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Candida guilliermondii isolated from HIV-infected human secretes a 50 kDa serine proteinase that cleaves a broad spectrum of proteinaceous substrates

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Abstract

Non-*albicans Candida* species cause 35–65% of all candidemias in the general population, especially in immunosuppressed individuals. Here, we describe a case of a 19-year-old HIV-infected man with pneumonia due to a yeast-like organism. This clinical yeast isolate was identified as *Candida guilliermondii* through mycological tests. *C. guilliermondii* was cultivated in brain heart infusion medium for 48 h at 37 °C. After sequential centrifugation and concentration steps, the free-cell culture supernatant was obtained and extracellular proteolytic activity was assayed firstly using gelatin-SDS–PAGE. A 50 kDa proteolytic enzyme was detected with activity at physiological pH. This activity was completely blocked by 10 mM phenylmethylsulphonyl fluoride (PMSF), a serine proteinase inhibitor, suggesting that this extracellular proteinase belongs to the serine proteinase class. E-64, a strong cysteine proteinase inhibitor, and pepstatin A, a specific aspartic proteolytic inhibitor, did not interfere with the 50 kDa proteinase. Conversely, a zinc-metalloproteinase inhibitor (1,10-phenanthroline) restrained the proteinase activity released by *C. guilliermondii* by approximately 50%. Proteinases are a well-known class of enzymes that participate in a vast context of yeast–host interactions. In an effort to establish a functional implication for this extracellular serine-type enzyme, we investigated its capacity to hydrolyze some serum proteins and extracellular matrix components. We demonstrated that the 50 kDa exocellular serine proteinase cleaved human serum albumin, non-immune human immunoglobulin G, human fibronectin and human placental laminin, generating low molecular mass polypeptides. Collectively, these results showed for the first time the ability of an extracellular proteolytic enzyme other than aspartic-type proteinases in destroying a broad spectrum of relevant host proteins by a clinical species of non-*albicans Candida*.

Keywords: *Candida guilliermondii*; Extracellular proteolytic activity; Extracellular matrix; HIV infection; Serine proteinase; Serum proteins

1. Introduction

Fungi are ubiquitous organisms that live in soil, water, and on animals and human beings. In human beings they can inhabit a variety of habitats such as the skin and the mucosa and generally live as innocuous commensals. However, when the body defenses are weak these commensals transform into parasitic pathogens. Furthermore, the emergence of new species as the cause of fungal infections, especially the yeast-like organisms, as much in a hospital environment as outside the hospital, as well as the variety of pathological processes that they can cause, some of which are particularly serious in individuals with low immunity levels, currently require special attention [1].

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Candida yeasts are the major pathogens that frequently cause life-threatening systemic infections in immunocompromised hosts, particularly in patients infected by human immunodeficiency virus (HIV), in neutropenic patients treated for cancer or lymphoproliferative disorders, in patients suffering from infectious complications after serious surgery, and in organ transplant recipients [1,2]. They have been associated with increased morbidity and mortality rates and with increased lengths of hospital stay for the affected patients [1,2]. Numerous studies on the prevalence of different *Candida* species have led to general consensus that *C. albicans* is the most commonly isolated species. However, there has been a growing trend of recovery of non-*albicans Candida* species [1–5]. One possible explanation is the increased use of fluconazole and other azoles, which has positively selected for some less sensitive *Candida* species, such as *C. glabrata* and *C. krusei*. Amphotericin B resistance has also been increasingly reported, most notably in isolates of *C. lusitaniae* and *C. guilliermondii* [3,4].

C. guilliermondii is an extremely rare cause of infection in humans. It has been isolated from specimens of human respiratory secretions, bronchial secretions and urine of patients [5], from dentoalveolar abscess [6], from vaginal smear [7], from infectious crystalline keratopathy [8], from a patient with osteomyelitis [9] and, in one report, it was described as a mycoflora on the dermal surfaces with pathogenic potential [10]. Dissemination of this fungus has been reported in immunocompromised or cancer patients, infertile humans, and asymptomatic individuals [11–16]. *C. guilliermondii* originates in the flora of the gastrointestinal tract and candidemic patients are generally infected with an endogenous strain [3,16].

The conversion of the otherwise non-virulent and mildly pathogenic *Candida* microorganism to an invasive one is being connected to several secreted virulence factors [17]. In this sense, *Candida* possesses a multiplicity of virulence factors that could be involved in the enhancement of pathogenicity [17]. There is accumulating evidence of the direct involvement of *Candida* proteinases in different phases of the yeast–host interactions. The physiological role of this class of enzymes during colonization of the host organism by the fungus is thought to be degradation of skin and mucosal barriers that augments the adherence of yeast cells to host tissues, digestion of host proteins in order to supply nutrients, and breaking the immune barrier by attacking lymphocytes and macrophages [17–20]. Additionally, the relationship between the expression of secreted aspartic proteinases (Saps) and invasiveness has been established for *C. albicans*, *C. tropicalis* and *C. parapsilosis* by biochemical, genetic and immunochemical evidences [17–20]. *C. albicans*, *C. tropicalis*, *C. dubliniensis*, *C. parapsilosis*, *C. lusitaniae* and *C. guilliermondii* possess *SAP* gene families [21]. The Saps of *C. albicans*, *C. parapsilosis*, *C. tropicalis* and *C. lusitaniae* have been characterized, and their inhibitors have been tested as potential antimycotic drugs [22]. Information on the extracellular proteolysis of other medically important *Candida* species, such as *C. guilliermondii*, *C. stellatoidea*, *C. glabrata*, *C. krusei* and *C. kefyr*, is extremely limited [23,24].

In contrast with Saps, the correlation between the existence of extracellular serine, cysteine or metalloproteinases and the *Candida* pathogenesis process is unclear [19]. Recently, our research group described the occurrence of four different proteolytic patterns (designated as A, B, C and D) composed of distinct extracellular serine (30–58 kDa) and metalloproteinase (64–95 kDa) activities from 44 oral clinical isolates of *C. albicans* in HIV-infected (29/50) and healthy (15/50) children [25]. In that study, our survey indicated that those oral clinical isolates of *C. albicans* had complex extracellular proteolytic profiles, which illustrates the heterogeneity of this species. However, two of those exocellular proteolytic profiles (B and D) were exclusively detected in *C. albicans* isolated from HIV-infected infants. Moreover, within the group of HIV-seropositive children, we found no statistical differences in *C. albicans* proteinase activity between isolates from patients at various stages of HIV infection. We also showed that the extracellular proteolytic activity expression, composed by serine and metalloproteinases in those *C. albicans* isolates, were independent on both $CD4^+$ T lymphocyte counts and plasma viral load [25]. Furthermore, the biological roles of those extracellular serine and metalloproteinases are completely unknown.

The present study identified a serine proteinase activity released to the extracellular medium by a clinical strain of *C. guilliermondii* isolated from the respiratory tract of a young HIV-infected man. Additionally, in an effort to establish a functional relevance for this enzyme, we investigated its capacity to hydrolyze some serum proteins such as human serum albumin and immunoglobulin G (IgG) as well as proteinaceous constituents of the extracellular matrix including human fibronectin and human laminin.

2. Materials and methods

2.1. Chemicals

Gelatin, human serum albumin, human fibronectin, human placental laminin, non-immune human immunoglobulin G (IgG), and all proteinase inhibitors (*trans*-epoxysuccinyl L-leucylamido-(4-guanidino) butane [E-64], phenylmethylsulphonyl fluoride [PMSF], 1,10-phenanthroline, and pepstatin A) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Reagents used in electrophoresis were purchased from Amersham

Life Science (Little Chalfont, England). All other reagents were analytical grade.

2.2. Case report

A 19-year-old HIV-infected man was admitted in a Hospital from Rio de Janeiro, Brazil, due to pneumonia leading to acute respiratory failure. Initially, the patient was submitted to antibiotic therapy. After two weeks, clinical features demonstrated a failure in the treatment. Bronchial washings performed through the endotracheal tube disclosed few granulocytes without bacteria. The bronchoalveolar lavage specimen (a purulent secretion) was inoculated onto blood agar plate and incubated at 35 °C for 48 h in an atmosphere of 5% CO_2. The culture sample revealed a single yeast-like organism. The yeast strain was maintained on Sabouraud agar plates (Difco, Detroit, USA) medium at 4 °C.

2.3. Identification tests

The yeast isolate was firstly tested on CHROMagar Candida medium (CHROMagar Candida® Company, Paris, France). The plate was incubated for 24–96 h at 37 °C under aerobic conditions. Metabolic properties, such as sugar assimilation and enzymatic reactions, were analyzed by the API–Candida–20C identification kit (API system, bioMérieux, La Balme-less-Grottes, Montalieu Vercieu, France), which was used according to the manufacturer's instructions. In addition, germ-tube test was performed by inoculating 1 ml fresh, pooled, normal human serum with a fresh colony of this yeast and incubating at 37 °C for 3 h. In this set of experiments, *C. albicans* (ATCC 36801) was used as a positive control of yeast to germ-tube transformation. At the end of the incubation, samples were used for microscopic assessment of germ-tube production in a Zeiss microscope.

2.4. Culture conditions and evaluation of cell growth

A *Candida* strain from the Sabouraud agar plate was inoculated into a 250 ml Erlenmeyer flask containing 50 ml of brain heart infusion (BHI) medium, and grown at 37 °C for 48 h in an orbital incubator shaker (200 rpm). Cell growth was estimated by counting the yeast cells in a Neubauer chamber. Cell viability was assessed by exclusion of trypan blue and by measurement of lactate dehydrogenase activity in the culture supernatant as previously described by Costa et al. [25].

2.5. Cell culture supernatant

The culture was centrifuged (4000*g*, 10 min, 4 °C) and the supernatant was filtered in a 0.22-μm membrane (Millipore). The free-cell culture supernatant was concentrated 50-fold in a 10,000 molecular weight cut-off Centricon micropartition system (AMICON, Beverly, MA, USA). Concentrated culture supernatant was treated with an equal volume of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer (125 mM Tris, pH 6.8, 4% SDS, 20% glycerol, 0.002% bromophenol blue). The same volume of BHI medium was also concentrated and used as a control to check for possible proteolytic activity [25].

2.6. Gelatin-SDS–PAGE

Proteolytic activities were assayed and characterized by electrophoresis on 10% SDS–PAGE with 0.1% (w/v) gelatin as substrate incorporated into the gel. The gels were loaded with 10 μl of concentrated supernatant (equivalent to 1.0×10^8 cells) and, following electrophoresis at a constant current of 120 V at 4 °C for 90 min, they were incubated in 10 volumes of 1% (v/v) Triton X-100 in the absence and in the presence of the following proteolytic enzyme inhibitors: 10 mM PMSF (serine proteinase inhibitor), 10 mM 1,10-phenanthroline (metalloproteinase inhibitor), 10 μM pepstatin A (aspartic proteinase inhibitor), and 10 μM E-64 (cysteine proteinase inhibitor) for 1 h at room temperature under constant agitation. The gels were then incubated for 40 h at 37 °C in 10 mM sodium phosphate buffer, pH 7.2, in the presence and in the absence of the same proteolytic inhibitors. The gels were stained overnight with 0.2% (w/v) Coomassie brilliant blue R-250 in methanol–acetic acid–water (50:10:40) and destained in methanol–acetic acid–water (5:10:85), to intensify the digestion halos. Prior to electrophoresis, Gibco BRL (Grand Island, NY, USA) molecular weight standards were boiled in SDS–PAGE sample buffer and then applied in the gels. The gels were dried, scanned, and densitometric analysis was performed with the use of the Kodak Digital Science EDAS 120 software [25].

2.7. Cleavage of soluble proteins

Twenty microliter of the concentrated culture supernatant was mixed with an equal volume of the following proteinaceous substrates: human fibronectin, human placental laminin, non-immune human IgG and human serum albumin. These proteins were diluted in 10 mM sodium phosphate buffer, pH 7.2, to obtain a final concentration of 5 μg/ml. These preparations were incubated for 16 h at 37 °C, in the absence or in the presence of proteinase inhibitors (PMSF, pepstatin A, E-64 and 1,10-phenanthroline). Reactions were terminated by freezing the samples, which were kept at –20 °C until their use in further analysis. The mixture reactions were then added of 10 μl SDS–PAGE sample buffer supplemented with 5% (v/v) β-mercaptoethanol, followed by boiling at 100 °C for 5 min. The degradation protein

profiles were analyzed on 10% SDS–PAGE. Electrophoresis was carried out at 4 °C, at 100 V for 90 min, and the gels were stained with Coomassie brilliant blue R-250 in methanol–acetic acid–water (50:10:40) and destained in the same solvent solution. To control for possible degradation of the proteinaceous substrates independent of candidal proteolytic enzymes, a second aliquot of the concentrated supernatant was heat-inactivated before the substrates were added. In addition, a control for each protein substrate was made by replacing concentrated supernatant with the same volume of PBS [26].

3. Results and discussion

The isolate grown on the primary culture was identified by mycology methods. The clinical specimen yielded small cream-colored yeast colonies during the first 24 h, changed to pink-colored colonies after 96 h at 37 °C on CHROMagar Candida. In this chromogenic medium, it is not possible to distinguish this *Candida* isolated at the species level. The isolate did not produce germ-tubes, indicating that it was a non-*albicans Candida* species. In order to identify this yeast species, this clinical isolate was subjected to substrate assimilation profile analysis with the API–Candida–20C identification system, and the results were summarized in Table 1. The profile corresponded to excellent identification of *C. guilliermondii*.

As previously reported, *C. guilliermondii* can undoubtedly cause invasive infections in human [5–16]. No specific risk factors have been described for *C. guilliermondii*. However, the majority of deaths occurred in cancer patients, who constitute nearly three-quarters of the patients reported [15,16]. To summarize the currently published data, the overall proportion of *C. guilliermondii* has increased worldwide from 0–0.7% in 1950–1990 to 0.7–5.5% in 1991–1998, being isolated mainly from cancer patients and among intensive care unit and surgical patients [27]. Moreover, in contrast with other non-*albicans Candida* species such as *C. tropicalis*, *C. glabrata*, and *C. paraspsilosis* little is known about the virulence and pathogenicity of *C. guilliermondii* [27].

Proteolytic enzymes are ubiquitous in occurrence, being found in all living organisms, and are essential for cellular growth and differentiation [28]. Additionally, there is renewed interest in the study of proteolytic enzymes, mainly due to the recognition that these class of enzymes not only play an important role in the cellular metabolic processes but have also gained considerable attention in the involvement in different phases of the microorganism–host interaction [17–19]. Proteinases are classified into various groups, dependent on whether they are active under acidic, neutral or alkaline conditions and on the characteristics of the active site group of the enzyme. Classes include metallo-, aspartic-, cysteine- and serine-type proteinases. Microorganisms produce a large array of proteinases, which are intracellular and/or extracellular. Intracellular proteinases are significant for various cellular and metabolic processes, such as cell growth, differentiation, death, protein turnover, maturation of enzymes, and maintenance of the cellular protein pool. Extracellular proteinases are important for the hydrolysis of proteins in cell-free environments and enable the cell to absorb and utilize hydrolytic products [28].

We identified the secretory proteolytic profile in this clinical strain of *C. guilliermondii* when grown in a complex medium (BHI) for 48 h (middle-exponential phase) at 37 °C. Initially, using substrate-SDS–PAGE analysis we detected a 50 kDa proteolytic enzyme with gelatinolytic activity at neutral pH (Fig. 1(a)). To better characterize this enzymatic activity, we incubated the concentrated supernatant in the absence and in the presence of proteolytic inhibitors of the four major proteinase classes. The use of 10 mM PMSF revealed that the 50 kDa extracellular proteinase belongs to the serine proteinase class, since this inhibitor promoted a complete inhibition of the proteolytic activity (Fig. 1(a)). Pepstatin A (an aspartyl proteolytic enzyme inhibitor) and E-64 (a potent cysteine proteinase inhibitor) did not alter the behavior of the 50 kDa enzyme (Figs. 1(a) and (b)). Conversely, 1,10-phenanthroline (a strong zinc chelator able to inhibit metalloproteinases or metal-dependent enzymes) inhibited the 50 kDa proteolytic enzyme by approximately 50%, as judged by densitometrical analysis of the digestion halos of proteolysis

Table 1
Some morphological and biochemical characteristics of the *Candida* strain isolated from the respiratory tract of a HIV-infected man

Colony color on CHROMagar Candida		Germ-tube or hyphae differentiation	API–Candida–20C identification kit	
24 h	96 h		+[b]	–[c]
Cream	Pink	Nd[a]	GLU, GAL, SAC, RAF, βMAL, βXYL	TRE, αAMY, βGUR, URE, βNAG, βGAL

[a] Non detected neither germ-tube nor true hyphae structures from the yeast cells.
[b] Positive results for glucose (GLU), galactose (GAL), saccharose (SAC), raffinose (RAF), β-maltosidase (βMAL), and β-xylosidase (βXYL).
[c] Negative results for trehalose (TRE), α-amylase (αAMY), β-glucuronidase (βGUR), urease (URE), *N* -acetil-β-glucosaminidase (βNAG), and β-galactosidase (βGAL).

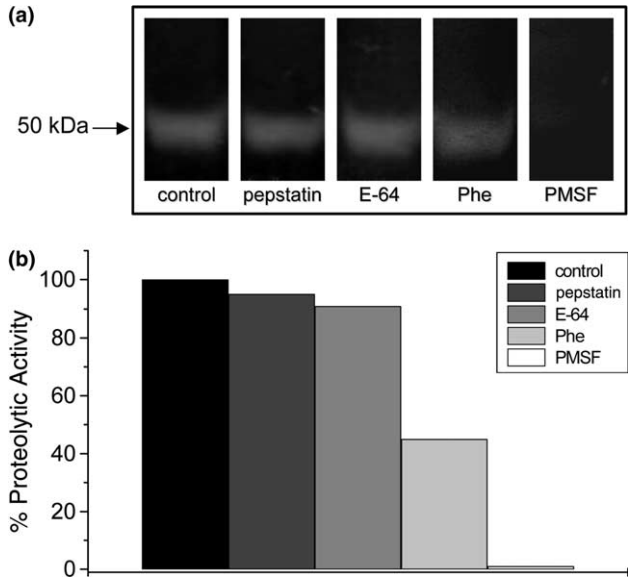

Fig. 1. (a) Gelatin-SDS–PAGE showing the extracellular proteolytic profile released by a clinical strain of *C. guilliermondii* isolated from the respiratory tract of a HIV-infected individual. Gel strips containing 50-fold concentrated BHI supernatant fluid were incubated for 40 h at 37 °C in 10 mM sodium phosphate buffer, pH 7.2, without inhibitor (control) or in the presence of 10 μM pepstatin, 10 μM E-64, 10 mM 1,10-phenanthroline (Phe) and 10 mM PMSF. Molecular mass of the proteinase, expressed in kilodaltons (kDa), is represented on the left. (b) Graphic representation of the percentage of the 50 kDa serine proteolytic enzyme activity. Gel strips were densitometrically analyzed, and each bar represents the value of three independent measurements.

on gelatin-SDS–PAGE (Figs. 1(a) and (b)). This may indicate that divalent zinc ions are involved in the structural stability of this proteolytic enzyme.

Serine proteinases constitute a very large and complex group of enzymes, with both nutritional and regulatory roles in nature. They are generally active at neutral and alkaline pH and show broad substrate specificity. Alkaline proteases are also used for developing products of medical importance. Kudrya and Simonenko [29] exploited the elastolytic activity of *Bacillus subtilis* strain 316M for the preparation of elastoterase, which was applied for the treatment of burns, purulent wounds, carbuncles, furuncles, and deep abscesses. A large number of microorganisms belonging to bacteria, actinomycetes, fungi and yeast are known to produce neutral-alkaline proteinases of the serine-type [28]. For instance, in *Paracoccidioides brasiliensis*, a dimorphic fungus known to cause a frequently encountered systemic mycosis in Latin America, extracellular collagenase, elastase and gelatinase activities have been reported [30]. *Cryptococcus neoformans* is an opportunistic fungus that causes disease in 6–8% of AIDS patients [31]. Rodrigues et al. [26] described an exocellular enzymatic activity capable to hydrolyze basement membrane-associated proteins (fibronectin, laminin and type IV collagen) by a *C. neoformans* serine proteinase of 75 kDa, which suggests that the fungus produces and secretes enzymes capable of tissue disruption. In addition, a 43 kDa secreted serine proteinase was recently reported for *C. neoformans*, which is able to hydrolyze several natural substrates including hemoglobin, beta-casein, and gamma-globulin [32]. Our group previously described the ubiquitous secretion of serine proteinases in *C. albicans* isolated from oral cavity of HIV-infected and healthy children, which presented activity in a broad pH range (from acidic to alkaline values) [25].

Candida infections involve a very complex interaction between a wide range of host factors and yeast virulence determinants that may be differentially expressed depending on the prevailing environmental conditions. The identification and determination of the contribution of specific virulence factors to various *Candida* infections is currently a topic of major interest to medical mycologists. One of the most important virulence factors of *Candida* is its ability to adhere to human tissue. Following yeast adherence and migration across the endothelial layer, *Candida* yeasts gain direct access to the extracellular matrix [17]. At this step, enzymes like proteinases may be involved in the penetration of the extracellular matrix. Morschhäuser et al. [33] demonstrated that *C. albicans* isoenzyme Sap2 degrades human subendothelial proteins and could subsequently facilitate the invasion of target organs.

In this context, we investigated the ability of the concentrated culture supernatant of *C. guilliermondii*, which is rich in 50 kDa serine proteinase, to hydrolyze some extracellular matrix components at physiological pH. Fibronectin is a large glycoprotein, consisting of two 220 kDa subunits linked by disulfide bonds. It circulates in the plasma in large quantities as a dimer and is also found in the extracellular matrix surrounding interstitial cells. In addition, soluble fibronectin is also found in most body fluids and secretions such as inflammatory exudates [34]. After 16 h of incubation with the *C. guilliermondii* supernatant fluid, the fibronectin represented on the SDS–PAGE pattern as a double band was degraded, generating some polypeptide bands of low molecular masses (Fig. 2). Penn and Klotz [35] described that *C. albicans* yeast cells bind soluble fibronectin through a glycoprotein receptor (adhesin) located on the cell surface. They also proposed that the interaction of soluble plasma fibronectin with *C. albicans* might be a relevant in vivo event especially in the case of disseminated candidiasis where the microorganism gains access to the intracellular space.

Laminin is another extracellular matrix glycoprotein that is present in basement membranes, and in the lung this glycoprotein can be exposed after tissue damage resulting either from inflammatory processes or from the lytic activity of microorganism or drugs [36]. Interactions with laminin are crucial for a number of biological processes that require cell adhesion, including diapedesis,

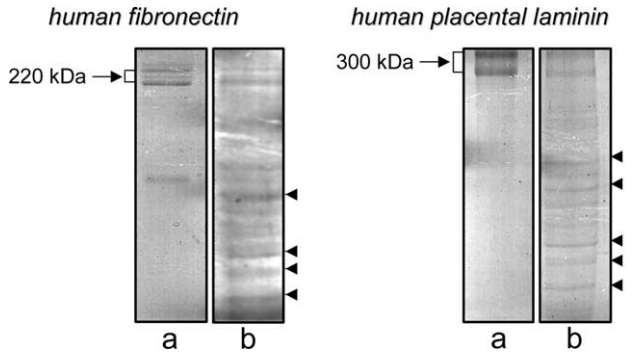

Fig. 2. Cleavage of extracellular matrix components by *C. guilliermondii* culture supernatant, which is rich in 50 kDa serine proteinase. The degradation profile was analyzed by 10% SDS–PAGE and the gels were stained with Coomassie brilliant blue R-250. (a) Control in which each proteinaceous substrate was supplemented only with PBS. (b) Candidal supernatant fluid was incubated in the presence of human fibronectin (left) and human placental laminin (right) for 16 h at 37 °C. The arrowheads show the fragmentation of the proteinaceous substrate after proteolysis.

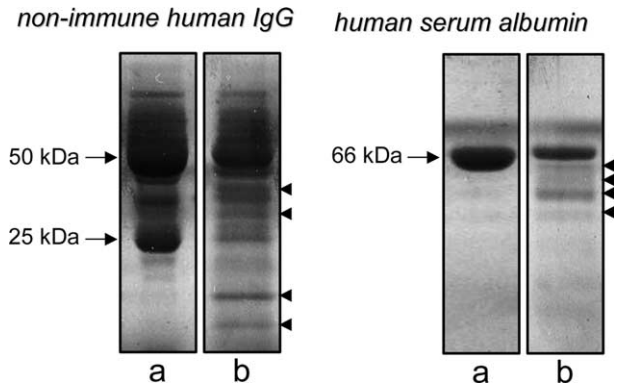

Fig. 3. Cleavage of serum proteins by *C. guilliermondii* culture supernatant, which is rich in 50 kDa serine proteinase. The degradation profile was analyzed by 10% SDS–PAGE and the gels were stained with Coomassie brilliant blue R-250. (a) Control in which each proteinaceous substrate was supplemented only with PBS. (b) Candidal supernatant was incubated in the presence of non-immune human immunoglobulin G (IgG; left) and human serum albumin (right) for 16 h at 37 °C. The arrowheads show the fragmentation of the proteinaceous substrate after proteolysis.

cellular cohesion inside tissues, metastasis of cancer cells, and infections. In a similar way, the supernatant of *C. guilliermondii* degraded the human placental laminin substrate (Fig. 2). Collectively, our results suggest that the 50 kDa exocellular serine proteinase released to the extracellular environment by *C. guilliermondii* could facilitate the dissemination of yeast cells to deep organs by destroying at least two of the major important protein components of the extracellular matrix.

Several microorganisms express surface proteins that interact with immunoglobulins in a non-immune fashion. These molecules provide pathogens with the potential to evade or elude the host defenses by interfering with opsonization, phagocytosis, and complement activation and consumption [37]. It has been reported in some in vitro studies that antibody immunity may contribute to host defense by direct promoting an anti-candidal activity, by providing opsonins for more efficient phagocytosis, by binding to immunomodulating polysaccharides, by neutralizing extracellular proteinases, by inhibiting the yeast-to-mycelium transition, and by preventing yeast attachment [31,37]. We demonstrated that the culture supernatant of *C. guilliermondii* was able to cleave non-immune human IgG, especially its light chain (a polypeptide of approximately 25 kDa), which was extensively degraded (Fig. 3).

Albumin is the most abundant protein in human serum. It interacts reversibly with a wide variety of endogenous and exogenous compounds and serves as an important depot and transport protein [36]. The *C. guilliermondii* exocellular serine proteinase also digested this relevant and multifunctional serum protein (Fig. 3). Besides, this enzymatically-degrading albumin could provide amino acids to be used by the *Candida* as nutrients in a similar manner to that suggested occurring in other microorganisms.

The cleavage of these soluble proteinaceous substrates was completely inhibited when the culture supernatant of *C. guilliermondii* was pre-incubated for 30 min with 10 mM PMSF or when the supernatant was heated by boiling for 5 min at 100 °C (data not shown). Additionally, a single proteolytic band with an apparent molecular mass of 50 kDa was observed when these extracellular matrix components and serum proteins were individually incorporated into the SDS–PAGE as co-polymerized substrates, which was also completely inhibited when the gels were incubated in the presence of 10 mM PMSF (data not shown). No proteolytic activity was observed when only concentrated BHI medium was applied on gelatin-SDS–PAGE as well as when tested with soluble proteinaceous substrates alone (data not shown). Finally, we believe that the 50 kDa extracellular serine proteinase is a true secretion based on the fact that within 48 h of *C. guilliermondii* cultivation in BHI medium more than 98% of cells were viable when assessed by the trypan blue exclusion method, and no lactate dehydrogenase activity was measured in the culture supernatant in this time (data not shown).

Among potential virulence factors of *Candida*, enzymes seem to play a relevant role. Many studies concern the Saps, and the degradation of some components of the subendothelial extracellular matrix by the isoenzyme Sap2 has been proved at least to *C. albicans* isolates [33]. Nevertheless, other proteolytic enzymes could be involved in the pathogenicity of this yeast. In a recent study of our laboratory, we described that a clinical strain of *C. albicans* isolated from human infected urine secreted two distinct extracellular proteolytic enzymes: a 50 kDa serine proteinase and a 60 kDa

metalloproteinase. The former activity was capable in degrading albumin, IgG, hemoglobin, fibronectin and laminin at physiological pH (unpublished observations).

The results presented here describe for the first time the secretion of serine proteinase to the extracellular environment by a clinical strain of *C. guilliermondii* isolated from a HIV-infected individual. This 50 kDa exocellular serine proteinase was able to hydrolyze a broad spectrum of proteinaceous substrates, some of them with great relevance in the context of microorganism–host interface. Further investigations are necessary to determine the biological significance of this proteolytic enzyme in vivo as well as the possibility to use this enzyme as a new target for the development of an antifungal peptidyl drug strategy based on proteinase inhibitors.

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