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1,10-Phenanthroline Inhibits the Metallopeptidase Secreted by *Phialophora verrucosa* and Modulates its Growth, Morphology and Differentiation

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Abstract *Phialophora verrucosa* is one of the etiologic agents of chromoblastomycosis, a fungal infection that affects cutaneous and subcutaneous tissues. This disease is chronic, recurrent and difficult to treat. Several studies have shown that secreted peptidases by fungi are associated with important pathophysiological processes. Herein, we have identified and partially characterized the peptidase activity secreted by *P. verrucosa* conidial cells. Using human serum albumin as substrate, the best hydrolysis profile was detected at extreme acidic pH (3.0) and at 37 °C. The enzymatic activity was completely blocked by classical metallopeptidase inhibitors/chelating agents as 1,10-phenanthroline and EGTA. Zinc ions stimulated the metallo-type peptidase activity in a dose-dependent manner. Several proteinaceous substrates were cleaved, in different extension, by the *P. verrucosa* metallopeptidase activity, including immunoglobulin G, fibrinogen, collagen types I and IV, fibronectin, laminin and keratin; however, mucin and hemoglobin were not susceptible to proteolysis. As metallopeptidases participate in different cellular metabolic pathways in fungal cells, we also tested the influence of 1,10-phenanthroline and EGTA on *P. verrucosa* development. Contrarily to EGTA, 1,10-phenanthroline inhibited the fungal viability (MIC 0.8 μg/ml), showing fungistatic effect, and induced profound morphological alterations as visualized by transmission electron microscopy. In addition, 1,10-phenanthroline arrested the filamentation process in *P. verrucosa*. Our results corroborate the supposition that

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metallopeptidase inhibitors/chelating agents have potential to control crucial biological events in fungal agents of chromoblastomycosis.

Keywords *Phialophora verrucosa* · Chromoblastomycosis · Metallopeptidase · 1,10-Phenanthroline · Growth · Differentiation

Introduction

Phialophora verrucosa is a dematiaceous fungus that, among other pathogens such as *Fonsecaea* spp., *Cladophialophora carrionii* and *Rhinocladiella aquaspersa*, causes a chronic disease called chromoblastomycosis (CBM) [1]. CBM is a progressive, subcutaneous mycosis, characterized by several types of lesions, including nodular, tumoral and verrucous, usually involving the lower limbs [2]. The mycosis begins with a transcutaneous trauma allowing hyphal fragments and conidial forms to penetrate, producing initial lesions which consist of plaques or nodules that become verrucous [3]. Complications include secondary bacterial infections, which contribute to lymphatic damage and elephantiasis. The disease is usually insidious, and the lesions increase slowly [2]. This mycosis is most common in the tropical and subtropical regions of Latin America, the Caribbean, Africa, Asia and Australia, with particular foci in Brazil, Madagascar, Mexico, Dominican Republic, Venezuela and India [4]. CBM usually affects male agricultural workers, who are not adequately protected while handling soil, vegetables and decomposing organic matter that are natural habitats of the fungi [1]. If not diagnosed early, the course of CBM evolves into a chronic state that may cause several problems, such as difficulty in managing therapy due to the recrudescent nature of the disease, potential association with the growth of epidermoid carcinoma in affected areas, a poor quality of life and work incapacity for the infected individuals [3]. *P. verrucosa* is also associated with phaeohyphomycosis and mycetoma [5, 6]. There are cases of fatal hemorrhage due to the invasive *P. verrucosa* [7] and endophthalmitis caused by this fungus [8]. Different therapies have been used throughout the years, but long-term efficacy is still too low to allow specialists to elect a drug of choice [2].

The factors involved in the virulence of *P. verrucosa* are poorly understood. The pathogenesis of fungal diseases is related to various factors and/or molecules, including the ability to adhere to both biotic and abiotic substrates, dimorphism, cell surface composition and hydrolytic enzyme production [3]. However, human fungal pathogens produce secreted peptidases that are directly involved in several central biological processes [9–13]. There are also evidences that proteolytic enzymes are able to attack cells and molecules of the host immune system to avoid or resist the antimicrobial activity [11, 12]. In this context, our research group detected both aspartic- and metallo-type peptidases secreted by *Fonsecaea pedrosoi*, another etiologic agent of CBM [3, 14–16]. Moreover, both peptidase classes were able to degrade key host proteinaceous molecules as well as participate in central fungal physiological events as growth and differentiation [14, 15]. In the present work, we have investigated the ability of conidial cells of *P. verrucosa* to produce extracellular proteolytic enzymes as well as the influence of proteolytic inhibitors on fungal development.

Materials and Methods

Chemicals

Itraconazole, human serum albumin (HSA), bovine serum albumin (BSA), human fibronectin, human laminin, human fibrinogen, keratin, collagens (types I and IV), mucin, hemoglobin, nonimmune human immunoglobulin G (IgG), phenylmethylsulfonyl fluoride (PMSF), ethylene glycol tetraacetic acid (EGTA), 1,10-phenanthroline (1,10-PHEN), tosyllysine chloromethyl ketone hydrochloride (TLCK), iodoacetamide and pepstatin A were obtained from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). Reagents used in electrophoresis and buffer components were purchased from Bio-Rad (Hercules, CA, USA) and Merck (Darmstaldt, Germany). Roswell Park Memorial Institute (RPMI) 1640 medium was obtained from Invitrogen (Camarillo, CA, USA). All other reagents were of analytical grade.

Growth Conditions

The pathogenic strain of *P. verrucosa* (FMC.2214 strain) isolated from a human patient with CBM was

used in all parts of the present work. Stock cultures were maintained on Sabouraud dextrose agar (SDA) under mineral oil and kept at 4 °C. Transfers were made at 6-month intervals. The conidial cells were cultivated for 7 days under constant agitation (130 rpm) at 26 °C in 50 ml of Czapek–Dox, a chemically defined medium [14], containing per liter: 30 g sacarose; 3 g $NaNO_3$; 0.5 g $MgSO_4 \cdot 7H_2O$; 0.5 g KCl; 1 g KH_2PO_4; 0.01 g $FeSO_4 \cdot 7H_2O$, pH 5.5. Conidia were obtained after hyphae removal by filtering cultures through gauze followed by centrifugation (4,000×*g*/10 min). For experiments, conidia were washed three times in saline (0.85 % NaCl) and the number of cells was estimated by counting in a Neubauer chamber.

Secretion Assay

P. verrucosa conidial cells (2.5×10^9) were resuspended in 0.2 ml of sterile phosphate-buffered saline (PBS; 150 mM NaCl, 20 mM phosphate buffer, pH 7.2) supplemented with 2 % glucose and incubated for 2 h at 26 °C with constant agitation (130 rpm). After this interval, the fungal cells were removed by centrifugation (4,000×*g*/10 min) and the cell-free PBS–glucose supernatant was directly submitted to peptidase activity assay as described below [13].

Proteolytic Activity Assays

The extracellular proteolytic activity was measured spectrophotometrically using the substrate HSA, according to the method described by Buroker-Kilgore and Wang [17]. Briefly, 15 µl of supernatant (10 µg of protein), HSA (0.1 mg/ml) and 20 mM sodium acetate buffer, pH 3.0, was added to a microcentrifuge tube (50 µl) and incubated at 37 °C. After incubation for 20 h, the reaction mixture (50 µl) was transferred to wells on a microtiter plate containing 50 µl of water and 50 µl of a Coomassie solution (0.025 % Coomassie brilliant blue G-250, 11.75 % ethanol and 21.25 % phosphoric acid). A control, where the substrate was added just after the reactions were stopped, was used as blank. After 10 min to allow dye binding, the plate was read on a Bio-Rad (model 680) microplate reader at an absorbance of 595 nm [17]. One unit of enzymatic activity was defined as the amount of enzyme that caused an increase of 0.001 in absorbance unit under standard assay conditions [16]. In parallel, the reaction mixtures were also applied on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) to demonstrate the fragments that correspond to the HSA hydrolysis. To this end, the reaction mixtures (30 µl) were treated with SDS-PAGE sample buffer (125 mM Tris–HCl, pH 6.8, 4 % SDS, 20 % glycerol, 0.002 % bromophenol blue) supplemented with 10 % β-mercaptoethanol, followed by heating at 100 °C for 5 min. Prior to electrophoresis, molecular mass standards (Thermo Scientific, Rockford, IL, USA) were boiled in SDS-PAGE sample buffer and then applied to the same gel. The degradation protein profiles were analyzed on 12 % SDS-PAGE as described by Laemmli [18]. Electrophoresis was carried out at 4 °C, and at 120 V for 90 min. The proteins were detected by silver staining [19].

Influence of pH, Time, Temperature, Divalent Cations and Proteolytic Inhibitors on the Peptidase Activity

The effect of pH was determined by incubating the reaction mixtures under standard assay conditions described above in the following buffer systems: 20 mM KCl/HCl, pH 2.0, 20 mM sodium acetate buffer, pH 3.0–6.0 or 20 mM Tris base pH 7.0–9.0. The proteolysis was also tested in two distinct temperatures (28 and 37 °C) as well as during different reaction time (2, 5 and 20 h), both at pH 3.0. The requirement of divalent cations and the effect of proteolytic inhibitors were determined by incubating the reaction mixtures in 20 mM sodium acetate buffer, pH 3.0, in the absence (control) or in the presence of divalent cations (20 mM of $MnCl_2$, $CaCl_2$ and $MgCl_2$, and different concentrations (5, 10 and 20 mM) of $ZnCl_2$) or in the presence of different proteolytic inhibitors (10 µM pepstatin A, 10 mM PMSF, 1 mM iodoacetamide, 1 mM TLCK, 10 mM EGTA and 10 mM 1,10-PHEN).

Cleavage of Proteinaceous Substrates

Twenty microliters of the PBS–glucose supernatant was mixed with an equal volume of the following proteinaceous substrates: IgG, fibrinogen, keratin, laminin, fibronectin, collagen types I and IV, hemoglobin and mucin. These proteins were diluted in 20 mM sodium acetate buffer (pH 3.0) in order to obtain a final concentration of 5 µg/ml in the reaction mixture. In addition, a control for each proteinaceous substrate was made by replacing PBS–glucose supernatant with the same volume of acetate buffer. These

preparations were incubated for 20 h at 37 °C, and the degradation protein profiles were analyzed on 12 % SDS-PAGE after silver staining.

Effects of 1,10-PHEN and EGTA on *P. verrucosa* Cellular Viability

Determination of antifungal activity was performed as described in the M38-A2 document published by Clinical and Laboratory Standards Institute (CLSI) for filamentous fungi [20], with some modifications [21, 22]. Briefly, the broth microdilution method was performed by 96-well microtiter assay plate containing RPMI 1640 medium at pH 7.0 buffered with 0.16 M MOPS. 1,10-PHEN and EGTA were diluted in DMSO to obtain final concentrations ranging from 0.1 to 200 μg/ml for both compounds. Next, *P. verrucosa* suspensions were incubated for 5 days at a final concentration of 10^5 conidia/ml. The minimum inhibitory concentration (MIC) of each drug was determined visually and by spectrophotometric reading at 490 nm. The lowest concentration inhibiting 100 % growth of the organism was recorded as the MIC. Itraconazole (ITC) was used (0.04–100 μg/ml) as reference antifungal compound. Each experiment was performed in triplicate. The minimum fungicidal concentration (MFC) was also tested. After determination of MIC, each well was homogenized and 2.5 μl was transferred onto SDA drug-free medium. The plates were incubated at 26 °C for 7 days. The MFC was determined as the lowest concentration without visual growth of fungal colonies. A fungicidal effect was defined as the range from the MFC value equal or until four times the MIC value. Above four times the MIC value, the antifungal effect is considered fungistatic [23]. IC_{50} was defined by logarithmic regression after MIC determination. The antifungal activity was calculated by applying the formula: % antimicrobial activity = 100 − (absorbance at 490 nm (ABS_{490}) sample − ABS_{490} mean of 100 % growth inhibition) × 100)/(ABS_{490} mean of 100 % growth − ABS_{490} mean of 100 % growth inhibition).

Effects of 1,10-PHEN on the ultrastructure of *P. verrucosa*

Conidia of *P. verrucosa* (5×10^7 cells) were incubated in RPMI 1640 medium, pH 7.0, for 15 h in the absence (control) or presence of 5 μg/ml 1,10-PHEN. After that, conidia were washed in PBS, pH 7.2, and fixed in a solution of 2.5 % glutaraldehyde and 4 % freshly prepared formaldehyde in 0.1 M cacodylate buffer, pH 7.2, for 2 h at room temperature. After fixation, cells were post-fixed for 1 h in 1 % osmium tetroxide containing 1.25 % potassium ferrocyanide and 5 mM $CaCl_2$ in cacodylate buffer, pH 7.2, washed in the same buffer, dehydrated in ethanol and embedded in Spurr [24]. Ultrathin sections were stained with uranyl acetate and lead citrate, and images were obtained in a Zeiss 900 transmission electron microscope equipped with a CCD Camera (Mega view III model, Soft Image System, Germany). Images were processed with iTEM software (Soft Image System, Germany).

Effects of 1,10-PHEN on the Morphogenesis of *P. verrucosa*

Conidia of *P. verrucosa* (10^7 cells/ml) were incubated at 37 °C in the absence (control) or in the presence of different concentrations of 1,10-PHEN (0.2, 0.4 and 0.8 μg/ml) in RPMI medium, pH 7.0 (without agitation) for induction of *P. verrucosa* filamentous form. The cells were observed for each 24 h at optical microscope Zeiss Axiostar Plus. Conidial viability in the presence of different concentrations of 1,10-PHEN was accessed by resazurin staining and colony-forming unit (CFU) assay [16].

Statistical Analysis

All experiments were performed in triplicate, in three independent experimental sets. The data were analyzed statistically by means of Student's *t* test using EPI-INFO 6.04 (Database and Statistics Program for Public Health) computer software.

Results

Secretion of Peptidase from *P. verrucosa* Conidia

Initially, we have investigated the possible secretion of peptidase from *P. verrucosa* conidial cells by testing the capability of the cell-free PBS–glucose supernatant, obtained after growth in a chemically defined medium, to hydrolyze soluble human albumin. Peptidase activity was detected only under extreme acidic

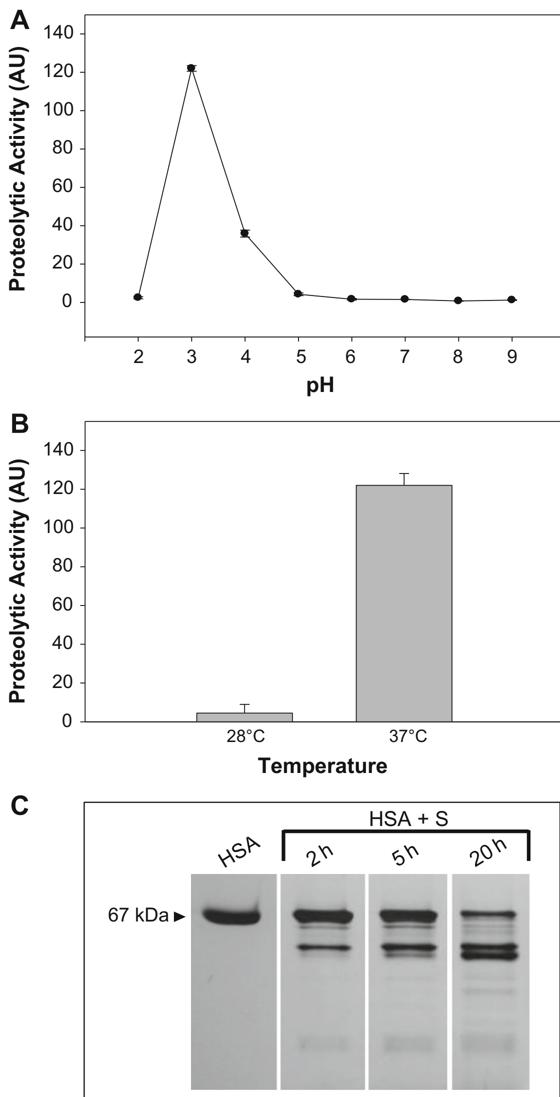


Fig. 1 Influence of pH, temperature and time course on the extracellular peptidase activity of *P. verrucosa*. **a** PBS–glucose supernatant was incubated in the presence of HSA and buffers with different pH values, ranging from 2.0 to 9.0, for 20 h at 37 °C. Then, the reaction medium was read at 595 nm. **b** The effect of temperature on the proteolytic activity was also measured by spectrophotometer in two distinct temperatures (28 and 37 °C) at pH 3.0. The proteolytic activity was expressed as arbitrary unit (AU), which was defined as the amount of enzyme that caused an increase of 0.001 in absorbance under standard assay conditions. **c** SDS-PAGE showing the HSA degradation profile after incubation with cell-free culture supernatant (S) of *P. verrucosa* for 2, 5 and 20 h at pH 3.0. The *first slot* represents a control in which HSA was supplemented only with buffer

conditions, presenting highest hydrolysis level at pH 3.0 (Fig. 1a). In addition, the peptidase activity secreted by *P. verrucosa* was more active at 37 °C when compared to 28 °C (Fig. 1b), and the degradation of soluble HSA was a time-dependent event (Fig. 1c).

Effects of Different Inhibitors and Cations on the Proteolytic Activity

P. verrucosa acidic extracellular peptidase activity was not significantly inhibited by PMSF, TLCK (two serine peptidase inhibitors), pepstatin A (an aspartic peptidase inhibitor) and iodoacetamide (a cysteine peptidase inhibitor) (Table 1). On the contrary, the metallopeptidase inhibitors EGTA and 1,10-PHEN powerfully blocked the peptidase activity (Table 1). As well known, metallo-type peptidases require divalent cations to their efficient hydrolytic activity. In this context, the influence of different divalent cations was tested on the peptidase activity secreted by *P. verrucosa* (Table 2). Calcium, manganese and magnesium ions did not significantly modulate the peptidase activity; however, zinc ions showed a dose-dependent stimulation on the metallopeptidase activity of *P. verrucosa* (Table 2).

Cleavage of Different Proteinaceous Substrates

We tested the ability of the fungal proteolytic activity to cleave a broad spectrum of proteinaceous substrates, including important host serum proteins and

Table 1 Effect of proteolytic inhibitors on the peptidase activity of *P. verrucosa*

Proteolytic inhibitor	Concentration	Relative activity (%)[a]
None	–	100.0 ± 8.95
1,10-Phenanthroline	10 mM	11.6 ± 0.14*
EGTA	10 mM	1.0 ± 0.05*
Pepstatin A	10 μM	80.0 ± 0.73
Iodoacetamide	1 mM	70.0 ± 8.35
TLCK	1 mM	91.5 ± 4.60
PMSF	10 mM	122.0 ± 1.39

* The proteolytic activity measured in the presence of inhibitors showed hydrolysis significantly different from control ($P < 0.05$, Student's t test)

[a] The proteolytic activity was measured in the standard assay described in "Materials and methods" using HSA as soluble substrate. Proteolytic activity was expressed as a percentage of that measured under control conditions, i.e., without addition of proteolytic inhibitors. The peptidase activity of the control (122 ± 6.1 arbitrary units) was taken as 100 %

Table 2 Influence of divalent cations on the proteolytic activity of *P. verrucosa*

Compounds	(mM)	Relative activity (%)
None	–	100.0 ± 3.5
$MnCl_2$	20.0	98.0 ± 8.6
$MgCl_2$	20.0	110.9 ± 5.2
$CaCl_2$	20.0	95.3 ± 9.8
$ZnCl_2$	20.0	732.5 ± 8.8
	10.0	240.1 ± 1.1
	5.0	154.2 ± 3.0

Reactions were carried out with conidia incubated for 20 h at 37 °C in a medium containing sodium acetate buffer, pH 3.0 and HSA substrate. The level of proteolytic activity seen in controls (producing 122.0 ± 6.1 arbitrary units) was taken as 100 %. The proteolytic activity in the presence of zinc was significantly different from control ($P < 0.05$, Student's t test)

extracellular matrix components. The secreted peptidase activity of *P. verrucosa* was able to degrade IgG, fibrinogen, fibronectin, keratin, laminin and collagen types I and IV (Fig. 2), while mucin and hemoglobin were not degraded under the employed experimental conditions (data not shown).

Effects of Metallopeptidase Inhibitors on the Viability of *P. verrucosa*

To investigate whether the metallopeptidase inhibitors might have any effect on *P. verrucosa* development, we performed experiments in which conidial cells were grown on RPMI in the presence of 1,10-PHEN and EGTA in order to determine MIC and MFC. 1,10-PHEN showed high antifungal activity, presenting MIC of 0.8 μg/ml ($IC_{50} = 0.5$ μg/ml) as demonstrated in Fig. 3. Conversely, EGTA was not able to inhibit the fungal viability (MIC > 200 μg/ml) (Fig. 3). MFC value of 6.2 μg/ml to 1,10-PHEN indicated a fungistatic effect for this compound (Fig. 3).

Effects of 1,10-PHEN on the ultrastructure of *P. verrucosa*

Based on the efficacy of the 1,10-PHEN in diminishing the viability of *P. verrucosa*, we next investigated the effect of this inhibitor on the fungal ultrastructure by transmission electron microscopy. In contrast to non-treated conidial cells, which presented the cell wall preserved and homogenous cytoplasm, the treatment with 1,10-PHEN at 5 μg/ml induced some morphological alterations, including detachment of the cell wall, intense vacuolization and reduced electron density of the cytoplasm, which are indicative of cell death (Fig. 4).

Effects of 1,10-PHEN on the differentiation of *P. verrucosa*

P. verrucosa conidial cells were able to germinate when incubated in RPMI medium for 60 h at room temperature, generating long filamentous cells as demonstrated by optical microscopy (Fig. 5). The treatment of conidia with 1,10-PHEN at sub-MIC concentration (½ × MIC = 0.4 μg/ml) was able to disturb the differentiation process of *P. verrucosa* (Fig. 5), promoting a considerably reduction on the size of the filamentous cells. On the contrary, the treatment of conidia with 1,10-PHEN at 0.2 μg/ml (¼ × MIC) did not alter the differentiation dynamics. As expected, 1,10-PHEN at both 0.2 and 0.4 μg/ml did not affect the fungal viability along the 60 h of in vitro incubation, as confirmed by both resazurin and CFU assays (data not shown). Conversely, 1,10-PHEN at 0.8 μg/ml inhibited the growth of *P. verrucosa,* and for this purpose, this concentration was not considered in the differentiation assay.

Discussion

Fungal infections are common in tropical countries and responsible for a significant impact on public health [25]. Among them, dematiaceous fungi are involved in a wide variety of infectious syndromes [26]. These fungi are a heterogenic group that present melanin associated to the cell wall. They are often found in soil and generally distributed worldwide, suggesting that most if not all individuals are exposed to dematiaceous fungi presumably from inhalation or trauma [26]. In recent years, several studies have reported a significant increasing in the number of diseases caused by these fungi such as CBM, mycetoma and phaeohyphomycosis that can result in considerable morbidity and mortality in several countries, including Brazil [4, 27, 28].

Little is known about the probable virulence attributes expressed by dematiaceous fungi during their life cycle and in the pathogenesis of the disease

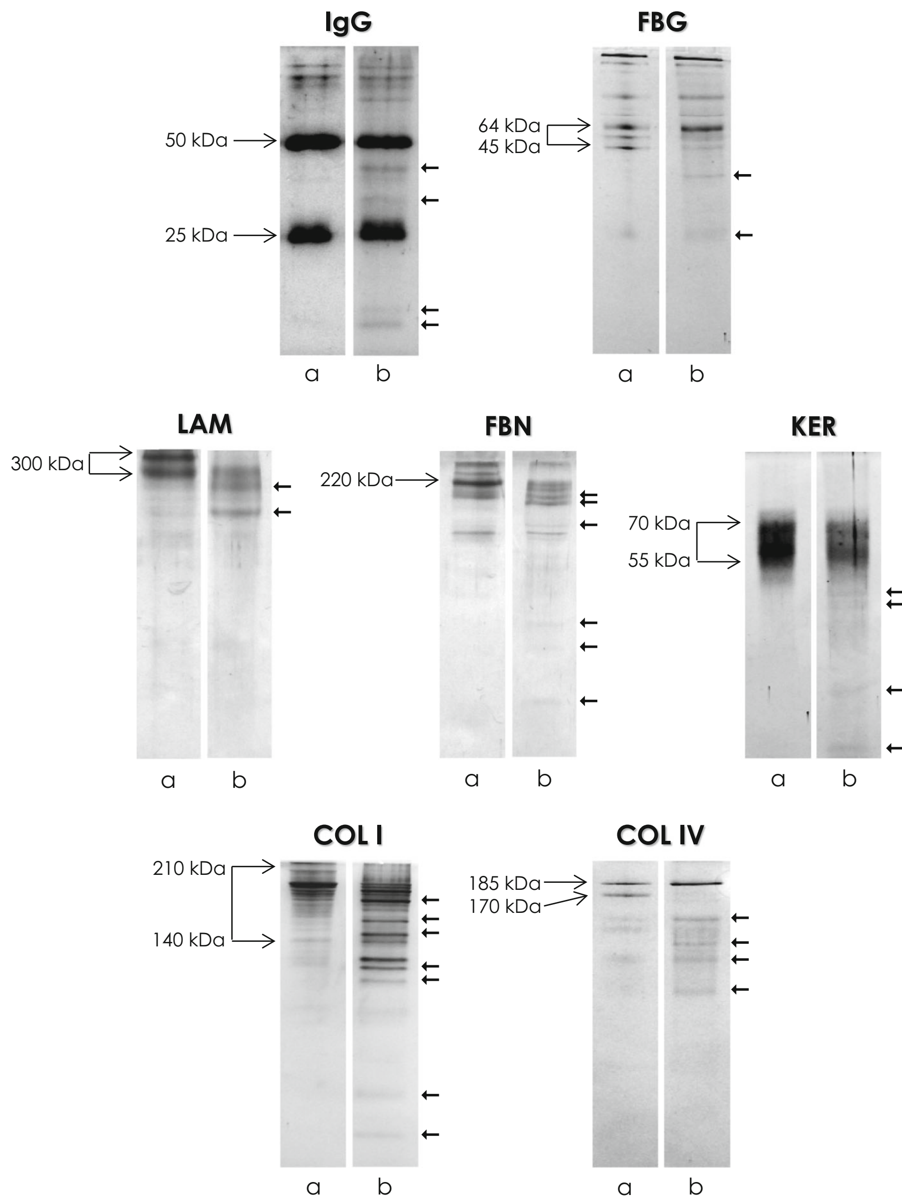
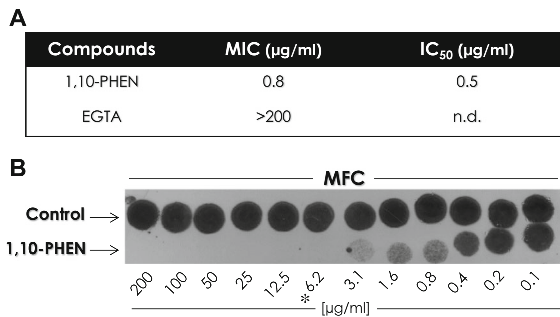


Fig. 2 Cleavage of different proteinaceous substrates by the extracellular peptidases of *P. verrucosa*. The degradation profile was analyzed by 12 % SDS-PAGE, and the gels were stained with silver. Human protein components were mixed with 20 mM sodium acetate, pH 3.0, and incubated for 20 h at 37 °C in the absence (**a**) or in the presence (**b**) of cell-free PBS–glucose supernatant from *P. verrucosa* conidial cells. The *numbers on the left* represent the molecular masses, expressed in kDa, of the following proteinaceous substrates: immunoglobulin G (IgG), fibrinogen (FBG), keratin (KER), laminin (LAM), fibronectin (FBN) and collagen type I (COL I) and type IV (COL IV). The *arrows on the right* show the fragmentation of substrates after proteolysis

[3, 29, 30]. Conversely, it is well known that direct virulence factors for pathogenic fungi include hydrolysis of protein of host cell membranes and extracellular matrix components to facilitate adhesion and tissue invasion or damaging cells [10–12]. Peptidases are believed to contribute to microbial virulence by destroying host tissues and digesting immunologically important proteins, such as antibodies, complement factors and antimicrobial peptides [9, 12]. Peptidases produced by fungal pathogens play also important



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Compounds	MIC (μg/ml)	IC_{50} (μg/ml)
1,10-PHEN	0.8	0.5
EGTA	>200	n.d.

Fig. 3 Effect of 1,10-PHEN and EGTA on *P. verrucosa* viability. **a** MIC assay was performed as described in the CLSI document M38-A2 for the filamentous fungi, with some modifications as detailed in "Materials and methods." Itraconazole (ITC) used as reference antifungal standard showed MIC of 3.12 μg/ml. The IC_{50} was determined by logarithmic regression using Microsoft Excel. **b** Evaluation of MFC of 1,10-PHEN on *P. verrucosa* growth. MFC was defined as the lowest concentration that resulted in no visual fungal growth, as indicated by *asterisk*. *P. verrucosa* showed fungistatic effect since the ratio value of MFC (6.2 μg/ml) by MIC (0.8 μg/ml) was above four times, as established by Pfaller et al. [23]. *n.d.* not determined

physiological roles as nutrition, growth, proliferation, signaling, death pathways, cell cycle progression and cellular differentiation [9–12]. Thereby, proteolytic enzymes are potential targets for antifungal drug development, since these enzymes play essential metabolic and regulatory functions in many central biological processes of fungal cells [12, 31]. In this context, we showed that *P. verrucosa* conidial cells were able to secrete peptidase activity to the extracellular environment, which was active at extremely acidic conditions of pH similar to several filamentous fungi like *F. pedrosoi* [3, 14–16] and *Pseudallescheria boydii* [32, 33]. It seems surprising that secreted peptidase activity of *P. verrucosa* conidia was active only in acidic pH. However, as described for *F. pedrosoi*, this may be compensated by the ability of fungus to actively acidify its surrounding environment in order to provide microniches optimal for peptidase activity during infection [15]. The forms of CBM fungi observed in infected tissues are comprised of hyphal segments/conidia and thick-walled sclerotic cells which come into contact with phagocytic cells such as macrophages [3]. The acidic peptidase activity produced and secreted by *P. verrucosa* could facilitate its survival within the acidic environment of phagolysosome. The virulence of several microorganisms is directly correlated with their ability to survive and multiply intracellularly [15]. Moreover, the hypha or

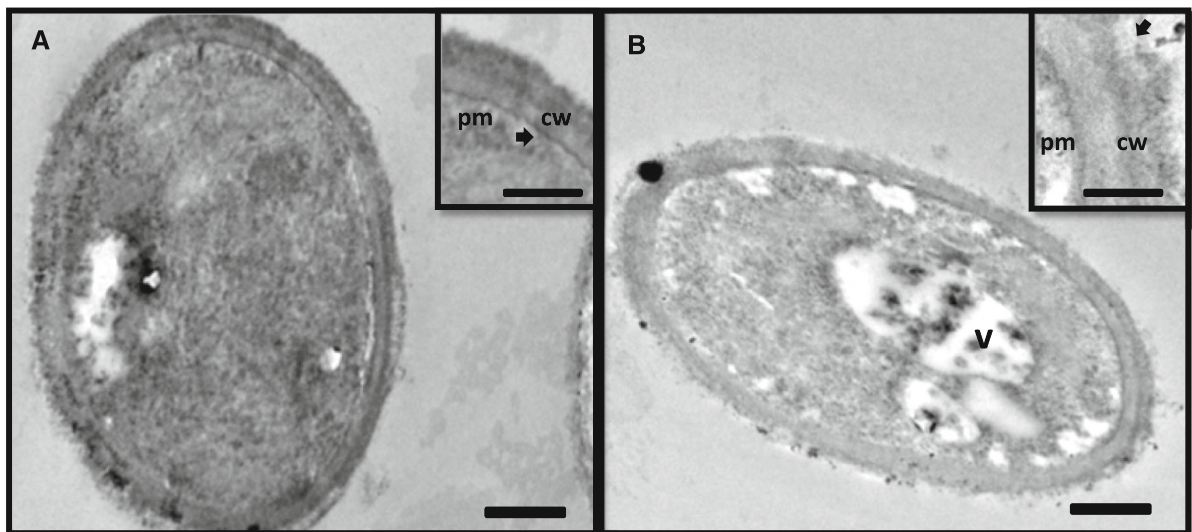

Fig. 4 Effect of 1,10-PHEN on the ultrastructure of *P. verrucosa*. Conidia of *P. verrucosa* (5×10^7 cells) were incubated in the absence (control) or presence of 5 μg/ml 1,10-PHEN for 15 h at 26 °C. Conidial cells were washed, fixed and processed as detailed in "Materials and methods." **a** Control cells present round shape morphology with an homogenous cytoplasm, linear plasma membrane (pm) and a cell wall (cw) with two distinct layers: an inner electron dense and outer fibrillar layer. **b** 1,10-PHEN treatment lead to an increase of electron lucent vacuoles (v) as well as the detachment of the outermost fibrillar layer (*inset*). *Bars* 0.5 μm; *inset bars* 0.25 μm

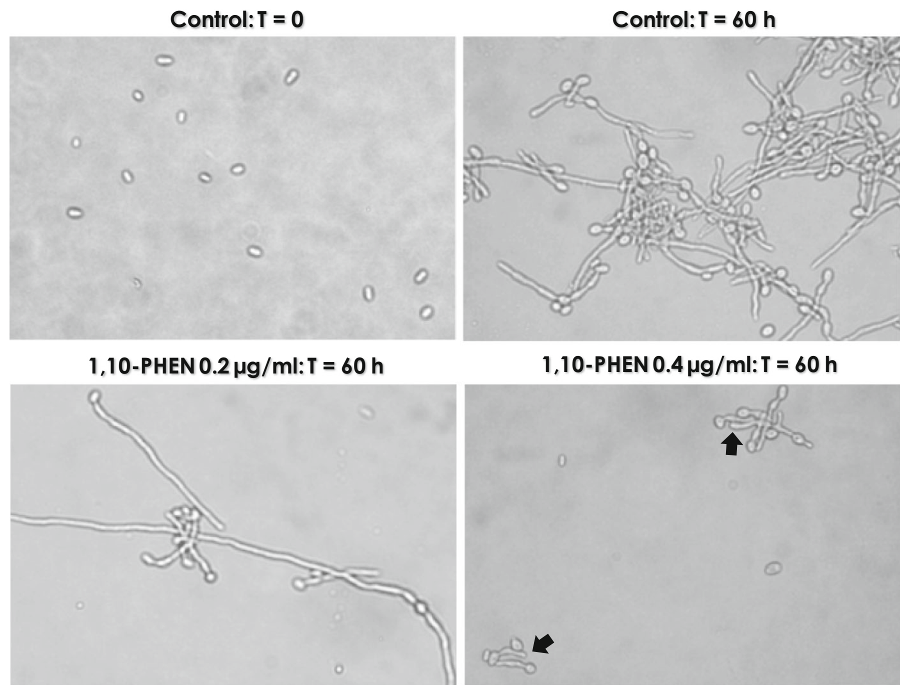

Fig. 5 Influence of 1,10-PHEN on the differentiation process of *P. verrucosa*. Conidia (10^7 cells/ml) were incubated in the absence (control) or presence of 1,10-PHEN at sub-inhibitory concentrations in RPMI medium, pH 7.0, without agitation, at 37 °C. Conidial cells were observed before ($T = 0$: zero time) and after incubation of 60 h ($T = 60$ h) at optical microscope Zeiss Axiostar Plus. The inhibition of the fungal morphological transition by 1,10-PHEN was determined by reduction of size of the filamentous cells (*arrows*)

conidia forms of CBM fungi convert into sclerotic cells in vitro when incubated in defined conditions only at extremely low pH [3]. Therefore, the acidic peptidase could participate directly or indirectly in this essential fungal biological process.

The proteolytic inhibition profile revealed that the peptidase activity released by *P. verrucosa* conidia belongs to the metallopeptidase class due to the sensibility to 1,10-PHEN and EGTA. Metallopeptidases have been described in different human fungal pathogens, including *F. pedrosoi, P. boydii*, *Paracoccidioides brasiliensis* and *Candida albicans*, participating in several fundamental biological processes [14, 33–35]. Inhibition of metallopeptidases by 1,10-PHEN occurs by removal and chelation of the metal ion required for catalytic activity, leaving an inactive apoenzyme [36]. Thus, these enzymes are characterized by the requirement of a divalent metal ion for their full activity. In *P. verrucosa*, among the metals tested, only Zn^{2+} was able to stimulate the proteolytic activity, suggesting an activity of zinc metallopeptidase as described in other fungi, such as *P. boydii* and *F. pedrosoi* [13, 14, 35]. Metallopeptidases differ widely in their sequences and structures, but the great majority of these enzymes contain a zinc atom that is catalytically active [37]. This is expected, since the prevalence of zinc is surpassed only by iron among the transition metals in biological systems [38]. The ability of 1,10-PHEN to sequester essential trace metals from biological environments has also been demonstrated to inhibit the survival of microbial pathogens [39]. It has been demonstrated that the inhibitory action of 1,10-PHEN toward a range of fungi such as *Penicillium roqueforti, Trichoderma harzianum, Paecilomyces variotii, Aspergillus niger* and *Aspergillus nidulans* depends significantly on the chelation of endogenous Zn^{2+} [35]. In this context, the trace element Zn^{2+} is required for proper function of a large number of eukaryotic proteins, including transcription factors capable of binding DNA and various metallo-type enzymes, as metallopeptidases [35, 37]. As expected, the sequestration of this important element provokes perturbation in the cell homeostasis, leading to various metabolic dysfunctions including cell death [35]. The effective inhibition of proteolytic activity by 1,10-PHEN corroborates the results showed that *P. verrucosa* secretes Zn^{2+}-metallopeptidase activity.

Metallopeptidases produced by fungi effectively participate in different steps of the interaction to host, including nutrition, adhesion, invasion, dissemination and escape, by destroying functional host proteins

located on cells and tissues [14, 32, 40]. For instance, fungalysin metallopeptidase (family M36) of *Aspergillus flavus* is capable of hydrolyzing laminin, elastin and collagen, helping the fungal cells to disseminate across the extracellular matrix [41]. Silva et al. [32] showed that metallopeptidases of *P. boydii* cleaved fibronectin, laminin, mucin, IgG and hemoglobin. *F. pedrosoi* conidial cells also degraded different host proteinaceous substrates due to the hydrolytic activity of extracellular metallopeptidase [14]. Similarly, metallopeptidase activity secreted by *P. verrucosa* cleaved important human serum proteins, such as HSA, IgG and fibrinogen, essential components of the extracellular matrix, such as collagen types I and IV, fibronectin and laminin, and the intermediate filament keratin. In a comparative perspective, the data presented herein suggest that peptidase activity secreted by *P. verrucosa* may also be involved with dissemination and escape from the host immune system. Nothing is known about the mechanism by which *P. verrucosa* invades tissue and disseminates from the cutaneous and/or subcutaneous tissues to the other parts of the body. However, these reports suggest and are consistent with the fungal secretion of tissue-degrading enzymes, such as extracellular peptidases [14].

P. verrucosa metallopeptidase activity, as described in other fungi, may be involved in protein nutrient acquisition as well as in the maintenance of physiological and metabolic processes such as control of cell growth [13, 33, 41, 42]. 1,10-PHEN and EGTA presented antifungal activity against a broad spectrum of pathogenic fungi [35, 42]. Our results showed that EGTA was not able to inhibit *P. verrucosa* viability until concentration of 200 μg/ml. So, considering the low antimicrobial activity of EGTA, this inhibitor was not used in other assays. On the contrary, 1,10-PHEN effectively inhibited the *P. verrucosa* conidial viability in the range of nanogram. This inhibitory concentration was comparable to that described for itraconazole (0.8 μg/ml) in in vitro study against a clinical isolated of *P. verrucosa* [7]. As proposed by Pfaller et al. [23], 1,10-PHEN can be considered as a fungistatic compound against *P. verrucosa*, since MFC value (6.2 μg/ml) was higher than four times the MIC (0.8 μg/ml). Palmeira et al. [14] showed that the growth of *F. pedrosoi* was completely abrogated by 10 mM of 1,10-PHEN. Furthermore, treatment with 1,10-PHEN also reduced the in vitro growth of *Cryptococcus neoformans* and *Candida parapsilosis* [42]. McCann et al. [43] showed that a clinical isolate of *C. albicans* was sensitive to 1,10-PHEN presenting MIC value established as 1.25–2.5 μg/ml. 1,10-PHEN was fungicidal against fungi belonging to the *P. boydii*/*Scedosporium apiospermum* complex, and the antifungal activity was directly dependent on both cell density and drug concentration [13]. Larvae of the insect *Galleria mellonella* were also employed to assess in vivo antifungal efficacy of 1,10-PHEN. Larvae pre-inoculated with this metallopeptidase inhibitor were protected from a subsequent lethal infection by the yeast *C. albicans*, while larvae inoculated post-infection showed significantly increased survival compared to control larvae [42].

1,10-PHEN have the ability to selectively sequester essential metal ion for cell metabolism by interfering in acquisition and availability of crucial metals for enzymatic reactions. However, besides metallo-type enzymes, other targets may be affected by these chelating agents. McCann et al. [35] proposed that 1,10-PHEN chelator can affect vital fungal biological processes, such as: disruption of cell membrane and withdrawal of cytoplasmic membrane; damage of mitochondrial function and uncoupling of cellular respiration; drug-induced circumvention on the control of cell division; chelation or sequestering essential trace metal ions inhibiting glycosylphosphatidylinositol synthesis; degradation of nuclear DNA and ruptured internal organelles and enlarged nucleus. All these interferences on the homeostasis of a fungal cell can culminate in cell death. Ultrastructural analyses demonstrated that *P. verrucosa* conidia treated with 1,10-PHEN presented important morphological changes, such as extensive vacuolization and detachment of the cell wall, which corroborate the anti-proliferative properties of the 1,10-PHEN.

In this study, we also evaluated the effect of 1,10-PHEN on the *P. verrucosa* differentiation. After 60 h of treatment, 1,10-PHEN perturbed the fungus morphogenesis, reducing considerably the size of the filamentous cells. Several studies have reported the differential expression of peptidases during fungal morphogenesis and that proteolytic inhibitors may control this crucial biological event [9, 14]. For instance, 1,10-PHEN (1 mM) was also able to block the differentiation process (conidia into mycelia) in *F. pedrosoi* [3, 14]. 1,10-PHEN also inhibited the transformation of *S. apiospermum* conidia into hyphae

[33]. It was also demonstrated that 1,10-PHEN completely inhibited the transformation of *C. albicans* yeast into hyphal form, as well as restricting the bud formation [42]. In *C. albicans,* the inhibition of the morphological switch from yeast to hyphal growth could inhibit adhesion, invasion and damage of both epithelial and endothelial cells [44]. In this way, a successful infection depends on the attachment of infectious propagules to host cells and their subsequent morphological transition. So, the elucidation of the mechanisms that impair *P. verrucosa* differentiation is of crucial significance and may allow the development of new antifungal therapies.

Taken together, our results revealed for the first time the presence of metallopeptidase activity secreted by conidial cells of *P. verrucosa*. Some of our results may help us to better understand the physiology of this dematiaceous fungus. In this sense, 1,10-PHEN, a potent metallopeptidase inhibitor/chelating agent, inhibited the extracellular metallo-type peptidase activity released by *P. verrucosa* conidial cells as well as blocked essential fungal biological processes such as growth and differentiation. 1,10-PHEN and its derivative compounds have been the subject of intense research due to their versatile coordination chemistry and recognized function in diverse biological systems as reagents for both biotechnology and medicine perspectives [35, 42]. In conclusion, our findings suggest that 1,10-PHEN has potential to be used for the synthesis of novel compounds with anti-*P. verrucosa* activity in order to be used as future alternative therapeutic intervention against infections caused by this opportunistic fungus.

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Conflict of interest None.

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