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Effect of high methoxyl pectin on pea protein in aqueous solution and at oil/water interface

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ABSTRACT

The effect of the addition of high methoxyl pectin on the stability of pea protein isolate emulsions was investigated. Except for low pectin concentrations at acidic pHs where bridging flocculation occurred the addition of pectin improved emulsion stability to pH changes and depletion flocculation induced by maltodextrin addition. The mechanism of pectin induced stability was probed by measuring protein–pectin complex formation in solution, zeta potential of the emulsions droplets and the change in surface viscoelasticity on pectin addition. The phase diagrams of pectin–pea protein isolate in solution and pectin–pea protein-stabilized emulsions were established based on the obtained experimental results. These diagrams showed that under acidic conditions and at low pectin concentrations, electrostatic bridging phenomena leads to the formation of high size pectin–protein complexes causing an increase in turbidity in solution and oil droplet flocculation in emulsions. It was concluded that the pectin induced stability could be mainly assigned to steric repulsion and oil–water interfacial membrane rigidity improvement after pectin adsorption. In fact, the emulsions formed after pectin adsorption could consist of oil droplets surrounded by multilayer interfacial coatings, which are comprised of an inner interfacial protein film and an outer pectin layer.

1. Introduction

Aqueous mixtures of plant proteins and polysaccharides are susceptible to interact by one of two major alternative mechanisms: complex coacervation or thermodynamic incompatibility (Tolstoguzov, 2003). When attractive forces are strong, complexation results, whereby the protein–polysaccharide complexes can be soluble or can lead to aggregative phase separation. On the other hand, thermodynamic incompatibility takes place if both the protein and the polysaccharide are uncharged or similarly charged so that there is a relatively strong steric or electrostatic repulsion between them. The polysaccharide complex formation could be used to improve plant protein functionality. For example, it has been shown that complex formation of seed globulins and anionic polysaccharides improves the globulin solubility in the vicinity of their isoelectric points (PI) and at lower pH values (Braudo, Plashchina, & Schwenke, 2001). The interactions between other proteins and hydrocolloids in aqueous solutions have been deeply investigated (Benichou, Aserin, & Garti, 2002; Dickinson, 2008; Larichev, Gurov, & Tolstoguzov, 1983; Lutz, Aserin, Portnoy, Gottlieb, & Garti, 2009; Tolstoguzov, 2003). Under certain conditions (such as pH, ionic strength, and protein/hydrocolloid ratio) interactions between proteins and negatively charged biopolymers, even at pH above the protein PI, can form charged complexes that own good functional properties (Lutz et al., 2009). It has been demonstrated, for example, that whey protein isolate (WPI) is capable of forming water-soluble complexes with certain hydrocolloids (xanthan, and guar and locust bean gums) (Benichou, Aserin, & Garti, 2007). The thermodynamic aspects of protein–polysaccharide complex formation as well as their structural and functional properties were recently reviewed (Turgeon, Schmitt, & Sanchez, 2007).

It is also possible to use the understanding of these protein–polysaccharide interactions to improve functional properties in colloidal systems, such as suspensions, emulsions or foams. Particularly, protein-stabilized emulsions are sensitive to environmental conditions such as pH and ionic strength and will tend to flocculate at pH values close to the PI of the adsorbed proteins and when the ionic strength exceeds a certain level. Droplet flocculation is usually prevented in emulsions stabilized by adsorbed proteins due to the relatively strong electrostatic repulsion between the charged droplets at pH values far from the PI. For their part, and despite they are less surface active, polysaccharides, such as pectin, gum arabic and modified starch, produced emulsions that are often more resistant to changes in pH, ionic strength, and high temperatures than those stabilized by proteins (McClements, 2004). This was attributed to the fact that the interfacial polysaccharide membrane is more porous and thicker

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than that made by proteins, which increases the steric repulsion and reduces the van-der-Waals attraction between oil droplets (Dickinson, 2003). However, in order to increase their effectiveness at producing stable emulsions, polysaccharides which act as surface active material through their hydrophobic residues, must be used in concentrations much higher than proteins. One wise idea that could help to produce emulsions with good environmental stability consists of combining the positive attributes of proteins and polysaccharides by using protein–polysaccharide complexes as emulsifier. The resulting multilayer emulsions consist in oil droplets coated by protein–polysaccharide interfacial complexes (Dickinson, Semenova, Antipova, & Pelan, 1998). These changes in interfacial characteristics alter the sign, magnitude, and range of the colloidal interactions acting between the droplets (Guzey & McClements, 2006a) as well as altering the susceptibility of the interfacial membrane to rupture. The stabilization of O/W emulsions by a mixture of proteins and hydrocolloids has long been used to improve emulsion stability (Cornec et al., 1998; KobersteinHajda & Dickinson, 1996). Guzey and McClements (2006b) have shown that under certain conditions, emulsions containing oil droplets surrounded by multilayered interfacial membranes have been found to have better stability to environmental stresses than conventional oil/water emulsions with single-layered interfacial membranes. To stabilize emulsions by protein–polysaccharide complexes, two methods were often used: (i) emulsification of oil in an aqueous complex dispersion, or (ii) emulsification of oil with an aqueous dispersion of one biopolymer, followed by adsorption of the second biopolymer at bulk conditions (mainly pH) allowing complex formation. When the latter method is used, charged polysaccharides have been shown to interact, under specific conditions, via electrostatic forces with proteins adsorbed at oil–water interfaces (Dickinson, 2008; McClements, 2006). Moreover, interfacial biopolymer association could be used to create emulsions with novel functional properties such as encapsulation or controlled release of lipophilic food and drug components (McClements, Decker, & Weiss, 2007).

Particularly, pea proteins may be used to stabilize oil-in-water emulsions because of the surface properties of their constitutive protein units: the storage globulins 7S (vicilin) and 11S (legumin) (Guéguen, 1989). It has been shown recently that pea proteins are able to decrease the interfacial tension between the water and oil and help to stabilize emulsions by forming a rigid membrane at the oil–water interface (Ducel, Richard, Popineau, & Boury, 2004a; Ducel, Richard, Saulnier, Popineau, Boury, 2004b). However, environmental factors such as pH, ionic strength, or the presence of other ingredients will affect the stabilizing behavior of pea proteins in food emulsions (Guéguen, Chevalier, Barbot, & Schaeffer, 1988). Recently, Ducel, Saulnier, Richard, and Boury (2005) used the soft particle analysis to show that pea globulins have a spherical shape and high positive charge density at low pH. In these conditions, they have demonstrated that a relatively poorly charged polysaccharide having a rod-like structure is more convenient to allow a good overlapping of the charges of the two polymers than a strongly charged and spherical polysaccharide. Thus, high methoxyl pectin was selected in this work as a potential stabilizer of PPI-stabilized emulsions because it is a linear anionic polyelectrolyte (Thakur, Rakesh, & Handa, 1997) usually used as a thickening agent in food products. Moreover, it has been shown recently that pectin can strongly interact with interfacial adsorbed proteins at different physico-chemical conditions. Thus, it has been demonstrated that, at pH values below their PI, soy proteins (Lam, Shen, Paulsen, & Corredig, 2007) and whey proteins (Zaleska, Ring, & Tomasik, 2000) are able to form complexes with pectin chains. The presence of high methoxyl pectin in the aqueous phase during the formation of emulsions stabilized by caseins leads to some incorporation of polysaccharide into the interfacial stabilizing oil/water film at pH 5.5, but not at pH 7 (Dickinson et al., 1998). In the same way, by using diffusing wave spectroscopy, it has been shown that high methoxyl pectin is able to adsorb onto the sodium caseinate stabilized oil droplet under acidification conditions (Bonnet, Corredig, & Alexander, 2005). This adsorption was shown to allow, in the studied concentrated systems, to changes in oil droplet size and to emulsion stabilization by forming a network of oil droplets loosely connected by strands of pectin. Interfacial protein–polysaccharide complex formation (Dickinson, 2003), as well as properties of multilayer emulsions (Guzey & McClements, 2006a), were recently well reviewed. Particularly, more details about emulsions stabilized by proteins, hydrocolloids, and their combination can be found in a recent review (Dickinson, 2009).

The purpose of the present work is to understand the origin of high methoxyl pectin–pea protein interactions in aqueous solutions and emulsions. Therefore, the behavior of pectin–PPI mixtures and their emulsion-stabilizing properties were evaluated in buffer solutions and in 5 wt.% oil-in-water emulsions, respectively. Changes caused by the addition of pectin to aqueous solutions and oil-in-water emulsions were studied in details at two pH values: below (pH 2.4) and above (pH 7) the isoelectric point of pea globulins (∼4.3). However, pectin-PPI interaction diagrams were constructed for a larger pH range from 2.4 to 8. In order to provide a deeper insight into the consequences of pectin–pea protein interactions allowing to stabilization of oil–water interfaces at low pH values, drop tensiometer measurements are used to understand the effect of these interactions on the mechanical properties of the oil–water interface.

2. Materials and methods

2.1. Materials

Powdered pea protein isolate (PPI) and maltodextrins having different dextrose equivalence (DE) (6, 12, 19 and 28) were obtained from Roquette-frères SA, (Lestrem, France). As measured by Kjeldahl method, the total protein content of the powder was 91.7 wt.% (dry matter basis), with globulin fraction up to 95% as stated by the manufacturer. Miglyol 812 Neutraloel, a medium chain triglyceride oil was obtained from Sasol (Germany GmbH). Analytical grade imidazole ($C_3H_4N_2$), acetic acid (CH_3COOH), sodium azide (NaN_3), sodium hydroxide (NaOH), hydrochloric acid (HCl), high methoxy pectin (degree of esterification ∼60) was purchased from Sigma Chemical Co (Germany). Deionized water was used for the preparation of all solutions and emulsions.

2.2. Complex solution preparation

A stock imidazole-acetate buffer solutions (5 mM) containing 0.04 wt.% NaN_3 (used as an antimicrobial) were prepared at different pH values. Biopolymer stock solutions were prepared by dissolving either 0.5 wt.% PPI or 0.5 wt.% pectin in the stock buffer solutions and stirring for at least 6 h to ensure complete hydration. The solutions were first centrifuged (model Sorvall® Rc6-Plus, Thermo electron Corp.) at 4000*g*-15 min for PPI solutions and 10,000*g*-30 min for pectin ones to remove any insoluble particles and then only the supernatants were used. Biopolymer mixtures containing PPI (0.25 wt.%) and pectin (0–0.0875 wt.%) were prepared by mixing different ratios of the stock solutions with buffer at the desired pH value. The pH of each of the solutions was adjusted to the appropriate value prior to mixing. The resulting solutions were mixed for 1 min using a vortex mixer, adjusted again to the desired pH value, and then stored at room temperature for 24 h prior to analysis.

2.3. Emulsion preparation

An aqueous emulsifier solution containing 0.555 wt.% PPI was prepared by dispersing powdered pea protein isolate into imidaz-

ole-acetate buffer (5 mM) containing 0.044 wt.% NaN_3 (as an antimicrobial agent) and adjusted at the desired pH. This protein solution was then stirred for at least 6 h to ensure complete hydration of the protein, and the pH was adjusted if necessary. 10 wt.% Miglyol was then blended with 90 wt.% aqueous emulsifier solution using an Ultra-Turrax T25 high-speed blender (IKA, Staufen, Germany) operated at 17,500 rpm for 90 s. The resultant pre-emulsion (10 wt.% oil, 0.5 wt.% protein isolate, and 0.04 wt.% NaN_3) was further homogenized at 500 bar with three recirculations using a high pressure homogenizer (Niro Soavi NS 1001 L, Parma Italy). Imidazole-acetate buffer (5 mM) and pectin solution (1 wt.%, imidazole-acetate buffer (5 mM)) were slowly added to the obtained emulsions at the same pH value to give emulsions (5 wt.% oil, 0.25 wt.% protein, 0.02 wt.% NaN_3) containing 0–0.25 wt.% pectin. For stability studies, imidazole-acetate buffer (5 mM, pH 2.4) and pectin solution (1 wt.%, in imidazole-acetate buffer (5 mM, pH 2.4)) were slowly added to the obtained emulsions to give primary (5 wt.% oil, 0.25 wt.% protein, 0.02 wt.% NaN_3, pH 2.4) and secondary (5 wt.% oil, 0.25 wt.% protein, 0.2 wt.% pectin, 0.02 wt.% NaN_3, pH 2.4) emulsions. To study the ζ-potential and creaming stability of primary and secondary emulsions as a function of pH changes, the pH was gradually adjusted to 3, 4, 5, 6, 7 and 8 using NaOH solution (0.1 M). The resulting emulsions were slowly stirred for 1 min, the pH was adjusted, and then stored at room temperature for 24 h prior to analysis.

2.4. Particle size measurement

Emulsion particle size distributions were measured by a laser diffraction instrument (Malvern Mastersizer S, Malvern Instruments, Worcs., UK). To avoid droplet flocculation, the emulsions were diluted with imidazole-acetate buffer (5 mM) at the suitable pH prior to making the measurements. The emulsions were stirred continuously throughout the measurement to ensure the samples were homogeneous. The volume particle diameter (d_{43}) was calculated from three injections of three separate samples with two readings per sample:

$$d_{43} = \sum n_i d_i^4 / \sum n_i d_i^3 \quad (1)$$

where n_i is the number of droplets of diameter d_i.

2.5. Zeta potential measurement

The electrophoretic mobility of oil droplets or pectin–PPI complexes was measured using a particle electrophoresis instrument (Zetacompact, CAD Instruments, France). The ζ-potential is determined by measuring the direction and velocity of particle movement in the applied electric field. The emulsions and complex solutions were diluted to a concentration of approximately 0.015 wt.% with imidazole-acetate buffer 5 mM adjusted to the suitable pH prior to measurements. The diluted solutions and emulsions were mixed thoroughly and then injected into the measurement chamber of the particle electrophoresis instrument. The ζ-potential measurements are reported as the average and standard deviation of measurements made on three freshly prepared samples, with three readings made per sample.

2.6. Turbidity measurements

The turbidity of complex solutions or emulsion serum contained in 10 mm path-length optical cells was measured using an UV–visible spectrophotometer (V-530, JASCO) at 600 nm and room temperature against imidazole-acetate buffer 5 mM adjusted to the suitable pH.

2.7. Microscopical analysis

A Nikon TE-2000 U microscope was used in the fluorescence mode to examine the microstructure of the samples. The proteins were stained with Nile Blue (12.5 μl of 1 wt.% dye solution added to emulsion or pectin-PPI solution to obtain a dye-protein ratio of 1–100). Observations were performed at least 30 min after diffusion of the dye into the matrix. Emulsions and pectin-PPI solutions were poured between a slide and a coverslip and then observed by a 100×-oil immersion objective lens. Samples were excited at 630 nm. Fluorescence intensity data were collected in a channel corresponding to 610–660 nm. Digital image files were acquired in .tif format and in 512 × 512 pixel resolution with a CRI Nuance camera connected to a Digital image processing system (Lucia).

2.8. Interfacial properties

An automated drop volume tensiometer (Tracker, IT Concept, Longessaigne, France) was used in a dynamic mode for measuring interfacial tensions and viscoelastic modulus at the oil–water interface at 20 ± 0.1 °C. In those measurements, the aqueous phases were PPI solution adjusted at suitable pH value and containing 0.04 wt.% PPI. This protein concentration was experimentally determined (data not shown) to make the oil–water interface fully covered and only a very small amount of protein remained in the bulk phase. The oily phase was Mygliol 812 N. During the experiment, the aqueous protein solutions were continuously agitated with a magnetic stirrer. After 10,000 s, 100 μl of pectin solution supernatant (0.1 wt.%) were delicately added to the protein solution without interrupting the measurement. Throughout the experiment, we measured the surface dilatational storage modulus (elastic component), ε', and the surface dilatational loss modulus (viscous component), ε'', by subjecting the oil–water interface to sinusoidal area deformation (10% of drop volume). It is thus possible to determine both the absolute value of the complex modulus, ε, the phase angle θ between changes in the interfacial tension and changes in interfacial area. More details about the used method have been previously published (Cases, Rampini, & Cayot, 2005).

2.9. Critical flocculation concentration (CFC) determination

The lowest unadsorbed biopolymer concentration required to cause depletion flocculation is referred to as the critical flocculation concentration (CFC), which depends on the dimensions of the biopolymer molecules and the size of the emulsion droplets. We therefore examined the trend of pectin and maltodextrins having various DE to promote depletion flocculation in primary (5 wt.% oil, 0.25 wt.% PPI, 0.02 wt.% NaN_3) and secondary (5 wt.% oil, 0.25 wt.% PPI, 0.2 wt.% pectin, 0.02 wt.% NaN_3) emulsions. The CFC of pectin were determined precisely by measuring turbidity on the serum layer of the creamed emulsions after 24 h storage. Fifty microliter of liquid was withdrawn from the middle of the serum layer of a creamed emulsion and then diluted with buffer solution at the suitable pH to five milliliter. The transmittance of the serum layer was then measured at 600 nm (Klinkesorn, Sophanodora, Chinachoti, & McClements, 2004) as mentioned above. The CFC was defined in this work as the lowest concentration of pectin that gave a transmittance less than that of a non-creamed 4 wt.% oil-in-water emulsion (prepared by diluting the emulsion before polymer addition). The CFC defined in this work corresponds to pectin or maltodextrin concentration where at least 20% of the droplets in the original emulsion had flocculated and creamed to the top.

2.10. Stability measurements

Primary and secondary emulsions were transferred into a cylindrical glass test tube (internal diameter 15 mm, height 180 mm) until an emulsion height of 135 mm, tightly sealed with a plastic cap, and then stored at room temperature. The extent of creaming was characterized by a creaming index (CI) that represents the serum layer formed at the bottom of the tubes expressed as a percentage of the total volume of emulsion in the tube. The creaming index provided indirect information about the extent of droplet aggregation in an emulsion: the higher the creaming index, the greater the aggregation. Measurements were carried out on three separate samples (replicates) and reported as the mean and standard deviation.

2.11. Statistical analysis

All experiments were performed using at least three freshly prepared samples. The results presented are the averages and standard deviations that were calculated from these replicate measurements. Statistical differences between samples were calculated using Student's *t* test for independent samples (Microsoft Excel, Microsoft Corporation, Redmond, WA).

3. Results and discussion

3.1. Pectin–pea protein interactions in solution

Aqueous solutions were prepared containing 0.25 wt.% of PPI and different concentrations of pectin. The solutions were left to settle overnight and the formation of the complexes was assessed by turbidity measurement and classified as very turbid solution (turbidity > 0.5 cm^{-1}), turbid solution (0.5 cm^{-1} > turbidity > 0.2 cm^{-1}), or clear solution (turbidity < 0.2 cm^{-1}). From these measurements, a phase diagram of pectin concentration against pH was constructed as shown in Fig. 1. Moreover, in Fig. 1, the curve giving the electric equivalence points (EEP) (ζ-potential of the pectin-PPI mixtures ∼0 mV) at different pH values was plotted and consequently, the phase diagram shows two distinct regions: a positively charged particle region below the EEP line and a negatively charged particle region above the EEP line.

At pH values above 6, clear solutions were obtained and, as the pH was gradually lowered to values below 6 the mixtures became turbid and then very turbid with precipitate formation. A high turbid solutions containing solid precipitates were observed both (i) at low pectin concentrations (<0.05 wt.%) and low pH (2.4, 3 and 4) and (ii) at pH 3 and pH 4 at high pectin concentrations (⩾0.075 wt.%). The formation of these precipitates can be explained by three different mechanisms. (i) at pH 4 and low pectin concentrations, the overall net charge on the protein molecules is low, the precipitate can be attributed to the protein aggregation at pH values near the protein's PI (∼4.3). (ii) at pH 2.4 and 3, the precipitate and high solution turbidity can be attributed to the formation of insoluble pectin–protein complexes, due to strong interactions between the two oppositely charged biopolymers near the EEP curve. It can therefore be assumed that the low charged pectin molecules could form bridges between highly charged protein ones in this diagram region. (iii) at pH 3 and 4 and high pectin concentrations, turbidity reached very high values indicating that extensive interaction between the two biopolymers took place because of the low protein positive charge at pH values close to the pk_a of pectin (∼3.5) (Thakur et al., 1997). We can therefore suppose, in this case, that the low charged protein molecules could form bridges between highly charged pectin ones at high pectin concentrations. Moreover, the results given by this phase diagram could be also interpreted in relation to pea protein globulin structure. In fact, it has been shown that pea legumin exhibits a hexameric structure (11S type) which is maintained only in the pH range from 7 to 9. Decreasing the pH leads to a dissociation with a mixture of intermediary conformations at pH 3.4 and a complete dissociation leading to 3S components at pH 2.4 (Guéguen et al., 1988). This dissociation is able to modify the protein surface hydrophobicity (Guéguen, 1989) and probably the surface charge distribution, leading to make interactions with pectin possible even at pH values above the PI (turbid solutions obtained at pH 5 and 6) (Turgeon et al., 2007).

In the following section, these pectin–protein interactions will be detailed in two different conditions: below (pH 2.4) and above (pH 7) the PI of pea globulins (∼4.3). Whereas both the PPI and pectin solutions were clear to slightly hazy, a largely increased turbidity could be visually observed in some biopolymer mixtures (Fig. 2). The high initial turbidity of about 0.75 cm^{-1} at pH 2.4, when pectin concentration was 0.0125 wt.%, denotes the presence of a large amount of dispersed high size pectin-PPI complexes as was confirmed by microscopy. This observed high size complex formation is in agreement with the previously evocated hypothesis of the formation of pectin bridges between protein molecules at low pH and low pectin concentrations. As can be derived from the drop of the turbidity value to reach a plateau of about 0.5 cm^{-1} when pectin concentration reaches 0.05 wt.% at pH 2.4, the particulate pectin-PPI complexes were gradually dissociated to give smaller complexes having a pectin concentration independent size. Both the high initial turbidity and the decrease in this parameter upon more pectin addition clearly indicate that at pH 2.4, where protein and pectin are oppositely charged, large electrostatically associated biopolymer aggregates were formed at all pectin to protein ratios.

To monitor how the net charge of formed complexes changes upon titration with pectin, the ζ-potential of the complexes was measured as a function of pectin concentration. At pH 2.4, the ζ-potential of the formed complexes (Fig. 3) changed from positive to negative as the pectin concentration was increased, indicating that the anionic polysaccharide molecules adsorbed to the cationic protein molecules through electrostatic attraction. The negative charge of the complexes reached a plateau value at a pectin concentration of approximately 0.0375 wt.%, which indicated that the protein molecules were "saturated" with pectin chains around this concentration (Harnsilawat, Pongsawatmanit, & McClements, 2006). It is important to note that the pectin concentration of 0.0125 wt.% giving the largest volume complexes at pH 2.4 (Fig. 2) is the closest ratio to the electric equivalence point at this pH (Fig. 3) leading to precipitation of insoluble pectin–protein complexes.

At pH 7 (Fig. 3), the ζ-potential of the mixture decreases from −15.6 mV (protein alone) to −18.6 mV at pectin concentration of 0.0875 wt.%, which is close the ζ-potential of pectin molecules alone at this pH. In addition, the turbidity slightly increased (Fig. 2) after pectin addition probably due to the increase of the total biopolymer concentration leading to the intensification of biopolymer self-association and no or only a limited amount of biopolymer complexes were formed. It is important to note that similar results were obtained in solutions containing only increased pectin concentrations (0 wt.% PPI) (data not shown).

It can be concluded from Figs. 1–3 that the increase of turbidity upon addition of pectin to protein together with a reduction of positive net charge indicates that protein molecules effectively bind pectin added to the solution at pH from 2.4 to 6 through mainly electrostatic interactions. Similar results have showed that formation of pea globulin–arabic gum complexes with a dense network are mainly due to electrostatic interactions but hydrogen bonds and hydrophobic interactions were shown to reinforce the packing of these complexes (Ducel, Pouliquen, Richard, & Boury, 2008).

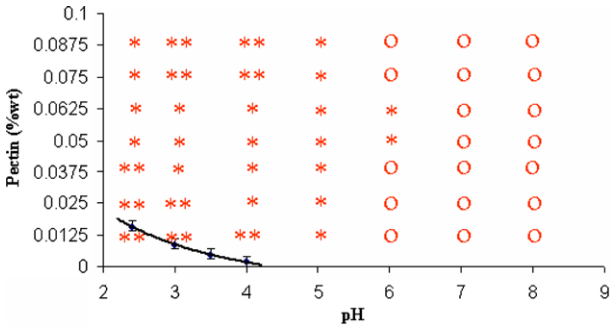

Fig. 1. Phase diagram in aqueous solution of the pea protein–pectin system, expressed in terms of the pH and the pectin concentration at a constant pea protein isolate concentration of 0.25 wt.% in 5 mM imidazole-acetate buffer. Key: (o) clear solution, (∗) turbid solution, (∗∗) very turbid solution. Line: electric equivalence points (ζ-potential ∼ 0 mV).

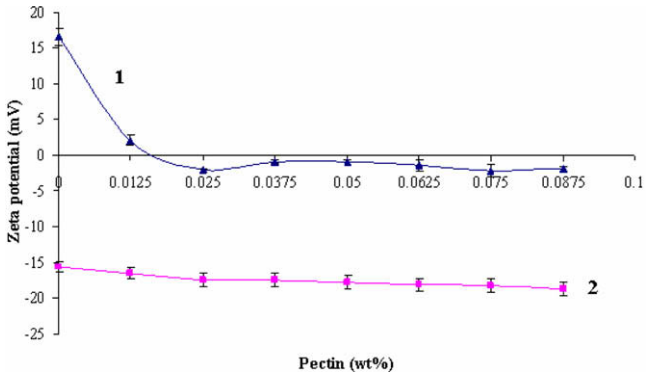

Fig. 3. Dependence of the ζ-potential of mixed biopolymer solutions on pectin concentration when 0.5 wt.% pectin was titrated into 0.5 wt.% pea protein isolate solution (5 mM imidazole-acetate buffer, pH 2.4 (1) and pH 7 (2)).

3.2. Pectin–pea protein interactions in emulsions

Oil-in-water emulsions were prepared containing 0.25 wt.% of PPI and different concentrations of pectin at pH ranging from 2.4 to 8 and a phase diagram of pectin concentration against pH was established as shown in Fig. 4. This phase diagram shows seven distinct regions: (1) at low pH and low pectin concentrations, negatively charged pectin molecules can interact with positively charged protein-stabilized oil droplets but there is no enough pectin to neutralize all protein positively charged groups. A single pectin molecule could adsorb to the surface of more than one emulsion droplet, thus acting as a polymeric link that promotes bridging flocculation (Guzey & McClements, 2006a); (2) above the EEP curve (curve a), the global electric charge of pectin–oil droplet complexes is negative but oil droplets are not completely saturated with pectin and bridging flocculation still occurs; (3) when pectin reached concentrations permitting to obtain monomodal particle size distribution (curve b), fine negatively charged pectin–protein complex coated oil droplets are obtained. Pectin concentration necessary to obtain stable multilayer emulsions decreased with increasing the pH mainly because of the decrease of the protein positive charge; (4) if pectin concentration exceeds certain levels (curve c), depletion flocculation caused by unadsorbed pectin molecules occurs. The amount of pectin able to induce depletion flocculation of pectin–protein complex stabilized emulsions was shown to slightly increase with increasing the pH (curve c) probably because of the increase of the adsorbed pectin negative charge when the pH increases. This could result in an increase in the repulsive forces between pectin coated oil droplets and consequently higher bulk pectin concentrations are needed to cause flocculation; (5) at pH values close to the protein PI (∼4.3), it was not possible to obtain protein-stabilized emulsions; (6, 7) at pH values above the protein PI, the unadsorbed anionic polysaccharide induced depletion flocculation of the negatively charged protein-coated droplets. In this latter case, the pectin CFC increases with increasing the pH (curve d) probably because of the increase of

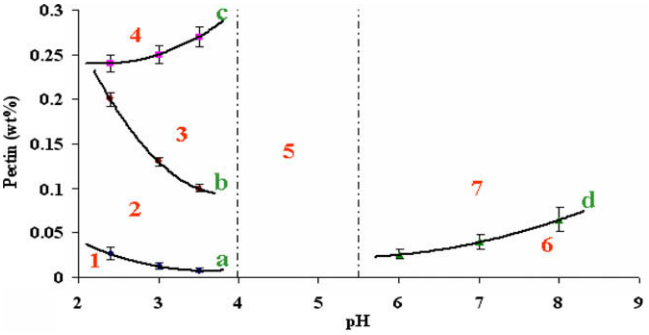

Fig. 4. Phase diagram in 5 wt.% oil-in-water emulsions stabilized by 0.25 wt.% PPI (5 mM imidazole-acetate buffer), expressed in terms of the pH and the pectin concentration. Key: (a) electric equivalence line (ζ-potential ∼ 0 mV), (b) end of bridging flocculation giving fine homogeneous secondary emulsions, (c) pectin CFC line of secondary emulsions, (d) pectin CFC line of primary emulsions. 1–7 (see text).

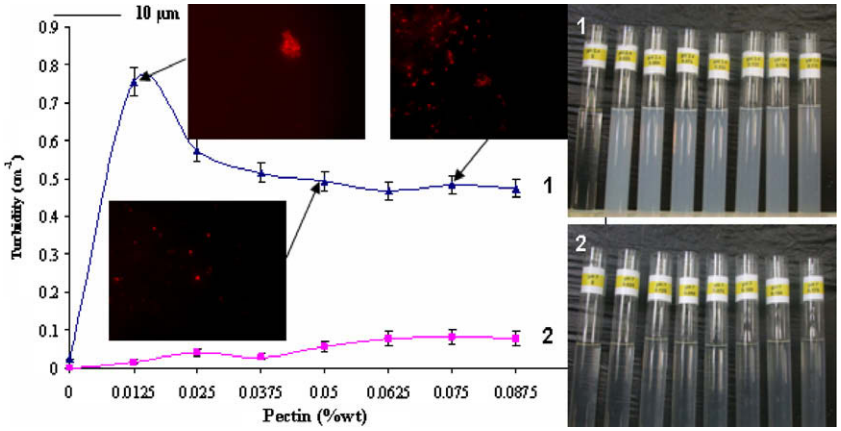

Fig. 2. Dependence of the turbidity (at 600 nm), visual, and microscopic aspects of mixed biopolymer solutions on pectin concentration when 0.5 wt.% pectin was titrated into 0.5 wt.% pea protein isolate solution (5 mM imidazole-acetate buffer, pH 2.4 (1) and pH 7 (2)).

protein negative charge when pH is increased. Moreover, at pH 6, flocculation occurs at relatively low pectin concentration because probably of an added bridging effect due to possible pectin–PPI interactions at this pH as it was suggested for aqueous mixtures of pectin and PPI in this range of pH (Fig. 1). Specifically, the behavior of emulsions at pHs 2.4 and 7 will be detailed in the paragraphs that follow.

At pH 2.4, emulsion stability decreased rapidly (serum separation) for pectin concentration ranged between 0.01 and 0.05 wt.% and then increased again (Fig. 5). A slight destabilization was also observed at high polysaccharide concentrations (~0.25 wt.%). However, at pH 7, as the concentration of pectin increased, the serum turbidity decreased gradually to reach a steady state (~2.5 cm^{-1}). The serum phase of pectin destabilized emulsions at pH 2.4 was clear compared to the turbid one of the destabilized emulsions at pH 7. This observation indicates the presence of individual polydisperse droplets in the serum phases of emulsions at pH 7, resulting from depletion flocculation phenomena, whereas at pH 2.4 the droplets can be linked into flocs as seen in the photomicrographs (Fig. 5). Indeed, at pH 2.4 and low pectin concentration, there was insufficient pectin present to completely saturate the droplet surfaces so bridging flocculation occurred. This type of bridging mechanism has been reported previously for protein-stabilized emulsions containing low concentration of ionic polysaccharides like carrageenan (Dickinson & Pawlowsky, 1997; Singh, Tamehana, Hemar, & Munro, 2003), dextran sulphate (Jourdain, Leser, Schmitt, Michel, & Dickinson, 2008) or pectin (Guzey, Kim, & McClements, 2004). The turbidity decrease at the highest pectin concentrations observed at pH 2.4 could be due to depletion flocculation phenomena.

In order to elucidate the causes for the observed differences in creaming stability, both the particle size distribution and the electrophoretic mobility of the emulsions were determined in emulsions containing different pectin concentrations (0–0.25 wt.%) after 24 h storage at pH 2.4 and pH 7 (Fig. 6). Comparing these results to the creaming profiles (Fig. 5), a strong correlation was found between creaming, particle size distribution, and ζ-potential.

At pH 2.4, the ζ-potential of the emulsion droplets changed from positive to negative as the pectin concentration was increased (Fig. 6), indicating that the anionic polysaccharide molecules adsorbed to the surface of the cationic protein-coated droplets through electrostatic attraction. The negative charge on the droplets reached a plateau value at a pectin concentration above 0.05 wt.%, which indicated that the oil–water interface charges were completely screened with polysaccharide around this concentration (Mun, Cho, Decker, & McClements, 2008). Fig. 6 shows also that the largest average diameter of oil droplets ($d_{43} \sim 15.5$ μm) was obtained at a pectin concentration close to the EEP at pH 2.4. Above 0.15 wt.% pectin, finer emulsions were formed ($d_{43} \sim 2.2$ μm). From the confrontation of Fig. 5 and Fig. 6, it follows that the reduced creaming tendency upon pectin addition coincided with a pronounced decrease in average particle size upon addition of at least 0.15 wt.% of pectin suggesting that individual oil droplets were saturated by polysaccharide chains at this pectin concentration. This stability of the pectin–protein complex coated oil droplets cannot be only attributed to electrostatic repulsions because the magnitude of the droplet ζ-potential is relatively low at pH 2.4 ($|\zeta| \sim 2.8$ mV), so that the electrostatic repulsion between the droplets could not be sufficient to stabilize secondary emulsion and we postulate that the two mainly stabilization mechanisms are: (i) a screening of some protein groups responsible to the attractive droplet–droplet interactions (e.g. van-der-Waals and hydrophobic attraction), and (ii) the thickness of the interfacial membranes that would have been greater in the pectin–protein-coated oil droplets than in the protein coated ones, which would have increased the steric repulsion between the droplets (Surh, Decker, & McClements, 2006).

At pH 7, the oil droplets have an initial negative ζ-potential (−35.3 mV) that increased gradually as pectin concentration was increased to reach a plateau value of −18 mV at polysaccharide concentration above 0.05 wt.% (Fig. 6). Due to the absence of bridging flocculation (Fig. 2) and the two separate negative ζ-potential families (data not shown) observed at pH 7, we can assume that pectin was not adsorbed to the droplet surfaces because of the relatively large electrostatic repulsion between the negatively charged droplets and negatively charged pectin molecules. As a result pectin would be expected to be present primarily in the aqueous phase of the emulsions at relatively high pH values in agreement with Moreau, Kim, Decker, and McClements (2003).

3.3. Stability of primary and secondary emulsions

3.3.1. Stability upon ageing

The purpose of these experiments was to examine the influence of the adsorbed pectin layer on the stability of protein-coated oil droplets as a function of time. Less droplet aggregation and less creaming were observed in the secondary emulsion than in the primary one (Fig. 7), which suggested that the adsorption of pectin to the surfaces of the protein-stabilized droplets increased their stability in this studied period (25 days). Ageing stability results indicate that the droplets in the secondary emulsion were highly stable to flocculation which may be due to the fact that the pectin–protein complex membrane is highly resistant to rupture and generates repulsive steric and electrostatic interactions between the

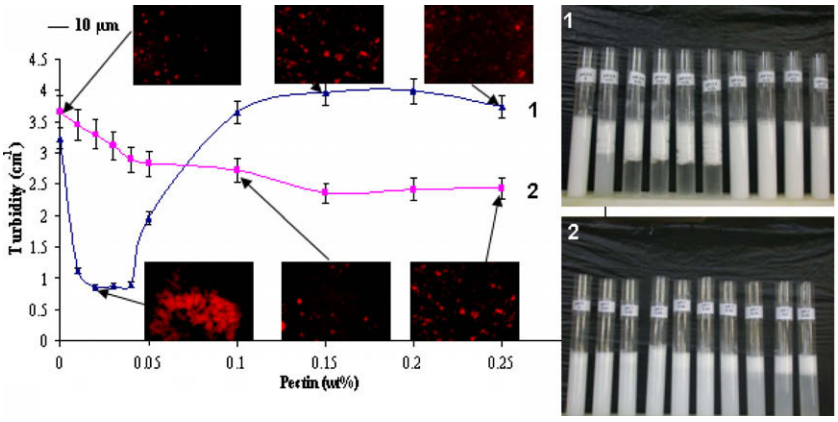

Fig. 5. Influence of pectin concentration (0–0.25 wt.%) on physical properties of 5 wt.% oil-in-water emulsions stabilized by 0.25 wt.% PPI (5 mM imidazole-acetate buffer, pH 2.4 (1) and pH 7 (2)) .

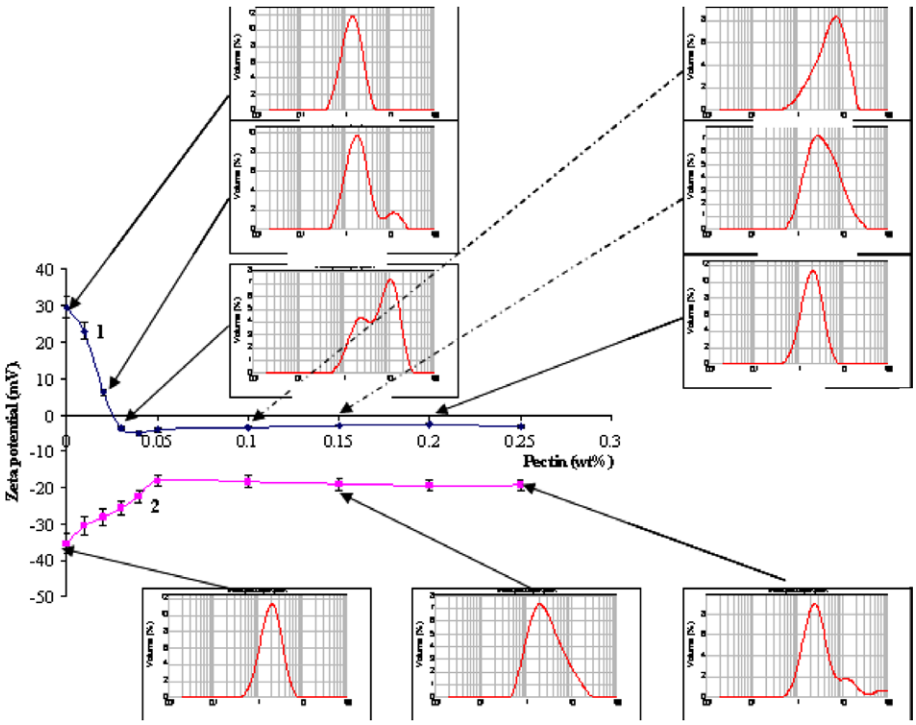

Fig. 6. Influence of pectin concentration (0–0.25 wt.%) on ζ-potential and particle size distribution of 5 wt.% oil-in-water emulsions stabilized by 0.25 wt.% PPI (5 mM imidazole-acetate buffer, pH 2.4 (1) and pH 7 (2)).

droplets. As shown above, steric repulsions seemed to be predominant at low pH values since the electrostatic charge of the multilayer stabilized droplets was simultaneously found to be relatively low. On one other hand, electrostatic repulsions could be considered as the major factor of emulsions stabilized by PPI alone at pH values far from the PI. In addition, hydrophobic attraction and disulfide bond formation between proteins adsorbed onto different droplets would be prevented in secondary emulsion because protein groups are screened by pectin chains.

3.3.2. Stability upon pH changes

In Fig. 8A the pH induced creaming was measured for primary and secondary emulsions after 24 h storage. The creaming measurements indicated that the emulsion prepared with PPI alone was unstable when the pH exceeded 3. This pH range is near the PI of pea globulins (∼4.3), and consequently, electrostatic repulsions become not strong enough (Fig. 8B) to overcome attractive interactions between droplets. At relatively high pH values, the ζ-potential is high enough to avoid droplet flocculation but primary emulsion remains unstable. In fact, in order to increase the emulsion pH from 2.4 to 6, 7 and 8 it was necessary to go through the PI of the adsorbed proteins. It can be therefore postulated that extensive droplet flocculation occurred at the PI range as the result of loss in protein solubility, which also promoted coalescence because the droplets were brought into close proximity to each other in agreement with Surh et al. (2006).

The secondary emulsion was stable to creaming and droplet aggregation over a large pH range (pH 2.4–6) (Fig. 8A) probably because of the relatively strong repulsions associated with the relatively thick and electrically charged double layer interfacial membrane. The ζ-potential in the secondary emulsions as a function of pH decreases as the same way as that of pectin alone (Fig. 8B). This behavior suggests that pectin has not detached completely from the surface of oil droplets even at pH values above the

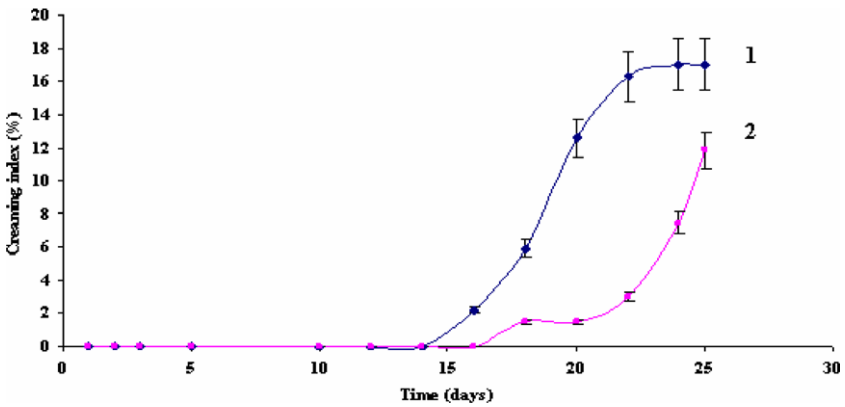

Fig. 7. Creaming stability of primary (5 wt.% oil, 0.25 wt.% PPI, 5 mM imidazole-acetate buffer) (1) and secondary (5 wt.% oil, 0.25 wt.% PPI, 0.2 wt.% pectin, 5 mM imidazole-acetate buffer) (2) emulsions as a function of time.

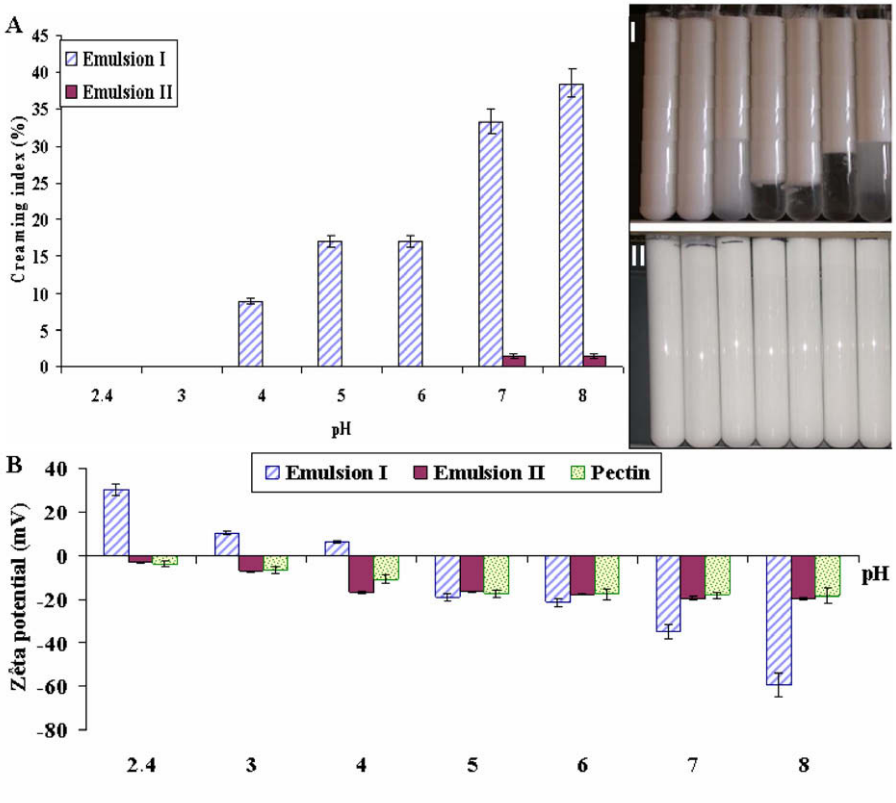

Fig. 8. (A) Creaming stability of primary (5 wt.% oil, 0.25 wt.% PPI, 5 mM imidazole-acetate buffer) (1) and secondary (5 wt.% oil, 0.25 wt.% PPI, 0.2 wt.% pectin, 5 mM imidazole-acetate buffer) (2) emulsions when pH was changed from 2.4 to 8. (B) ζ-potential of primary emulsion (5 wt.% oil, 0.25 wt.% PPI, 5 mM imidazole-acetate buffer) (1), secondary emulsion (5 wt.% oil, 0.25 wt.% PPI, 0.2 wt.% pectin, 5 mM imidazole-acetate buffer) (2), and pectin aqueous solution (5 mM imidazole-acetate buffer) (3) when pH was changed from 2.4 to 8.

protein PI. This would have increased the repulsion between the droplets, thereby reducing their tendency to aggregate after 24 h at various pH values. As the pH is increased, the droplets of the secondary emulsion become more negatively charged because of the increase in negative charge of the pectin and reduction of positive charge/increase of negative charge of the proteins (Fig. 8B). This suggests that when the pH was increased, either some of the negatively charged pectin molecules desorbed from the surface of the emulsion droplets. This desorption was shown to cause slight droplet aggregation at pH values above 6 (Fig. 8A). A possible explanation for this observation is that the interaction between the pectin molecules and the droplet surfaces became weaker as the pH was increased due to the decrease in positive charge of the adsorbed protein layer. Consequently, it may have become easier for part of a pectin molecule to detach itself from one droplet and bind to another droplet, promoting bridging flocculation in agreement with Guzey and McClements (2006b). Moreover, the non-adsorbed pectin in the aqueous phase could promote droplet flocculation through a depletion mechanism (Guzey et al., 2004) and above all that concentration of pectin (0.2 wt.%) used to stabilize the secondary emulsion is relatively high (Fig. 6).

3.3.3. Resistance to depletion flocculation

After maltodextrin addition, spectrophotometric results showed that maltodextrin did not promote droplet flocculation or coalescence at a concentration below 11 wt.% for the two studied emulsions (Fig. 9). For the entire studied DE, maltodextrin had less effect on the creaming stability of the secondary emulsions than that of the primary one. For example, the CFC of maltodextrin having a DE of 28 was 23.6 and 28.3 wt.% for primary and secondary emulsions at pH 2.4, respectively. In other words, the amount of maltodextrin which was needed to destabilize the emulsions prepared at pH 2.4 by depletion flocculation was higher in the secondary emulsion than in the primary one for all studied DE.

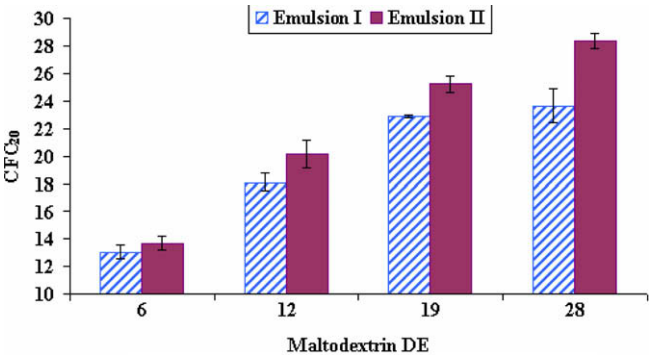

Fig. 9. Critical flocculation concentration (CFC_{20}) of different maltodextrins in primary emulsion (5 wt.% oil, 0.25 wt.% PPI, 5 mM imidazole-acetate buffer, pH 2.4) (1) and secondary emulsion (5 wt.% oil, 0.25 wt.% PPI, 0.2 wt.% pectin, 5 mM imidazole-acetate buffer, pH 2.4) (2) after 24 h storage at room temperature.

Two main factors may account for the ability of an adsorbed pectin layer to improve the resistance to depletion flocculation of protein-coated lipid droplets. First, the pectin–protein layer surrounding the lipid droplets may have been thicker than the protein layer, thereby generating a greater short-range steric repulsion between the droplets that may prevent them from coming close enough together to flocculate (Guzey & McClements, 2006a). Second,

the presence of a pectin layer around the protein-coated lipid droplets may have prevented attractive interactions occurring between protein molecules adsorbed onto different lipid droplets, which might otherwise occur when the droplets are forced into close proximity during the depletion.

The increase of the stability of emulsions stabilized by pea legumin through their complex formation with polysaccharides such as chitosan has been observed earlier (Braudo et al., 2001). This fact has been explained by the high exclusion volume of the polysaccharide and the high viscosity of both the surface layer and the serum.

3.4. Influence of protein–pectin interactions on oil–water interfacial properties

3.4.1. Interfacial properties before pectin addition

The interfacial tension and viscoelastic modulus measurements of PPI at oil–water interfaces was measured before (below 10,000 s) and after (above 10,000 s) excess pectin addition at different pH values and results are only given in details for pH 2.4 and pH 7 (Fig. 10A). It can be observed that the interfacial tension $\gamma(t)$ between oil and PPI aqueous solutions decreases throughout the whole range of measurement time, thus indicating that the pea globulins adsorb at the oil–water interface at the two pH values (curves 1 and 2 in Fig. 10A). We can also see that a less decrease rate in $\gamma(t)$ is observed and adsorption equilibrium is reached after about 8000 s.

Regarding the interpretation of the results dealing with the viscoelastic modulus developed by the rearrangement of proteins adsorbed onto the interface, we had observed that $\varepsilon'(t)$ was very close to $\varepsilon(t)$ and that $\varepsilon''(t)$ was constant and negligible over the time scale of the experiment before and after pectin addition (data not shown), indicating purely elastic behavior and no relaxation phenomena of adsorbed proteins. So, as $\varepsilon'(t)$ was always similar to $\varepsilon(t)$ for all studied pH values, only values of $\varepsilon(t)$ will be discussed. This pure elastic rheological behavior indicates that compression of the interfacial film induces only protein segment compression in the plane of the interface, but no major reorganization such as the expulsion of protein segments toward adjacent phases (Saulnier et al., 2001). At the two considered pH values, the first stage of $\varepsilon(t)$ occurred rapidly (curves 3 and 4 in Fig. 10A), suggesting that the interfacial rearrangement of pea globulins leading to first layer formation would be easy because probably of the relatively lower level of protein–protein interactions. The second stage of $\varepsilon(t)$ is less rapid and led slowly to a relatively rigid film ($\sim$ 24 mN m^{-1}) at pH 2.4 and a less rigid one ($\sim$18.6 mN m^{-1}) at pH 7 after 10,000 s of adsorption. This result could be explained by the change in pea globulin dissociation state as a function of pH in agreement with Guéguen et al. (1988) which in turn can influence protein hydrophobicity and its interfacial adsorption properties.

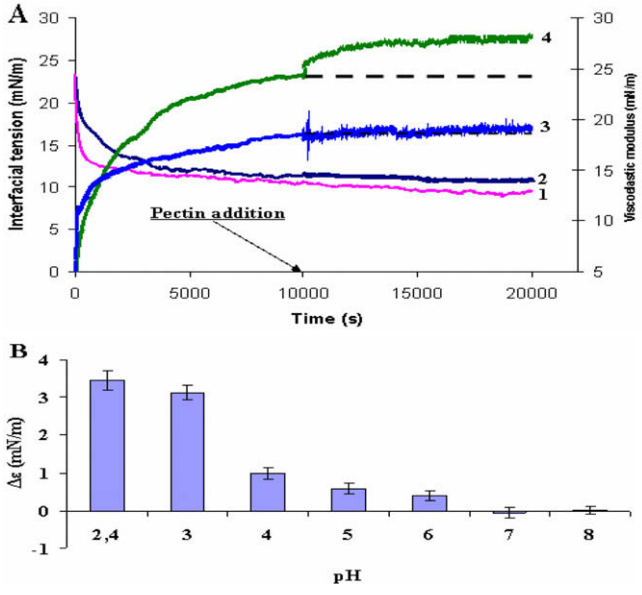

Fig. 10. (A) Time-dependant changes in interfacial tension (γ) and interfacial viscoelastic modulus (ε) of pea protein isolate in 5 mM imidazole-acetate buffer at the oil–water interface at 20 °C (γ at pH 7 (1); γ at pH 2.4 (2), ε at pH 7 (3), ε at pH 2.4 (4)). Excess pectin was added after 10,000 s adsorption. Dotted line corresponds to ε at pH 2.4 without pectin. (B) Changes in interfacial viscoelastic modulus when pectin was added after 10, 000 s adsorption at pH ranged from 2.4 to 8.

3.4.2. Interfacial properties after pectin addition

First of all, it is important to emphasize the fact that the added pectin has no significant effect on the interfacial tension profile at all studied pH values. Moreover, At pH 7, pectin addition has a marginal influence on the $\varepsilon(t)$ compared to that without pectin, and just small fluctuations, that could be due to weak interactions between protein and pectin molecules, has been shown (curves 3 Fig. 10A). Meanwhile, at pH 2.4 (curves 4 in Fig. 10A) the addition of pectin leads to a sudden and rapid increase in the interfacial film rigidity to reach approximately 28 mN m^{-1}. Actually, after pectin injection in the bulk solution, it would be more favorable for polysaccharide to form rapidly complexes with interface adsorbed proteins, which have already lost much of their entropy, as compared to residual protein molecules in the bulk (Ganzevles, Zinoviadou, vanVliet, CohenStuart, & de Jongh, 2006). It can be assumed that although pectin adsorption has not contributed to a better interfacial activity of the pea proteins, this adsorption increases the interfacial film rigidity at pH 2.4.

Even though it is known that changes in $\varepsilon(t)$ reflected internal frictions and hence was a measure of the extent of protein–protein interactions at the interface (Benjamins, Cagna, & Lucassen-Reynders, 1996), in our case, the increase in viscoelastic modulus would be considered as a reflection of an increase in the strength of lateral interactions between the adsorbed protein and pectin molecules resulting in a more cohesive and less compressible film in agreement with Bos and van Vliet (2001). In similar studies, it has been shown that adsorption of β-lactoglobulin–pectin complexes at oil–water interface leads to formation of interfacial membranes having larger dilatational elasticity than with the pure protein (Ganzevles, Cohen Stuart, van Vliet, & de Jongh, 2007). This pectin layer adsorption was found to be able to increase salt stability at low pH of oil-in-water emulsion stabilized by β-lactoglobulin (Guzey et al., 2004). In the same way, it has been recently found that adsorption of polysaccharide to a previously formed (dense) β-lactoglobulin layer can reinforce the latter and result in a higher dilatational modulus than that of the pure protein layer (Ganzevles, Kosters, vanVliet, CohenStuart, & deJongh, 2007).

The highly viscoelastic modulus value at pH 2.4 reached a short time after pectin addition would reflect both a rapid adsorption and a high packing density of pectin–protein adsorbed complexes as compared with the packing of protein layer alone. This adsorption would be caused by electrostatic intermolecular interactions between pectin and already adsorbed protein molecules. Thus properties of the composite adsorbed layer could be controlled by the strength of pectin–protein interactions by changing pH or ionic strength for example. In order to detect the magnitude of these interfacial pectin-adsorbed protein interactions, we plotted the increase of the viscoelastic modulus after pectin addition as a function of pH (Fig. 10B). The improvement of the interface rigidity by pectin addition decreases gradually as the pH increases because of the adsorbed protein charge changes. However, measurements

were particularly carried at pH 5, 6, 7 and 8 to assess whether interfacial complexation had occurred at pH values above the protein PI where both biopolymers carry the same net charge. Results presented in Fig. 10 showed that, whereas no detectable complexation between protein and pectin occurs at pH 7 and 8, some interactions were detected at pH 5 and 6 causing an increase in the interfacial layer elasticity. These results could thus partly used to explain the secondary emulsion destabilization induced when pH reached 6 in Fig. 8A. In fact, the absence or the very weak interfacial pectin protein interactions at oil–water interface detected by interfacial rheological properties measurements at pH values above 6 could lead to pectin chain desorption from the oil droplet surfaces and therefore bridging and/or depletion flocculation phenomena could easily occur as explained in Section 3.4.2. On one other hand, it is obvious that extrapolation of the results dealing with the protein/pectin complex formation in solution are difficult to extrapolate in order to explain the mechanisms of interactions that occur between the two polymers at the O/W interfaces. Indeed, proteins in solutions have a relatively intact tertiary conformation, while at interfaces, the adsorbed globular proteins tend to unfold, and this is commonly known as interfacial denaturation. In our case, the only common result between the interactions in solution and that at interfaces that could be considered is the formation of electrostatic complexes at pH above the pI.

4. Conclusion

The objective of this study was to examine the formation of pectin–pea protein complexes in aqueous solution and in oil-in-water emulsions as a function of pH and pectin concentration. Our results showed that protein–pectin complexes can be formed in solution and at oil–water interfaces under acidic condition, and probably even at pH values above the protein PI, through mainly electrostatic interactions. When the pectin concentration is just enough to cover all oil droplets, secondary stable emulsions were obtained at low pH values. Our experiments have also shown that creating a pectin layer around the lipid droplets in an oil-in-water emulsion can improve emulsion stability to ageing, pH changes, and can also increase the resistance to depletion flocculation caused by maltodextrin addition. This stability was mainly assigned to the steric repulsion effects of pectin at the oil–water interface because of the relatively low electrostatic repulsions. Thus, forming a pectin layer around the lipid droplets in an oil-in-water emulsion could appreciably widen the use pH range of food emulsions stabilized by plant proteins and increase the amount of added maltodextrin without destabilizing the system. This latter case may have important consequences for the development of dry emulsions because microencapsulation efficiency is known to increase with increasing wall material concentration.

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