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Physicochemical study of mixed systems composed by bovine caseinate and the galactomannan from *Gleditsia amorphoides*

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ABSTRACT

Model systems formed by sodium caseinate (NaCAS) and espina corona gum (ECG) were studied. There was no evidence of attractive interactions between NaCAS and ECG macromolecules. Aqueous mixtures of NaCAS and ECG phase-separate segregatively over a wide range of concentrations. According to the images obtained by confocal laser scanning microscopy, NaCAS particles form larger protein aggregates when ECG is present in the system. An increase in the hydrodynamic diameter of NaCAS particles, as a result of ECG addition, was also observed by light scattering in diluted systems. A depletion–flocculation phenomenon, in which ECG is excluded from NaCAS surface, is proposed to occur in the concentrated mixed systems, resulting in NaCAS aggregation. ECG raises the viscosity of NaCAS dispersions without affecting the Newtonian flow behaviour of NaCAS. These results contribute to improve the knowledge of a barely-studied hydrocolloid which may be useful in the development of innovative food systems.

1. Introduction

Protein-polysaccharide mixtures are widely used in the food industry in order to impart desirable texture and sensory characteristics (Dickinson, 1998; Mohajer, Rezaei, & Hosseini, 2017). Understanding the behaviour of the systems composed either by milk proteins or hydrocolloids is of great importance, since the functional properties of milk proteins are usually affected by polysaccharides (Matignon et al., 2014; Mende et al., 2013; Rosa-Sibakov et al., 2016).

Due to their physicochemical and functional properties, sodium caseinates (NaCAS) are useful ingredients added to different food products as emulsifiers, gelling, texturizing and water and fat binding agents (Gaucheron, Le Graet, Boyaval, & Piot, 1997; Perrechil, Braga, & Cunha, 2009).

Vegetable galactomannans are a group of environment-friendly polysaccharides obtained from seeds of some Leguminosae tree and consist of chains of mannose residues with randomly attached galactose units as side-chains (Tavares, Monteiro, Moreno, & Da Silva, 2005). Many plant gums (e.g. carrageenan, pectin, starch, guar and sodium carboxyl methyl cellulose) are broadly used in food systems as thickeners, gelling and suspending agents, texture modifiers, emulsifiers and emulsion stabilizers (Balaghi, Mohammadifar, & Zargaraan, 2010; Dickinson, 1989; Saha & Bhattacharya, 2010). Mixed systems composed of food proteins and many galactomannans like guar gum, locust bean gum and tara gum, among others, have been extensively studied (Tavares & Da Silva, 2003).

Espina corona gum (ECG), the polysaccharide used in this work, is a galactomannan extracted from the seeds of *Gleditsia amorphoides*, a leguminous tree (Pavón, Lazzaroni, Sabbag, & Rozycki, 2014) and its chemical composition was described by Cerezo (1965). The molecular weight of ECG is 1390 Da and the galactose/manose ratio in ECG is 2.5 (Perduca et al., 2013), similar to that of guar gum, one of the galactomananns of the utmost commercial importance. It is a non-gelling biopolymer that may be used as a food thickener or stabilizer. In fact, the mechanical and microstructural properties of milk whey protein/ECG mixed gels were studied (Spotti, Santiago, Rubiolo, & Carrara, 2012) and a recent investigation reported the addition of ECG in cholesterol-reduced probiotic

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yoghurts (Pavón et al., 2014). However, the applications of this galactomannan have not yet been well studied (Albuquerque et al., 2014) and its effects on aqueous suspensions of NaCAS have not been reported so far.

The aim of this work was to study, from a physicochemical point of view, a model system containing NaCAS and ECG in order to explore future applications of this galactomannan in food products.

2. Materials and methods

2.1. Materials

Bovine sodium caseinate (NaCAS) and 1-anilino-8-naphtalene-sulfonate (ANS) were purchased from Sigma–Aldrich (Steinheim, Germany). ECG was gently donated by Idea Supply Argentina S.A. (Chaco, Argentina), and was used without further purification.

2.2. Preparation of the stock solutions

NaCAS powder was dissolved in distilled water in order to obtain a 10 wt.% NaCAS solution. A 1 wt.% ECG stock solution was prepared by dispersing ECG powder in distilled water at room temperature and was kept in stirring until complete solubilization of the polysaccharide. Diluted systems were prepared for spectroscopic techniques to ensure the correct dissolution of both biopolymers and to avoid an inner filter effect. A 1% w/v NaCAS and a 0.5% w/v ECG stock solutions were prepared. A small amount of sodium azide, purchased from Mallinckrodt Chemical (St. Louis, USA) was added to protein and polysaccharide suspensions in order to inhibit microbial development.

2.3. Fluorescence spectroscopy

2.3.1. Intrinsic fluorescence spectra

Fluorescence emission spectra of NaCAS (0.01% w/v) were obtained at 35 °C, exciting at 280 nm, at increasing concentrations of ECG (0–0.3% w/v). The scanning rate was 10 nm/min and data acquisition was performed every 0.1 nm with a 0.1 nm slit from 300 to 400 nm in an Amico Browman spectrofluorometer Series 2000 using a thermostatized quartz cell of 1 cm path length. The inner filter effect, caused by the absorption of the incident light or the emitted light, was corrected in all fluorescence intensity (FI) measurements (Lakowicz, 2007).

2.3.2. Quenching of the intrinsic fluorescence

The fluorescence quenching of NaCAS tryptophan residues, when excited at 280 nm, was carried out using 4 M acrylamide, which is a collisional quencher. Protein concentration was fixed at 0.01% w/v, while ECG concentration ranged from 0 to 0.3% w/v. Emission intensities were recorded at 340 nm in the absence of acrylamide (FI_0) and after the addition of successive aliquots of 10 μL of quencher solution (FI).

2.3.3. Surface hydrophobicity

Surface hydrophobicity (S_0) was estimated according to the method described by Kato and Nakai (1980), using the ammonium salt of the fluorescent probe 1-anilino-8-naphtalene-sulfonate (ANS). FI of samples containing 4 mM ANS and different concentrations of ECG/NaCAS mixtures were determined. Measurements were carried out at 35 °C using the excitation and emission wavelength set at 380 and 484 nm, respectively. The initial slope of FI vs. protein concentration plot was used to estimate NaCAS surface hydrophobicity.

2.4. Zeta potential and hydrodynamic diameter measurements

Zeta potential and the hydrodynamic diameter (HD) of NaCAS were determined at 35 °C in a Nano Particle Analyzer Horiba SZ-100. Protein concentration was fixed at 0.01% w/v whereas ECG concentration varied over the range of 0–0.03% w/v. Each solution was filtered through a glass microfiber paper with a cut-off of 1.6 μm, in order to avoid powders in suspensions. Such diluted systems were prepared in order to avoid interferences with the dynamic light scattering technique (Anema & Klostermeyer, 1996).

2.5. Confocal scanning laser microscopy

Immediately after mixing and after a 24-h incubation at 35 °C, the effect of ECG on 3% NaCAS dispersions was observed using confocal scanning laser microscopy (CSLM). Rhodamine B (Sigma–Aldrich, St. Louis, USA) was added to those systems at a final concentration of 0.1 mg L^{-1}. Each sample (100 μL) was placed in a compartment of LAB-TEK II cells. Representative images were captured using a confocal microscopy (Nikon Eclipse TE-2000-E, Japan), with an objective of 60× (oil immersion lens), a magnification of 5× and a numerical aperture of 1.4. Digital images were acquired with a pixel resolution of 1024 × 1024.

2.6. Evaluation of the biopolymer concentration ranges for phase separation

Samples were prepared by carefully mixing weighed amounts of ECG and NaCAS stock solutions in order to obtain systems with different biopolymer composition (NaCAS and ECG concentrations ranged from 1 to 9 wt.% and between 0.1 and 0.9 wt.%, respectively). The systems were mixed in vortex and were placed under controlled temperature at 35 °C. The occurrence of phase separation was verified after 24 h of incubation, according to Hidalgo et al. (2015).

2.7. Viscosity measurement

The rheological measurements of NaCAS, NaCAS/ECG and ECG systems were performed at 35 °C using a viscometer Brookfield LVDV-II$^+$CP (USA) in steady shear with a cone-plate geometry (diameter 48 mm, angle 0.8°).

2.8. Statistical analysis

The determinations were made at least in triplicate. The significance of the effect of ECG on each parameter was determined by means of *t*-test.

3. Results

3.1. Spectroscopic studies of the NaCAS/ECG diluted mixed systems

Fluorimetric techniques allow the assessment of the global structure of proteins and therefore to determine the effect of ECG on NaCAS conformation in solution.

3.1.1. Intrinsic fluorescence spectra

The emission spectra of NaCAS at increasing ECG concentrations are shown in Fig. 1. It is to be noted that Trp fluorescence contributes the most to protein spectra due not only to its higher quantum yield but also to the energy transfer from Phe to Tyr and from Tyr to Trp. The FI is usually related to the solvent exposition of this fluorescent amino acid. There was a progressive increase in the FI values as the

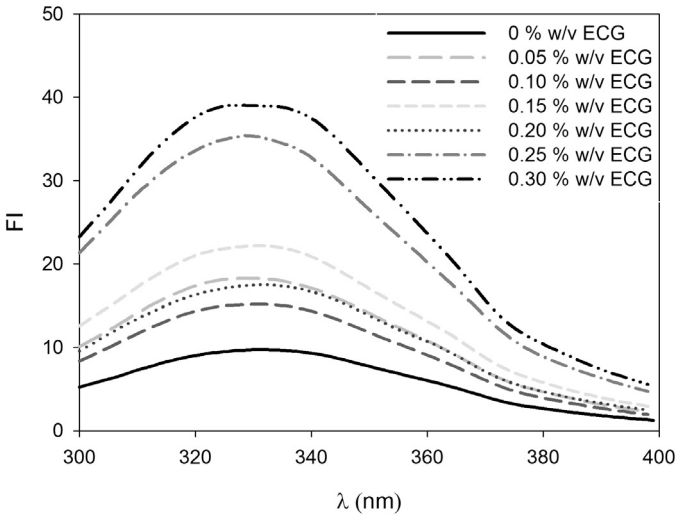

Fig. 1. Effect of ECG (0–0.3% w/v) on the fluorescence emission spectra of NaCAS (0.01% w/v).

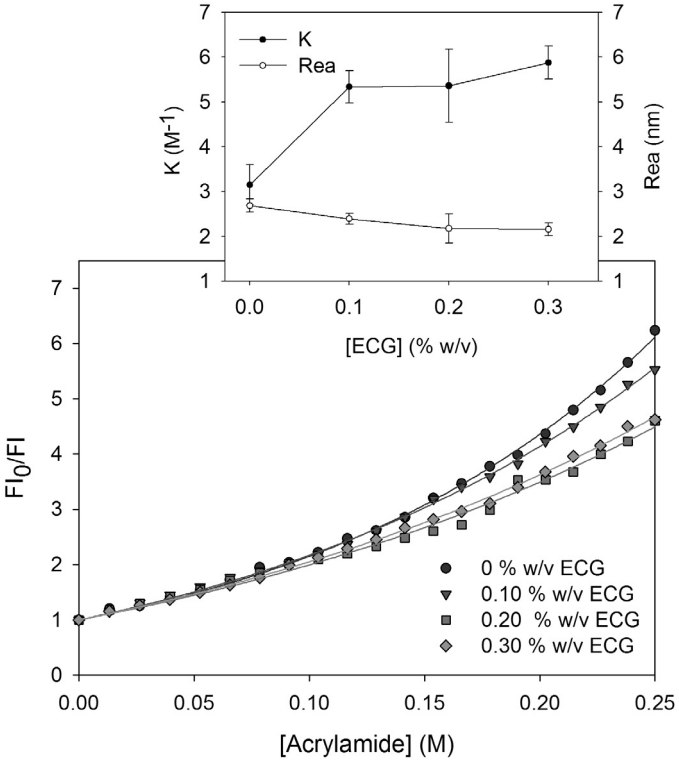

Fig. 2. Quenching of intrinsic fluorescence of NaCAS (0.01% w/v) in the presence of ECG (0–0.3% w/v).

ECG concentration increased. On the other hand, maximum emission wavelength of the intrinsic fluorescence spectrum has been recurrently related to the hydrophobicity of the Trp microenvironment. Changes in the wavelength corresponding to the emission peak were not observed.

3.1.2. Quenching of the intrinsic fluorescence

Quenching data were presented as Stern–Volmer plots and are shown in Fig. 2. The upward-curved resulting plots were interpreted in terms of a modified Stern–Volmer equation, the "sphere of action" model, in which the probability of effective quenching in a sphere of volume v, is unity. This situation is described by:

$$FI_0/FI = (1 + KD * [Q]) * \exp([Q] * v * N/1000) \quad (1)$$

where v is the volume of the sphere, N is the Avogadro number, [Q] is the quencher concentration and KD is the dynamic extinction constant (Lakowicz, 2007). Therefore, the Stern–Volmer curves obtained were fitted using Eq. (1) in order to obtain the sphere radius (Rea) and the KD parameter. The KD value represents the reciprocal value of the quencher concentration necessary to obtain half of the initial florescence of the fluorophores, and Rea represents the average distance between the fluorophore and the quencher necessary to assure effective quenching.

The presence of ECG did not affect Rea value ($p = 0.0571$). On the other hand, KD increased from $3.1 \pm 0.4\,M^{-1}$ to $5.5 \pm 0.3\,M^{-1}$ in the presence of ECG, with no differences among the different concentrations studied ($p = 0.2542$).

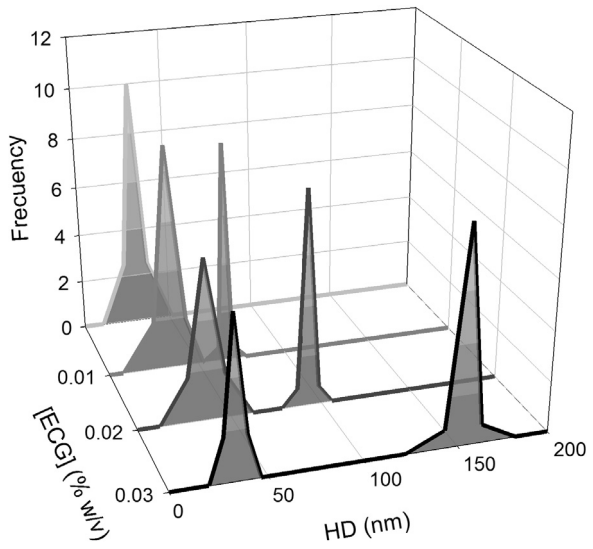

Fig. 3. Hydrodynamic diameter distributions of NaCAS aggregates (0.01% w/v) in the presence of increasing concentrations of ECG (0–0.03% w/v).

3.1.3. Surface hydrophobicity

An increase of $20 \pm 2\%$ in the S_0 of NaCAS particles was found in the presence of ECG. There was no statistical difference between the effects of the different ECG concentrations studied ($p = 0.333$).

3.2. Determination of hydrodynamic diameter and zeta-potential

The hydrodynamic diameter (HD) distributions of NaCAS aggregates in the presence of increasing concentrations of ECG are shown in Fig. 3. All samples showed a submicellar form whose size averaged 20 nm, in agreement with the studies reported by Chu, Zhou, Wu, and Farrell (1995). In the presence of ECG, another population with a higher HD was found. Its HD increased with increasing the concentration of the polysaccharide.

Zeta potential of NaCAS was -10.5 ± 0.7 mV and it was not affected by the presence of the ECG concentrations studied ($p = 0.6786$).

3.3. Visualization of the NaCAS/ECG mixed systems by CSLM

The effect of the presence of ECG on 3% NaCAS dispersions was visualized by CSLM. Fig. 4 shows the images obtained for NaCAS and NaCAS/ECG mixtures. Protein appears as bright areas due to Rhodamine labeling, while the aqueous phase corresponds to dark regions.

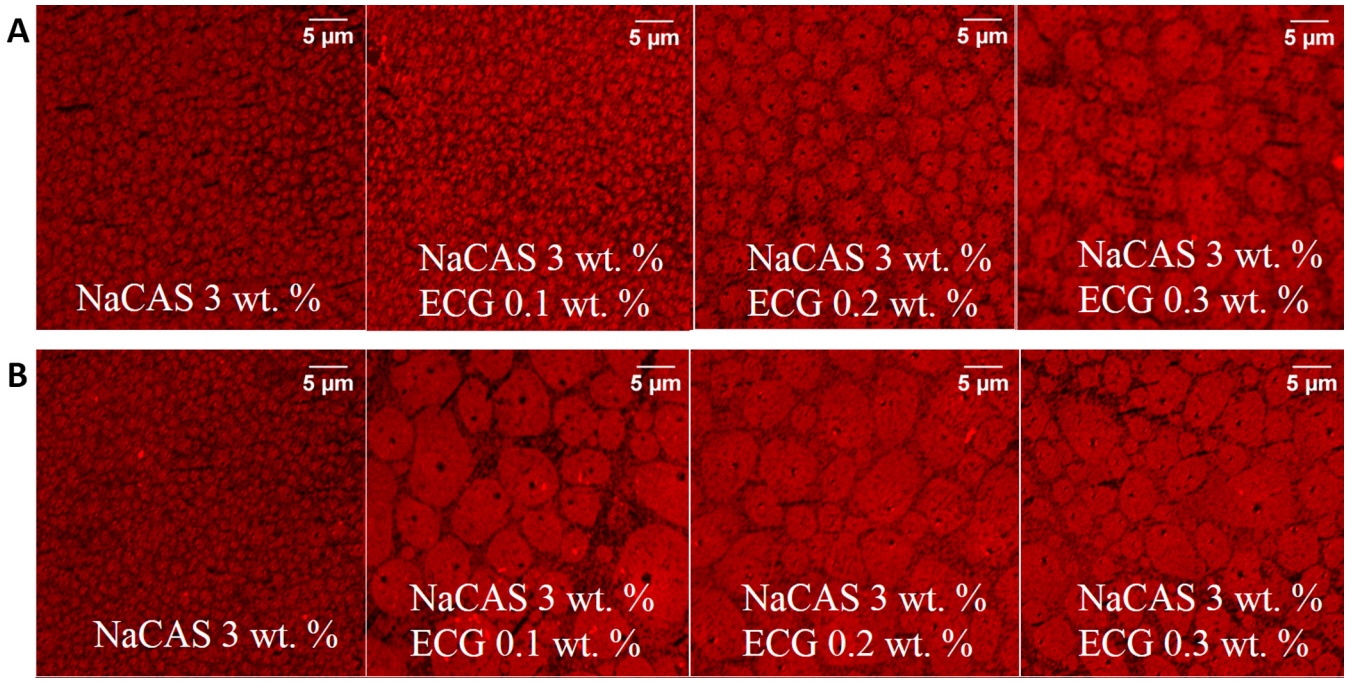

Fig. 4. Confocal laser scanning microscopy of NaCAS and NaCAS/ECG mixed systems, (A) before and (B) after 24-h incubation at 35 °C. The scale bar attached to the images represents 5 μm.

As seen from the images obtained, the distribution of NaCAS particles (in the absence of ECG) did not change throughout the 24-h incubation at 35 °C. On the other hand, when ECG was added to the systems, an increase in the particle size of NaCAS was observed when the systems were visualized after mixing. This was more evident at higher ECG concentrations. In addition, the systems visualized after 24-h incubation showed the presence of protein aggregates larger than those observed prior to incubation. All the ECG concentrations assayed led to NaCAS aggregates of similar size after 24-h incubation.

3.4. Evaluation of the biopolymer concentration ranges for phase separation

In order to determine the phase diagram of NaCAS/ECG, several systems composed by different concentrations of NaCAS (up to 9% every 1%) and ECG (up to 0.9%) were prepared and incubated at 35 °C for 24 h. Fig. 5 shows that there is only one homogeneous phase in the systems containing low biopolymer concentrations (up to ~2% NaCAS and up to ~0.3% ECG), and that there are two liquid phases in the systems containing higher biopolymer concentrations (Tolstoguzov, 2004).

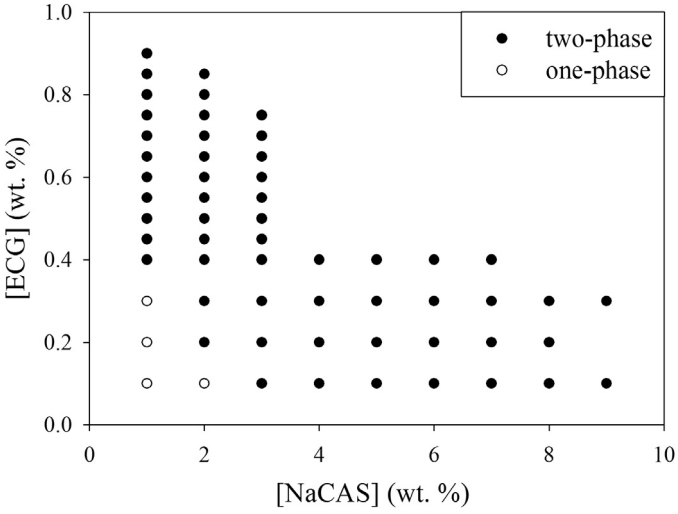

Fig. 5. Phase separation of NaCAS/ECG mixed systems. Incubation: 24 h at 35 °C. (Black dots represent systems which exhibited phase separation whereas white dots represent one-phase systems.)

3.5. Viscosity

Fig. 6 shows the flow curves displayed by NaCAS and NaCAS/ECG solutions (immediately after mixing) and the apparent viscosity (η) values for NaCAS, NaCAS/ECG and ECG systems at a fixed shear rate of 75 s^{-1}.

NaCAS solutions with and without the addition of ECG exhibited Newtonian behaviour at the range of shear rate tested, with no significant differences between the flow curves before and after shearing and resting. Our results also show that viscosity increased as ECG concentration increased. Moreover, a synergistic effect was observed, since the η of the NaCAS/ECG mixture was higher than the arithmetic sum of the η of NaCAS and ECG at each concentration (inset Fig. 6).

4. Discussion

4.1. Spectroscopic studies of NaCAS/ECG diluted mixed systems

4.1.1. Intrinsic fluorescence spectra

As pointed out in Section 3.1.1, there was an increase in FI. However, as NaCAS lacks a defined tertiary structure (Marinova et al., 2009), the increase in FI values could not be clearly related to a change in the environment of the intrinsic protein fluorophores. Therefore, other factors must be responsible for this behaviour. The changes observed in the emission spectra would be associated to the fact that ECG raised the viscosity of the media (Perduca et al., 2013) so that relaxation process (non-radiant) may be disfavoured.

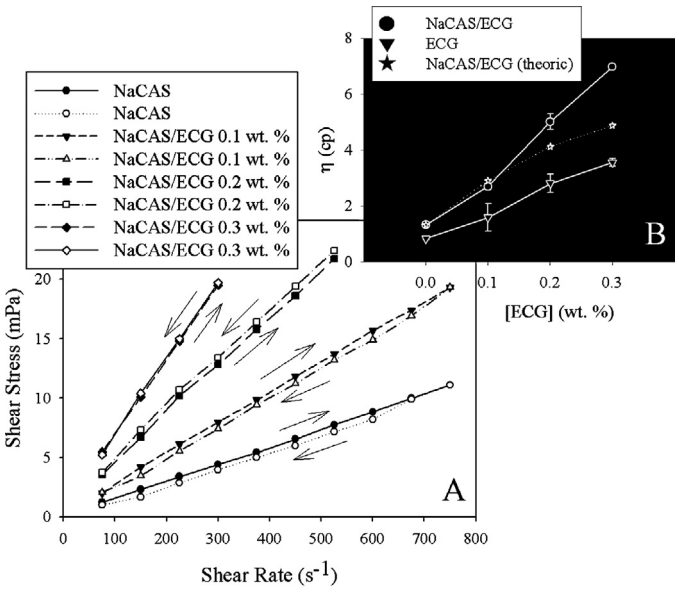

Fig. 6. Effect of ECG (0–0.3 wt.%) on the flow behaviour of NaCAS systems (3 wt.%). Temperature: 35 °C. (Inset) Effect of ECG (0–0.3 wt.%) on the viscosity of NaCAS systems (3 wt.%). In addition, the predicted viscosity value for the NaCAS/ECG mixtures was represented. This value was obtained from the sum of the viscosity values of NACAS and ECG in aqueous systems.

4.1.2. Quenching of the intrinsic fluorescence

Since the Rea values did not change in the presence of ECG, this may suggest that the protein intrinsic fluorophore, i.e. the Trp residues of NaCAS, and the quencher (acrylamide), proved to be at the same distance with and without the addition of ECG. On the other hand, the effect of ECG on the KD values means that a lower concentration of acrylamide was required to produce the same quenching effect when ECG was present. This may indicate that the presence of ECG produces a higher interaction between the fluorophores in the protein and the water molecules, probably due to a preferential exclusion of ECG from the protein surface.

4.1.3. Surface hydrophobicity

The increase in the S_0 of NaCAS when ECG was added suggests a higher exposition of hydrophobic residues to the surface, so that ANS/NaCAS interaction was favoured when ECG is present. Cheng, Ma, Li, Yan, and Cui (2015) reported that if the S_0 of a milk protein suspension increased in the presence of a polysaccharide, it could suggest that these biopolymers do not interact effectively. This is in agreement with previous results that indicate that ECG is excluded from the protein surface. Hence, ECG would be likely to favour the aggregation of NaCAS.

4.2. Hydrodynamic diameter and zeta potential

These results suggest that the presence of ECG may induce the formation of NaCAS aggregates, whose sizes depend on ECG concentration.

Since electrostatic repulsion is one of the stabilization mechanisms of NaCAS aggregates in solution (Horne, 2002; Partanen et al., 2008), it is important to determine the effect of the ECG on the protein surface charge distribution. Zeta potential of NaCAS was not affected by the presence of the ECG concentrations studied, suggesting that there was no modification in the surface charge of the NaCAS particles in the presence of ECG. These results confirm that even if ECG favours NaCAS aggregation, charge density distribution on the protein surface is not modified.

4.3. Visualization of the NaCAS/ECG mixed systems by CSLM

The increase in the particle size of the NaCAS due to the addition of ECG suggests that the presence of ECG favours NaCAS self-aggregation. Moreover, the increase in the size of the protein particles after 24-h incubation suggests that ECG affects not only the aggregation of NaCAS but also the stability of NaCAS in solution (Sharafbafi, Alexander, Tosh, & Corredig, 2015). These results obtained for concentrated systems are in agreement with those obtained for diluted systems, discussed in Section 4.2.

As several systems composed of galactomannans and caseins were known to phase-separate (Antonov, Lefebvre, & Doublier, 1999; Kasapis, 1995; Neirynck, Dewettinck, & Van Der Meeren, 2007), it is important to determine the biopolymer concentrations which produce phase separation.

4.4. Evaluation of the biopolymer concentration ranges for phase separation

A relatively low concentration of ECG is needed to induce phase separation in NaCAS systems. However, even lower concentrations of guar gum were needed to induce phase separation in NaCAS/guar gum mixtures (Hidalgo et al., 2015). The excluded volume effect is often considered to be responsible for segregative phase separation in polymer mixtures (Samy Gaaloul & Corredig, 2010). The differential effects of ECG and guar gum on phase separation can be explained by taking into account that ECG showed lower molecular weight than guar gum (Perduca et al., 2013). In agreement with this, Bourriot, Garnier, and Doublier (1999) had reported that a low molecular weight hydrocolloid induces a lesser extent of exclusion from the protein surface. Thus, the phase separation of NaCAS/ECG mixtures occurred at higher biopolymer concentrations than those in NaCAS/guar gum systems.

In two-phase systems, a clear difference between the lower and upper phase could be distinguished: a clear upper phase and a relatively opaque lower phase were formed after separation. The concentrations of NaCAS and ECG were determined in each phase in order to determine the critical point, the tie lines and the binodal curve. The lower phase was enriched in NaCAS and the upper one in the polysaccharide. This result was also seen in different biopolymer mixtures, where the upper phase was mostly enriched in the galactomanann (Hidalgo et al., 2015; Perrechil et al., 2009). This phenomenon is caused by the different density of each biopolymer phase. However, it was not possible to determine the equilibrium concentrations of these two biopolymers in each phase since the composition and the volume of each phase varied with incubation time (from 24 h to 72 h).

Biopolymer mixtures are known to phase-separate segregatively due to two possible mechanisms: either to depletion flocculation or to thermodynamic incompatibility (Goh et al., 2008). The main difference between these phenomena is that the former is of a non-equilibrium nature (Parris, Kato, Creamer, & Pearce, 1996). According to our results, depletion flocculation takes place in NaCAS/ECG mixed systems.

4.5. Viscosity

The synergism observed between ECG and NaCAS regarding the viscosity of the systems could be due to changes in the NaCAS structure in solution as a result of the presence of ECG. Comparing our results with those obtained by Bourriot et al. (1999) in guar gum/casein micelles systems, it could be concluded that the flocculation of NaCAS as a result of the exclusion of the ECG from the protein surface is responsible for the behaviour observed.

5. Conclusion

In this work we propose that ECG addition causes NaCAS aggregation trough depletion flocculation, since the galactomannan is excluded from the NaCAS surface. A better understanding of these mixed systems could lead to the development of improved food formulations.

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