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Acid proteinase enzyme activity in *Candida albicans* strains: a comparison of spectrophotometry and plate methods

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Abstract: In recent years, the incidence of fungal infections has been rising all over the world. Although the amount of research in the field of pathogenic fungi has also increased, there is still a need for studies on fungal virulence. In this investigation, we focused on testing bloodstream-identified *Candida albicans* in experimental in vitro virulence assays. The proteinase enzyme activities of 30 *C. albicans* strains isolated from the bloodstream and sterile body fluids were investigated (15 colonizations, 15 infections). The spectrophotometric and plate methods were used to determine proteinase activity. The correlations between the 2 methods were compared. This study indicates that, in terms of proteinase activity, there were no statistically significant differences between strains obtained from infection and colonization sites by either method ($P > 0.05$).

Key words: *Candida albicans*, secreted aspartyl proteinase (SAP) activity, virulence

Candida albicans suşlarında asit proteinaz enzim aktivitesi: spektrofotometri ve petri metodlarının karşılaştırılması

Özet: Son yıllarda bütün dünyada fungal enfeksiyon olgularında artış gözlenmektedir. Patojenik funguslarla ilgili araştırmalar artmış olmasına rağmen, hala virulansla ilgili çalışmalara ihtiyaç vardır. Bu çalışmada kandan izole edilen *Candida albicans* suşlarında in vitro virulans faktörlerinin araştırılması amaçlanmıştır. Steril vücut sıvılarından izole edilen 30 *C. albicans* (15'i kolonizasyon, 15'i enfeksiyon) suşlarının proteinaz enzim aktivitesi spektrofotometrik ve petri plak yöntemi ile değerlendirilmiştir. İki yöntem karşılaştırıldığında, aspartil proteinaz enzim aktivitesi açısından izole edilen suşlarda enfeksiyon ve kolonizasyon gruplarında istatistiksel olarak anlamlı bir farklılık bulunmamıştır ($P > 0,05$).

Anahtar sözcükler: *Candida albicans*, sekrete aspartil proteinaz aktivitesi (SAP), virulans

Introduction

Although *C. albicans* is a normal commensal inhabitant of mucosal surfaces, it frequently causes surface infections when certain host factors are imbalanced. Under certain circumstances, these superficial infections may disseminate and cause serious systemic infections.

During the past 2 decades, the frequency of nosocomial yeast infections has increased dramatically with a consequent rise in related mortality and prolonged hospitalizations (1-3). This trend was reported to be 219%-467% in some tertiary care hospitals and 75%-350% in community hospitals (4). The *Candida* species can cause not only

superficial mucosal and skin-associated infections, but also invasive bloodstream infections that are associated with the highest rates of morbidity and mortality (5). In immunocompromised patients, the *Candida* species have the potential to invade all host organs and systems and cause severe systemic infections. Case mortality rates can increase up to 33%-55% when associated with *Candida* infections (6).

The increase in the proportion of bloodstream infections due to opportunistic fungal pathogens is likely associated with the increasing number of critically ill patients, surgical procedures, cytotoxic therapies with prolonged neutropenia, other immunosuppressive therapies, the use of broad-spectrum antibiotics, indwelling invasive medical devices, and intensive care supports (7).

Although *C. albicans* is still the most commonly isolated organism from bloodstream cultures, other *Candida* species have emerged as clinically important pathogens in their own right (8). Different *Candida* isolates can play a role in invasive infections and colonizations. Multiple characteristics of the *Candida* species have been proposed to be virulence factors that enable the organism to cause disseminated infections in a susceptible host. These factors can cause rapid changes in phenotype, hyphal formation, molecular mimicry**,** and the ability to produce enzymes (proteinases and phospholipases) that cause proteolysis (9-11).

In *C. albicans*, 4 types of phospholipases have been reported: phospholipases A, B, C, and D (12). Phospholipases play a role in the destruction of phospholipids on the cell surface, and proteinases destroy some important immune proteins of the host cell such as immunoglobulin, lactoperoxidase, lactoferrin, mucin, and pepstatin A. The enzymes facilitate invasion by destroying host cells (13), and they are very important in pathogenesis. Some defective mutants that have lost these enzymes are less virulent and are more easily phagocytosed (14). The expression of the enzyme in vivo was shown by Borg and Rüchel (15).

Some of the most important virulence factors are the secreted aspartyl proteinases (SAPs), which are encoded by 10 SAP genes (16). The proteinases possess distinct differences in pH, with Sap1-Sap3 (yeast-associated) having optimum activity at lower pH values and Sap4-Sap6 (hyphal-associated) having optimum activity at higher pH values, with a pH range of activity between 2.0 and 7.0 (12). Although the consequences of proteinase secretion during human infection are not precisely known, in vitro, animal, and human studies suggest that the proteinases may influence *C. albicans* virulence by a few probable mechanisms: 1) by facilitating adhesion through the proteolysis of host surface proteins, resulting in host tissue damage; 2) through defects in the host immune response due to deterioration of host proteins; 3) by increasing fungal nitrogen resources via peptide degradation products; 4) by damaging endothelial cells; and 5) by stimulating the host's proteolytic mechanisms.

In normal conditions, candidal colonization of mucocutaneous surfaces is very rare. Adherence and persistence (colonization) of *Candida* spp. on mucosal surfaces is the first step in the process of candidosis. The adherence capabilities of *C. albicans* and *C. tropicalis* are stronger than those of other *Candida* spp. (17,18). In hospitalized patients, mucosal colonization by *C. albicans* may reach 80%, but this rate is much lower in healthy adults, at 2%-37% (17,19).

Materials and methods

The study was conducted with a total of 30 *C. albicans* isolates in order to compare the proteinase activities of the different strains. Of these, 15 strains were obtained from the bloodstream and sterile body fluids (infectious strains), and the other 15 isolates were obtained from throat, sputum, and feces samples of hospitalized patients (colonization strains). All yeasts were isolated from different patients.

The Centers for Disease Control and Prevention (CDC) definition for bloodstream infection (BSI) was used in this study, and the detection of *Candida* in at least one blood culture specimen was considered to represent candidemia (20). Yeast growth was confirmed by observation of typical cells on gram-stained films, and all isolates were identified by germ tube tests, observation of corn meal agar chlamydospore formation, and the conventional assimilation reaction kit, ID-32-C (bioMerieux, Marcy l'Etoile, France). *C. albicans* (CBS 2730), *C.*

albicans (ATCC 36802), *C. albicans* (ATCC 10231), *C. krusei* (ATCC 6258), *C. parapsilosis* (ATCC 22019), and *C. kefyr* (ATCC 1012) reference strains were also included in this study. Their well-known proteinase activities, as reported by Rüchel et al. (21), were added to the study to optimize the timing of the controls for the plate and spectrophotometric methods.

This study was performed in order to evaluate any possible differences in the proteinase activities of different infectious and colonization strains of *C. albicans* isolates, and to compare the 2 methods (spectrophotometric and plate methods) that were used in the in vitro experiments to measure proteinase activity.

Plate method

Proteinase activities of the strains were evaluated according to the suggestions of Cassone et al., with slight modifications (22). All isolates were evaluated for their ability to secrete aspartyl proteinase on solid medium containing bovine serum albumin (BSA). Briefly, the yeast were precultured in YEPD medium (2% glucose, 1% yeast extract, and 2% Bacto peptone) and induced to secrete proteinases onto the BSA agar. The medium, containing 1.17% yeast carbon base (Difco, Detroit, MI, USA), 0.01% yeast extract (Biolife, Milan, Italy), and 0.2% BSA (BDH, Poole, UK), was adjusted to a pH of 5.0, sterilized by filtration, and added to a stock solution of autoclaved (2%) agar. Filter paper disks, with a diameter of 6 mm, were dipped into a suspension of yeast culture at a density of 10^7 yeast mL^{-1} in YEPD medium and applied to the plate. A maximum of 4 disks were used for each 90-mm-diameter plate. The plates were incubated at 28 °C for 7 days. The plates were observed each day for increased opacity around the disks, caused by growing fungi. The opacity caused by precipitated albumin was observed for subsequent clearing due to hydrolysis by the acid proteinases of the fungi. The millimetric zone measurements were evaluated as negative (–) for no clearance, positive (+) for mild activity (a lysis zone of 1-2 mm around the disk), and double-positive (++) for strong activity (a lysis zone of 3-5 mm around the disk). The standard strains were used as positive controls, and the experiment was completed in triplicate. BSA proteolytic activity was visualized by staining with Amido black as described by Rüchel et al. (23). Each strain was analyzed in duplicate. The diameter of the colony and the total diameter of the colony with the precipitation zone (pz) were measured (24-26).

Spectrophotometric method

Candida proteinase activity was determined spectrophotometrically following the digestion of the BSA substrate, as described by Cassone et al. (21) and De Bernardis et al. (26,27).

To determine proteinase activity in the liquid medium by the spectrophotometric method, yeast cells were inoculated in 5 mL of YEPD medium (1% yeast extract, 2% peptone, and 2% dextrose) and incubated at 37 °C overnight. Culture supernatants (0.1 mL) were added to 0.9 mL of 0.1 M citrate buffer (pH 3.2) containing 0.2% BSA with and without the proteinase inhibitor pepstatin A (0.5 mg mL^{-1}, Sigma), and then incubated at 37 °C. The reaction was stopped after 3 min by the addition of 500 µL of 15% trichloroacetic acid. The mixture was centrifuged and the supernatant was read in a spectrophotometer at 280 nm. Aspartyl proteinase activity is expressed as the change in the optical density (OD) per milliliter of culture, with the OD values of the samples containing pepstatin (in the range of 0.01-0.03) subtracted from the readings of samples without pepstatin. Each isolate was tested in duplicate. Quantitative values were calculated according to the following formula: Protein mg mL^{-1} = $1.55 \times Abs_{280} - 0.77 \times Abs_{260}$ (28,29).

Statistical analysis

The differences in the proteinase activities of the isolated colonization and infection strains were compared with the Mann Whitney-U test for independent samples, and the correlation between the 2 methods was assessed by Pearson's chi-square test (30).

Results and discussion

Measurements of the proteinase activities of standard strains were used as controls for both the spectrophotometric and plate method. Proteinase activity results for the colonization and infection strains measured by the spectrophotometric and plate methods are shown in Tables 1 and 2 and Figures 1 and 2, respectively.

Table 1. Proteinase activity of colonization[a] and infection[b] strains measured by the spectrophotometric method.

Cases (n = 15)	ΔAb280[a]	ΔAb280[b]
1	0.272	0.272
2	0.194	0.169
3	0.120	0.164
4	0.119	0.152
5	0.101	0.151
6	0.087	0.139
7	0.083	0.127
8	0.073	0.111
9	0.065	0.084
10	0.063	0.054
11	0.052	0.038
12	0.025	0.023
13	0.010	0.017
14	0.009	0.017
15	0.002	0.016
Mean value	0.085	0.102

Table 2. Proteinase activity of colonization[a] and infection[b] strains measured by the plate method.

Cases (n = 15)	Zone diameter (cm)[a]	Zone diameter (cm)[b]
1	0.5	0.9
2	1.0	0.8
3	0.7	1.5
4	0.7	0.8
5	0.7	0.6
6	1.3	0.5
7	0.6	0.7
8	0.5	1.1
9	0.5	0.0
10	0.5	0.8
11	0.8	0.7
12	0.0	1.3
13	0.5	1.3
14	1.0	0.0
15	1.4	0.5
Mean value	0.71	0.77

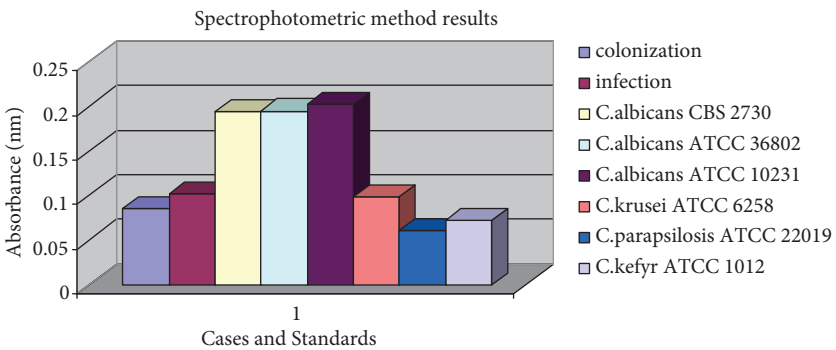

Figure 1. Proteinase activity of colonization and infection strains measured by the spectrophotometric method.

The protein concentrations in the culture supernatant of the colonization and infection strains were also determined and are shown in Figure 3. In the group of colonization strains, the average ΔAbs value was 0.077 ± 0.019 and the average concentration (mg mL^{-1}) was 0.119 ± 0.131 (read at 280 nm). The average zone diameter in the same strains studied with the plate method was 0.728 ± 0.096 cm.

In the infection strain group, the average ΔAbs value was 0.102 ± 0.019 and the average concentration (mg mL^{-1}) was 0.068 ± 0.010 (read at 280 nm). The average zone diameter in the same strains studied with the plate method was 0.821 ± 0.103 cm.

According to these results, there was no statistically significant difference in the mean values of the proteinase activities of the 2 groups of strains,

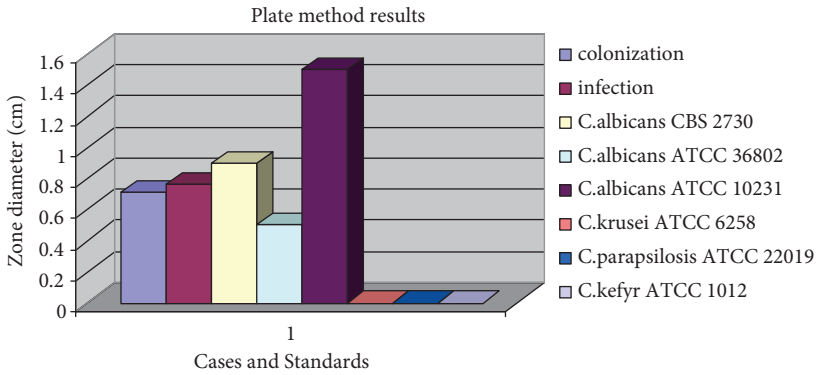

Figure 2. Proteinase activity of colonization and infection strains measured by the plate method.

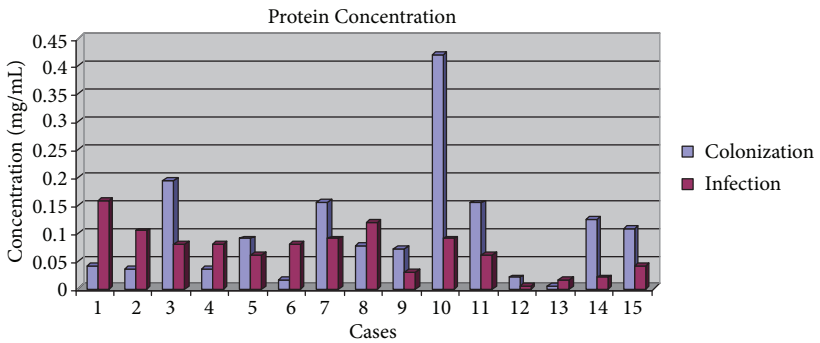

Figure 3. The protein concentration in culture supernatant of colonization and infection strains.

as measured by either the spectrophotometric or plate methods ($P > 0.05$, Mann-Whitney) (30).

Bloodstream infections due to *Candida* spp. have become an important cause of mortality and morbidity among patients. Yeasts in the genus *Candida* continue to be among the most important etiologic agents of nosocomial infection. The considerable increase in deep-seated candidosis is most commonly observed in patients in ICUs, those with indwelling catheters or those receiving oncological treatment, organ transplant recipients, and other immunocompromised individuals subject to heavy therapeutic protocols.

The secreted proteinases (SAPs) are responsible for the adhesion tissue damage and invasion of host immune responses. Their proteolytic activity has been associated with tissue invasion.

Several studies dealing with proteinase secretion and proteinase activity have shown a clear correlation between the ability of *C. albicans* strains to secrete SAPs and their ability to cause disease (16,22,31,32). The extracellular proteinases produced by *C. albicans* are some of the virulence factors associated with this organism, and the degree of virulence is correlated with the level of proteinase produced.

The theory that there is an association between extracellular proteinase secretion and virulence in *C. albicans* was postulated by MacDonald and Odds (33). The enzymes are secreted in vitro when the organism is cultured in the presence of exogenous protein as the nitrogen source (34).

Proteinase production by *C. albicans* depends not only on the strain type or the type of infection, but also on the phenotypic switch type, environmental conditions, and even the stage of infection (35).

Therefore, caution must be exercised when interpreting the results of proteinase assays.

A considerable amount of information is now available regarding the extracellular proteinases produced by fungi, especially of the genus *Candida*. *C. albicans*, which is the major human pathogen of this genus, is a commensal organism in healthy individuals and seems to be almost universally present.

De Bernardis (26) found that cutaneous isolates of *C. parapsilosis* had uniformly elevated SAP activity, which was more than 4 times higher than the enzyme activity in the blood isolates.

SAPs are also important virulence factors in *C. lipolytica*, *C. parapsilosis*, and *C. tropicalis*, and are important during mucosal and disseminated infections caused by *C. albicans* (36).

Strains with higher proteolytic activity were shown to be more virulent (37). De Bernardis et al. (35) reported high activity of secreted acid proteinases in vitro for all of the *C. parapsilosis* strains isolated from outpatients with vaginitis.

Similar studies investigating phospholipase and protease activities recorded different enzyme activities in *Candida* spp. (38-40).

Matsumoto et al. (41) isolated 80 yeast strains from hospitalized children, 59 from blood cultures and 21 from vascular catheter cultures. The prevalent species in both blood and catheter isolates was *C. albicans* (16.9% and 28.6%, respectively), followed by *C. parapsilosis* (32.2% and 48.9%, respectively).

Kantarcıoğlu et al. (36) investigated 60 strains of *C. albicans* and found that 56 strains (93.3%) produced phospholipases and 57 strains (95%) produced proteases. The nonalbicans *Candida* species *C. kefyr*, *C. lipolytica*, *C. parapsilosis*, and *C. tropicalis* were also found to be protease producers.

Kalkancı et al. (42) investigated the adherence abilities, enzyme production, and susceptibility patterns of the *Candida* strains. All isolates of *C. albicans* expressed an enzymatically active SAP. However, no production was observed in the nonalbicans *Candida* species.

Ozkan et al. (43) studied the slime production and proteinase activities of 54 strains, consisting of 19 *C. parapsilosis* and 35 *C. albicans* strains, in isolated blood samples by the method of Staib (34).

The extracellular proteinases of eukaryotic microbial pathogens have attracted the attention of many laboratories due to their potential role in pathogenesis. In the genus *Candida*, *C. albicans* is the major human pathogen and it has extracellular proteinases.

Fotedar and Al-Hedaithy (44) tested 87 isolates of *C. dubliniensis* and 52 isolates of *C. albicans* recovered from routine clinical specimens of HIV-negative patients. Of the 87 *C. dubliniensis* isolates tested, 59 (68%) were negative for proteinase activity, while the remaining 28 (32%) produced moderate amounts of proteinase. It is interesting to note that a higher percentage (88%) of *C. dubliniensis* isolates recovered from the bloodstream were positive for proteinase activity compared to the isolates recovered from skin (29%), urine (27%), high vaginal swabs (HVS) (32%), and respiratory secretions (23%) (43). In contrast, high proteinase activity was observed for 41 of 52 of the *C. albicans* isolates. A significant majority of the *C. albicans* isolates from the bloodstream (88%), HVS (82%), and respiratory (80%) specimens showed high proteolytic activity. The production of these enzymes by *C. albicans* is well known to cause localized and systemic infections (22,31,45).

Girish Kumar et al. (25) conducted a study with 67 isolates of *Candida* collected from immunocompromised patients (HIV-seropositive and cancer patients). Of the 24 bloodstream isolates tested, all 6 of the *C. albicans* bloodstream isolates produced both proteinases and phospholipases, whereas 20% and 80% of the *C. tropicalis* (n = 15) bloodstream isolates produced phospholipase and proteinase activity, respectively.

Oksuz et al. (40) aimed to determine the in vitro phospholipase and proteinase activities of 122 *Candida* spp. isolated from several anatomically distinct sites of healthy adults. Of the proteinase-positive isolates, 46 (56.7%) were *C. albicans* and 18 (43.9%) were non-*C. albicans* isolates.

Gokce et al. (2) analyzed the proteinase, phospholipase, and biofilm production of 68 *C. albicans* and 31 nonalbicans *Candida* strains (11 *C. tropicalis*, 8 *C. parapsilosis*, 6 *C. glabrata*, 4 *C. quillermondii*, and 2 *C. krusei*) isolated from

bloodstream cultures. In total, 61 (89.7%) *C. albicans* strains were detected as proteinase-positive, whereas 8 (25%) nonalbicans *Candida* strains were proteinase-positive.

It has become especially clear that yeast of the genus *Candida* continue to be important etiologic agents of nosocomial bloodstream infections. The enzyme production of *C. albicans* and other species isolated from different clinical conditions and anatomical sites was studied, and variations of 62.5%-100% for proteinase activity were determined by Rüchel et al. (23).

In a study conducted in Turkey, it was reported that proteinases and phospholipases can act as virulence factors that contribute to host tissue invasion by digesting proteins such as hemoglobin, keratin, and collagen, and can also degrade cell membranes. Cerikçioğlu et al. (46) suggested that exhibition of strong hydrolytic enzyme activities for long durations of colonization might contribute to the development of candidemia in preterm infants. Meanwhile, some authors report that infection strains produce a high level of proteinases (47,48).

We found no statistically significant difference in the in vitro proteinase activity between colonization and infection strains. Our results seem paradoxical when compared with other reports in the literature. It has been reported that genes found in *C. albicans* strains that are responsible for proteinase production are only expressed under suitable conditions. Many factors, such as host hormone concentration, nutrition, the pH of mucosal surfaces, and serum Fe concentrations, are thought to have a role in gene expression (49). With this information, it is not surprising that we did not see differences between the 2 groups in our study. In the presence of stable in vitro conditions for proteinase production, more than 90% of *C. albicans* strains produce proteinases. Consequently, different properties of the in vitro studies must be debated and resolved with in vivo studies of the proteinase enzymes in terms of the virulence of *C. albicans*. Several studies dealing with proteinase secretion have shown a clear correlation between the ability of *C. albicans* strains to secrete SAPs and their ability to cause disease (22,31,50,51). The 2 methods for the measurement of SAPs were both tried in order to determine if there is a possible correlation between them. It was determined, by both methods, that proteinases were expressed in all *C. albicans* strains, but no positive correlation was evaluated. This may be due to using the same structure proteins as substrate. The spectrophotometric method has the advantage of being able to measure low values and also has the ability to create a threshold value. On the other hand, the plate method is less technically difficult. The results reported here warrant further study with more strains to evaluate whether there is a correlation between the 2 methods.

We conclude that the extracellular proteinases produced by *C. albicans* are important virulence factors associated with this organism. The absence or lowered expression of these enzymes may indicate the less virulent nature of *Candida* spp. when compared with *Candida* species with higher expression of these enzymes, particularly in immunosuppressed patients, and the degree of virulence and pathogenicity are correlated with the level of secreted proteinases. Determination of these factors might be a helpful tool to inform the clinicians about the possible virulence of the strain.

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