Short Communication

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Biosynthesis of chondroitinase and hyaluronidase by different strains of *Paracoccidioides brasiliensis*

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The biosynthesis of chondroitinase and hyaluronidase by different isolates of *Paracoccidioides brasiliensis* was investigated in 20 strains isolated from patients (17 strains), a penguin (*Pygoscelis adeliae*, one strain), an armadillo (*Dasypus novemcinctus*, one strain) and the environment (dog food, one strain). All the *P. brasiliensis* isolates studied had the ability to produce chondroitinase and hyaluronidase, although differences in colony morphology and enzyme production were detected among them. These results suggest that further investigations should be carried out in the clinical field in order to clarify the potential role of *P. brasiliensis* enzyme production in the pathogenesis of paracoccidioidomycosis.

Introduction

Paracoccidioides brasiliensis is one of the most prevalent invasive fungal pathogens in Latin America. The chronic form of paracoccidioidomycosis afflicts the lungs and can disseminate to other organs and tissues, forming secondary lesions in the mucous membranes, skin, lymph nodes and adrenal glands. The acute form is characterized by a significant rate of mortality, due primarily to reticuloendothelial system organ hypertrophy (Borges-Walmsley *et al.*, 2002).

Molecular aspects of *P. brasiliensis* dimorphism, diagnosis, epidemiology, taxonomy, genetics and immunology have been the focus of intensive research (San-Blas & Niño-Vega, 2001; San-Blas *et al.*, 2002). It produces various enzymes, such as proteases (Mendes-Giannini *et al.*, 1990; Puccia & Travassos, 1991; Vaz *et al.*, 1994; Carmona *et al.*, 1995; de Assis *et al.*, 1999), elastase and collagenase (Bedoya-Escobar *et al.*, 1993), phospholipases (Heise & Cardoso de Almeida, 1994; San-Blas & Niño-Vega, 2001) and urease (Nogueira, 1959). Several micro-organisms produce hydrolytic enzymes and structures that help in their pathogenicity (Schaechter *et al.*, 1993). Chondroitinase and hyaluronidase are believed to play a role in the pathogenicity of fungi, particularly species of the genus *Candida* (Shimizu *et al.*, 1995). These enzymes are also considered as important pathogenic factors of bacteria that cause oral infections (Hershon, 1971; Takeuchi *et al.*, 1982; Tam *et al.*, 1982).

In this study, we investigated whether *P. brasiliensis* was able to secrete chondroitinase and hyaluronidase, using chondroitin sulphate type A and sodium hyaluronate as substrates.

Methods

Twenty different isolates of *P. brasiliensis* were studied, of which 17 were recovered from patients, one from the environment (dog food probably contaminated with soil in Uberlândia, Brazil) (Ferreira *et al.*, 1990), one from an Adelie penguin (*Pygoscelis adeliae*) (Garcia *et al.*, 1993) and one from a nine-banded armadillo (*Dasypus novemcinctus*) (Vidal *et al.*, 1995). Sixteen of these isolates were provided by the Fungal Culture Collection of the Institute of Tropical Medicine of São Paulo, São Paulo, Brazil. Cultures were grown on agar slants of bacto-peptone/glucose, pH 5.5, at 27 °C for 30 days and then transferred onto Petri dishes with basic medium to evaluate enzyme production. A strain of *Candida albicans* from our stock (ICB-12A) was used as a positive control for chondroitinase and hyaluronidase production.

Secretion of chondroitinase and hyaluronidase (Smith & Willett, 1968; Shimizu *et al.*, 1995) by *P. brasiliensis* isolates was tested by cultivation on medium consisting of 15 g Noble agar (Difco), 10 g neopeptone (Difco) and 40 g glucose (Merck) dissolved in water to 1000 ml final volume. The medium was autoclaved at 121 °C for 15 min and cooled to 46 °C. Aqueous solutions of 5 % bovine albumin fraction V (Sigma) and either 4 mg bovine trachea chondroitin sulphate type A ml⁻¹ (Sigma) for the chondroitinase assay or 2 mg human umbilical cord sodium hyaluronate ml⁻¹ (Sigma) for the hyaluronidase assay were added to the medium. All the solutions were previously sterilized by Millipore filtration (0.20 µm pore size). The substrates were added to cooled media to give a final concentration of 400 µg ml⁻¹; bovine albumin fraction V was added to a final concentration of 1 %. Final culture pH was 6.8 ± 0.1. Chondroitin agar was poured onto Petri dishes and left at room temperature. Plates were inverted at 4 °C in a moist atmosphere for up to 1 month before use. The test medium was initially translucent.

Photographs showing halo formation by *Paracoccidioides brasiliensis* isolates on chondroitin and hyaluronic acid agar plates are available as supplementary material in JMM Online.

Plates were inoculated with *P. brasiliensis* and *C. albicans* and subsequently incubated at 27 °C for 1 month. All the isolates were placed on test media in duplicate. Petri dishes were observed daily and checked for the presence of a transparent ring around the colonies. The formation of a halo (zone of precipitation) around the colony was considered to indicate enzyme production. Enzyme activities for hyaluronidase (Hz) and chondroitin sulphatase (Cz) were determined as the ratio of the colony diameter to the total diameter of the colony and zone of precipitation, as described by Price *et al.* (1982) for the production of phospholipase by *C. albicans*. Therefore, Hz or Cz = 1.00 would be an enzyme activity-negative strain (index = 1); Hz or Cz < 1.00 and ≥ 0.64 would be a positive strain (index = 2) and Hz or Cz < 0.64 would be a strongly positive strain (index = 3) for the production of the enzyme. Total degraded chondroitin or hyaluronidase was visualized by flooding the plates with 2 M acetic acid for 10 min. Photographs showing halo formation are available as supplementary material in JMM Online.

Results and Discussion

Hyaluronidase and chondroitin sulphatase are considered virulence factors for either dissemination or tissue penetration, favouring the dispersion of infecting micro-organisms. These virulence factors catalyse depolymerization of hyaluronic acid and chondroitin sulphate, basic constituents of tissue (Schaechter *et al.*, 1993).

Twenty isolates of *P. brasiliensis* were grown on chondroitin agar and hyaluronic acid agar at 27 °C for 30 days. Colonies of *P. brasiliensis* isolates 1–8 and 10–20 showed an ivory-coloured, smooth morphology and exhibited wrinkled surfaces, with no aerial mycelia. Some isolates of *P. brasiliensis* 1, 3–8, 10 and 20, however, grew as cotton colonies with unwrinkled, flat, woolly, white surfaces and aerial mycelia.

All the *P. brasiliensis* isolates revealed chondroitinase and hyaluronidase activity when grown on chondroitin agar or hyaluronic acid agar as appropriate. The chondroitinase activity index of *P. brasiliensis* isolates 1–6 and 9–19 was 2 and that of isolates 7, 8 and 20 was 3 (Table 1). The hyaluronidase activity index of isolates 1–6, 8 and 11–20 was 2, while that of isolates 7, 9 and 10 was 3 (Table 1).

Production of these enzymes is a major virulence factor for some bacteria, for instance *Streptococcus* species. Some *Streptococcus* strains are highly invasive, mainly due to hyaluronidase production (Schaechter *et al.*, 1993). It was verified that, amongst oral bacteria, production of hyaluronidase and chondroitin sulphatase is associated with perio-

Table 1. Morphology and enzyme activities of *P. brasiliensis* strains

Cz and Hz values of enzyme activity were determined according to Price *et al.* (1982). Cz or Hz = 1 indicates absence of enzyme activity (index = 1); Cz or Hz ≥ 0.64 and < 1.0 indicates positive enzyme activity (index = 2); Cz or Hz < 0.64 indicates strongly positive enzyme activity (index = 3).

Strain	Colonies		Chondroitinase		Hyaluronidase	
	Morphology*	Colour†	Index	Cz	Index	Hz
1	C/G/M	W/I	2	0.78 ± 0.05	2	0.78 ± 0.05
2	G/M	I	2	0.71 ± 0.05	2	0.68 ± 0.02
3	C/G/M	W/I	2	0.80 ± 0.04	2	0.65 ± 0.03
4	C/G/M	W/I	2	0.92 ± 0.03	2	0.73 ± 0.02
5	C/G/M	W/I	2	0.87 ± 0.04	2	0.76 ± 0.05
6	C/G/M	W/I	2	0.88 ± 0.05	2	0.87 ± 0.04
7	C/G/M	W/I	3	0.41 ± 0.01	3	0.41 ± 0.01
8	C/G/c	W/I	3	0.48 ± 0.02	2	0.73 ± 0.02
9	C	W	2	0.67 ± 0.05	3	0.38 ± 0.02
10	C/G/c	I	2	0.66 ± 0.02	3	0.50 ± 0.02
11	G/M	I	2	0.66 ± 0.03	2	0.87 ± 0.05
12	G/M	I	2	0.68 ± 0.02	2	0.65 ± 0.04
13	G/M	I	2	0.72 ± 0.04	2	0.83 ± 0.04
14	G/M	I	2	0.79 ± 0.04	2	0.71 ± 0.05
15	G/M	I	2	0.82 ± 0.03	2	0.67 ± 0.03
16	G/M	I	2	0.87 ± 0.04	2	0.82 ± 0.03
17	G/M	I	2	0.83 ± 0.02	2	0.66 ± 0.04
18	G/W	I	2	0.67 ± 0.02	2	0.69 ± 0.03
19	G/c	I	2	0.85 ± 0.05	2	0.85 ± 0.04
20	C/G/M	W/I	3	0.29 ± 0.02	2	0.86 ± 0.05
<i>C. albicans</i> (control)	–	–	3	0.43 ± 0.01	3	0.49 ± 0.02

*C, Cotton; c, coriaceous; G, glabrous; M, membranous; W, wrinkled.

†I, Ivory; W, white.

dontal disease (Tam *et al.*, 1982). Shimizu *et al.* (1995) suggested that the production of such enzymes is related to the pathogenicity of some *Candida* species.

In conclusion, the 20 isolates studied presented chondroitinase and hyaluronidase production, although differences among them were observed with respect to colony morphology and enzyme activity. We believe that the production of these enzymes might be associated with the pathogenicity of *P. brasiliensis* strains, mainly in seriously compromised patients. Our results suggest that further investigations should be carried out in the clinical field in order to clarify the potential role of *P. brasiliensis* enzyme production in the pathogenesis of paracoccidioidomycosis.

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