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Caleosin of *Arabidopsis thaliana*: Effect of Calcium on Functional and Structural Properties

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A non-radioactive blot binding assay has proved the capacity of a purified recombinant form of *Arabidopsis thaliana* caleosin (AtClo1), a key protein of this plant oil body, to bind calcium. Calcium affected recombinant caleosin aggregation state, solubility, and electrophoretic mobility on SDS-PAGE. The effect of calcium on interfacial behavior of recombinant caleosin was studied at three interfaces: air/water (A/W), purified oil/water (O/W), and air/phosholipid/water (A/PLs/W). Recombinant caleosin was able to decrease interfacial tension (IFT) at A/W and O/W interfaces as a function of concentration and calcium, whereas no interaction was detected at the A/PLs/W interface. Effect of calcium was time dependent, and its amplitude strongly varied with the interface considered. Reconstituted oil bodies were used to prove the involvement of recombinant caleosin in their calcium-driven aggregation and coalescence. Calcium ions at concentration as low as 100 nM were able to strongly modify the shape and aggregation state of purified oil bodies, as well as their behavior within a monolayer, reflecting potentially profound changes in their structure and dynamic.

KEYWORDS: *Arabidopsis thaliana*; emulsion; oil bodies; lipid bodies; caleosin; calcium; interfaces; dynamic light scattering; interfacial properties

INTRODUCTION

In oilseeds, lipids are stored in specialized organelles called lipid or oil bodies (OB). They comprise a nonpolar core composed of neutral lipids covered by a monolayer of phospholipids and various proteins (*1, 2*). Most abundant OB proteins are called oleosins. These proteins, unique to plants, belong to a multigenic family and are found in various organs (*3*). Their conserved triblock structure comprises N- and C-terminal hydrophilic regions of various lengths and a central conserved hydrophobic region. This conserved amphiphilic organization is reflected by their capacity to insert themselves at various interfaces (*4*) and to stabilize artificial oil bodies (*5*) and, finally, by the importance of oleosins to control OB structure and seed lipid content (*6*).

Another type of protein, called caleosins, are also important in the structure and stability of OBs. Among caleosins, AtClo1, a 27 kDa protein encoded by *At4g26740*, constitutes <10% of total OB proteins (*7, 8*). It also belongs to a multigenic family and shares a conserved amphiphilic organization with oleosins, its central hydophobic region being shorter (30 residues versus 70−80 for oleosins). *Arabidopsis thaliana* seed caleosin 1 (AtClo1) harbors a conserved EF hand domain, probably involved in calcium binding and post-translational modifications such as disulfide bridge and partial serine phosphorylation (*9*). Chen et al. (*10*) have proved that caleosin was capable of stabilizing emulsions composed of triacylglycerol (TAG), phospholipids (PLs), and water. The size of the droplets was apparently smaller than those containing oleosins. Poxleitner (*11*) demonstrated that during germination, AtClo1, involved in the interaction of oil bodies with vacuoles, plays a role in the degradation of storage lipid in oil bodies.

Calcium binding capacity of caleosins has been proved by Takahashi et al. (*12*) for AtClo3 (encoded by *At2g33380*) isolated from *A. thaliana* and by Chen et al. (*13*) for caleosin from *Sesamum indicum*. Calcium ions are capable of promoting aggregation of purified OB (*14*) and are predicted to play an important role in OB aggregation and regulation during biogenesis processes (*15*). Calcium is known as an important cellular second messenger and, thus, it was proposed that calcium and in consequence caleosin could play a role in the biogenesis or degradation of lipid bodies (*16*).

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Oil bodies, when dispersed in aqueous media, represent a natural emulsion system, and an understanding of its stability is necessary before strategies can be developed to use them in food or cosmetic preparations. We have checked the capacity of the recombinant purified form of caleosin to bind calcium and have studied the impact of the binding of this ion on the solubility of recombinant caleosin. To better understand how AtClo1 interacts with the various components of OB, and to check whether this behavior was affected by calcium, we have performed a detailed study of the insertion of recombinant caleosin at various liquid interfaces using the pendant drop method and Langmuir balance. We also have studied the influence of calcium on the interfacial behavior of purified oil bodies and on the aggregation of purified or artificial oil bodies containing recombinant caleosin.

MATERIALS AND METHODS

Purification of Recombinant Caleosin. Cloning, bacterial expression, purification, and immunodetection of recombinant caleosin have already been described in Purkrtova et al. (*9*). Recombinant caleosin was expressed in two forms, as a recombinant protein bearing six histidines at its C terminus (rClo1.his) or as a recombinant protein without any fusion tag (rClo1).

Purification of Lipid Bodies. OB were purified (FC4, fraction 4) from *A. thaliana* mature seeds (Colombia) as described in Jolivet et al. (*7*).

Artificial Oil Body (AOB) Reconstitution. The sonication process was adapted from the protocol of Tai et al. (*17*) to reconstitute AOB solution. Purified oil (7.5 mg) and 0.5 mL of caleosin solution in water were sonicated on ice in a 2 mL Eppendorf tube with a 3 mm titan probe connected to a digital sonicator (Branson Digital Sonifier). Amplitude was set at 10% for 30 s (pulse on, 2 s, 3−5 W; pulse off, 0.5 s), the sample was left on ice for 5 min, and then sonication was repeated twice. Microscopy was performed with an Olympus BX 51 light microscope with a 100× oil immersion objective. For recording the Photometrics CoolSNAP software was used. Size measurements were performed using ImageJ (http://rsb.info.nih.gov/ij/).

Calcium Binding. *Electrophoretic Mobility Shift Assay.* A mobility shift assay was carried out using the protocol of Chen (*14*), with slight modifications. Final concentration of all effectors was 2.5 mM. Ten microliters of rClo1 (10 μg) in 50 mM Tris buffer, pH 8, was mixed with 0.53 μL of 50 mM EDTA (Promega, Madison, WI), pH 8, or with 0.26 μL of 0.1 M $CaCl_2$, pH 8 (Fluka, Steinheim, Germany), and incubated for 10 min at room temperature. The EDTA-treated rClo1 was further incubated with 0.27 μL of 0.1 M $CaCl_2$, $MgCl_2$, or KCl for 10 min at room temperature. Effects of the diverse treatments on rClo1 migration were analyzed on 4−12% gradient SDS-PAGE gel using MES buffer system (Invitrogen, Cergy Pontoise, France), followed by Coomassie blue staining.

Fluorescence Assay. We used the capacity of Quin2 (Calbiochem, Nottingham, U.K.), a dye capable of fluorescing in the presence of Ca^{2+}, to assess calcium binding by rClo1.his bound to PVDF membranes, as described by Tatsumi (*18*). Proteins were resolved by SDS-PAGE and transferred on PVDF membrane (Millipore Corp., Saint Quentin Yvelines, France). The membrane was washed at 4 °C overnight with a solution containing 60 mM KCl, 5 mM $MgCl_2$ and 10 mM imidazole, pH 6.8. Washing was repeated for 2 h with fresh solution, and the membrane was incubated with the same solution containing 1 mM $CaCl_2$. The membrane was rinsed three times for 6 min in 20% ethanol and finally in Milli-Q grade water. Finally, the membrane was incubated with a solution containing 1 mM Quin2 for 1 h at room temperature. Membrane fluorescence was revealed using UV light at 305 nm.

Effect of Calcium Ions on Caleosin Aggregation State and Solubility. *Dynamic Light Scattering.* Dynamic light scattering was carried out with a Malvern HPPS (High Performance Particle Sizer, Worcestershire, U.K.). The scattering angle was fixed at 173°, the laser wavelength was at 633 nm, and the temperature was set at 25 °C. The scattered light intensity was also recorded as a function of time. Instrument accuracy was checked with 200 nm latex beads (Duke Scientific, Palo Alto, CA).

Solubility Study. rClo1.his (10 μg) was mixed with aliquot amounts of 1 M $CaCl_2$ solution to reach the desired final calcium concentration. After 5 min of incubation at room temperature, the solution was spun at 20000*g* for 1 min at room temperature. The presence of rClo1.his in the supernatant and pellet was checked by SDS-PAGE analysis using 4−12% NuPAGE gel (Invitrogen, Cergy Pontoise, France) and MES running buffer.

Surface Tension Measurements at Various Interfaces Using Pendant Drop Technique. Experiments were carried out at 23 °C on a contact angle DSA10, Drop Shape Analysis system from Kruss (Hamburg, Germany). Data were analyzed with the drop shape analysis DSA1 version 1.9 (Kruss) software. When needed, a solution of $CaCl_2$ or EDTA (in 20 mM Tris buffer, pH 8) was added to the protein solution just before measurement or after incubation at 4 °C, the final concentration being 1 mM. 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), buffer density (1.126 g/cm^3), and oil density (0.912 g/cm^3). Results are given with an accuracy of ±0.5 mN/m at the A/W interface and 1 mN/m at the O/W interface. Experiments were performed as duplicates.

A/W Interface. An 18 μL drop was created from rClo1.his (concentration ranging from 0.01 to 0.26 g/L) in 20 mM Tris buffer, pH 8. Images were recorded every 3 min for the first 0.5 h and then every 15 min for the next 90 min.

O/W Interface. A 15 μL drop of rClo1.his in 20 mM Tris buffer, pH 8 (protein concentration ranging from 0.01 to 0.24 g/L), was suspended in 2 mL of rapeseed oil (commercial, Auchan, France) purified using a Sep-Pak Silica Cartridge (Waters Corp., Milford, MA). Images of the drop were recorded every 10 min.

Characterization of A/W and A/PLs/W Interfaces Using Langmuir Balance. Pressure−area isocycles were recorded at a compression rate of 50 cm^2/min on a NIMA 601 trough (NIMA Technology, Coventry, U.K.) equipped with a Wilhelmy plate for monitoring the surface pressure. The entire balance is housed to avoid contamination and 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 three to four times with Milli-Q grade water. All solutions were prepared from grade water filtered through 0.22 μm filters. Experiments were performed as duplicates.

Behavior of Recombinant Caleosin at A/PLs/W Interface. A 500 μL aliquot of 100 g/L phospholipids in chloroform [soy lecithin (PC) > 99%] was spread by using a microsyringe on the aqueous surface. After 30 min of equilibration, proteins were added directly on the surface. After 30 min of equilibration, rClo1.his (100 μL of concentration 0.5 g/L dissolved in 20 mM Tris buffer, pH 8) was spread at different locations on the compressed phosholipid monolayer (5 mN/m). After a new equilibration period (30 min), the pressure isotherms were recorded. When the effect of calcium ions was investigated, 600 μL of 1 M $CaCl_2$ stock solution (in 20 mM Tris, pH 8) was spread on the equilibrated surface of lecithin with rClo1.his. After 30 min of equilibration, the pressure−area isocycle was recorded.

Behavior of Purified Oil Bodies at the A/W Interface. The trough was filled with either 20 mM Tris, pH 8, or Milli-Q grade water. A suspension of OB (fraction 4, 18.9 μg/μL) (*7*) or of lipids extracted from OB was spread on the liquid surface. The system was equilibrated until pressure reached a constant value (usually 30−60 min). Isocycles of compression then proceeded between 350 and 50 cm^2. Barrier displacement was engaged to decrease the surface. Barriers were moved backward before any collapse of the monolayer appeared.

RESULTS

Binding of Calcium Ions Affects Recombinant Caleosin Electrophoretic Mobility and Aggregation State. The calcium binding ability of caleosin was verified by using complementary electrophoretic mobility shift and fluorescence assays. As shown in **Figure 1A**, we proved the capacity of rClo1.his to fluoresce

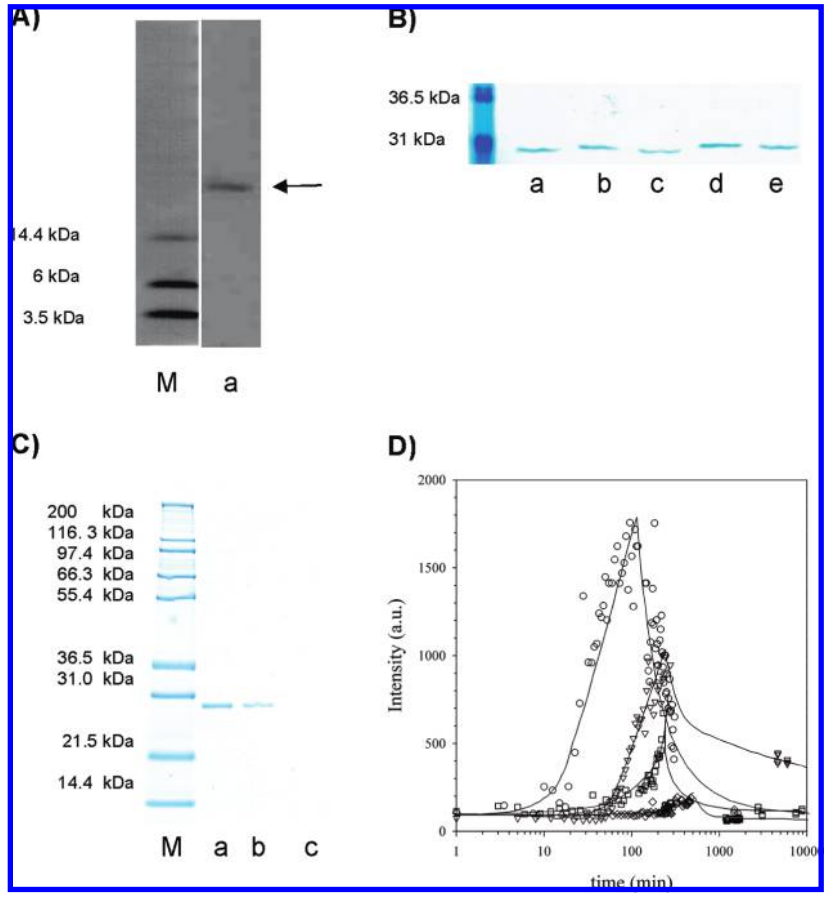

Figure 1. Effect of calcium ions on electrophoretic mobility and aggregation state of caleosin. (**A**) Fluorescence assay of calcium binding. Ten micrograms of purified rClo1.his (a) was separated using 12% gel. M indicates molecular weight standard. Arrow highlights positive signal of rClo1.his. (**B**) Electrophoretic mobility shift assay. Ten micrograms of purified rClo1 (a) was treated with 2.5 mM EDTA (b) or with 2.5 mM Ca^{2+} (f). EDTA-treated rClo1 was further incubated with various cations: Ca^{2+} (c), Mg^{2+} (d), K^{+} (e) at a final concentration of 2.5 mM. Label on the left indicates the molecular mass of proteins. (**C**) Influence of calcium ions on solubility of rClo1.his. Ten micrograms of purified rClo1.his (a) was treated with 20 mM Ca^{2+} and spun at 20000*g*, pellet (b), supernatant (c).(**D**) Influence of calcium ions on aggregation of rClo1.his. Average scattered intensity as a function of incubation time for different calcium chloride concentrations: (◇) 0.02 mM, (□) 0.1 mM, (▽) 1 mM and (○) 10 mM, caleosin concentration being fixed at 0.1 mg/mL.

in the presence of a specific fluorescence dye, Quin2, upon incubation with calcium. In **Figure 1B**, we proved that EDTA-treated rClo1 (lane b) migrated more slowly than nontreated protein (lane a). The mobility of rClo1 was recovered by the addition of Ca^{2+} ions to EDTA-treated rClo1 (lane c). Treatment with other divalent cations such as Mg^{2+} or K^{+} ions did not affect rClo1.his mobility (lanes d and e).

Although the electrophoretic mobility shift and fluorescence assays have proved the ability of rClo1.his to bind calcium, CD experiments realized previously in our laboratory revealed the absence of the effect of calcium binding on its secondary structure (*9*). Calcium ions, due to their divalent properties, can provoke aggregation of calcium binding proteins (*19, 20*). This is why we have investigated the effect of Ca^{2+} ions on the aggregation state of rClo1.his. The presence of rClo1.his in the pellet obtained after centrifugation of protein incubated with 20 mM Ca^{2+} revealed that this divalent cation promoted complete and immediate aggregation of the protein (**Figure 1C**). Similar results were obtained with 1 mM calcium (data not shown).

Caleosin in solution can aggregate in the presence of calcium ions, and precipitation of caleosin flocs may even occur. We have explored the influence of calcium chloride concentration (from 0.02 to 10 mM) on the aggregation of rClo1.his by static light scattering. In **Figure 1D** we have reported the average scattered intensity as a function of calcium chloride concentration, rClo1.his concentration being fixed at 0.1 g/L. The evolution of light intensity always exhibited a similar pattern. The intensity of scattered light first slightly varied with time (period I), then it sharply increased (period II), and finally an abrupt decrease of the intensity (period III) was observed. For concentrations varying from 0.02 to 1 mM maximal intensity varied 9−10-fold, and maximal intensity occurred between 100 and 500 min. The scattered intensity increases due to the formation of calcium-induced aggregates of rClo1.his. The subsequent growing of these aggregates leads to their precipitation. Consequently, they disappear from the investigated volume, and the scattered intensity decreases.

Calcium Ions Influence the Behavior of rClo1.his at Interfaces. *Air/Water Interface.* The interfacial behavior of rClo1.his at the air/water (A/W) interface was investigated in 20 mM Tris buffer, pH 8, to avoid possible protein aggregation in water. The interfacial tension (IFT) value for buffer was very close to the standard value of distilled water (73 mN/m at 20 °C) and was stable with time (**Figure 2**). As can be seen from **Figure 2A**, rClo1.his decreased IFT at the A/W interface in a time-dependent manner. The IFT decrease was dependent on the protein concentration (**Figure 2A**) and gets a maximum from a caleosin concentration of 0.1 g/L. Further increase influenced very slightly the caleosin ability to decrease IFT. By plotting IFT versus log time, it is possible to see that the process was at least biphasic (**Figure 2B**), with a first phase (I) of diffusion

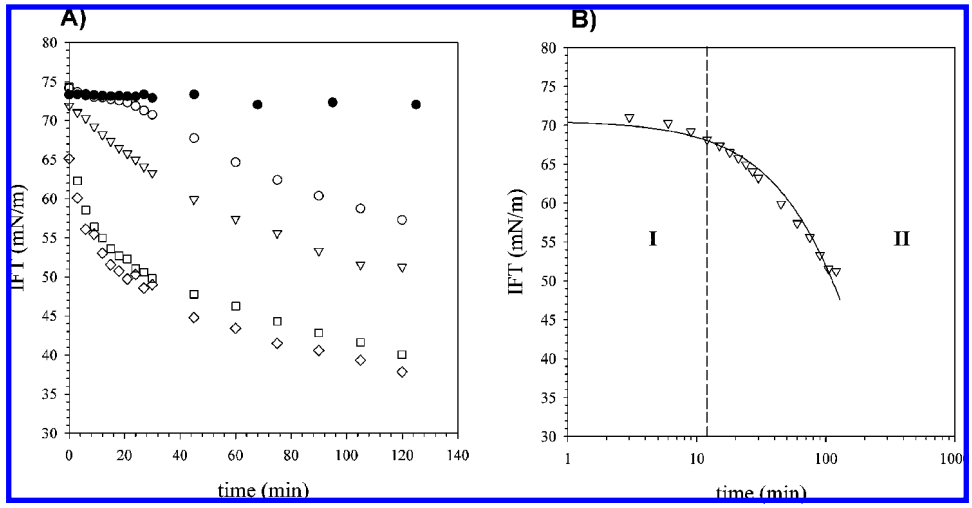

Figure 2. Effect of caleosin concentration on the time-dependent decrease of IFT at the air/water (A/W) interface. (**A**) rClo1.his concentration was 0 (●), 0.01 (○), 0.05 (▽), 0.1 (□), or 0.26 g/ L (◇). (**B**) rClo1.his (0.05 g/L) at the A/W interface was expressed using time logarithmic scale. I indicates the first phase of diffusion, and II indicates the second phase of adsorption.

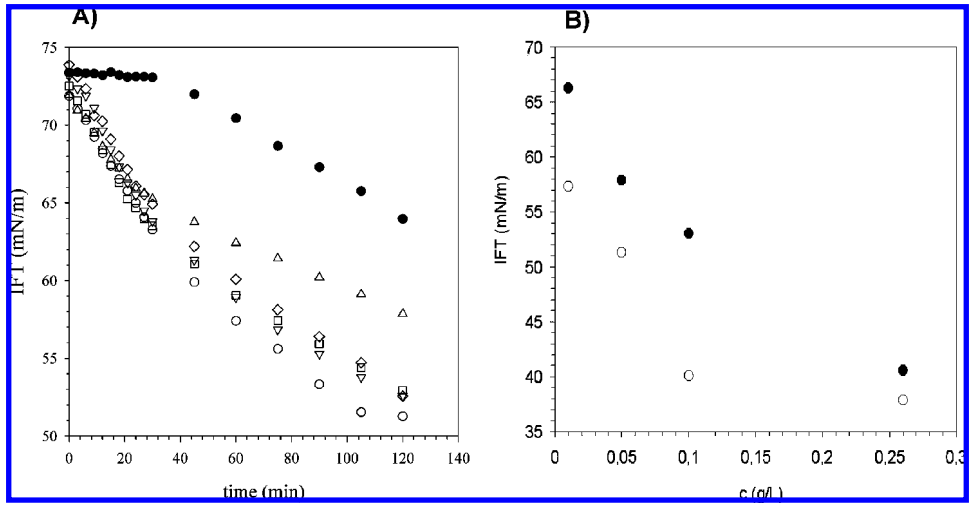

Figure 3. Influence of incubation time and protein concentration on calcium effect on the interfacial properties of caleosin at the air/water (A/W) interface. (**A**) Influence of incubation time. rClo1.his (0.05 g/L) was incubated with 1 mM calcium for 1 (▽), 8 (□), 24 (◇), or 120 (△) h before IFT measurement. Controls, IFT versus time curve for rClo1.his without calcium (○) and buffer with calcium ions (●), are shown for comparison. (**B**) Influence of protein concentration on calcium effect. rClo1.his was incubated for 120 h with (●) or without 1 mM calcium (○) before IFT measurement. IFT values are given after 120 min.

(until 1−2 min) and second phase (II) of adsorption. We can note that, in our experimental conditions, the third phase of rearranging and equilibrium could not be totally observed.

The effect of calcium ions on rClo1.his interfacial properties was studied. Short-time incubation with 1 mM calcium ions did not affect the behavior of rClo1.his at the A/W interface. rClo1.his was incubated with 1 mM calcium for 1, 8, 24, or 120 h (**Figure 3A**). After an incubation of 120 h with calcium, caleosin partially lost its interfacial properties. The IFT value (at 120 min) was 57.9 mN/m for protein incubated with calcium and 51.3 mN/m for the nontreated protein.

To summarize the effect of calcium on rClo1.his interfacial properties, we reported the IFT versus the concentration with or without calcium (**Figure 3B**). In the presence of calcium, rClo1.his is less efficient in decreasing IFT whatever the concentration.

Oil/Water Interface. Purified rapeseed oil containing only nonpolar lipids was used as a model interface mimicking the TAG core of oil bodies. As can be seen in the **Figure 4A**, rClo1.his possessed a good capacity to decrease IFT at the O/W interface, with a slower kinetics than at the A/W interface. This capacity was dependent on protein concentration (data not shown). The time-dependent IFT curve of rClo1.his at O/W exhibited two phases, with a transition point located around 80 min (**Figure 4A**).

Addition of calcium ions (1 mM) immediately before measurement did not affect the interfacial properties of rClo1.his (data not shown). However, when the protein solution was incubated for 18 h with calcium ions before interfacial tension measurements (**Figure 4B**), the protein partially lost its capacity to place itself at the O/W interface. This phemenon was reversible, as the incubation with EDTA, a calcium chelator, led to a time-dependent increase of rClo1.his capacity to decrease IFT at the O/W interface (data not shown).

Behavior of Caleosin at A/PLs/W Interface Is Not Affected by Calcium Ions. The study of the insertion of rClo1.his in a PLs monolayer ($P = 5$ mN/m) did not lead to any pressure increase and modification of the compression isotherms (data not shown). Addition of calcium (2 mM) and overnight incubation did not affect the isotherm. This behavior is surprising

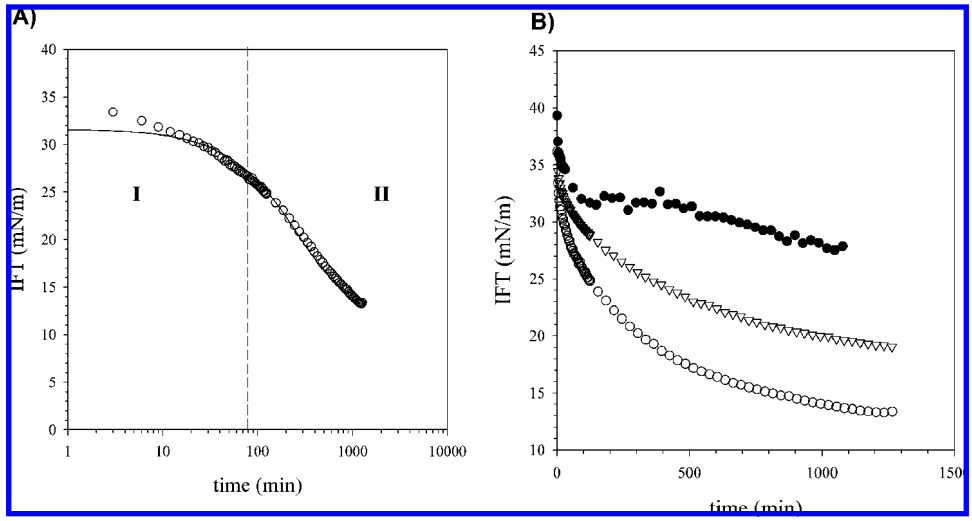

Figure 4. Interfacial properties of caleosin at the interface purified oil/water (O/W): (**A**) time dependence, rClo1.his (c = 0.24 g/L); (**B**) influence of calcium ions, rClo1.his (0.24 g/L) (○), rClo1.his incubated for 18 h with calcium ions (1 mM final concentration) (▽), or buffer (●).

in comparison to the interfacial structure of oil bodies comprising caleosins at the surface of the globular oil body. It can be explained by the fact that, in physiological conditions, the other proteins such as oleosin may help caleosin to anchor at the OB surface. Furthermore, the physiological mechanisms of caleosin anchorage (routing enzymes) are totally different from that existing in our model conditions based uniquely on physicochemical interactions.

Caleosin Stabilizes Artificial Oil Bodies. The effect of the addition of increased rClo1.his amounts to O/W emulsions is described in the lower panel of **Figure 5**. As deduced from droplet size measurement performed with ImageJ, emulsion mean diameter decreased with addition of rClo1.his (from 2.1 to 0.5 μm). Moreover, dispersity decreased, too. This is coherent with caleosin's ability to reduce interfacial tension at the O/W interface: interface stabilization with caleosin molecules leads to smaller size of droplets and thus to a better stabilization against creaming.

Calcium Ions Induce Caleosin-Mediated AOB and OB Aggregation. We previously observed the aggregation and precipitation of rClo1.his in solution induced by calcium chloride. We have studied the fate of AOB prepared with 0.1 or 0.05 g/L rClo1.his and then incubated with 1 mM calcium chloride. Microscopic observations (**Figure 5**, upper panel) were achieved for different incubation times. For AOB reconstituted with 0.1 g/L rClo1.his, the droplets tended to flocculate. For rClo1.his concentration equal to 0.05 g/L, droplet coalescence and flocculation were observed as well as a more important phase separation (creaming). These data confirmed that calcium induced AOB aggregation was due to the presence of rClo1.his. The same results have been obtained with native oil bodies isolated and purified from *A. thaliana* (FC4) incubated in the presence of 1 mM Ca^{2+}. Rapidly, and as already reported by Tzen (*13*), calcium induced aggregation of oil bodies (data not shown).

Calcium Influences the Behavior of Purified Oil Bodies at the A/W Interface. Compression isotherms of purified lipid bodies (fraction 4) spread on Tris surface are shown in **Figure 6A**. Isocycle exhibited between two and three phases, defined by the slopes of the curves. Collapse was not reached under our conditions. The OB compression isotherm comprised a transition located around 12 mN/m, which could be attributed to triglycerides (*21*). Isocycles performed with neutral lipid purified from OB gave rise to similar profiles, especially; the transition around 12 mN/m was still visible. This could be explained by the rupture of the OB structure at the surface, already described for egg yolk LDL (*22*).

Addition of calcium (final concentration = 100 nM) immediately before the isocycle was performed did not modify its shape (**Figure 6A**). The shape of isocycles recorded after 1 h, 4 h, or overnight incubation revealed the effect of calcium on the behavior of OB in monolayer and the slow kinetics of this process as well. Isocycles were shifted toward higher areas and pressures; the final pressure increase was dependent on the time of incubation. The shape of the isocycles was also modified. The transition associated with the triglyceride collapse disappeared. The lack of transition and the shift in area suggests the formation of small triglyceride domains surrounded by a network of proteins linked by calcium.

The reversibility of the process was assayed by removing calcium with the use of a specific chelator. A solution of EGTA (300 nM) was added to the lipid bodies (18 μL), which were already incubated with calcium ions (100 nM) overnight, and isocycle was performed (**Figure 6B**). No changes were visible upon addition of EGTA, even after overnight incubation. Calcium effect was not reversed in our conditions. To verify that the calcium effect was not contingent on Tris, we performed similar isocycles in water (**Figure 6C**). Eighteen microliters of OB were spread on the water surface. The corresponding isocycle differed strongly from the isocycle recorded for lipid bodies spread on Tris buffer. We could observe only a steep increase of pressure at the end of compression (from 5.4 to 12.8 mN/m). The effect of calcium (100 nM) on the isotherm was clearly visible even when lipid bodies were spread on the water surface.

DISCUSSION

The protein coat of lipid bodies is made up of integral proteins, predominantly oleosins, which stabilize OB and maintain their integrity. Caleosin is also an integral OB protein (*7, 8, 14, 16*), but its role remains unknown. In a recent work Chen et al. (*10*) demonstrated the capacity of sesame caleosin to stabilize AOB (i.e., a triphasic emulsion made up of oil, water, and phospholipids). Apparently, the diameter of caleosin-stabilized oil droplets was smaller than that of oleosin-stabilized ones, reflecting a better capacity of caleosin to stabilize emulsions (*23*). Calcium is known to be capable of inducing

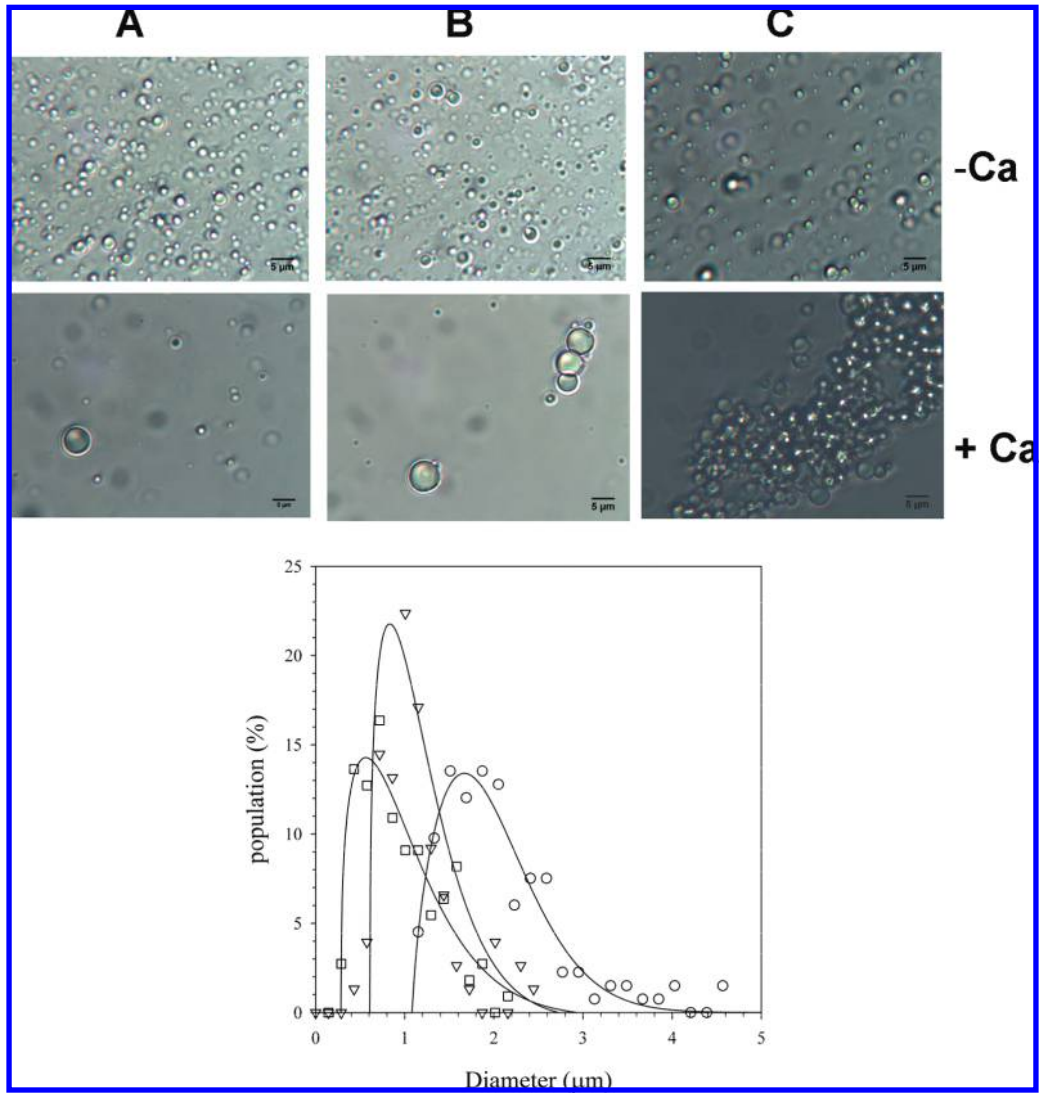

Figure 5. Stabilization of artificial oil bodies (AOB) by caleosin. (Upper panel) Microscopic observations (100× oil immersion) of reconstituted AOB: (**A**) without rClo1.his, (**B**) 0.05 g/L rClo1.his, and (**C**) 0.1 g/L rClo1.his and incubated for 24 h plus or minus 1 mM calcium chloride. (Lower panel) Effect of caleosin concentration on AOB diameter distribution. Caleosin concentration: (○) 0 g/L; (▽) 0.05 g/L; (□) 0.1 g/L.

lipid body aggregation (*13, 24*) and also increasing droplet size (*24*), but until now, the nature of the molecules interacting with this divalent ion has not been described. To better understand how caleosin is inserted within lipid bodies, and its behavior is modulated with calcium, we have studied its behavior at three model interfaces: A/W, O/W, and A/PLs/W.

Caleosin was capable of inserting itself within A/W and O/W interfaces, as reflected by the IFT decrease recorded in hanging drop experiments, but not within the A/PLs/W interface (Langmuir balance experiments). Insertions at A/W and O/W interfaces were both biphasic phenomenon, undergoing a rapid diffusion phase, followed by an adsorption phase. Equilibrium could not be reached within our experiment time (120 min). The overall kinetics of adsorption at the O/W interface was slower than for adsorption at the A/W interface, even for higher protein concentration. The diffusion phase occurred within minutes for the A/W interface and within 1 h for the O/W interface, the transition point being located at around 80 min. Contrary to oleosins (*4*), in our hands, caleosin was not able to interact with the phospholipid monolayer.

Caleosin shares with oleosins a triblock structural organization, allowing its insertion at various hydrophilic/hydrophobic interfaces. It is then possible to hypothesize that caleosin may anchor within the neutral core of OB, but the hydrophilic part of the protein is, contrary to oleosins, unlikely to interact with the surrounding PLs.

Caleosin is a protein possessing one calcium half-binding site. Using either labeled compounds or electrophoretic migration shift assays, Takahashi et al. (*12*) and Chen et al. (*14*) proved the calcium binding capacity of caleosins isolated from *A. thaliana* (AtClo3, encoded by *At2g33380*) or *S. indicum*. We prove the ability of rClo1 to bind calcium, using fluorescence assay, and we prove using gel shift assay that binding was specific to this divalent ion, this ion affecting protein tertiary structure. The faster migration of caleosin with bound calcium can be explained by a more compact structure of caleosin upon calcium binding. Caleosin bearing a histidine tag at its C terminus exhibited similar features (data not shown).

Aggregation of caleosin was dependent on time and calcium concentration. Calcium concentrations as low as 0.02 mM were capable of inducing aggregation. Increasing the calcium chloride concentration modified the aggregation kinetics (decrease of the time of formation of big aggregates and acceleration of precipitation) and increased the number of aggregates. Caleosin aggregation was a process essentially driven by the calcium

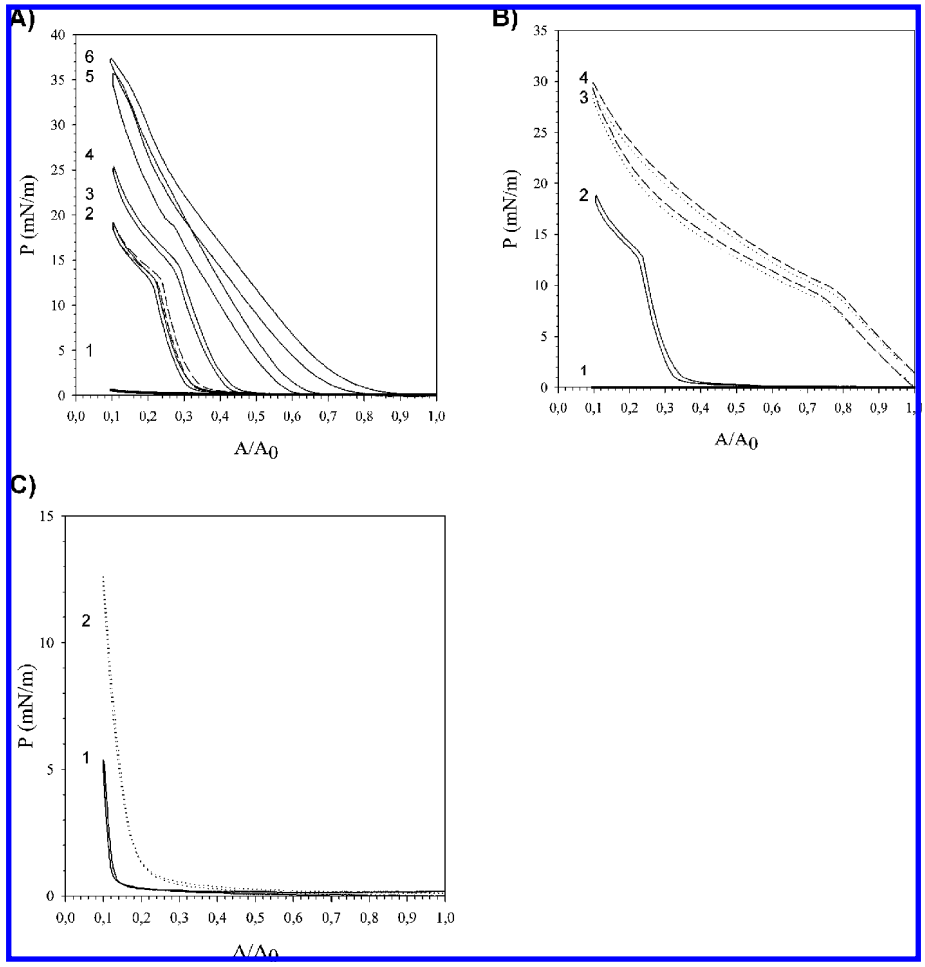

Figure 6. Effect of calcium ions on the behavior of purified lipid bodies at air/water interface. (**A**) Influence of incubation time. Isocycle of purified oil bodies without calcium (curve 2), with calcium spread on the surface of lipid bodies system (final concentration = 100 nM), performed immediately (curve 3) and after 1 h (curve 4), 4 h (curve 5), or overnight (curve 6). Control isocycle of 20 mM Tris buffer in the presence of 100 nM calcium ions is shown (curve 1). (**B**) Influence of EGTA. EGTA (final concentration = 300 nM) was added to the interface prepared as in **A**: curve 1, buffer + calcium + EGTA; curve 2, oil bodies; curve 3, oil bodies + calcium; curve 4, oil bodies + calcium + EGTA. (**C**) Influence of solubilization system. Oil bodies were spread on the surface of ultrapure water, calcium ions were added (final concentration = 100 nM), and the system was incubated until relaxation was reached. Control isocycle with water only was run (curve 1). After isocycle, the system was incubated overnight, and another isocycle was recorded (curve 2).

chloride concentration; on the other hand, protein concentration had negligible effects on aggregation kinetics and intensity.

The presence of calcium ions decreased slowly caleosin's capacity to decrease IFT at A/W and O/W interfaces, indicating that calcium binding decreased the capacity of the protein to reach interfaces. The effect was almost 5 times faster at A/W than at O/W interfaces. These facts indicate that calcium binding influences the interface's saturation by protein. Calcium binding may cause a decrease of the protein net charge and/or structural changes (such as bridging), which enable more compact folding of protein at the surface. As a consequence, a higher protein amount is required for the surface saturation. The presence of calcium did not favor interaction of caleosin with PLs.

The role of caleosin as target for calcium binding in AOB and purified OB, and as an effector for their overall stability, has been evidenced for the first time to our knowledge. AOB containing rClo1.his were aggregating in the presence of Ca^{2+}; this was not the case when rClo1.his was omitted. rClo1 secondary structure was not affected by Ca^{2+} (*25*). Aggregation phenomenon may tentatively be explained by either modification of electrostatic interactions between OB, or by the possible bridging of caleosin from different oil bodies (interdroplets bridges) with calcium ions. The fact that rClo1.his lost its capacity to insert itself at various interfaces upon incubation with Ca^{2+} makes it an interesting trigger to modulate OB stability. The behavior and structure of OB monolayers on the water surface are profoundly affected in a time-dependent manner by the presence of calcium concentrations as low as 100 nM. At this time, we have no evidence of either aggregation or disruption of OB on the water surface in Langmuir balance, so it is difficult to infer the fate of OB after long times of incubation with Ca^{2+}.

Our data support the role of calcium in the aggregation state of rClo1 that could have an impact in the caleosin-mediated dynamics of OB. The binding of calcium ions to rClo1 induces aggregation of rClo1. This aggregation has two consequences: the interfacial properties are decreased, and calcium induces bridging between artificial oil bodies. Further investigation is required to confirm the formation of a calcium-linked network of rClo1 surrounding domains of triglycerides.

ABBREVIATIONS USED

AtClo1, *Arabidopsis thaliana* seed caleosin 1; *A. thaliana*, *Arabidopsis thaliana*; AOB, artificial oil bodies; A/PLs/W, air/phospholipids/water interface; A/W, air/water interface; Ca,

calcium ions; IFT, interfacial tension; MES, 2-(*N*-morpholino)-ethanesulfonic acid; OB, oil (lipid) bodies; O/W, purified oil/water interface; *P*, surface pressure; PC, phosphatidylcholine; PLs, phospholipids; PVDF, poly(vinylidene fluoride); rClo1, recombinant caleosin; rClo1.his, recombinant caleosin bearing six histidine tags; TAG, triacylglycerol.

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