Carbohydrate Polymers 110 (2014) 345–353

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Rheological and structural characterization of agar/whey proteins insoluble complexes

Cristina M.R. Rocha[a,*], Hiléia K.S. Souza[a], Natália F. Magalhães[a,b], Cristina T. Andrade[b], Maria Pilar Gonçalves[a]

[a] REQUIMTE, Departamento de Engenharia Química, Faculdade de Engenharia, Universidade do Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal
[b] Instituto de Macromoléculas Professora Eloisa Mano, Universidade Federal do Rio de Janeiro, Centro de Tecnologia, Bloco J, 21945-970 Rio de Janeiro, RJ, Brazil

ARTICLE INFO

Article history:
Received 5 August 2013
Received in revised form 3 April 2014
Accepted 4 April 2014
Available online 18 April 2014

Keywords:
Whey proteins
Agar
Insoluble complexes
Coacervation
Rheology

ABSTRACT

Complex coacervation between whey proteins and carboxylated or highly sulphated polysaccharides has been widely studied. The aim of this work was to characterise a slightly sulphated polysaccharide (agar) and whey protein insoluble complexes in terms of yield, composition and physicochemical properties as well as to study their rheological behaviour for better understanding their structure. Unlike other sulphated polysaccharides, complexation of agar and whey protein at pH 3 in the absence of a buffering agent resulted in a coacervate that was a gel at 20 °C with rheological properties and structure similar to those of simple agar gels, reinforced by proteins electrostatically aggregated to the agar network. The behaviour towards heat treatment was similar to that of agar alone, with a high thermal hysteresis and almost full reversibility. In the presence of citrate buffer, the result was a "flocculated solid", with low water content (75–81%), whose properties were governed by protein behaviour.

1. Introduction

Protein–polysaccharide interactions are the basis for many important biological processes. In foods, proteins and polysaccharides are the most important structure-forming ingredients (Tolstoguzov, 1991) and their use in mixed systems can improve or modify their functional behaviour (Dickinson & Galazka, 1991; Dickinson & Izgi, 1996; Gurov & Nuss, 1986; Turgeon & Beaulieu, 2001).

Mixing a protein with a polysaccharide into an aqueous solution may drive to one of several situations (de Kruif & Tuinier, 2001; Doublier, Garnier, Renard, & Sanchez, 2000; Syrbe, Bauer, & Klostermeyer, 1998; Tolstoguzov, 1991) depending on the polymer–polymer and solvent–polymer attractive or repulsive interactions present and on the polymer molecular masses:

(a) Co-solubility, though this is the least typical situation;
(b) Incompatibility: in this case the system may have one or two phases. In the former, the biopolymers concentrations are lower than the phase-separation threshold. In the latter each phase is enriched in one of the polymers (segregation), when the concentrations are above that threshold;
(c) Complexation: in this case also one or two phase systems can be formed. The soluble complexes are stabilised through electrostatic interactions and hydrogen bonding resulting in one phase system. Two phases are formed when polysaccharides are adsorbed onto the protein or bridge between several protein molecules, therefore concentrating both polymers in one phase leading to the exclusion of the solvent from their vicinity. From the two phases formed one of them is thus enriched in both polymers while the other is depleted in both polymers and solvent enriched. This phenomenon has been called aggregative phase separation or coacervation and is typical of oppositely charged anionic polysaccharides and proteins.

In fact, coacervation between an anionic polysaccharide and a protein is a widely known phenomenon and many examples are described in the literature (e.g. (Burgess, Kwok, & Megremis, 1991; de Kruif, Weinbreck, & de Vries, 2004; Galazka, Smith, Ledward, & Dickinson, 1999; Imeson, Ledward, & Mitchell, 1977; Laneuville, Paquin, & Turgeon, 2000; Mendanha et al., 2009; Onder, Sarier, & Cimen, 2008) due to both their biological relevance and promising industrial applications (Aberkane, Jasniewski, Gaiani, Scher, & Sanchez, 2010; Turgeon, Schmitt, & Sanchez, 2007). These

* Corresponding author. Tel.: +351 220414838; fax: +351 5081440.
E-mail addresses: cmrocha@fe.up.pt, cmrvicente@gmail.com (C.M.R. Rocha).

http://dx.doi.org/10.1016/j.carbpol.2014.04.015
0144-8617/

applications include encapsulation, protein recovery, multilayer structures (such as packaging films), biomimetic systems and new food gels or emulsions. It is usually a reversible process, driven by a large entropy increase due to the release of counterions, that depends on the properties of the biopolymers involved (charge density, molecular weight, total biopolymer concentration and protein to polysaccharide ratio), on the environmental conditions (pH, ionic strength, type of ions) and on external factors such as temperature, pressure and shearing (Schmitt & Turgeon, 2011). Functional properties of polysaccharide-protein complexes are usually improved, probably by combination of the properties of each component (Schmitt, Sanchez, Desobry-Banon, & Hardy, 1998).

Whey proteins (WP) are widely used in food formulations due to their nutritional and functional properties (de Wit, 1998; Turgeon & Beaulieu, 2001). Besides their classical nutritional benefits, they are well known for their versatile functionality, including emulsifying, gelling or foaming abilities. Whey proteins are the group of milk proteins that remain soluble in "milk serum", or whey, after precipitation of caseins at pH 4.6 and 20 °C. They possess high levels of secondary, tertiary and, in most cases, quaternary structures. Most of them are globular proteins (feature responsible for many of their functional properties) that can be denatured on heating (e.g. completely at 90 °C for 10 min) and contain intramolecular disulphide bonds that stabilise their structure. Their isoelectric point typically varies between 4.5 and 5.5.

Agar is a biopolymer extracted from red seaweeds extensively used in food and pharmaceutical industries as gelling and stabilising agent. It is built up on two main fractions: agarose, the linear neutral fraction responsible for agar's good gelling ability, and agaropectin, the charged polymer fraction that results from the presence of several substituent groups such as sulphates, methyl ethers and pyruvates. Although this polyanionic polysaccharide is a sulphated polysaccharide and has been considered as a strong polyelectrolyte (Boral & Bohidar, 2010), its sulphate content is much lower than in dextran sulphate and carrageenans. This fact can be beneficial when coacervating since, though the charges of polyelectrolytes should be large enough to allow significant electrostatic interactions, too many charges can cause precipitation of the complex instead of coacervation (Boral & Bohidar, 2010). In fact, precipitation has been referred in whey proteins and strong polyelectrolytes complexation, such as sulphated carrageenans or dextran sulphate (de Kruif et al., 2004; Weinbreck, Nieuwenhuijse, Robijn, & de Kruif, 2004).

Numerous studies have focused on whey protein/polysaccharide interactions including complex coacervation applications (e.g. (Aberkane, Jasniewski, Gaiani, Hussain, Scher, & Sanchez, 2012; Weinbreck, Tromp, & de Kruif, 2004)). One particular application is the separation of β-lactoglobulin and α-lactalbumin (e.g. Hidalgo & Hansen, 1971), as they have slightly different isoelectric pH values (5.2 and 4.1, respectively). At pH 4.5, and under adequate conditions, β-lactoglobulin forms a coacervate and α-lactalbumin remains in solution (Capitani, Pacheco, Gumerato, Vitali, & Schmidt, 2005). Other applications include their use as functional agents (stabilisers, fat replacers, texturising agents) or microencapsulation. Coacervation between a sulphated polysaccharide and a globular protein has been described in the literature (e.g. (Jones, Handschin, Adamcik, Harnau, Bolisetty, & Mezzenga, 2011; Klassen, Elmer, & Nickerson, 2011; Weinbreck, Nieuwenhuijse, et al., 2004; Weinbreck, Tromp, et al., 2004)). However, applications involving agar complexation are very rare and, to our knowledge, mostly gelatine-agar systems have been studied (Boral & Bohidar, 2010; Singh, Aswal, & Bohidar, 2007, 2011), being gelatine a structural non-globular protein, unlike e.g. whey proteins. A previous study on the interactions between bovine whey protein isolate (WPI) and agars (commercial or obtained by microwave-assisted extraction) with different physicochemical properties was performed by the authors' group, using spectroscopic and calorimetric techniques (Souza, Sousa, Gómez, & Gonçalves, 2012). Optical dispersion (O.D.) and isothermal titration calorimetry (ITC) results revealed that molecular mass and sulphate content of different agars had a determinant effect on coacervate formation. No studies on WPI/agar insoluble complexes' characterization were made. Therefore, the present paper aims at characterizing a low sulphate content polysaccharide (agar) and WPI insoluble complexes in terms of yield, composition and physicochemical properties as well as studying their rheological behaviour in order to better understand their structure.

2. Materials and methods

2.1. Materials

Whey protein isolate (WPI) was obtained as a commercial sample (Lacprodan DI-9224) from Arla Foods Ingredients, Ambh (Denmark), and used as protein source without further purification. As specified by the manufacturer, the isolate contains a minimum of 92% 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, 69 kDa). The isolate further contains lactose and fat (each at a maximum content of 0.2%), and minerals such as sodium (0.5%), potassium (1.3%), and calcium (0.1%). Commercial agar (ref. A-7002 with an ash content of 2–4.5%) was obtained from Sigma-Aldrich Co. (St. Louis, MO). The average molecular mass (M_v) of the polysaccharide was obtained from viscosity measurements in a previous work (Souza et al., 2012) and was estimated to be 137.5 kDa.

All other chemicals (citric acidic, sodium citrate, sodium chloride, etc.) were of analytical grade and used without further purification.

2.2. Preparation of solutions

Commercial agar and WPI stock solutions were separately prepared by dissolving the amounts of their corresponding solids in 10, 20 or 50 mmol dm^{-3} citrate buffer or in water. When necessary, pH (measured using a Crison pHmeter, model GLP 21, Spain) was adjusted to 3 by addition of citric acid, sodium citrate or, in the case of water solutions, with concentrated hydrochloric acid.

Agar dispersions were heated at 95 °C for 30 min with gentle stirring to ensure complete dissolution. They were subsequently incubated at 40 °C to avoid gelation, till further use. WPI dispersions were gently stirred at room temperature till complete dissolution and left to hydrate for 6 hours before use.

2.3. Turbidity measurements

Turbidity (optical dispersion, OD) of mixed WPI/agar dispersions was measured using a UV–visible spectrometer (Jasco, V-630 Bio), at a wavelength of 400 nm using quartz cells with a light path of 10 mm. Turbidity values were only recorded when the signal became stable and were corrected using buffer as blank. Each reported value is the average of three consecutive readings. In order to study the effect of 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%, w/w) and increasing concentrations of protein delivered from a common stock solution (0.4%, w/w). The mixed dispersions were prepared at pH 3 and 35 °C and were allowed to stand at this temperature for at least 30 min to ensure that the reaction was completed before recording their turbidity. Three replicas were made for each buffer.

2.4. Yield and physicochemical properties of the insoluble complexes

Insoluble complexes were prepared by pouring 38 mL of WPI stock solution (1.2%, w/w) drop by drop into 190 mL of agar stock solution (0.6%, w/w), at 40 °C, in a 250 mL centrifuge flask. Stock solutions were prepared in 10, 20, 50 mmol dm^{-3} citrate buffer and in water, at pH 3.0, as described in 2.2. After complexation, the solutions were allowed to stand for 20 min at 40 °C, gently centrifuged at 2000 × *g* for 10 min at 40 °C, and the supernatant was removed. Wet and dry yields were determined by weighting and drying the insoluble complex for 24 h in a vacuum chamber at 40 °C. At least three flasks were prepared for each rheological analysis, which means that wet yield values are an average of nine measurements. At least four replicas were made for the dry yield values. Protein and agar in the coacervate phase were determined indirectly from protein concentration in the supernatant and this concentration was determined using the Bradford method. The supernatants from samples with no buffer or low concentrated citrate buffer were diluted with 200 mmol dm^{-3} NaOH. This was supposed to destroy eventual electrostatically formed soluble complexes by changing the overall protein charge, thus avoiding their interference with the protein quantification. Salt content was considered to be the same in the coacervate and in the supernatant phases. At least three replicates were made for each value.

Zeta potential of particle suspensions was determined by laser Doppler velocimetry in a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK), at 25 °C, using a disposable capillary cell (DTS 1060, Malvern, UK). Agar and WPI solutions were prepared as in turbidity experiments. Zeta potential was calculated from the electrophoretic mobility of the charged particles. Three different samples were used for each point and three automatic measurements were made for each sample.

2.5. Rheological measurements

For rheological measurements, insoluble complexes were prepared as described in Section 2.4.

The viscoelastic properties of WPI/polysaccharide complexes were evaluated in a controlled stress ARG2 rheometer (TA Instruments, USA) fitted with a parallel plate geometry (cross hatched, 40 mm diameter, gap 800 μm) and a Peltier system, for fast and accurate temperature control. All tests were made at least in triplicate and the surface of samples in contact with air was covered with a thin layer of liquid paraffin to prevent evaporation. Standard deviations were below 10%.

For each coacervate, oscillatory tests were performed in two different sets of experiments:

a) *Large amplitude oscillatory shear (LAOS) tests* were made both to analyse non-linear response and to ensure that further tests were made within the linear viscoelastic region; strain sweeps from 0 to 120%, at 1 Hz and 20 °C, were performed before and after the temperature ramps described below;
b) *Small amplitude oscillatory shear (SAOS) tests*: The sample was placed into the measuring device and allowed to equilibrate for 5 min, at 35 °C, before performing a frequency sweep from 0.01 to 10 Hz at this temperature. Then, a temperature ramp from 35 °C to 20 °C (rate 2 °C min^{-1}) was applied; the temperature was maintained at 20 °C for 30 min, after which another frequency sweep (0.01–10 Hz) was recorded. The sample was then heated from 20 °C to 95 °C, maintained at 95 °C for 5 min and cooled back to 20 °C. After equilibration at 20 °C for 10 min, another frequency sweep (0.01–10 Hz) was recorded. Both frequency sweeps were performed with a strain amplitude of 0.5%, which was within the linear viscoelastic region as determined in LAOS tests. Temperature and time sweeps (equilibration steps) were performed at a frequency of 1 Hz and a strain amplitude of 0.5%. Temperature sweeps were performed at a rate of 2 °C min^{-1}.

2.6. Microstructure

The microstructure of coacervates was confirmed by Cryogenic Scanning Electron Microscopy (CryoSEM), at CEMUP, Porto, Portugal.

For each sample, a small volume of coacervate (approx. 1–3 mm^3) was frozen in slushy nitrogen (−210 °C), transferred to an ALTO 2500 cryo preparation chamber and placed onto a cool stage (−150 °C), where it was fractured exposing the internal surface. The ice formed on the exposed fractured surface was removed by sublimation at −90 °C for 1.5 min. The sample was then coated with sputtered Au–Pd thin film at −150 °C for 40 s, from a sputter head using ultrapure argon gas. Analyses were performed at -150 °C in a JEOL JSM 6301F scanning electron microscope (Japan) equipped with a Gatan ALTO 2500 cryo preparation chamber using an accelerating voltage of 15 kV and working distances of 15 mm.

3. Results and discussion

The results section is divided in three parts. Firstly, results of turbidity measurements are presented. These results served as a basis for the choice of working conditions in the subsequent studies. pH 3 was chosen based on previous work (Souza et al., 2012). In the second part, insoluble complexes prepared at the chosen pH and protein to polysaccharide ratio were characterised in terms of yield and physicochemical properties. Finally, rheological and structural properties are discussed.

3.1. Effect of the WPI concentration on the interaction with agar

Considering that at pH 3 agar is negatively charged and WPI is positively charged (below the isoelectric point (pI) of WPI, at 4.7–5.2), the establishment of electrostatic interactions between agar and WPI is strongly expected. Agar/WPI complexes are expected to be formed through the interaction of the positive amine groups from whey proteins and the negatively charged sulphate groups of agar. However agar electrostatic behaviour is also strongly dependent on its overall hydrophobicity which is a function of the 3,6-anydrogalactose content and its molecular weight (Souza et al., 2012). The interaction between WPI and agar was studied measuring optical dispersion (OD) at 400 nm.

Fig. 1 presents the evolution in OD of an agar solution (0.1%, w/w) during the addition of aliquots of a WPI solution (0.4%, w/w). The solutions were kept at 35 °C and pH 3 (either with citrate buffer, 10 and 50 mmol dm^{-3}, or with HCl, for the experiments without buffer). In the initial stage of titration the turbidity of solutions shows a growth trend across the whole range of wt.% ratios, reaching a maximum when the ratio is around 0.3 and 0.6 for solutions buffered with citrate buffer and with water, respectively. Beyond these values, OD values remain almost constant, though with a slight decrease trend. The increase in OD is associated with the formation of an increasing amount of insoluble protein–polysaccharide complexes, large enough to diffuse light, that remain suspended in the liquid matrix (Wang, Wang, Ruengruglikit, & Huang, 2007). The constant OD values attained after reaching a maximum occur when no more insoluble WPI–agar aggregates are formed (agar is becoming saturated with protein) and the ones formed remain stable, for further increases of WPI concentration. The slight decrease obtained after reaching the plateau, more visible without the presence of citrate, can be interpreted as

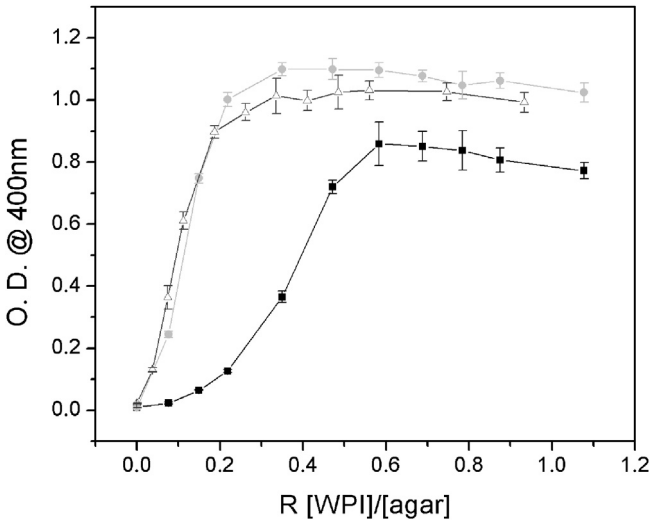

Fig. 1. Effect of the WPI/agar weight ratio on the turbidity of the samples. The concentration of agar was 0.1% (w/w) and the concentration of WPI was varied from 0 to 0.12% (w/w). Turbidity (optical dispersion at 400 nm) was measured at 35 °C, pH 3 (■ – no buffer; ● – 20 mmol dm^{-3} citrate buffer; Δ – 50 mmol dm^{-3} citrate buffer).

the re-dissolution of some protein-polyelectrolyte complexes due to segregative phase separation.

The OD behaviour found with citrate buffer was similar to that found with formate buffer in a previous work (Souza et al., 2012). Furthermore, the overall OD is slightly lower for more concentrated buffers, which is in accordance with the generally accepted idea that interactions become weaker with the increase of ionic strength. The presence of more salt reduces the effective charge of polymers and can overcome complex coacervation as a result of counterion screening of the polyelectrolytes. However, this difference is much weaker than the difference reported in literature when adding NaCl to coacervate-forming solutions due to the buffering nature. Additionally, in the absence of buffer (pH correction was made with HCl), the maximum OD values achieved were much lower. This usually means a much lower WPI/agar interaction and thus less complexes formation (mostly smaller and more water-soluble WPI/agar complexes) than in the former case. In the presence of buffer, water exclusion in the coacervate phase seems to be much stronger, leading to big aggregates of WPI/agar complexes, thus forming a solid precipitate or flocculant complex. In the absence of buffer, the coacervate retains much more water. The WPI/agar ratio that corresponds to maximum turbidity, indicating electrical equivalence, is also shifted from ca. 0.3 in the presence of buffer to 0.6 in its absence, which may indicate a preferential bonding of citrate with agar, thus competing with protein molecules.

The low critical WPI/agar ratios achieved also reflect the low charged nature of the agar molecule when compared to other sulphated polysaccharides (e.g. carrageenans) commonly used in literature for coacervation with milk proteins, tipically well above 1 (Jones et al., 2011; Stone & Nickerson, 2012; Weinbreck, Nieuwenhuijse, et al., 2004; Weinbreck, Tromp, et al., 2004).

3.2. Yield and physicochemical properties of the coacervates

The yields and composition of the different coacervates are presented in Table 1. Without buffer higher yields are achieved, which probably reflects a screening effect of buffering solutions, once ionic interactions are the drive mechanism for association. Also a much higher water content results in this case.

The results for protein and agar contents in the complexes are in accordance with the plateau detected in turbidimetry measurements in the absence and in the presence of citrate buffer (WPI/agar ratio around 0.6 and 0.3, respectively). Stoichiometric precipitation occurs when complexating a protein and a strong polyelectrolyte, in the absence of salt (Cooper, Dubin, Kayitmazer, & Turksen, 2005).

It is important to note that soluble complexes probably exist in the solvent-rich phase and the yields presented refer only to the insoluble sedimentable part. In fact, when determining protein concentration in the supernatant phase (which was used to calculate coacervate composition), for the 10 mmol dm^{-3} and no buffer samples, the concentrations achieved when samples were diluted in water where extremely low (particularly in the latter case). When samples were diluted in 200 mmol dm^{-3} NaOH, aiming at impairing electrostatic interactions, higher protein contents were achieved. For 20 mmol dm^{-3} and 50 mmol dm^{-3} buffers, the same protein concentration was achieved in both cases. Thus, it can be inferred that in the first case (no buffer or 10 mmol dm^{-3}) significant soluble complexes were present, interfering with the protein quantification method, whereas in the second case no soluble complexes existed. For this reason, the protocol for protein determination was slightly changed to include a preliminary step to destroy soluble complexes by increasing the pH. The presence of soluble complexes was already predictable by the difference observed in the shape and height of OD curves (Fig. 1). OD is lower for the complexes prepared without buffer.

Another important fact is related with protein and agar contents in the coacervate phase without buffer. When the WPI/agar ratio used (e.g. 0.4%, w/w) is significantly below the stoichiometric mixing ratio (in the absence of buffer it seems to be around 0.6%, w/w), agar is in excess and more soluble complexes are obtained due to the presence of non-neutralised charges (the complex is insoluble when the net charge is close to neutrality). Therefore, coacervation yield is 40%, lower than for 0.7 WPI/agar. The presence of more soluble complexes was confirmed both by the lower turbidity and the higher difference in protein content between the samples diluted with NaOH and with water (protein contents in the supernatant determined with samples diluted in water were up to 70% lower than protein contents determined with samples diluted NaOH solution, for the experiments made in the absence of buffer or in 10 mmol dm^{-3} citrate buffer – results not shown).

In spite of the screening effect of the buffer, differences in zeta potential are visible for all samples (Table 2). WPI solutions have a positive zeta potential and agar solutions a negative zeta potential, as expected. WPI and agar zeta potential values (25.0 and −16.3 mV, respectively) with no buffer are in accordance with the literature values: 17–32 mV for WPI and pH ranging from 4 to 2.5 (Bedie, Turgeon, & Makhlouf, 2008; Benichou, Aserin, Lutz, & Garti, 2007) and ≈−20 mV at pH 4 for agar (Boral & Bohidar, 2010). This value also reflects the lower agar sulphate content in comparison with k-carrageenan, which presents a zeta potential of −63 ± 6 mV (Jones, Adamcik, Handschin, Bolisetty, & Mezzenga, 2010). Complexes with a WPI:agar ratio of 0.4 possess a zeta potential close to electroneutrality, thus indicating that association has occurred. However, the complex in water produced with 0.4 WPI/agar ratio has a slightly negative value which may arise from the excess of agar (with negatively charged sulphate groups) used in comparison with the plateau indicated by the optical dispersion tests (near 0.7 WPI/agar). This fact is confirmed by the decrease in the absolute value of zeta potential for the complex in water produced with 0.7 WPI/agar ratio, which is much nearer to zero.

A soluble complex, a coacervate or an amorphous precipitate can result from the association of a protein and a polysaccharide, depending on their properties and on solution conditions (Dickinson, 1998). For non-gelling polysaccharides, usually a coacervate results in the case of carboxylated polysaccharides (pectin, alginate) and precipitates or flocculated particles appear in the

Table 1
Yield and composition of the coacervates in different buffer solutions.

Buffer concentration (mmol dm^{-3})	Wet yield (%)	Protein (%)	Agar (%)	Solids (%)	Dry yield (%)
0 (*WPI/agar ratio* = 0.3)	3.3 ± 0.9	1.7 ± 0.1[a]	2.9 ± 0.2	4.3 ± 0.8	27.1 ± 3.0
0 (*WPI/agar ratio* = 0.7)	4.5 ± 0.8	1.8 ± 0.3[a]	3.1 ± 0.1	4.9 ± 1.0	45.8 ± 6.4
10	0.52 ± 0.04	4.2 ± 0.3[a]	12.9 ± 2.6	19.3 ± 3.6	22.7 ± 2.7
20	0.59 ± 0.07	4.8 ± 0.6	17.3 ± 3.2	24.1 ± 3.0	18.6 ± 2.0
50	0.56 ± 0.05	5.0 ± 0.5	16.5 ± 0.6	21.6 ± 5.9	19.1 ± 3.6

[a] Sample of supernatant diluted with 200 mmol dm^{-3} NaOH.

Table 2
Zeta potential for the different solutions/suspensions at pH 3 (in water and in citrate buffer).

	Zeta potential (mV)			
	0 mmol dm^{-3}	10 mmol dm^{-3}	20 mmol dm^{-3}	50 mmol dm^{-3}
0.1% (w/w) agar	−16.3 ± 1.5	−5.2 ± 2.2	−3.2 ± 0.3	−3.3 ± 0.6
0.4% (w/w) WPI	25.0 ± 0.8	17.4 ± 0.7	12.5 ± 1.1	11.1 ± 0.3
Complex at WPI:agar = 0.3	−3.6 ± 1.4	−0.7 ± 0.4	−0.9 ± 0.3	−1.7 ± 0.1
Complex at WPI:agar = 0.7	−1.2 ± 0.1	–	–	–

case of sulphated carbohydrates (e.g. carrageenans). This is usually ascribed to the fact that attraction between amine groups of proteins is much stronger towards sulphate groups than towards carboxylate groups. In the present case, a highly hydrated gel seems to be formed in the absence of citrate buffer and a flocculated solid appears in the presence of citrate. Though agar is a sulphated polysaccharide, it has much less sulphate groups than, e.g., carregeenan, leading to a much lower charge density, resulting in a highly hydrated complex. In the presence of citrate, agar seems to behave like a stronger polyelectrolyte than agar alone (probably due to agar/citrate interactions), water exclusion is much higher and the behaviour becomes similar to that of carrageenan complexes.

3.3. Rheological and structural properties

LAOS tests were made to ensure that SAOS tests were conducted within the region of linear viscoelasticity and to assess the non-linear behaviour. SAOS tests were made to continuously monitor the evolution of viscoelastic properties either in frequency sweeps or with temperature, avoiding any modification of the molecular structure caused by shear. These results were correlated with the microstructure of insoluble complexes.

The insoluble complexes enriched phase formed in the presence of a citrate buffering solution revealed a high sensitivity to strain (Fig. 2), with the non-linear region starting at very low strains (below 1%). This was particularly critical for samples in 50 mmol dm^{-3} citrate buffer, and results of SAOS tests for these samples should be viewed with precaution, as the plateau of linear viscoelasticity was very limited. Coacervates formed without citrate buffer are much more strain resistant (critical strain at ≈50%) and show a weak strain overshoot. This behaviour changes significantly with temperature (Fig. 2B). In the presence of buffer, strain sensitivity is reduced possibly due to some stabilization of the structure ascribed probably to the formation of covalent bonds between denatured proteins. In the case of no buffer, strain sensitivity slightly decreases, probably due to some loss in the flexibility of the structure that can be attributed to the establishment of protein–protein hydrophobic bonds, and the weak strain overshoot is lost.

Mechanical spectra at 20 °C (Fig. 3) reveal a viscoelastic solid behaviour as G' is much higher than G''. However, there is a slight dependency on the frequency typical of gel networks involving physical bonds, where electrostatic forces are important. This frequency dependency is significantly lower for the coacervate with no buffer, possibly indicating a more entangled network, though less stiff as both moduli are lower. Also, at very low frequencies the moduli seem to rapidly increase. This could mean that for very low frequencies (below the experimental range used) there is a relaxation point and for even lower frequencies the samples would behave like liquids, which is a typical behaviour for entanglement networks. Though the complex in buffer seems to be much stronger after heating, the relative contribution of physical bonds seems to remain almost the same as the slope only slightly decreased. This decrease could be associated with the establishment of more permanent WPI/WPI protein interactions. The complex in water is slightly weakened by the heat treatment. Its frequency dependence is the same, except for the lower frequencies (0.01–0.1 Hz) where the complex seems to be more frequency-dependent after heating.

Fig. 4 presents the thermal profile of the elastic modulus obtained. The behaviour of coacervates in water is typical of a continuous agar matrix, with almost reversible high thermal hysteresis. Coacervates without buffer have already a strong solid character at 35 °C, which strengthens with the decrease of temperature. The temperature ramp from 20 to 95 °C results in a very slow response of G' until almost 80 °C and then a sharp decrease when approaching the agar melting temperature. However, a slight inflexion point can be seen around 85 °C together with a tinny inversion during the time sweep at 95 °C, probably due to protein denaturation and protein–protein interactions. However, this behaviour is not dominant (probably because protein concentration in the coacervate is not high enough; <4%), and with the decreasing temperature ramp the elastic behaviour is again reinforced (more sharply below 40 °C). Thus, interactions of WPI with agar apparently do not change significantly with the heat treatment and the coacervates' behaviour is still similar to that of agar. Gelling temperature of agar solutions is around 30–40 °C (depending on the type of agar, agar concentration, cooling rate and so on) and melting temperature can be near 85–95 °C. When temperature decreases, agar changes from a disordered random coil conformation to a double helical state, due to hydrogen bonds. These helices interact among themselves forming aggregates (fibre bundles) with a typical size of seven to eleven double helices, through extensive intermolecular hydrogen bonding. Aggregates interact at junction zones and form a three dimensional network. Agar is characterised by a substantial thermal hysteresis in solution-gel and gel-solution transitions. The reverse melting process, with increasing temperature, happens at much higher temperatures due to the stabilization of helices within the aggregates (Cooke, Gidley, & Hedges, 1996).

In the presence of citrate buffer the behaviour is completely different. The complex has already a strong solid character at 35 °C, but it is only slightly strengthened with the further decrease of

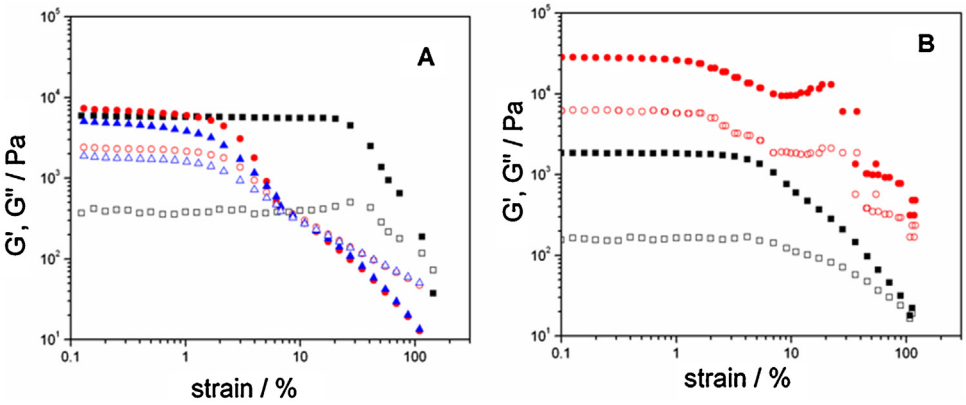

Fig. 2. Strain sweep at pH 3.0, 20 °C: (A) before heating; (B) after heating (■ – no buffer; ● – 20 mmol dm^{-3} citrate buffer; ▲ – 50 mmol dm^{-3} citrate buffer; filled symbols – G'; open symbols – G'').

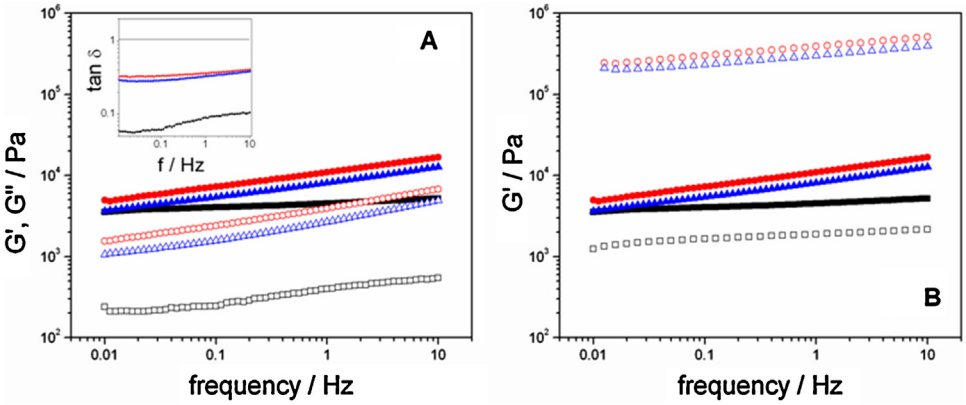

Fig. 3. Mechanical spectra at: (A) pH 3.0, 20 °C (the insert shows the changes in tan δ versus oscillation frequency; filled symbols – G'; open symbols – G''); (B) G' at pH 3.0, 20 °C, before (filled symbols) and after (open symbols) the heating step (■ – no buffer; ● – 20 mmol dm^{-3} citrate buffer; ▲ – 50 mmol dm^{-3} citrate buffer).

temperature, up to 20 °C. The temperature ramp results in an almost reverse G' decrease, and thermal hysteresis is not observed. At ca. 70 °C whey proteins start to denature and the elastic modulus starts to invert the decreasing trend. Between 80 and 85 °C, the elastic modulus rises sharply. One could think that rising temperature would result in agar solubilisation and that the coacervate would gain a strong "liquid" character. However, apparently at this point a whey protein network is becoming important and the elastic modulus follows the typical behaviour of heat-set whey protein gelation (Rocha, Teixeira, Hilliou, Sampaio, & Goncalves, 2009). The higher temperature also provokes irreversible denaturation of whey proteins exposing their inner regions, thus allowing protein aggregation or even gelation (if the protein concentration and heat treatment are high enough). Whey proteins' aggregation occurs mainly due to hydrophobic interactions and intermolecular disulphide bridges between thiol groups at neutral pH. For more acidic pH values, aggregates are mainly formed through physical interactions, such as hydrogen bonding and electrostatic and/or hydrophobic interactions, being the contribution of thiol groups much lower (Verheul, Roefs, & de Kruif, 1998). Thus, as the temperature further increases, the elastic modulus also increases. There is a slight inflection point near 90 °C, probably corresponding to

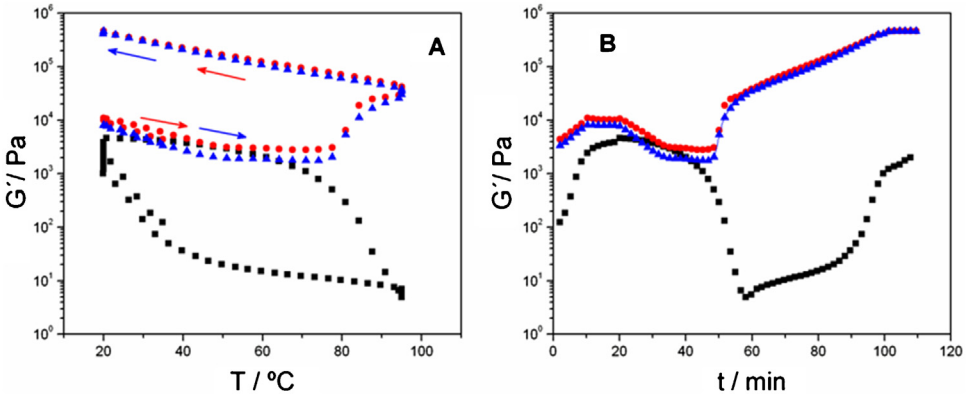

Fig. 4. Thermal profile of elastic modulus (G'): (A) as a function of temperature; (B) as a function of time with the same temperature profile of A (■ – no buffer; ● – 20 mmol dm^{-3} citrate buffer; ▲ – 50 mmol dm^{-3} citrate buffer).

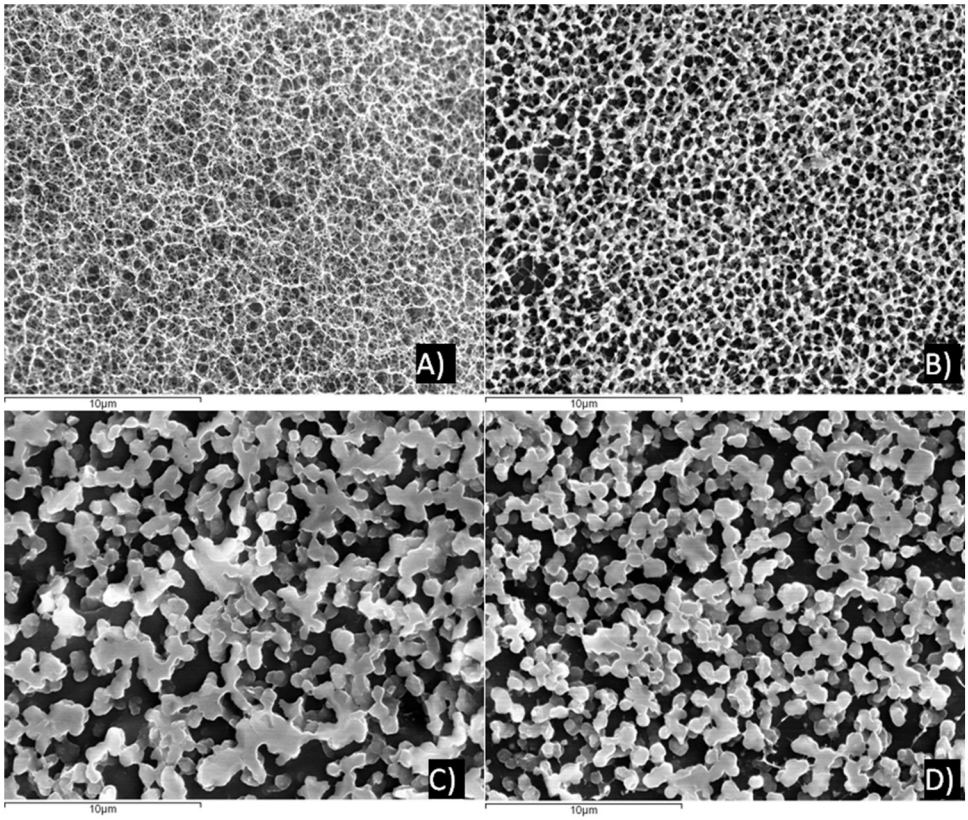

Fig. 5. Structure of non-heated coacervates produced at 35 °C and pH 3: (A) agar (1%, w/w); (B) coacervate in water; (C) coacervate in 20 mmol dm^{-3} citrate buffer; (D) coacervate in 50 mmol dm^{-3} citrate buffer.

the agar solubilisation temperature. The inverse process (temperature decrease) does not correspond to a reversible process and the complex network is further reinforced. The increase of *G'* during the cooling period is probably due to the strengthening of inter-particle attractive non-covalent bonds such as van der Waals interactions and hydrogen bonds, as for whey protein gels (e.g. Doucet et al., 2001; Ould Eleya et al., 2004). When testing gelation properties of k-carrageenan and β-lactoglobulin mixtures at pH 4, a behaviour similar to that of protein gel properties has been reported (Eleya & Turgeon, 2000). The presence of k-carrageenan was only detected by the enhancement of protein gel strength.

In theory, coacervate formation is a reversible process due to its ionic interactions-based nature. Changing pH to a region that is not favourable to ionic interactions allows coacervates' solubilisation. However, these WPI/agar insoluble complexes revealed to be completely insoluble over a wide range of pH values (2–12; results not shown). In the absence of buffer, the solid nature of the network was probably conferred by agar and high temperatures are needed to allow complex solubilisation. In fact, increasing the temperature up to 95 °C resulted in a whitish creamy liquid. However in the presence of buffer, the complex-enriched phase is depleted in water and, thus, highly concentrated in both biopolymers. In this case, whey protein concentration seems to be high enough in order to allow the occurrence of denatured protein–protein interactions and thus the establishment of a protein irreversible network. Thus the coacervate has always a solid nature even with the increase of temperature. Protein–polysaccharide electrostatic complexes can have interesting functional properties but their application is sometimes hindered by their pH reversibility. Thermal treatment of whey protein–pectin complexes has been used to stabilise them in a wider range of pH values (Gentes, St-Gelais, & Turgeon, 2010). In the case of coacervates prepared in the absence of citrate buffer, reversibility is possible by changing pH at high temperatures.

3.4. Cryo-SEM

Micrographs obtained with cryo-SEM are in accordance with the rheological results. When no buffer was used, the coacervate network (Fig. 5B) seems to be based on the structure of simple agar gels (Fig. 5A) slightly reinforced by proteins electrostatically aggregated to the agar network. Whey proteins seem to be distributed randomly and homogeneously through the agar gel matrix. As the solvent concentration is depleted in the coacervate phase and agar concentration becomes quite higher than in the original solution, agar gels and the resulting protein–polysaccharide insoluble complex is probably made of an agar network containing globular proteins entrapped within the agar gel matrix. Similar behaviour is described by Burova et al. (2007) for carrageenan/casein complexes. These authors propose that carrageenan random coil-double helix transitions occur with similar mechanisms in the presence or in the absence of casein. However, in the presence of casein there is a slight reduction of carrageenan helicity without changing the helix stability, with the inclusion of protein monomers in the helical structure. They also refer that transition temperature is not affected by protein content, as happens in the case of WPI/agar complexes.

In the presence of citrate buffer (Fig. 5C and D), coacervates present a much coarser and less specific network, with big aggregates "attached" to one another, and some agar bridges every one and then. The presence of citrate seems to impair agar gelation. In fact, heat solubilised agar in 10 mmol dm^{-3} citrate buffer formed only a weak gel after cooling to room temperature and in 20 or

50 mmol dm^{-3} formed no gel at all (results not shown). Though agar-based structure zones are still detectable in the complex, protein–protein aggregation seems to be much more significant. Citrate is a trivalent acid with one extra hydroxyl group at the main backbone. It has, thus, several hydrogen bonding sites that can compete/bond with agar, thus interfering with the gelling mechanism. Normally, complexes from polyelectrolytes with long flexible chains and/or low charge density, and/or dissolved in high ionic strength media may stay in solution whereas for other systems there are no stable soluble complexes formed (de Vries & Stuart, 2006). Therefore, the presence of soluble complexes when no buffer or very dilute buffer is used can be another evidence for this: when no buffer is present, agar can be considered a long chain polysaccharide whereas in the presence of buffer the association of agar is impaired resulting in shorter chains. Though at pH 3 citrate is mainly present in the fully protonated form, multivalent anions can also be present. These can interact with the positive domains of whey proteins and this fact can be responsible for the change in the equivalent ratio from ca. 0.6, when no citrate buffer is used, to ca. 0.3 in the presence of citrate. Also, citrate can bridge between proteins and be responsible for the formation of big aggregates, probably composed by agar-protein complexes, where proteins are already in a highly aggregated form. Unlike the case of the absence of buffer, here proteins participate actively in defining the final solid structure. Besides the electrostatical attractive forces between agar and whey proteins, other physical bonds between protein molecules also influence the structure of the complexes, such as hydrogen binding or hydrophobic interactions. A more deep insight with a more amplified scale is needed to assess the structure inside these big aggregates.

4. Conclusion

Although agar is only a slightly charged polymer, the formation of insoluble complexes between whey proteins and agar molecules is possible, with dry yields higher than 18% (up to 46% when no buffering agent was used). These complexes have a “solid-like” behaviour at temperatures below 35 °C. Coacervates’ behaviour with temperature depends on the presence or absence of citrate buffer, as it strongly influences their final composition.

When no buffer was used, resulting gels have similar properties to those of agar gels, but incorporate in their structure electrostatically bound proteins. This can be particularly interesting in protein delivering systems. Also, as complexes are not solubilised with pH or with temperature, the range of functional applications can be further broadened.

In the presence of citrate buffer, proteins are determinant in the type of microstructure obtained. When this solid complex is heated and then re-cooled, crosslinking of proteins through hydrophobic interactions highly reinforces the final structure.

These results indicate that protein–sulphated polysaccharide interactions can be tuned aiming at optimal functional properties upon application in different processing conditions or food products.

Acknowledgments

This work was supported by the European Union (FEDER funds through COMPETE) and National funds (Fundação para a Ciência e Tecnologia, FCT) through project PEst-C-EQB-LA0006/2013 and postdoctoral grant to Hileia Souza (SFRH/BPD/37514/2007). The work also received financial support from QREN (European Union, FEDER funds) through Project NORTE-07-0124-FEDER-000069 and from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES; Brazil). The authors thank Prof. Maria do Carmo Pereira and Joana Loureiro for the use of the Zetasizer.

References

Aberkane, L., Jasniewski, J., Gaiani, C., Hussain, R., Scher, J., & Sanchez, C. (2012). Structuration mechanism of beta-lactoglobulin – Acacia gum assemblies in presence of quercetin. *Food Hydrocolloids*, *29*, 9–20.

Aberkane, L., Jasniewski, J., Gaiani, C., Scher, J., & Sanchez, C. (2010). Thermodynamic characterization of acacia gum-beta-lactoglobulin complex coacervation. *Langmuir*, *26*, 12523–12533.

Bedie, G. K., Turgeon, S. L., & Makhlouf, J. (2008). Formation of native whey protein isolate-low methoxyl pectin complexes as a matrix for hydrosoluble food ingredient entrapment in acidic foods. *Food Hydrocolloids*, *22*, 836–844.

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

Boral, S., & Bohidar, H. B. (2010). Effect of Ionic strength on surface-selective patch binding-induced phase separation and coacervation in similarly charged gelatin-agar molecular systems. *Journal of Physical Chemistry B*, *114*, 12027–12035.

Burgess, D. J., Kwok, K. K., & Megremis, P. T. (1991). Characterization of albumin-acacia complex coacervation. *Journal of Pharmacy and Pharmacology*, *43*, 232–236.

Burova, T. V., Grinberg, N. V., Grinberg, V. Y., Usov, A. I., Tolstoguzov, V. B., & de Kruif, C. G. (2007). Conformational changes in iota- and kappa-carrageenans induced by complex formation with bovine beta-casein. *Biomacromolecules*, *8*, 368–375.

Capitani, C. D., Pacheco, M. T. B., Gumerato, H. F., Vitali, A., & Schmidt, F. L. (2005). Milk whey protein recuperation by coacervation with polysaccharide. *Pesquisa Agropecuaria Brasileira*, *40*, 1123–1128.

Cooke, D., Gidley, M. J., & Hedges, N. D. (1996). Thermal properties of polysaccharides at low moisture. *Journal of Thermal Analysis*, *47*, 1485–1498.

Cooper, C. L., Dubin, P. L., Kayitmazer, A. B., & Turksen, S. (2005). Polyelectrolyte–protein complexes. *Current Opinion in Colloid & Interface Science*, *10*, 52–78.

de Kruif, C. G., & Tuinier, R. (2001). Polysaccharide protein interactions. *Food Hydrocolloids*, *15*, 555–563.

de Kruif, C. G., Weinbreck, F., & de Vries, R. (2004). Complex coacervation of proteins and anionic polysaccharides. *Current Opinion in Colloid & Interface Science*, *9*, 340–349.

de Vries, R., & Stuart, M. C. (2006). Theory and simulations of macroion complexation. *Current Opinion in Colloid & Interface Science*, *11*, 295–301.

de Wit, J. N. (1998). Nutritional and functional characteristics of whey proteins in food products. *Journal of Dairy Science*, *81*, 597–608.

Dickinson, E. (1998). Stability and rheological implications of electrostatic milk protein–polysaccharide interactions. *Trends in Food Science & Technology*, *9*, 347–354.

Dickinson, E., & Galazka, Y. B. (1991). Emulsion stabilization by ionic and covalent complexes of beta-lactoglobulin with polysaccharides. *Food Hydrocolloids*, *5*, 281–296.

Dickinson, E., & Izgi, E. (1996). Foam stabilization by protein–polysaccharide complexes. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, *113*, 191–201.

Doucet, D., Gauthier, S. F., & Foegeding, E. A. (2001). Rheological characterization of a gel formed during extensive enzymatic hydrolysis. *Journal of Food Science*, *66*, 711–715.

Doublier, J. L., Garnier, C., Renard, D., & Sanchez, C. (2000). Protein–polysaccharide interactions. *Current Opinion in Colloid & Interface Science*, *5*, 202–214.

Eleya, M. M. O., & Turgeon, S. L. (2000). The effects of pH on the rheology of beta-lactoglobulin/kappa-carrageenan mixed gels. *Food Hydrocolloids*, *14*, 245–251.

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

Gentes, M.-C., St-Gelais, D., & Turgeon, S. L. (2010). Stabilization of whey protein isolate-pectin complexes by heat. *Journal of Agricultural and Food Chemistry*, *58*, 7051–7058.

Gurov, A. N., & Nuss, P. V. (1986). Protein–polysaccharide complexes as surfactants. *Nahrung-Food*, *30*, 349–353.

Hidalgo, J., & Hansen, P. M. T. (1971). Selective precipitation of whey proteins with carboxymethylcellulose. *Journal of Dairy Science*, *54*, 1270.

Imeson, A. P., Ledward, D. A., & Mitchell, J. R. (1977). Nature of interaction between some anionic polysaccharides and proteins. *Journal of the Science of Food and Agriculture*, *28*, 661–668.

Jones, O. G., Adamcik, J., Handschin, S., Bolisetty, S., & Mezzenga, R. (2010). Fibrillation of β-lactoglobulin at low pH in the presence of a complexing anionic polysaccharide. *Langmuir*, *26*, 17449–17458.

Jones, O. G., Handschin, S., Adamcik, J., Harnau, L., Bolisetty, S., & Mezzenga, R. (2011). Complexation of beta-lactoglobulin fibrils and sulfated polysaccharides. *Biomacromolecules*, *12*, 3056–3065.

Klassen, D. R., Elmer, C. M., & Nickerson, M. T. (2011). Associative phase separation involving canola protein isolate with both sulphated and carboxylated polysaccharides. *Food Chemistry*, *126*, 1094–1101.

Laneuville, S. I., Paquin, P., & Turgeon, S. L. (2000). Effect of preparation conditions on the characteristics of whey protein - xanthan gum complexes. *Food Hydrocolloids*, *14*, 305–314.

Mendanha, D. V., Molina Ortiz, S. E., Favaro-Trindade, C. S., Mauri, A., Monterrey-Quintero, E. S., & Thomazini, M. (2009). Microencapsulation of casein hydrolysate by complex coacervation with SPI/pectin. *Food Research International*, *42*, 1099–1104.

Onder, E., Sarier, N., & Cimen, E. (2008). Encapsulation of phase change materials by complex coacervation to improve thermal performances of woven fabrics. *Thermochimica Acta*, *467*, 63–72.

Ould Eleya, M. M., Ko, S., & Gunasekaran, S. (2004). Scaling and fractal analysis of viscoelastic properties of heat-induced protein gels. *Food Hydrocolloids*, *18*, 315–323.

Rocha, C., Teixeira, J. A., Hilliou, L., Sampaio, P., & Goncalves, M. P. (2009). Rheological and structural characterization of gels from whey protein hydrolysates/locust bean gum mixed systems. *Food Hydrocolloids*, *23*, 1734–1745.

Schmitt, C., Sanchez, C., Desobry-Banon, S., & Hardy, J. (1998). Structure and technofunctional properties of protein–polysaccharide complexes: A review. *Critical Reviews in Food Science and Nutrition*, *38*, 689–753.

Schmitt, C., & Turgeon, S. L. (2011). Protein/polysaccharide complexes and coacervates in food systems. *Advances in Colloid and Interface Science*, *167*, 63–70.

Singh, S. S., Aswal, V. K., & Bohidar, H. B. (2007). Structural studies of agar-gelatin complex coacervates by small angle neutron scattering, rheology and differential scanning calorimetry. *International Journal of Biological Macromolecules*, *41*, 301–307.

Singh, S. S., Aswal, V. K., & Bohidar, H. B. (2011). Internal structures of agar-gelatin co-hydrogels by light scattering, small-angle neutron scattering and rheology. *European Physical Journal E*, *34*.

Souza, H. K. S., Sousa, A. M. M., Gómez, J., & Gonçalves, M. P. (2012). Complexation of WPI and microwave-assisted extracted agars with different physicochemical properties. *Carbohydrate Polymers*, *89*, 1073–1080.

Stone, A. K., & Nickerson, M. T. (2012). Formation and functionality of whey protein isolate-(kappa-, iota- and lambda-type) carrageenan electrostatic complexes. *Food Hydrocolloids*, *27*, 271–277.

Syrbe, A., Bauer, W. J., & Klostermeyer, N. (1998). Polymer science concepts in dairy systems – An overview of milk protein and food hydrocolloid interaction. *International Dairy Journal*, *8*, 179–193.

Tolstoguzov, V. B. (1991). Functional properties of food proteins and role of protein–polysaccharide interaction. *Food Hydrocolloids*, *4*, 429–468.

Turgeon, S. L., & Beaulieu, M. (2001). Improvement and modification of whey protein gel texture using polysaccharides. *Food Hydrocolloids*, *15*, 583–591.

Turgeon, S. L., Schmitt, C., & Sanchez, C. (2007). Protein–polysaccharide complexes and coacervates. *Current Opinion in Colloid & Interface Science*, *12*, 166–178.

Verheul, M., Roefs, S., & de Kruif, K. G. (1998). Kinetics of heat-induced aggregation of beta-lactoglobulin. *Journal of Agricultural and Food Chemistry*, *46*, 896–903.

Wang, X., Wang, Y.-W., Ruengruglikit, C., & Huang, Q. (2007). Effects of saft concentration on formation and dissociation of beta-lactoglobulin/pectin complexes. *Journal of Agricultural and Food Chemistry*, *55*, 10432–10436.

Weinbreck, F., Nieuwenhuijse, H., Robijn, G. W., & de Kruif, C. G. (2004). Complexation of whey proteins with carrageenan. *Journal of Agricultural and Food Chemistry*, *52*, 3550–3555.

Weinbreck, F., Tromp, R. H., & de Kruif, C. G. (2004). Composition and structure of whey protein/gum arabic coacervates. *Biomacromolecules*, *5*, 1437–1445.