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Differential Sensitivity of Oleosins to Proteolysis During Oil Body Mobilization in Sunflower Seedlings

Hamid Reza Sadeghipour and Satish Chander Bhatla [1]

Department of Botany, University of Delhi, Delhi 110007, India

Until now, there has been no conclusive demonstration of any in vivo oleosin degradation at the early stages of oil body mobilization. The present work on sunflower (*Helianthus annuus* L.) has demonstrated limited oleosin degradation during seed germination. Seedling cotyledon homogenization in Tris-urea buffer, followed by SDS-PAGE, revealed three oleosins (16, 17.5 and 20 kDa). Incubation of oil bodies with total soluble protein from 4-day-old seedlings resulted in oleosin degradation. In vitro and in vivo degradation of the 17.5-kDa oleosin was faster than the other two, indicating its greater susceptibility to proteolysis. Oleosin degradation by the total soluble protein resulted in a transient 14.5-kDa polypeptide, followed by an 11-kDa protease-protected fragment, which appeared post-germinatively and accumulated corresponding to increased rate of lipid mobilization. A 65-kDa protease, active at pH 7.5–9.5, was zymographically detected in the total soluble protein. Its activity increased along with in vivo accumulation of the protease-protected fragment during seed germination and accompanying lipid mobilization. Protease-treated oil bodies were more susceptible to maize lipase action. Differential proteolytic sensitivity of different oleosins in the oil body membranes could be a determinant of oil body longevity during seed germination.

Keywords: *Helianthus annuus* — Oil body mobilization — Oleosins — Protease — Seed germination — Sunflower.

Abbreviations: IS, imbibed seeds; PPF, protease-protected fragment; TAGs, triacylglycerols; TSP, total soluble protein.

Introduction

During seed maturation, oilseeds accumulate a relatively high proportion of triacylglycerols (TAGs) in oil bodies of variable sizes (0.2–2.0 μm). Oil bodies consist of a hydrophobic matrix of TAG, surrounded by a half-unit phospholipid membrane and low molecular mass (14–26 kDa) proteins called oleosins as the major protein constituent (Huang 1996). Oleosins consist of an N-terminal amphipathic domain of 40–60 amino acid residues exposed to the cytosol, a central hydrophobic domain of 70 amino acid residues inserted into the oil body matrix and a C-terminal amphipathic domain of 30–40 amino acid residues, facing the cytosol (Frandsen et al. 2001). By providing steric hindrance and electrostatic interactions, oleosins help oil bodies maintain their individuality and integrity during seed maturation and drying (Murphy and Cummins 1989, Tzen and Huang 1992) or following seed rehydration (Leprince et al. 1998). Oleosins might also be involved in oil body mobilization by acting as lipase receptors (Wang and Huang 1987) or as docking sites for glyoxysomes, thereby facilitating oil body TAG mobilization by glyoxysomal lipase (Chapman and Trelease 1991). In addition, oleosins provide surface area for lipase activity by regulating the size of oil bodies (Ting et al. 1996).

A gradual disappearance of oleosins concomitant with TAG mobilization has been reported in maize (Fernandez et al. 1988), rapeseed (Murphy et al. 1989), anise (Radetzky et al. 1993, Radetzky and Langheinrich 1994), soybean (Herman 1995) and sesame (Tzen et al. 1997). However, the role of oleosins during oil body mobilization has remained speculative. Murphy (1990) hypothesized that a protease might hydrolyze oleosins during oil body mobilization, prior to lipolytic action. A soybean oil body membrane protein, with molecular mass of 34 kDa, was reported to be processed into a slightly smaller peptide of 32 kDa at the onset of oil body mobilization (Herman et al. 1990). The 34-kDa protein was subsequently, however, localized in the protein storage vacuoles. Hence, the observed association of this protein with oil bodies was attributed to an artifact of extraction procedure (Kalinski et al. 1992). Treatment of oil bodies with a protease, like trypsin, can make oil bodies susceptible to degradation by lipolytic enzymes, such as phospholipase A_2 (Tzen and Huang 1992) and 13-lipoxygenase, in some oilseeds (Matsui et al. 1999). Oil bodies extracted from mid to late developmental stages of various seedlings have, in general, been shown to be better substrates for lipolytic enzymes as compared to those extracted from ungerminated seeds (Lin et al. 1982, Hoppe and Theimer 1997, Matsui et al. 1999). These observations led Matsui et al. (1999) to propose a model for oil body mobilization, whereby degradation of oil body proteins, more remarkably oleosins, is the first step for the initiation of oil body mobilization.

There has been no clear-cut in vivo demonstration of proteolytic action on oleosins at the onset of TAG matrix mobilization in germinating oilseeds. Working on the regulation of lipid mobilization in sunflower seedlings, the rapid disappear-

[1] Corresponding author: E-mail, satrita@tatanova.com; Fax, +91-11-7666744.

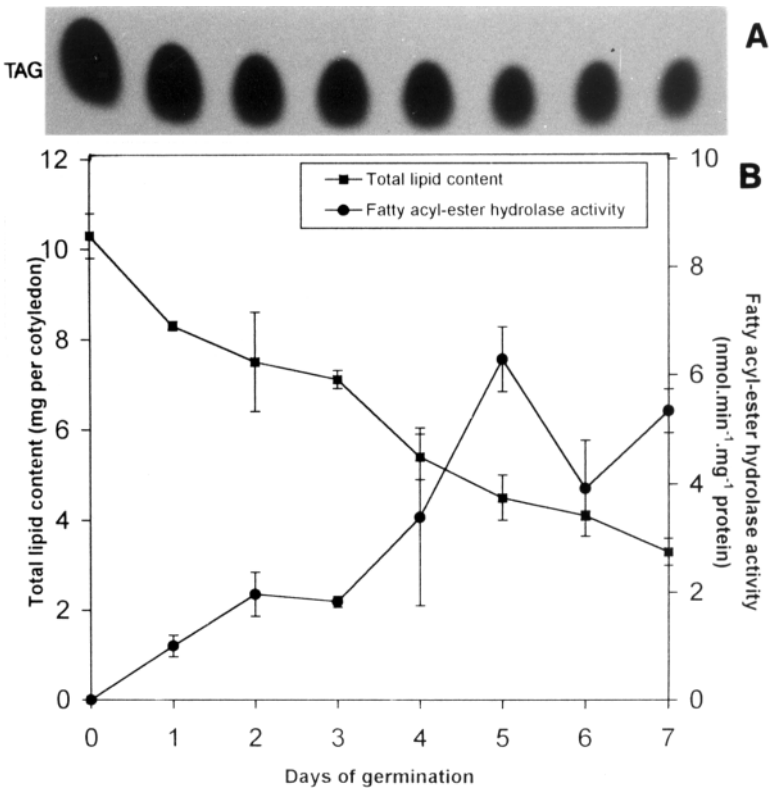

Fig. 1 (A) Changes in the content of cotyledonary TAGs in dark-grown sunflower seedlings as resolved by thin layer chromatography. Total lipid from the cotyledons was extracted in chloroform. Aliquots equivalent to 0.02 cotyledons were chromatographed on each spot on silica gel plates and visualized with iodine vapours. The eight spots from left to right correspond to imbibed seeds (IS) and 1- to 7-day-old seedlings. (B) Changes in total lipid and fatty acyl-ester hydrolase activity in the cotyledons of germinating seedlings of sunflower. Each value in (B) is the mean of three independent experiments ± SE.

ance of oleosins at early stages of seedling growth was observed in the present investigation. This prompted work on the proteolytic activity responsible for oleosin degradation during seed germination. Analysis of seedling extracts revealed a protease with features that indicate specific action on oleosin degradation. Based on the present observations, the functional significance of proteolytic activity on oil bodies of germinating seeds is discussed. Finally, a role for oleosins as the determinants of oil body longevity in germinating seedlings is suggested.

Results

Lipid mobilization during seed germination in the dark

The changes in total lipid and TAGs of cotyledons along with the principal enzyme responsible for TAG mobilization (lipase) can be used as a measure of the dynamics of oil body mobilization. A gradual depletion of TAGs was observed during seed germination in sunflower (Fig. 1A). It was not possible to detect lipase activity in cotyledons at any of the seedling developmental stages, using a lipase-specific chromogenic substrate, i.e. 1,2-*O*-dilauryl-rac-glycero-3-glutaric acid-resorufin ester. This is in agreement with earlier reports (Wang and Huang 1984, Chapman 1987). Therefore, fatty acyl-ester hydrolase activity has been used as a criterion of the extent of lipolysis in the present work. Enzyme activity was negligible in imbibed seeds (IS), low in the cotyledons of 1-day-old seedlings and gradually increased with a peak of activity in 5-day-old seedlings (Fig. 1B). The most intense period of lipid mobilization occurred between 3 and 5 d of seedling growth, so that by 5 d of germination, about 60% of total lipid present in IS was metabolized.

Changes in the pattern of oleosins and other oil body-associated proteins

Detection of changes in the composition of oil body membrane proteins during seed germination is significantly affected by the washing procedure. Carbonate washing is suitable for extracting proteins not firmly embedded in oil body membranes (Fujiki et al. 1982). In contrast, urea-washed oil bodies are devoid of any contaminating proteins (Thoyts et al. 1996) and only oleosins remain resistant to urea washing (Millichip et al. 1996). Carbonate-washed oil bodies, prepared after tissue homogenization in HEPES-sucrose buffer, showed significant

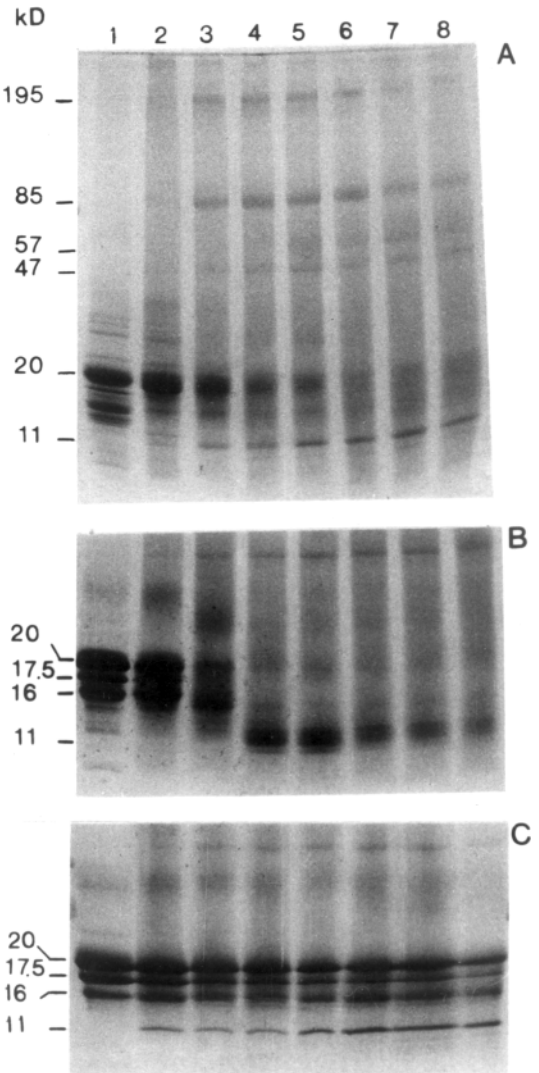

Fig. 2 Changes in the oil body membrane polypeptide patterns during seed germination in dark. Oil bodies obtained after tissue homogenization in HEPES-sucrose buffer, were washed in 0.1M $NaHCO_3$ (A) or in Tris buffer (50 mM, pH 7.5) containing 9 M urea (B). Alternatively, Tris-urea buffer was used as a homogenization medium to isolate oil bodies, followed by washing of oil bodies in the same buffer (C). Lane 1, imbibed seeds (IS); lanes 2–8, oil body preparations from cotyledons of 1- to 7-day-old seedlings. Protein equivalent to 150 μg was loaded in each lane of a linear gradient (5–20%) resolving SDS gel.

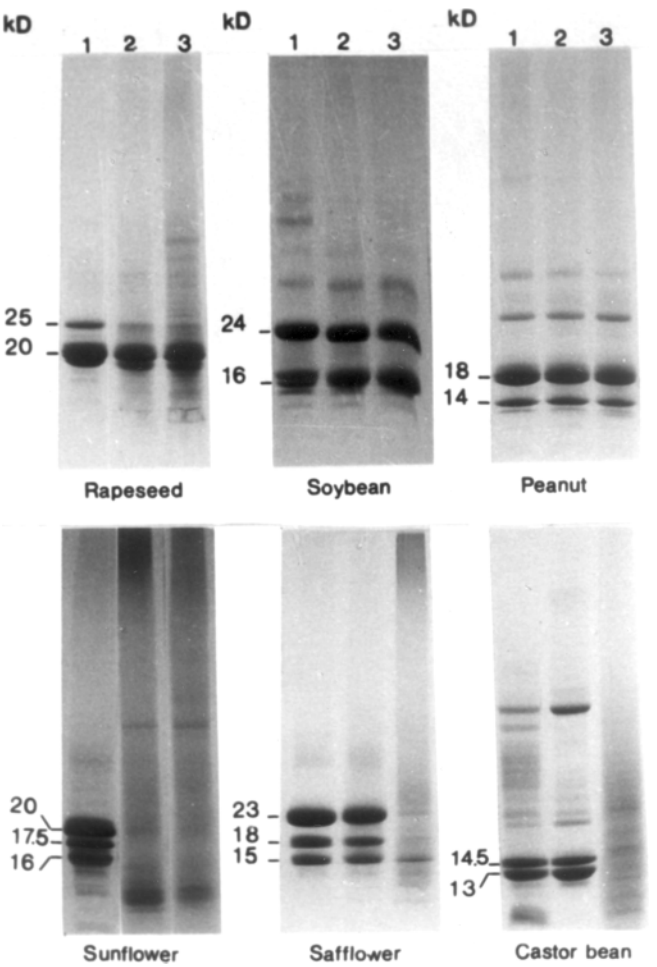

Fig. 3 Changes in the oil body membrane polypeptide (putative oleosins) patterns of rapeseed, soybean and peanut, safflower and castor bean as compared with sunflower, at different stages of seed germination. Lane 1, imbibed seeds (IS); lane 2, 3-day-old seedlings; lane 3, 6-day-old seedlings. Oil bodies from tissue homogenized in HEPES-sucrose buffer were washed in Tris-urea buffer. Protein equivalent to 150 μg was loaded in each lane of a linear gradient (5–20%) resolving SDS gel.

changes in membrane protein composition during seedling development and new polypeptides with molecular masses of 47, 57, 85 and 195 kDa were detected (Fig. 2A). The appearance of a 11-kDa polypeptide occurred just after germination, and levels increased with the age of seedlings. A polypeptide of 20 kDa started disappearing soon after germination (Fig. 2A). Urea-washed oil bodies (prepared following tissue homogenization in HEPES-sucrose buffer) revealed three oleosins of 16, 17.5 and 20 kDa during the early stages of seed germination and they were not detectable after 3 d of germination (Fig. 2B). The 11-kDa polypeptide was consistently present in both carbonate- and urea-washed oil bodies from germinating seeds, indicating its firm association with oil bodies.

The oil body membrane polypeptide pattern, analyzed after tissue homogenization and washing in Tris-urea buffer, showed oleosins of 16, 17.5 and 20 kDa. While the 16-kDa oleosin largely remained unchanged, the 17.5- and 20-kDa oleosins gradually disappeared following germination (Fig. 2C). Mobilization of the 17.5-kDa oleosin was faster than that of the 20-kDa oleosin. The 11-kDa polypeptide appeared within 1 d of germination and accumulated between 3 and 5 d of germination. Thereafter, it remained unchanged.

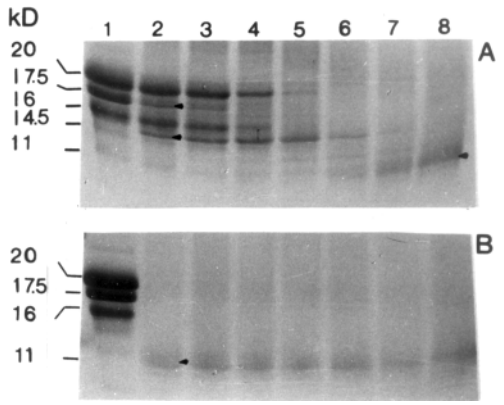

Fig. 4 Kinetics of sunflower oleosin degradation by the protease source of the TSP. Oil bodies from IS were isolated in HEPES-sucrose buffer and washed with Tris-urea buffer. Oil bodies equivalent to 50 mg were digested with 50, 100, 200, 400, 800, 1600 and 3200 μg of TSP obtained from the cotyledons of 4-day-old seedlings as a source of protease (lanes 2–8) for 1 h (A). Alternatively, they were digested with 3200 μg TSP added for 1–7 h (lanes 2–8) (B). Oil bodies with no sunflower TSP added were used as control (lane 1). Oleosins and their cleavage products were separated in a linear gradient (10–20%) resolving SDS gel. The arrowheads in lane 2 of (A) refer to 17.5-kDa oleosin and 14.5-kDa oleosin degradation product. The arrowhead in lane 8 refers to 11-kDa PPF. In (B) the arrowhead refers to 11-kDa PPF.

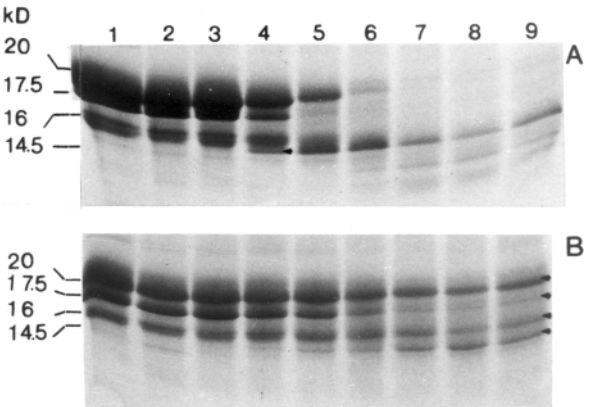

Fig. 5 Assay of protease activity capable of oleosin degradation in the germinating sunflower seedlings as analyzed by SDS-PAGE. Oil bodies from IS were isolated in HEPES-sucrose buffer and washed with Tris-urea buffer. Oil bodies equivalent to 50 mg lipid were digested with 200 μg of TSP from IS (lane 2) or 1- to 7-day-old seedlings (lanes 3–9) for 1 h in the presence (A) or absence (B) of 2-mercaptoethanol. Oil bodies with no sunflower TSP added were used as control (lane 1). A linear gradient (10–20%) resolving SDS gel was used to separate oleosins and their cleavage products. The arrowhead in lane 4 of (A) refers to 14.5-kDa oleosin degradation product and the arrowhead in lane 8 of (B) represents the three oleosins along with 14.5-kDa oleosin degradation product.

Comparison of sunflower oil body polypeptides with other oilseeds

Oil bodies isolated in HEPES-sucrose buffer and washed with urea (according to Millichip et al. 1996), were used to analyze oil body polypeptide patterns in other oilseeds during three developmental stages: imbibed seeds, 3-day-old and 6-day-old seedlings (Fig. 3). The major oil body proteins, 20 kDa in rapeseed, 16 and 24 kDa in soybean and 14 and 18 kDa in peanut, remained unchanged until 6 d of germination (Fig. 3). A 25-kDa polypeptide of rapeseed oil body, however, disappeared following germination. The above observations indicate that the oleosin degradation pattern in these oilseeds differs from that in sunflower. However, patterns of oleosin degradation similar to that in sunflower in terms of mobilization of specific polypeptides with progress of germination, were observed for safflower and castor bean (Fig. 3).

Kinetics of oleosin degradation by sunflower protease

Tris-urea washed oil bodies obtained from imbibed seeds of sunflower, were used to investigate oleosin degradation by total soluble protein (TSP, 10,000×*g* supernatant), from the cotyledons of 4-day-old seedlings as a source of protease. As little as 50 μg of TSP resulted in a drastic depletion of 17.5-kDa oleosin, with simultaneous appearance of a 14.5-kDa polypeptide after 1 h of incubation (Fig. 4A, lane 2, arrowhead). Increasing the amount of TSP led to a rapid disappearance of the 17.5-kDa oleosin, followed by degradation of other two oleosins (16 and 20 kDa) and the transient accumulation of a 14.5-kDa polypeptide. At high TSP concentrations (3,200 μg ml^{-1}), only a polypeptide with molecular mass of 11 kDa was detected as a result of oleosin degradation (Fig. 4A, lane 8, arrowhead). Varying the incubation period from 1 to 7 h showed that 3,200 μg of TSP led to complete degradation of oleosins, and the appearance of an 11-kDa polypeptide remained unaltered (Fig. 4B, arrowhead). This polypeptide (11 kDa) is referred to as protease-protected fragment (PPF) to denote that portion of the oleosin molecule inserted into the oil body TAG matrix and it is, therefore, protected against proteolytic actions. This polypeptide was also detectable as a persistent component of oil body membranes following germination (Fig. 2).

Assay of protease activity capable of oleosin degradation in germinating seedlings of sunflower

The extent of oleosin degradation (from the oil bodies of IS) and formation of its cleavage products by identical quantities of TSP from the cotyledons of different developmental stages of sunflower seedlings, was analyzed by SDS-PAGE and used to estimate protease activity in the germinating seedlings (Fig. 5). There was no oleosin degradation with respect to the control when TSPs from IS or 1-day-old seedlings were used as a source of protease. Protease activity increased in 2-day-old seedlings, as was evident by the transient formation of a 14.5-kDa polypeptide (Fig. 5A, lane 4, arrowhead). The greatest increase in protease activity occurred between 3 and 5 d of seedling growth. During this period, oleosins (16, 17.5 and 20 kDa) disappeared. Beyond 5 d of germination, protease

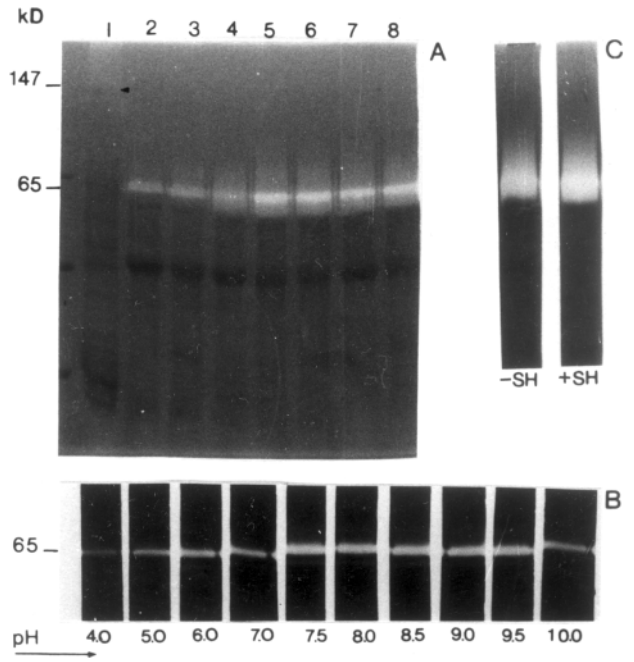

Fig. 6 Zymographic detection of protease activity and its partial characterization in sunflower seedlings. (A) TSP (100 μg) from IS (lane 1) or from the cotyledons of 1- to 7-day-old seedlings (lanes 2–8) were resolved by SDS-PAGE on a gel containing 0.1% gelatin. After removal of SDS and protein renaturation, the gel was incubated in a suitable buffer (pH 7.5) for 2.5 h at 37°C. Transparent gelatin-free zones representing protease activity, were visualized in the gel stained with amido black. (B) Effect of pH on the activity of 65-kDa protease was investigated by resolving TSP (100 μg) from 4-day-old seedlings in a gelatin containing gel. Following electrophoresis, the gel was cut into slices, renatured and incubated in 50 mM sodium acetate (pH 4 and 5), morpholinoethane sulfonic acid (MES; pH 6), HEPES (pHs 7 and 7.5), Tris (pH 8, 8.5 and 9) and glycine (pH 9.5 and 10). Protease activity visualized as in (A). (C) The 65-kDa protease activity from TSP (100 μg) of 4-day-old seedlings in the absence (−SH) or presence (+SH) of 2-mercaptoethanol (0.1%; v/v).

activity remained more or less constant and the 14.5-kDa polypeptide was the major oleosin cleavage product (Fig. 5A). The above experiment was performed in the presence of 5 μl ml^{-1} of 2-mercaptoethanol in the reaction mixture in order to maintain protease activity. In the absence of this reducing agent, protease from TSP remained ineffective in complete degradation of oleosins (Fig. 5B, lane 8, arrowhead).

Zymographic detection of protease activity

Samples equivalent to 100 μg protein from cotyledonary TSP fractions were resolved by electrophoresis in 10 to 20% gradient polyacrylamide SDS gels, containing 0.1% (w/v) gelatin. Following renaturation of resolved protein bands by incubating the gel in 50 mM Tris (pH 7.5) containing 2% (v/v) Triton X-100, transparent, gelatin-free zones created by protease action, were visualized in the gel stained with Amido black. A protease, with an apparent molecular mass of 65 kDa, was identified (Fig. 6A). The activity appeared from 1 d of germination and increased gradually during next 2 d. The maximum increase in enzyme activity occurred between 3 and 5 d of germination. During subsequent days of seedling growth, protease activity remained more or less constant. These observations are in agreement with the oleosin degradation pattern in sunflower seedlings (Fig. 2C). A high molecular mass 147-kDa protease activity zone was evident only in the TSP of IS. It disappeared in the germinating seeds, indicating its significance at the developmental stages other than germinating seedlings.

Effects of pH and 2-mercaptoethanol on protease activity

Protein samples (100 μg) from cotyledonary TSP fraction of 4-day-old seedlings were resolved on gelatin-containing SDS gels. After electrophoresis, each gel lane was cut, the protein renatured, and incubated in developing buffer solutions of varying pH or in developing buffer (pH 7.5) in the presence or absence of 2-mercaptoethanol. A 65-kDa protease exhibited relatively low activity at pH 4.0 (Fig. 6B). Its activity increased gradually at less acidic to neutral pH, being highest at pH 7.5. Enzyme activity remained more or less constant at alkaline pH. Enzyme activity was reduced at pH 10. The protease from TSP exhibited partial activation by the thiol reagent, 2-mercaptoethanol (Fig. 6C), indicating that it might be a thiol protease.

Kinetics of oleosin degradation by trypsin

Earlier experiments on the kinetics of sunflower oleosin degradation by the protease (TSP) in sunflower seedlings showed faster degradation of 17.5-kDa oleosin as compared to 16- and 20-kDa oleosins (Fig. 4A), indicating greater specificity of the endogenous protease for 17.5-kDa oleosin. Alternatively, the 17.5-kDa oleosin might be more prone to degradation by proteolytic action, compared with 16- and 20-kDa oleosins. To answer this question, degradation of sunflower oleosins in the presence of an exogenous protease, namely trypsin, was investigated. Tris-urea washed oil bodies obtained from IS of sunflower were used to investigate oleosin degradation with trypsin concentrations ranging from 0.01 to 500 μg ml^{-1} (Fig. 7A, B). As little as 0.01 μg ml^{-1} trypsin led to the formation of polypeptides with molecular masses of about 12, 13 and 14 kDa (Fig. 7A, lane 2, arrowhead). Increased trypsin concentration in the incubation mixture (up to 0.1 μg ml^{-1}) resulted in complete degradation of 17.5-kDa oleosin. Oil bodies exhibited a gradual, trypsin concentration-dependent oleosin degradation, so that at 0.5 μg ml^{-1} trypsin, 16- and 20-kDa oleosins were completely degraded. The 8- and 8.8-kDa polypeptides appeared to be the final oleosin degradation products by trypsin (Fig. 7B, lanes 3 and 4, arrowheads). A 24-kDa polypeptide showed increased accumulation at high concentrations (250 and 500 μg ml^{-1}) of trypsin (Fig. 7B).

Sunflower oil body susceptibility as a substrate for maize lipase

Different viewpoints exist for the significance of proteolytic action prior to lipase action. Action of proteolytic

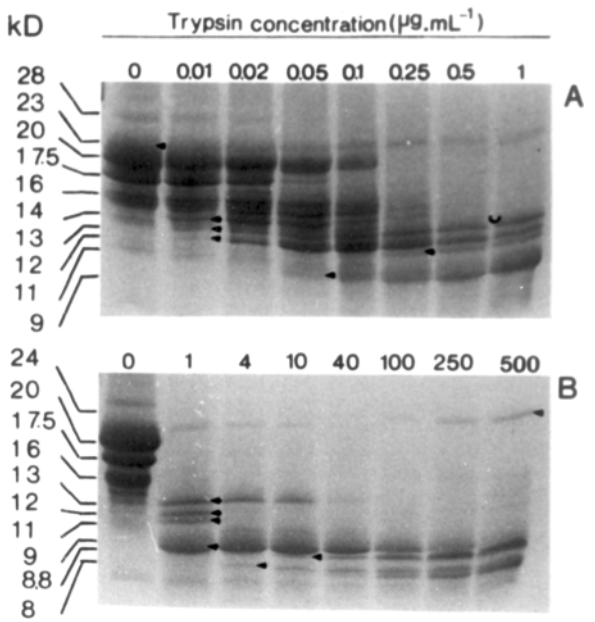

Fig. 7 Kinetics of sunflower oleosin degradation by trypsin. Oil bodies from IS were isolated in HEPES-sucrose buffer and washed with Tris-urea buffer. Oil bodies equivalent to 50 mg lipid were digested with trypsin in the concentration range of 0.01–1 μg ml^{-1} (A) and 1–500 μg ml^{-1} (B). Oleosins and their cleavage products were separated on a linear gradient (10–20%) resolving SDS gel.

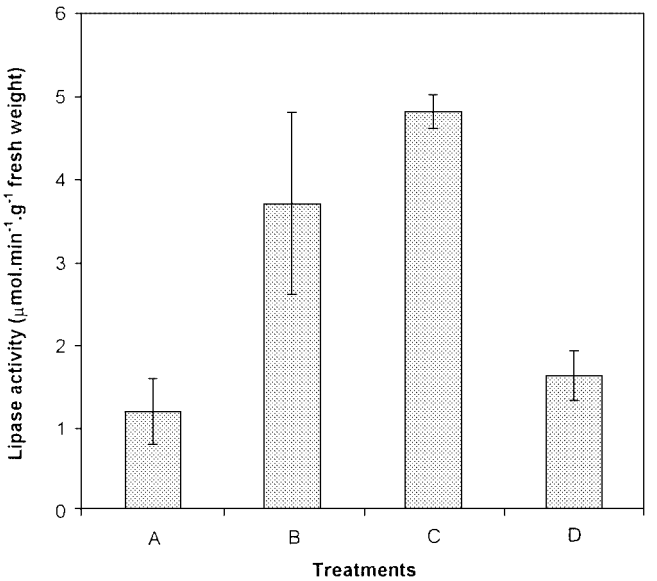

Fig. 8 Susceptibility of sunflower oil bodies as a substrate for maize lipase. Tris-urea-washed oil bodies (25 mg) from sunflower IS (A), digested with 0.25 mg ml^{-1} trypsin (B) or 3.2 mg ml^{-1} of TSP of 4-day-old sunflower seedlings (C) and also oil bodies from 3 d old seedlings (D) were used as a substrate for maize lipase. The liberated Fatty acids were quantified spectrophotometrically by using Rhodamine 6G reagent, according to Chakrabarty et al. (1969). Each value is the mean of three independent experiments ± SE.

enzymes, such as trypsin, on the oil bodies, increased their susceptibility as a substrate for lipid metabolizing enzymes, such as phospholipase A_2 (Tzen and Huang 1992) or lipoxygenase (Matsui et al. 1999). More recently, however, Beisson et al. (2001) concluded that oil body mobilization in the germinating seedlings can be achieved by the action of lipase(s) without prior action of a protease. Oil bodies from maize scutella are a rich source of lipase. Due to our failure to detect lipase activity in sunflower, we tested the susceptibility of oil bodies from sunflower to lipase from maize. Pretreatment of urea-washed oil bodies with trypsin or endogenous protease (TSP) resulted in a 3–4 times higher rate of lipolysis as compared to control (Fig. 8). However, significant activity was observed on untreated oil bodies, consistent with the observations of Beisson et al. (2001). Compared to oil bodies from IS, oil bodies from 3-day-old seedlings were more susceptible (about 20%) to lipolytic action, as was evident from higher lipase activity obtained, when they were used as a substrate.

Discussion

Protein degradation has been shown as a major factor regulating the activity of many enzymes involved in lipid catabolism in germinating oilseeds (McFadden and Hock 1985, Eising and Gerhardt 1987, Matsui et al. 1993). Matsui et al. (1999) hypothesized that proteolysis of oleosins is the first step that triggers oil body mobilization. There is, however, no supportive evidence for the in vivo oleosin degradation at the onset of oil body mobilization. In comparison with some other oilseeds, oleosins in the oil body membranes of sunflower (as analysed in the present work) display significant changes during seed germination. Using a stringent urea-washing method that removes all proteins except oleosins from the oil body membranes (Millichip et al. 1996), present investigations have shown that the three protein bands (16, 17.5 and 20 kDa) represent true sunflower oleosins.

Homogenization of cotyledons in Tris-urea buffer resulted in a significantly different oil body polypeptide pattern as compared with HEPES-sucrose buffer (Fig. 2A, C). As a strong chaotropic agent, urea is expected to denature any enzyme as the cells rupture. Therefore, the apparent disappearance of oleosins observed (following tissue homogenization in HEPES-sucrose buffer) at very early stages of seedling growth, may be due to the presence of a protease activity in the extracts of germinating seedlings. This protease may act specifically on oleosins as no proteolytic effect on non-oleosin proteins of higher molecular masses is observed (Fig. 2A). Sunflower oleosins displayed differential degradation when analyzed after tissue (cotyledon) homogenization in Tris-urea buffer. Kinetics of in vitro oleosin degradation by sunflower protease in the TSP (Fig. 4A) simulates the in vivo pattern (Fig. 2C). Degradation of the 17.5-kDa oleosin was faster than that of the other two (16 and 20 kDa), implying that the protease responsible for in vivo oleosin degradation has retained its activity in seedling

extracts. Whereas sunflower, safflower and castor bean exhibit differential oleosin degradation with progress in germination, rapeseed, soybean and peanut did not reveal any significant changes (Fig. 3). Transient formation of an oleosin degradation product in sunflower (14.5 kDa, Fig. 4A) indicates that the protease acts at specific exposed portions of N- and/or C-terminal domains of oleosin, these being more prone to proteolysis. The final in vitro oleosin degradation product, i.e. PPF, is observed as a 11-kDa polypeptide in vivo just 1 d after germination (Fig. 2C, 4B). Its relative proportion increases between 3 and 5 d of germination (Fig. 2C). Earlier work has not revealed any detectable accumulation of degradation products of oleosins, as inferred from investigations using antibodies raised against intact oleosins (Herman 1995), indicating their complete mobilization during seed germination.

Analysis of sunflower oleosin degradation by the action of protease from the cotyledonary extracts of sunflower seedlings has shown a substantial increase in protease activity between 3 and 5 d of germination (Fig. 5). This is in agreement with in vivo results of 11-kDa PPF accumulation during this period (Fig. 2C). Zymographic analysis revealed a protease with an apparent molecular mass of 65 kDa and exhibiting maximal activity in neutral to alkaline pHs (Fig. 6). The 65-kDa protease exhibits some features also suggested by Shutov and Vaintraub (1987) which make it a good candidate for future investigations on an enzyme responsible for limited oleosin degradation in the germinating sunflower seedlings. The formation of an 11-kDa PPF in vivo and 65-kDa protease activity coincide during different stages of seed germination (Fig. 2C, 6A). The enzyme is activated by 2-mercaptoethanol (Fig. 6C), as it was observed that oleosin degradation is also enhanced by 2-mercaptoethanol with the protease source present in the TSP (Fig. 5). Finally, any proteolytic activity on the cytosol-facing oil body surface is expected at neutral to slightly alkaline cytosolic pH. While degradation of many cytosolic proteins is accomplished by the ubiquitin–proteosome pathway, the degradation of cytosol-exposed domains of oleosins is expected to be achieved by a non-ubiquitin–proteosome pathway. Purification and further characterization of the 65-kDa protease will clarify its role in oleosin mobilization. Besides 65-kDa protease, we have identified six other proteases in the dark-grown seedlings, but all of them display optimal activity at acidic pH (data not shown). This indicates that they belong to lytic compartments with acidic pH, such as vacuoles or protein storage vacuoles (Swanson et al. 1998).

Can limited proteolysis of oleosins be considered a prerequisite for TAG degradation in the oil bodies? Considering the 11-kDa PPF as a marker of oil bodies that have undergone proteolysis, it starts increasing between 3 and 5 d of germination (Fig. 2C), corresponding with the period at which seedlings display the highest in vivo rate of lipolysis along with an increase in the activity of fatty acyl-ester hydrolase (Fig. 1). These observations imply that oleosin degradation and lipid mobilization are interrelated. Moreover, protease-treated oil bodies are 3–4 times more susceptible to the action of maize lipase, as is evident from the enhanced release of fatty acids in the incubation mixture (Fig. 8), indicating that prior proteolysis of the oleosin coat is necessary for efficient TAG hydrolysis. The hydrolysis of the IS oil bodies by lipase in the absence of a protease in the present work could be due to loss of integrity of a fraction of oil bodies during the isolation and washing steps. Our observations on sunflower, however, do not exclude the existence of other mechanisms of oil body mobilization operating in other oilseeds.

Compared to 16- and 20-kDa oleosins, the 17.5-kDa oleosin is more prone to proteolysis by the endogenous protease (Fig. 4A) as well as trypsin (Fig. 7A). This differential susceptibility to proteolytic action observed in the present study on sunflower oleosins is also manifested in their cleavage products. Thus, the 11- and 12-kDa polypeptides are degraded faster than 9- and 13-kDa polypeptides (Fig. 7B). Accordingly, formation of two PPFs (8- and 8.8-kDa polypeptides) might represent these differences. Two oleosin isoforms have been reported earlier in some oilseeds (Tzen et al. 1990). These isoforms differ in their amino acid sequences (Tzen et al. 1992, Chuang et al. 1996) which might confer upon them differential sensitivity to proteolytic actions.

Oleosin isoforms coexist on oil body surface (Tzen et al. 1998). Had every individual oil body been coated uniformly with different oleosin isoforms and mobilized instantly, while others remained intact in the course of germination, as suggested by Tzen et al. (1997), any changes in the relative quantities of sunflower oleosins should not have been observed during the progress of germination (Fig. 2C). Thus, to reconcile the results of the present study on sunflower oil body mobilization with the model of oil body mobilization suggested by Tzen et al. (1997), it is supposed that oil bodies in a fully mature sunflower seed are not identical with respect to their oleosin composition. In this regard, the physiological significance of having different oleosin isoforms is to achieve temporal regulation of oil body mobilization and distribution of energy and carbon resources of TAGs at every stage of seedling development (Huang 1996, Frandsen et al. 2001). In other words, oleosin isoforms can be considered as determinants of oil body longevity in the germinating sunflower seedling. In agreement with the expected differential coating of oil bodies in sunflower with low and high molecular mass oleosins, early and mid-late expression patterns of two sunflower oleosin transcripts (PSO5 and PSOM, respectively) have earlier been reported during embryo development (Thoyts et al. 1995, Mazhar et al. 1998).

In summary, the present work has shown limited oleosin degradation during oil body mobilization in sunflower seedlings. The in vitro enhancement of TAG degradation from oil bodies by lipase action following protease action, along with the appearance of 11-kDa PPF on the first day of germination (when oil body TAG matrix has not yet begun to get mobilized), supports the hypothesis of Matsui et al. (1999) that ole-

osin mobilization is a prerequisite for subsequent TAG matrix mobilization in some oilseeds. Different isoforms of oleosins exhibit different sensitivities to protease action. The 65-kDa protease has features which make it a good candidate for investigations on an enzyme responsible for oleosin degradation. Work is in progress in the author's laboratory with regard to further characterization of the 65-kDa protease and 11-kDa PPF. Thus, the present work offers further interesting research opportunities on the early events involved in oil body mobilization during seed germination in oilseeds.

Materials and Methods

Plant material

Sunflower (*Helianthus annuus* L. cv Morden) seeds were soaked for 4 h in water and placed on moist germination paper at 25°C in dark. Radicle emergence was taken as the start of germination. Following germination, cotyledons were harvested from seedlings having similar chronological age and hypocotyl length. Seeds of peanut (*Arachis hypogea* L. cv JL 24), rapeseed (*Brassica campestris* L. cv PUSA Jai Kissan), soybean (*Glycin max* L. cv JS-335) and maize (*Zea mays* L.) were likewise soaked in water and germinated similar to sunflower. Seed storage tissues from seedlings of different plant species at various stages of development after germination, and 6-day-old maize scutella were frozen and stored in liquid nitrogen for subsequent analyses.

Preparation of tissue homogenate

Cotyledons (20 pairs) from IS of sunflower or seedlings grown for 1–7 d, and also other plant materials, were ground to a fine powder in liquid nitrogen and homogenized with cold HEPES-sucrose homogenization buffer [0.1 M *N*-2-hydroxyethyl piperazine-*N*′-2-ethane sulfonic acid (HEPES)-KOH, pH 7.5, 0.4 M sucrose, 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1% (v/v) 2-mercaptoethanol] in a proportion of 3 ml $(g\ FW)^{-1}$ using a pre-chilled pestle and mortar (Lin et al. 1983, Hammer and Murphy 1993). The homogenate was filtered through five layers of muslin cloth and centrifuged at 10,000×*g* for 30 min at 4°C. Oil body pad (top layer of the homogenate in the centrifuge tube) was collected with a spatula and used for subsequent analyses. The TSP in the 10,000×*g* supernatant was used for protease assay and other analyses. While using TSP for the assay of sunflower fatty acyl-ester hydrolase, PMSF and 2-mercaptoethanol were excluded from the homogenization buffer (Teissere et al. 1995). In one experiment sunflower cotyledons from IS or seedlings of different ages were homogenized in Tris-urea buffer (50 mM Tris, pH 7.5 containing 9 M urea) to obtain oil body pad as above.

Measurement of fatty acyl-ester hydrolase activity

Activity of fatty acyl-ester hydrolase (EC 3.1.1.1) was measured spectrophotometrically, according to Winkler and Stuckmann (1979). Thirty mg of *p*-nitrophenyl palmitate (pNPP) was dissolved in 10 ml isopropanol and mixed with 90 ml of a solution containing 0.11% (w/v) gum arabic, 0.23% (w/v) sodium deoxycholate and buffered with 55 mM Tris (pH 8.0). Freshly prepared substrate solution (2.4 ml) was mixed with aliquots (up to 100 µl) of TSP in a total final volume of 2.5 ml and the enzyme-catalyzed release of nitrophenyl anions at 37°C was monitored at 410 nm, during 2–5 min after the start of the reaction. The extinction coefficient (ε) was assumed to be 15 $cm^2\ \mu mol^{-1}$.

Washing of oil bodies

The oil body pad obtained after centrifugation was resuspended in 1.25 ml Tris-urea buffer (50 mM Tris, pH 7.5 containing 9 M urea) and incubated for 1 h at room temperature with frequent vortexing. The oil bodies were recovered by centrifugation at 10,000×*g* for 30 min at room temperature. This washing procedure was repeated three times (Millichip et al. 1996). Alternatively, the oil body pad from the first step was resuspended in 1.25 ml of 0.1 M $NaHCO_3$ and incubated for 30 min at 4°C (Fujiki et al. 1982, Matsui et al. 1999). Following centrifugation at 10,000×*g* for 30 min at 4°C, the oil pad was saved. Urea-washed or carbonate-washed oil bodies were resuspended in Tris-sucrose buffer (20 mM Tris, pH 7.5 and 0.2 M Suc) in order to wash out excess urea and carbonate, respectively. After centrifugation at 10,000×*g*, the oil pad was resuspended in the same buffer or in other buffers, depending on the experimental design, in order to get a concentration of about 200 mg lipid ml^{-1}.

Solubilization of oil body membrane proteins

In order to remove neutral lipids, washed oil body suspension in Tris-sucrose buffer was extracted five times with four volumes of diethyl ether (Moreau and Huang 1981). Residual diethyl ether in the aqueous fraction was evaporated with a stream of nitrogen. To the aqueous suspension, SDS was added from a 10% (w/v) stock in order to obtain a final concentration of 2% SDS. The sample was heated in a water bath at 90°C for 5 min in order to solubilize membrane proteins. Following centrifugation at 10,000×*g* for 15 min, the supernatant containing solubilized proteins was mixed with reducing Laemmli sample buffer for subsequent SDS-PAGE analyses (Laemmli 1970).

SDS-PAGE analysis of proteins

SDS-PAGE was carried out according to Laemmli (1970), using a 5% stacking gel and a 10 to 20% linear gradient resolving gel. For the analysis of oil body proteins from seedlings of different developmental stages, a 5 to 20% linear gradient resolving gel was used. Urea (6 M) was included in both stacking and resolving gels in case of oil body protein analyses. Gels were stained with 0.2% (w/v) Coomassie blue R-250 in methanol : acetic acid : water (4 : 1 : 5; v/v/v). Loading was based upon equal protein quantities from each sample.

Treatment of oil bodies with sunflower protease and kinetics of oleosin degradation

A suspension of urea-washed oil bodies from IS (50 mg) was taken in HEPES-sucrose homogenization buffer (pH 7.5) containing 50–3,200 µg of TSP from 4-day-old seedlings in a final volume of 1 ml and incubated for 1 h at 25°C. Oil body digestion was stopped by rapid transfer of reaction vials to ice bath (0°C), followed by centrifugation at 16,000×*g* for 10 min at –10°C and transfer of oil body pad to Tris-urea buffer for washing, according to Millichip et al. (1996). The washed oil bodies were used to study the kinetics of oleosin degradation by SDS-PAGE and other analyses.

Assay of sunflower protease activity during seedling development

TSP (200 µg) from IS or seedlings of different ages was used as a source of protease to digest urea-washed oil bodies from IS, as described. The extent of oleosin degradation and the resulting cleavage products were analyzed by SDS-PAGE.

Trypsin treatment of oil bodies and kinetics of oleosin degradation

A suspension of urea-washed oil bodies from IS (50 mg lipid) was taken in 0.1 M Tris buffer (pH 8.0) containing 0.01–500 µg Trypsin (Sigma, U.S.A.) to make a final volume of 1 ml and incubated for 1 h at 37°C. Oil body digestion was stopped by the addition of PMSF to a final concentration of 1 mM. Following trypsin treatment,

oil bodies were recovered and washed (Millichip et al. 1996) for subsequent SDS-PAGE analyses of oleosin degradation products by trypsin.

Zymographic detection of sunflower protease

A 10 to 20% linear polyacrylamide gradient, resolving SDS gel containing 0.1% (w/v) gelatin as protease(s) substrate was used to detect protease(s) in the TSP of germinating sunflower seedlings (Heussen and Dowdle 1980). Aliquots from TSP, equivalent to 100 μg protein, were mixed with non-reducing Laemmli sample buffer solution and incubated at 40°C for 20 min before loading on the gel (Jiang et al. 1999). Following electrophoresis at 7°C, protein bands were renatured by incubating the gel for 40 min in a solution of 50 mM Tris buffer (pH 7.5) containing 2% (v/v) Triton X-100, with one change of solution after 20 min. The gel was transferred to developing buffer (50 mM Tris, pH 7.5 or other buffers as indicated in the text) containing 0.1% (v/v) 2-mercaptoethanol in a water bath shaker for 2.5 h at 37°C. Bands representing protease were visualized as transparent zones after staining with 0.1% (w/v) amido black in methanol : acetic acid : water (3 : 1 : 6; v/v/v/) solution, followed by destaining in methenol : acetic acid : water (1.5 : 0.5 : 9.5; v/v/v/).

Susceptibility of oil bodies as a substrate for lipase

A suspension of urea-washed oil bodies (25 mg lipid) from IS, IS treated with trypsin or endogenous protease from TSP of 4-day-old seedlings and also oil bodies from 3-day-old seedlings, was taken in 0.1 M Tris buffer (pH 7.5) in a final volume of 980 μl. Lipolysis was started by the addition of 20 μl of a maize oil body membrane preparation containing lipase activity (Lin et al. 1983). Aliquots between 30 and 50 μl were taken from the reaction mixture at 15 min intervals, heated at 90°C for 5 min to inactivate the enzyme and extracted with 1.5 ml benzene. To 1 ml of the benzene extract, 0.5 ml Rhodamine 6G (Sigma, U.S.A.) reagent was added and the fatty acids thus released were quantified by reading absorbance at 535 nm (Chakrabarty et al. 1969). Fatty acid production was found to be linear during the first 75 min after the start of the reaction. A standard curve was made using palmitic acid. Lipase activity was expressed as μmol fatty acids released min^{-1} $(g\,FW)^{-1}$ of scutellum.

In the preparation of maize lipase, oil bodies obtained from 1 g maize scutella of 6-day-old seedlings were washed once with the HEPES-sucrose homogenization buffer. After centrifugation at 10,000×*g* for 30 min the resulting oil pad was resuspended in 1 ml homogenization buffer and extracted three times with double volume of cold diethyl ether. The residual diethyl ether was evaporated under a stream of nitrogen. The aqueous suspension obtained was oil body membrane or solubilized membrane components containing lipase activity (Lin et al. 1983).

Other analytical methods

The protein in 10,000×*g* supernatant (TSP fraction) was measured according to Bradford (1976). SDS-solubilized oil body membrane proteins were quantified according to Markwell et al. (1981). Total lipids were extracted, gravimetrically determined and resolved by thin layer chromatography, according to Hara and Radin (1978).

All experiments were performed at least three times.

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