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Oleosins of *Arabidopsis thaliana*: Expression in *Escherichia coli*, Purification, and Functional Properties

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The interfacial behavior of oleosins, the most abundant proteins from seeds oil bodies, was investigated using the pendant drop method at water/oil interfaces and compared to the behavior of β-casein and lysozyme, proteins with contrasted emulsifying properties. Recombined high (rS3) and low (rS4) molecular weight oleosins comprising N-terminal histidine tags were purified to electrophoretic homogeneity. rS3 decreased the interfacial tension at the oil/water interface better than rS4, oleosins being more efficient than β-casein. Oleosins formed aggregates when spread on noncompressed phospholipid (PL) films at the air/water interface as observed using a Langmuir−Blodgett balance equipped with a Brewster angle microscope. Oleosin spread at the surface of a compressed PL monolayer (5−20 mN/m) did not aggregate. Pressure increased immediately and proportionally to the amount of protein spread on the monolayer. The results stress the capacity of oleosins to be inserted in oil and in PL monolayers, which is of particular relevancy to their potential uses as water/oil emulsifiers.

KEYWORDS: Oleosins; *Arabidopsis thaliana*; water/phospholipid/air interface; air/lipid interface

INTRODUCTION

Plant seeds subjected to desiccation store triacylglycerols (TAGs) in oil bodies called oleosomes. Their mean diameter is comprised between 0.2 and 3 μm (*1*). They are composed of a central core of TAGs surrounded by a monolayer of phospholipids (PLs) in which different proteins are embedded (*2*−*4*).

Oleosins represent the most abundant proteins found at the surface of the lipid body. In oleaginous plant species, oleosins represent up to 10% of the seed proteins in weight (*5*, *6*). Located in vivo at the lipid/water interfaces, they would act as natural emulsifying/stabilizing agents (*7*−*12*), preventing the coalescence of oil bodies even during seed imbibition (*8*) and maintaining a high oil body surface accessible to lipases during germination. Oleosins are amphipatic proteins with a constitution of three domains. N- and C-terminal domains of variable length are amphiphilic, while the central domain of 72 highly conserved amino acids is highly hydrophobic and constitutes the longest hydrophobic fragment known to date (*7*, *13*). *Arabidopsis thaliana* (*A. thaliana*) genome contains 16 sequences coding for oleosins differing by the length of the N- and C-terminal regions (*12*). The conserved size of the central hydrophobic regions makes it likely that these proteins, with molecular masses between 10.7 and 53.2 kDa (*12*), will constitute a reservoir of emulsifying proteins with contrasted interfacial properties.

Numerous works have focused on the structure of oleosins and on their ability to be inserted into lipid particles, in vivo and in vitro. No data are however available in the literature about the surface properties of individual oleosins at the air/water or oil/water interfaces. Few individual oleosins have been purified to date from seeds (almond and peanut), and their strong tendency to polymerize (*14*) is very likely to hamper the purification of individual native oleosin from oil-rich seeds. *A. thaliana* is a model plant for oleaginous crop (rape), but the abundancy of oleosin in its seeds is offset by the miniature size of these plant seeds, which contain only four oleosins (*4*). Recombined purified proteins appear thus as a unique tool to investigate in vitro the functional properties of oleosins with different molecular weight and in vivo abundancy.

This paper reports on the surface properties of two bacterially expressed oleosins from *A. thaliana*, at the water/oil interface and on their insertion and effect on the surface tension of model air/PLs interfaces, to evaluate the potential of such abundant proteins as emulsifiers or foaming agents.

The interfacial properties of the two recombinant oleosins, one being the most abundant in *A. thaliana* oil bodies (S3) and a minor one (S4), were compared to those of two commercial proteins with distinct structures: an amphiphilic one (*15*), with loose structure and excellent emulsifying properties exploited in the food industry (β-casein), and a very compact one, with poor emulsifying properties (lysozyme).

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MATERIALS AND METHODS

Cloning of S3 and S4 Oleosins. DNA sequences coding for S3 (EMBL X62353) and S4 oleosins (EMBL X91956) were obtained by amplification using PCR and YAP clones (nonoriented, inserted in λZAPII, gifts from L. Lepiniec and V. Guyon, INRA, Versailles, France) as target. Primers OleoAT1ec1-1 (5′-g gaa ttc cat atg gcg gat aca gct aga gga-3′) containing a NdeI restriction site (underlined) and OleoAT1ec1-2 (5′-ggt ggc cag cac act act ctc gag cgg-3′) containing a XhoI restriction site (underlined) were designed for amplifying S3. primer N-terminal OleoAT2ec1-1 (5′-g gaa ttc cat atg gcg gat aca cac cgt-3′) containing a NdeI restriction site (underlined) and primer C-terminal OleoAT2ec1-2 (5′-ggg ggg acg acg gct gca ctc gag cgg-3′) containing a XhoI restriction site (underlined) were designed for amplifying S4. Amplification was performed in a minicycler apparatus (MJ Research), using Ready to go PCR bead (Amershan Pharmacia Biotech, Orsay, France), 1 U of Tfu DNA polymerase (Appligene, Illkirch, France), and 20 pmol of each primer. The program cycling conditions were as follows: 25 cycles of 1 min at 94 °C, 30 s at 60 °C for S3, or 52 °C for S4, 40 s at 72 °C. The amplified PCR fragments were inserted into the NdeI-XhoI cloning site of a pET-20b(+) vector from Novagen, so as to add a histidine tag at the C-terminal extremity of the protein. Plasmids were multiplied in JM109 strains. Transformants were selected on LB ampicilin medium, and plasmids were then sequenced. The resulting plasmids were then used to transform competent BL21pLys cells for expression of the protein, leading to rS3 and rS4 expression strains. Stocks of the saturated cultures were sampled and kept in glycerol 25% (w/v) at −80 °C.

Bacterial Expression of Oleosins in *Escherichia coli*. A 5 mL aliquot of a saturated preculture was used to inoculate 250 mL of LB medium containing 100 μg/mL ampicilin (LBA). The culture was performed at 37 °C in baffled Erlenmeyers under shaking (250 rpm) in a Infors shaking incubator. Protein expression was induced at 0.6 $A_{600\,nm}$ by the addition of IPTG (0.1 mM; Euromedex, Mundolsheim, France). Cells were harvested by centrifugation at 5000 g for 5 min and washed with 50 mL of 0.9% NaCl and then suspended in 8 mL of 0.1% Triton X-100 and kept at −20 °C at least overnight.

Oleosin Purification. The purification procedure was based on affinity for the polyhistidine tag from a resin containing nickel (Ni−NTA technology from Qiagen).

A bacterial pellet from a 200 mL culture was thawed at 30 °C in 25 mL of buffer B (0.1 M NaH_2PO_4, 10 mM TrisHCl, pH 8 containing 8 M urea), containing one tablet of antiproteases (EDTA free, from Roche) for 30 min under shaking (250 rpm). The supernatant obtained upon centrifugation 20 min at 4 °C and 9000*g* of a bacterial lysate was supplemented with 2 mL of ethanol and mixed with 6 mL of Ni−NTA slurry. Binding of the protein to the resin was obtained by successive incubations of the supernatant with the resin, followed by washes (three times) with 20 mL of buffer D (0.1 M NaH_2PO_4, 10 mM TrisHCl, pH 5.9 containing 8 M urea).

Oleosins were eluted with 10 mL of buffer E (buffer D acidified to pH 4.5). Fractions of 0.5 mL each were collected, until $A_{280\,nm}$ was nil. The quantity of protein purified was estimated using $\epsilon_{280\,nm} = 14\,650$ $M^{-1}\cdot cm^{-1}$ for rS3 and $\epsilon_{280\,nm} = 15\,930$ $M^{-1}\cdot cm^{-1}$ for rS4 oleosin.

Western Blotting. Proteins were separated by SDS−PAGE according to the method of Laemmli (*16*) using Nu-Page Bis Tris 12% gels (Novex) in the presence of 2% (w/v) SDS and 5% (w/v) β-mercaptoethanol. The gels were either stained with Coomassie blue G-250 according to Neuhoff et al. (*17*) or transferred to a membrane immobilon-P from Millipore according to the method of Laurière (*18*). The recombinant oleosins were then detected using antibodies raised in a mouse against pentahistidine proteins (pentahistidine antibody BSA free, QIAgen) (working dilution, 1/200000) with goat anti-mouse immunoglobulins coupled with phosphatase alkaline (Biosys, working dilution, 1/800) as secondary antibodies. Blots were revealed using NBT/BCIP (Roche; nitrotetrazolium blue/5-bromo-4-chloro-3-indolyl phosphate).

Protein Solubilization. Recombined oleosins were either solubilized in buffer E during the purification process or in chloroform/methanol (95/5 (v/v)) (*11*). To solubilize oleosins in chloroform/methanol, one volume of purified proteins in urea was incubated with one volume of chloroform/methanol at 50 °C during 30 min in a dry bath. After centrifugation the upper aqueous phase was discarded.

Surface Tension Measurements at Oil/Water Interfaces. Experiments were carried out on a contact angle DSA10, drop shape analysis system from Krüss. The 15 μL drops of proteins solubilized in 8 M urea (protein concentration ranging from 0.025 to 0.2 g/L) were suspended in 2 mL of commercial sunflower oil filtered on a Sep-Pak silica cartridge (Waters Corp.) at 20 °C. Images of the drops were recorded every 10 min during 2 h. Data were analyzed with the drop shape analysis DSA1 version 1.65 (Krüss) software. The interfacial tension (γ) was determined by fitting the drop profile according to the Laplace equation. Parameters used for analysis were the diameter of the syringe (1.54 mm), the oil density (0.912), and the buffer density (1.126).

Time-dependent variation of interfacial tension was analyzed upon fitting the experimental data with the phenomenological parameters of the following equation: $\gamma(t) = \gamma_0 + \gamma_1 e^{-(t/T_1)} + \gamma_2 e^{-(t/T_2)}$. Fitting was performed using the Marquard algorithm of the nonlinear fitting routine of the Slide Write for Windows software (Encinitas, CA).

Characterization of Air/Lipid Interfaces. *Compression Isotherms*. Compression isotherms were recorded for the monolayers on a NIMA 601 trough (NIMA) equipped with a Wilhelmy plate for monitoring the surface pressure (*P*). The entire balance is housed to avoid contamination, air currents, and to reduce surface evaporation. The temperature of the trough was maintained at 20 °C. Before each experiment, the Teflon trough (trough areas: $A_{min} = 30$ cm^2; $A_{max} = 700$ cm^2) and the barriers were carefully cleaned with chloroform and rinsed 3−4 times with water. The surface was compressed with two coupled barriers at a compression rate of 50 cm^2/min. The balance was filled with ultrapure water filtered trough 0.22 μm filters (Millipore, Molsheim, France).

A 70 μL aliquot of 1 g/L phospholipids in chloroform (soy lecithin (PC) > 99%, Fluka) were spread by using a microsyringe on the aqueous surface. After 30 min equilibration, proteins were added directly either on the noncompressed or on the compressed PL monolayer. In this last case, the PLs surface was compressed until the pressure reached 5 or 20 mN/m. After 30 min equilibration, the rS3 oleosins dissolved in chloroform were spread at different locations on the film (100 μL at 0.5 g/L). After a new equilibration period (30 min) the pressure isotherms were recorded.

Brewster Angle Microscope. The adsorbed monolayer texture was investigated by coupling a Brewster angle microscope (BAM; NFT, Nanofilm Technologie, Göttingen, Germany) to the film balance. The observation at the Brewster angle (53.1°) of the reflected intensity allowed one to follow changes in the thickness or density of the monolayer. The Brewster angle microscope was equipped with a visible laser diode (wavelength, 688 nm) as the light source and an optical unit that includes a polarizer, an analyzer, and a CCD camera. The image resolution was about 10 μm; the size of the surface observed was 4 × 5 mm.

RESULTS

Expression Purification of rS3 and rS4 Recombinant Oleosins. Oleosins were found to be expressed in *Escherichi coli* (*E. coli*) as inclusion bodies as deduced from the SDS−PAGE analysis of bacterial pellets obtained upon bacterial lyses (data not shown). Attempts to solubilize oleosins from these pellets with 8 M urea failed, unless inclusion bodies were incubated for 20 min at 4 °C. Both oleosins became soluble under these conditions and could be subjected to further purification using Ni−NTA gels. The use of successive binding/washing cycles on the gel allowed finally purification of both proteins to 95% purity for rS3 and 98% for rS4 (**Figure 1**). In typical purification 2 mg of oleosins was obtained from 150 mL cultures. Anti-pentahistidine antibodies recognized the purified proteins upon Western blotting (bands corresponding to 19 and 30 kDa peptides for rS3 and proteins bands corresponding to 22 and 35 kDa rS4 upon; **Figure 1**).

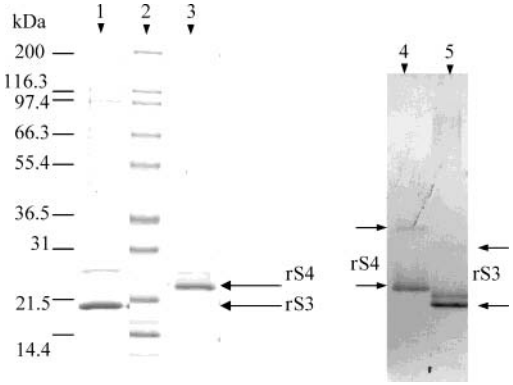

Figure 1. Bacterially expressed histidine-tagged rS3 and rS4 oleosins were purified using metal chelate chromatography. Purified proteins were analyzed using SDS–Page. (1) rS3, 12 μg; (2) molecular weight marker; (3) rS4, 8 μg [upon transfer, proteins were detected using antibodies anti-pentahistidine (arrows indicate position of monomers and dimers of oleosins)]; (4) Western blot performed on purified rS4; (5) Western blot performed on purified rS3.

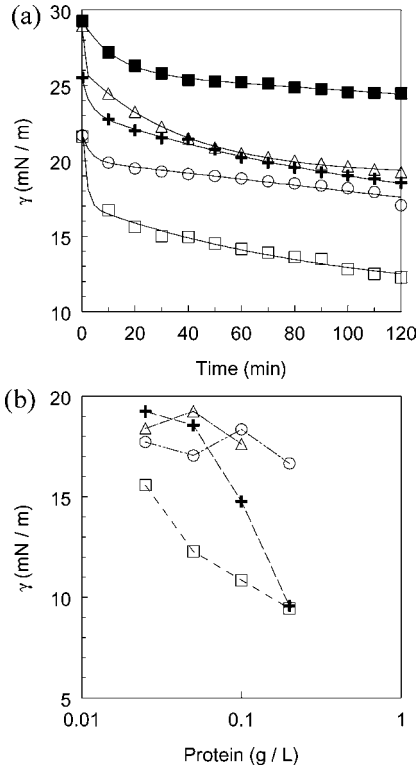

Figure 2. Interfacial tension measurements at the sunflower oil/buffer E interface using the pendant drop technique. Oleosins: rS3 and rS4, β-casein, and lysozyme, solubilized in buffer E, were introduced in a 3 mL glass cuvette containing 2 mL of sunflower oil. (**a**) Time dependence of γ (drop images were recorded every 10 min during 2 h) at 0.05 g/L proteins; (**b**) concentration dependence of the "equilibrium" value of γ. Buffer E, ■; lysozyme, △; rS3, □; rS4, +; β-casein, ○.

Surface Tension of Oleosins at Water/Oil Interfaces. Pendant drops of protein solutions in buffer E were suspended in oil in order to investigate the influence of the protein bulk concentration and to compare the kinetics relative to different proteins (oleosins rS3 and rS4, β-casein, and lysozyme, **Figure 2**). At a concentration of 0.05 g/L (**Figure 2a**), oleosins rS3 and rS4 and β-casein affect the interfacial tension (γ) within the first 10 s following the drop formation. Indeed, the first measurement performed indicated a decrease of γ from 30 mN/m (value corresponding to milli-Q water/sunflower oil) to 25.5, 21.7, and 21.7 mN/m for S4, β-casein, and S3, respectively. During these first 10 s no significant effect was observed with the buffer alone or with the lysozyme solution. The almost constant γ value or "equilibrium" value reached after 2 h was much lower for rS3 (12.3 mN/m) than for lysozyme, rS4 oleosin, and β-casein (respectively 19.2, 18.5, and 17.0 mN/m). A slight evolution concerning the buffer alone was observed (18% lower than the initial value), which was negligible compared to the effect of the proteins.

As shown in **Figure 2a** all the experimental data could be fitted reasonably well with the equation [$\gamma(t) = \gamma_0 + \gamma_1 e^{-(t/T_1)} + \gamma_2 e^{-(t/T_2)}$]. The introduction of a second exponential term in the equation was necessary to fit β-casein and rS3 and rS4 oleosins. The two exponential decays are interpreted as a rapid coverage of the interface followed by a slower step corresponding to the reorganization of proteins at the interface. The amplitude of this second term became important for both oleosins, especially for rS3. As already pointed out, the interfacial tension was decreasing within seconds for all proteins but lysozyme. Considering the influence of the bulk protein concentration on the "equilibrium" γ value (**Figure 2b**), both oleosins exhibited singular behavior compared to the two reference proteins. While no significant change in γ was observed for lysozyme and β-casein in the concentration range considered, γ was still a decreasing function of the bulk protein concentration for both oleosins. At 0.2 g/L the same value of γ = 9.5 mN/m was reached for rS3 and rS4.

Compression Isotherms and BAM Images of Oleosins at Water/Phospholipid/Air Interfaces. Purified rS3 oleosins solubilized in chloroform/methanol were carefully spread on the clean water surface of the Langmuir balance. No surface tension modification could be recorded under compression of the surface, while white patches were simultaneously observed with the BAM (**Figure 3a**).

The compression isotherm of the phosphatidylcholine monolayer is presented in **Figure 4**, curve 1. During the compression step, the texture of the image observed with the BAM remained uniform but the overall intensity increased from dark gray to light gray (not shown). When 50 μg of oleosins, solubilized in chloroform/methanol, were spread on the PC gas-phase monolayer (P = 0 mN/m), a slight increase of the pressure was observed. The corresponding compression isotherm, **Figure 4**, curve 2, was shifted upward with respect to curve 1 and developed an inflection point at 26 mN/m. On the BAM images, recorded simultaneously, white stripes appeared with increasing pressure as illustrated in **Figure 3b** at P = 15 mN/m.

A 50 μg amount of oleosins, solubilized in chloroform/methanol, was spread onto a compressed PC monolayer at P = 5 mN/m or P = 20 mN/m. In both cases, an immediate increase of the surface pressure, +7 or +5 mN/m respectively, was noticed. The two compression isotherms shown in Figure 4, curves 3 and 4, exhibited an inflection point around 28 and 30 mN/m. The BAM images recorded on compression remained uniformly gray even for a pressure of 25 mN/m (**Figure 3c**). When 100 μg of oleosin was spread on a compressed PC monolayer at P = 5 mN/m, an immediate increase of 17 mN/m was observed, i.e. about two times the value obtained with 50 μg. The inflection point at 28 mN/m was more visible (**Figure 4**, curve 5).

The spreading of a mixture of oleosins and PC incubated overnight in pure chloroform on the water surface led to the same compression isotherms as those recorded for oleosins spread onto a noncompressed PL film (**Figure 4**, curve 2). The

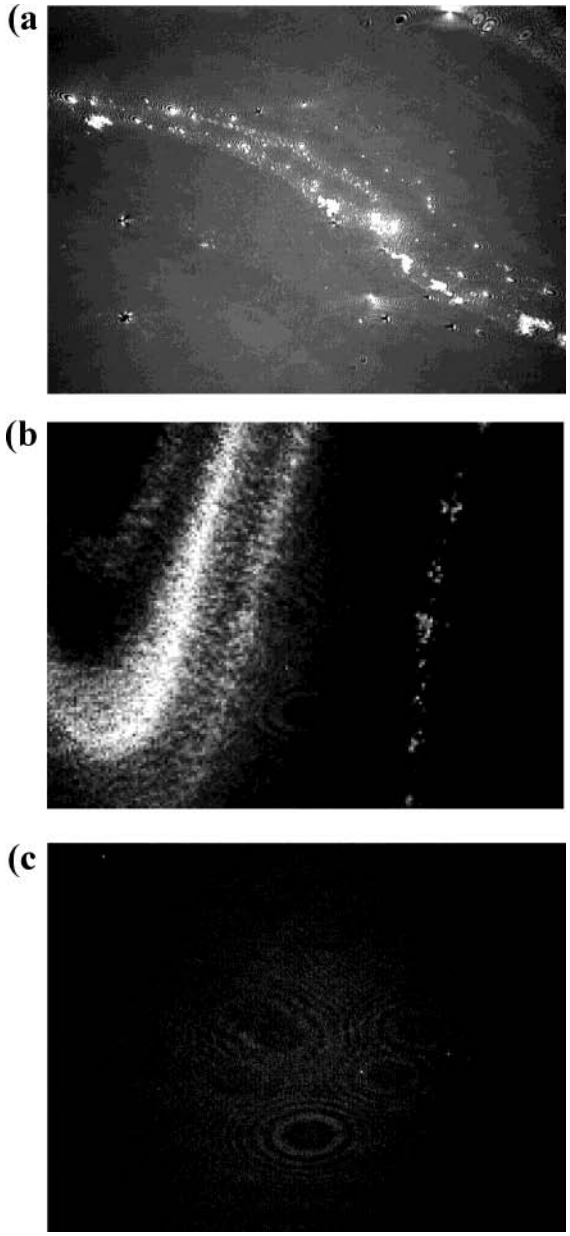

Figure 3. BAM images for rS3 solubilized in chloroform/methanol and spread on different surfaces: (**a**) rS3 on the water surface; (**b**) rS3 on a noncompressed PC monolayer (image taken at a pressure of 15 mN/m during the compression); (**c**) rS3 on a compressed film of PC monolayer ($P = 5$ mN/m; image taken at a pressure equal to 15 mN/m during the compression).

BAM images recorded also showed white stripes, as already obtained in **Figure 3b**.

DISCUSSION

Oleosins were mainly expressed in *E. coli* as inclusion bodies. These proteins were hard to solubilize in concentrated urea solutions, except at low temperature. This temperature-dependent solubilization reflects preeminent hydrophobic interactions, which arise from the structure of oleosins themselves, especially as these proteins contain the longest hydrophobic region known to date (*4*). The existence of dimeric oleosins solid enough to resist the denaturating conditions of SDS−PAGE is not surprising and may be due to interactions between the long hydrophobic regions of the protein. S3 oleosin contained 1 GG7, and 2 GA4, while S4 oleosins contained 1GG7, 4 GG4, and 5 GA4

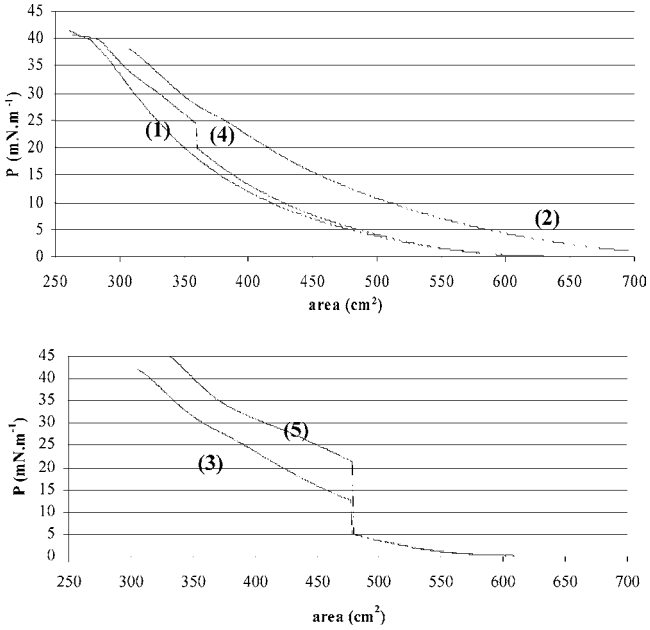

Figure 4. Compression isotherms: (1) PC alone on water; (2) 50 μg of rS3 added to a PC monolayer at an initial pressure of 0 mN/m; (3) 50 μg of rS3 added to a PC monolayer at an initial pressure of 5 mN/m; (4) 50 μg of rS3 added to a PC monolayer at an initial pressure 20 mN/m; (5) 100 μg of rS3 added to a PC monolayer at an initial pressure of 5 mN/m.

motifs, which constitute a framework for transmembrane helix--helix association (*19*). Such dimeric oleosins have already been described by Pons et al. (*14*). The extremely hydrophobic character of these proteins is confirmed by the fact that they could be solubilized in chloroform/methanol (95/5). Such behavior has already been described by Beisson et al. (*11*) to solubilize an almond oleosin (about 15 kDa). Almond oleosin has shorter hydrophilic domains (e.g. N- and C-terminal regions) than *A. thaliana* oleosins. *A. thaliana* oleosins described in this study are less hydrophobic than almond oleosin and consequently were also found at the urea/chloroform interface.

Commercial sunflower oil, even after filtration, was certainly not completely free of surfactant molecules, which led to a slow and limited decrease of the interfacial tension at the urea/oil interface in the absence of any added protein. This effect could be considered almost negligible compared to the effect upon addition of proteins. The behavior of β-casein, rS4, and especially rS3 oleosin was clearly at least biphasic, with two steps exhibiting different amplitude and relaxation times. The rapid decrease of the interfacial tension observed for oleosins, especially for rS3 with respect to the other proteins described in this study, is remarkable. This may be interpreted as the result of a fast insertion in the lipid of oleosins already present close to the oil/water interface. This high affinity for the oil/water interface is in good agreement with their natural position in oleosom. S3 has been classified as a low molecular weight oleosin and S4 as a high molecular weight oleosin (*20*). The better capacity of rS3 oleosin (19.6 kDa) to decrease γ at the water/oil interface with respect to rS4 (22.4 kDa) confirms the finding of Tai et al. (*21*) who observed a better stabilization of oil/PL/water emulsions by low molecular weight oleosins. Moreover, it is remarkable that S3 oleosin, the most abundant in oil bodies (*4*), is exhibiting the best capacity to decrease γ at the water/oil interface. Oleosins were able to interact with TAGs, as they decreased γ at the oil/water interface, in the presence of urea. It is possible to hypothesize that the central hydrophobic

domain of the protein is capable of penetrating into the oil bodies and reaching the TAGs, confirming the structural model proposed by Qu and Huang (*22*). As the critical micellar concentration (CMC) is generally increased in urea solutions (*23*), the observed capacity of oleosin to decrease γ may certainly be improved in a system containing less urea.

Oleosins were not capable of inserting themselves at air/water interfaces. Due to their extremely poor solubility in water, they rapidly formed aggregates of high polymerization degree, visible using BAM, and certainly sunk to the bottom of the Langmuir balance reservoir. To better understand the behavior of oleosin at interfaces, we choose to study their insertion in PL films. When oleosins were added on a gas phase of PLs, they formed aggregates. These results were presumably due to the presence of water at the surface of the interface. On the other hand, when oleosins were added to a liquid or solid phase of PLs, no more water being available at the surface of the interface, no aggregates were observed using the BAM. The interactions of oleosins with PLs stabilized the water/air interface and modified its mechanical properties. The impossibility to obtain films from a preincubated PL/oleosin mixture in chloroform/methanol stresses the fact that oleosins can only be introduced in PLs if PLs are already organized in monolayer.

A thaliana is a model organism for oil seeds, with entirely sequenced genome, freely available to the scientific community. Oleosin, an abundant protein in oil seeds, belongs to a multigenic family, comprising 16 members in the *A. thaliana* genome. This work is the first step in the use of a multigenic family of proteins with a conserved central hydrophobic region, and N- and C-terminal variable amphiphilic regions, aimed at analyzing the correlation between interfacial properties and structural features of oleosins.

To improve our understanding of the interfacial properties of oleosins, more experimental work will be needed using individual low and high molecular weight oleosin, soluble in organic solvents or in aqueous media. These proteins will be obtained either from oil seeds or from recombined microorganisms.

ABBREVIATIONS USED

BAM, Brewster angle microscope; CMC, critical micellar concentration; *P*, surface pressure; PC, phosphatidylcholine; PLs, phospholipids; rS3, recombined oleosin S3; rS4, recombined oleosin S4; TAG, triacylglycerol; γ, interfacial tension.

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