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Catalase activity in developing seedling of opium poppy *Papaver somniferum* L.

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Abstract

Catalase is an enzyme unique to glyoxysomes in developing poppy seedlings. Catalase activity is very low in endosperm and in embryo of germinating poppy seeds. During postgerminative growth and development the enzyme activity increases rapidly with maximum in endosperm on day 2 and in developing seedling on day 3. A rapid decline of enzyme activity parallells the extension growth of poppy seedlings. Three electrophoretic forms of catalase have been detected in isolated glyoxysomes and partially purified catalase preparation. Electron microscopic observation indicates the presence of catalase in glyoxysomes of parenchyma cells of poppy seedling cotyledons. Numerous lipid bodies and electron-dense deposits in vacuoles are the most characteristic feature of these cells.

Additional key words: cotyledons, endosperm, glyoxysomes, sacharose gradient, seedlings.

Introduction

Seeds of opium poppy, *Papaver somniferum* L. belong to a group of oil seeds. The content of reserve lipids in endosperm of poppy seeds is about 40 - 50 % of dry seed mass (Bewley and Black 1978) and the seed reserve lipids are stored in lipid bodies (Huang *et al.* 1983). The amount of lipid bodies in the cells of outer endosperm layer is higher than in embryo of imbibed seeds of *Papaver bracteatum* Lindl. (Pšenák *et al.* 1985).

The mobilization of lipid reserves during seed germination and early seedling growth is realized by the function of glyoxysomes (Beevers 1982). Fatty acids released are degraded via the beta-oxidation pathway (Hutton and Stumpf 1969), and

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acetyl-CoA formed is converted by glyoxylate cycle to a substrate of gluconeogenesis (Fusseder and Theimer 1984, Reddy and Mannaerts 1994).

Catalase, H_2O_2 oxidoreductase (EC 1.11.1.6) is a characteristic enzyme of glyoxysomes and has been continually used as a biochemical and histochemical marker enzyme for glyoxysomes and peroxisomes (Kunce *et al.* 1984, Kunce and Trelease 1986, Kindl 1992). Catalase is responsible for the breakdown of potentially harmful hydrogen peroxide released by beta-oxidation pathway in glyoxysomes.

To correlate the biochemical and developmental processes with the accumulation of thebaine in poppy seedlings, first the rate of seedlings growth and the developmental profile of thebaine have been determined (Pšenák *et al.* 1987b). In the following experiments the developmental profile and organ distribution of catalase during poppy seed germination and seedling growth is reported. Glyoxysomal catalase activity was electrophoretically and histochemically characterized.

Material and methods

Plant material: Opium poppy seeds, *Papaver somniferum* L., cv. Amarín germinated in Petri dishes on a polyurethane foam layer (thickness 1 cm) covered with nylon cloth (at 25 °C, in the dark, 75 - 80 % relative humidity, 0.15 g of seeds and 100 cm^3 redistilled water per dish).

Catalase extraction and purification: For determination of developmental profile of catalase, 150 seedlings (embryos and endosperms separated) were extracted after homogenization (teflon-glass) in 2 cm^3 extraction media, pH 7.5. This media contained 0.05 mol dm^{-3} HEPES, 0.4 mol dm^{-3} saccharose, 0.01 mol dm^{-3} KCl, 0.003 mol dm^{-3} dithiothreitol, 0.001 mol dm^{-3} EDTA, 0.1 mol dm^{-3} $MgCl_2$ and 0.1 mmol dm^{-3} cysteine (grinding medium). After centrifugation (30 000 g, 0 °C, 15 min), the supernatant obtained was used as a crude enzyme solution.

For purification of catalase 1500, 3-d-old, endosperm free seedlings were used. Catalase purification was performed as it was described (Bezáková *et al.* 1995).

Catalase activity and protein determination: For assay of catalase activity the amount of oxygen released was measured by a Clark-oxygen electrode (*GILSON Oxygraf K-TC*). The reaction mixture in a final volume of 1.7 cm^3 contained: 22 mmol dm^{-3} H_2O_2, 0.01 - 0.05 cm^3 of enzyme solution (15 - 75 μg proteins) and 0.05 mol dm^{-3} Na-phosphate buffer, pH 7.0. The reaction was started by enzyme solution at 25 °C. The amount of oxygen released was calculated according to Heisler (1991). The enzyme activity is expressed in nkat of oxygen evolved. The protein content was determined according to Bradford (1976).

Isolation of glyoxysomes: Glyoxysomes were isolated from 1500, 3-d-old, endosperm-free seedlings by the method of Kunce and Trelease (1986) with minor modifications.

Nondenaturing PAGE: Disc electrophoresis was performed according to Maurer (1971) using 7.0 % polyacrylamide supporting gel (acrylamide:bisacrylamide 28:0.735) and 3.0 % stacking gel (acrylamide:bisacrylamide 2:0.5). On each disc (5 × 80 mm) 100 - 150 μg protein in 0.01 mol dm^{-3} HEPES buffer, pH 7.0 with 20 % saccharose was applied. As electrode buffer 0.05 mol dm^{-3} Tris-glycine solution, pH 8.5, was used, and protein separation took place at 4 °C and 3 - 4 mA/disc. The gels were stained for catalase activity as it was described by Gregory and Fridovich (1974).

Electronmicroscopy and cytochemistry: Samples from cotyledons of 3-d-old seedlings were fixed for 4 h with 5 % glutaraldehyde in 0.1 mol dm^{-3} Na-phosphate buffer, pH 7.2. After washing in the same buffer, catalase was demonstrated using the standard 3,3′-diaminobenzidine (DAB) medium (Fahimi 1969). Control samples were preincubated with propanediol buffer containing 0.02 mol dm^{-3} 3-amino-1,2,4-triazole before incubation with standard DAB medium. Aminotriazole, an inhibitor of catalase activity was used for elimination of glyoxysome staining (Frederick and Newcomb 1969). Following these cytochemical reactions all samples were postfixed with 2.0 % osmium tetroxide in 0.1 mol dm^{-3} Na-phosphate buffer pH 7.2 for 2 h. After dehydration in acetone the samples were embedded in *Durcupan ACM* (*Fluka* Buchs, Germany). Unstained thin sections were viewed with *TESLA BS 613* (*Tesla*, Brno, Czech Republic) and *JEOL 2000 FX* (Tokyo, Japan) electron microscopes.

Results

Catalase activity is relatively low in germinating poppy seed, *i.e.* until up to the radicle protrussion (24 h post-imbibition) (Fig. 1). During seed germination the enzyme activity is higher in endosperm compared to that in embryo. After radicle emergence, there is a rapid increase in catalase activity in endosperm-free seedlings particularly (Fig. 1).

Table 1. Purification of catalase from 3-d-old poppy seedlings

Fractions	Total units [nkat]	Total protein [mg]	Specific activity [nkat mg^{-1}]	Purification [fold]
Crude extract	4 882.06	125.00	39.05	1.00
0 - 40 % $(NH_4)_2SO_4$	2 250.70	22.50	100.03	2.56
Sephadex G-200	103.23	0.30	344.10	8.81
Phenyl-Sepharose CL-4B	49.51	0.05	990.20	25.35

In the endosperm the maximal activity was found on day 2, in endosperm-free seedlings on day 3. After day 2 or 3 a rapid decline in catalase activity was observed in endosperm and particularly in seedlings. In 3-d-old seedlings 95 % of catalase activity was in cotyledons. After day 4 and 5 there was still a low level of catalase activity present in cotyledons (Fig. 2).

Partially purified catalase preparation (Table 1) with a specific activity 990.2 nkat mg^{-1} protein was used for its isoforms determination by PAGE.

Glyoxysomes were prepared from 3-d-old seedlings on the discontinual gradient of sucrose sedimented on the interphase of 50 % sucrose (density 1.20 - 1.25 g cm^{-3}). Broken glyoxysomes and the partially purified catalase were subjected tonondenaturing PAGE. When the gels were negativelly stained three achromatic bands were detected in purified enzyme (results not shown) and in isolated glyoxysomes (Fig. 3).

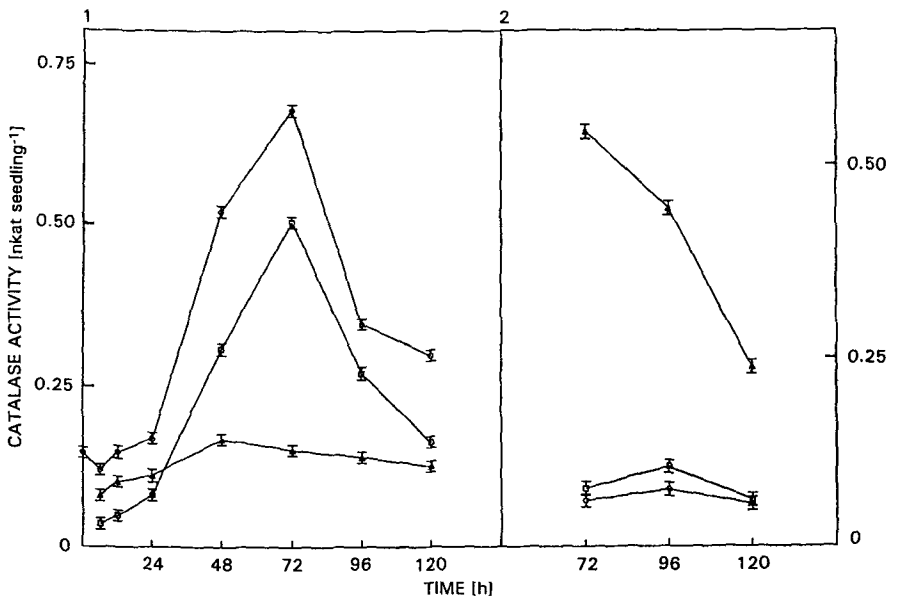

Fig. 1. Developmental profile of catalase in whole poppy seedlings (*circles*), in separated endosperm (*triangles*) and in endosperm-free embryos or seedlings (*squares*).

Fig. 2. Distribution of catalase in endosperm-free poppy seedlings: cotyledons (*triangles*), hypocotyls (*squares*) and roots (*circles*).

Lipid bodies and vacuoles with electron-dense deposits inside were the most apparent features of parenchyma cells of cotyledons from 3-d-old poppy seedlings (Fig. 4 a,b,c). This electron-dense material was located either along the tonoplast or inside of vacuoles in the form of solid electron-dense bodies (Fig. 4 b). Well developed mitochondria, network of endoplasmatic reticulum, etioplast and glyoxysomes are visible in cytoplasm (Fig. 4 c). Glyoxysomes with pleiomorphic shape were localized among lipid bodies, mitochondria and etioplast. After cytochemical staining of catalase, glyoxysomes were strongly stained by reaction products (Fig. 4 a). This reaction was not observed when aminotriazol was used (Fig. 4 d).

Discussion

Developmental profiles of glyoxysomal enzymes activities reflect basically the process of reserve lipids reutilization during oil seeds germination and subsequently during seedlings growth and development.

In the poppy seeds the reserve lipids and proteins are localized in endosperm and embryo. The amount of lipid bodies found in embryo of imbibed seeds is significantly less than in endosperm (Pšenák *et al.* 1985).

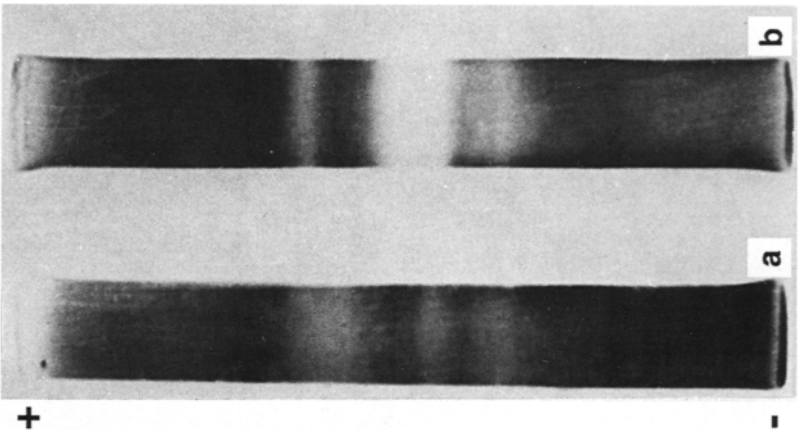

Fig. 3. Nondenaturing polyacrylamide gels (7 %) showing multiple forms of catalase in glyoxysomes from cotyledons of 3-d-old poppy seedlings (*line a*) and in commercially available catalase from beef liver (*line b*).

There is a low catalase activity in both parts of poppy seeds during germination (up to 20 - 24 h subsequent to imbibition).

The activities of other glyoxysomal enzymes are also very low during poppy seed germination (Pšenák *et al.* 1987 a). These results indicate that during this time period fatty acids released from reserve lipids are not the main sources of carbon and energy.

Saccharose was found to be the main soluble sugar in endosperm and embryo of imbibed poppy seeds (Pšenák *et al.* 1981). The content of raffinose is only one fifth of that of saccharose. These soluble sugars are completely reutilized during the period of poppy seeds germination.

The total activity of catalase is increasing in endosperm and in developing seedlings particularly after radicle protrussion (20 - 24 h post-imbibition). The catalase activity in endosperm represents only one third of that in cotyledons. Identical distribution and activity profiles were found for isocitrate lyase and fructose-1,6-bisphosphatase (a marker enzyme of gluconeogenesis) (Pšenák *et al.*

1987a) during postgerminative growth and development of poppy seedlings (between day 1 and 3 post-imbibition) (Pšenák *et al.* 1987 b). These results indicate, that in case of poppy seeds the process of lipid reserve reutilization and gluconeogenesis takes place in endosperm as well as in seedling cotyledons. The extent of metabolic communications between endosperm and cotyledons during poppy seedling growth and development remains to be established. Similar developmental profiles for total

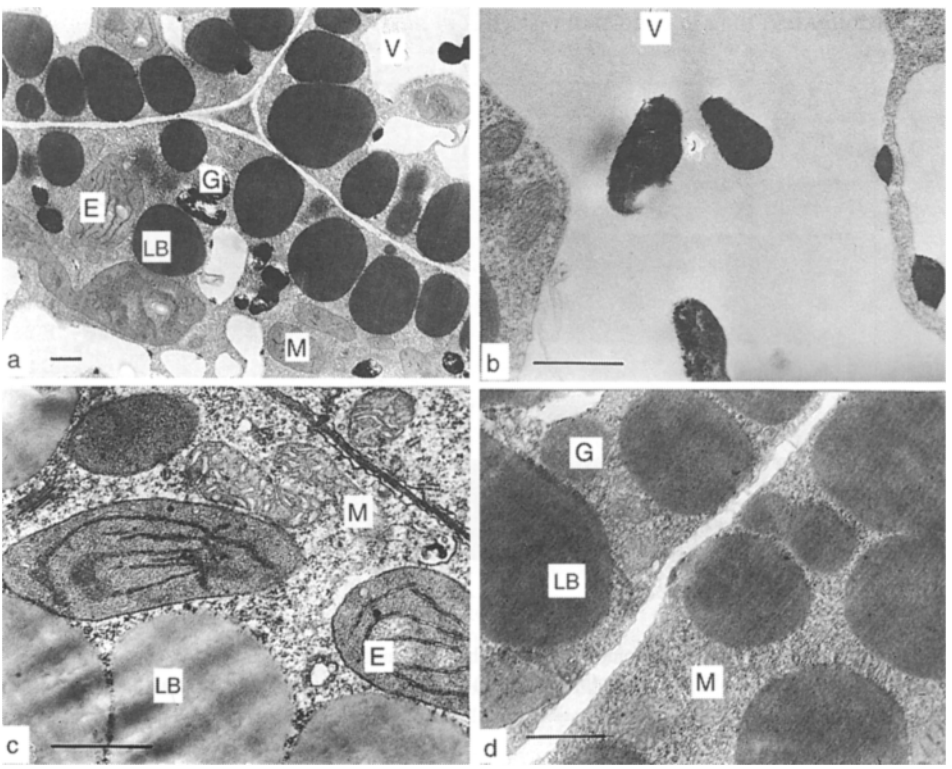

Fig. 4. Electron micrographs of cells from cotyledons of 3-d-old poppy seedlings treated with diaminobenzidine (DAB): *a* - cell of cotyledon with numerous lipid bodies (LB), glyoxysomes (G), etioplasts (E), mitochondria (M) in cytoplasm and with vacuoles (V) containing electron-dense deposits inside. (× 6 300, *bar* = 1 μm); *b* - detail of electron-dense bodies localized inside of vacuole (V). (× 17 200, *bar* = 1 μm); *c* - mitochondria (M) with well developed cristae and lipid bodies (LB). (× 18 600, *bar* = 1 μm); *d* - aminotriazole inhibited DAB staining of glyoxysomes (G) (× 15 600, *bar* = 1 μm).

catalase activity were reported in pumpkin (Yamaguchi *et al.* 1986), cucumber (Davies and Chapman 1979) and cotton seedling cotyledons (Ni *et al.* 1990). This postgerminative rise of glyoxysomal activities in fat storing seeds is a result of their *de novo* synthesis and compartmentalization (Lazarow and Fujiki 1985, Trelease 1984).

Differential expression of tetrameric catalase subunits and selective degradation of individual enzyme isoforms were found to be responsible for postgerminative time course of catalase activity (Ni *et al.* 1990). However in maize three separate genetic loci were identified for catalase. Their differential expression is responsible for catalase isoforms in postgerminative maize seedlings (Redinbaugh *et al.* 1988).

In partially purified enzyme preparation and in isolated glyoxysomes from 3-d-old poppy seedlings three electrophoretic forms of catalase have been observed. Characteristics of these isoforms are presently under investigation. Multiple forms of catalase have been reported for cotton (Kunce and Trelease 1986) and sunflower (Eising and Gerhardt 1986) seedlings. A true isoenzyme system of catalase has been reported in maize seedlings (Redinbaugh *et al.* 1990) and cotton seeds (Ni and Trelease 1991).

Vacuoles observed in parenchyma cells of cotyledons contained electron dense deposits (Fig. 4 a, b). Similar material was reported in vacuoles of cultured cells of *Papaver bracteatum* Lindl. (Cline and Coscia 1989). It is assumed that these electron dense bodies are the sites of alkaloid accumulation. Similar deposits in vacuoles were reported in laticifers of *Papaver somniferum* and *Papaver bracteanum* (Roberts *et al.* 1983, Kutchan *et al.* 1986).

The presence of electron-dense material in vacuoles of cotyledons of poppy seedlings has not yet been reported. Thebaine was found to be the main alkaloid in poppy seedlings. The longitudinal growth of poppy seedlings started between day 3 and 4 post-imbibition and in this period the accumulation of thebaine has been the largest (Wieczorek *et al.* 1986, P enák *et al.* 1987 b). Whether the vacuolar deposits can be related to the thebaine accumulation in poppy seedlings remains to be established.

Numerous glyoxysomes identified by cytochemical reaction of catalase with DAB, are localized close to lipid bodies and/or mitochondria which was also reported for maize seedlings (Bosabalides and Tsaftaris 1987).

These histochemical and biochemical findings indicate the essential role of glyoxysomal function in reserve lipids utilization in post-germinative poppy seedlings growth and development.

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