Rev. Inst. Med. trop. S. Paulo
50(5):269-272, September-October, 2008

SECRETION OF FIVE EXTRACELLULAR ENZYMES BY STRAINS OF CHROMOBLASTOMYCOSIS AGENTS

Thais Furtado de SOUZA(2), Maria Lúcia SCROFERNEKER(1,2), Juliana Mônica da COSTA(1), Mariana CARISSIMI(2) & Valeriano Antonio CORBELLINI(3)

SUMMARY

The gelatinase, urease, lipase, phospholipase and DNase activities of 11 chromoblastomycosis agents constituted by strains of *Fonsecaea pedrosoi*, *F. compacta*, *Phialophora verrucosa, Cladosporium carrionii*, *Cladophialophora bantiana* and *Exophiala jeanselmei* were analyzed and compared. All strains presented urease, gelatinase and lipase activity. Phospholipase activity was detected only on five of six strains of *F. pedrosoi.* DNase activity was not detected on the strains studied. Our results indicate that only phospholipase production, induced by egg yolk substrate, was useful for the differentiation of the taxonomically related species studied, based on their enzymatic profile.

KEYWORDS: Chromoblastomycosis; *Fonsecaea pedrosoi*, Lipase; Phospholipase, DNAse; Urease; Gelatinase.

INTRODUCTION

Chromoblastomycosis is a subcutaneous fungal disease caused by dematiaceous fungi being characteristic of tropical and subtropical areas[18,23]. The disease occurs mainly among male agricultural laborers and primarily affects the lower limbs[26,39]. Infection begins with the traumatic implantation of conidia or hyphal fragments into subcutaneous tissues, producing initial lesions consisting of papules or nodules that become verrucous[12]. In Brazil, the most frequent etiological agents are *Fonsecaea pedrosoi, Phialophora verrucosa* and *Cladosporium carrionii*[27,45]. More rarely, *Fonsecaea compacta*, *Exophiala jeanselmei* and *Rhinocladiella aquaspersa* can also be isolated[40].

The secretion of enzymes to the extracellular environment might be an important adaptive mechanism during the life cycle of fungi[29]. The first studies of fungal enzymatic activities aimed at establishing the role of enzymes in fungal pathogenicity, as well as their capacity to induce inflammatory reactions in the host[40]. It is logical to assume that these enzymes could act by making tissue invasion easier, but they could also participate in infection by eliminating some mechanisms of the immune defense and/or helping in the obtaining of nutrients, thus causing injuries to the human host[2,3,10,38,43].

The enzymatic characterization of clinical isolates of chromoblastomycosis agents is important due to the fact that few studies have focused on this subject[14,17,44]. In the present study, we investigated the activity of five hydrolytic enzymes of 11 strains of chromoblastomycosis agents. The aim was to characterize the enzymatic profile of different species of chromoblastomycosis agents.

MATERIALS AND METHODS

Storage and cultivation of strains: The strains of chromoblastomycosis agents *Fonsecaea pedrosoi* ATCC 46428, ATCC 46422, IMTSP 49, IMTSP 674, IQE 444.62 (19)*, MA; *F. compacta* IMTSP. 373; *Phialophora verrucosa* FMC.2214 (8); *Cladosporium carrionii* IMTSP. 680; *Cladophialophora bantianum* 2907-78 (13); *Exophiala jeanselmei* CROMO.HC8 (45) are patient isolates and were obtained from the Laboratory of Pathogenic Fungi of the Department of Microbiology of the Institute of Basic Health Sciences of Universidade Federal do Rio Grande do Sul, and stored on Sabouraud agar slants at 25 ºC. For all enzyme assays, the strains were previously cultured for 14 days on Sabouraud agar slants at 25 ºC.

Candida albicans ATCC 10231, *Malassezia furfur* IMTSP. 225, *Nocardia brasiliensis* IMTSP. 739 and *Staphylococcus aureus* ATCC 25923 were utilized as positive controls in the enzyme assays. *C. albicans* and *S. aureus* were previously cultured for 24h and *M. furfur* and *N. brasiliensis* for 14 days. *S. aureus* was cultured on nutrient agar. *C. albicans, M. furfur* and *N. brasiliensis* were cultured on Sabouraud agar.

Enzymatic assays: Chromoblastomycosis agents strains previously cultured on Sabouraud agar were transferred to potato dextrose agar (PDA) slants at 35 ºC for 10 days. Conidial suspension was prepared by means of sterile saline solution (0.85%). Standard suspension was adjusted in sell UV-visible spectrophotometer (Spectrum Instruments Co. China, Shanghai) at 530 nm to 80-82%. After a 1:10 dilution, cell debris concentration yield corresponded to 10x0.4 - 5x10^4 UFC/mL. The suspension was added to Petri dishes containing specific medium to each

(1) Graduate Program in Medical Sciences, Universidade Federal do Rio Grande do Sul, Rio Grande do Sul State, Brazil.
(2) Department of Microbiology of the Institute of Basic Health Sciences of Universidade Federal do Rio Grande do Sul, Rio Grande do Sul State, Brazil.
(3) Department of Physics and Chemistry of Universidade de Santa Cruz do Sul, Rio Grande do Sul State, Brazil.
Correspondence to: Maria Lúcia Scroferneker, Rua Vasco da Gama 176/604, 90420-111 Porto Alegre, RS, Brasil. Fax: +55 51 3308 3155. E-mail: scrofern@ufrgs.br

enzyme tested for previous stimulation. After complete fungal growth on the surface of the agar plate (seven to 14 days), 5-mm culture discs were purchased and applied in triplicate onto other plates containing the specific medium. For the gelatinase assay, strains were grown in tubes containing 5 mL of test medium according to the method reported by KURTZMAN & FELL[22]. The strains were considered negative when the medium remained solid. They were considered positive when the gelatin liquefied. *N. brasiliensis* IMTSP 739 was utilized as positive control.

Urease activity was determined according to the method proposed by CHRISTENSEN[6] and SEELIGER[43]. The strains were grown for seven days at 25 ºC and were considered positive when the medium became pinkish. *N. brasiliensis* IMTSP 739 was utilized as positive control.

The lipase assay was carried out according to the method described by MUHSIN *et al.*[31]. *M. furfur* IMTSP 225 was used as positive control. The lipase medium consisted of peptone 1%, sodium chloride 5%, calcium chloride 0.01% and agar 2%. Tween 80 was autoclaved separately and added to the medium at a final concentration of 1%. After inoculation, the plates were incubated at 25 °C for up to 30 days. Formation of a precipitate around the colonies demonstrated lipase production.

Phospholipase activity was determined according to the method described by PRICE *et al.*[37]. *C. albicans* ATCC 10231 was used as positive control. The medium consisted of peptone 1%, glucose 2%, sodium chloride 5.73%, calcium chloride 0.05% and agar 2%. The medium was autoclaved and then cooled to approximately 50 °C before the addition of sterile egg yolk at a final concentration of 4%. The plates were incubated at 25 °C for up to 30 days. Precipitation around the colonies attested phospholipase production.

DNase activity was determined according to the method proposed by LOPEZ-MARTINEZ *et al.*[24]. *S. aureus* ATCC 25923 was utilized as positive control. For the DNase assay, the isolates were inoculated on DNase plates, which were then incubated at 25 °C for up 30 days. The assay was considered positive when a degradation halo was visualized around the colonies after the addition of 5N HCl.

RESULTS

All strains of chromoblastomycosis agents presented lipase, urease and gelatinase and lack of DNase activity. Table 1 shows the presence/absence of enzymatic activities of the tested strains and controls.

Phospholipase was produced by most of *F. pedrosoi* strains; only strain 19 did not excrete the enzyme. The strains of other chromoblastomycosis agents were not able to degrade the substrate used.

DISCUSSION

The strains from chromoblastomycosis agents were phenotypically characterized by means of the secretory capacity of DNase, urease, lipase, phospholipase and gelatinase. The degradation pattern varied among different substrates.

The DNase assay was not useful in this study for the detection of extracellular activity of this enzyme, differently from the observation made by LOPEZ-MARTINEZ *et al.*[24], when secretion levels could be evaluated by dermatophytes isolated from acute and chronic cases.

Phospholipases hydrolyze phospholipids, molecules common to all cell membranes[1,36,37]. Phospholipase has been used as a virulence marker for some pathogenic fungi such as *Candida albicans*[16,19,20,21,37,46], *Cryptococcus neoformans*[5,8,45], *Aspergillus fumigatus*[2], *Trichophyton rubrum*[13] and *Microsporum canis*[25]. According to their phospholipase activity, the strains analyzed can be placed in two groups. The first one, which is characterized by positive phospholipase activity, includes *F. pedrosoi* MA, IMTSP. 674, ATCC (46422, 46428) and IMTSP 49. The negative results of the other strains (*F. pedrosoi* 19, *F. compacta* 373, *P. verrucosa* 8, *C. carrionii* 680, *C. bantianum* 13 and *E. jeanselmei* 45) could be related to the enzyme-substrate specificity rather than to the absence of this enzyme on the profile of these strains. The genotypic expression of the enzyme was not investigated, but negative results of phospholipase production, described for other pathogenic fungi, were associated with the restriction of enzyme activity to the intracellular environment[11].

Table 1
Enzyme activity profile of chromoblastomycosis agents

Strain	Lipase	Phospholipase	Urease	Gelatinase	DNAse
F. pedrosoi 46428	+	+	+	+	-
F. pedrosoi 46422	+	+	+	+	-
F. pedrosoi 49	+	+	+	+	-
F. pedrosoi 674	+	+	+	+	-
F. pedrosoi MA	+	+	+	+	-
F. pedrosoi 19	+	-	+	+	-
F. compacta 373	+	-	+	+	-
P. verrucosa 8	+	-	+	+	-
C. carrioni 680	+	-	+	+	-
C. bantianum 13	+	-	+	+	-
E. jeanselmei 45	+	-	+	+	-
Control strain *	+	+	+	+	+

*Control strain for the lipase assay: *Malassesia furfur* IMTSP.225; control strain for the phospholipase assay: *Candida albicans* ATCC 10231.

Extracellular proteinases of saprophytic microorganisms are primarily secreted to break down or decompose complex materials into nutrients readily available to the cells or to compete with other environmental bacteria, parasites or fungi[9]. Direct virulence factors for pathogenic microorganisms include hydrolyzing proteins of host cell membranes to facilitate adhesion and tissue invasion, or damaging cells and molecules of the host defense system to avoid or resist antimicrobial attack[30,32,41]. Peptidases are believed to contribute to microbial virulence by destroying host tissues and digesting immunologically important proteins, such as antibodies and complement factors[4,42]. Gelatinase activity was only qualified in this study, and its potential for differentiating chromoblastomycosis agents is controversial, since its use for this purpose has already been described, particularly for the characterization of *Cladosporium carrionii* and *Exophiala jeanselmei*[15], showing no significant differences between known pathogenic and nonpathogenic isolates[14]. PALMEIRA *et al.*[34] found that medium composition could induce the synthesis and secretion of distinct proteolytic enzymes by *F. pedrosoi* conidia.

All analyzed strains presented precipitation haloes for lipolytic activity. Tween 80, utilized as carbon source, accumulated efficiently as biomass and stimulated lipase excretion by the fungus. The use of lipase activity for differentiating fungal genera closely related to mycotic infection is well reported in the literature, especially concerning dermatophytes[33]. According to other studies, an elevated rate of seborrheic dermatitis was related to lipase production on different substrates and under pH effect, for instance on *Mallassezia furfur*[35], or to the virulence of *Candida albicans* strains[43]. Greater lipase activity was also verified frequently on isolates from chronic mycotic infections[24]. Nevertheless, further investigation is necessary to confirm these findings, with inclusion of more strains of the same species as well as of other genera (*Wangiella, Rhinocladiella*).

All strains presented urease activity in this work, showing that urea is a suitable nitrogen source to these strains. These results are in agreement with those observed in dematiaceous fungi[17]. Urease is a metalloenzyme that catalyzes the hydrolysis of urea to ammonia and carbamate. This reaction can result in an increase in pH under physiological conditions[28]. In *Cryptococcus neoformans,* a significant human pathogenic fungus that produces large amounts of urease, this activity is involved in the pathogenesis of cryptococcosis, but its importance may be specific to the species and/or infection site[7].

Our results indicate that for differentiation among the taxonomically related species studied based on their enzymatic profile, only phospholipase production, induced by egg yolk substrate, was useful. Nevertheless, the production of enzymes that degrade substrates found in animal tissues is an indicative of this activity during an infectious process.

RESUMO

Secreção de cinco enzimas extracelulares por amostras de agentes da cromoblastomicose

As atividades gelatinase, urease, lipase, fosfolipase e DNase de 11 agentes da cromoblastomicose constituídos por amostras de *Fonsecaea pedrosoi*, *F. compacta*, *Phialophora verrucosa, Cladosporium carrionii*, *Cladophialophora bantiana* e *Exophiala jeanselmei* foram analisadas e comparadas. Todas as amostras apresentaram atividade urease, gelatinase e lipase. A atividade fosfolipase foi detectada apenas em cinco das seis amostras de *F. pedrosoi*. A atividade DNase não foi detectada nas amostras estudadas. Os resultados indicam que para a diferenciação entre espécies taxonomicamente relacionadas estudadas, baseado no seu perfil enzimático, apenas a produção de fosfolipase, induzida pelo substrato com gema de ovo, foi útil.

ACKNOWLEDGEMENTS

The authors are grateful to Propesq/UFRGS for their financial support.

REFERENCES

1. BARRET-BEE, K.; HAYES, Y.; WILSON, R.G. & RYLEY, J.F. - A comparison of phospholipase activity, cellular adherence and pathogenicity of yeasts. **J. gen. Microbiol., 131:** 1217-1221, 1985.

2. BIRCH, M.; DENNING, D.W. & ROBSON, G.D. - Comparison of extracellular phospholipase activities in clinical and environmental *Aspergillus fumigatus* isolates. **Med. Mycol., 42:** 81-86, 2004.

3. BLANCO, J.L.; HONTECILLAS, R.; BOUZA, E. *et al.* - Correlation between the elastase activity index and invasiveness of clinical isolates of *Aspergillus fumigatus*. **J. clin. Microbiol., 40:** 1811-1813, 2002.

4. CASSONE, A.; DE BERNARDES, F.; MONDELLO, F.; CEDDIA, T. & AGATENSI, L. - Evidence for a correlation between proteinase secretion and vulvovaginal candidosis. **J. infect. Dis., 156:** 777-783, 1987.

5. CHEN, S.C.A.; MÜLLER, M.; ZHOU, J.Z.; WRIGHT, L.C. & SORRELL, T.C. -Phospholipase activity in *Cryptococcus neoformans*: a new virulence factor? **J. infect. Dis., 175:** 414-420, 1997.

6. CHRISTENSEN, W.B. - Urea decomposition as a means of differentiating *Proteus* and paracolon cultures from each other and from *Salmonella* and *Shigella* types. **J. Bact., 52:** 461-466, 1946.

7. COX, G.M.; McDADE, H.C.; CHEN, S.C.A. *et al*. - Extracellular phospholipase activity is a virulence factor for *Cryptococcus neoformans*. **Molec. Microbiol., 39:** 166-175, 2001.

8. COX, G.M.; MUKHERJEE, J.; COLE, G.T.; CASADEVALL, A. & PERFECT, J.R. - Urease as a virulence factor in experimental cryptococcosis. **Infect. Immun., 68:** 443-448, 2000.

9. CUNNINGHAM, E.L. & AGARD, D.A. - Disabling the folding catalyst is the last critical step in alpha-lytic protease folding. **Protein Sci., 13:** 325-331, 2004.

10. DA SILVA, B.C.M.; AULER, M.E.; RUIZ, L.S. *et al. - Trichophyton rubrum* isolated from AIDS and human immunodeficiency virus-infected patients in São Paulo, Brazil: antifungal susceptibility and extracellular enzyme production. **Chemotherapy, 51:** 21-26, 2005.

11. DAS, S.K. & BANERJEE, A.B. - Lipolytic enzymes of *Trichophyton rubrum*. **Sabouraudia, 15:** 313-323, 1977.

12. DE HOOG, G.S.; QUEIROZ-TELLES, F.; HAASE, G. *et al*. - Black fungi: clinical and pathogenic approaches. **Med. Mycol., 38**(suppl. 1): 243-250, 2000.

13. DOS SANTOS, J.I.; VICENTE, E.J.; PAULA, C.R. & GAMBALE, W. - Phenotypic characterization of *Trichophyton rubrum* isolates from two geographic locations in Brazil. **Europ. J. Epidem., 17:** 729-734, 2001.

14. ESPINELL-INGROFF, A.; GOLDSON, P.R.; McGINNIS, M.R. & KERKERING, T.M. - Evaluation of proteolytic activity to differentiate some dematiaceous fungi. **J. clin. Microbiol., 26:** 301-307, 1988.

15. FISCHER, F. & COOK, N.B. - **Micologia: fundamentos e diagnóstico**. Rio de Janeiro, Revinter, 2001.

16. GHANNOUM, M.A. - Potential role of phospholipases in virulence and fungal pathogenesis. **Clin. Microbiol. Rev., 13:** 122-143, 2000.

17. GUGNANI, H.C. & OKEKE, C.N. - Physiological characteristics of environmental isolates of pathogenic dematiaceous fungi. **Mycoses, 32:** 78-83, 1989.

18. HAMZA, S.H.; MERCADO, P.J.; SKELTON, H.G. & SMITH, K.J. - An unusual dematiaceous fungal infection of the skin caused by *Fonsecaea pedrosoi*: a case report and review of the literature. **J. cutan. Path., 30:** 340-343, 2002.

19. IBRAHIM, A.S.; MIRBORD, F.; FILLER, S.G. *et al*. - Evidence implicating phospholipase as a virulence factor of *Candida albicans*. **Infect. Immun., 63:** 1993-1998, 1995.

20. KOTHAVADE, R.J. & PANTHAKI, M.H. - Evaluation of phospholipase activity of *Candida albicans* and its correlation with pathogenicity in mice. **J. med. Microbiol., 47:** 99-102, 1998.

21. KREJER VAN-RIJ, N.J.W. - **The yeasts, a taxonomic study**. Amsterdam, Elsevier, 1984. p. 1081.

22. KURTZMAN, C.P. & FELL, J.W. - **The yeast, a taxonomic study**. Amsterdam, Elsevier, 1998. p. 1055.

23. LACAZ, C.S.; PORTO, E.; MARTINS, J.E.C.; HEINS-VACCARI, E.M. & MELO, N.T. - **Tratado de micologia médica Lacaz**. São Paulo, Sarvier, 2002. p. 441-461.

24. LOPEZ-MARTINEZ, R.; MANZANO-GAYOSSO, P.; MIER, T.; MENDEZ-TOVAR, L.J. & HERNÁNDEZ-HERNÁNDEZ, F. - Exoenzimas de dermatofitos aislados de tiñas agudas y crónicas. **Rev. lat.-amer. Microbiol., 36:** 17-20, 1994.

25. MAIA, M.L.S. - **Caracterização de biótipos de *Microsporum canis* isolados de animais**. São Paulo, 1997. (Dissertação de Mestrado em Microbiologia - Instituto de Ciências Biomédicas da Universidade de São Paulo).

26. McGINNIS, M.R. - Chromoblastomycosis and phaeohyphomycosis: new concepts, diagnosis and mycology. **J. Amer. Acad. Derm., 8:** 1-16, 1983.

27. MINOTTO, R.; BERNARDI, C.D.; MALLMANN, L.F.; EDELWEISS, M.I. & SCROFERNEKER, M.L. - Chromoblastomycosis: a review of 100 cases in the state of Rio Grande do Sul, Brazil. **J. Amer. Acad. Derm., 44:** 585-592, 2001.

28. MOBLEY, H.L.; ISLAND, M.D. & HAUSINGER, R.P. - Molecular biology of microbial ureases. **Microbiol. Rev., 59:** 451-480, 1995.

29. MONOD, M. & BORG-VON-ZEPELIN, M. - Secreted proteinases and other virulence mechanisms of *Candida albicans*. **Chem. Immunol., 81:** 114-128, 2002.

30. MONOD, M.; CAPOCCIA, S.; LECHENNE, B. *et al*. - Secreted proteases from pathogenic fungi. **Int. J. med. Microbiol., 292:** 405-419, 2002.

31. MUHSIN, T.M.; AUBAID, A.H. & AL-DUBOON, A.H. - Extracellular enzyme activities of dermatophytes and yeast isolates on solid media. **Mycoses, 40:** 465-469, 1997.

32. NAGLIK, J.R.; CHALLACOMBE, S.J. & HUBE, B. - *Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis. **Microbiol. Molec. Biol. Rev., 67:** 400-428, 2003.

33. NOBRE, G. & VIEGAS, M.M.P. - Lipolytic activity of dermatophytes. **Mycopathologia (Dan Haag), 46:** 319-323, 1972.

34. PALMEIRA, V.F.; KNEIPP, L.F.; ALVIANO, C.S. & SANTOS, A.L.S. - Secretory aspartyl peptidase activity from mycelia of the human fungal pathogen *Fonsecaea pedrosoi*: effect of HIV aspartyl proteolytic inhibitors. **Res. Microbiol., 157:** 819-826, 2006.

35. PLOTKIN, L.I.; SQUIQUERA, L.; MATHOV, I.; GALIMBERT, R. & LEONI, J. - Characterization of the lipase activity of *Malassezia furfur*. **J. med. vet. Mycol., 34:** 43-48, 1996.

36. PRICE, M.F. & CAWSON, R.A. - Phospholipase activity in *Candida albicans*. **Sabouraudia, 15:** 179-185, 1977.

37. PRICE, M.F.; WILKINSON, I.D. & GENTRY, L.O. - Plate method for detection of phospholipase activity in *Candida albicans*. **Sabouraudia, 20:** 7-14, 1982.

38. REMENTERIA, A.; LÓPEZ-MOLINA, N.; LUDWIG, A. *et al*. - Genes and molecules involved in *Aspergillus fumigatus* virulence. **Rev. iberoamer. Micol., 22:** 1-23, 2005.

39. RICHARD-YEGRES, N.; YEGRES, F. & ZEPPENFELDT, G. - Chromomycosis; endemia rural, laboral y familiar en Venezuela. **Rev. iberoamer. Micol., 9:** 38-41, 1992.

40. RIPPON, J.W. - **Medical mycology: the pathogenic fungi and the pathogenic actinomycetes**. Philadelphia, W.B. Saunders, 1982. p. 277-302.

41. SAJID, M. & McKERROW, J.H. - Cysteine proteases of parasitic organisms. **Molec. Biochem. Parasit., 120:** 1-21, 2002.

42. SANTOS, A.L.S; PALMEIRA, V.F.; ROZENTAL, S. *et al.* - Biology and pathogenesis of *Fonsecaea pedrosoi*, the major etiologic agent of chromoblastomycosis. **FEMS Microbiol. Rev., 31:** 570-591, 2007.

43. SCHALLER, M.; BORELLI, C.; KORTING, H.C. & HUBE, B. - Hydrolytic enzymes as virulence factors of *Candida albicans*. **Mycoses, 48:** 365-377, 2005.

44. SEELIGER, H.P.R. - Use of a urease test for the screening and identification of cryptococci. **J. Bact., 72:** 127-131, 1956.

45. SILVA, J.P.; SOUZA, W. & ROZENTAL, S. - Chromoblastomycosis: a retrospective study of 325 cases on Amazonic region (Brazil). **Mycopathologia, 143:** 171-175, 1999.

46. VIDOTTO, V.; LEONE, R.; SINICCO, A.; ITO-KUWA, S. & CRISEO, G. - Comparison of phospholipase production in *Cryptococcus neoformans* isolates from AIDS patients and bird droppings. **Mycopathologia, 142:** 71-76, 1998.

47. WILLIANSON, M.I.; SAMARANAYAKE, L.P. & MAcFARLANE, T.W. - Phospholipase activity as a criterion for biotyping *Candida albicans*. **J. vet. med. Mycol., 24:** 415-417, 1986.

Received: 1 November 2007
Accepted: 1 September 2008