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Induction and secretion of elastinolytic and proteolytic activity in cultures of *Paracoccidioides brasiliensis*

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SUMMARY

***P. brasiliensis* parasitizes various human tissues and proteinases exported by this fungus may allow it to metabolize and invade host tissues. The influence of the culture medium on the production of proteinases by *P. brasiliensis* isolates was studied and the export of these enzymes was followed as a function of culture time. The fungus was grown in neopeptone, BSA, elastin or collagen medium. The culture medium was assayed for azocollytic, elastinolytic and caseinolytic activity. Proteolytic activity was also analysed by electrophoresis of the culture medium on gelatin and casein substrate gels. *P. brasiliensis* expressed relatively high levels of azocoll, elastin and casein degrading activity in all types of medium, except in neopeptone medium. Generally, expression of azocollytic activity peaked during the third week of culture and caseinolytic activity during the fourth week of culture. Azocollytic activity was highest at pH 4.0 and caseinolytic activity at pH 8.0. Elastinolytic activity was also highest at pH 8.0. This activity, as well as the others, may provide the fungus with a source of carbon and nitrogen and may also be responsible for the invasion of host tissues, such as pulmonary elastic fiber, by *P. brasiliensis*.**
Keywords: Proteolytic activity; collagenolytic activity; elastinolytic activity; *Paracoccidioides brasiliensis*; paracoccidioidomycosis.

INTRODUCTION

Paracoccidioides brasiliensis is a dimorphic fungus that causes paracoccidioidomycosis, a frequently progressive and chronic disorder. The infectious process is started by fungal conidia from the mycelial form, which penetrate the host through the upper airways and lodge in the lungs. The existence of different clinical forms of this disease and the occurrence of asymptomatic infection may be a result of host-related factors, immunological status and characteristics of the infecting agent, especially its virulence (Franco et al., 1994).

Specific features of the fungus that may play a critical role in infection include cell-wall composition (α-1,3 glucan), ability to grow at 37 °C, dimorphism, production of 43 kDa glycoprotein (gp43), proteinases, and the ability to adhere to host tissues (San-Blas, 1982; Kashino et al., 1987, Mendes-Giannini et al., 1994, Mendes-Giannini et al., 2005). Proteinases produced by certain microorganisms have been associated with their pathogenicity and in some fungi it has been suggested that tissue invasion is facilitated by proteolytic enzymes, produced *in vivo* by the pathogen (Cassone et al., 1987; De Bernardis et al., 1999; De Bernardis et al., 2001; Taylor et al., 2005; Jackson et al., 2007). There is presumptive evidence of a role for proteinases in the pathogenesis of infection with *Sporothrix schenckii* (Tsuboi et al., 1987; Lei et al., 1993), dermatophytes (Apodaca & Mckerrow, 1990; Kaufman et al., 2007) and *Aspergillus fumigatus* (Kothary et al., 1984; Frosco et al., 1992; Kolattukudy et al., 1993; Monod et al., 1993) and more definitive evidence of their action as virulence factors in candidiasis (Naglik et al., 2004; Taylor et al., 2005; Lian & Liu, 2007; Jackson et al., 2007).

These proteinases have been reported to have a collagenolytic, elastinolytic or keratinolytic activity. Extracellular enzymes are probably essential for these organisms to degrade the structural barriers of the host. Since the major natural mode of entry of *P. brasiliensis* into the host is via inhalation, the barriers in the lung would be the first targets for the fungal extracellular enzymes (Franco et al., 1993). Some studies of *P. brasiliensis* have also pointed to the presence of proteinases and according to the authors, this may be related to pathogenicity (Mendes-Giannini et al., 1990; Bedoya-Escobar et al., 1993; Mendes-Giannini & Toscano, 1994; Vaz et al., 1994; Carmona et al., 1995; De Assis et al., 1999; Matsuo et al., 2006). However, elastinolytic activity has never been detected in *P.brasiliensis*. The presence of elastase activity has been described as particularly relevant because elastin constitutes a significant proportion of total lung proteins and it is an important virulence factor in other lung pathogens such as *Pseudomonas aeruginosa* and *Aspergillus fumigatus* (Garcia et al., 2006).

In this paper, we report the expression of elastinolytic and, additionally, azocollytic and caseinolytic activity by cultures of yeast and mycelial phases of *P. brasiliensis* and we assess the effect of various culture media on the production of these enzymes as a function of time in culture.

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MATERIAL AND METHODS

Fungal strains

Isolates of *P. brasiliensis* (Pb) from seven different patients, which had been maintained in the mycology laboratory of Faculdade de Ciências Farmacêuticas UNESP University at Araraquara, Brazil, were used for the assays. Three isolates originated from Brazil (strains 18, 113, 265), two from Venezuela (5778, 8228) and two from Colombia (JP, FO). The isolates were maintained routinely on Fava-Netto's medium (Kashino et al.,1987) and subcultured every five and 10 days in the yeast phase and mycelial phase, respectively.

All the strains in the mycelial phase of growth were used to determine azocollytic activity and one isolate (113) was chosen for further study. The mycelial extracts were obtained in neopeptone medium maintained at 25ºC for periods of seven, 14, 21 and 28 days of growth.

Methodology

The production of extracellular proteinases by the yeast form of the fungus was studied in the following induction media: yeast carbon base (Difco Laboratories, Detroit, USA) + collagen (Sigma Aldrich, St Louis, Mo) + vitamin solution; yeast carbon base + BSA (Sigma Aldrich) + vitamin solution; 1% neopeptone (w:v); 1% neopeptone + 0.5% glucose; 1% neopeptone + 2% glucose, and BHI + elastin (Apodaca & Mckerrow, 1990). All culture media were sterilized by autoclaving and then supplemented with glucose (Sigma Aldrich, St Louis, MO) or proteins. All ingredients were sterilized at the same time.

An aliquot of the yeast cell suspension, typically 10.0mL, was added to 200mL of the different liquid media and incubated in a shaker (150 rpm) at 35°C for seven, 14, 21 and 28 days. Following this incubation period, an aliquot of the extract was collected and submitted to centrifugation in a microfuge (FANEN Ltda, Sao Paulo) for 5min. at 4°C and the clear supernatant was stored at -70°C prior to being assayed for azocollytic, caseinolytic and elastinolytic activities.

Azocoll degradation

Proteolytic activity was measured in a suspension of azocoll (Apodaca & Mckerrow, 1990) at various pH values in sodium phosphate buffer (100 mM, pH 4-6.5) and in Tris-HCl buffer (100 mM pH 6.5 to 9.0) with 1 mM $CaCl_2$ added.

The different extracts, typically 50 - 100 µL, were incubated with 4mg of azocoll (Sigma) in a 1.6mL Eppendorf tube for 4-24h at 37°C. The final reaction volume was 1mL. Following incubation, the samples were centrifuged for 5min in a microfuge. Degradation of azocoll was measured by determining the A_{520} of the supernatant in a Micronal spectrophotometer. One unit of azocollytic activity degrades 0.1 A_{520} unit of azocoll h^{-1}.

Assay for casein hydrolysis

3.5mL of 1% casein solution in 0.1 M phosphate buffer, pH 6.0, was incubated with 1.5mL of enzyme solution for various periods at 37ºC. After specified intervals, 1.0mL was removed from the tubes and 1.0mL of 10% trichloroacetic acid (TCA 10%) was added. The tubes were stored in the refrigerator and the material was filtered. The supernatant protein content was then analyzed by Lowry's method (Lowry et al., 1951). As controls we used: 1. only buffer (To) and 2. casein plus 10% TCA and the enzyme source in buffer minus the substrate (T1). One unit of proteinase is the amount of enzyme that releases 1 µg tyrosine/min/mL at 700 nm.

Elastase assay

Elastase was assayed with elastin - Congo red (Sigma) as substrate, at 10mg/mL in 50 mM Tris-HCl, pH 6-10 (Kothary et al., 1984). A standard curve was prepared by reacting various amounts of the substrate with porcine pancreatic elastase (Sigma) until complete hydrolysis and plotting the increase in absorbance against milligrams of elastin-Congo red. This curve was used to estimate amounts of Congo-red released by *P. brasiliensis* elastase. One unit of elastase is the amount of enzyme releasing soluble dye equivalent to 1 mg of elastin - Congo red in 3h at 37ºC.

Identification of proteolytic activity

General proteinase activity was also determined in sodium dodecyl sulfate (SDS) - polyacrylamide gels copolymerized with a substrate in which proteinases can be detected and molecular weights can be determined simultaneously (Vaz et al., 1994). The resolving gel, 10% (w:v) polyacrylamide, was polymerized in the presence of 0.1% (w:v) pig skin, type I gelatin (Sigma) or casein (Sigma); the stacking gel (5%, w:v, polyacrylamide) did not contain substrate.

Approximately 150 µg of each sample was solubilized separately in 40 µL of the sample buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 0.025% bromophenol blue, and 20% glycerol. No heating or reducing agents were used. After the electrophoretic run, the casein gel was left in distilled water for 10 minutes, and then immersed in 0.05 M Tris-HCl buffer, pH 6.0, with 1% Triton X-100. Preliminary experiments were performed to select the optimum pH. The gel was left in 0.05 M Tris-HCl buffer for one additional hour at pH 6.0 and then at 37°C for 18 hours. After the protein renaturation steps, the gel was stained with 0.1% (w/v) Coomassie brilliant blue R-250 (Bio-Rad) in water: methanol: acetic acid (5:5:1, v:v) for 30 minutes and then destained in 45% (v/v) methanol and 3% (v:v) acetic acid until the proteolytic bands could be visualized.

The gelatin gel was washed in 2.5% Triton X-100 (Sigma) for 10 min. The gels were then incubated with 50 mM NaCl and 25 mM Tris HCl, pH 7.5, for 5min and Hanks

solution, pH 7.4, was finally added and left overnight at 37ºC. The gel was then stained and destained as described above. Molecular weight standards (BioRad) were used.

Protein concentration

Protein concentration was measured by the method of Lowry et al. (1951).

Electrophoresis

All extracts were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (1970) using a separation gel of 10% polyacrylamide. The gels were stained with silver nitrate staining solution.

Inhibitor studies

The following proteinase inhibitors were tested for activity against the Pb culture collagen extract: 1 mM pepstatin, 1,10-phenanthroline, 1 mM E-64, 5 mM E-64, 1.0 mM phenylmethylsulfonyl fluoride (PMSF), and 1.0 mM ethylenediamine tetraacetic acid (EDTA). In the assay, 60 µg of Pb collagen extract was dissolved in enzyme buffer and preincubated with the specific inhibitor at room temperature for one to two hours. A longer incubation time was necessary for the PMSF inhibitor, to ensure a complete reaction. At the end of this period, sample buffer was added to each tube and aliquots were applied to the electrophoresis gel with substrate (gelatin and/or casein). Inhibitor-free samples and samples with the solvents used for inhibitor solubilization were applied simultaneously.

RESULTS

Mycelial culture filtrates obtained from all of the isolates produced extracellular azocollytic activity, although the amount of activity produced varied (Table 1). One isolate (Pb 113) was chosen for further study. Azocollytic activity peaked during the third week of mycelial culture and was highest at pH 6.0 (4.2 U/mL). Low elastinolytic activity and high caseinolytic activity (3.9 U/m'/mL) were observed in mycelial extract.

The mycelial culture filtrate showed a broad zone of high-molecular weight activity on gelatin substrate gel. This was demonstrable from the 7th day of fungal incubation and more intense proteolysis was observed after 14 and 21 days of culture (Figure 1).

Cultures of yeast cells of *P. brasiliensis* were grown in neopeptone broth (1% [w:v]), supplemented or not with 0.5 or 2% glucose yeast carbon base, and in collagen, BHI-elastin and BSA medium. Table 2 summarizes the proteinase activities expressed by *P.brasiliensis* in the above–described media, while analyses of these extracts by SDS-PAGE are presented in Table 3. The electrophoretic patterns of the proteins differed in number and intensity of bands, which ranged from 200 to 10 kDa, irrespective of the medium. All extracts showed common major bands at 72 and 43 kDa, except the one obtained in neopeptone medium without glucose, which contained the 43kDa fraction weakly. Comparison of neopeptone medium with and without 0.5 and 2% of glucose showed that glucose did not interfere with the main components released into the culture filtrate, except for the expression of the 72 kDa fraction.

The effect of glucose, protein source and time of culture on the expression of proteolytic activity was assessed. No azocollytic or elastinolytic activity was detected in neopeptone medium with or without glucose or in BSA extracts. There was no clear-cut effect of glucose on proteinase production by these cultures of *P. brasiliensis*.

On the other hand, the source of protein influenced the appearance of various fractions. Cultures of the *P. brasiliensis* yeast phase, grown in collagen medium, showed azocollytic and caseinolytic activity, respectively peaking during the third and fourth week of culture (Table 4). No elastinolytic activity was observed in this extract. The pH activity curves showed that the azocollytic and caseinolytic activities have sharp optima at pH 4.0 and pH 8.0, respectively (Table 5).

When the yeast extract from *P. brasiliensis* cultured in collagen medium was analyzed on gelatin substrate gels several proteinase species were detected. The 7-day extract showed bands of apparent molecular mass ranging from 220 to 20 kDa. The 14, 21 and 28 day extracts showed more intense proteolysis with a very broad band of digestion of high molecular mass (220 to 130 kDa) and additional activities at Mr 116, 90, 70, 30, 25 and 20 kDa (Figure 2). The amount of these proteinases appeared to increase with time.

The casein gel showed a smaller number of proteinase species compared with the gelatin gel (Figure 3). Five proteolytic activities were observed at Mr 220, 116, 90, 52 and 31 kDa. The 7th day extract showed two areas of proteolysis, a high molecular mass and 52 kDa. The other bands were more visible in the 14, 21 and 28-day extracts. Four species were apparently influenced by time: a broad zone of high molecular mass activity, 116, 90 and 31 kDa.

Further characterization of the collagen medium was carried out with specific inhibitors. Most of the fractions were inhibited by PMSF (Figure 4) and 78% of the azocollytic activity was inhibited by PMSF, 45.6% by EDTA and 6.2% by phenanthroline.

The expression of elastase activity depended on medium composition and on time in culture (data not shown). The yeast elastin extract showed high elastinolytic activity at pH 8.0 and azocollytic activity at pH 6.0 and caseinolytic activity was similar at pH 6.0 and 8.0. The mycelial extract showed high elastinolytic and caseinolytic activity at pH 8.0 and no azocollytic activity (Table 6).

When yeast and mycelial extracts cultured in the elastin media were analyzed on gelatin substrate gels, a broad zone of clearing from approximately 100 kDa to the top of the gel was detected. No individual species could be distinguished (data not shown).

Table 1 - Expression of azocollytic activity by mycelial cultures of *P. brasiliensis* grown in neopeptone medium.

Isolates	Enzymatic activity U/mL
18 BR*	1.4
113BR	1.4
265BR	0.6
JP/CO*	1.0
FO/CO	1.6
5778/Ve*	0.6
8228/Ve	0.9

*Isolates from Brazil (Br), Colombia (Co) and Venezuela (Ve), respectively.

Table 2 - Summary of the effects of culture medium on the expression of proteolytic activity.

	Substrate		
Culture medium	Azocoll	Elastin	Casein
Yeast form			
1% Neo*	-	-	-
1% Neo + 0,5 glu**	-	-	-
1% Neo + 2% glu	-	-	-
Collagen	+	-	+
Elastin	++	+++	+
BSA	-	-	-
Mycelial form			
Neo	+	+/-	+
Elastin	-	+++	+++

* neopeptone ** glu = glucose

Table 3 - Analysis of extracts of the various *P. brasiliensis* yeast –form cultures by SDS-PAGE.

	Number of bands 7 to 28 days	Range (kDa)	Main bands
Neopeptone 1%	14 - 17	12 - 140	52, 72
Neo + 0.5% glu*	20 - 26	15 - 140	72, 60, 50, 43, 35, 28
Neo + 2 % glu	21 - 24	14 - 140	72, 60, 43, 35
BSA medium	8 - 10	16 - 140	74, 72, 62, 52, 43, 14
Collagen medium	11 - 20	20 - 200	72, 43, 38, 26, 20
Elastin medium	7 - 12	10 - 100	100, 72, 43, 30, 12

* neopeptone + glucose.

Table 4 - Expression of proteolytic activity by cultures of *P. brasiliensis* strain 113 grown in collagen medium (yeast form) after various periods of growth.

Days	Azocollytic activity U/mL	Caseinolytic activity U/ m'/mL
7	1.0	6.7
14	0.9	6.4
21	1.4	7.0
28	1.2	8.0

Table 5 - Expression of proteolytic activity by cultures of *P. brasiliensis* strain 113 (yeast form) after 21 days of growth in collagen medium at various pH.

pH	Azocollytic activity U/mL	Caseinolytic activity U/m'/mL
4	2.0	11.2
6	1.4	14.2
8	1.5	14.5

Table 6 - Expression of proteolytic activity by cultures of *P. brasiliensis* grown in BHI - elastin medium (yeast and mycelial form) at three different pH values.

	Yeast extract substrate			Mycelial extract substrate		
	Elastin	Casein	Azocoll	Elastin	Casein	Azocoll
pH	U/mL	U/m'/mL	U/mL	U/mL	U/m'/mL	U/mL
4.0	1.5	2.5	4.3	3.4	3.8	-
6.0	1.8	3.0	5.3	3.8	4.7	-
8.0	12.6	3.0	0.9	10.0	12.0	-

- no substrate degradation

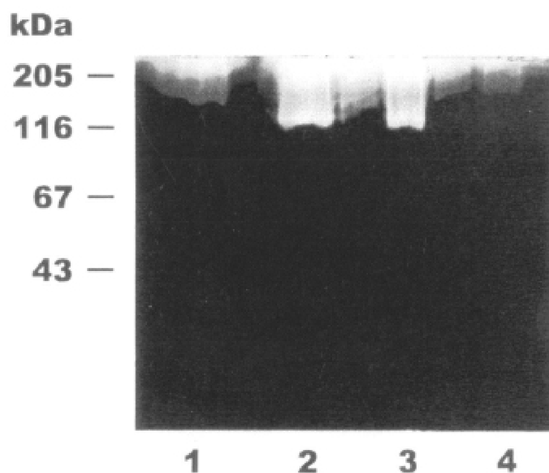

Figure 1. Gelatin substrate gel analysis of *P. brasiliensis* 113 mycelial extract obtained in neopeptone medium after culture incubation periods of: 7 (1); 14 (2); 21 (3); 28 (4) days.

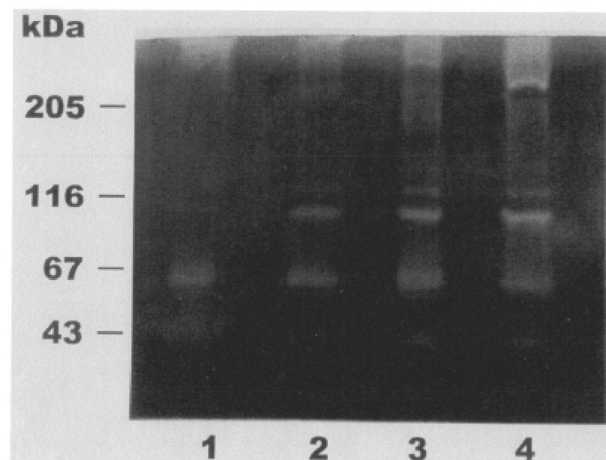

Figure 3. Casein substrate gel analysis of *P. brasiliensis* yeast extract obtained in collagen medium after culture incubation periods of: 7 (1); 14 (2); 21 (3); 28 (4) days cultivation.

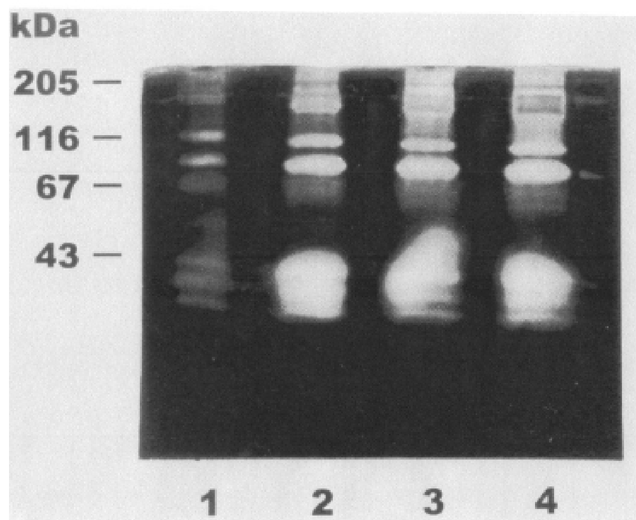

Figure 2. Gelatin substrate gel analysis of *P. brasiliensis* yeast extract obtained in collagen medium after culture incubation periods of: 7 (1); 14 (2); 21 (3); 28 (4) days.

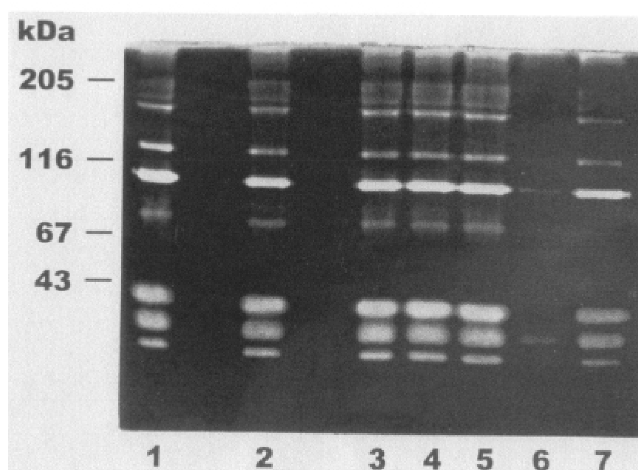

Figure 4. Analysis of enzymatic activity of *P. brasiliensis* yeast extract obtained in collagen medium, with and without an inhibitor, by gelatin substrate electrophoresis. (1) 20 day extract without inhibitor; (2) 20 day extract with 1,10 phenanthroline; (3) with 1mM E-64; (4) with 5 mM E-64; (5) with 1 mM pepstatin; (6) with PMSF; (7) with EDTA.

DISCUSSION

Pathogenic fungi utilize different mechanisms to establish themselves in a host and to cause disease. Some factors such as tolerance to temperature, mechanisms of adhesion and evasion of host defenses, dimorphism and biochemical properties have been pointed out as potential virulence factors among fungi. Proteinase production has been indicated as one of the factors possibly related to the pathogenesis and later dissemination of the infecting fungi in the host (Cassone et al., 1987; Resnick et al., 1987; Ray & Payne, 1988; Puccia et al., 1998; Naglik et al., 2004; Taylor et al., 2005; Jackson et al., 2007; Lian & Liu, 2007).

In the present study we observed that all seven isolates of the *P. brasiliensis* mycelial form tested exhibited protease activity in neopeptone medium. Brazilian and Colombian isolates produced more protease activity, with one exception. The 265 extract showed a low activity compared to the 18 and 113 isolates. This isolate was considered to be an avirulent strain for mice and exhibited diminished adhesion to Vero and HeLa cells (Hanna et al., 2000). On the other hand, extract of the yeast form in neopeptone medium did not produce detectable proteolytic activity. In the mycelial form of *P. brasiliensis*, these enzymes could be important for fungal survival in the environment, metabolic processes and host invasion, as shown for other fungi such as *Trichophyton* and *Candida* species (Apodaca & Mckerrow, 1990).

The utilization of exogenous proteins by fungi requires the enzymatic degradation of proteins to peptides and amino acids before uptake by the organism. The enzymes involved in this action are mainly extracellular proteinases. Regarding the role of exogenous proteins as inducers of protease, at least two mechanisms appear to be operative in fungi. Either protease repression or induction occurs in certain fungi, depending on the substrate. In *T. rubrum*, exogenous proteins serve as inducers of protease (Apodaca & Mckerrow, 1990). An interesting result of the present study was the expression of proteinases by the mycelial form. This does not depend on the culture medium, suggesting that this is a proteinase with a broad spectrum of activity, whereas the proteinases of the yeast-like form were obtained only by means of inducing substrates.

The present report draws attention to the presence of exocellular proteases in *P. brasiliensis*. Our results show that the yeast form of *P. brasiliensis*, like other pathogenic fungi, is capable of releasing elastinolytic and collagenolytic enzymes when grown on inducing medium. It was found that azocollytic and elastinolytic activities were only expressed in the collagen type I and elastin salt media. No azocollytic or elastinolytic activity was expressed in any of the neopeptone media tested, except in the mycelial phase of this fungus.

By comparing the extracts in which collagen and elastin media induced the expression of substances with enzymatic activity, we may conclude that an enzyme with acid azocollytic activity was expressed in both media but that an alkaline elastase was obtained only in the medium with elastin, since only caseinolytic activity, but no elastinolytic ativity, was detected in the medium with collagen.

Even though a variety of proteases have been produced by *P. brasiliensis*, our results indicate that both forms of this fungus produce an extracellular elastinolytic protease when grown on elastin. This result was not observed by Bedoya-Escobar et al. (1993). In their work only the mycelial form expressed elastinolytic and collagenolytic enzymes. None of the isolates in the yeast form exhibited elastinolytic activity.

The expression of azocollytic activity was maximal in the third week of culture in collagen medium. The gelatin substrate gel patterns were similar, regardless of time, suggesting that variations in the levels of total proteinase expression, or variations in the amount of actual enzyme present in the total secreted protein, or the presence of proteinase inhibitor accounted for the changes in expression of azocollytic activity from week to week. Preliminary analysis with inhibitors showed that the large number of the fractions present in gel with gelatin was inhibited by PMSF. This result suggests that these are serine proteinases.

At present we do not know if azocollytic or elastinolytic activity is due to a specific fungal collagenase or elastase or if it results from a protease with broad specificity. The expression of different numbers of proteinase(s) over time and the degradation of collagen type I and elastin show that these probably are true collagenase and elastase activities. However, their identification will require further experimentation.

The proteinases of this fungus may facilitate the invasion of host tissue in cases of disseminated disease. These proteinases appear to be multifunctional and may play several roles in the host-parasite relationship. There is an impressive body of data linking the production of proteinases in bacteria and fungi with their virulence (Tsuboi et al., 1987; Apodaca & Mckerrow, 1990; Kolattukudy et al., 1993; Lee & Kolattukudy, 1995; Harrington, 1996; De Bernardis et al., 2001; Naglik et al., 2004; Taylor et al., 2005; Jackson et al., 2007; Kaufman et al., 2007; Lian & Liu, 2007; Silva et al., 2008). However, this subject has been little explored, except for the serine proteinase described by the Puccia group. Soluble components of *P. brasiliensis* are detected in culture filtrates, among them the 43 kDa glycoprotein, whose role as a diagnostic tool and as a serological marker to determine the efficacy of treatment has been demonstrated (Puccia & Travassos, 1991; Mendes-Giannini et al., 1994) and which is and which are also involved in cell immune reactivity (Benard et al., 1997) and possesses proteolytic activity (Mendes-Giannini et al., 1990). This protein can also be released into the circulation during the course of the pathological process, possibly contributing to the worsening of the disease (Mendes-Giannini et al., 1989). An exocellular proteinase activity has been characterized in *P. brasiliensis* culture filtrates (Carmona et al., 1995), and this exocellular serine-thiol proteinase was able to cleave, *in vitro*, proteins

related to the basal membrane, such as human laminin and fibronectin, type IV collagen and proteoglycans, and could be associated with the fungal tissue invasion (Puccia et al., 1998). However, this proteinase apparently was not able to hydrolyse trypsin-like, elastin-like and chymotrypsin-like chromogenic peptides.

Since the major route of entry of *P. brasiliensis* is by inbalation, the lungs may be the first targets of these extracellular enzymes. Emphasis has been placed on the study of extracellular proteinases since the pulmonary barriers are proteinaceous. Since elastin makes up 30% of pulmonary tissue, elastinolytic enzymes may play a significant role in fungal invasion. Recently, Gonzalez et al. (2008) described the presence of the elastic fiber system and an elastolytic process in lungs of BALB/c mice infected with *P. brasiliensis* conidia. Those results also suggested the presence of elastase activity *in vivo* and reinforced the need to isolate the component responsible for elastinolytic activity described here.

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RESUMO

Indução e secreção de atividade elastinolítica e proteolítica em culturas de Paracoccidioides brasiliensis

Paracoccidioides brasiliensis* pode parasitar vários tecidos humanos e proteinases produzidas por este microrganismo podem permitir que este fungo as utilize para invadir os tecidos do hospedeiro. O efeito de meio de cultura sobre a produção de proteinases por isolados de *P. brasiliensis* foi estudado. O fungo foi cultivado em meio de neopeptona, albumina bovina, elastina ou colágeno. Os extratos foram avaliados quanto atividade azocolítica, elastinolítica e caseinolítica. Atividade proteolítica também foi analisada por eletroforese em géis usando como substrato gelatina e caseína. *P. brasiliensis* expressou níveis elevados de atividade azocolítica, elastinolítica e caseínolítica em todos os meios ensaiados, exceto em meio de neopeptona. Geralmente, a expressão da atividade azocolítica foi maior na terceira semana de cultura e a caseinolítica durante a quarta semana. Atividade azocolítica foi mais elevada em pH 4,0 e caseinolítica em pH 8,0. Atividade elastinolítica também foi mais elevada em pH 8,0. A observação desta atividade, realizada pela primeira vez na literatura, bem como as outras, pode estar relacionada a captação de nutrientes pelo fungo e pode também ser responsável pela invasão de tecidos, como as fibras elásticas do tecido pulmonar, por *P. brasiliensis.

Palavras-chave: atividade proteolítica; atividade colagenolítica; atividade elastinolítica; *Paracoccidioides brasiliensis*; paracoccidioidomicose.

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