Arch Microbiol (2007) 188:159–166
DOI 10.1007/s00203-007-0232-y

ORIGINAL PAPER

Mycelial forms of *Pseudallescheria boydii* present ectophosphatase activities

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Received: 28 February 2007 / Revised: 28 February 2007 / Accepted: 5 March 2007 / Published online: 12 April 2007
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Abstract Phosphatase activities were characterized in intact mycelial forms of *Pseudallescheria boydii*, which are able to hydrolyze the artificial substrate *p*-nitrophenylphosphate (*p*-NPP) to *p*-nitrophenol (*p*-NP) at a rate of 41.41 ± 2.33 nmol p-NP per h per mg dry weight, linearly with increasing time and with increasing cell density. $MgCl_2$, $MnCl_2$ and $ZnCl_2$ were able to increase the (*p*-NPP) hydrolysis while $CdCl_2$ and $CuCl_2$ inhibited it. The (*p*-NPP) hydrolysis was enhanced by increasing pH values (2.5-8.5) over an approximately 5-fold range. High sensitivity to specific inhibitors of alkaline and acid phosphatases suggests the presence of both acid and alkaline phosphatase activities on *P. boydii* mycelia surface. Cytochemical localization of the acid and alkaline phosphatase showed electron-dense cerium phosphate deposits on the cell wall, as visualized by electron microscopy. The product of *p*-NPP hydrolysis, inorganic phosphate (Pi), and different inhibitors for phosphatase activities inhibited *p*-NPP hydrolysis in a dose-dependent manner, but only the inhibition promoted by sodium orthovanadate and ammonium molybdate is irreversible. Intact mycelial forms of *P. boydii* are also able to hydrolyze phosphoaminoacids with different specificity.

Keywords *Pseudallescheria boydii* · Phosphatase activities · Ecto-enzymes

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Introduction

Pseudallescheria boydii belongs to the class Ascomycetes and is the perfect (sexual) form of *Scedosporium apiospermum.* It is a natural saphrophyte that is commonly found in soil and polluted water (Rippon 1998). The most common clinical condition in immunocompetent patients involving *P. boydii* is mycetoma (chronic infection of the skin and subcutaneous tissues) (Bakerspiegel 1971; Baxter et al. 1996). Immunocompromised patients are at higher risk for locally invasive or disseminated *Pseudallescheria* infection due to increased incidence of human immunodeficiency virus infection, antineoplasic or immunosuppressive therapy (Berenguer et al. 1989; Patterson et al. 1990).

Several kinds of therapies against *P. boydii* infection have been used but the treatment is problematic, since this fungus frequently exhibits resistance to amphotericin B (Nielsen 1967; Ginter et al. 1999). In this context, the characterization of cell wall and other surface components are relevant to the development of new antifungal drugs and it is of great relevance for the understanding of the pathogenicity mechanisms. The composition of the cell wall is of primary importance in the microbial adherence and the establishment of infection (Calderone et al. 1994). The mechanism of adherence and invasion have been studied in some fungi, such as *Candida albicans* (Sundstrom 2002), *Histoplasma capsulatum* (Bullock and Wright 1987), *Aspergillus fumigatus* (Bouchara et al. 1995), *Paracoccidioides brasiliensis* (Hanna et al. 2000), *Sporothrix schenckii* (Lima et al. 2001) and *Fonsecaea pedrosoi* (Limongi et al.

1997). These events have not been well characterized in *P. boydii.* Recently, the presence of a peptidorhamnomannan antigen was demonstrated on the cell wall of *P. boydii*, which is involved in the interaction process with HEp2 cells (Pinto et al. 2004).

The surface of cells contains enzymes facing the external medium rather than the cytoplasm. The activities of these enzymes, referred to as ecto-enzymes, can be measured using living cells (Meyer-Fernandes et al. 1997; Furuya et al. 1998; Peres-Sampaio et al. 2001). A number of fundamental processes in fungi such as cell cycle control, gene transcription and mating have been shown to require protein phosphorylation (Madhani and Fink 1998; Zhan et al. 2000; Dickmann and Yarden 2000). Reversible phosphorylation of protein is controlled by the coordinated action of protein kinases and phosphatases (Hunter 1995). These protein kinases and phosphatases have been detected in several microbes including protozoa (Gottlieb and Dweyer 1981; Fernandes et al. 1997; Meyer-Fernandes et al. 1999), bacteria (Bliska et al. 1991, 1993) and fungi (Mildner et al. 1975; Arnold et al. 1986, 1987; Kneipp et al. 2003, 2004). Phosphorylation–dephosphorylation of serine, threonine and tyrosine residues can trigger conformational changes in protein that alter their properties (Cohen 1989; Zhan et al. 2000). Several biological roles for extracytoplasmic phosphatases have been proposed. In *Candida parapsilosis*, a surface phosphatase activity was described to be involved with fungal adhesion to host cells (Fernanado et al. 1999) and in *C. albicans*, the endocytosis by vascular endothelial cells is associated with tyrosine phosphorylation of specific host cell proteins (Belanger et al. 2002). These ecto-enzymes have also been associated with cell differentiation (Nakagura et al. 1985; Bakalara et al. 2000) and may also have a role as "safeguard" enzymes to protect the cells from acidic conditions, by buffering the periplasmic space with phosphate released from polyphosphates (Touati et al. 1987).

In the present work we show that phosphatase activities on the external surface of *P. boydii* can be identified by biochemical methods and localized at the mycelial surface by cytochemical analysis.

Materials and methods

Chemicals

All reagents were purchased from Sigma Chemical Co., St. Louis, MO, USA or from E. Merck, D-6100 Darmstadt, Germany (divalent cations and phosphatase inhibitors). Distilled water was deionized using a MilliQ system of resins (Millipore Corp., Bedforrd, MA) and used in the preparation of all solutions.

Microorganisms and growth conditions

P. boydii, isolated from eumycotic mycetoma, was kindly supplied by Dr Bodo Wanke, Evandro Chagas Hospital, Instituto Oswaldo Cruz, Rio de Janeiro, Brazil. It was maintained in a medium containing (g l^{-1}): Difco peptone, 10; Difco yeast extract, 5; Difco agar, 20; glucose, 40 at pH 7.4. Cells were grown on Sabouraud solid medium for 7 days at 25°C and inoculated into Erlenmeyer flasks (500 ml) containing liquid Sabouraud medium (200 ml), which was incubated for 7 days at 25°C with orbital shaking at 200 rpm. The mycelium was filtered with filter paper, washed three times with distilled water and dried.

Measurement of enzymatic activity

Mycelium (10 mg wet weight which correspond to 1.2 mg dry weight) of *P. boydii* was incubated at 25°C for 60 min with continuous shaking in a reaction mixture (0.5 ml) containing 116.0 mM NaCl, 5.4 mM KCl, 4.0 mM glucose, 20.0 mM HEPES pH 7.4 and 5 mM *p*-NPP, as substrate, unless otherwise stated in the legend of figures. The reaction was terminated by the addition of 1 ml of 1 N NaOH to the reaction mixture. The phosphatase activity was determined measuring the rate of *p*-NP production. For determining the rate of *p*-NP formed in the hydrolysis reaction, the tubes were centrifuged at 1,500*g* for 20 min (4°C). The supernatant was measured spectrophotometrically at 405 nm, using a *p*-NP curve as standard (Meyer-Fernandes et al. 1999). The phosphatase activity was calculated by subtracting the non-specific *p*-NPP hydrolysis measured in the absence of cells as previously established for other fungi (Kneipp et al. 2003, 2004; Collopy-Junior et al. 2006a, b) and expressed per mg of dry weight cells. In the experiments where other phosphatase substrates (β-glycerophosphate or phosphoaminoacids) were used, the hydrolytic activity measured under the same conditions described earlier were assayed spectrophotometrically by measuring the release of inorganic phosphate (Pi) from these substrates by the modified Fiske and Subbarow methods (Lowry and Lopez 1946). The values obtained for *p*-NPP hydrolysis measured using both methods (Lowry and Lopez 1946; Meyer-Fernandes et al. 1999) were exactly the same. When the reversibility of the inhibition caused by different agents was assessed, the cells were previously incubated with them, washed and tested for phosphatase activity. Cellular viability was assessed before and after incubations by the Trypan blue method. The viability of the cells was not affected by the conditions used in this work.

Cytochemistry and electron microscopy

Cells were fixed in 1% glutaraldehyde in 100 mM cacodylate buffer (pH 7.2) with 100 mM sucrose (CS buffer) for

10 min on ice. Cells were washed 4–5 times over the course of 1 h with one CS buffer and then submitted to the cytochemical methods, which will be described later. The samples were washed first in in CS buffer and later, they were washed twice in 100 mM Tris–maleate buffer, pH 5.0 or 8.0 for acid phosphatase and alkaline phosphatase, respectively, containing 100 mM sucrose. The acid phosphatase medium contained 2 mm $CeCl_3$, 2 mM substrate (β-glycerophosphate), and 100 mm Tris–maleate buffer (pH 5.0) containing 100 m sucrose. The alkaline phosphatase medium consisted of 2 mm $CeCl_3$, 2 mM substrate (β-glycerophosphate), and 100 mm Tris–maleate buffer (pH 8.0) containing 100 m sucrose. Substrate-free media served as control to assess the specificity of the reaction. The detergent Triton X-100 (0.0001%) was added to the cytochemical media in order to permeabilize the cells. All media were prepared and filtered through a Millipore filter (0.22 μm) just prior to use. Incubations were carried out at 37°C with gentle agitation for 1 h (media were replaced with fresh media after 30 min). Following the cytochemical reaction, the cells were washed once in the respective cytochemical buffer and then washed twice in CS buffer.

After the cytochemical reactions the two samples and their respective controls were fixed again in 2.5% glutaraldehyde in CS buffer for 1 h at room temperature and postfixed in 1% osmium tetroxide in 100 mM cacodylate buffer, pH 7.2, for 1 h at room temperature. Cells were then rinsed in 100 mM cacodylate buffer, dehydrated in acetone and embedded in Polybed 812. Thin sections obtained with a Reichert Ultracut S were observed unstained, using a FEI-Morgani 268 transmission electron microscope operating at 80 kV.

Statistical analysis

Data are means of three determinations using different cell suspensions. The results were analyzed statistically using the Student's *t*-test. The maximal velocity (V_{max}) and Michaelis constant (K_m) for *p*-NPP were calculated using a computerized non-linear regression analysis of the data to the Michaelis–Menten equation (Meyer-Fernandes et al. 1997).

Results

Ecto-phosphatase activity

The characterization of the ecto-phosphatase activity was determined using intact mycelial forms of *P. boydii*, which was able to hydrolyze, at pH 7.4, the artificial substrate for phosphatases, *p*-NPP at rate of 41.41 ± 2.33 nmol *p*-NP h^{-1} mg^{-1} of dry weight. The time course of *p*-NPP hydrolysis was linear for at least 90 min ($r^2 = 0.9744$) (Fig. 1a). Similarly, in assays to determine the influence of cell density on the phosphatase activity measured over 60 min, it was also observed a linear profile over a nearly 5-fold range of cell density ($r^2 = 0.9860$) (Fig. 1b). To check the possibility that the observed *p*-NPP hydrolysis was the result of secreted soluble enzymes, as seen in other parasites (Rodrigues et al. 1999; Dutra et al. 2001), cells were incubated in the absence of *p*-NPP. Subsequently, the suspension was centrifuged to remove the cells and the supernatant failed to show *p*-NPP hydrolysis (data not shown) in the same conditions used in the cell-based assay. These data also rule out the possibility that the phosphatase activity here described could be from lysed cells and a result of secreted enzymes.

Effect of pH and phosphatase inhibitors on the phosphatase activity

The influence of pH was also analyzed. This activity was enhanced by increasing pH values (2.5–8.5) (Fig. 2). Different phosphatase inhibitors were tested and the results are shown in Table 1. An alkaline phosphatase inhibitor, levamisole (1 mM), was able to inhibit approximately 40% of maximal activity. In addition, inhibitors of acid phosphatases, such as ammonium molybdate (Na_2MoO_4), sodium

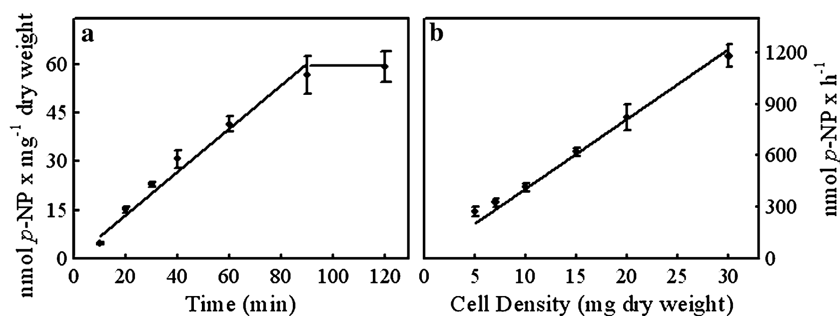

Fig. 1 Time course and cell density dependence of the ecto-phosphtase activities of *Pseudallescheria boydii*. Intact cells were incubated at 25°C in the reaction medium described in Materials and methods section for different times (**a**) or for 60 min under conditions of increasing cell density (**b**). Data are means ± SE of three determinations using different cell suspensions

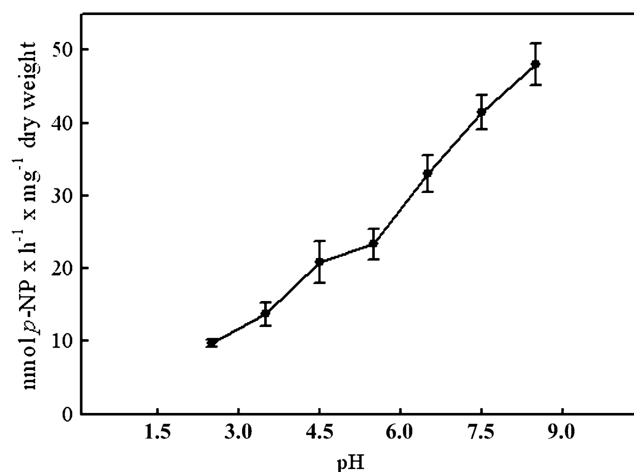

Fig. 2 Effect of pH on the ecto-phosphtase activities of *P. boydii.* Intact cells were incubated for 60 min at 25°C in a reaction medium (final volume 0.5 ml) containing 116.0 mM NaCl, 5.4 mM KCl, 4.0 mM glucose, 5 mM *p*-NPP as substrate, 10 mg cells (wet weight) and 116 mM MES-HEPES-CITRATE buffer adjusted to pH values between 2.5 and 8.5. Data are means ± SE of three determinations using different cell suspensions

Table 1 Kinetic parameters of *P. boydii* ecto-phosphatase activities for different phosphatase inhibitors

Addition	Relative activity (%)
–	100.00 ± 5.53
Levamisole (1 mM)	65.10 ± 4.42[a]
P_i (10 mM)	28.40 ± 0.71[a]
Sodium orthovanadate (1 mM)	15.24 ± 1.88[a]
Ammonium molybdate (1 mM)	15.38 ± 0.94[a]
Sodium fluoride (10 mM)	20.09 ± 3.60[a]
Sodium β-glycerophosphate (10 mM)	70.63 ± 8.93[b]

Note. Intact cells were incubated in a reaction medium (final volume 0.5 ml) as described in Materials and Methods, in the absence or in the presence of different phosphatase inhibitors. Data are means ± SE for three determinations with different cell suspensions

[a] Denotes statistically significant difference ($P < 0.001$)

[b] Denotes statistically significant difference ($P < 0.014$)

orthovanadate (Na_3VO_4) and sodium fluoride (NaF), inhibited approximately 85, 85 and 80% the enzyme activity, respectively (Table 1). Figure 3 details the kinetic of inhibition for each compound, where it is shown that the *p*-NPP hydrolysis was inhibited in a dose-dependent manner by sodium orthovanadate with K_i value of 4.23 µM (Fig. 3, panel a), ammonium molybdate with K_i of 7.5 µM (Fig. 3, panel b) and sodium fluoride with K_i of 126.78 µM (Fig. 3, panel c). Moreover, the effect of P_i, the product of the enzymatic reaction, was also tested. P_i was able to reduce the activity in a dose-dependent manner with K_i value of 1.5 mM (Fig. 3, panel d). The ecto-phosphatase activity was reversibly inhibited by inorganic phosphate as well as sodium fluoride, while the inhibition promoted by ammonium molybdate and sodium orthovanadate was irreversible (insets of Fig. 3). From these data we could not rule out the presence of alkaline phosphatase activities insensitive to levamizole or that the inhibitors of acid phosphatase activities could also interfere with the alkaline phosphatase activities present on the external surface of *P. boydii*.

Influence of different cations and redox regulation on the phosphatase activity

The effect of divalent cations on *p*-NPP hydrolysis was analyzed. Our results show that the addition of EDTA did not influence this phosphatase activity (Fig. 4). However, it was remarkably stimulated by $MgCl_2$, $MnCl_2$ and $ZnCl_2$ and inhibited by $CuCl_2$ and $CdCl_2$. None of the other cations tested, $CaCl_2$, $SrCl_2$ and $CoCl_2$ had any effect on the activity (Fig. 4). Reductive agents (DTT and β-mercaptoethanol) had no effect on the ecto-phosphatase activity, as well as cysteine, an amino acid with reducing characteristics, which presents a thiol group in its structure (data not shown).

Determination of substrate specificity of the ecto-phosphatase activity

The dependence on *p*-NPP concentration showed a normal Michaelis–Menten kinetics for phosphatase activities measured at pH 2.5 and pH 8.5 (data not shown). The values of V_{max} and apparent K_m for *p*-NPP hydrolysis were 12.25 ± 0.33 nmol *p*-NP per h per mg dry weight and 0.65 ± 0.07 mM *p*-NPP and 103.0 ± 8.6 nmol *p*-NP per h per mg dry weight and 6.66 ± 1.0 mM *p*-NPP, respectively. As well as *p*-NPP, β-glycerophosphate was also substrate for these phosphatase activities (data not shown). Intact cells of *P. boydii*, at the same pH used in the culture conditions, were also able to hydrolyze phospho amino acids such as phosphoserine, phosphotyrosine and phosphothreonine. As depicted in Fig. 5, only the hydrolysis of the tyrosine-phosphorylated amino acid was comparable to *p*-NPP hydrolysis. Although the hydrolysis of phosphoserine and phosphothreonine was also observed, it occurred at lower levels.

Cytochemical detection of the enzymatic activity

Cytochemical detection of acid and alkaline phosphatase activities in mycelial forms of *P. boydii* was performed in order to corroborate the biochemical findings. In cells that had been assayed for the detection of acid (Fig. 6a) and alkaline phosphatase (Fig. 6b, c) activities, a homogeneous electron-dense precipitate of cerium phosphate was observed over the cells' surface. All cells in both samples

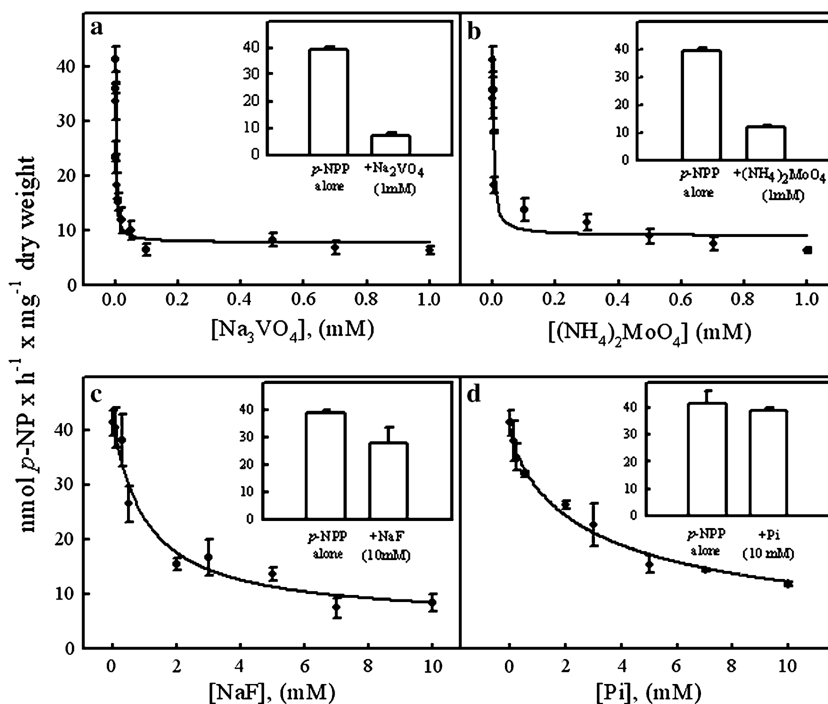

Fig. 3 Effect of sodium orthovanadate (Na_3VO_4 —**a**), ammonium molybdate (Na_2MoO_4 **b**), sodium fluoride (NaF **c**) and inorganic phosphate (Pi **d**) on the ecto-phosphtase activities of *P. boydii*. Intact cells were incubated for 60 min at 25°C in the absence or in the presence of increasing concentration of the inhibitors. Inset of figures: reversible inhibition, the cells were incubated in the same reaction medium as described in Materials and Methods section but in the absence of substrate, for 60 min, in the presence of inhibitors. Then, cells were washed three times and tested for *p*-NPP hydrolysis. Data are means ± SE of three determinations using different cell suspensions

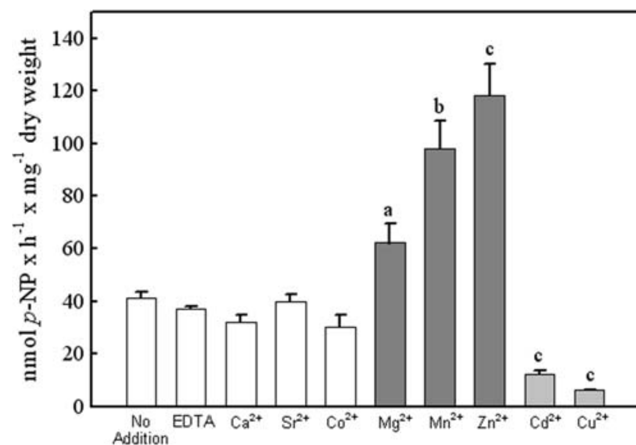

Fig. 4 Effect of different metals on the ecto-phosphtase activities of *P. boydii*. Intact cells were incubated for 60 min at 25°C in the absence or in the presence different metals, as described in Materials and Methods section. Data are means ± SE of three determinations using different cell suspensions. [a]Denotes statistically significant difference ($P < 0.003$). [b]Denotes statistically significant difference ($P < 0.013$). [c]Denotes statistically significant difference ($P < 0.001$)

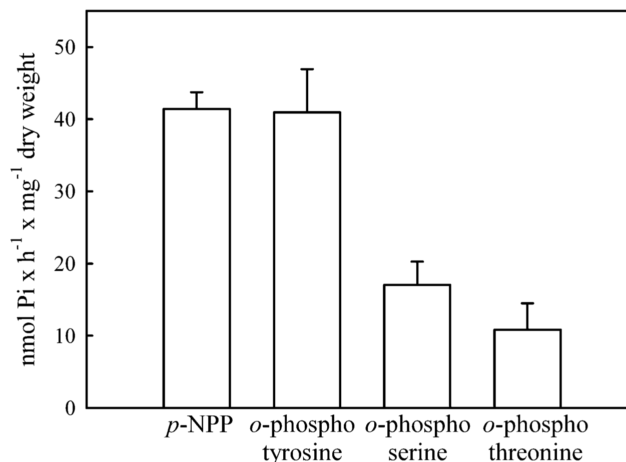

Fig. 5 Hydrolysis of different phosphoaminoacids by the ecto-phosphtase activities of *P. boydii*. Intact cells were incubated for 60 min at 25°C as described in Materials and Methods section in the presence of *p*-NPP 5 mM or different phosphorylated amino acids as substrate. The ecto-phosphatase was assessed by measuring the release of P_i from these substrates by the modified Fiske and SubbaRow methods. Data are means ± SE of three determinations using different cell suspensions

were labeled. The alkaline phosphatase activity, made in evidence by a large area of electron-dense precipitates on the cell wall, was stronger than that observed for acid phosphatase. In some cells the electron-dense precipitate indicative of the alkaline phosphatase activity were also found apart from the cells (Fig. 6c), however, no enzymatic activity was biochemically detected (data not shown). No reaction products were seen when parasites were incubated in the acid phosphatase and alkaline phosphatase medium without β-glycerophosphate.

Discussion

In this work, we described ecto-phosphatase activities present on the cell surface of mycelial forms of *P. boydii*, an emergent human pathogen frequently found in soil and polluted water that is involved in subcutaneous and disseminated mycosis (Rippon 1998). Phosphatase activities were ectolocalized by the cytochemical methods, as visualized

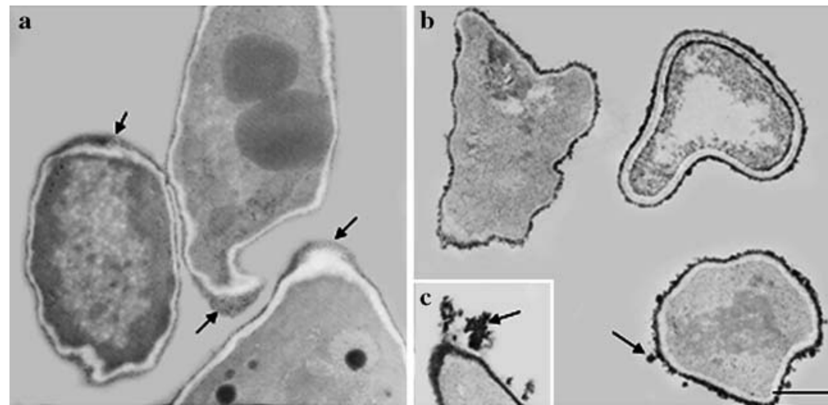

Fig. 6 Cytochemical detection of phosphatase activities in mycelial forms of *P. boydii*. Acid (**a**) and alkaline phosphatase (**b, c**) activities were localized on the cell wall. Acid phosphatase activity is less intense than that observed for alkaline phosphatase activity and was well observed in tangential sections of different cells (*arrows* in **a**). The electron-dense deposits indicative of both cytochemical reactions were observed in all cells analyzed. Occasionally electron-dense precipitates indicative of the alkaline phosphatase activity were observed projecting from the cell wall (*arrows* in **b** and **c**). Bar = 1 µm

by the cerium phosphate electron-dense deposits on the whole surface of the fungus. This experiment was performed in different pH values in order to verify the coexistence of acid and alkaline phosphatase activities, since partial sensitivity to inhibitors of alkaline phosphatases (levamisole) was observed and a dose-dependent inhibition of acid phosphatases (orthovanadate, fluoride and molybdate). In both conditions, the detection of cerium precipitates occurred however the alkaline milieu favored the detection, suggesting the predominance of phosphatases with alkaline characteristics on the cell surface of this fungus. This data was confirmed by assessing the activity in a pH range of 2.5–8.5, where alkaline pH stimulated the ecto-phosphatase activity of *P. boydii*. This kind of phenomenon was observed in *Mycobacterium bovis*, where it was characterized a non-specific phosphomonoesterase activity, produced by acid and alkaline phosphatases present on the cell surface of the same microorganism (Braibant and Content 2001).

Our experiments were performed at neutral range of pH, 7.4, the same pH used in the fungi growth, where we could observe a total ecto-phosphatase activity produced by the acid and the alkaline enzymes. This total activity was linear with time and cell density increment, indicating that all experiments were processed in correct kinetic conditions for the enzymes function. Taken together, the following observations seemingly excluded the participation of extracellular enzymes from lysed cells or their disruption in the assay condition: (1) mycelia were viable during all incubation period, according to trypan blue dye exclusion assays; (2) the hydrolysis of *p*-NPP was linear with time, suggesting that eventual cell disruption during the course of incubation was not adding appreciably to the total enzymatic activity; (3) there was no detectable phosphatase activity in the supernatants of cells incubated for 60 min at 25°C in the assay buffer. The ecto-phosphatase activity was able to hydrolyze several phosphorylated substrates, including β-glycerophosphate and phosphoaminoacids. Phosphotyrosine hydrolysis level was comparable to the *p*-NPP hydrolysis and the level of dephosphorylation of phosphothreonine and phosphoserine occurred at a lower extent, suggesting preference for the tyrosine residues, probably result of action of a phosphotyrosine phosphatase. However, the threonine and serine phosphorylated residues can also be recognized by the ecto-phosphatase activities. The influence of divalent metal ions was verified. Our reaction medium was not contaminated by divalent ions since EDTA, a metal chelator, was not able to modulate the ecto-phosphatase activities. On the other hand, the *p*-NPP hydrolysis was stimulated by $MgCl_2$, $MnCl_2$ and $ZnCl_2$, and was inhibited by $CuCl_2$ and $CdCl_2$. Although the majority of protein phosphatases described (Jesus et al. 2002; Lemos et al. 2002) are not stimulated by divalent metals, metallo-phosphatases were characterized in *Fonsecaea pedrosoi* (Kneipp et al. 2003, 2004).

The prototype phosphotyrosine phosphatases (PTP1B family) are classically modulated by reductive and oxidizing agents, because cysteine residues are critical to its catalytic cycle and sulphydril groups are susceptible to regulation by the redox state of the medium (Hernandez-Hernandez et al. 1999; Barret et al. 1999; Denu et al. 2002). In this way, reducing conditions, generally maintained by thiol reagents, are necessary to keep the enzyme active, since the oxidation of such residues occurs spontaneously in the presence of oxygen (Huyer et al. 1997). On the basis of this statement, the data obtained in this work rule out the existence of a classical phosphotyrosine phosphatase on the cell surface of *P. boydii*, since none of the reducing agents

tested exerted any effect on the activity, including cysteine, an amino acid which presents the same sulphydryl group in its structure (data not shown). However, we observed irreversible inhibition of the ecto-phosphatase activity by sodium orthovanadate and ammonium molybdate, a common characteristic among the phosphotyrosine phosphatases (Almeida-Amaral et al. 2006), since this anion is similar to inorganic phosphate, the product of the reaction, and the bond between vanadate and sulfur is not disrupted by nucleophilic attack of water molecule (Walton and Dixon 1993; Stone and Dixon 1994). Taken together, our results reveal for the first time the presence of ecto-phosphatase activities in *P. boydii* but further experiments are necessary to establish their biological functions.

Acknowledgments We acknowledge Dr. Martha Sorenson (Instituto de Bioquímica Médica, Universidade Federal do Rio de Janeiro) for recommending needed changes in the English. This work was partially supported by grants from the Brazilian Agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível superior (CAPES) and Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ). Marcia R. Pinto is recipient of a fellowship from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP)—Grants Nos. 05/02776-0 and 05/56161-6.

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