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Phospholipid monolayer of plant lipid bodies attacked by phospholipase A_2 shows 80 nm holes analyzed by atomic force microscopy

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Abstract

In plant storage tissue, lipid bodies are composed of triacylglycerides and surrounded by a phospholipid monolayer which is stabilized by oleosins. At the onset of lipid body mobilization, cells express phospholipase A_2, which partially degrades the monolayer and thus provides access for the subsequently acting triacylglyceride degrading enzymes. Analyzing the lipid body surface by atomic force microscopy we show that, at the stage of maximal phospholipase A_2 expression, the monolayer contains holes of approximately 80 nm in width and 2.45 ± 0.46 nm in depth. Non-contact mode imaging was performed with a lateral resolution of approximately 10 nm and a vertical resolution of less than 0.1 nm. The depth of the holes corresponds to the width of the monolayer, while the width of the channels is sufficiently large to provide access to 100 kDa enzymes, such as lipoxygenase and lipases.

Keywords: Atomic force microscopy; *Cucumis sativus*; Degradation; Oleosome; Phospholipase A_2; Phospholipid monolayer

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1. Introduction

Many plants build storage tissues and especially provide seeds with energy and carbon sources for heterotrophic growth during germination [1,2]. If triacylglycerides represent the form of reserves, they are confined to organelles called lipid bodies. Lipid bodies, also designated as oil bodies or oleosomes, consist primarily of triacylglycerides in which linoleoyl moieties may constitute more than 60% of the acyl groups [2]. On the surface, a monolayer of phospholipids, together with partially hydrophobic proteins [3,4], designated as oleosins, serves as a stabilizing principle which prevents the fluid lipids from fusing together. Thus, lipid bodies are rather stable and do not tend to aggregate as would be the case if their surface were not isolated by a protein net and a polar lipid coat.

It has been hypothesized that oleosins embedded in the monolayer and partially extending into the triacylglyceride phase may serve as binding sites for a particular lipoxygenase and a transiently active lipase [5]. However, the high specificity of the lipoxygenase acting on linoleoyl moieties of triacylglyceride [6,7] rather than on the linoleoyl moieties of phospholipids, and the specificity of a particular lipase hydrolyzing only acyl moieties modified by hydroperoxidation, causes us to attribute the primary action of lipid body mobilization to an enzyme which initiates the mobilization phase by destructing the phospholipid layer. Thus, the recruitment of phospholipase A_2 on the lipid body surface [8] and its action have to be postulated as primary events, and the consequence of this process needs to be demonstrated.

Atomic force microscopy (AFM) has been successfully applied to scan the surface of phospholipid layers and the defects within them [9–11]. It was demonstrated [9] that phospholipase A_2 forms channels of a comparable size to the diameter of a single enzyme in phospholipid bilayers.

We set out to analyze the lipid body surface with regard to the changes brought about by the action of an endogenous phospholipase A_2. Noncontact AFM with washed lipid bodies prepared from cucumber seeds showed that at a stage of development where only oleosins and phospholipase A_2 are present at this site, holes of a defined size are created.

2. Materials and methods

2.1. Plant material and lipid body preparation

Cucumber (*Cucumis sativus*) seeds were either imbibed for 4 h or germinated at 26°C for 2 days. The method for lipid body preparation had been described in detail by Sturm et al. [12].

2.2. Analysis of lipid body-associated proteins

Proteins were precipitated with trichloroacetic acid, dissolved in 2% sodium dodecyl sulfate (SDS) and subjected to polyacrylamide gel electrophoresis (PAGE). Analysis by immunodetection on Western blots was performed with antibodies raised against the purified cucumber lipid body lipoxygenase [12,13] or phospholipase A_2 [8], obtained by the bacterial expression of the corresponding cDNA. Lipid body proteins solubilized as SDS micelles were subjected to molecular sieving chromatography on Ultrogel AcA44 in the presence of 0.1% SDS. The subfractions were evaluated after analytical SDS-PAGE.

2.3. Preparation of lipid bodies on microscope slides

The lipid bodies had been washed with double-distilled water. A drop (10 μl) of the suspension was deposited on a clean microscope slide, which was glued to a steel disc for magnetic fixation on the AFM stage. Usually the lipid bodies were floating on the water drop surface, and in order to fix the lipid bodies on the glass surface the microscope slides had been stored in a desiccator over P_4O_{10} prior to AFM experiments. The samples were placed on the AFM stage immediately before scanning.

2.4. Atomic force microscopy

Images of dry lipid bodies were collected at ambient conditions (i.e. a relative humidity of

approx. 65%) with an Explorer AFM (TopoMetrix, Santa Clara, USA) equipped with a 2 μm tube scanner. The scanner was calibrated in the *z*-direction (height) by scanning colloidal gold, and in the *x*- and *y*-directions by scanning the TopoMetrix and the Nanosensors (L.O.T.-Oriel GmbH, Darmstadt, Germany) calibration grid. In order not to damage the surface of the lipid bodies at ambient conditions, we performed non-contact mode imaging. In non-contact AFM, the cantilevered tip oscillates near its resonance frequency at a sample distance of 10–100 nm, and does not touch or damage the surface [14]. Due to attractive forces near the surface, the effective force constant of the AFM tip decreases, and as a consequence, its resonance frequency decreases too. This results in a dampening of the tip amplitude at the excitation frequency, depending on the tip–sample distance. At a given frequency, the amplitude of the oscillation decreases with decreasing tip–sample distance. In our experiments, amplitude damping was used for control of the tip–sample distance and feedback. Silicon HRF probes (TopoMetrix) were used with resonance frequencies of approximately 230 kHz, and typical force constants between 30 and 50 N/m. Typical cantilever dimensions were: 130 μm in length, 4.0 μm in thickness, and 30 μm in width; and the tip heights varied from 10 to 15 μm. All of the images shown are raw data, except for horizontal flattening.

2.5. Scanning electron microscopy

Scanning electron microscopy (SEM) on a Hitachi S-4100 SEM, equipped with a field emission cathode, was used to check the quality of the AFM-tips.

3. Results

3.1. Analysis of the predominant proteins present in lipid bodies during germination

At the onset of germination, the protein fraction of the lipid bodies mainly consists of oleosins, either in the range of 20 kDa, or larger species in the range of 30–40 kDa. In cucumber seeds, the predominant oleosin is a 38 kDa protein, hereafter designated p38. It persists up to day 2–3 of germination. At that stage, with the oleosin still present but the newly formed lipoxygenase accumulating, both the p38 oleosin and the 100 kDa lipoxygenase together represent more than 70% of the total lipid body protein (Fig. 1) [6,12].

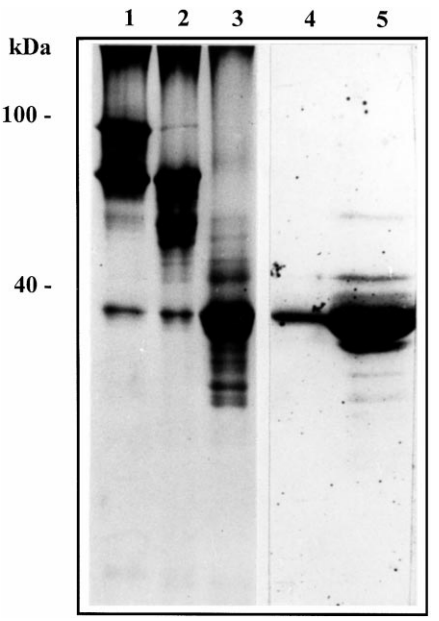

Fig. 1. Electrophoretic analysis of protein fractions prepared from the lipid bodies of cucumber cotyledons. Cotyledons were harvested from germinating seeds at day 2 after imbibation. Following the isolation of the lipid bodies, SDS-micelles were prepared and separated according to size. Lanes 1–3: subfractions after chromatography; lanes 4 and 5: various amounts of purified p38.

3.2. Small amounts of phospholipase A_2 transiently occurring at the lipid body surface

Lipid body fractions were prepared from cotyledons of seedlings germinated for various time periods. The protein profile of phospholipase A_2 was analyzed by immunoblotting using antiserum raised against the patatin-like protein from cucumber seeds (Fig. 2). Determining the intensities of the immunostain bands, and drawing the intensities against time of germination, provided a profile of the transient appearance of

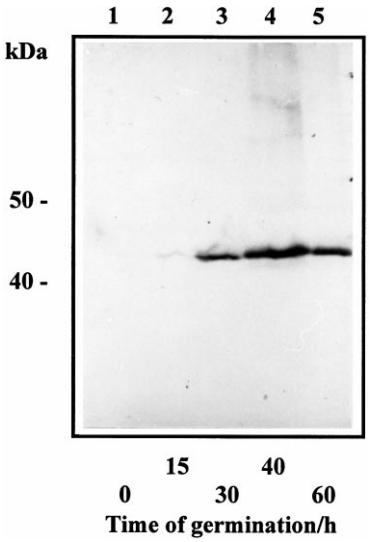

Fig. 2. Profile of phospholipase A_2 present in the lipid body fraction of germinating cucumber seedlings. Each lane represents the lipid body protein prepared from 0.5 g cotyledons, germinated for the indicated time. The protein levels are visualized by immunodecoration with antiserum raised against the overexpressed protein.

phospholipase A_2 on the lipid bodies. The very pronounced increase of this protein can also be seen in Fig. 2. The data indicate that small amounts of phospholipase A_2 are expressed between 15 and 30 h of germination and directly transferred to the lipid bodies.

3.3. Images of the lipid body surface after 4 and 60 h of germination

To gain an overview of the objects to be studied in detail, we conducted AFM imaging on freshly isolated lipid bodies, prepared as mentioned in Section 2.3. This provides data on the size of the organelles and hints as to their behavior on a glass surface. Fig. 3 displays an AFM image of a typical lipid body preparation on a microscope slide. Because of the small maximum scan area of the used AFM scanner, we performed AFM imaging on small lipid bodies. The volume of the lipid bodies imaged can be calculated from the measured lateral and vertical size, this yields diameters of initially spherical lipid bodies of approximately 400 nm. This is in good agreement with the data presented in the literature, taking into account that small lipid bodies had been chosen for imaging. In maize, the lipid body sizes varied between 0.5 and 2.5 μm [1,5], and even smaller lipid bodies can be found in different cell types [15].

Measurements were made to characterize the surface and surface degradation at the stage of maximal activity of phospholipase A_2. Fig. 4a,b shows two typical images of a lipid body prepared from cucumber seeds imbibed for 4 h. The whole lipid body is shown in Fig. 4a. Defects can be identified, which may be due to the preparation. An analysis of the height profile shows that, within these defects, the AFM-tip scans the substrate surface. For a better analysis of the lipid body surface, the magnification and resolution were increased during scanning (Fig. 4b). Except for the damage, indicated by the arrow, no holes due to phospholipase A_2 action can be found.

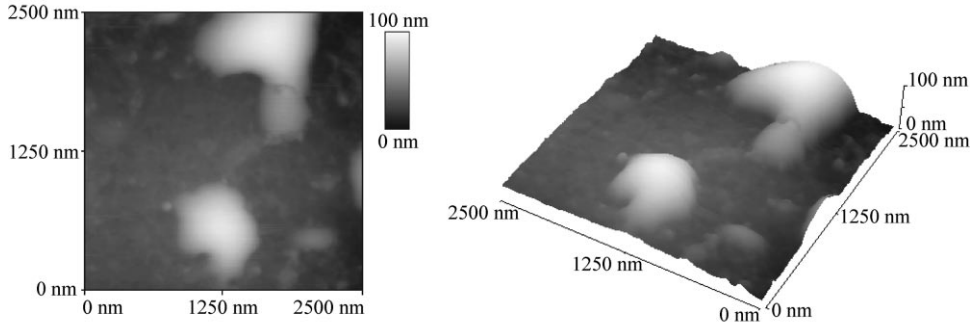

Fig. 3. Non-contact AFM image of a typical lipid bodies preparation from cucumber seeds on a microscope slide taken at ambient conditions. Left: top view; right: three-dimensional presentation.

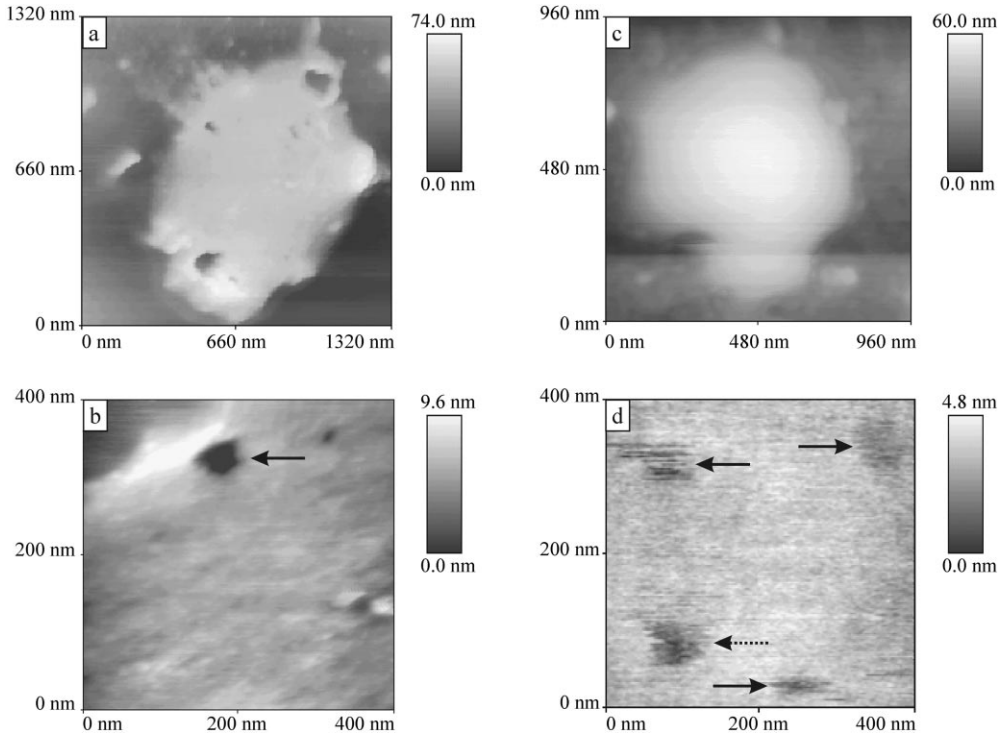

Fig. 4. (a) Non-contact AFM image of a whole lipid body deposited on a microscope slide prepared from imbibed cucumber seeds. (b) Magnification of (a), showing no holes in the phospholipid monolayer of the lipid body due to degradation. The black arrow marks a defect due to preparation. (c) Non-contact AFM image of a whole lipid body deposited on a microscope slide prepared from germinated (60 h) cucumber seeds. (d) Zoom in a single lipid body prepared as in Fig. 4c, showing a few holes (marked by arrows) in the phospholipid monolayer of the lipid body due to degradation. A single line through the hole marked by the dotted arrow is shown in Fig. 5.

In contrast to the situation at the early stage, we observed quite a different picture at the later stage when phospholipase A_2 is highly active.

A typical lipid body from the cotyledons of seedlings which had been germinated for 60 h is shown in Fig. 4c. In order to identify single holes in the phospholipid monolayer, the magnification had to be increased. Therefore, in Fig. 4d, a zoom image with increased resolution of the surface of a lipid body germinated for 60 h is presented. In contrast to the lipid body shown in Fig. 4a,b, holes in the phospholipid monolayer can be identified and are indicated by arrows. The depth of the holes was 2.45 nm, with a S.D. of ± 0.46 nm. This value was measured from a lot of different holes, and is in good agreement with the thickness of a phospholipid monolayer. The measured minimum value was 1.92 nm and the maximal value was 3.03 nm. The width of the holes varied from 45 to 135 nm, and is typically 80 nm. Fig. 5 shows a horizontal line scan through the hole

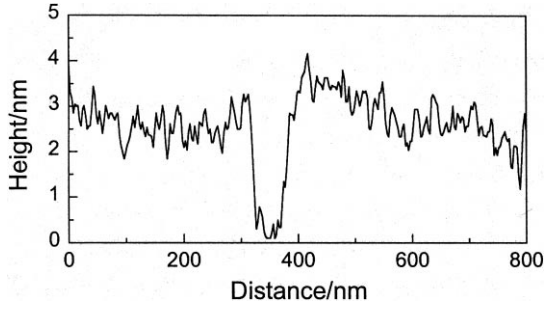

Fig. 5. Line scan through the hole marked by the dotted arrow in Fig. 4d, showing the dimensions of the hole in the lipid body surface.

marked with the dotted arrow in Fig. 4d, demonstrating the hole properties.

The findings demonstrated that the lipid bodies taken from seedlings at the later stage differed from those of the early stage by the very characteristic holes.

4. Discussion

Our biochemical analysis showed marker proteins for lipid bodies at three different stages in the degradation phase of the lipid bodies. We started out with dry seeds or shortly imbibed seeds, revealing a 38 kDa oleosin as the predominant protein (stage I). Secondly, the stage of maximal expression of a membrane-destructing phospholipase A_2 was attributed to day 1.5–2 days of germination (stage II). Finally, a period of time within the germination sequence was analyzed (stage III) where the amount of phospholipase A_2 declined, while lipoxygenase reached its highest activity levels.

Interestingly, the most marked changes in the appearance of the lipid body surface were observed at stage II, when phospholipase A_2 had already acted on the phospholipid monolayer. Except for black membranes or continuous monolayers which can close a hole by immediately expanding their fluid phase, the monolayer of lipid bodies is made distinct by the imbedded oleosins. Thus, even at the stage of maximal phospholipase A_2 activity, the lipid body monolayer is decorated by oleosins which reduce the fluidity and might transiently stabilize the holes formed by the action of the phospholipase. In addition to the very particular point of time when the holes appeared, the characteristics of the holes, i.e. primarily their depth of 2.45 nm, clearly indicated that they agreed with the properties of a phospholipid monolayer, and the diameter of the holes were sufficient for a subsequent entry by enzymes of more than 100 kDa molecular mass. To our knowledge, this is the first report on lesions or holes in biological monolayers. For bilayers, filipin-induced lesions were found in dipalmitoylphosphatidylamine/cholesterol bilayers [11], and protrusions with a mean diameter of 19 nm were detected. Furthermore, the degradation of a supported dipalmitoylphosphatidylcholine (DPPC) bilayer by phospolipase A_2 in an aqueous buffer solution was also observed by AFM, showing channels of well-defined width ($\sim 15 \pm 3$ nm) and different lengths, possibly due to the activity of a single enzyme [9]. These channels remained stable until enough hydrolysis products had been produced that reorganization of the non-hydrolyzed DPPC was able to occur.

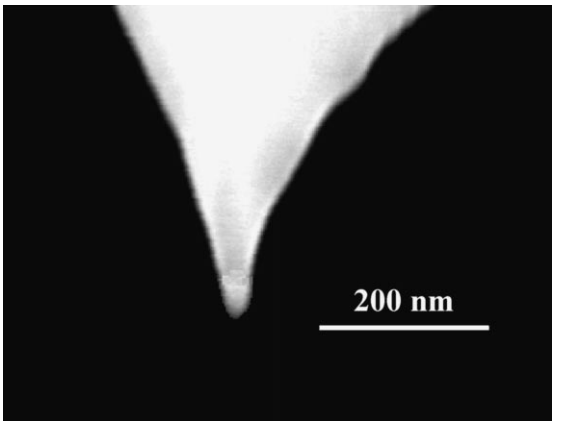

Fig. 6. SEM image of the apex of a TopoMetrix AFM tip usually used in non-contact AFM measurements.

The coincidence of the transient appearance of the phospholipid monolayer-attacking enzyme and the changes in the phospholipid monolayer strongly support the hypothesis that the subsequent attack by the lipid body degrading enzymes would not be feasible without the previous action of phospholipase.

Fig. 6 shows a SEM-image of a TopoMetrix AFM tip used for non-contact imaging. From this figure, a tip radius of approximately 10 nm can be estimated. From geometrical considerations, this could lead to a difference in the measured hole diameters, which could be slightly larger (13 nm). This is a calculated value for contact-mode AFM. In non-contact mode AFM, the situation is quite different due to the tip–sample distance. Furthermore, the true geometry of the apex of the tip cannot be resolved by SEM. It should be mentioned that standard Si_3N_4 contact AFM tips were found to have radii of curvature at the apex of the tip as small as 1 nm [16].

Therefore, the influence of the tip geometry on the size of the holes in non-contact AFM can only be estimated, with a maximum difference in the lateral dimension of approximately 13 nm. This would lead to an increase in the measured hole diameters, but it does not change the statement that the hole diameters are sufficiently large to provide access for 100 kDa enzymes, such as lipoxygenase or lipase.

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