Available online at www.sciencedirect.com
ScienceDirect
Plant Physiology and Biochemistry 44 (2006) 714–723
Plant Physiology and Biochemistry
www.elsevier.com/locate/plaphy

Research article

Evidence for the probable oil body association of a thiol-protease, leading to oleosin degradation in sunflower seedling cotyledons

S. Vandana, S.C. Bhatla*

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

Received 11 February 2006; accepted 29 September 2006
Available online 27 October 2006

Abstract

The activity of a 65 kDa, cytosolic protease from sunflower seedling cotyledons coincides with the degradation of oleosins during seed germination. Further investigations carried out in this laboratory have demonstrated the probable association of a thiol-protease with oil bodies, leading to gradual degradation of oleosins during seedling growth. Evidence to this effect have been brought out through zymographic detection of protease activity from oil bodies, degradation of oleosins by electrophoretically eluted protease from the seedling cotyledons and inhibition of protease activity by thiol-protease inhibitor, such as N-ethylmaleimide (NEM). In addition to these biochemical evidence, visualization of thiol-protease activity has also been achieved by a novel fluorescence microscopic method and confocal imaging. It involves the uptake and binding of a fluorogenic thiol-protease inhibitor (fluorescein mercuric acetate, FMA) at the intracellular thiol-protease activity sites in protoplasts, leading to fluorescence emission at 523 nm following excitation at 499 nm. Maximum protease activity is observed in 4-d-old seedling cotyledons, coinciding with the phase of active triacylglycerol (TAGs) hydrolysis. All these observations provide evidence for the expression of the said thiol-protease activity on the oil body surface, leading to gradual proteolysis of oleosins during seed germination.

Keywords: Confocal microscopy; Fluorescein mercuric acetate; Fluorescence photomicroscopy; *Helianthus annuus* L.; Seed germination; Thiol-protease activity

1. Introduction

Oilseed crops have tremendous potential as sources of edible oils and renewable oil-based raw materials for the chemical and pharmaceutical industries. During development, oilseeds synthesize storage oils, principally triacylglycerols (TAGs), as food reserves for germination and post-germinative growth of seedlings. TAGs are present in small, discrete, intracellular organelles called 'oil bodies', 'lipid bodies', 'oleosomes' or 'spherosomes'. Seed oil bodies are 0.5–2 μm in diameter and comprise of a TAG matrix shielded by a monolayer of phospholipids (PL) and proteins. These proteins include abundant structural proteins–oleosins, and at least two minor proteins–caleosins and steroleosins [29,36]. Oleosins are alkaline proteins with molecular mass of 15–26 kDa in most cases, depending on the isoform and plant species in which they occur. Oleosins are considered to play a role in the biogenesis, stability and mobilization of oil bodies [4,23,29]. Caleosins are a novel class of oil body proteins with calcium-binding domain. They are believed to be involved in oil body fusion and lipid trafficking [12,37]. Steroleosins, on the other hand, are proposed to be involved in signal transduction, regulating certain specialized biological functions related to seed oil bodies [28].

A variety of enzymes are expected to get associated with oil bodies in the seedling cotyledons during seed germination. Feussner and Kindl [9] have reported a lipoxygenase to be a constituent of the main lipid body proteins in cucumber and soybean cotyledons during TAG mobilization. Lipoxygenase is activated when it binds to lipid body membrane [10] and oxygenates the storage lipids containing linoleic acid, paralleled by a specific release of the oxidized fatty acids from the lipid bodies into the cytosol [11]. Regulated proteolysis of membrane proteins is observed to be associated with various

Abbreviations: FDA, fluorescein diacetate; FMA, fluorescein mercuric acetate; NEM, N-ethylmaleimide; PMSF, phenylmethanesulfonyl fluoride; TAGs, triacylglycerols.

* Corresponding author. Tel./fax: +91 11 2766 6744.
E-mail addresses: shwetavandana@hotmail.com (S. Vandana), scbhatla@hotmail.com (S.C. Bhatla).

0981-9428/$ - see front matter © 2006 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.plaphy.2006.09.022

aspects of plant development [7]. Matsui et al. [32] observed that trypsin-assisted digestion of isolated oil bodies from the cotyledons of cucumber seedlings renders them more susceptible to lipoxygenase action. Oil bodies extracted from mid- to late stages of seedling development are much better substrates for lipoxygenase action as compared to those from early germinating seedlings. These results lead Matsui et al. [32] to hypothesize that the processing of lipid body proteins, particularly oleosins, is the first step for the initiation of oil body mobilization. More recently, Murphy [35] observed partial cleavage of some oleosin isoforms prior to full breakdown of storage lipid bodies in rape seedlings. In this case, oleosins are cleaved from 19 kDa to about 16 kDa fragments but retain their recognition by antibodies and binding to lipid bodies. This limited proteolytic processing of oleosins does not immediately affect the integrity of lipid bodies but it may render the latter more susceptible to lipase action on TAG matrix [36].

Further evidence was reported from our laboratory to support the processing of oleosins prior to oil body mobilization in sunflower [44]. The activity of a 65 kDa, cytosolic protease is enhanced, coinciding with the cleavage of oleosins into smaller fragments. Oil bodies containing the processed oleosins are expected to be susceptible to lipase-mediated mobilization of the TAGs stored in the oil body matrix. The process of oleosin processing and TAG mobilization in sunflower is further enhanced in light-grown seedlings [45]. A novel fluorescence microscopic method has also been developed for the in situ localization of temporal and spatial changes in lipase activity in protoplasts of sunflower seedling cotyledons [18,19]. Employing thiol-protease inhibitors, zymographic analyses and a novel in situ localization method, present work provides evidence for the probable action of a 65 kDa, cytosolic, thiol-protease on oleosin degradation in sunflower seedling cotyledons.

2. Results

2.1. Oleosins are differentially degraded during the course of seed germination

A comparison of the SDS-PAGE profile of the total soluble protein (TSP) from the 10,000 × *g* supernatant (Fig. 1A) and the oil body membrane polypeptide pattern (Fig. 1B), analyzed after tissue homogenization and washing of oil bodies in Tris–urea buffer, showed oleosins of 16, 17.5 and 20 kDa. While 20 and 17.5 kDa oleosins largely remained unchanged, the 16 kDa oleosin gradually disappeared following germination. Mobilization of 16 kDa oleosin was faster than 20 and 17.5 kDa oleosin. No major polypeptide bands were detected in the TSP in this range.

2.2. A 65 kDa, cytosolic protease is expressed coinciding with lipolysis in seedling cotyledons

Zymographic analysis of the TSP subsequent to SDS-PAGE on a 10–20% gradient gel containing 0.1% gelatin, showed protease action corresponding to 65 kDa. This was evident on the basis of creation of a gelatin-free zone after staining the gel with amido black. A protease with an apparent molecular mass of 65 kDa was, thus, identified (Fig. 2A). The enzyme activity was found to be negligible in 1-d-old seedlings and it increased in the seedling cotyledons at 4 and 6 d stage of germination. Findings from the above two experiments are in conformity with our earlier observations on the expression of a 65 kDa protease in TSP, coinciding with oleosin degradation in the oil body fraction [44].

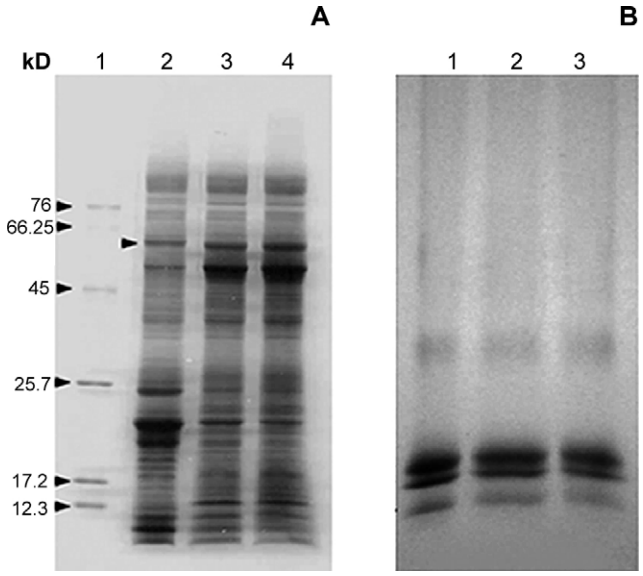

Fig. 1. Protein profiles of TSP and oleosin polypeptides. **A**, Polypeptide pattern of TSP from the cotyledons of dark-grown sunflower seedlings at 1 (lane 2), 4 (lane 3) and 6 d (lane 4) stage of growth. Lane 1 represents the molecular weight marker. **B**, The pattern of oleosin polypeptides during seed germination in dark at 1 (lane 1), 4 (lane 2) and 6 d (lane 3) stage of developing sunflower seedlings. Oil body pad was washed with 9 M urea followed by resuspension in Tris–sucrose buffer to remove excess urea, prior to subsequent treatments for SDS-PAGE analysis of oleosins. Other details as in 'Section 4' (Section 4.3).

2.3. The activity of 65 kDa protease is also evident on the oil body surface

The 65 kDa protease detected from the TSP of seedling cotyledons (Fig. 2A), was also detectable in the oil body fraction from the seedling cotyledons of different developmental stages (Fig. 2B). Oil bodies obtained after centrifugation were resolved on 10–20% gradient SDS gel, followed by zymography, as detailed in 'Section 4'. Association of protease activity with oil bodies from 1-d-old seedling cotyledons was negligible. Its activity was enhanced in 4-d-old seedling cotyledons and remained more or less same in 6-d-old seedlings, coinciding with observations from the TSP of seedling cotyledons at the respective stages of seedling development. The non-expression of a band equivalent to 65 kDa protease during electrophoretic analysis of oleosins (Fig. 1B) is not an anomaly because in this case oil bodies were subjected to washing with 9 M urea in order to principally obtain oleosins (free from any other loosely bound proteins). In contrast, oil bodies were sub-