,000*g* for 30 min at 5 °C and the nitrogen content determined in the supernatant by the Kjeldahl method ($N \times 6.38$). The percentage of native and denatured protein was calculated as the difference of protein concentration in the original WPC sample and in the supernatant after precipitation at pH 4.6. The average value of two replicates is reported. The results reveal the presence of 84% native and 16% denatured protein in the WPC sample.

2.2. Preparation of WPC/PS mixed systems

Whey protein concentrate and polysaccharide were dissolved in Milli-Q ultrapure water at room temperature and the pH was adjusted to 7 with a >99.5% Trizma buffer solution ($(CH_2OH)_3CNH_2$/$(CH_2OH)_3CNH_3Cl$; Sigma, St. Louis, MO) at 0.05 M of ionic strength (I). Polysaccharide dispersions were stirred for at least 1 h at 80 °C to ensure complete dispersion, and then they were left overnight at 4–5 °C to hydrate appropriately. The WPC concentration was kept constant at 1.0% in most experiments (except those indicated otherwise). The effect of PS (within the range 0.0–1.0%) on the magnitude of the protein–polysaccharide interactions was analysed. WPC/PS mixed systems were obtained by mixing appropriate volumes of each double concentrated biopolymer solution up to the final required concentration. Aqueous solutions of WPC and WPC/PS mixed systems were stirred for 30 min at room temperature (20–23 °C) before measurements of biopolymer interactions were performed. It should be noted that there were very slight differences in the ionic strengths of solutions, due to ions contained in the biopolymer samples.

2.3. Measurements in the aqueous phase

The interactions in the aqueous phase of WPC/PS mixed systems were determined by intrinsic and extrinsic fluorescence spectroscopy, absorption spectroscopy in the presence of methylene blue (MB), and confocal laser scanning microscopy at room temperature (20−23 °C). It is important to point out that in the present work protein–polysaccharide interactions in mixed systems of relatively high concentration in both biopolymers were studied, with

the purpose of finding a direct application in food dispersion formulation.

2.3.1. Fluorescence spectroscopy

Fluorescence measurements were performed using an F2000 spectrofluorimeter (Hitachi, Tokyo, Japan) equipped with a 1.0 cm quartz cell. The widths of both the excitation slit and the emission slit were set at 2.5 nm. For the intrinsic tryptophan (Trp) fluorescence measurements, the excitation wavelength at 295 nm was used, to avoid excitation of tyrosine residues. The emission spectra of WPC/PS mixed systems were recorded between 310 and 450 nm. The blank corresponding to the Trizma buffer solution was subtracted from these spectra, to correct background fluorescence. Extrinsic fluorescence measurements were determined with the hydrophobicity fluorescence probe 1-anilino-8-naphthalenesulfonic acid (ANS, Fluka Chemie AG, Buchs, Switzerland) according to Kato and Nakai (1980), with some modifications. Serial dilutions in Trizma buffer were obtained from WPC/PS dispersions. Dilutions were prepared at pH 7 up to a final concentration of 0.01–0.50 mg/ml. Ten microlitres 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 WPC surface hydrophobicity (S_0). Each spectrum was obtained in triplicate. The average of at least three measurements was included in figures by means of error bars.

2.3.2. Absorption spectroscopy

In order to determine the attractive interactions between WPC and PS, absorption spectroscopy in methylene blue (MB) solution was used, according to the method developed by Michon, Vigouroux, Cuvelier, and Launay (2000). Aqueous solutions of MB showed a maximum in optical density (OD) at 664 nm, which corresponds to the absorption of free MB molecules. When anionic PS (such as carrageenan and other hydrocolloids) were added to the MB aqueous solution, the peak at 664 nm decreased and a shoulder at lower wavelengths was detected, which was enhanced with increasing PS concentration. This absorption shift could be attributed to the presence of MB molecules that interact with PS to form water-soluble metachromatic complexes (Soedjak, 1994). If a protein, which interacts associatively with the PS is introduced into a MB/PS solution, some MB molecules will be removed from the surface of the metachromatic complexes and released into the aqueous phase. The peak intensity at 664 nm will then increase and the shoulder at lower wavelengths will decrease. A methylene blue (MB) solution with a concentration of about 0.001% was prepared by dissolving the correct amount in Trizma buffer at pH 7. WPC/PS mixed systems were prepared in the same way as described above, but using MB aqueous solution. Methylene blue was purchased from Merck (Darmstadt, Germany). The OD measurements were performed with a Lambda-20 spectrophotometer (Perkin–Elmer, Norwalk, CT). All the spectrophotometric experiments were performed in triplicate.

2.3.3. Confocal laser scanning microscopy

The ultrastructure of WPC/PS mixed systems was analysed by confocal laser scanning microscopy (CLSM) using an inverted microscope (Nikon TE2000E, Japan) equipped with a Nikon C1siR confocal system. CLSM was used combining epi-fluorescence and differential interferential contrast (DIC) modes. For ultrastructure observations the WPC was labelled with ANS. It is important to highlight that ANS does not covalently bind to the protein. Therefore, there is no chemical modification of the structure of the system, as is the case with covalent labelling (Albani, 2004). The excitation using the UV laser was performed at 374 nm and the emission was recorded between 415 and 485 nm. Aliquots of WPC/PS mixed systems were placed between a slide and a cover slip and were examined at 40× objective. In some cases, the zoom tool of the instrument was applied, to obtain the ultrastructure of the mixed systems at 200×.

3. Results and discussion

3.1. Effect of biopolymer interactions on intrinsic tryptophan fluorescence

One of the intrinsic fluorophores that has been the most studied among proteins is tryptophan amino acid (Trp). Trp fluorescence is highly sensitive to local environmental conditions of the proteins. For this reason the analysis of Trp fluorescence has become an excellent tool for the molecular dynamic characterisation of systems which include proteins. The interactions between proteins and polysaccharides can induce perturbations or modifications in Trp fluorescence parameters, such as intensity, quantum yield or lifetime. Thus, the analysis of these perturbations can be used to determine the nature and origin of the interactions between these biopolymers (Albani, 2004).

In order to study protein–polysaccharide interactions in the aqueous phase, the effect of PS on the WPC intrinsic fluorescence was firstly analysed. Fig. 1 shows the effect of PS type and its con-

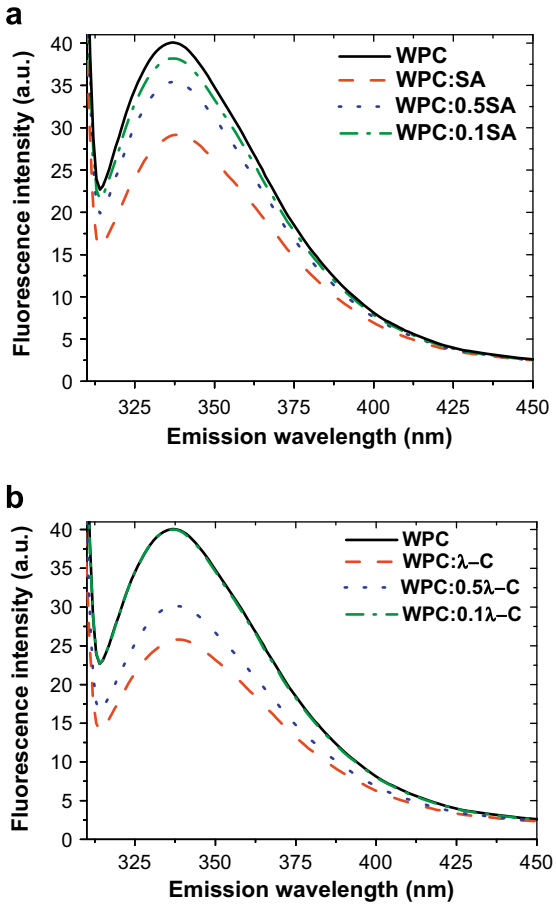

Fig. 1. Effect of PS concentration on the WPC intrinsic fluorescence. (a) WPC/SA systems: WPC:SA (at 1.0:1.0% weight ratio), WPC:0.5SA (at 1.0:0.5% weight ratio), and WPC:0.1SA (at 1.0:0.1% weight ratio). (b) WPC/λ-C systems: WPC:λ-C (at 1.0:1.0% weight ratio), WPC:0.5λ-C (at 1.0:0.5% weight ratio), and WPC:0.1λ-C (at 1.0:0.1% weight ratio). pH 7 and *I* 0.05 M.

centration in the aqueous phase (within the range 0.0–1.0%) on the Trp fluorescence emission intensity of 1.0% WPC solution. It was observed that after the PS addition the fluorescence intensity of WPC decreased regularly with the increase in PS concentration. This behaviour shows the existence of significant biopolymer interactions in the aqueous phase for WPC/PS mixed systems. The reduction in the fluorescence intensity can be related to the quenching effect of the protein molecular environment on Trp emission characteristics (Albani, 2004; Lakowicz, 1999). The quenching effect can be produced by a large variety of molecular interactions, including excited-state reactions, molecular rearrangement, energy transfer, ground-state complex formation, and collisional quenching. The magnitude of the collisional quenching effect can be evaluated by the Stern–Volmer equation (Albani, 2004; Lakowicz, 1999):

$$F_0/F = 1 + K_{SV}[PS] \quad (1)$$

where F_0 corresponds to the maximum value of the protein fluorescence intensity peak and F is the maximum value of the fluorescence intensity peak of the protein in the presence of PS. The slope of the Stern–Volmer (K_{SV}) plot may be considered as an index of the collisional quenching effect on WPC intrinsic fluorescence, due to the presence of PS in the aqueous phase, which in this particular case could be acting as a quencher.

Fig. 2 shows the application of Eq. (1) to the fluorescence emission profiles of WPC/PS mixed systems. As can be seen, λ-C produced a collisional quenching effect of higher magnitude than SA, because the K_{SV} value for WPC/λ-C mixed systems ($0.589\%^{-1}$, $r = 0.9886$) was higher than K_{SV} for the WPC/SA system ($0.362\%^{-1}$, $r = 0.9841$). This behaviour can be due to a higher perturbation in the emission fluorescence intensity of Trp as a consequence of greater number of macromolecular collisions in the presence of λ-C compared to SA. Intermolecular interactions between these biopolymers could be due to protein–protein and/or protein–polysaccharide collisions. Thus, these preliminary observations reveal some differences exist in the molecular dynamics of WPC/PS mixed systems, depending on the polysaccharide and WPC fractions. Tryptophan fluorescence would be more sensitive to the native fraction. Nevertheless, the thermodynamic characterisation of their nature by intrinsic fluorescence measurements requires further experiments at different temperatures (Hu, Liu, Zhang, Zhao, & Qu, 2005; Lakowicz, 1999).

3.2. Effect of biopolymer interactions on extrinsic ANS fluorescence

The extrinsic fluorescence study was carried out by the evaluation of the protein surface hydrophobicity (S_0) in WPC/PS mixed systems. Fig. 3 shows S_0 dependence on PS concentration (within a 0.0–1.0% range) for the mixed systems. It can be seen that S_0 for WPC decreased in the presence of λ-C. At the highest λ-C concentration, a lower S_0 value was obtained. However, the addition of SA produced a reduction in the value of S_0 for WPC, but as the concentration of SA increased the values of S_0 progressively increased so that, the highest value of S_0 was recorded at 1.0%. These results indicate that the dependence of S_0 on PS concentration was a characteristic of each WPC/PS mixed system, which can give information about the existence of different macromolecular interactions between these biopolymers. The occlusion of the WPC hydrophobic surface sites in the presence of polysaccharides at high λ-C concentrations could be associated with the existence of attractive interactions between protein and polysaccharides (Benichou et al., 2007). A possible explanation to account for the initial reduction of S_0 at low SA concentration might be that the non-native protein fraction first aggregates, due to the segregative effect of the polysaccharide, and that the native fraction is probed subsequently. Nevertheless, the increment in the concentration of SA produced a higher exposure of the protein hydrophobic segments. This technique is probably giving an average behaviour of the aggregated and native fractions.

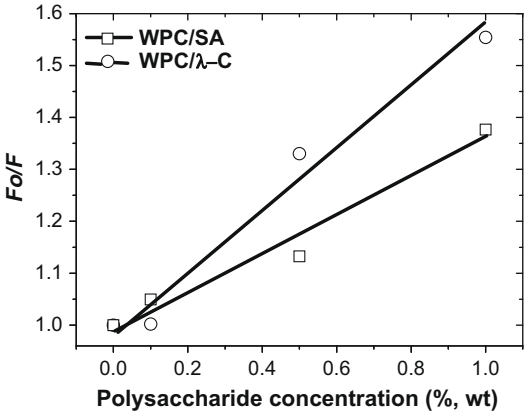

Fig. 2. Stern–Volmer plots for WPC/PS mixed systems. WPC concentration in the aqueous phase 1.0%, pH 7, and I 0.05 M.

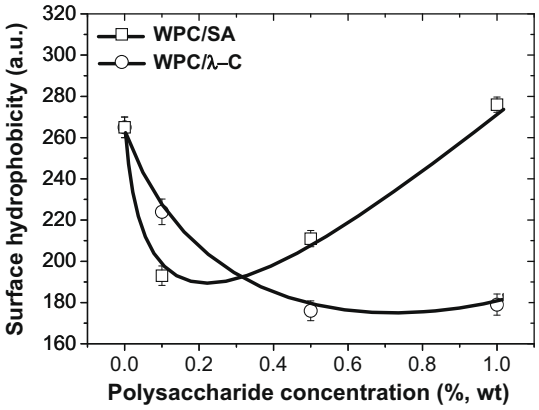

Fig. 3. Effect of PS concentration on the WPC surface hydrophobicity. WPC concentration in the aqueous phase 1.0%, pH 7, and I 0.05 M.

3.3. Effect of biopolymer interactions on the absorption behaviour in MB solutions

Interactions between WPC and PS were also studied evaluating the spectral response of the mixed systems in the presence of methylene blue (MB). Fig. 4 shows the absorption spectra for MB/SA and MB/λ-C mixed systems (Fig. 4a and b, respectively). These spectra showed a peak at 584 and 559 nm, respectively, which was related to metachromatic complex formation. On the other hand, the intensity of the peak at 664 nm decreased when PS concentration increased, indicating that free MB molecules in the aqueous phase decreased.

An alternative representation of these data can be constructed with the optical density ratio, $OD_{664}/OD_{MB/PS}$, as a function of biopolymer concentration, where OD_{664} is the optical density at the wavelength corresponding to free MB (664 nm) and $OD_{MB/PS}$ are the optical density values at the metachromatic complex (MB–PS) absorption wavelength (Benichou et al., 2007). These plots were inserted in Fig. 4. In general, a decrease in the optical density ratio with PS concentration was observed. This behaviour could be related to the progressive saturation of the biopolymer charge (Michon, Konaté, Cuvelier, & Launay, 2002). For the MB/WPC system, an increment of the secondary peak of MB absorption spectra (at 613 nm) was observed when the protein concentration was in-

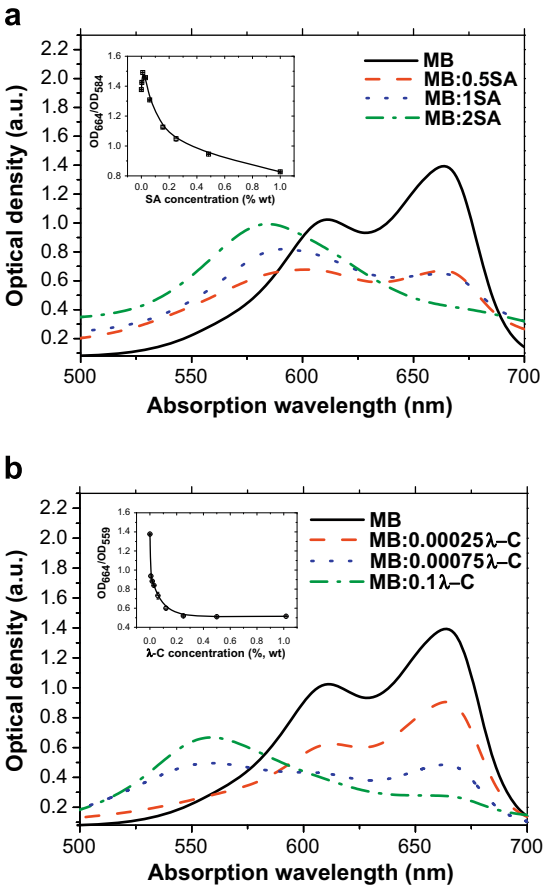

Fig. 4. Absorption spectral behaviour for (a) MB/SA and (b) MB/λ-C systems. The inserts correspond to the effect of PS concentration on the optical density ratio, $OD_{664}/OD_{MB/PS}$. MB concentration 0.001%, pH 7, and *I* 0.05 M.

creased (data not shown). Since the protein is also an electrolyte, it could interact with MB, but these attractive interactions should not result in metachromatic complex (MB–WPC) formation (Benichou et al., 2007; Michon et al., 2000).

Fig. 5 shows competitive assays at 1.0% WPC constant concentration and variable PS concentrations. The addition of SA did not produce any changes in the optical density ratio up to 0.5%, but this optical density ratio decreased at higher SA concentrations. This decrease could be attributed to the formation of the MB–SA metachromatic complex (increase in OD_{584}), indicating that there are not attractive interactions between WPC and SA. The confirmation of this hypothesis requires further research into WPC–SA interactions in the aqueous phase.

On the other hand, after the addition of λ-C at low concentrations to 1.0% WPC solution in the presence of MB, the optical density ratio decreased, which could suggests the formation of a MB/λ-C metachromatic complex (Michon et al., 2002; Soedjak, 1994). This behaviour coincides with the fast reduction in the OD_{664}/OD_{559} value (insert of Fig. 4b). Moreover, it can be seen the presence of a plateau in the λ-C concentration range of 0.1–0.25%, after which the value of OD_{664}/OD_{559} increases slightly, but significantly. Above 0.25%, the increase in the OD_{664}/OD_{559} value is due to an increase of free MB molecules, which could be related to the disruption of the metachromatic complex, as a consequence of the attractive interactions between WPC and λ-C. A similar phenomenon was observed by Michon et al. (2002) for gelatine–carrageenan mixed system.

3.3.1. Effect of NaCl concentration on metachromatic complex formation (MB/PS)

Associative interactions may be formed locally between proteins and polysaccharides, depending on the magnitude of attractive forces between both biopolymers, which are modulated by the aqueous environment conditions, such as pH, ionic strength and temperature (Tolstoguzov, 2003b). In order to prove the existence of electrostatic complexes in WPC/PS systems at neutral pH, their spectral behaviour was evaluated as a response of increasing NaCl concentration in the presence of MB.

Fig. 6 shows the effect of the NaCl addition on the spectral behaviour of metachromatic complexes MB–SA and MB–λ-C. It can be seen that both metachromatic complexes behave in a different way when NaCl concentration is increased. The addition of low salt concentrations to the MB/SA system produced an increase in the optical density ratio, which could be attributed to MB release, resulting from metachromatic complex breakdown (increase in OD_{664}). Nevertheless, the steady increase in salt concentration produced the reduction of the optical density ratio up to values similar to the initial one, which suggests that a re-association of MB and SA molecules takes place at high NaCl concentrations, giving a metachromatic complex. One possible explanation for this behaviour must consider the chemical structure of SA. The structural homogeneity in the polysaccharide may have an effect on the interactions between MB and SA, with some regions along the SA chain that may be more adequate for the metachromatic complex formation. Moreover, the change of the ionic strength in SA aque-

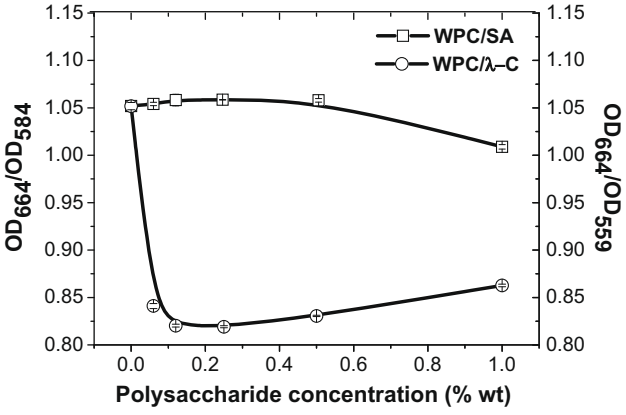

Fig. 5. Effect of PS concentration on the optical density ratio ($OD_{664}/OD_{MB/PS}$) for WPC/SA and WPC/λ-C mixed systems. WPC concentration in the aqueous phase 1.0% weight, MB concentration 0.001%, pH 7, and *I* 0.05 M.

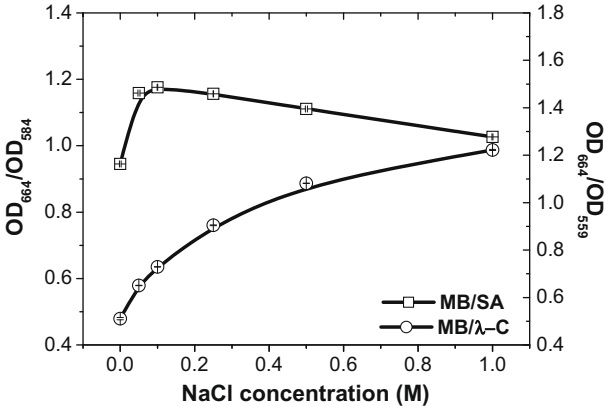

Fig. 6. Effect of NaCl concentration on the absorption spectral behaviour for MB/PS systems. MB concentration 0.001%, pH 7, and *I* 0.05 M.

ous solution has a significant effect on the behaviour of this biopolymer, especially on the reorientation of the chain polar groups and on the aqueous solution viscosity (Moe et al., 1995). The effect of ionic strength on the molecular characteristics of SA and its behaviour in solution would have a complex role in the formation of the MB–SA metachromatic complex.

On the other hand, a progressive increase of salt in the MB/λ-C system produced a continuous increment in the optical density ratio. The changes in the metachromatic properties of the MB/λ-C system, as a consequence of NaCl addition, mainly derive from the competence between Na^+ and MB for the negative sites in the polysaccharide molecule. Thus, an increase in the ionic strength could produce a progressive displacement of MB molecules from the surface of the MB–λ-C metachromatic complex. It is important to remark that the same profiles of optical density ratio, as a function of salt concentration, were obtained for different PS concentrations (data not shown).

3.3.2. Effect of NaCl concentration on WPC/PS interactions

Fig. 7 shows the effect of NaCl addition on the spectral behaviour of the WPC:0.5PS (at 1.0:0.5% weight ratio) mixed system in the presence of MB, as an example. The optical density ratio for the WPC:0.5SA system in the presence of MB did not depend on salt concentration, which confirms that segregative conditions prevails between WPC:SA. Nevertheless, a progressive increase in NaCl concentration added to the WPC:0.5λ-C mixed system produced a steady increment in the optical density ratio, which might be related to a higher amount of free MB in the aqueous phase.

In order to observe the net effect of NaCl addition on electrostatic interactions between WPC and PS, the optical density profile of Fig. 6 can be subtracted from the optical density profile of Fig. 7. In this way, the possible effect produced by PS and MB interactions could be avoided and the results would only reveal the nature of WPC and PS interactions (Michon et al., 2000). This result was plotted in Fig. 8 as the change in optical density ratio (ΔOD). In general, when the concentration of NaCl increased, the behaviour of WPC/PS mixed systems mainly depended on the PS type. For the WPC:0.5SA system, a negative value of ΔOD was obtained, suggesting that repulsive interactions prevail between these biopolymers (Harnsilawat et al., 2006). A progressive increase of NaCl concentration produced a steady increment in ΔOD, indicating that an increasing addition of electrolyte will shield charges in both protein and polysaccharide, inducing stronger segregative interactions and protein aggregation.

On the other hand, for the WPC:0.5λ-C system, low salt concentrations produced a ΔOD positive maximum, suggesting the existence of attractive interactions between WPC and λ-C. From this maximum, a further increase in NaCl concentration produced a ΔOD reduction to negative values. This observation is evidence of

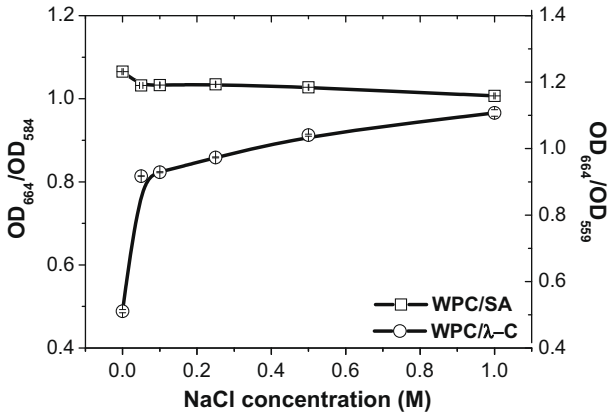

Fig. 7. Effect of NaCl concentration on the optical density ratio ($OD_{664}/OD_{MB/PS}$) for WPC/SA and WPC/λ-C mixed systems (at 1.0:0.5%, weight ratio). MB concentration 0.001%, pH 7, and *I* 0.05 M.

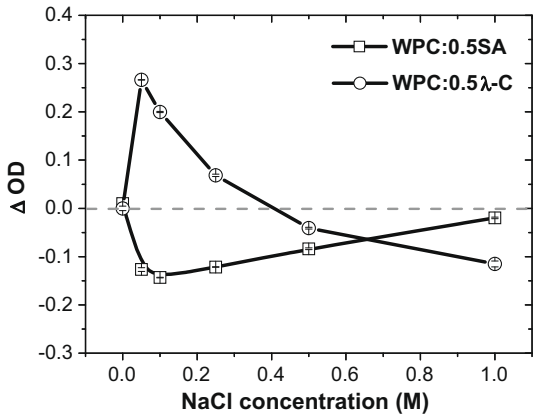

Fig. 8. Effect of NaCl concentration on the WPC/PS interactions represented by the subtraction between the $OD_{664}/OD_{MB/PS}$ profiles (ΔOD) for (□) WPC/0.5 SA and (○) WPC/0.5 λ-C mixed systems (at 1.0:0.5%, weight ratio). MB concentration 0.001% weight, pH 7, and *I* 0.05 M.

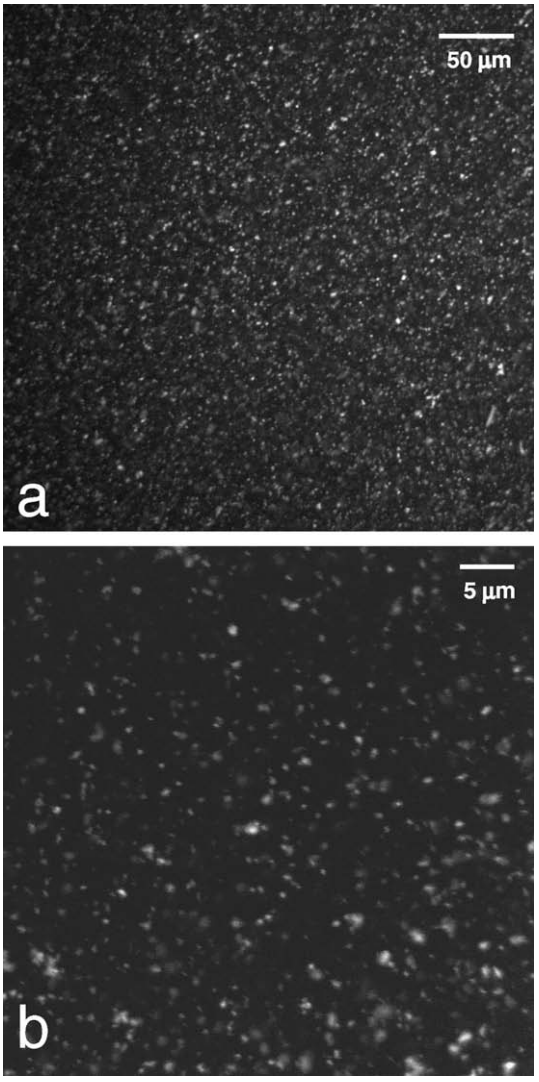

Fig. 9. Confocal micrograph of WPC dissolution. (a) 40× and (b) 200×. WPC concentration in the aqueous phase 1.0%, pH 7, and *I* 0.05 M.

the reduction of the attractive interactions between WPC and λ-C as the NaCl concentration increased. At high salt concentrations, the evolution of ΔOD with the addition of NaCl for the WPC:0.5λ-C mixed system might be driven by the molecular dynamics of the metachromatic complex MB–λ-C. Under these conditions, an increasing addition of salt will shield charges in both WPC and λ-C, preventing the attractive interactions.

In the literature, there is evidence concerning the carrageenan molecules' capacity to interact locally with the positive "patches" of milk whey proteins (especially with bovine serum albumin), even at pH >p*I*, where repulsive interactions between these biopolymers is assumed (Galazka, Smith, Ledward, & Dickinson, 1999). Furthermore, at neutral pH and low ionic strength, the affinity of carrageenan sulphate groups for attractive interactions is higher than those of sodium alginate carboxylic groups. Thus, based on the latter evidence, the existence of local attractive interactions between WPC and λ-C might be assumed, which could promote the formation of hybrid biopolymer associations in the aqueous phase for WPC/λ-C mixed systems.

3.4. Effect of biopolymer interactions on the ultrastructure of WPC/PS mixed systems

The ultrastructure of WPC/PS mixed systems was visualised by confocal fluorescence microscopy. Fig. 9 shows the ultrastructure of 1.0% WPC solution. Fig. 9a shows a confocal micrograph of WPC at 40× objective lens. Little protein aggregates homogenously distributed through the whole field of vision were distinguished with a higher magnification (200×) (Fig. 9b). Furthermore, WPC analysis by size-exclusion HPLC revealed the existence of protein aggregates of high molecular weight. These results could be explained by considering that the aggregation of WPC protein takes place as a consequence of the thermal denaturation of protein during the industrial process of WPC production.

Figs. 10 and 11 show the ultrastructural characteristics of WPC/SA and WPC/λ-C mixed systems, respectively. In these images, bright areas correspond to the fluorescence of ANS and thus reveal the presence of protein. On the contrary, dark areas correspond to the localisation of the polysaccharide in the aqueous solution. Fig. 10a shows the confocal micrograph for a WPC:0.1SA (at 1.0:0.1% weight ratio) mixed system at 40×, which reveals the existence of protein aggregates on a fluorescence field homogeneously distributed. A higher magnification (200×) allowed the distinction of protein aggregates of a size slightly larger than the aggregates in the WPC sample (Fig. 10b). Fig. 10c presents the ultrastructural characteristics of the WPC:SA (1.0:1.0% weight ratio) mixed system at 40×. The existence of large protein aggregates spread out on an attenuated fluorescence field can be observed. The field fluorescence attenuation could be related to a micro-segregated phase, as a result of different affinities for the solvent and the thermodynamic incompatibility between both biopolymers (Bourriot, Garnier, & Doublier, 1999; Hemar, Tamhana, Munro, & Singh, 2001). A higher magnification showed that the protein aggregate size in this system was much larger than the aggregates size of the WPC sample (Fig. 10d). These observations confirm the protein aggregation trend and biopolymer segregation in separated

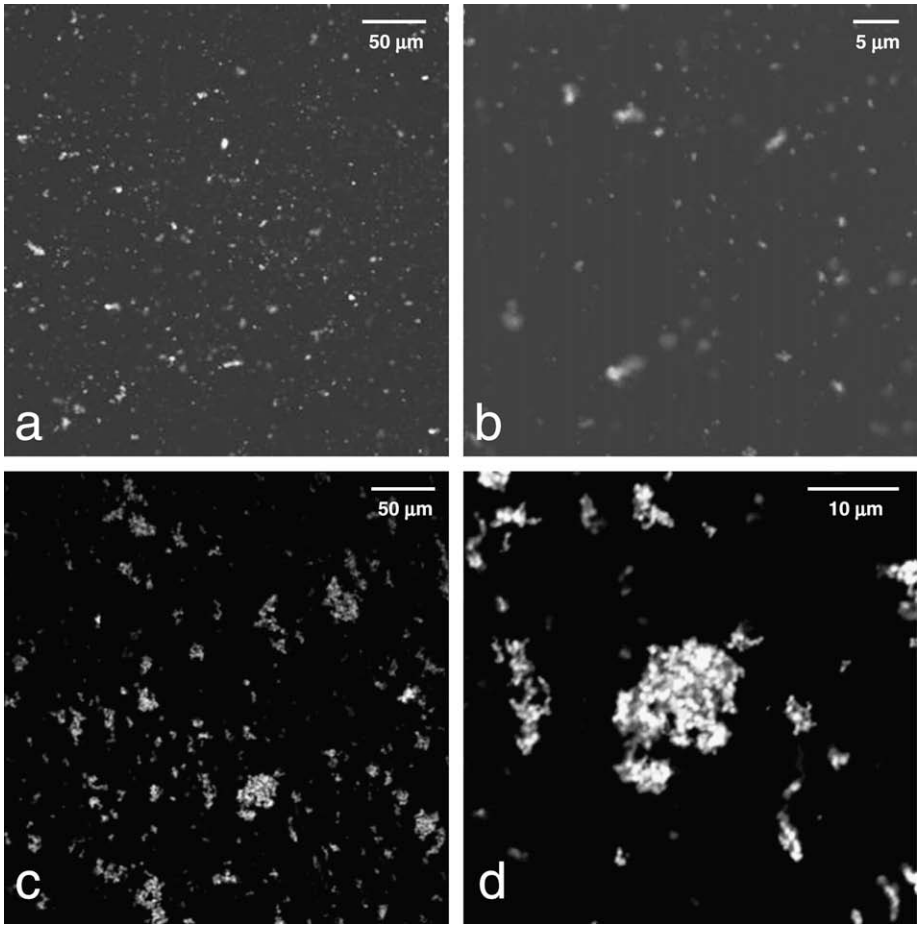

Fig. 10. Confocal micrographs of WPC/SA mixed systems: (a) and (b) WPC:0.1SA mixed system (at 1.0:0.1% weight ratio) at 40× and 200×, respectively, (c) and (d) WPC:SA mixed system (at 1.0:1.0% weight ratio) at 40× and 200×, respectively.

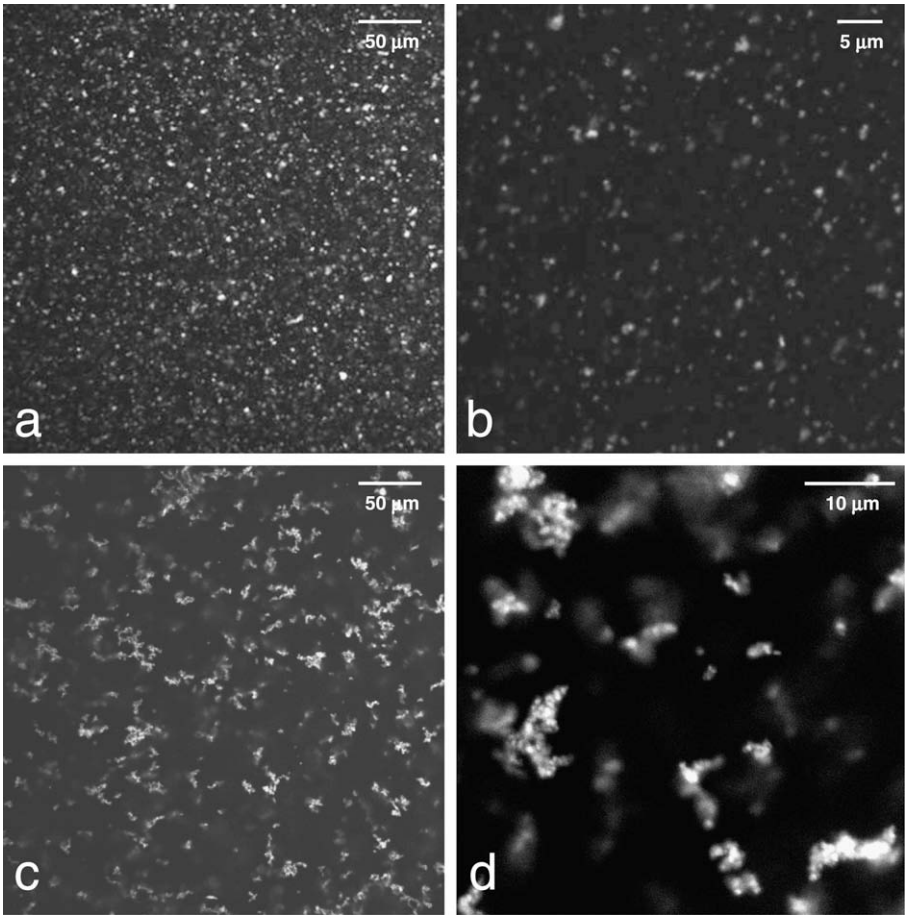

Fig. 11. Confocal micrographs of WPC/λ-C mixed systems: (a) and (b) WPC:0.1λ-C mixed system (at 1.0:0.1% weight ratio) at 40× and 200×, respectively, (c) and (d) WPC:λ-C mixed system (at 1.0:1.0% weight ratio) at 40× and 200×, respectively.

microdomains discussed in previous sections. Thus, confocal microscopy visualisation of WPC/SA mixed systems was consistent with the analytical data of absorption spectroscopy in the presence of MB discussed in preceding sections.

On the other hand, Fig. 11a shows the confocal micrograph of the WPC:0.1λ-C (1.0:0.1% weight ratio) mixed system at 40×, where a homogeneous distribution of the fluorescence through the whole visualisation field can be observed. The existence of small protein aggregates was observed with higher magnification (200×) (Fig. 11b). The size and shape of these aggregates were like those for 1.0% pure WPC. Fig. 11c shows the ultrastructure of the WPC:λ-C (1:1) mixed system at 40×. The field fluorescence was homogeneously distributed through the whole field of vision, but the intensity was lower than that observed for 1.0% pure WPC solution (Fig. 9a). The three-dimensional appearance of the field was visualised with higher magnification (200×) (Fig. 11d). On the lens focal plane, protein aggregates of variable sizes and undefined forms can be observed.

3.4.1. Effects of NaCl on the ultrastructure of WPC/PS mixed systems

Fig. 12 shows the effect of adding increasing amounts of NaCl on the ultrastructure of WPC:0.5SA (Fig. 12a–c) and WPC:0.5λ-C (Fig. 12d–f) mixed systems. Fig. 12a–c, shows the ultrastructure of WPC:0.5SA with 0, 0.5 and 1.0 M NaCl at 40×, respectively. Big protein aggregates spread on a low fluorescence field were observed in the absence of salt (Fig. 12a). This report was in accordance with the trend to phase micro-separation that WPC/SA systems showed. In the presence of 0.5 M NaCl (Fig. 12b), the mean size of the protein aggregate did not change, but the field fluorescence decreased. The increase in salt concentration at 1.0 M (Fig. 12c) resulted in a fluorescence field increment and a closer distribution of the microdomains occupied by protein aggregates, which suggests that, at this salt concentration, the system tends to segregative interactions and protein aggregation. This observation was also in agreement with the results of absorption spectroscopy in the presence of MB at high NaCl concentrations, previously analysed.

On the other hand, Fig. 12d–f, shows the ultrastructure of the WPC:0.5λ-C mixed system with 0, 0.5 and 1.0 M NaCl at 40×, respectively. Without salt (Fig. 12d), this system showed a similar appearance to the WPC:λ-C system (Fig. 11c). The addition of 0.5 M NaCl (Fig. 12e) leads both to a reduction in the field fluorescence and to the beginning of the hybrid macromolecular entities' deterioration with evident phase micro-separation. At 1.0 M NaCl (Fig. 12f) small protein aggregates in a field with low fluorescence and completely deteriorated hybrid macromolecular entities were visualised. In general, the ultrastructural analysis of the WPC/λ-C mixed system was consistent with the results of absorption spectroscopy in MB when NaCl was added. The high content of native WPC in the sample would be involved in the formation of soluble WPC/λ-C hybrid macromolecular entities, while the WPC aggregated fraction would be involved in the micro-phase separation. This last observation was also reported by Baussay, Nicolai, and Durand (2006) in the evaluation of the phase behaviour of β-lactoglobulin clusters and κ-carrageenan mixtures. This hypothesis, which requires further molecular and structural confirmation,

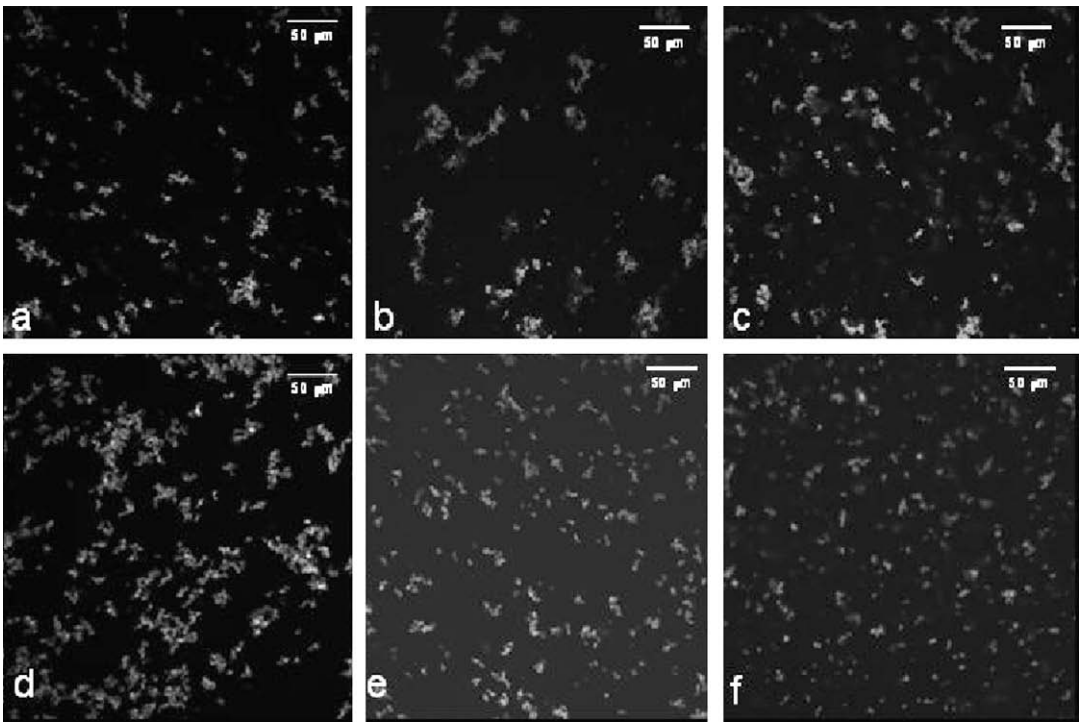

Fig. 12. Effect of NaCl concentration on the ultrastructure of WPC/PS mixed systems. Confocal micrographs for WPC:0.5SA mixed system (at 1.0:0.5% weight ratio) at 40×: (a) without NaCl, (b) 0.5 M NaCl, and (c) 1 M NaCl. Confocal micrographs of WPC:0.5λ-C mixed system (at 1.0:0.5% weight ratio) at 40×: (d) without NaCl, (e) 0.5 M NaCl and (f) 1 M NaCl.

would explain the better interfacial properties of WPC/λ-C mixed system compared to pure WPC (Perez et al., 2008).

3.5. Effect of biopolymer interactions in solution on the kinetics of WPC-adsorption at the air–water interface

The main features of the protein adsorption at fluid interfaces include (MacRitchie, 1990): (i) diffusion of the protein from the aqueous phase onto the interface, (ii) adsorption (penetration) and interfacial unfolding, and (iii) aggregation (rearrangement) within the interfacial layer, multilayer formation and even interfacial gelation.

The effect of biopolymer interactions in solution on the diffusion rate of WPC at the air–water interface can be analysed by the surface hydrophobicity (S_0) dependence of (k^a_{diff}) (Fig. 13). In fact, at low S_0 values, which coincide with the existence of attractive interactions between WPC and PS and/or with a WPC aggregation in the presence of PS (at high λ-C or at low SA concentrations) (Perez et al., 2008), the diffusion rate of WPC to the air–water interface is also low (Fig. 13). However, at high S_0 values, which coincide with a higher exposure of the protein hydrophobic segments at high SA concentration, the diffusion rate of WPC to the air–water interface is also high (Fig. 13). The results indicated that the greater the surface hydrophobicity of the WPC/PS mixed systems is, the greater is the diffusion rate of WPC to the air–water interface. That is, the existence of interactions between WPC and PS in the aqueous phase has a significant effect on the WPC diffusion to the air–water interface.

On the other hand, there exists a direct relationship between the amount of WPC-adsorbed at the air–water interface (i.e. on the surface pressure at long adsorption time, π_{10800}) and S_0 (Fig. 13), which indicates that the factors that promote a high hydrophobicity in WPC–PS mixed systems (Perez et al., 2008) also favour a high amount of WPC-adsorbed at the air–water interface at long-term adsorption (i.e. high π_{10800} values).

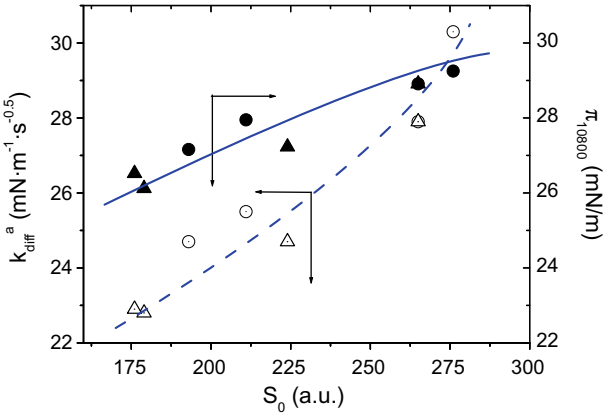

Fig. 13. The surface hydrophobicity (S_0) dependence of (open symbols) the apparent rate of diffusion to the interface (k^a_{diff}) and (filled symbols) the surface pressure at 10,800 s of adsorption time (π_{10800}) for (○,●) WPC/SA and (△,▲) WPC/λ-C adsorbed film at the air–water interface. WPC concentration 1.0%, temperature 20 °C, pH 7, and *I* 0.05 M.

Finally, a close relationship has been observed between foaming capacity and interfacial characteristics of adsorbed films at short adsorption time (k^a_{diff}) on the one hand, and between foam stability and the interfacial characteristics of adsorbed films at long-term adsorption (π_{10800}), on the other hand (unpublished results to be published in a future paper). Thus, the interfacial and foaming properties for WPC/SA mixed systems are driven by segregative phenomena between these biopolymers in the aqueous phase and at the air–water interface vicinity; while for WPC/λ-C mixed systems are driven by the formation of hybrid macromolecular entities in solution, giving a higher interfacial structuration of WPC-adsorbed films.

4. Conclusions

Interactions between WPC and PS in the aqueous phase were analysed by different techniques that probed the two WPC fractions (native and non-native) with different weight but altogether were useful to make a global evaluation of the commercial WPC. At neutral pH and low ionic strength, the results suggest that the molecular dynamics of WPC/SA and WPC/λ-C mixed systems were highly affected by the interactions between biopolymers in the aqueous phase. Complementary studies showed significant differences between both mixed systems, which depended on the PS type, and their relative concentrations in the aqueous phase. The SA in the WPC/SA mixed system promotes protein–protein interactions, which became apparent, (i) from a high trend to protein aggregation due to the increment of WPC surface hydrophobicity and (ii) from the trend to segregation of protein and SA into separated microdomains. On the other hand, λ-C produces high structuration of WPC/λ-C mixed systems, which breaks down with increasing salt in solution. The analytical techniques revealed the existence of attractive interactions in WPC/λ-C mixed systems over the whole range of concentrations. This may be explained by the existence of "patches" (positively charged domains) on the protein, which are able to interact associatively with λ-C forming hybrid biopolymer entities. The ultrastructural analysis of the WPC/λ-C mixed system suggests also the existence of a micro-phase separation, involving the WPC aggregated fraction. The hybrid biopolymer entities become the basis of the excellent interfacial viscoelastic properties and foaming characteristics of the WPC/λ-C system. These aspects are presented and discussed in previous contributions.

Acknowledgements

This research was supported by the following projects: CYTED 105PI0274, CAI+D 2005 (12H429) from Universidad Nacional del Litoral (UNL, Argentina), PICTO 36237 (UNL-ANCyPT, Argentina), CICYT AGL2007-60045 (Spain), and Consejería de Educación y Ciencia, Junta de Andalucía (Spain), through grant PO6-AGR-01535. The authors also acknowledge the contribution of Jose M. Pellegrino for the confocal laser scanning microscopy study and the Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina (CONICET) for a postgraduate fellowship to Adrián A. Perez.

References

AACC (1983). *Methods of American Association of Cereal Chemists*. Method 46-23.

Albani, J. R. (2004). *Structure and dynamics of macromolecules: Absorption and fluorescence studies*. Paris: Elsevier.

Baeza, R., Carrera, C., Rodríguez Patino, J. M., & Pilosof, A. M. R. (2006). Adsorption and rheological properties of biopolymers at the air–water interface. *AIChE Journal, 52*, 2627–2638.

Baeza, R., Carrera Sánchez, C., Pilosof, A. M. R., & Rodríguez Patino, J. M. (2004). Interactions of polysaccharides with β-lactoglobulin spread monolayers at the air–water interface. *Food Hydrocolloids, 18*, 959–966.

Baeza, R., Carrera Sánchez, C., Pilosof, A. M. R., & Rodríguez Patino, J. M. (2005). Interactions of polysaccharides with β-lactoglobulin adsorbed films at the air–water interface. *Food Hydrocolloids, 19*, 239–248.

Baussay, K., Nicolai, T., & Durand, D. (2006). Effect of the cluster size on the micro phase separation in mixtures of β-lactoglobulin clusters and κ-carrageenan. *Biomacromolecules, 7*, 304–309.

Benichou, A., Aserin, A., Lutz, R., & Garti, N. (2007). Formation and characterization of amphiphilic conjugates of whey protein isolate (WPI)/xanthan to improve surface activity. *Food Hydrocolloids, 21*(3), 379–391.

Bourriot, S., Garnier, C., & Doublier, J. L. (1999). Phase separation, rheology and microstructure of micellar casein–guar gum mixtures. *Food Hydrocolloids, 13*(1), 43–49.

de Wit, J. N., Hontelez-Backx, E., & Adamse, M. (1988). Evaluation of functional properties of whey protein concentrates and whey protein isolate. 3. Functional properties in aqueous solution. *Netherlands Milk Dairy Journal, 42*, 155.

Dickinson, E. (2003). Hydrocolloids at interfaces and the influence on the properties of dispersed systems. *Food Hydrocolloids, 17*(1), 25–40.

Galazka, V. B., Smith, D., Ledward, D. A., & Dickinson, E. (1999). Complexes of bovine serum albumin with sulphated polysaccharides effects of pH, ionic strength and high pressure treatment. *Food Chemistry, 64*(3), 303–310.

Ganzevles, R. A., Cohen Stuart, M. A., van Vliet, T., & de Jongh, H. H. J. (2006). Use of polysaccharides to control protein adsorption to the air–water interface. *Food Hydrocolloids, 20*(6), 872–878.

Girard, M., Turgeon, S. L., & Gauthier, S. F. (2002). Interbiopolymer complexing between β-lactoglobulin and low- and high-methylated pectin measured by potentiometric titration and ultrafiltration. *Food Hydrocolloids, 16*(6), 585–591.

Harnsilawat, T., Pongsawatmanit, R., & McClements, D. J. (2006). Characterization of β-lactoglobulin–sodium alginate interactions in aqueous solutions: A calorimetry, light scattering, electrophoretic mobility and solubility study. *Food Hydrocolloids, 20*(5), 577–585.

Hemar, Y., Tamhana, M., Munro, P. A., & Singh, H. (2001). Viscosity, microstructure and phase behaviour of aqueous mixtures of commercial milk protein products and xanthan gum. *Food Hydrocolloids, 15*(4–6), 565–574.

Hu, Y. H., Liu, Y., Zhang, L. X., Zhao, R. M., & Qu, S. S. (2005). Studies of interaction between colchicine and bovine serum albumin by fluorescence quenching method. *Journal of Molecular Structure, 750*(1–3), 174–178.

Kato, A., & Nakai, S. (1980). Hydrophobicity determined by a fluorescence probe methods and its correlation with surface properties of proteins. *Biochimica et Biophysica Acta, 624*, 13–20.

Lakowicz, J. R. (1999). *In principles of fluorescence spectroscopy* (2nd ed.). New York: Plenum Press. p. 237.

MacRitchie, F. (1990). *Chemistry at interfaces*. San Diego, CA: Academic Press.

Martinez, K., Baeza, R. I., Millán, F., & Pilosof, A. M. R. (2005). Effect of limited hydrolysis of sunflower protein on the interactions with polysaccharides in foams. *Food Hydrocolloids, 19*, 361–369.

Martínez, K., Carrera, C., Pizones, V., Rodríguez Patino, J. M., & Pilosof, A. M. R. (2007). Effect of limited hydrolysis of soy protein on the interactions with polysaccharides at the air–water interface. *Food Hydrocolloids, 21*, 813–822.

Martinez, K., Carrera, C., Pizones, V., Rodríguez Patino, J. M., & Pilosof, A. M. R. (2007). Soy–protein–polysaccharide interactions at the air–water interface. *Food Hydrocolloids, 21*, 804–812.

Michon, C., Konaté, K., Cuvelier, G., & Launay, B. (2002). Gelatin/carrageenan interactions in coil and ordered conformations followed by a methylene blue spectrophotometric method. *Food Hydrocolloids, 16*(6), 613–618.

Michon, C., Vigouroux, F., Cuvelier, G., & Launay, B. (2000). Gelatin/iota-carrageenan interactions in non-gelling conditions. *Food Hydrocolloids, 14*(3), 203–208.

Moe, S. T., Graget, K. I., Skjak-Braek, G., & Smidsrod, O. (1995). Alginates. In A. M. Stephen (Ed.), *Food polysaccharides and their applications* (pp. 245–286). New York: Marcel Dekker.

Perez, A. A., Carrara, C., Carrera, C., Santiago, L. G., & Rodríguez Patino, J. M. (2008). Interfacial dynamic properties of whey protein concentrate/polysaccharide mixtures at neutral pH. *Food Hydrocolloids, 23*(5), 1253–1262.

Piculell, L. (1995). Gelling carrageenans. In A. M. Stephen (Ed.), *Food polysaccharides and their applications* (pp. 205–244). New York: Marcel Dekker.

Rodríguez Patino, J. M., Rodríguez Niño, M. R., & Carrera, C. (2007). Physico-chemical properties of surfactant and protein films. *Current Opinion in Colloid and Interface Science, 12*, 187–195.

Rodríguez Patino, J. M., Rodríguez Niño, M. R., & Carrera, C. (2008). Implications of interfacial characteristics of food emulsifiers in foam formulations. *Advances in Colloid Interface Science, 140*, 95–113.

Sharma, S. C. (1981). Gums and hydrocolloids in oil–water emulsion. *Food Technology, 41*, 59–67.

Soedjak, H. S. (1994). Colorimetric determination of carrageenans and other anionic hydrocolloids with methylene blue. *Analytical Chemistry, 66*(24), 4514–4518.

Stephen, A. M. (Ed.). (1995). *Food polysaccharides and their applications*. New York: Marcel Dekker.

Spahn, G., Baeza, R., Santiago, L. G., & Pilosof, A. M. R. (2008). Whey protein concentrate/λ-carrageenan systems: Effect of processing parameters on the dynamics of gelation and gel properties. *Food Hydrocolloids, 22*(8), 1504–1512.

Tolstogusov, V. B. (2003b). Some thermodynamics considerations in food formulation. *Food Hydrocolloids, 17*(1), 1–23.

Tolstoguzov, V. B. (2003a). Thermodynamic considerations on polysaccharide functions. Polysaccharides came first. *Carbohydrate Polymers, 54*(3), 371–380.

Weinbreck, F., de Vries, R., Schroogen, P., & de Kruif, C. G. (2003). Complex coacervation of whey proteins and gum arabic. *Biomacromolecules, 4*(2), 293–303.