Acid Phosphatase Distribution and Localization in the Fungus *Humicola lutea*

Penka Aleksieva, Dimitrina Spasova*, and Spasimira Radoevska

Bulgarian Academy of Sciences, Institute of Microbiology, Acad. G. Bonchev str., bl 26, 1113 Sofia, Bulgaria. Fax: +35 92 70 01 09. E-mail: nava@abv.bg

* Author for correspondence and reprint requests

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Acid phosphatase activities in a culture liquid and mycelial extract were studied in submerged cultures of the filamentous fungus *Humicola lutea* 120-5 in casein-containing media with and without inorganic phosphate (Pi). The Pi-repressible influence on the phosphatase formation was demonstrated. Significant changes in the distribution of acid phosphatase between the mycelial extract and culture liquid were observed at the transition of the strain from exponential to stationary phase. Some differences in the cytochemical localization of phosphatase in dependence of Pi in the media and the role of the enzyme in the release of available phosphorus from the phosphoprotein casein for fungal growth were discussed.

Key words: Acid Phosphatase, Localization, Fungus

Introduction

Acid phosphatases have been reported to occur in fungi, such as, *Aspergillus* (Nozawa *et al.*, 1998), *Penicillium* (Yoshida *et al.*, 1989; Haas *et al.*, 1991), *Fusarium* (Yoshida and Tamiya, 1971) and *Neurospora* (Nahas *et al.*, 1982). Most of the fungal phosphatases indicated above were produced in media containing an inorganic nitrogen source ($NaNO_3$, $(NH_4)_2SO_4$, NH_4NO_3) and a very low concentration of inorganic phosphate (Pi). Much less information is available on the production of phosphatases by soil fungi using organophosphorus compounds as a source of phosphate (Tarafdar *et al.*, 1988). The behaviour of acid and alkaline phosphatase in the culture liquid (extracellular enzymes) and mycelial extract (cytoplasmic and cell-bound enzymes) has been investigated in seven fungi grown as stationary cultures in a mineral medium (Reyes *et al.*, 1990). The production of extracellular and cellular acid phosphatases by *Aspergillus* and *Rhizopus* species during growth in the presence or absence of cooper ions in the medium has been studied (Tsekova *et al.*, 2000, 2002). The cytochemical localization of the phosphatases has been made by a few workers (Garrison and Arnold, 1983; Arnold *et al.*, 1988; Cherepova and Spasova, 1996; Spasova and Galabova, 1998).

The fungal strain *Humicola lutea* 120-5 utilizes the phosphoprotein casein through biosynthesis of extracellular enzymes: acid proteinases (Aleksieva and Mutafov, 1997; Aleksieva and Peeva, 2000) and acid phosphatases (Aleksieva and Micheva-Viteva, 2000; Micheva-Viteva *et al.*, 2000). The predominant phosphatase production by *H. lutea* cells proceeds in a casein-containing medium lacking in mineral orthophosphates (Aleksieva and Micheva-Viteva, 2000).

The present study was carried out in an attempt to clarify the distribution of *H. lutea* acid phosphatase between the culture liquid and mycelial extract depending on the presence and the absence of Pi in a casein-containing medium used for submerged cultivation of the filamentous fungus. The localization of acid phosphatase in mycelia obtained in media with and without Pi was also investigated cytochemically at electron microscope level.

Materials and Methods

Strain and culture conditions

H. lutea 120-5, a mutant fungal strain (National Bank for Industrial Microorganisms and Cell Cultures: 391, Bulgaria) was derived from the parent strain *H. lutea* 72 after radiation (^{60}Co) mutagenesis (Grigorov *et al.*, 1983). The culture was maintained on 1.5% (w/v) beer agar at 28 °C for 7 days to obtain dense sporulation. The submerged cultivation of the fungus was carried out in two types of media: *Medium 1* (with Pi or Pi-sufficient) containing (grams per liter): glucose, 20; casein, 4; KH_2PO_4, 1; KCl, 0.5; $MgSO_4$, 0.5; $FeSO_4.7H_2O$, 0.004; $ZnSO_4.7H_2O$, 0.003; $MnSO_4.H_2O$, 0.001 and

Medium 2 (without Pi or Pi-deficient) containing the same components without KH_2PO_4. The pH was adjusted to 6.0 and media were autoclaved at 115 °C for 15 min. A portion (1.0 ml) of the spore suspension containing 10^7 to 2×10^7 spores per ml was used to inoculate 50 ml of the media in 500 ml Erlenmeyer flasks. The cultures were incubated on a rotary shaker (250 rpm) at 28 °C. Each flask was considered as a sample. The samples were taken in different phases of the growth. The mycelia were separated from the fermentation broth by filtration and the clear culture liquid (CL) was used for assaying acid phosphatase. The mycelia were washed twice with distilled water and crushed with quartz sand. The crushed mycelia were suspended in acetate buffer with pH 4.8. The cell suspension was centrifugated (13,000 × *g*, 20 min) and the supernatant fluid or mycelial extract (ME) was used for enzyme determination.

Enzyme assay

Acid phosphatase (nonspecific orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) in CL and ME was determined by the method of Andersch and Szezypinski (1947) using *p*-nitrophenyl phosphate (pNPP, Merck, Germany) as the substrate. One unit of phosphatase activity is defined as the release of 1 μmol 4-nitrophenol in 1 min at 37 °C at a pH of 4.8.

Dry weight measurment

Some samples were assayed for dry mycelium weight (dmw). The fungal 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 distribution of acid phosphatase between CL and ME were repeated at least twice and the samples were assayed in triplicate. The data points represented the mean values within ± 4 to 5% of the individual values.

Ultracytochemical methods

Acid phosphatase in 36 h-old mycelia was demonstrated by the method of Gomori, modified by Miller and Palade (1964) using pNPP as the 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 suspension was 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 sucrose. The final pH was adjusted to 5.0 with 0.2 M CH_3COOH. In control experiments the incubation mixture lacked the enzyme substrate (pNPP) for the reaction.

Transmission electron microscopy

The samples were postfixed 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 with a Zeiss electron microscope (model 10C).

Results and Discussion

The mycelial growth (expressed as g dry weight per flask) in the course of *H.lutea* cultivation in both Pi-sufficient and Pi-deficient casein-containing media was similar. Using *Medium 1* the cell concentration increased from 0.41 up to 0.54 g/flask at the transition of the culture from exponential (36 h) to stationary phase (72 h). The corresponding values of the biomass were approximately equal (0.37 and 0.59 g/flask, respectively) when *Medium 2* was used. Considerable differences were observed only in the beginning of the growth. In contrast to the Pi-deficient culture, there was a two times larger amount of biomass (0.16 g/flask) in 12 h-old Pi-sufficient culture which was due to the presence of available phosphorus for fungal development. An omisson of KH_2PO_4 from the medium (*Medium 2*) left the casein as the sole phosphate source that provoked the ability of the fungus to dephosphorylate casein molecules providing orthophosphate for cell growth. This process of organic phosphorus mineralization was probably the cause of slower biomass formation in the first hours of the cultivation. These results agree with the conclusion that some pro- and eukaryotic microorganisms play an important role in the release of available phosphorus from organophosphorus compounds through the production of

Table I. Acid phosphatase productivity of *H. lutea* mycelium in Pi-sufficient and Pi-deficient casein-containing medium. Enzyme distribution between mycelial extract (ME) and culture liquid (CL) in exponential (36 h) and stationary phase (72 h) of growth.

Incubation time [h]	Medium	Total acid phoshatase activity U/flask	Specific activity of mycelia U/gdw/flask	Distribution of total enzyme activity in ME and CL %	
				ME	CL
36	Pi-sufficient	4.9	11.9	74	26
	Pi-deficient	38.7	101.3	43	57
72	Pi-sufficient	6.4	11.9	36	64
	Pi-deficient	74.7	126.5	11	89

phosphatases (Tarafdar *et al.*, 1988; Abd-Alla, 1994).

The data for the total acid phosphatase activity (U/flask) in CL and ME and specific activity of mycelia (U/g dw/flask) at the exponential (36 h) and the stationary phase (72 h) of *H. lutea* growth in *Medium 1* and *Medium 2* are presented in Table I. In Pi-sufficient culture the total enzyme activity increased slightly from 4.9 up to 6.4 U at the transition of the cells from exponential to stationary phase of growth. The corresponding values of the specific activity of mycelia were the same (11.9 U/g). The use of Pi-deficient medium resulted in an approximately eight times higher phosphatase yield (38.7 U) and a ten times higher specific activity of mycelia (101.3 U/g) as early as the exponential phase. At the stationary phase the total phosphatase increased doubly (74.7 U) and the specific activity of mycelia reached a value of 126.5 U/g. These results illustrating the Pi-repression influence on the phosphatase formation are in agreement with those reported by Shieh *et al.* (1969) when the regulation of the production of two *Aspergillus ficuum* acid phosphatases by Pi was investigated. Contrary to the situation observed in the fungi indicated above, the expression of *Mycobacterium bovis* phosphatase is not regulated by the environmental inorganic phosphate concentration (Braibant and Content, 2001).

The effect of Pi on the distribution of *H. lutea* acid phosphatase between ME and CL was investigated (Table I). After 36 h of cultivation in medium containing KH_2PO_4, 74% of the total enzyme activity were in ME and 26% in CL. At the transition of the culture from exponential to stationary phase the distribution of phosphatase in ME and CL was 36% and 64%, respectively. In Pi-deficient conditions after 36 h of cultivation, 43% of the total phosphatase activity were in ME and 57% in CL. The corresponding values in a 72 h-old culture were 11% and 89%. Consequently, the majority of the enzyme at the stationary phase was excreted in the culture broth, since only trace amounts of the phosphatase were found in the mycelia.

The presence of higher phosphatase activity in CL than in ME has been confirmed by Arnold *et al.* (1988) in *Thermoascus crustaceus*: the distribution of enzyme in the 72 h-old culture at 37 °C was 67% for CL and 33% for ME. A correlation between the degree of autolysis and acid phosphatase increase in CL and decrease in ME was observed in fungi of the genera *Ascomycotina*, *Basidiomycotina* and *Zygomycotina* (Reyes *et al.*, 1990).

The localization of *H. lutea* acid phosphatase was studied by a cytochemical electron microscopy. In the case of Pi-sufficient culture the reaction product was observed on the cytoplasmic membrane (Fig. 1 A,B,C). Lead phosphate granules situated on the entire cytoplasmic membrane. When the cells were cultivated in a Pi-deficient medium, electron dense deposits in form of amorphous irregular granules were found on the outside of the cell wall and in the intracellular space (Fig. 2 A,B,C). Single lead phosphate granules were localized on the cell surface. There are no electron dense precipitates of lead phosphate in control fungal cells, incubated without substrate (Fig. 1 D and Fig. 2 D). The results with *H. lutea* are different from those previously documented for *Th. crustaceus* (Arnold *et al.*, 1988). In the later case the authors concluded that there were no significant trends with regard to acid phosphatase ac-

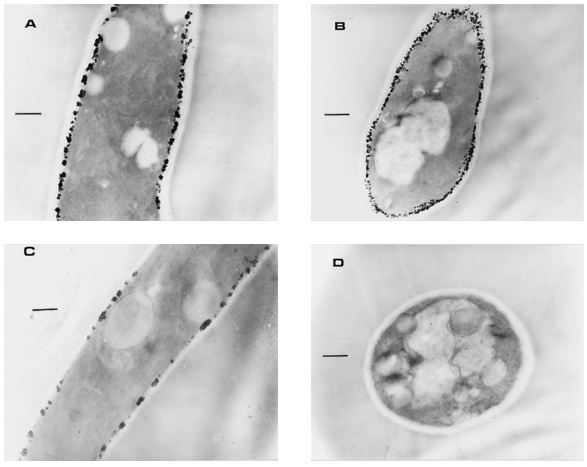

Fig. 1. Cytochemical localization of acid phosphatase in ultrathin sections of Pi-sufficient *Humicola lutea* culture. Very well expressed reaction with lead phosphate granules along the surface of the cytoplasmic membrane (A,B,C). Control incubation without an enzyme substrate (D). All marker bars, 0.2 μm.

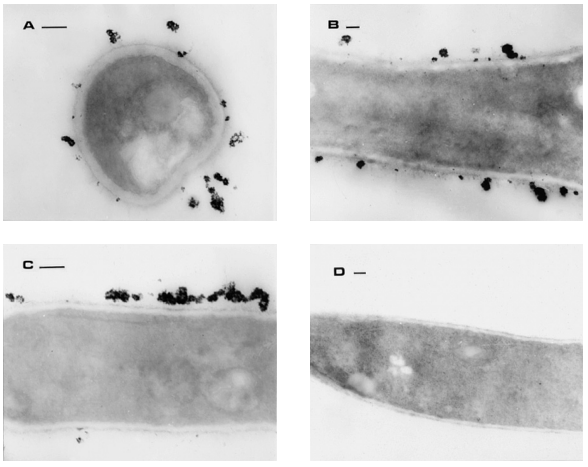

Fig. 2. Cytochemical localization of acid phosphatase in ultrathin sections of Pi-deficient *Humicola lutea* culture. The product of the reaction is found on the surface of the cell wall (A,B,C). Hyphal filament incubated in the absence of a substrate (D).

tivity sites with and without phosphate supplement in the growth medium: the Gomori reaction revealed enzyme activity on the surface of the cell wall, in the periplasmic space and within the cytoplasm. A multiplicity of acid phosphatase activity sites in both yeast-like and mycelial phase cells of the dimorphic fungus *Sporothrix shenckii* was observed by Garrison and Arnold (1983). Transmission electron micrographs of thin sections of this fungal culture show that an electron-opaque deposit of lead phosphate was disposed at and along the entire cell wall surface. Other active sites were in the intermost aspect of the cell envelope (the periplasmic space and/or the plasma membrane) and in vacuoles.

From our observation, it is concluded that the high level of *H. lutea* acid phosphatase in a 36 h-old Pi-deficient culture (Table I) and its localization completely on the outside of the cell wall (Fig. 2 A,B,C), favour of view that the fungal phosphatases have an important nutritional role in the vegetative cells, providing available phosphorus from organic phosphorus sources, as it has been discussed before (Abd-Alla, 1994; Gonzales *et al.*, 1994) for some prokaryotic microorganisms.

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