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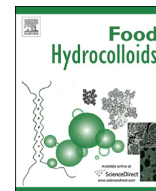

Design and characterization of soluble biopolymer complexes produced by electrostatic self-assembly of a whey protein isolate and sodium alginate

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The aim of this study was to design and characterize soluble biopolymer complexes formed by self-assembly of an anionic polysaccharide (sodium alginate, SA) and a whey protein isolate (WPI). First, conditions for producing stable protein aggregates were studied by analysing the effect of concentration (2, 4, 6% w/w) and heating temperature (55, 70, 85 °C) on WPI structural characteristics, using spectroscopic techniques UV–Vis and fluorescence. As heat treatment of WPI at the highest temperature induced the formation of soluble protein aggregates with a greater exposure of hydrophobic patches on their surface, this condition was selected to form protein particles. Secondly, from aqueous solutions of 6% w/w heat-treated WPI at 85 °C, WPI/SA systems were obtained at different ratios (2:1, 4:1 and 6:1) and transmittance measurements as a function of pH (6.0–3.0) were made. Dynamic behaviour of biopolymers in WPI/SA systems was discussed in terms of transition pHs, pH_c and pH_ϕ, corresponding to the formation of WPI-SA soluble complexes and the beginning of associative phase separation (coacervation), respectively. Finally, with these pH values, phase diagrams were constructed for each system. From these diagrams, pH 4.0 was selected to perform deposition of SA onto WPI aggregates surface, as at this pH value formation of WPI-SA soluble complexes was observed for all WPI:SA ratios. Resultant self-assembled biopolymer complexes were then characterized by the same set of spectroscopic techniques. Higher WPI:SA ratios produced soluble self-assembled biopolymer particles where hydrophobic patches of protein aggregates would be more occluded ensuring the protection of potential lipophilic bioactive agents attached inside.

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1. Introduction

Self-assembly and interactions among biomacromolecules (DNA, RNA, proteins and polysaccharides) are an essential prerequisite for the development of life (Gaaloul, Turgeon, & Corredig, 2009; Tolstoguzov, 2004; Turgeon, Schmitt, & Sanchez, 2007; Weinbreck, 2004). However, from a technological point of view, this macromolecular phenomenon could be exploited in many processes as a basis for the creation of new biopolymeric materials (Murillo-Martínez, Pedroza-Islas, Lobato-Calleros, Martínez-Ferez, & Vernon-Carter, 2011; Rodríguez Patino & Pilosof, 2011; Schmitt, Sanchez, Desobry-Banon, & Hardy, 1998). Regarding the development of these materials, the study of interactions between proteins and polysaccharides has gained great interest in several industrial sectors (food, agricultural, pharmaceutical, biomedical, cosmetics, etc.), mainly due to the high availability, biocompatibility and biodegradability presented by these natural resources (Alizadeh-Pasdar & Li-Chan, 2001; Chen, Yuan, Song, Wu, & Li, 2008; Li, Wu, Chen, & Wang, 2009). Biopolymer particles generated from interactions between proteins and polysaccharides could be used for nano and microencapsulation technologies so as to protect, transport and deliver bioactive agents, such as minerals, peptides, vitamins, ω-3 fatty acids or lipophilic drugs (Chen & Subirade, 2006; Jones & McClements, 2011; Schmitt et al., 1998). However, obtaining them requires a systematic study of macromolecular interactions and the variables that modulate those interactions in response to possible changes both in structure and in the aqueous medium.

Whey proteins are milk proteins widely used as food ingredients because of not only their high nutritional quality but also their excellent functional properties (Dalgleish, Senaratne, & Francois,

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1997; De Wit, 2001; Foegeding, Davis, Doucet, & McGuffey, 2002). Whey protein isolates (WPI) contain about 90% of protein, which primarily consist of β-lactoglobulin (~75%) and alpha-lactalbumin (~15%) (De Wit, 2001; Stone & Nickerson, 2012). Since β-lactoglobulin (β-Lg) is the main protein in WPI, it strongly affects both WPI functional properties and WPI thermal behaviour (Alting et al., 2004; Baeza & Pilosof, 2002; Kazmierski & Corredig, 2003). According to literature, this structurally compact globular protein (18.3 kDa) contains 162 amino acid residues, from which the more hydrophobic ones are buried in the inner part of the molecule (Harnsilawat, Pongsawatmanit, & McClements, 2006). There are also two Trp residues among the amino acid sequence that confer β-Lg intrinsic fluorescence properties (Bhattacharjee & Das, 2000). The isoelectric point of the protein has been reported to be within the range of 4.7–5.2 while its denaturation temperature is around 70.7 °C (Fessas, Iametti, Schiraldi, & Bonomi, 2001; Santipanichwong, Suphantharika, & McClements, 2008; Sawyer & Kontopidis, 2000; Weinbreck, 2004). β-Lg belongs to the lipocalin protein family and it has been studied for its carrier properties as it can bind small hydrophobic molecules (Loch et al., 2012; Zimet & Livney, 2009). However, conformational changes in the protein structure induced by heat treatment could affect protein–ligand interactions, thereby modifying its binding affinity for hydrophobic molecules (Liang & Subirade, 2012). When a β-Lg solution is heated beyond its denaturation temperature at physiological pH, the proteins may undergo complex conformational changes throughout the unfolding process, adopting a "molten globe" conformation as an intermediate during thermal transition (Bhattacharjee & Das, 2000; Doi, 1993). As a result, the protein dissociates from a dimer to a monomer, exposing reactive surface sites hidden inside the core of the molecules in the native state, such as hydrophobic regions and –SH groups, thus promoting aggregation through protein–protein interactions and sulfhydryl/disulphide interchange reactions (Gimel, Durand, & Nicolai, 1994; Hoffmann & van Mil, 1997; Kazmierski & Corredig, 2003). Although β-Lg thermal behaviour has been extensively reported by many researchers, there is little information about structural changes of heat treated WPI, through surface hydrophobicity measurements. This knowledge could be useful as it would provide valuable data about modifications in the carrier properties of these proteins.

Alginates are salts of alginic acids, which are a group of naturally occurring polysaccharides that are extracted from the cell wall of brown marine algae (*Phaeophyceae*). Their main commercial availability is the sodium salt that is widely used in the food industry for its safety and GRAS (*generally recognized as safe*) status. Alginates are linear binary copolymers of (1 → 4) linked β-D-mannuronate (M) and α-L-guluronate (G) residues arranged in nonregular homopolymeric blocks along the chain. Since dissociation constants for mannuronic (M) and guluronic acid (G) monomers are 3.38 and 3.65, respectively, alginates tend to be negatively charged across a wide range of pH values (Harnsilawat et al., 2006; Mancini, Moresi, & Sappino, 1996; Soares, Santos, Chierice, & Cavalheiro, 2004; Zhong, Huang, Yang, & Cheng, 2010).

Depending on the conditions of the aqueous medium (such as pH, ionic strength and biopolymer relative concentration) different interaction behaviours between proteins and polysaccharides could be distinguished. At neutral pH and low ionic strength, both proteins and polysaccharides are negatively charged and they remain cosoluble in the aqueous medium (on a diluted regime). Moreover, at pH values near the protein isoelectric point (pI), soluble protein-polysaccharide complexes could be formed due to self-assembly between the two biopolymers through attractive electrostatic interactions. As pH falls below pI, electrostatic attraction forces between biopolymers increase, and aggregation of self-assembled particles takes place, giving rise to the phenomenon of associative phase separation or complex coacervation (De Kruif, Weinbreck, & De Vries, 2004; Jones & McClements, 2011; Perez, Carrara, Carrera Sanchez, Rodriguez Patino, & Santiago, 2009; Weinbreck, 2004).

In this context, complex formation with a polysaccharide could provide additional protection for the potential hydrophobic ligand bound to the protein. Even though there are some works that reported interactions studies between WPI and different polysaccharides, such as carrageenans, chitosan, etc. (Alizadeh-Pasdar & Li-Chan, 2001; Perez et al., 2009), changes in hydrophobic properties that may result from whey proteins and sodium alginate interactions have not been studied yet.

The objective of this paper is to study the influence of thermal treatment on the structure of a whey protein isolate and the effect of protein-polysaccharide ratio on the formation of soluble self-assembled mixed complexes, by applying spectroscopic and optical techniques. These complexes will be prepared by following a two-step assembly method: (i) formation of soluble protein aggregates by controlled heat treatment of WPI (ii) performing electrostatic deposition of an anionic polysaccharide (sodium alginate) onto the surface of soluble protein aggregates. These findings would be of great interest to design biopolymer particles for transport and/or for delivery strategies of bioactive lipophilic agents linked to hydrophobic sites of the protein.

2. Materials and methods

2.1. Materials

Milk whey protein isolate (WPI) was provided by Davisco Food International, Inc. (Minnesota, USA) and its composition (% w/w) was: 96.18% protein (N × 6.38) (dry basis), 0.20% fat, 1.90% ash, 5.57% moisture, 1.72% others. A commercial sample of low density sodium alginate (SA) was provided by Cargill (Buenos Aires, Argentina) (PM 135 kDa). Glucono-Delta-Lactone (GDL) (SIGMA, St. Louis, USA) (purity 99.9%, PM 178.14 g/mol) was used as a modifier of the pH value of the aqueous medium.

2.2. Biopolymer dispersion preparation

Stock dispersions of WPI (2, 4 and 6% w/w) were prepared using Milli-Q ultrapure water at pH 7.0 at room temperature (25–27 °C). They were stirred for 2 h and then stored at 4 °C overnight. Sodium alginate (SA) was dispersed in Milli-Q ultrapure water and stirred at 70 °C for 35 min to promote solubilization. The pH value of all dispersions was adjusted to 7.0 with HCl and NaOH (0.1 mol l^{-1}) and then it was stored at 4 °C overnight.

2.3. Soluble WPI aggregates formation

To evaluate the effect of a heat treatment on WPI aggregation at pH 7, a 3 × 2 factorial design was used. The factors studied were protein concentration and WPI heating temperature. Experimental design was run in 6 blocks, where the order of assays had been completely randomized (Montgomery & Runger, 2006). A volume of WPI stock dispersion (2, 4 and 6% w/w) was subjected to heat treatment in a water bath at different temperatures (55, 70 and 85 °C) for 30 min. Finally, heated dispersions were allowed to cool down to room temperature and then they were stored at 4 °C overnight. WPI aggregates obtained were evaluated through characterization techniques described below.

2.4. Differential scanning calorimetry (DSC)

A Mettler Toledo 820 DSC was used to assess thermal transitions of heat-treated WPI dispersions. About 50 mg of 12.5% w/w WPI

solutions were poured into 160 μL aluminium pans and they were heated from 5 to 95 °C, at a scanning rate of 5 °C/min. An empty aluminium pan was used as reference and all measurements were made at least in duplicate (Baeza & Pilosof, 2002). Thermal transition temperatures were determined from DSC curves: onset temperature (T_{onset}), peak temperature (T_{peak}), endset temperature (T_{endset}); and the transition peak was integrated to obtain the area which corresponds to the overall enthalpy change associated with the protein unfolding process.

2.5. Formation of self-assembled WPI-SA complexes

From aqueous solutions of 6% w/w heat-treated WPI at different temperatures, WPI/SA mixed systems at pH 7 were prepared at different ratios (WPI:SA 2:1, 4:1 and 6:1) by mixing appropriate volumes of each dispersion and maintaining a total biopolymer concentration of 0.3% w/w. WPI/SA systems containing non-heat-treated WPI were also prepared as a control. Formation of self-assembled WPI-SA complexes was performed by lowering the pH to 4 using HCl 0.5 N.

2.6. Dynamic behaviour of macromolecular interactions

To study the dynamic interaction behaviour between biopolymers under experimental conditions of variable pH, GDL was added (0.35% w/w) to WPI/SA mixed systems (containing both native and heat-treated WPI, respectively) under stirring. After dissolution of GDL, stirring was stopped and 7 mL of the system under study were transferred into glass tubes so as to perform transmittance measurements; the rest was used for pH measurements. Transmittance measurements at 850 nm (TM TURBISCAN 2000; France) and pH measurements (Sper Scientific, USA) were registered simultaneously, as a function of time. From these data, transmittance (T, %) vs. pH curves were constructed.

2.7. Spectroscopic characterization of soluble WPI aggregates and self-assembled WPI-SA complexes

To study both the influence of thermal treatment on the formation of WPI aggregates and the effect of WPI:SA ratio on the formation of soluble self-assembled WPI-SA complexes, a set of complementary spectroscopic techniques was used.

2.7.1. Turbidity measurements

Optical density (OD) of 0.3% w/w WPI and WPI/SA dispersions were registered at 400 nm, using an UV–Vis spectrometer (Perkin Elmer Lambda 20, USA), as a measure of the turbidity of the systems (Vardhanabhuti, Yucel, Coupland, & Foegeding, 2009). Each system was poured into 1-cm pathlength quartz cuvettes and data were collected at room temperature (25 °C).

2.7.2. Fluorescence spectroscopy

Fluorescence measurements were performed using an F2000 spectrofluorimeter (Hitachi, Tokyo, Japan) equipped with a 1-cm pathlength quartz cell. The widths of both the excitation and the emission slits were set at 2 nm. Intrinsic tryptophan (Trp) fluorescence measurements were made at 295 nm (excitation wavelength) to avoid excitation of tyrosine residues. Emission spectra of both 0.3% w/w WPI dispersions and WPI/SA mixed systems were recorded between 250 and 450 nm (Perez et al., 2009). Both the maximum emission fluorescence intensity (FI_{Trp}) and the wavelength corresponding to this maximum (λ_{Trp}) were identified from analysis of the spectra. Extrinsic fluorescence measurements were performed using the hydrophobic fluorescence probe 1-anilino-8-naphtalenesulphonic acid (ANS, Fluka Chemie AG, Buchs, Switzerland) according to Perez et al. (2009). Proteins were marked according to the following relationship: 10 μL of 8 mM ANS solution were added to 2 mL of 0.05% w/w protein dispersion. Extrinsic fluorescence measurements were made at 350 nm (excitation wavelength) and emission spectra of both 0.3% w/w WPI solutions and WPI/SA mixed systems were recorded between 400 and 600 nm. From analysis of the spectra, both the maximum emission fluorescence intensity (FI_{ANS}) and the wavelength corresponding to this maximum (λ_{ANS}) were identified. FI_{ANS} was used as a protein surface hydrophobicity index.

2.8. Statistical analysis

All assays were measured at least in duplicate. Means and standard deviations were calculated and differences between means were determined with LSD test at $p < 0.05$ significance level (Statgraphics Centurion XV).

3. Results and discussion

3.1. Effect of thermal treatment on WPI structure

First, we explored the heat treatment conditions that would produce WPI aggregates, by applying the experimental design described before to study the effect of both concentration and heating temperature on structural characteristics of WPI. Fig. 1 shows the effect of the two factors on the OD of WPI aqueous dispersions. On the one hand, it was observed that the range of WPI concentrations assayed did not produce significant ($p < 0.05$) differences on the turbidity of the solutions. On the other hand, only at 85 °C, there was a significant ($p < 0.05$) increase in the turbidity of WPI solutions, with regard to the unheated control, which would suggest the formation of soluble protein aggregates, probably of greater molecular size than native WPI. These results are consistent with the analysis of DSC thermograms, where WPI denaturing temperature resulted to be close to 73 °C. Table 1 shows thermal transition temperatures and enthalpy changes related to the unfolding process of heat-treated WPI. Although heat treatment of WPI did not produce any relevant shift on thermal transition temperatures, it caused a significant effect on the enthalpy changes corresponding to those transitions. Non-heat-treated WPI showed an endothermic ΔH value of 5.20 J/g, which was in agreement with

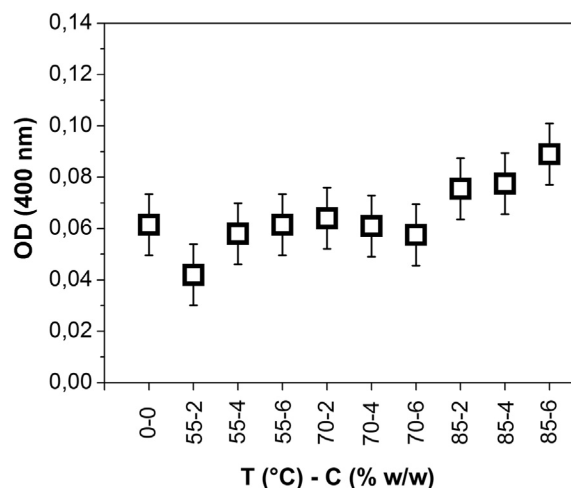

Fig. 1. Effect of WPI concentration and heating temperature (T-C) on the OD of WPI dispersions. Non-heat-treated WPI is included as control (0-0). Error bars represent 95% confidence intervals (LSD Test).

Table 1
Thermal transition temperatures and enthalphy changes of heat-treated WPI at different temperatures at pH 7.

	T_{onset}	T_{peak}	T_{endset}	ΔH (J g^{-1})
Control WPI	64.87 ± 2.93^a	73.29 ± 0.35^b	79.64 ± 1.85^c	5.20 ± 0.11^d
WPI 55 °C	66.81 ± 0.04^a	73.13 ± 0.06^b	78.69 ± 0.18^c	4.52 ± 0.06^e
WPI 70 °C	67.23 ± 0.25^a	73.15 ± 0.25^b	78.62 ± 0.39^c	1.24 ± 0.06^f
WPI 85 °C	No transition was observed			

Data represents means ± standard deviations. Different letters indicate significant statistical differences.

results reported by several authors (Pilosof & Baeza, 2002). Besides, the higher the temperature of WPI heat treatment, the lower the ΔH values observed from DSC measurements. Since the enthalpy is directly related to the amount of native proteins, lower values of ΔH would indicate partial unfolding of WPI due to gradual heat denaturation (Yong Ju, Hettiarachchy & Kilara, 1999). So, the protein would be completely unfolded after heat treatment at 85 °C, since no transition was observed at this temperature. The relationship between turbidity and molecular size of different colloidal species is well known in the literature, including protein aggregates induced by thermal denaturation (Baier, Decker, & McClements, 2004; Baussay, Nicolai, & Durand, 2006; Ryan et al., 2012). It is assumed that heat treatment of milk whey proteins could result in exposure of hydrophobic patches and −SH groups, previously hidden in the interior of proteins in the native state, thereby promoting the formation of soluble aggregates by mechanisms involving protein–protein interactions (hydrophobic interactions) and disulphide bond formation via SH–SS interchange reactions (Galani & Owusu Apenten, 1999; Iametti, De Gregori, Vecchio, & Bonomi, 1996).

Furthermore, Fig. 2 shows the effect of heating temperature of WPI on Trp fluorescence intensity (FI_{Trp}) and on wavelengths corresponding to the maximum values of FI_{Trp} (λ_{Trp}) (Fig. 2A). There is an increase in FI_{Trp} when increasing heating temperature, reaching the highest value at 85 °C. These results would suggest that rising temperatures could promote a greater exposure of Trp residues which would be more available to emit fluorescence. In addition, Fig. 2A shows that an increase of heating temperature shifts λ_{Trp} towards longer wavelengths (red shift), indicating that the molecular microenvironment of Trp residues would become more polar when increasing temperature, this suggesting a greater exposure of these aminoacids to the aqueous medium (Albani, 2004; Cairoli, Iametti, & Bonomi 1994; Iametti et al., 1996). Besides, Fig. 2B shows the effect of WPI heating temperature on both ANS fluorescence intensity (FI_{ANS}) and on λ_{ANS}. The temperature increase caused a gradual increment in FI_{ANS}, finding a maximum value at the highest evaluated temperature (85 °C). This behaviour would suggest that rising temperatures could promote greater exposure of hydrophobic patches on the WPI surface that would become available for non-covalent interaction with ANS molecules. The increment of hydrophobic patches might indicate that the heat-treated protein would expose molecular segments that are normally occluded within the native WPI (Batthacharjee & Das, 2000; Cairoli et al., 1994; Iametti et al., 1996). In addition Fig. 2B shows a significant shift towards lower wavelengths (blue shift) of λ_{ANS}, this suggesting the binding of ANS molecules to increasingly hydrophobic regions on WPI surface as temperature rises (Albani, 2004).

As described so far, it can be summarized that the range of WPI solution concentrations assayed did not produce significant ($p < 0.05$) effects on the parameters studied. Nevertheless, the temperature increment would cause formation of protein aggregates (mainly at 85 °C) mediated by hydrophobic interactions between segments of proteins, which might be related to gradual denaturation of WPI. The more exposed hydrophobicity of WPI aggregates could promote an increased binding capacity of bioactive lipophilic ligands (vitamins, drugs, essential fatty acids, peptides, etc.). Because of these characteristics, the dispersion of 6% w/w heat-treated WPI at 85 °C was selected for further evaluation in WPI/SA mixed systems.

3.2. Effect of WPI:SA ratio on dynamic behaviour of WPI/SA mixed systems in variable-pH conditions

Considering that protein-polysaccharide interactions strongly depend on the charge density of each biopolymer, and that pH plays a fundamental role in the formation of self-assembled biopolymer particles (Jones & McClements 2011; Rodriguez Patino, & Pilosof, 2011; Santipanichwong et al. 2008; Turgeon et al., 2007; Weinbreck, 2004), the effect of both structural changes induced by WPI aggregation (85 °C) and WPI:SA ratio on the dynamic behaviour of WPI/SA mixed systems under conditions of variable pH will be discussed in this section.

Electrostatic complexes formation is extensively reported in the literature for protein/polyelectrolyte systems (Chen & Subirade, 2006; Dubin, Gao, & Mattison, 1994; McClements, 2006; Schmitt & Turgeon, 2011), where complexation appeared as a two-step process upon pH change, which can be monitored via turbidity measurements (Weinbreck, de Vries, Schrooyen, & De Kruif, 2003). However, in this experimental approach we attemped to study interactions between biopolymers by following transmittance measurements, as shown in Fig. 3. Indeed, two pH-induced transitions were identified by drawing the tangent lines in the T% vs. pH curves, between the areas determined by their inflection points. The first turning point (from left to right) is called pH_c, and the

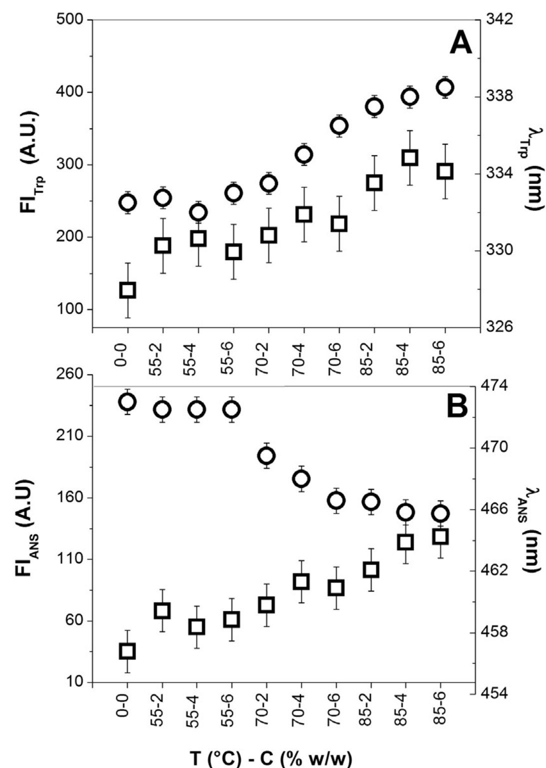

Fig. 2. Effect of WPI concentration and heating temperature (T-C) on: (A) Maximum emission fluorescence intensity of Trp (□) and the wavelength corresponding to this maximum, λ_{Trp} (○); (B) Maximum emission fluorescence intensity of ANS (□) and the wavelength corresponding to this maximum, λ_{ANS} (○). Non-heat treated WPI is included as control (0-0). Error bars represent 95% confidence intervals (LSD Test).

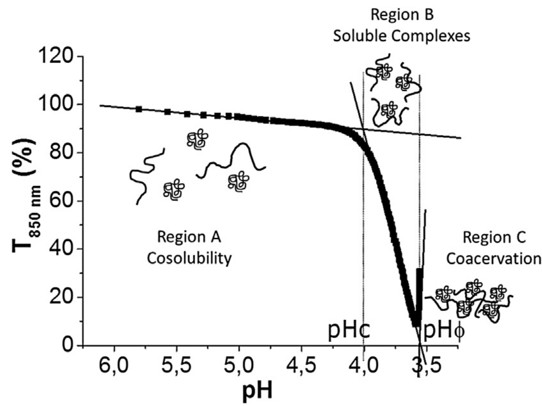

Fig. 3. Influence of pH on the transmittance (%) at 850 nm of a typical protein-polysaccharide mixture: 0.2% w/w WPI, 0.1% w/w SA.

second turning point is called pH_ϕ (De Kruif et al., 2004; Weinbreck, 2003). At pH_c, the formation of soluble complexes is initiated, and below pH_ϕ visual phase separation occurs (coacervation). Thus, in Fig. 3 it is observed that at pH > pH_c biopolymers remain cosoluble (Region A); at pH values between pH_c < pH < pH_ϕ soluble complexes formation begins (Region B); and below pH_ϕ, coacervation or associative phase separation takes place (Region C). Fig. 4A and C show the evolution of transmittance (T,%) with the decrease of pH in WPI/SA mixed systems containing both control and heat-treated WPI (85 °C), respectively. It was observed that under conditions of nearly neutral pH (to about pH = 5.0) WPI/SA systems showed high T% values, indicating no interaction between the two biopolymers. As pH fell below 5.0, there was a decrease in T%, this being probably related to an increase in WPI-SA electrostatic attraction and, consequently, to self-assembled biopolymer particles formation (electrostatic soluble complexes). From this area, it was possible to determine characteristic transition-pH values for each system: pH_c and pH_ϕ (as described before), which were then used to construct phase diagrams (pH vs. WPI:SA ratio).

Phase diagrams for WPI/SA mixed systems containing both non-heat-treated and heat-treated WPI at 85 °C are shown in Fig. 4B and D, respectively. In Fig. 4B it can be seen that the experimental method used enabled us to set a well-defined soluble complexes region (Region B) for WPI/SA mixed systems containing native WPI. However, for WPI/SA systems containing heat-treated WPI (85 °C), pH_ϕ values for 2:1 and 6:1 WPI:SA ratios could not be determined because no associative phase separation was observed after seven days of analysis. These results could be explained taking into account that when WPI/SA mixed systems reached pH values nearby 3.0, cold-set gels were formed at room temperature preventing phase separation from occurring. Several authors have reported that WPI tend to form soluble aggregates with a strandlike morphology when heating below the critical protein concentration (12% w/w), above the denaturation temperature, at a pH distant from its isoelectric point (Alting et al., 2004; Alting, Hamer, de Kruif, Paques, & Visschers, 2003; Santipanichwong et al., 2008; Verheul & Roefs, 1998). These filamentous aggregates do not gel upon cooling and remain soluble. However, acid cold-gelation could be induced at room temperature by gradually lowering the pH below the WPI isoelectric point, thereby reducing electrostatic repulsion between aggregates and promoting gel formation (Alting et al., 2003, 2004; Bryant & McClements, 2000). Nevertheless, further research is needed to understand the influence of WPI:SA ratio in the formation of these cold-set gels and future studies should be encouraged by applying analytical techniques that can elucidate the structure of this type of gels.

In addition, from the overall analysis of these phase diagrams it could be pointed out that: (i) The magnitude of pH_c and pH_ϕ values in WPI/SA systems containing non-heat-treated WPI mainly depended on WPI:SA ratio. At higher WPI:SA ratios there was an

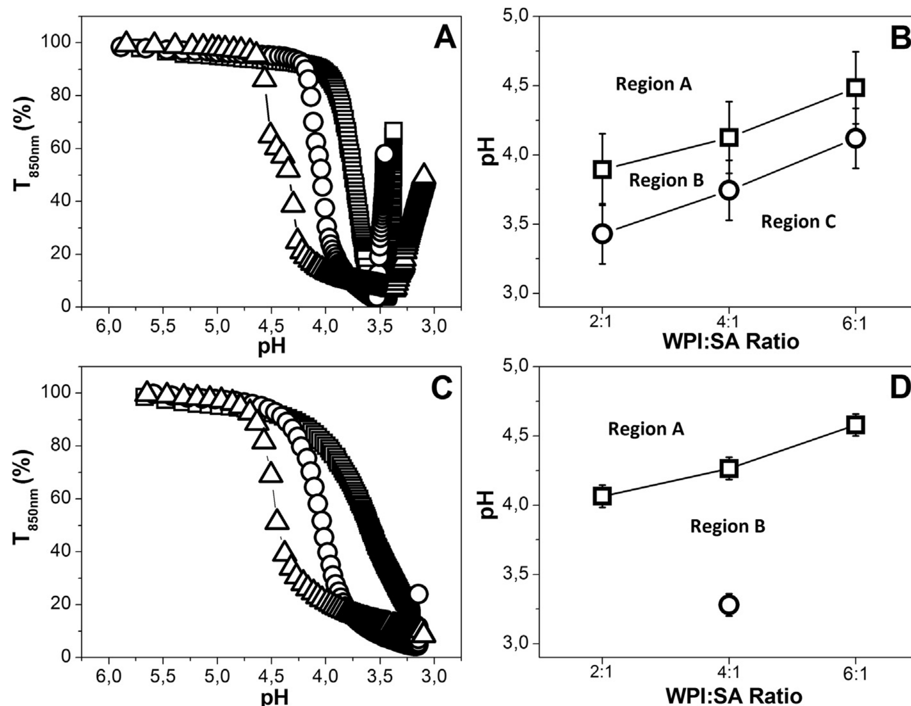

Fig. 4. Effect of WPI:SA ratio (2:1 △, 4:1 ○, 6:1 □) on the transmittance (850 nm) (T, %) of WPI/SA mixed systems containing non-heat-treated WPI (A) and heat-treated WPI at 85 °C (C) as a function of pH. Phase diagrams constructed from representative pH_c (□) and pH_ϕ (○) of WPI/SA mixed systems containing non-heat treated WPI (B) and heat-treated WPI at 85 °C (D). Region A: cosolubility between WPI and SA, Region B: soluble complexes formation, Región C: coacervation or associative phase separation. Error bars represent 95% confidence intervals (LSD Test).

increase in transition-pH values (pH_c and pH_ϕ), this phenomenon being probably associated with a greater tendency to charge neutralization in WPI/SA mixed systems, which could accelerate the associative phase separation. (ii) The same trend was observed for pH_c values in WPI/SA mixed systems containing heat-treated WPI. This behaviour would indicate that although heat treatment has a significant ($p < 0.05$) effect on the molecular structure of WPI (favouring the exposure of hydrophobic regions and the formation of protein aggregates), these structural changes would not have a considerable influence on the dynamic behaviour of WPI/SA mixed systems under variable pH conditions, at least at critical pH level (pH_c), where formation of soluble protein-polysaccharide complexes is initiated. (iii) The magnitude of pH_ϕ value seemed to be influenced not only by WPI:SA ratio but also by the changes induced in WPI structure by heat treatment. As shown in Fig. 4D, pH_ϕ value determined for 4:1 ratio is lower than pH_ϕ corresponding to the same WPI:SA ratio of systems containing non-heat treated WPI (Fig. 4B). This could indicate there was a delay in the associative phase separation. It might be possible that WPI heat treatment could retard phase separation when interacting with SA (at a fixed WPI:SA ratio) by lowering pH_ϕ values. This would probably be related to the changes undergone in WPI surface charge when heating above its denaturation temperature. The formation of WPI aggregates at 85 °C may promote a decrease of the protein surface area for interacting with SA molecules (Zaho, Li, Carvajal, & Harris, 2009). Therefore, it would be necessary to reach lower pH values to achieve stoichiometric charge neutralization and, consequently, associative phase separation.

Since it was not possible to determine pH_ϕ values for 2:1 and 6:1 WPI:SA ratios, a broad region of WPI-SA soluble complexes formation can be observed in Fig. 4D, starting from pH 4.0. To induce the formation of biopolymer particles it is necessary to determine the conditions where protein-polysaccharide interactions are promoted, so that a layer of anionic polysaccharide could be adsorbed onto the surface of cationic heat-denatured protein aggregates (Santipanichwong et al., 2008). Based on preceding results, electrostatic deposition of the polysaccharide on the surface of the protein aggregates (formed at 85 °C) will be performed at pH 4.0, since at this pH value self-assembled soluble biopolymer complexes could be obtained at all WPI:SA ratios (Fig. 4D).

3.3. *Effect of WPI:SA ratio on self-assembled biopolymer complexes formation*

Finally, we evaluated the effect of WPI:SA ratio on both the formation and molecular characteristics of soluble self-assembled biopolymer complexes generated by electrostatic deposition of SA onto the surface of WPI aggregates at pH 4.0. In all cases, the discussion of the characteristics of these hybrid complexes was compared to those containing non heat-treated WPI (control). Fig. 5 shows the effect of WPI:SA ratio on the turbidity of WPI/SA mixed systems at pH 4.0. It should be noted that there was an increase in the OD of mixed systems with the increment of WPI:SA ratio. Considering the close relationship between particle size and turbidity (Weinbreck et al., 2003), the latter behaviour would suggest an increase in the size of self-assembled biopolymer complexes at higher WPI:SA ratios probably due to a greater charge neutralization. Moreover, in mixed systems containing heated WPI (85 °C), there was a slight decrease in the OD values regarding mixed systems containing non heat-treated WPI (control), mainly at higher WPI:SA ratios, thus suggesting that self-assembled WPI-SA complexes of smaller size might have been formed with aggregated WPI. In addition, heat treatment could also induce the exposure of local positively charged regions (*patches*) on the surface of WPI aggregates, previously buried in the interior of the native

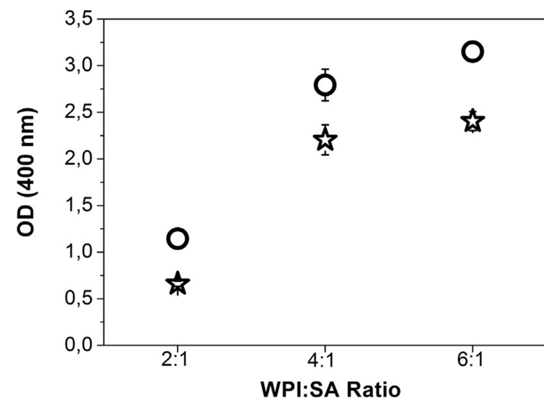

Fig. 5. Effect of WPI:SA ratio on the OD of WPI/SA mixed systems containing heat-treated WPI at 85 °C (☆) and non-heat-treated WPI (○), as a control. Error bars represent 95% confidence intervals (LSD Test).

protein, which would be available to promote strong attractive interactions with negatively charged SA molecules (Schmitt et al., 1998; Zaho et al., 2009). Therefore, WPI-SA complexes generated by heat-treated WPI might be denser packed with regard to their native counterparts, providing WPI/SA systems with lower turbidity values.

The influence of WPI:SA ratio on FI_{Trp} of WPI/SA mixed systems at pH 4.0 is shown in Fig. 6A. It can be observed that FI_{Trp} decreases significantly ($p < 0.05$) when increasing WPI:SA ratio of mixed systems, thus indicating a greater occlusion of Trp residues inside the self-assembled biopolymer complexes at higher WPI:SA ratios. Furthermore, higher FI_{Trp} values observed in WPI/SA mixed

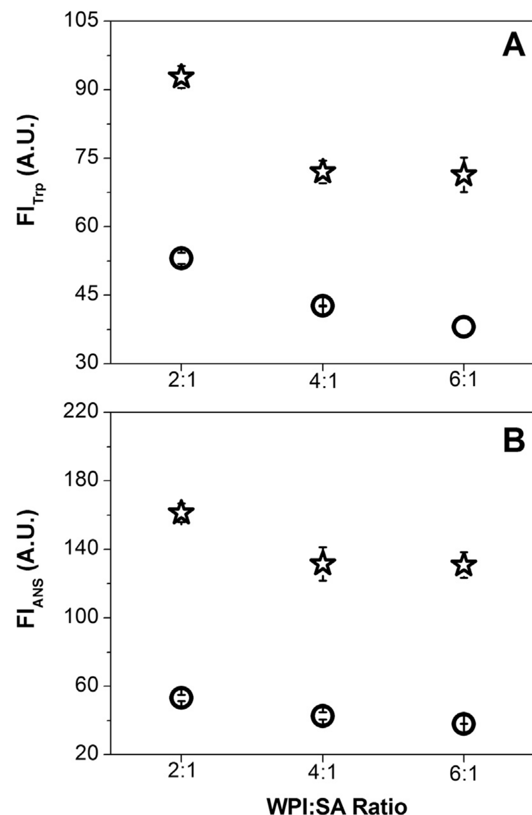

Fig. 6. Effect of WPI:SA ratio on both FI_{Trp} (A) and FI_{ANS} (B) of WPI/SA mixed systems containing heat-treated WPI at 85 °C (☆) and non-heat treated WPI (○), as a control. Error bars represent standard deviations.

systems containing heat-treated WPI (85 °C), with regard to those containing control WPI, could be mainly related to the protein structural changes induced by heat treatment, as shown in previous results. However, WPI:SA ratio did not significantly ($p < 0.05$) affect the λ_{Trp} of WPI/SA mixed systems (data not shown), suggesting there were no changes on the molecular environment of Trp inside self-assembled complexes induced by the presence of the polysaccharide.

In addition, Fig. 6B shows the effect of WPI:SA ratio on FI_{ANS} of WPI/SA mixed systems at pH 4.0. It should be noted that FI_{ANS} slightly decreased when increasing WPI:SA ratio of mixed systems, for native and heat-treated WPI. At higher WPI:SA ratios, the decrease in FI_{ANS} of mixed systems might be associated with a greater occlusion of hydrophobic patches inside of self-assembled biopolymer complexes, this behaviour being consistent with intrinsic fluorescence results discussed above. Besides, the highest FI_{ANS} values in the systems containing biopolymer particles formed from heat-treated WPI at 85 °C, could be associated with structural changes of the protein induced by heat treatment (Bhattacharjee & Das, 2000; Cairoli et al., 1994), as we discussed before. However, WPI:SA ratio did not produce significant shifts in the λ_{ANS} of WPI/SA mixed systems (data not shown).

From the results of this section, it can be deduced that the greater occlusion of hydrophobic sites produced by the polysaccharide at higher WPI:SA ratios could ensure the safety and possible transport of lipophilic bioactive agents linked within these biopolymer particles.

4. Conclusions

The dynamic interaction behaviour between WPI and SA was dependent on both structural changes of the protein produced by heat treatment and the modification of aqueous environmental variables, pH and WPI:SA ratio. Heat-treatment of WPI at the highest temperature (85 °C) induced the formation of soluble protein aggregates with a higher exposure of hydrophobic patches on their surface, which would be more available to interact with potential lipophilic bioactive agents. Moreover, formation of WPI-SA soluble complexes was observed at pH 4 for all WPI/SA systems, and it was found that higher WPI:SA ratios could produce soluble self-assembled biopolymer complexes where protein hydrophobic patches would be more occluded, ensuring the protection and transport of those potential bioactive agents attached inside.

Results derived from this research could be useful to determine structural and environmental (pH, WPI:SA ratio) conditions in order to: (i) control polysaccharide electrostatic deposition on the surface of soluble protein aggregates (ii) develop self-assembled biopolymer particles with specific characteristics and multiple applications. Nevertheless, it would be useful to carry out additional studies in the future both to provide more information about the lipid-binding capacity and to evaluate the stability of these self-assembled biopolymer particles containing bioactive molecules linked within them.

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