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Interfacial properties of oleosins and phospholipids from rapeseed for the stability of oil bodies in aqueous medium

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ABSTRACT

Oleosins are plant proteins associated with phospholipids in seed oil bodies. The ability of oleosins to aid in the emulsification and stabilization of oil bodies is well known, but little information is available on their interaction with phospholipids at the interface between oil bodies and aqueous medium. Oil body reconstitution at various phospholipid/oleosin ratios was carried out to observe how rapeseed oleosins of 20 kDa and rapeseed phospholipids affect oil body stability. Phospholipids are needed to stabilize oil droplets, but oleosins are mandatory to avoid coalescence. We thus characterized how phospholipids affect the interfacial properties of oleosins at pHs 5.5 and 8.5, by analyzing the adsorption kinetics and interfacial dilational rheology. We observed a synergic effect between oleosins and phospholipids in increasing surface pressure at both pHs. This kind of effect was also observed for the dilational modulus at pH 5.5. A thermodynamic approach highlights these synergic interactions between oleosins and phospholipids through a positive deviation from ideality.

1. Introduction

In plants, lipids are stored in spherical organelles named oil bodies or oleosomes, which consist of small triglyceride droplets surrounded and stabilized by a layer of phospholipids associated with unique proteins named oleosins. Oleosins are usually present as two or more isoforms. These isoforms are commonly classified as high and low molecular weight forms with a molecular weight range between 15 000 and 26 000 Da [1]. In rapeseeds, these proteins represent around 3% of the total oleosome weight [2] and between 8 and 20% of the total number of seed proteins [3,4]. Protein with molecular weight around 19 ± 0.5 kDa, is the most representative type of oleosins in rapeseed oils [5].

Oleosins are amphipathic alkaline proteins [6,7]. Their structure consists of three domains. The first is a highly conserved hydrophobic central domain with a predominant β-strand structure embedded in the non-aqueous phase of lipid bodies. Other researchers have also demonstrated the presence of an α-helical hairpin structure [8]. This hydrophobic region is flanked by putative α-helical structures in the polar N- and C-terminal domains, which are probably located within the lipid/water interface [9–11]. In rapeseed, the central hydrophobic domain contains about 70 amino acid residues [10] with a high proportion of very hydrophobic residues (isoleucine, leucine and valine) [7]. The N- and C-terminal domains comprise around 60 and 50 residues, respectively, with a scattered distribution of hydrophilic and hydrophobic residues. Positively charged residues face the interior of the oil body and negatively charged residues face the exterior [7].

These proteins are predicted to irreversibly bind to the lipid/water interface [11], behaving similarly to animal serum apolipoproteins [12].

Phospholipids, along with proteins, are major components of the biological membrane. They are characterized by the presence of a polar or hydrophilic head group and a non-polar or hydrophobic fatty acid region. In the presence of water, phospholipids adopt various structures based on their nature. Phospholipids form mainly lamellar and hexagonal structures, to build membranes and micelles, respectively [13]. Phospholipids in oil bodies are organized into a monolayer membrane, in which the two acyl moieties interact with the triglyceride matrix. An example of composition of rapeseed phospholipids is given by Tzen et al. with 59.9% phosphatidylcholine (PC), 5.9% phosphatidylethanolamine (PE), 14.0% phosphatidylinositol (PI) and 20.2% phosphatidylserine (PS) [2]. These amphipathic molecules, especially PC, are widely used as surfactants in industry [14].

Harada et al. [15] have previously demonstrated the use of oleosin as an emulsifying agent. This property, including the fact

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that it is a natural product, is of great interest to the food, cosmetic and pharmaceutical industries. The exact mechanism involved in generating oleosin-stabilized emulsions has not been extensively studied. However, two studies [7,10] have shown that, in reconstituted emulsions formed from isolated native rapeseed components, oleosins require phospholipids to obtain new oleosomes as stable as primary oleosomes. The fundamental aspects of these phenomena have never been clearly explained.

Interactions between proteins and phospholipids in emulsions have been widely studied [16–23]. Asynergic effects can occur if a surfactant replaces the less active phospholipids at the interface [19]. However, oleosin adsorption at the interface is relatively irreversible, and these proteins are unlikely to be removed by the presence of phospholipids. Several synergic effects between phospholipids and proteins have been reported. Formation of hydrogen bond [16] and hydrophobic interactions [20] between the two compounds provides significant stability to the emulsion by improving viscoelasticity [17] and molecular flexibility.

We studied the effects of the ratio of phospholipids to oleosins on the stability of reconstituted oil bodies. We analyzed the adsorption kinetics and interfacial dilational rheology at the oil/water (O/W) interface for oleosins and mixed phospholipid/oleosin systems. The molecular interactions between oleosins and phospholipids are discussed based on a simple thermodynamic analysis. We also evaluated the effects of pH on the medium, as it can modify the net charge of the protein. Thus, the system was evaluated at two pHs: 5.5 (pH of rapeseed crushed in water—native pH) and 8.5. The relationships between possible structures adopted by oleosins at the interface and phospholipids are discussed.

2. Materials and methods

2.1. Materials

The rapeseed (Kosto) came from Momont (Compiègne, France). Rapeseed oil was commercially obtained from Lesieur (Asnières-sur-Seine, France). Rapeseed phospholipids were kindly provided from Lasenor Emul S.L. (Barcelona, Spain). Diethyl ether (≥99%), methanol (99.8%), sodium chloride (≥99%), acetone (analytical grade ≥99%), triolein (practical grade ∼65%), florisil (100–200 mesh), hexafluoroisopropanol (≥99%) and tris(hydroxymethyl)aminomethane (≥99.5%) were purchased from Sigma–Aldrich (L'Isle d'Abeau Chesnes, France). Standard phospholipids (purity ≥99%) were purchased from Avanti Polar Lipids Inc. (Alabaster, USA). Water was purified by means of a Millipore (Milford, USA) filtration device (Milli-Q).

2.2. Oleosins extraction, purification and characterization

Oleosins were extracted and purified from rapeseeds. Oilseeds were crushed in distilled water (20/80, w/w). This crushing was carried out with a high shear rate device (Silverson L4RT, Silverson Machines Ltd., Waterside, England) for 10 min at maximum speed, followed by two passes through a high-pressure homogenizer (Lab 1000, APV, Evreux, France) at 350 bar. At the end, phase separation was performed with a centrifuge (Sigma 6K15 Fisher Bioblock Scientific, Illkirch, France) at 5000 × *g* for 10 min at 6 °C with a free-angle rotor. The upper layer containing oleosomes was recovered with a fine mesh (100 μm × 100 μm of porosity). Two volumes of 4% sodium chloride solution were added to one volume of emulsion, to solubilize non-membranous proteins. The solution was gently stirred and then centrifuged at 10 000 × *g* for 10 min at 0 °C. A compact cream was obtained. The cream was then washed with two volumes of distilled water. The solution was stirred at 5000 rpm with a high shear rate crusher, and centrifuged at 15 000 × *g* for 10 min at −5 °C, to recover the cream. This procedure was repeated twice. Afterwards, the cream was delipidated five times with three volumes of diethyl ether until total discoloration of the organic phase. Then, 30 mL of chloroform and 15 mL of methanol were added to the delipidated cream (2/1, v/v), to remove phospholipids. After gentle shaking, the tubes were centrifuged at 5000 × *g* for 10 min and the interfacial material, containing oleosins, was collected. This procedure was repeated three times. Two volumes of distilled water were added to the cream and this solution was placed under nitrogen gas to eliminate all the remaining ether. The solution containing oleosins was then freeze-dried to obtain oleosins in powder form. Oleosin purity was verified by the Kjeldahl method [24], and SDS PAGE (12.5% polyacrylamide) electrophoresis. After electrophoresis, the gel was stained with Coomassie blue and destained.

2.3. Phospholipid purification

Phospholipid purification consisted of using five cycles of precipitation with cold acetone and centrifugation at 4750 rpm for 20 min at 4 °C. Phospholipid analysis was performed by HPLC–ELSD. Retention times were used for their identification by comparison with standard phospholipids.

2.4. Triolein purification

Triolein was used as model rapeseed oil in adsorption kinetics and interfacial dilational rheology measurements. Triolein was purified according to the procedure described by Gaonkar [25], by percolation through a column packed with florisil first activated by heating at 160 °C for 16 h. About 20 g of florisil were used per litre of triolein. As the procedure took several hours, a flow of nitrogen was maintained above the balloon flask used for collection. The flask was wrapped in aluminium foil to avoid further triolein deterioration.

2.5. Oil body reconstitution

We reconstituted oleosomes at their native pH, i.e. 5.5. We used various concentrations of oleosins, phospholipids and rapeseed oil (Table 1).

The nature of the interface is directly dependant on the order of mixing [26] due to the high adsorption capacity of the molecules. Oleosins and phospholipids were first introduced in the recipient flask, which was followed by oil and water to allow competitive adsorption of the molecules. The mixtures were homogenized with an ultrasound probe (Sonics Vibracell, Sonics & Materials, Newton, USA) at 20 kHz and 200 W; the solutions were sonicated – 5 s, with a 25 s interval between pulses – for a total of 3 min. The samples were

Table 1
Percentage of the different components in the reconstituted emulsions (weighted phospholipid/oleosin (PL/OL) ratios are indicated).

	PL = 0	OL = 0	PL/OL = 50	PL/OL = 5	PL/OL = 0.5	PL/OL = 0.015
Oleosin	2.00	0.00	0.04	0.33	1.33	2.00
Phospholipid	0.00	2.00	1.96	1.67	0.67	0.03
Rapeseed oil	38.00	38.00	38.00	38.00	38.00	38.00
Water	60.00	60.00	60.00	60.00	60.00	60.00

then stored at ambient temperature for two weeks and regularly observed.

2.6. Changes to the droplet size and creaming rate of the reconstituted oil bodies

Oil body sizes were analyzed using optical light microscopy (Nikon Eclipse E600, Nikon France S.A.S., Champigny Sur Marne, France). Surface coverage was calculated using the average size of the emulsion droplets with rapeseed oil density of 0.915. We calculated the creaming rate by monitoring the stability of the reconstituted oil bodies over time. At the end of the observation period, samples were centrifuged at 2000 × *g* for 5 min to determine the amount of oil released.

2.7. Zeta potential

Zeta potential was measured by dynamic light scattering (DLS, Zetasizer NanoZS, Malvern Instruments S.A., Orsay, France). The samples were diluted in distilled water (1/100, v/v) and the measurements were repeated three times.

2.8. Dynamic surface tension measurement at the oil/water interface

A dynamic drop tensiometer (Tracker, IT Concept, Longessaigne, France) was used to measure changes in the interfacial tension in oleosin and oleosins–phospholipids dispersions with time.

A triolein droplet containing oleosins at 0.1 g/L or phospholipid/oleosin mixture (PL/OL = 0.02; 0.2; 2, w/w with a constant oleosin concentration at 0.1 g/L) was delivered from the tip of a curved capillary (PS20, IT Concept, Longessaigne, France) attached to a 250 μL syringe and was placed in a cell containing 7 mL of 75 mM Tris buffer at pH 5.5 or 8.5. The interfacial tension was determined by analyzing the profile of the droplet according to the Laplace equation, by means of a CCD camera connected to a computer. Triplicate measurements were performed at 25.0 ± 0.5 °C.

2.9. Surface rheology characterization at the oil/water interface

Rheological properties of oleosins and phospholipid/oleosin interfacial layer were characterized using a Tracker dynamic drop tensiometer (IT Concept, Longessaigne, France) in oscillatory mode [27]. The principle was to measure the surface stress ($\Delta\gamma$) resulting from a small sinusoidal deformation of the interfacial area (ΔA).

A triolein droplet containing oleosins at 0.1 g/L or phospholipid/oleosin (PL/OL = 0.02; 0.2; 2, w/w with a constant oleosin concentration at 0.1 g/L) was delivered from the tip of a curved capillary (PS20) attached to a 250 μL syringe and was placed in a cell containing 7 mL of 75 mM Tris buffer at pH 5.5 or 8.5. The drop area changed by sinusoidal fluctuations in the drop volume of 0.5 μL at a frequency of 0.05 Hz. The interfacial tension is determined by analyzing the profile of the droplet according to the Laplace equation, by means of a CCD camera connected to a computer. Fluctuations of the area and the resulting sinusoidal interfacial tension are recorded every second. The interfacial viscoelasticity modulus ε is defined as the complex quantity:

$$\varepsilon = \varepsilon' + i\varepsilon''$$

where the real part ε' is the elastic component and the imaginary part ε'' is the viscous component. They are calculated from the following equations [27]:

$$\varepsilon\prime = |\varepsilon| \cdot \cos\phi$$

$$\varepsilon'' = |\varepsilon| \cdot \sin\phi$$

where ϕ is the phase angle and $|\varepsilon|$ is the dilational viscoelastic modulus. All the measurements were performed at 25.0 ± 0.5 °C and were repeated at least three times.

2.10. Monolayer characterization at the air/water interface

The oleosin solution was prepared by dissolving the oleosins in hexafluoroisopropanol (HFIP). Phospholipids were dissolved in chloroform/methanol (2/1, v/v) mixture. We used 75 mM Tris with the pH adjusted to 5.5 or 8.5 with concentrated HCl as subphase. Milli-Q water (resistivity < 18 MΩ cm and surface tension = 72.01 mN/m) was used to prepare the buffer.

Monolayer experiments were carried out using an automated Langmuir film balance (KSV instruments Ltd., Helsinki, Finland) equipped with two movable barriers. The minimum and maximum areas of the through were 26×10^{-4} m^2 and 2.5×10^{-2} m^2, respectively. It was filled with the buffer solution and temperature was kept at 25.0 ± 0.1 °C by circulating water.

The entire subphase was cleaned to ensure zero surface pressure before spreading the sample. We used a 50 μL micrometric syringe to spread 3 mg/mL oleosin and/or phospholipid solution onto the surface of the subphase; the solution was dispensed as tiny droplets to produce a uniform monolayer. The solutions were allowed to sit for 30 min before further analysis was carried out, ensuring evaporation. The compression speed for each measurement was maintained constant at 0.06 nm^2 mol^{-1} min^{-1}, which was determined to be sufficiently low for the protein to be in thermodynamical equilibrium at the interface. The surface pressure was recorded via a platinum plate immersed in the subphase and linked to a microbalance. All experiments were repeated at least three times.

3. Results and discussion

3.1. Oleosins and phospholipids characterization

The SDS PAGE analysis confirmed a protein of 20 kDa (Fig. 1), corresponding to the size reported for oleosins in rapeseed [5]. We analyzed the protein content of the oleosin sample in powder form using the Kjeldahl method; oleosin was greater than 95% pure. HPLC–ELSD analysis revealed that phospholipid composition in rapeseeds was of 7.3% of phosphatidic acid (PA), 23.0% of phosphatidylethanolamine (PE), 11.6% of phosphatidylinositol (PI) and 40.2% of phosphatidylcholine (PC). The remaining 17.2% were various phospholipids, each of them being less than 3%. PC was the major phospholipid which is consistent with previous reports [2].

3.2. Oil body reconstitutions

Rapeseed oil body reconstitutions were carried out using various phospholipid/oleosin ratios, to observe which surface-active components are involved in oleosome stability. Experiments were performed at the native pH (5.5). Reconstituted oil bodies were characterized by their size just after their formation. Their stability was estimated by monitoring the creaming and coalescence phenomena over the time.

3.2.1. Droplet sizes of the various oil body reconstitutions

We observed significant difference in droplet sizes through optical microscopic analysis of the various samples (Table 2). Small droplets of 1.44 μm were obtained in the presence of phospholipids alone, whereas samples with greater proportions of oleosins formed a polydisperse collection with bigger droplets. This demonstrates the better emulsifying properties of phospholipids than oleosins. This will be discussed later with dynamic interfacial tension experiments.

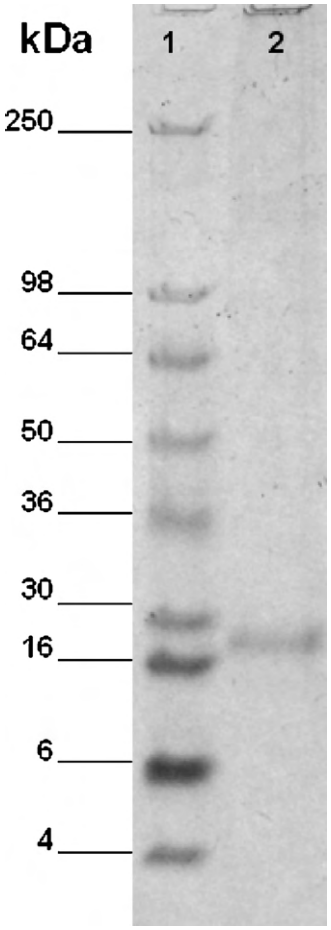

Fig. 1. SDS PAGE (12.5% polyacrylamide): (1) molecular mass markers and (2) oleosins in powder.

3.2.2. Destabilization by creaming

Creaming was observed in the reconstitution trials (Fig. 2). Reconstituted oil bodies made up mostly of oleosins (PL = 0, PL/OL = 0.015 and PL/OL = 0.5, w/w in Fig. 2) were observed to form large aggregates quickly. This high flocculation phenomenon is due to gravitational collisions of the larger size droplets. According to the Stokes equation (Eq. (1)), the creaming rate is then directly related to large droplets or the size of aggregates:

$$v = \frac{2r^2 g(\rho_{oil} - \rho_{water})}{9\eta} \quad (1)$$

where v is the creaming rate, r is the droplet size, g is the gravitational acceleration constant, ρ is the density of the phase and η is the viscosity of the continuous phase.

In contrast to a previous study with maize oil body reconstitutions [28], phospholipids alone (Fig. 2, OL = 0) do allow the emulsion to remain stable against creaming.

Table 2
Average droplet size and oleosin surface coverage in the reconstituted emulsions with various phospholipid/oleosin weighted ratios.

PL/OL ratio	Average droplet size (μm)	OL surface coverage (g/m²)
PL = 0	47 ± 32	0.377
OL = 0	1.44 ± 0.74	–
PL/OL = 50	1.15 ± 0.57	0.000
PL/OL = 5	1.70 ± 0.63	0.002
PL/OL = 0.5	7.15 ± 3.07	0.038
PL/OL = 0.015	110.45 ± 93.57	0.887

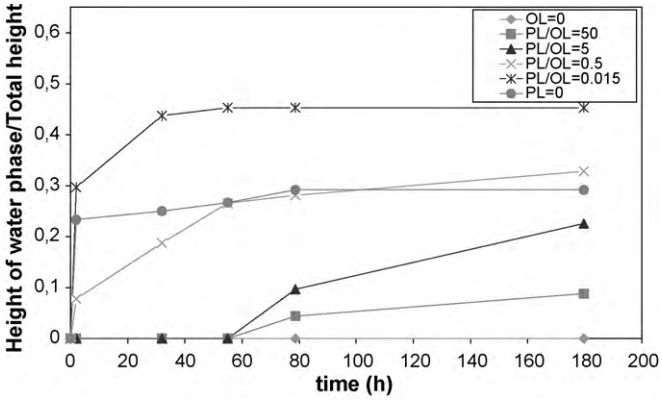

Fig. 2. Changes as a function of time (in hours) of the creaming of reconstituted oil bodies with different PL/OL weighted ratios.

Increase the concentration of oleosins in reconstituted oil bodies (PL/OL = 50, 5, 0.5, 0.015, w/w in Fig. 2) increases the creaming rate. We suggest that oleosins promote the phenomenon of flocculation. The existence of attractive forces between the oleosin molecules of the various droplet interfaces can be deducted.

3.2.3. Influence of the zeta potential in oil body reconstitution

At pH 5.5 the absolute zeta potential increases from 30 to 75 mV with the concentration of phospholipids (Fig. 3). In other words, the net surface charge density increases with the density of phospholipids on the surface. Under these conditions (pH of 5.5), phospholipids have an overall negative charge [29,30], increasing electrostatic repulsive forces. By contrast, oil bodies stabilized by oleosins alone present a low net charge (7 mV), indicating that at pH 5.5 we are close to the isoelectric point of oil bodies, which promotes aggregation. Thus, the zeta potential is directly dependant on the density of phospholipids on the surface.

3.2.4. Oil release by coalescence

After two weeks of observation, samples containing oleosins or sole phospholipids alone as surfactant, including samples for which oleosins were the main component (PL/OL = 0.015 and 0.5, w/w), did not produce a free oil phase. By contrast, samples with high phospholipid/oleosin ratios (PL/OL = 5 and 50, w/w) were completely unstable, and these mixtures produced a free oil phase within two weeks.

According to the DLVO theory [31], both attractive and repulsive interactions coexist in all dispersed systems. Oleosins are mainly responsible for hydrophobic [32] and van der Waals attractive forces. However, close to the isoelectric point, as revealed by zeta

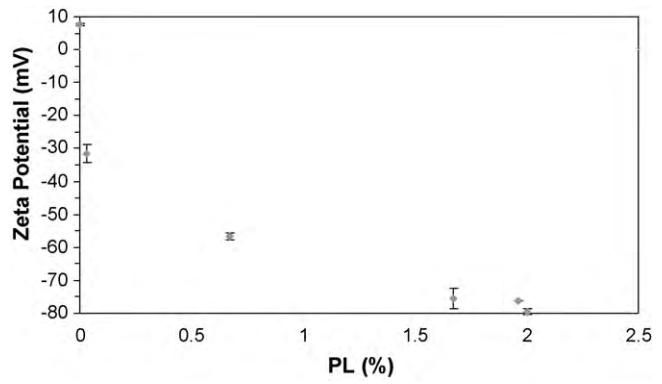

Fig. 3. Variation of the zeta potential as a function of the percentage of phospholipids weighted content on the reconstituted oil bodies.

Table 3
Flocculation, coalescence and demixing phenomena in oil body reconstitution with various phospholipid/oleosin ratios.

	OL = 0	PL/OL = 50	PL/OL = 5	PL/OL = 0.5	PL/OL = 0.015	PL = 0
Flocculation	–	+	++	+++	+++	+++
Coalescence	+++	+++	++	+	–	–
Global destabilization	–	+	++	+	–	–

+++: high; ++: medium; +: little; –: absence.

potential measurements, there is negligible electrostatic repulsion. However, steric repulsive forces dominate at very short range. Hence, at native pH, oleosins promote flocculation but prevent coalescence.

With phospholipids, hydrophobic forces are minimized. Van der Waals forces are also low as the size of the particles is generally smaller. An energetic barrier, for which height is directly linked to the squared zeta potential, prevents flocculation. However, there is no coalescence barrier associated with phospholipids. After overcoming the electrostatic repulsions, the resulting flocculation leads to quick coalescence.

In the presence of a large quantity of phospholipids and a non-negligible quantity of oleosins (PL/OL = 50 and 5, w/w), the zeta potential decreases slightly from 80 to 75 mV, related to the increasing amount in oleosins. Indeed, the quantity of oleosins is not sufficient to homogeneously cover and stabilize the interface; thus, coalescence occurs due to the break down of the interfacial film.

Table 3 described the various phenomena for the tested compositions in phospholipids and oleosins. According to the general principles of kinetic, the slowest process is rate controlling. As long as oleosins are more concentrated than phospholipids (PL/OL = 0.015 and 0.5, w/w), coalescence remains slow and no oil phase is created. When phospholipids are more concentrated than oleosins (PL/OL = 50 and 5, w/w), oleosins do not cover effectively the interface to prevent the coalescence while the repulsion electrostatic forces induced by the phospholipids are not enough to stabilize the emulsion. Calculations of surface coverage by oleosins are in accordance with the hypothesis for which oleosins prevent coalescence by coverage of oil/water interface. We see by comparison of Tables 2 and 3 that there is no coalescence when the surface coverage by oleosins is more important. The value of 0.8 g/m^2 is very higher to that quoted by Fang and Dalgleish [33] where 1 mg/m^2 of casein was sufficient to stabilize emulsions.

3.3. Dynamic interfacial tension measurements

We investigated the adsorption kinetics of oleosins and mixed phospholipid/oleosin systems at a triolein/water interface in aqueous medium at pHs 5.5 and 8.5. The kinetics are similar at both pH and we discussed the results at pH 8.5 to explore the possible polyelectrolyte behaviour of oleosins (Fig. 4). Note that a pure triolein/water interface has an interfacial tension stabilized at 10.0 ± 0.3 mN/m at 25 °C.

If oleosins are dispersed throughout the oil phase, their adsorption at the interface occurs very quickly. At a concentration of 0.1 g/L, the surface tension decreased instantaneously, reaching equilibrium in about 50 s. Although the mean molecular weight (20 kDa) of these oleosins is similar to that of β-lactoglobulin and β-casein [34], two well-known dairy surface-active proteins, oleosins present faster adsorption at an oil/water interface [35]. It emphasizes the highly amphipathic structure of oleosins and consequently their high affinity for interfaces. The adsorption kinetics of a polymer is dependent on the diffusion of the molecule from the bulk to the interface, but also on whether the molecule has the ability to unfold after its adsorption [36]. Proteins with little internal structure adsorb more rapidly at a liquid/liquid interface than globular proteins with a compact structure [37]. Our results suggest that oleosins have a less globular behaviour than β-casein and β-lactoglobulin.

In the presence of phospholipids, regardless of their proportion in the mixed system, the dynamic adsorption was reduced. It took more than 50 s to reach a plateau and complete stabilization of the surface tension occurred after 200 s. Similar behaviour was observed when the pH of the aqueous medium was 5.5.

In the absence of phospholipids, oleosins give rise to an equilibrium interfacial pressure of 5.8 and 4.8 mN/m at pHs 8.5 (Fig. 5A) and 5.5 (Fig. 5B), respectively. Adding phospholipids to an identical concentration of oleosins in the oil phase led to a significantly higher equilibrium interfacial pressure—between 7.2 and 8.5 mN/m (Fig. 5A and B, respectively). We can correlate the effectiveness of the synergic action of phospholipids and oleosins to increase the interfacial pressure with the decrease of droplet diameter in the reconstituted oil bodies (Table 2). The interfacial pressure was not significantly influenced by the proportion of phospholipids in the system or the pH of the aqueous phase. The equilibrium interfacial pressure value of mixed systems was also much higher than that obtained with phospholipids alone which was about 1.0 and 2.5 mN/m at pHs 8.5 (Fig. 5A) and 5.5 (Fig. 5B), respectively. Thus, there appears to be a synergic effect between phospholipids and oleosins.

3.4. Interfacial rheological properties

Sinusoidal deformation has been applied to the interface to measure the dilational modulus of the interfacial film. In the absence of phospholipids, the dilational modulus (ε) of oleosin was very low, and was comprised between 1.1 (Fig. 5A) and 3.2 mN/m (Fig. 5B). These values are much lower than the dilational modulus of other proteins like β-casein (13 mN/m) and β-lactoglobulin (62 mN/m) with similar concentrations [38] but our triolein/water system is different than the paraffin oil/water system of these studies.

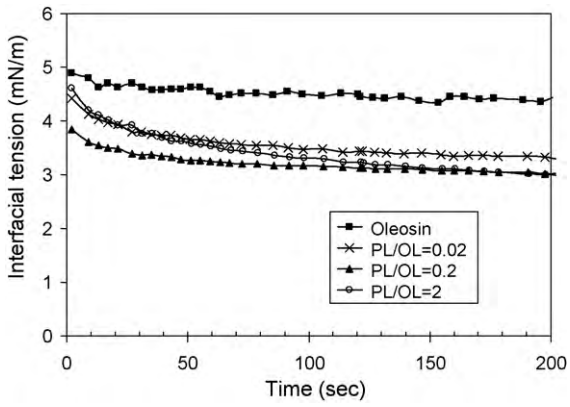

Fig. 4. Dynamic interfacial tension as a function of time for oleosins and phospholipid/oleosin mixed systems at the triolein/aqueous buffer interface. The buffer pH is 8.5. Components are initially dispersed in triolein phase. Oleosins concentration was 0.1 g/L. The phospholipid/oleosin (PL/OL) weighted proportions are 0.02, 0.2 and 2. The relative error in interfacial tension estimated from three repetitions is 7%.

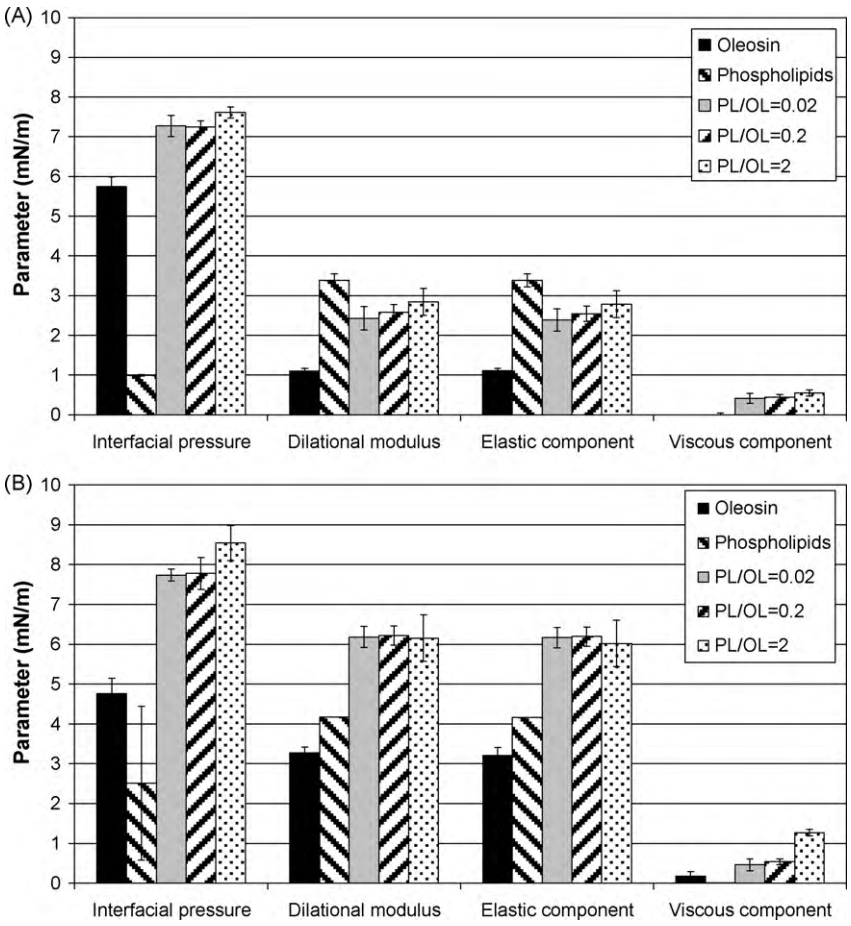

Fig. 5. Equilibrium interfacial pressure after the adsorption of oleosins or phospholipid/oleosin mixed systems, interfacial complex viscoelasticity, and the elastic and viscous components of these layers at a triolein/aqueous buffer interface. Components were initially dispersed in triolein phase. The oleosin concentration is 0.1 g/L. The phospholipid/oleosin weighted proportions PL/OL are 0.02, 0.2 and 2. The pH of the aqueous buffer is 8.5 (A) and 5.5 (B).

It is often difficult to make a relationship between interfacial elasticity and emulsion stability, but it can be speculated in some cases that when molecules like packed proteins are present on oil/water interfaces, the higher the elastic modulus, the higher the stability of the interface which forms a more elastic and rigid film. Note that a rigid interface has high elasticity [39].

The subphase pH exerts a significant effect on the rheological properties of oleosin layer. In our results, ε is higher at pH 5.5 than at pH 8.5 (comparison between Fig. 5A and B). We can think that oleosins at the triolein/water interface interact better at pH 5.5 giving a more rigid film. Generally, proteins interact better at their isoelectric point (IP), i.e. when their net charge is close to zero, and consequently, the intermolecular electrostatic repulsions are lower. There is significant variation between oleosin IPs, depending on their origin and the type of isoform. Most oleosins have a basic IP around 9–9.5 but some have neutral or even acid IP. For example, the sunflower oleosins have IPs that are between 5 and 6 according to White et al. [40]. Katavic et al. [6] have determined that rapeseed oleosins have an overall IP around 9.5. However, Roux et al. [41] have highlighted variation in IP values along the structure of oleosin. The central hydrophobic segment is not charged, but the terminal ends have an IP mostly between 6 and 9 with some zones with an IP around 5.

In our case at pH 5.5, we suggested a closer contact between neighbouring oleosin molecules than at pH 8.5. Although at this pH 5.5, the molecule should bear a higher net overall charge than at pH 8.5, the oleosins may have occupied a particular conformation at the triolein/water interface. This may have favored the interaction of segments with lower IP that are exposed in this conformation. This was consistent with the value of zeta potential close to zero at pH 5.5 for reconstituted oil bodies rich in oleosins.

When phospholipids are prior mixed with oleosins and then adsorbed at the triolein/water interface, ε was significantly higher than with oleosins alone regardless the pH (Fig. 5A and B). Again, the proportion of phospholipids in the system had no marked influence. One interpretation of this result is the existence of interactions between oleosins and rapeseed phospholipids, which may have led to the formation of a particular mutual organization. It is often explained that when proteins are present at interfaces and if a dilational deformation is applied, the deformation of the interface is not uniform and it can be expected holes and tears formation [42]. Phospholipids can insert between oleosins by combination with them. When deformation of interface occurs, the complex oleosin/phospholipid is able to respond quickly and prevent film rupture [42]. This synergic interfacial behaviour is not common, as low molecular weight surfactants have been generally observed to weaken viscoelasticity of the adsorbed protein, due to competition at the interface [43,44].

Under our experimental conditions, oleosins and phospholipid/oleosin mixed systems formed an almost complete elastic film at the oil/water interface with the elastic component very much larger than the viscous component whatever the pH (Fig. 5A and B). This indicated that either components, separate or in mixture, bind to the surface, stay firmly bound to the sur-

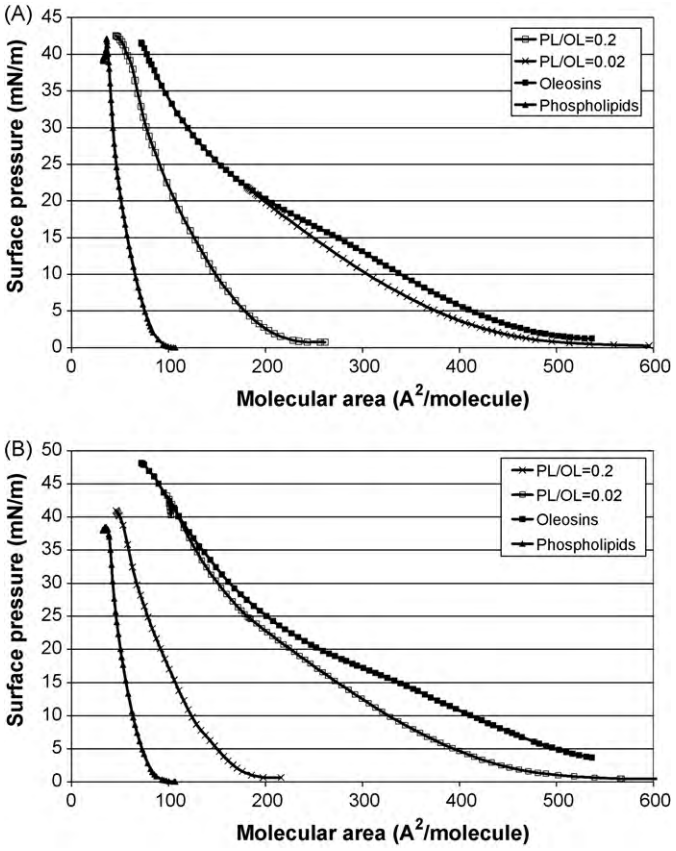

Fig. 6. Surface pressure versus mean molecular area (Π–A) isotherms, at the air–aqueous buffer interface, of pure oleosins monolayers, and of mixed phospholipid/oleosin monolayers at PL/OL molar ratios of 0.17 (0.2, w/w mixed phospholipid/oleosin systems) and 0.67 (0.02, w/w mixed phospholipid/oleosin systems); values were recorded at 25 °C with a Tris buffer subphase at pH 8.5 (A) and at pH 5.5 (B).

face and retain elasticity. Thus, there was no evidence of an exchange of material between the surface and the bulk solution, and/or molecule rearrangement within the film during sinusoidal oscillations, two relaxation phenomena usually involved [38,45].

3.5. Monolayer properties at air/water interface

Langmuir film balance experiments were performed at an air/water interface to get further insight into the interfacial organization of oleosins during presence or absence of phospholipids. Compression isotherms for pure oleosins, with a Tris–HCl at pHs 8.5 and 5.5 as subphase are shown in Fig. 6. For both pHs, the isotherm of pure oleosin has a sigmoidal shape. In the gaseous state, corresponding to the plateau at high molecular area, the surface pressure is not 0 mN/m as expected for an interface poor in surface-active molecules, but presented a value around 1.0 mN/m and around 3.7 mN/m for a subphase at pHs 8.5 and 5.5, respectively. This non-zero value may be attributed to intermolecular repulsive forces of an electrostatic nature [46]. The higher value at pH 5.5 suggests that oleosins exhibit a greater number of similarly charged species at the interface. This is consistent with the basic IP value (9.5) determined by Katavic et al. [6], suggesting a higher net overall charge of the protein at pH 5.5 than pH 8.5. However, this contrasts with the higher viscoelasticity modulus observed at pH 5.5 at the triolein/water interface, which suggested higher intermolecular attraction at this pH. The nature of the interface (oil/water versus air/water) may have been responsible for this discrepancy. Indeed, the conformation of a protein at an oil/water interface is different from that at an air/water interface [47].

For both pHs, a further compression of pure oleosin films gave rise to an inflection around 15 mN/m in the curve. This transition zone was attributed to a change in the molecular arrangement at the air/water interface [48]. When the film was compressed further, the surface pressure increased, reaching values around 48 and 42 mN/m at pHs 5.5 and 8.5, respectively, at the highest level of compression.

Mixed systems, in which phospholipids make up a small component (PL/OL = 0.02, w/w), had no effect on the overall shape of the isotherm, but they did effect specific changes. At pH 5.5, the initial plateau starts at zero, suggesting that electrostatic repulsions were abolished. The inflection point occurs at a higher surface pressure value around 20 mN/m and a clear collapse point appeared around 43 mN/m. All these changes show that the two compounds influence each other when they get organized at the interface and are thus in agreement with results from the study of kinetics and interfacial rheological properties. The isotherm shape was completely different for sample with higher proportion of phospholipids, confirming the significant effects of phospholipids on the interfacial organization of oleosins.

If the area is expressed as the mean molecular area of the components deposited at the interface, then mixed systems have mean molecular areas comprised between those of the single components.

We performed a thermodynamic analysis to get further information on the mixing behaviour and the intermolecular interactions between oleosins and phospholipids.

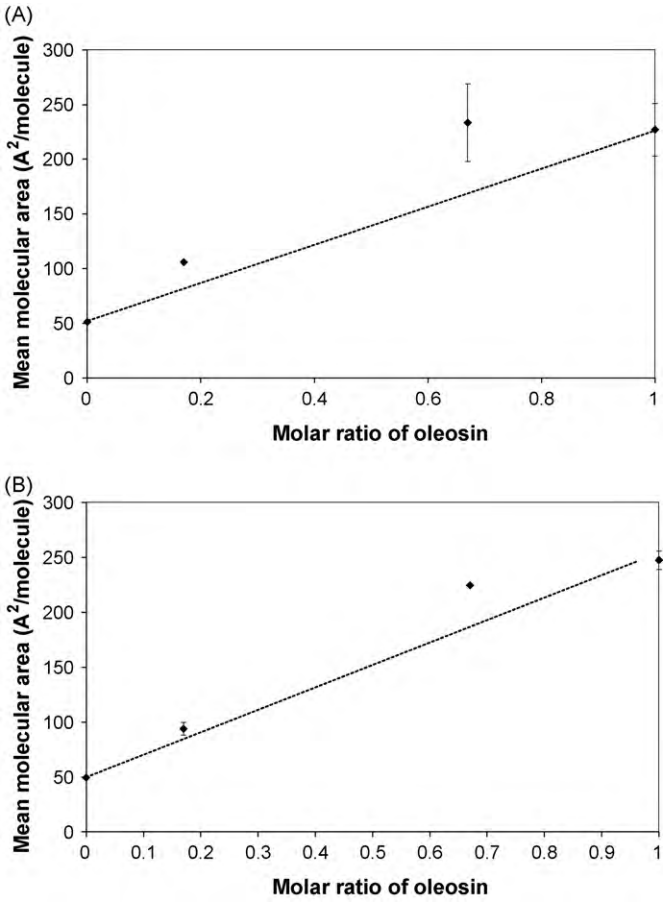

Fig. 7. Mean molecular area of mixed phospholipid/oleosin monolayers (Eq. (1)) as a function of the oleosin molar ratio. The line represents the additivity rule values. (A) Tris buffer subphase at pH 8.5 and (B) at pH 5.5.

For binary systems at interfaces, Eq. (2) defines the mean area occupied per molecule, at a defined surface pressure, in the case of an ideal behaviour (A_{id}) [48] (i.e. when the interfacial components are either immiscible or ideally miscible). This area corresponds to the sum of the molecular areas of the isolated components:

$$A_{id} = X_1 A_1 + (1 - X_1) A_2 \quad (2)$$

where A is the mean molecular area and X is the molar ratio.

In our case, subscripts 1 and 2 refer to oleosins and phospholipids, respectively. Any deviation of the observed molecular area from the A_{id} (dashed line in Fig. 7) for a defined surface pressure and a defined oleosins molar ratio may be attributed to specific interactions (i.e. excess interaction) between the two compounds [49].

The molecular area observed for mixed phospholipid/oleosin monolayers (X_1 = 0.17 for PL/OL = 0.2 and 0.67 for PL/OL = 0.02) at 20 mN/m revealed a significant positive deviation from the additivity rule (A_{id}) regardless of the pH of the subphase. This indicated partial mixing between the two components and the formation of a non-ideally mixed phospholipid/oleosin monolayer (Fig. 7).

Electrostatic interactions between the basic amino acid residues of oleosins and the overall negatively charged phospholipids may be at the origin of this miscibility and specific interfacial organization of the two components. They can also explain the synergic effect observed in kinetics and interfacial rheological properties studies.

4. Conclusion

Oil body reconstitutions and interfacial characterization of oleosins and phospholipids from rapeseed were performed to understand their role in the stability of oleosomes in aqueous medium. Oil bodies rich in oleosins were resistant to coalescence but quickly flocculate (low absolute zeta potential value), increasing the creaming rate. By adding phospholipids to oil bodies, the electrostatic repulsive forces increase (high absolute zeta potential value), avoiding flocculation. We have demonstrated that oleosins are needed to avoid coalescence. The synergic effect of oleosins and phospholipids at pH 5.5 can be explained by the increase of the dilational modulus making the interface more rigid and resistant to collapse.

It is unclear whether there is a direct correlation between dilational modulus and emulsion stability [50], but phospholipid/oleosin mixed systems stabilize emulsions with greater efficiency than oleosins or phospholipids separately. Adsorption kinetics and dilational modulus measurements highlighted the influence of phospholipids on the behaviour of oleosin at the interface. From the Langmuir monolayer experiment, we suggest that electrostatic interactions between the two compounds are at the origin of this effect and promote stable interfacial films.

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