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Research article

Oil body mobilization in sunflower seedlings is potentially regulated by thioredoxin *h*

Nahid Babazadeh [a], Maral Poursaadat [a], Hamid Reza Sadeghipour [a,*], Abasalt Hossein Zadeh Colagar [b]

[a] Department of Biology, Faculty of Science, Golestan University, Gorgan 4913815739, Iran
[b] Department of Biology, Faculty of Basic Science, University of Mazandaran, Babolsar, Iran

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ABSTRACT

Thioredoxins are believed to mediate starch and protein mobilization in germinating cereals and dicotyledons. Nothing is known about redox regulation of lipid mobilization in plants. The possible redox regulation by thioredoxin *h* (Trx *h*) of a thiol–protease which degrades the oleosin coat of the oil body and its impacts on lipid mobilization was investigated in sunflower (*Helianthus annuus* L.) seedlings. An alkaline proteolytic activity stimulated by light was detected in seedlings. In vitro, the activity of this alkaline protease increased after reduction by NADPH-thiordoxin reductase system (NTS). The expression pattern of an alkaline 65 kDa thiol protease detected by gelatin SDS–PAGE technique, corresponded to the activity profile of the NTS-activated protease. The thiol-specific fuorochrome monobromobimane (mBBr) showed that a 65 kDa protein was also in a reduced state in vivo and becomes reduced in vitro by NTS. Except for 17–20 kDa oleosins, other oil body associated mBBr-labeled proteins were disappeared within three days following germination. Treatments of sunflower oil bodies by the NTS-activated alkaline protease made them more susceptible to maize lipase action. Ascorbate application enhanced lipid mobilization of seedlings. A model for seedling oil body mobilization was proposed according to which Trx *h* or other Trx isoforms, reductively activates an oleosin degrading thiol–protease and some oil body proteins, thus renders the organelle more susceptible to subsequent lipolytic actions. For the first time the potential role of Trx in the mobilization of lipid reserves in plants has been shown.

1. Introduction

Triacylglycerols (TAGs) are one of the major food reserves of oilseeds which are mobilized post-germinatively to fulfill energy and carbon demands of growing embryos [1]. TAGs constitute the matrix of a lipid storing organelle called oil body or lipid body, the latter is encapsulated by a half-unit membrane of phosphorlipids and proteins like caleosins, steroleosins and most notably unique oleosins [2]. Oleosins serve to prevent oil bodies from coalescence while they are packed within storage tissues [3]. They may also function as determinants of oil body longevity during seedling growth [4].

Oil body mobilization at least in some plants, appears to require the integrated sequential action of proteases, phospholipases and lipases for the hydrolysis of oleosins, phospholipids and TAGs, respectively. Thus, the in vitro phospholipase activity on the isolated oil bodies requires prior treatment of the organelle by a protease [5]. Furthermore, proteases and phospholipases increase the accessibility of TAG matrix of the isolated oil bodies to either lipase or lipoxygenase, respectively [4,6]. Limited mobilization of oleosins during oil body mobilization has been reported in sunflower and cucumber seedlings [4,6]. The in vivo action of proteases and phospholipases on the oil body surfaces of sunflower and cucumber seedlings has also been reported [7–9]. These hydrolytic actions lead to the organelle membrane disruption and its increased susceptibility to lipolytic enzymes [4,6]. There are also other mechanisms for oil body mobilization in plants [1].

In germinating cereal grains, early events related to the inception of mobilization of both storage proteins and carbohydrates are mediated by thioredoxin *h* (Trx *h*). It is a low molecular weight protein containing a bicysteine conserved WCG/PPC motif with dithiol oxidoreductase activity [10]. After reduction by $NADP^+$–thioredoxin reductase (NTR), it reduces the oxidized

Abbreviations: DTT, dithiothreitol; EDTA, ethylene diamine tetra acetic acid; FFA, free fatty acids; IS, imbibed seeds; mBBr, monobromobimane; NEM, N-ethylmaleimide; NTR, $NADP^+$–thioredoxin reductase; NTS, $NADP^+$–thioredoxin reductase system; NTSP, non-reduced total soluble proteins; PMSF, phenylmethylsulfonylfluoride; PVPP, polyvinylpolypyrrolidone; TAGs, triacylglycerols; TCA, trichloroacetic acid; Trx *h*, thioredoxin *h*; TSPs, total soluble proteins.

* Corresponding author. Tel.: +98 9111775149; fax: +98 1714427040.
E-mail address: h.r.sadeghipour@gmail.com (H.R. Sadeghipour).

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inter-/intra-molecular disulfide bonds of the target proteins and in this way modulates their activity [11]. Thus the inception of storage protein mobilization by Trx *h* in germinating cereal grains is achieved through the reductive activation of proteases [12,13] and/or reductive unfolding of storage proteins accompanied with their increased solubility and susceptibility to proteolytic actions [14]. Reductive inactivation of protease inhibitors might also account for the increased mobilization of storage proteins by Trx *h* [15]. Trx *h* or other Trx-like proteins also activate storage starch mobilization in cereal endosperm [16]. The regulation of starch mobilization by reduced Trx *h* is seemingly brought about by the reductive activation of amylolytic enzymes like pullulanase [17], and the inactivation of amylase inhibitors [15]. Proteomic studies in the last decade have also established the potential role of thioredoxins in regulating starch and protein mobilization in plants [10,13,18].

Despite conclusive evidence for the initiating role of Trx *h* in the mobilization of storage proteins and carbohydrate during seed germination, nothing is known about the potential regulation of lipid mobilization by thioredoxins in plants. As oleosin proteolysis in sunflower seedlings is mediated by a thiol–protease and it leads to increased susceptibility of oil bodies to subsequent lipolytic actions [4], the possible regulation of this protease by Trx and its impacts on seedling lipid mobilization were investigated. Having establishment of the in vitro regulation of this thiol protease by Trx *h*, the physiological significance of this regulation in sunflower seedlings has been discussed.

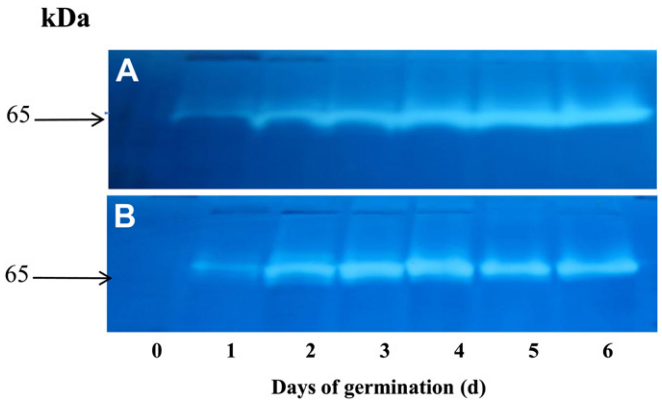

Fig. 2. Developmental changes in sunflower seedling protease activity active at pH 8.0 by gelatin SDS–PAGE technique. Total soluble proteins (TSPs) were extracted from either imbibed seeds (IS) and 1–6 days old dark- (A) and light- (B) grown sunflower seedlings, mixed with electrophoresis sample buffer and heated at 40 °C for 20 min. Proteins (100 μg) were separated on a gelatin containing 15% SDS gel, re-natured and stained by Amido Black. Bands of protease activity corresponding to gelatin-digested area were then visualized.

2. Results

2.1. Development of protease activity in sunflower seedlings

Azocasein is a synthetic chromogenic substrate for sensitive assay of endopeptidase activity over a wide range of pHs. Using azocasein, the proteolytic activity of total soluble proteins (TSPs, 10,000 × g supernatant) from 3 to 4 d old sunflower seedlings was investigated at various pHs [Fig. 1A]. The greatest activity occurred at pH 5.0 which declined with the increase of pH up to 7.0. A second rise in protease activity occurred at pH 8.0 and gradually declined toward pHs 9.0. The absolute protease activity at pH 8.0 was about one fifth of that observed at pH 5.0. Thus sunflower seedlings might have at least one acid and one alkaline protease.

The azocaseinolytic activity at pH 5.0 [Fig. 1B] was detectable in imbibed seeds (IS). This activity was greatest in both 3 d old dark- and light-grown seedlings and declined gradually thereafter. There were no remarkable differences between dark and light grown seedlings in the patterns of protease activity at pH 5.0. The azocaseinolytic activity at pH 8.0 was hardly detectable in IS [Fig. 1C]. In dark, the enzyme activity was greatest in 4–5 d old seedlings. In light, it increased sharply and was greatest in 3–4 d old seedlings. For the first four days, the enzyme activity of light grown seedlings was significantly greater than that of the corresponding dark grown ones. The proteolytic activity detected on azocasein at pH 8.0, was also able to degrade milk casein and the caseinolytic activity of light grown seedlings was much greater and appeared earlier than that of the dark grown ones (data not shown). For further characterization of seedling alkaline proteolytic activity, casein rather than azocasein was used as the substrate.

2.2. A 65-kDa thiol protease active at alkaline pHs is likely to represent the protease acting on azocasein at pH 8.0

Protein samples (100 μg) from TSPs of IS and seedlings were resolved by gelatin SDS–PAGE, re-natured and the transparent gelatin digested zones obtained following gel incubation at pH 8.0 were visualized by Amido Black staining. A 65-kDa band of protease activity formerly reported as a thiol–protease implicated in oleosin mobilization [4,8,19] was identified [Fig. 2A]. Based on the thickness of the gelatin digested zones, the activity was not detectable in IS, hardly detectable in 1 d old dark grown seedlings and gradually increased with the maximum activity occurred in 4–5 days old

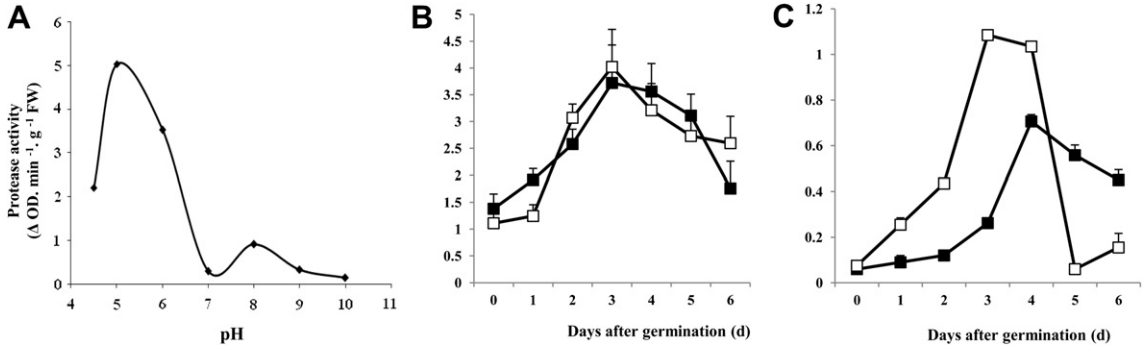

Fig. 1. Proteases of sunflower seedlings. The crude cotyledonary extract from 4 day old sunflower seedlings were used as a source of protease and its ability in azocasein digestion was assessed at different pHs (A). As the greatest protease activity in A occurred at pHs 5.0 and 8.0, changes in azocaseinolytic activity of both dark-grown (closed square) and light-grown (open square) sunflower seedlings were investigated at pHs 5.0 (B) and 8.0 (C) for 6 d of seedling growth. Each value in B and C are means of three independent experiments ± SE.