

Ultracytochemical Localization of Acid Phosphatase in *Humicola lutea* Conidia and Mycelia

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Electron microscopic cytochemical procedures were used to determine the cellular location of acid phosphatase in the fungus *Humicola lutea* grown in casein-containing medium lacking in mineral orthophosphates. In our investigations acid phosphatase in nongerminating conidia was localized on the outer side of the cell wall, in the cell wall, and on the exterior surface of the plasma membrane. The reaction product of acid phosphatase in germinating conidia was seen in the outer wall layer while in young mycelium on the cell surface and in the extracellular space. The relationship between phosphatase activities localized in the cell wall and their role in the enzymatic degradation of the phosphoprotein casein providing available phosphates for cell growth is discussed.

Key words: Localization, Acid Phosphatase, Fungi

Introduction

The production and characterization of fungal acid phosphatases, as well as their function in the cells have been reported in several studies (Haas *et al.*, 1991; Nozawa *et al.*, 1998; Tsekova *et al.*, 2000, 2002). Most of the phosphatases were produced in low-phosphate media containing inorganic forms of nitrogen sources. The information on the biosynthesis of phosphatases by fungi capable of utilizing organic compounds as a phosphorus source is insufficient (Tarafdar *et al.*, 1988).

It is known that the fungus *Humicola lutea* 120–5 produces acid phosphatases during submerged cultivation in a medium containing casein (a mixture of phosphoproteins with different molecular weights) as a sole phosphate source (Aleksieva and Micheva-Viteva, 2000). Our previous studies showed that *H. lutea* acid phosphatases could be determined as constitutive (AcPh I) and induced by casein two Pi-repressible forms (AcPh II and AcPh III) (Micheva-Viteva *et al.*, 2000). The distribution of acid phosphatase activities in the culture liquid and mycelial extract and some differences in the localization of the enzyme in the hyphal filaments were previously discussed (Aleksieva *et al.*, 2003).

The cytochemical localization of the microbial phosphatases has been made mainly in vegetative cultures by a few scientists (Garrison and Arnold,

1983; Arnold *et al.*, 1988; Cherepova and Spasova, 1996; Spasova and Galabova, 1998). Spectrophotometric and cytochemical methods were used for the quantification of acid phosphatase in ungerminated conidia from the fungus *Colletotrichum graminicola* (Schadeck *et al.*, 2000).

In the present study we investigate the cytochemical localization of acid phosphatase in thin sections of conidia and hyphae of the fungus *H. lutea* 120–5 cultivated in casein-containing medium lacking mineral orthophosphates. The main goal is to clarify the physiological role of acid phosphatase in the degradation of casein from the culture medium, releasing the available phosphorus for mycelial growth.

Materials and Methods

Fungus

The fungal mutant strain *Humicola lutea* 120–5 (National Bank for Industrial Microorganisms and Cell Cultures: 391, Bulgaria) isolated and selected (Grigorov *et al.*, 1983) in the Institute of Microbiology, Sofia, was maintained on 1.5% beer agar at 28 °C for 7 d to obtain dense sporulation.

Media and culture conditions

The submerged cultivation of the fungus was carried out in casein-containing medium (CM) without inorganic phosphate (Pi) (per litre): 20 g

glucose, 4 g casein, 0.5 g KCl, 0.5 g MgSO_4 , 0.004 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.003 g $\text{ZnSO}_4 \cdot \text{H}_2\text{O}$, 0.001 g $\text{MnSO}_4 \cdot \text{H}_2\text{O}$. As a control a medium containing the same components plus KH_2PO_4 (Pi) in the concentration 1 g/l was used. The pH value was adjusted to 6.0 and media were autoclaved at 115 °C for 15 min. A portion (4 ml) of the spore suspension containing 10^7 to 2×10^8 spores/ml was used as an inoculum for 50 ml of the nutrient media in 500 ml Erlenmeyer flasks. The submerged cultivation was at 28 °C for conidia germination and mycelia formation. The samples were taken at different times hours of the cultivation for determination of dry biomass, acid phosphatase activity and preparation of thin sections for transmission electron microscopy (TEM).

Enzyme assay

Acid phosphatase activity was assayed by measuring *p*-nitrophenylphosphate (pNPP) hydrolysis, using the method of Andersch and Szeypinski (1947). One unit of enzyme activity is defined as the release of 1 μmol *p*-nitrophenol in 1 min at 37 °C at pH 4.8.

Dry weight measurement

Some samples were assayed for dry mycelium weight (dmw). The biomass concentration was determined by drying at 105 °C until a constant weight was obtained.

Reproducibility

All the experiments concerning the formation of biomass and the determination of acid phosphatase activities were repeated at least twice and the samples were assayed in triplicate. The data points represented the mean values within \pm (2 to 3)% of the individual values.

Ultracytochemical methods

Acid phosphatase in 24- and 36-h-old cultures was demonstrated by the method of Gomori, modified by Miller and Palade (1964), using pNPP as a substrate. The cells were washed in cacodylate buffer (0.1 M, pH 7.2) containing 0.22 M sucrose, centrifugated and fixed preliminary in 2% (v/v) glutaraldehyde in cacodylate buffer at 4 °C for 1 h. The samples were centrifugated and washed in cacodylate buffer. The prefixed cells were incubated for 1 h at 37 °C in 0.5 M sodium acetate (pH 5.0) containing 3% pNPP, lead nitrate and 0.22 M su-

crose. The final pH value was adjusted to 5.0 with 0.2 M CH_3COOH . In the control the incubation mixture contained no substrate.

Transmission electron microscopy

The samples were post-fixed in 1% OsO_4 in cacodylate buffer at 4 °C for 2 h, then dehydrated by increasing concentrations of alcohol and embedded in Durcupan (Fluka). Thin sections were examined by a Zeiss electron microscope (model 10C).

Results and Discussion

Formation of mycelial biomass from *H. lutea* conidia

H. lutea spores were incubated directly in a medium containing casein (phosphoproteins) as a sole phosphate source. As a control, casein-containing medium plus inorganic phosphate (KH_2PO_4) were used. Some differences in growth behavior of the fungus during the course of shake flask cultivation in the two media were established. In the first 12 hours of incubation the mycelial dry weight in the control cultures was almost two-fold higher (0.155 g/flask) compared to the corresponding Pi-free cultures. In the next 24–36 hours the amount of biomass in the samples obtained in CM without Pi increased gradually (0.175 and 0.375 g/flask, respectively) reaching the same level as in the control experiment (0.525–0.530 g/flask) at the end (60 h) of the cultivation process. The biomass formation even in the initial hours of incubation of *H. lutea* conidia in the absence of Pi in CM medium could be explained with the presence of conidial phosphatase which probably hydrolyzed the casein ensuring inorganic phosphate, a key metabolite for cellular development (Biswas and Cundiff, 1991). When an extensive survey of the literature was carried out in order to associate phosphatases with spores or spores in germination, little information was found. According to Nahas (1989) these enzymes play a role in *Neurospora crassa* conidia as a result of the need to supply energy for the initial stages of fungal growth.

Ultracytochemical localization

Representative electron micrographs demonstrating the location of acid phosphatase in conidia (nongerminating and germinating) and young

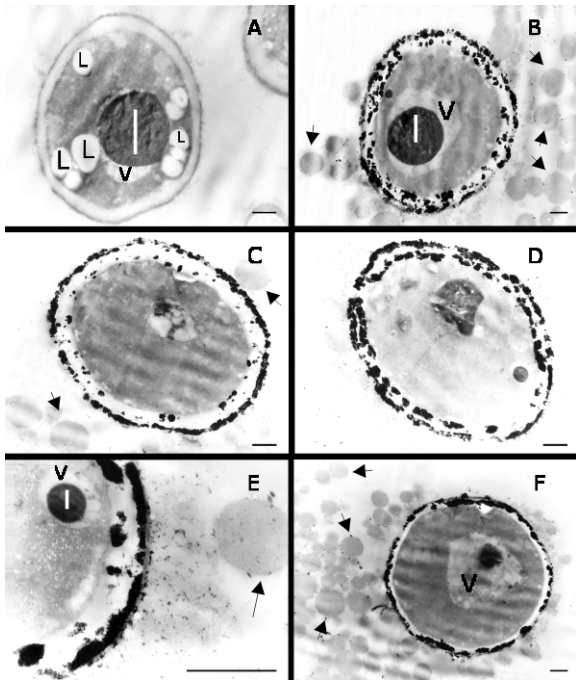


Fig. 1. Cytochemical localization of acid phosphatase in ultrathin sections of nongerminating conidia of *H. lutea*. (A) Section of cell incubated in the absence of enzyme substrate (pNPP); large lipid body (■) with electron-dense areas inserts in a vacuole (V); numerous lipid bodies (L) with electron-transparent matrix in the cytoplasm. (B) Note the clearly expressed reaction with lead phosphate granules on the exterior surface of the plasma membrane, in the inner electron-transparent zone and on the outer melanized layer of the conidial cell wall; large lipid body (■) in a vacuole (V); casein globules (arrows). (C, D) Electron-dense precipitates accumulated on the outer melanized layer of conidial cell wall; (C) individual and (D) numerous lead phosphate deposits on the exterior surface of the plasma membrane. (E) Single amorphous irregular granules on the plasma membrane; lipid body (■) with electron-dense matrix inserts in a vacuole (V); casein globules (arrows). (F) Localization of reaction product beneath the outer layer of conidial cell wall; large vacuole (V) filled with fibrous material; numerous casein globules (arrows). Bars: 0.5 μ m.

hyphae after 24–36 h of *H. lutea* cultivation in CM lacking in mineral orthophosphates are assembled in Figs. 1 and 2. Electron-opaque reaction product was not seen (Fig. 1A and Figs. 2A, C) when the substrate pNPP was deleted from the assay mixture. The sites of lead phosphate formation in nongerminating conidia are: on the outer wall layers, in the inner electron-transparent zone and on the surface of the plasma membrane (Figs. 1B–

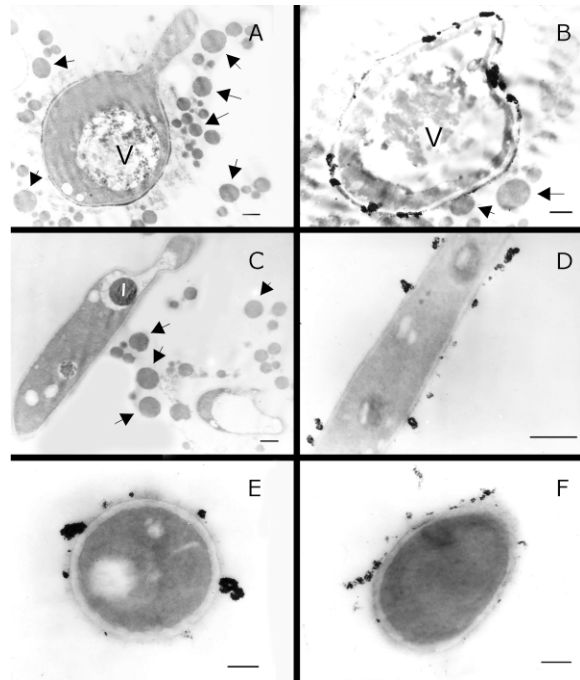


Fig. 2. Cytochemical localization of acid phosphatase in ultrathin sections of germinating conidia and young hyphae of *H. lutea*. (A) Conidial germ tube incubation without an enzyme substrate (pNPP); vacuole (V); numerous casein globules (arrows). (B) Electron-dense precipitate in the outer melanized wall layer of conidial germ tube; large vacuole (V); casein globules (arrows). (C) Section of early hypha (24 h); treatment with incubating medium without substrate (pNPP); lipid body (■) with electron-dense areas; casein globules (arrows). (D) Lead phosphate granules situated on the cell surface of early hyphal filament (24 h). (E, F) Cross section of a hyphal body (36 h); irregular granules (E) on the outside of the cell wall and (F) in the exocellular space. Bars: 0.5 μ m.

F). Similar results were obtained by TEM (Kneipp *et al.*, 2004) demonstrating a cell-wall-associated acid phosphatase in conidial forms of *Fonsecaea pedrosoi*, a fungal pathogen causing chromoblastomycosis. In contrast, acid phosphatase activity in ungerminated conidia from the fungus *Colletotrichum graminicola* was localized in the vacuoles and not on the cell surface (Schadeck *et al.*, 2000). Figs. 1E, F clearly demonstrate the reaction between the substrate (casein globules) and the enzyme (phosphatase) released by the outer conidial wall into the exocellular space. The localization of acid phosphatase in *H. lutea* germinating conidia and young mycelia is shown in Fig. 2. As can be seen, the electron-dense deposits of lead phosph-

phate were observed in the outer wall layer of the germ tube (Fig. 2B) and on the cell surface (Figs. 2D, E) as well as in the extracellular space (Fig. 2F) in the case of 24- to 36-h-cultures of hyphal bodies. These results differ from other observations showing intravacuolar localization of acid phosphatase in germinated spores and hyphae of the arbuscular mycorrhizal fungus *Gigaspora margarita* (Saito, 1995) as well as localization of the enzyme in peripheral cytoplasmic vesicles in the ascomycetous fungus *Claviceps purpurea* (Voříšek and Kalachová, 2003). The cytochemical demonstration of enzyme activity in the vicinity of the wall of *H. lutea* germinating conidia and hyphal filaments (Fig. 2) as the quantity of lead phosphate granules

was less in comparison with the reaction product presented in nongerminating conidia (Fig. 1). The cultivation of *H. lutea* in CM without Pi resulted in a high yield of acid phosphatase excreted in the culture broth. Approx. 60% of the total enzyme activity (40 U/flask) were registered in culture supernatant after 36 h of fermentation. For this reason, a consistent location of lead phosphate granules may not be expected (Figs. 2B, D, E and F). A similar conclusion was made by D'Silva *et al.* (2000) investigating the cytochemical localization of phytase in ruminal microorganisms. The outer membrane association of the phytase enzyme would easily explain the significant extracellular release of phosphate from phytate by pure ruminal bacteria (Yanke *et al.*, 1998).

- Aleksieva P. and Micheva-Viteva S. (2000), Regulation of extracellular acid phosphatase biosynthesis by phosphates in proteinase producing fungus *Humicola lutea* 120–5. *Enzyme Microb. Technol.* **27**, 570–575.
- Aleksieva P., Spasova D., and Radoevska S. (2003), Acid phosphatase distribution and localization in the fungus *Humicola lutea*. *Z. Naturforsch.* **58c**, 239–243.
- Andersch M. A. and Szezyński A. (1947), Use of *p*-nitrophenyl phosphate as the substrate in determination of serum acid phosphatase. *Am. J. Clin. Pathol.* **17**, 571–574.
- Arnold W. N., Garrison R. G., Man L. C., and Wallace D. P. (1988), The acid phosphatases of *Thermoascus crustaceus*, a thermophilic fungus. *Microbios* **54**, 101–112.
- Biswas T. and Cundiff C. (1991), Multiple forms of acid phosphatase in germinating seeds of *Vigna sinensis*. *Phytochemistry* **30**, 2119–2125.
- Cherepova N. and Spasova D. (1996), Ultrastructural localization of acid phosphatase in some bacteria, after treatment with Lubrol W₁. *Microbios* **88**, 199–204.
- D'Silva C. G., Bae H. D., Yanke L. J., Cheng K.-J., and Selinger L. B. (2000), Localization of phytase in *Selemonas ruminantium* and *Mitsuokella multiacidus* by transmission electron microscopy. *Can. J. Microbiol.* **46**, 391–395.
- Garrison R. G. and Arnold W. N. (1983), Cytochemical localization of acid phosphatases in the dimorphic fungus *Sporothrix schenckii*. *Curr. Microbiol.* **9**, 253–258.
- Grigorov I., Aleksieva P., Djerova A., Sheremetska P., and Tchobanov B. (1983), Selection of gamma-ray mutant from a strain *Humicola lutea* 72, producing acid proteases. *Eur. J. Appl. Microbiol. Biotechnol.* **17**, 355–357.
- Haas H., Redl B., Leitner E., and Stoffler G. (1991), *Penicillium chrysogenum* extracellular acid phosphatase: purification and biochemical characterization. *Biochim. Biophys. Acta* **1074**, 392–397.
- Kneipp L. F., Rodrigues M. L., Holandino C., Esteves F. F., Souto-Padron T., Alviano C. S., Travassos L. R., and Meyer-Fernandes J. R. (2004), Ectophosphatase activity in conidial forms of *Fonsecaea pedrosoi* is modulated by exogenous phosphate and influences fungal adhesion to mammalian cells. *Microbiology* **150**, 3355–3362.
- Micheva-Viteva S., Tchobanov B., Aleksieva P., and Lazarova V. (2000), Acid phosphatases excreted by *Humicola lutea* 120–5 in casein-containing medium. *World J. Microbiol. Biotechnol.* **16**, 859–863.
- Miller F. and Palade G. E. (1964), Lytic activities in renal protein adsorption droplets. *J. Cell. Biol.* **23**, 519–522.
- Nahas E. (1989), Control and localization of the phosphatases in conidia of *Neurospora crassa*. *Can. J. Microbiol.* **35**, 830–835.
- Nozawa S. R., Maccheroni W., Stabeli R. G., Thedei G., and Rossi A. (1998), Purification and properties of Pi-repressible acid phosphatases from *Aspergillus nidulans*. *Phytochemistry* **49**, 1517–1523.
- Saito M. (1995), Enzyme activities of the internal hypha and germinated spores of an arbuscular mycorrhizal fungus *Gigaspora margarita*. *New Phytol.* **129**, 425–431.
- Schadeck R. J. G., Buchi D. F., and Leite B. (2000), Acid phosphatase activity in ungerminated conidia from *Colletotrichum graminicola* as determined by spectrophotometric and cytochemical methods. *Braz. J. Morphol. Sci.* **17**, 81–85.
- Spasova D. and Galabova D. (1998), Yeast permeabilization as a tool for measurement of *in situ* enzyme activ-

- ity: localization of alkaline phosphatase. *Z. Naturforsch.* **53c**, 347–351.
- Tarafdar J. C., Rao A. V., and Bala K. (1988), Production of phosphatases by fungi isolated from desert soils. *Folia Microbiol.* **33**, 453–457.
- Tsekova K., Galabova D., and Todorova K. (2000), Copper accumulation and phosphatase activities of *Aspergillus* and *Rhizopus*. *Z. Naturforsch.* **55c**, 708–712.
- Tsekova K., Galabova D., Todorova K., and Ilieva S. (2002), Phosphatase activity and copper uptake during growth of *Aspergillus niger*. *Process Biochem.* **37**, 753–758.
- Voříšek J. and Kalachová L. (2003), Secretion of acid phosphatase in *Claviceps purpurea* – an ultracytochemical study. *Folia Microbiol.* **48**, 767–770.
- Yanke L. J., Bae H. D., Selinger L. B., and Cheng K.-J. (1998), Phytase activity of anaerobic ruminal bacteria. *Microbiology* **144**, 1565–1573.