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Complexation of WPI and microwave-assisted extracted agars with different physicochemical properties

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ABSTRACT

The complex formation between whey protein isolate (WPI) and agar has been investigated and their interactions were monitored as a function of the physicochemical properties of agar, the pH and the ionic strength of the medium. Agars from *Gracilaria vermiculophylla* were extracted under different MAE conditions and characterized according to their physicochemical properties. By using microwave irradiation a wide variety of agars was obtained, as different MAE conditions results in polyelectrolytes with distinct properties. UV–vis (in optical dispersion (O.D.) model) spectroscopy and isothermal titration calorimetry (ITC) have been used to study the formation of insoluble (coacervate) complexes. MAE agars revealed excellent properties for complex formation with WPI. The binding of WPI to MAE agar samples has been shown to be the result of different contributions. O.D. and ITC results showed that the molecular mass and the sulfate content of different agars had a determinant effect on coacervate formation.

1. Introduction

Interactions between polysaccharides and proteins in aqueous solutions have attracted significant interest because of their widespread applications and relatively complex behaviors. Numerous studies have provided new perspectives toward the understanding of the molecular determinants of these interactions (Aberkane, Jasniewski, Gaiani, Scher, & Sanchez, 2010; Doublier, Garnier, Renard, & Sanchez, 2000; Girard, Sanchez, Laneuville, Turgeon, & Gauthier, 2004; Kruif, Weinbreck, & Vries, 2004; Schmitt, Sanchez, Desobry-Banon, & Hardy, 1998). For systems composed of proteins and oppositely charged polyelectrolytes, the strong electrostatic attraction between the charged groups becomes the main driving force for the molecular recognition process. One of the most interesting, and studied, phenomena associated to these kind of electrostatic interaction, is the formation of complex coacervates between protein and anionic polysaccharides (Galazka, Smith, Ledward, & Dickinson, 1999; Girard, Turgeon, & Gauthier, 2003; Wang, Wang, Ruengruglikit, & Huang, 2007). For instance, pectin has been one of the most studied anionic polysaccharides involved in the formations of complex coacervates with β-lactoglobulin (β-Lg) (Girard et al., 2003; Wang et al., 2007). Other anionic polyelectrolytes consisting of sulfated saccharide monomers such as κ- and ι-carrageenans or dextran sulfate have proved to be excellent candidates for the formation of complex coacervates with proteins (Galazka et al., 1999; Stone & Nickerson, 2012; Weinbreck, Nieuwenhuijse, Robijn, & de Kruif, 2004).

The formation of coacervate complexes based on another sulfated polysaccharide as agar, is a subject that, to the best of our knowledge, has been addressed in a handful of reports (Boral & Bohidar, 2010; Levy, Horner, & Solomon, 1981; Singh, Aswal, & Bohidar, 2007; Singh, Bohidar, & Bandyopadhyay, 2007; Singh, Siddhanta, et al., 2007). In this context, different research teams have investigated the formation of coacervates between this specie and certain proteins as gelatin and monoclonal proteins.

Whey proteins are milk proteins, which in their original media are dispersed in a continuous phase containing ions of various salts and lactose. Whey protein isolate (WPI) is composed mainly of β-Lg and α-lactoalbumin (α-LA). Studies on the interaction of whey proteins with polysaccharides to form either soluble or insoluble complexes have been documented and are depending on the colloidal properties of protein/polysaccharide systems (Souza, Bai, Gonçalves, & Bastos, 2009; Weinbreck, de Vries, Schrooyen, & de Kruif, 2003; Weinbreck et al., 2004). These properties are related not only to the individual functionality of proteins and polysaccharides, but also to the nature and strength of the interactions between them.

Agar is a mixture of cell-wall polysaccharides extracted from marine red macroalgae, mainly of the genera *Gelidium and*

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Table 1
Extraction conditions and physicochemical properties of MAE agars and commercial sample used as standard.

Sample id	PTA1	PTW1	PTA2	PTW2	NA3	CA
Biomass type	IMTA	Wild	IMTA	Wild	IMTA	n.a.
Pre-treatment conditions	NaOH 6% (w/w) solution at 85 °C during 3.5 h and neutralization with acetic acid (0.5% (v/v) for 1 h at room temperature)[a]				Directly put into the microwave apparatus	n.a.
MAE conditions	20 min of extraction, 110 °C, 40 mL of water and no agitation (MAE1)[b]		5 min of extraction, 90 °C, 20 mL of water and maximum stirring speed (MAE2)[b]		15 min of extraction, 100 °C, 40 mL of water and no agitation (MAE3)	n.a.
Sulfate content (%)	2.3 ± 0.04	2.9 ± 0.5	1.7 ± 0.1	2.1 ± 0.1	3.9 ± 0.5	1.6 ± 0.3
3,6-AG content (%)	31.5 ± 0.8	28.3 ± 0.2	39.4 ± 0.3	33.1 ± 0.6	10.9 ± 0.3	29.8 ± 0.2
Molecular mass (kDa)	75.1	86.3	115.2	164.0	132.5	137.5

n.a. – not applied.
[a] Optimum conditions obtained in previous work (Villanueva et al., 2010).
[b] Optimum conditions obtained in previous work (Sousa et al., 2010).

Gracilaria, extensively used in the food industry as gelling, thickening and stabilizing agent. Its skeleton is build up of alternating 3-linked β-D-galactose and 4-linked α-L-galactose units where the latter, if existing in a neutral 3,6-anhydro form (agarose fraction) confers to agar its high gelling power. In contrast, sulfate ester groups commonly observed throughout the polymer chain will decrease the polymer's gel strength. The inclusion of an alkaline treatment previous to agar extraction, converts α-L-galactose 6-sulfate into 3,6-anhydro-α-L-galactose, thus improving its gelling ability (Armisen, 1995). Species, environmental and physiological factors as well as the extraction procedure (Rodríguez, Matulewicz, Noseda, Ducatti, & Leonardi, 2009; Stephen, 1995) will mold the substitution pattern of each polysaccharide.

Very recently, microwave-assisted extraction (MAE) technique was used for the first time in agar extraction with excellent results (Sousa, Alves, Morais, Delerue-Matos, & Gonçalves, 2010). Alkali pre-treated form of agar was extracted from *Gracilaria vermiculophylla* cultivated in integrated multitrophic aquaculture (IMTA) systems under microwave irradiation. No alteration in agar quality was induced by the use of microwave heating and extracts with significant better properties were obtained when compared with the traditional process (hot-water extraction under conventional heating).

The complexation between WPI and the recently introduced MAE agars has been investigated in this paper. To the best of our knowledge coacervation phenomena involving MAE agars have never been described before.

With this purpose, agars from *G. vermiculophylla* were extracted under different MAE conditions. Since different extraction conditions may yield products with distinct chemical properties, the physicochemical characterization of MAE agars becomes highly relevant being the molecular mass and the sulfate content the main parameters to be assessed. WPI/agar coacervation has been studied by monitoring the increase in solution turbidity under a variety of solvent conditions (pH, ionic strength, etc.) These studies were complemented with thermodynamic data on the amount of heat released when WPI was continuously titrated with agar until complete saturation of the protein by isothermal titration calorimetry (ITC) measurements.

2. Materials and methods

2.1. Materials

Two kinds of raw-material were used for microwave-assisted extraction of agar: wild *G. vermiculophylla*, harvested directly from Ria de Aveiro (northwestern Portugal), or after cultivation in an IMTA system located at A. Coelho & Castro aquaculture, Rio Alto (northwestern Portugal). In the latter case, samples were composed of a mixture of algal biomass of different tanks (subjected to different amounts of nutrients) with minimum period of cultivation of three weeks. Biomass was mixed, washed with freshwater and dried at 60 °C during 48–72 h in an oven. The algal material was, then, ready for microwave-assisted extraction. The extraction conditions used, with and without alkali pre-treatment of the seaweeds, where previously optimized (Sousa et al., 2010; Villanueva, Sousa, Gonçalves, Nilsson, & Hilliou, 2010) and are summarized in Table 1. Commercial agar (CA) from Sigma (ash 2–4.5%; A-7002, Lot 128K0068, Sigma–Aldrich Co., St. Louis, MO) was used as a reference.

A shorthand notation for the different agar samples was defined for making the paper of easier understanding. Briefly: (i) biomass type, wild *Gracilaria* (W) or cultivated in IMTA (A); (ii) samples with alkali pre-treatment (PT) or native (*i.e.* no pre-treatment prior to the extraction; N). Arabic numbers (*i.e.* 1–3) were chosen to distinguish the different MAE operational conditions (MAE1, MAE2 and MAE3). For instance, PTA1 agar corresponds to the pre-treated polysaccharide extracted from IMTA *Gracilaria* using MAE1 conditions while NA3 agar is the native agar isolated from IMTA *Gracilaria* using MAE3 conditions. A brief description of the biomass type, extraction and pre-treatment conditions as well as the chosen nomenclature is summarized in Table 1.

Whey protein isolate (WPI), was obtained as a commercial sample (LACPRODAN DI 9224) from Arla Foods Ingredients, Ambh (Denmark), and used as the protein source without further purification. As specified by the manufacturer, the isolate contains a minimum of 93.5% total protein content, and the major protein constituents are: 74% β-lactoglobulin (β-LG, 18.36 kDa), 18% α-lactalbumin (α-LA, 14.5 kDa), 6% bovine serum albumin (BSA, 6.9 kDa). The isolate further contains lactose and fat (both at a maximum content of 0.2%), and minerals such as sodium (0.5%), potassium (1%), and calcium (0.1%) ions. All chemicals (formic acid, sodium formate, sodium chloride, etc.) were analytical grade and used without further purification. Purified water produced by a Milli-Q filtration system was used for the preparation of all solutions.

Agar (both commercial and extracted) and WPI solutions were prepared by dissolving the amounts of their corresponding solids in 0.01 mol dm^{-3} formate buffer. When necessary, the pH was adjusted to a value of 3 (measured using a Crison pHmeter, model GLP 21) by addition of formic acid or sodium formate.

Agar solutions were heated at 90 °C with gentle stirring to ensure complete dissolution and incubated at 35 °C until their use in order to avoid partial gelification of agar.

2.2. Methods

2.2.1. Viscosimetry

The molecular mass of the agar samples was estimated by viscosimetry. Viscosity-average molecular mass (M) was determined using the Mark Houwink equation (Rochas & Lahaye, 1989), as previously described by Villanueva et al. (2010): $[\eta] = 0.07\,M^{0.72}$, where $[\eta]$ is the intrinsic viscosity (mL g^{-1}) and M is given in Da.

$[\eta]$ was determined by viscosimetry at 35 ± 0.1 °C. For this purpose, dilute solutions of agar were prepared in the presence of NaSCN at a concentration of 0.75 mol L^{-1}. The dilution factors were chosen in order to obtain relative viscosities between 1.2 and 2.0. Under these conditions the proper linearity is obtained in order to perform data extrapolation in a Huggins plot with high accuracy. For these measurements, a Cannon-Fenske viscometer (size 50; Comecta S.A., Barcelona) was used in all cases.

2.2.2. UV–vis measurements

The sulfate content of the different forms of agar used in this study was determined turbidimetrically as described by Jackson and McCandless (1978). Agar samples were hydrolyzed, at boiling temperature, in 1 mol L^{-1} HCl for at least 4 h, and the liberated sulfate subsequently precipitated with barium ion. The 3,6-anhydro-α-L-galactose content (3,6-AG) of the extracts was determined by the colorimetric method of Yaphe and Arsenault (1965) using the resorcinol–acetal reagent and with fructose as standard. All experiments were performed, at least, in triplicate.

The turbidity of WPI/agar solutions was measured using a Shimadzu UV-1603 UV–visible spectrophotometer, at a wavelength of 400 nm using quartz cells (HELMA) with a light path of 10 mm. The optical dispersion (O.D.) was only recorded when the signal became stable and was corrected using the buffer as background. Each of the values reported is the average of 3 consecutive readings. Four different experimental approaches were used to monitor the formation of WPI/agar coacervate complexes by the increase in the turbidity of the solution: (i) in order to study the effect of the WPI concentration on the extent of coacervate formation with agar, several solutions were prepared in 2 mL disposable centrifuge tubes containing a constant concentration of agar (0.1 wt%) and increasing concentrations of protein delivered from a common stock solution (0.4 wt%). All the solutions were prepared at pH 3 and handled at 35 °C. Measurements were performed after 30 min in order to ensure that the reaction was completed. (ii) Kinetic studies on the formation of coacevates were performed by addition of 150 μL WPI (0.4 wt%) into 900 μL of agar solution (0.1 wt%) at pH 3.0 and 35 °C. (iii) In order to account for the effect of the protein charge on the formation of WPI/agar complexes, solutions containing both types of agar (commercial or extracted) at a concentration of 0.1 wt% and WPI at the concentration ratio that provides maximum of turbidity at pH 3 ($R_{WPI/agar} = 0.57$) were equilibrated and, then, titrated with sodium formate (at a concentration of 0.1 mol dm^{-3}) in the range pH 3–6. pH and turbidity were measured after each single addition of sodium formate. (iv) For deeper insight into the nature of the interactions involved in the formation of the WPI/agar complexes, the mixtures were titrated with a concentrated NaCl solution (4 mol dm^{-3}) at pH 3 (with protein and polyelectrolyte concentrations of $R_{WPI/agar} = 0.57$).

2.2.3. Isothermal titration calorimetry (ITC)

The energetics of the interaction between agar and WPI was measured using an isothermal titration calorimeter (VP-ITC, MicroCal Inc., Northampton, MA) (Wiseman, Williston, Brandts, & Lin, 1989). Polysaccharide solution (0.3 wt%) was loaded into the calorimetric cell (1.4189 mL), equilibrated at 35 °C and, then, titrated by adding 20–30 successive 10 μL injections of protein solution (0.6 wt%) while continuously stirring the solution at 310 rpm. After each WPI addition, a certain amount of heat evolved as a consequence of the concomitant formation of the protein–polyelectrolyte complex and the corresponding temperature of the measuring cell, as compared to the temperature of the reference cell, increased for an exothermic reaction or decreased for an endothermic reaction. The amount of electrical power (μcal s^{-1}) sent to the reference cell (in the case of an exothermic reaction) or to the measuring cell (for an endothermic reaction) to maintain matched the temperatures of both cells, was monitored as a function of time. Typically, the injections were made at a constant flow rate of 0.5 μL s^{-1} (usually 10 μL in 20 s) in order to minimize both mechanical heat and temperature gradients within the calorimetric cell upon ligand injection. The time between injections was adjusted to 400 s since the kinetics of WPI–agar complex formation was shown to be fast enough to ensure the completion of the reaction within this time interval. The amount of power required to maintain the reaction cell at constant temperature after each injection was monitored as a function of time. The integration of each calorimetric peak, using the software package Origin 7.0 from MicroCal, yields the apparent enthalpy change for coacervates formation. All experiments were performed at 35 ± 0.1 °C and repeated at least three times to evaluate the reproducibility of the calorimetric results.

3. Results and discussion

3.1. Characterization of MAE agars

The molecular mass, chemical composition and charge density of interacting polymers are properties of crucial importance in the formation of insoluble complexes (coacervates) in solution (Weinbreck et al., 2003). Since the physicochemical properties of the MAE agars are of crucial importance when understanding the molecular determinants of their capability to be recognized by WPI, special attention was paid to the characterization of the different MAE agar samples. Microwave-assisted extracted and commercial agars were characterized in terms of their sulfate and 3,6-AG contents as well as molecular mass and the results are presented in Table 1.

As found in a previous work (Sousa et al., 2010), an inverse correlation between sulfate and 3,6-AG contents of MAE agars was observed. The conversion of α-L-galactose-6-sulfate to 3,6-anhydro-α-L-galactose is a highly specific process, as only sulfate in carbon C6 suffers an intramolecular nucleophilic displacement. Thus, sulfate contents exhibited by alkali-treated agars can be attributed to sulfates present in other positions (Navarro & Stortz, 2005). The results in Table 1 seem to corroborate this view as the native agar (NA3, a sample non subjected to the alkali-treatment) exhibited the lowest 3,6-AG content (10.9%) and, therefore, the highest sulfate fraction (3.9%). On the contrary, alkali-treated samples showed much higher 3,6-AG contents (~30–40%). Among these samples, PTA2 revealed the highest 3,6-AG content (~40%) and, consequently, the lowest fraction of negatively charged moieties (<2%). A slightly lower 3,6-AG content and similar fraction of sulfate was observed for commercial agar. Differences in 3,6-AG content may be due to the different source (usually *Gelidium* algae or a mixture of *Gelidium* and *Gracilaria* biomass) and extraction procedure in the commercial samples.

The remaining MAE agars exhibited chemical composition that fell within the limits stated by samples NA3 and PTA2.

The molecular mass of MAE agars has been estimated by means of viscosity measurements. Agars from *G. vermiculophylla* grown in IMTA systems showed lower molecular masses than agars extracted from the wild biomass using the same conditions (PTA1 *vs.* PTW1 and PTA2 *vs.* PTW2). Differences were more pronounced in

the samples extracted under MAE2 conditions as shown in Table 1 for PTA2 (115.2 kDa) and PTW2 (164.0 kDa). These results might be related to the different growth conditions applied to both types of *G. vermiculophylla* used (seaweeds grown in IMTA systems present typically higher tissue nitrogen content when compared to the wild ones). Another interesting point is that MAE2 conditions lead to agars with significantly higher molecular mass than those obtained using MAE1 conditions. Under microwave radiation, a polarized molecule rotates to align itself with the electromagnetic field at a frequency of 4.9×10^9 times per second (Srogi, 2006). Thus, variations in MAE conditions can explain the reduction in the polymer chain length. Several authors have previously shown that the heat treatment can induce a sharp decrease in polysaccharides molecular masses (polysaccharide degradation) (Wang et al., 2009, 2010; Wei et al., 2010). Typically, native agars from *Gracilaria* species extracted using conventional heating procedures present higher viscosities and consequently, higher molecular masses (Murano, 1995). This relation was observed with the native sample extracted from seaweeds produced in aquaculture systems (NA3 in Table 1) revealing higher molecular mass (132.5 kDa) than the alkali-treated agars extracted from the same biomass (PTA1 and PTA2, respectively, 75.1 and 115.2 kDa). The low molecular mass revealed by NA3 when compared to alkali-extract PTW2, can be simply attributed to the different algae used in this case (wild *G. vermiculophylla*) as well as MAE conditions (longer extraction times and higher temperatures). Despite the significant chemical differences, the molecular mass of native agar was similar to that of the commercial sample (137.5 kDa).

3.2. Insoluble complexes

3.2.1. Effect of MAE agar concentration on the WPI/agar interaction

The low solubility of the complexes formed between WPI and MAE agars leads to the formation of insoluble complexes (coacervates) as has been previously shown for many other natural polysaccharides (Aberkane et al., 2010; Schmitt et al., 1998). The formed coacervates are able to disperse the incident light and, as a consequence, UV–vis spectroscopy can be used to monitor the formation of these species by means of optical dispersion measurements.

Fig. 1 presents the evolution in the optical dispersion at 400 nm of MAE agar solution (0.1 wt%), buffered at pH 3 and kept at 35 °C, during the addition of aliquots of WPI solution (0.4 wt%). While CA and NA3 agars achieved a plateau at an O.D. around 1.1, much lower O.D. values were reached for the rest of the samples. Agar extracted under MAE2 conditions (PTA2 and PTW2) exhibited a very similar behavior with a plateau at O.D. in the order of 0.5. In a similar way, MAE1 extracted agars (PTA1 and PTW1) displayed similar reduced turbidity with O.D. values around 0.2. At a WPI/agar weight ratio of 0.5, the O.D. for the solutions of agars extracted under MAE2 and MAE1 conditions is reduced by a factor of 2 and 6, respectively, when compared with the complexes formed by CA and NA3. Beside this contrasting behavior, additional differences can be observed in the concentration ratio required to reach constant O.D. Whereas CA and NA3 achieved the plateau for [WPI]/[agar] ≥ 0.4, much lower values were found for agar extracted under MAE2 (∼0.2) or MAE1 (∼0.1) conditions.

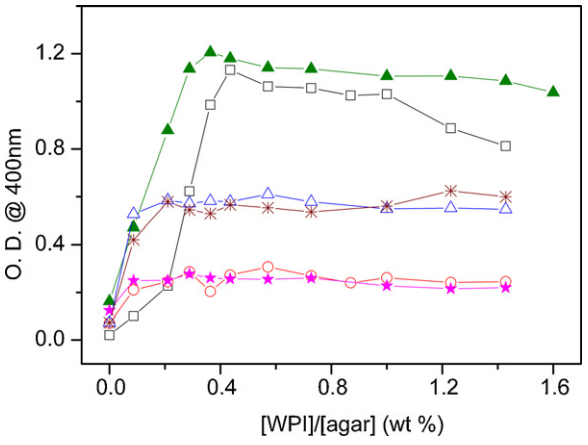

Fig. 1. Turbidity of the samples containing WPI to different agar wt% ratio (CA □, NA3 ▲, PTW2 △, PTA2 ✳, PTW1 ○ and PTA1 ⋆, respectively) at pH 3.0, 35 °C and buffered in formate buffer 0.01 mol dm^{-3}.

The results presented in Fig. 1 can be rationalized in terms of the interaction between the protonated amino groups of the protein and the negative sulfate groups of the different forms of agar at the pH investigated (Park, Muhoberac, Dubin, & Xia, 1992). In this respect, the sulfate content of the different agars was expected to be crucial for the behavior observed.

In great contrast with this expectation, the results in Fig. 1 revealed that certain forms of agar with low sulfate content, such as those extracted under MAE2 conditions, form coacervates to a larger extent than other agars with a larger sulfate content (as MAE1 agars). In line with this behavior, commercial agar also showed to lead to extensive formation of the highly dispersing coacervate even when its sulfate content is the lowest for all the investigated agars. Therefore, the ability of a certain MAE agar to form coacervates with WPI seems to be the result of a delicate balance between the sulfate content of the MAE agar (responsible for the polyelectrolyte nature of the polysaccharide and the electrostatic attraction between the complex counterparts) and the overall hydrophobicity of the complex (in which the 3,6-AG content of the agar sample must play a major role).

On the other hand, the molecular mass of the MAE agar seems to play also a significant role in its ability to form coacervates. The decrease in the tendency to form coacervates by a given polyelectrolyte and a protein as the molecular mass of the former is increased has been explained in terms of the entropy changes upon coacervation (Schmitt et al., 1998; Souza, Gonçalves, & Gómez, 2011) though, in some cases, a favorable correlation between the molecular mass of the polyelectrolyte and its ability to form coacervates has been described (Overbeek & Voorn, 1957). In this case, the molecular mass (Table 1) of the different agars seems to be also an important factor explaining the results in Fig. 1. In this respect, agars with lower sulfate content but larger molecular mass (MAE2 agar and CA) showed formation of complexes with enhanced dispersion of light. This is explained on the basis of the formation of particles of larger size when the agar employed has a high molecular mass. Many authors have claimed that the mechanism of formation of protein–polysaccharide complexes is driven by the gain in entropy resulting from the formation of a randomly mixed coacervate phase (Schmitt et al., 1998) and the release of an important number of water molecules as a result of the (partial) dehydration of the interacting protein and polyelectrolyte surfaces, with larger forms of agar generating larger entropic gains. On the other hand, agars with high sulfate content but low molecular mass (MAE1) lead to smaller aggregates with lower capacity of light dispersion. According to this view, a form of agar with molecular mass between those previously described, but with higher sulfate content (NA3) exhibited the largest value of O.D. However, there are other undetermined particularities that seem to be related to the source and/or the extraction procedures (beyond their effects in the physicochemical properties of the agars described here), that may have an effect on the coacervate formation. An example of this is illustrated by the different behavior exhibited by samples PTW2

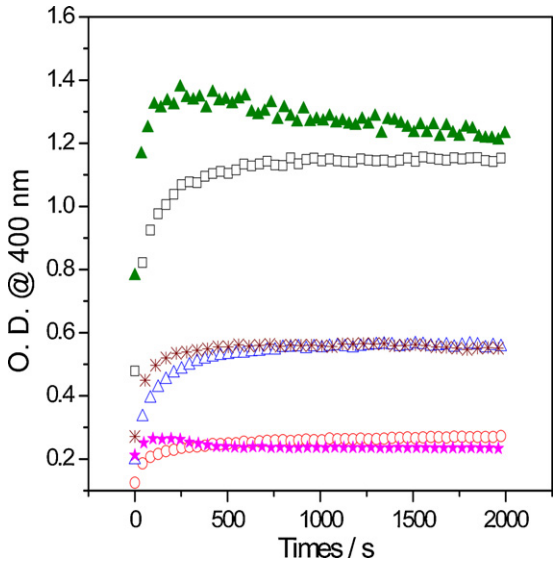

Fig. 2. Time dependence of turbidity change after protein addition to agar solution. The ratio of the coacervate was [WPI]/[agar] = 0.57. The turbidity (optical dispersion at 400 nm) was measured for coacervate samples containing (CA □, NA3 ▲, PTW2 △, PTA2 ✳, PTW1 ◯ and PTA1 ⋆, respectively) at pH 3.0, 35 °C and buffered in formate buffer 0.01 mol dm^{-3}.

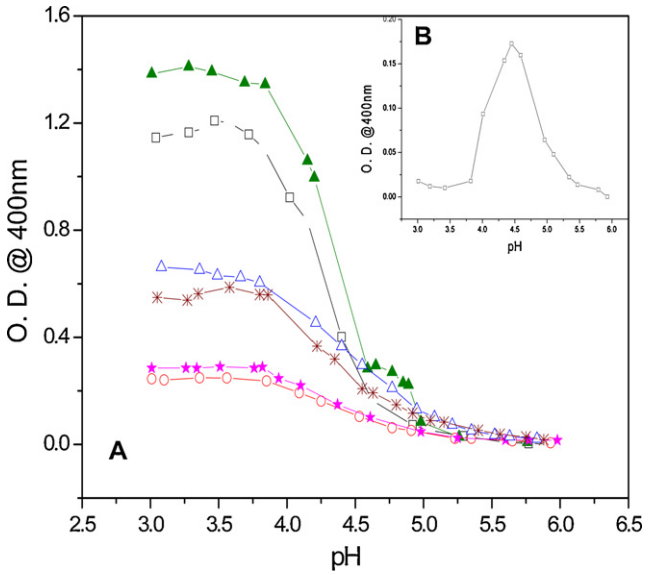

Fig. 3. pH dependence of the turbidity (at 400 nm) of (A) solutions containing WPI and agar at a weight ratio of 0.57, for which the maximum turbidity for the titration of agar with WPI was obtained. The solutions contained the agar samples CA (□), NA3 (▲), PTW2 (△), PTA2 (✳), PTW1 (◯) and PTA1 (⋆), respectively. (B) Solution of WPI (no agar).

and CA: in spite of the much larger molecular mass and sulfate content in PTW2, the O.D. reached at the plateau was half of that obtained for CA.

Another example illustrating that some other factors on agar extraction may also affect the formation of the coacervates is the reproducible decrease in the O.D. of CA samples for wt% ≥1.0, similarly to what is observed for chitosan coacervates under and excess of chitosan in solution (Souza et al., 2011), since excess of WPI may be related here with the redissolution of the complexes (dissolution of agar on the excess of protein). This behavior was just observed for CA sample, and could be related to the differences in the biomass source (environment growth, species, and so on). Thus, factors such as the higher purity of the CA agars, or the higher gelling power could account for these differences.

3.2.2. *Kinetics of coacervate formation*

In order to shed more light on complexation phenomena, the kinetics of coacervate formation was studied by registering the spectral transients at 400 nm for agar solutions (0.1 wt% in 0.01 mol dm^{-3} at pH = 3 and 35 °C) right after the addition of 150 μL of WPI 0.4 wt% (final molar ratio was 0.57 in all cases).

Fig. 2 shows the turbidity changes produced as a function of time for all the agar solutions investigated. As noticed in the figure, the O.D. rises sharply in all cases after WPI addition while the rate of turbidity increase, dOD/dt, decreases with time until reaching a constant value. Similarly to what was seen in the titration of agar with WPI (Fig. 1), the highest O.D. values were obtained for NA3 while CA reached similar O.D. values and MAE2 and MAE1 agar solutions yielded O.D. values lower by a factor of 2 and 6, respectively. According to our results, no significant differences are observed for the kinetics of coacervate formation between different MAE agar samples and WPI, the reaction being completed within 600 s in all cases.

3.2.3. *Effect of the pH on the complex coacervation process*

The effect of the pH on the complex coacervation process was also studied. For this purpose, the O.D. of mixtures of WPI and the different forms of agar with a wt% ratio of 0.57, at pH 3 and 35 °C, was monitored at 400 nm in the pH interval between 3 and 6, by addition of aliquots of 0.1 mol dm^{-3} sodium formate solutions.

Fig. 3 shows the evolution of the O.D. in these mixtures with increasing pH (Fig. 3A). Similar measurements were performed in pure protein solution (Fig. 3B), in control experiments.

Complex coacervation between protein and polyelectrolytes are expected to be favored at pH values for which the macromolecules bear opposite signs ($pK_{a\text{agar}} < pH < pI_{WPI}$).

Starting with the spectrum obtained for the WPI solution, large changes in the turbidity were observed in the whole pH window (Fig. 3B). The apparent isoelectric point of the protein mixture can be easily deduced from the pH value of maximum O.D. ($pI \sim 4.5$) due to the strong formation of self-aggregates of milk proteins in the surroundings of their p*I*. Both the shape of the curve and p*I* value agree reasonably well with those reported in the literature (Souza et al., 2009).

The results obtained for WPI/agar mixtures showed a similar shape in all cases (Fig. 3A). The curves show a maximum O.D. between pH 3.0 and 3.75 for all the systems investigated indicating either the achievement of a maximum number of particles with a certain size or the formation of the largest particles possible. For pH > 3.75, a large decrease in the O.D. of the mixture is observed until pH ∼ 4.5 is reached (the estimated p*I* of the protein mixture). As the pH gets closer to the p*I*, the charge of the whey protein is reduced and the coacervate dissolution is almost complete. In this case, pH = 4.5 corresponds to the minimum limit where coacervation can occur under the necessary conditions (sufficient protein charge to bind and neutralize the polyelectrolyte) (Antonov, Mazzawi, & Dubin, 2010). Obviously, the O.D. does not drop immediately to zero at pHs beyond 4.5 and from this value to pH = 5.0, a slight decrease in the turbidity is still observed. This is normal as 4.5 is just an estimate for the p*I* of the protein mixture and some of their components should have a larger p*I* and still remain positively charged (and forming the complexes) in this range of pH. But, even if some of the protein making part of the mixture had a $pI \geq 4.5$ (*e.g.* α-LA), the own self-association phenomena could be responsible for the slight dispersion of the light (Weinbreck et al., 2003) (such as observed for the whole mixture between pH 4.5 and 5). In these cases, and even near the p*I* for which the net charge of the protein is negative, positively charged domains should still exist in the protein for interaction with agars (surface selective

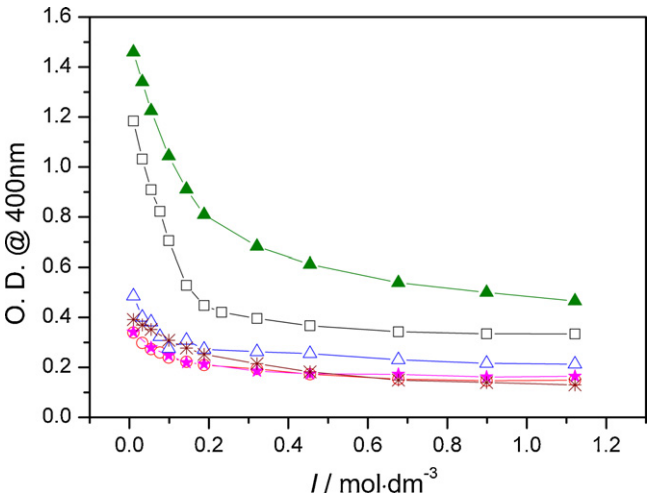

Fig. 4. Ionic strength dependence of the complex coacervates formation between WPI and agar (CA □, NA3 ▲, PTW2 △, PTA2 ✳, PTW1 ◯ and PTA1 ★, respectively) at pH 3.0, 35 °C and buffered in formate buffer 0.01 mol dm^{-3}.

charged patch binding) (Antonov et al., 2010; Boral & Bohidar, 2010; Wiseman et al., 1989).

The behavior observed for the different agar samples in the pH range for which coacervate formation is favorable, matches in the trends observed before in Figs. 1 and 2 with different MAE agar samples displaying a similar trend.

3.2.4. Effect of the ionic strength on the turbidity of WPI/MAE agar solutions

The ionic strength (I) has previously shown to be a very important parameter in the interaction established between polyelectrolytes since electrostatic attraction between oppositely charged macromolecules is expected to be the main driving force for the molecular recognition process and the increase in the concentration of salts present in the solution would lead to the screening of the electrostatic field between charges species (Schmitt et al., 1998).

The effect of I on the formation of WPI/agar complexes was studied by monitoring the addition of a NaCl solution (4 mol dm^{-3}) to a mixture with WPI/agar, wt% = 0.57, at pH 3 (Fig. 4). The same trend was identified for solutions containing the different types of agar. However, important differences in the O.D. values were found for the different agars studied. Once again, NA3 and CA exhibited a different behavior with respect to the other agar species. They showed the highest values of O.D. in the whole salt concentration range. In line with the results presented above, the spectral profiles registered in the presence of alkali-treated agars extracted under MAE1 and MAE2 conditions yielded significantly lower values of O.D. But, in this case, analogous behavior for the two different MAE1 or MAE2 agars was detected at low salt concentration.

While O.D. decreases fastly in the range $0.2 > I > 0$, much slower decrease is observed beyond this value, achieving a constant value for $I > 0.6$ mol dm^{-3} in all cases. These results indicate that the turbidity of the solution and, thus, the size of the complexes formed in the solution, are reduced by increasing the concentration of salt in the corresponding solutions independently of the type of agar. As previously seen for other complexes (Souza et al., 2011; Stone & Nickerson, 2012; Vasconcelos et al., 2006) this is explained considering that increasing the ionic strength results in an increased screening of the attractive electrostatic interaction established between WPI proteins and the different agars at the pH investigated.

Though a monotonic decrease in the turbidity of the solution as a result of the increase in the ionic strength of the solution is observed for all the tested samples, a significant amount of coacervates seems to remain in solution even at high ionic strength ($I > 0.6$). Again this behavior can be explained in terms of both the strength of the electrostatic attraction between agar and WPI (mainly electrostatic in nature) and the solubility of the formed complex which is mainly due to its hydrophobicity (in which the 3,6-AG content is expected to play an important role) and the overall molecular mass of the complex. According to this view, a significant amount of insoluble complexes remained in solution at high ionic strength for the forms of agar with the richest sulfate content (NA3) or with the highest molecular mass (CA and NA3). In the case of NA3, the net charge in a single agar molecule is larger due to the greater percentage of negative sulfate groups per molecule. On the other hand, the high molecular masses of CA and NA3 enable the extended establishment of WPI/agar hydrophobic and coulombic interactions. Thus, under similar screening conditions, these forms of agar are able to establish stronger interaction with WPI proteins. The small differences in the curves for MAE1 and MAE2 agars seem to be dictated by the differences in their molecular masses and sulfate content. In particular the higher molecular mass of MAE2 agars leads to enhanced formation of complexes despite their significantly lower sulfate content (Table 1).

Besides the physical characteristics of the agars, the higher amount of salt present in the systems containing alkali-treated samples extracted under MAE1 and MAE2 conditions (higher effective values of I), may have a negative impact on the formation of protein–polyelectrolyte coacervate complexes.

3.2.5. Thermodynamic description of the interaction process in WPI/MAE agar systems

In order to obtain important information on the thermodynamics of the interactions between WPI and agar, so as to delineate the best possible conditions for future applications, coacervate formation was also monitored using isothermal titration calorimetry (ITC). For this purpose, agar solutions were continuously titrated with WPI, at 35 °C, until complete saturation. The integrated heats of the interaction were corrected by subtracting the corresponding dilution heats resulting from injecting identical amounts of protein and agar in formate buffer.

The interactions established between the different forms of agar and WPI protein showed to be accompanied by the release of heat. The changes in enthalpy per mole of protein injected into the cell were plotted *versus* WPI/agar wt% ratio in Fig. 5. Dissimilar exothermic contributions to the overall energetics of the molecular recognition process were observed in all cases, especially to the lowest wt% ratio investigated. These differences showed to decrease as the agar becomes saturated with the protein (redissolution process). One needs to take into account the fact that during the redissolution process the increase in the concentration of WPI does not change the number of protein–polyelectrolyte contacts because the establishment of new interactions between agar and the WPI molecules added to the solution implies the disruption of similar interactions between agar molecules and protein molecules previously present in the solution. As a consequence, the enthalpy change due to the redissolution can be anticipated to be close to zero because of the similar energetics of the newly formed interactions and the ones previously disrupted. Therefore, the calorimetric titration monitors mainly the formation of the protein–polyelectrolyte complexes without the contribution of the redissolution process.

The formation of the complex between WPI and different forms of agar is enthalpically driven since, in all cases, the enthalpy change upon complex formation is exothermic. In this respect, the changes in enthalpy became much smaller for wt% ratio > 0.15. In fact, as anticipated, a quasi-plateau was achieved in all cases for wt% > 0.2. According to these changes, two different regions

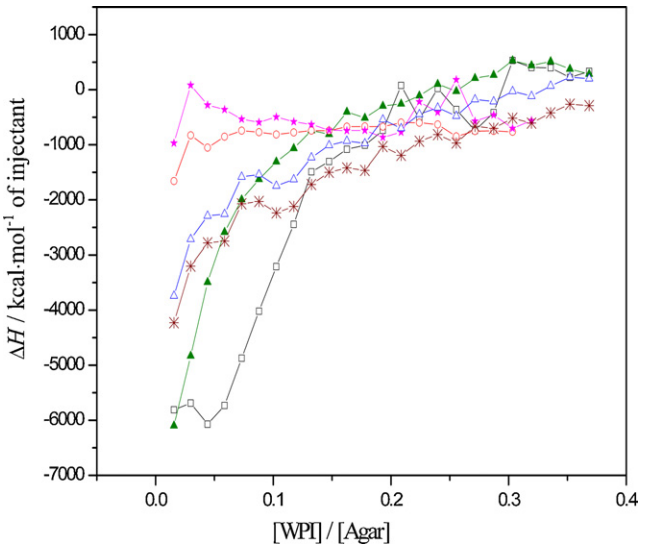

Fig. 5. Interaction enthalpies for the titration of agar at a concentration of 0.3 wt% with WPI solutions (0.6 wt%) (CA □, NA3 ▲, PTW2 △, PTA2 ✳, PTW1 ○ and PTA1 ⋆, respectively) at pH 3.0, 35 °C and buffered in formate buffer 0.01 mol dm^{-3}.

associated to two distinct regimes of interaction can be distinguished in the curves. In the first region (wt% = 0–0.15), the interactions are enthalpically driven mainly due to the attractive electrostatic forces established between both biopolymers (other weaker forces such as hydrophobic and Van der Waals, may also contribute to the enthalpy). Nevertheless, as the wt% ratio increases, further addition of WPI results in the release of an increasingly lower amount of heat (second region). This turning point may be established at the wt% ratio for which the agar is completely saturated with the protein and the redissolution process previous described takes place. From the data in Fig. 5, it seems to be around wt% 0.15 in all cases with the exception of MAE1 agar for which it seems to happen at a lower concentration ratio. Whether these enthalpic changes involves particle formation is not possible to asses, although it is strongly suggested with base on previous sprectroscopic data.

Significant differences were observed in the enthalpic response of the system in the presence of the different types of agars. Once again, these differences can be easily correlated with the physicochemical properties of the different forms of agar (as previously seen for the turbidity changes in Fig. 1). The high molecular mass and sulfate content of NA3 agar lead to a higher release of heat in its interaction with the WPI, due to a larger amount of negative charges per molecule of agar to interact with the protein. On the other hand, although the percentage of sulfate is lower in CA, its molecular mass is similar to that of the native sample, which seems to be the determinant factor in this case.

In contrast with this behavior, the curves obtained for alkali-treated samples extracted under MAE1 conditions achieved the plateau right after the addition of the first aliquots of protein. In addition, the values of enthalpy registered for these mixtures were much less exothermic than those obtained for CA and NA3. Also, in accordance with the spectroscopic results, MAE2 agars showed an intermediate enthalpic response. In spite of exhibiting high molecular masses like the CA sample, the large neutral fraction present in PTA2 and PTW2 agars could explain the difference in the behavior of these samples. However, the characteristics of their curves changed for wt% ratios of the same order of those observed for NA3 and CA. As previously explained, these differences may be attributed to the different physicochemical properties of the different agars.

4. Conclusions

MAE agars revealed excellent properties for complex formation with WPI. By using microwave irradiation a wide variety of agars can be obtained, as different MAE conditions can result in polyelectrolytes with significantly distinct properties.

The binding of WPI to different MAE agar samples and the subsequent phase-separation leading to the formation of coacervates has been shown to be the result of different contributions. On the one hand, electrostatic attraction between the interacting molecules seems to be a key determinant in the molecular recognition process. The contribution of electrostatics is dictated by the sulfate content of agar and strictly modulated by environmental conditions such as pH or ionic strength. On the other hand, the solubility of the formed WPI/agar complexes (which governs the phase separation process and therefore the formation of insoluble coacervates) has been shown to depend not only on the sulfate content of the agar sample (that may contribute to the polarity of the complex with the uncompensated charges) but also, mainly, on the hydrophobicity and the molecular mass of the complex, being the former strongly affected by the 3,6-AG content of agar.

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