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The Proteolytic System of *Candida dubliniensis*

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Abstract: Proteases of *Candida dubliniensis* have been scarcely studied, these enzymes may play an important role in nitrogen metabolism, post-translational processing, nutritional stress, dimorphism, virulence, etc. In this work, we report the presence of five different intracellular proteases and one extracellular proteolytic activity. The intracellular proteases are: aminopeptidase ycdAPE, carboxypeptidase ycdCP, dipeptidyl aminopeptidase ycdDAP, proteinases ycdPrA and ycdPrB, and extracellular protease Sap activity, measured under several nutritional conditions. *C. dubliniensis* produced the highest level of intracellular proteolytic enzymes, i.e., ycdAPE, ycdCP, ycdDAP, ycdPrA and ycdPrB in media with peptone during stationary growth phase. Chelating agents affected mainly APE activity; whereas ycdCp, ycdDAP, and ycdPrB were affected by serine protease inhibitors; ycdPrA was affected by pepstatin, an aspartyl protease inhibitor. We found Sap activity in *C. dubliniensis* in YCB-SBA medium, this activity was inhibited by pepstatin inhibitor. Southern analysis revealed the presence of at least four genes encoding Sap in the *C. dublinienis*is genome (using as probes *SAP1*, *SAP2*, *SAP3,* and *SAP4-6* genes from *C. albicans*).

Key words: Protease, Sap (secreted aspartyl proteinase), virulence factor, *Candida dubliniensis*.

INTRODUCTION

Proteolysis, a vital process for cell life, plays an important role in different physiological functions, such as protein digestion, hormone maturation, immune response, inflammation, fertilization, germination, as well as in other morphological processes. Some proteases are important in spores formation and germination, pathogenesis of several microorganisms, and post-translational regulation [1-3].

Candida dubliniensis was first described as a novel species in 1995. This organism is very close to the human yeast pathogen, *Candida albicans*. However, despite the close phylogenetic relationship between *C. albicans* and *C. dubliniensis*, phenotypic and molecular data suggest differences in virulence between these two species. The apparent low virulence of *C. dubliniensis* could be due to differential gene expression rather than to simple absence or divergence of particular genes [4].

The study of *Saccharomyces cerevisiae* has been important in the elucidation of proteinase multiplicity and proteinase function in eukaryotic cells, numerous proteolytic enzymes have been biochemically and genetically characterized [5]. There are several proteases associated with yeast proteasomes that participate in stress-dependent and ubiquitin-mediated proteolysis. They are involved in the degradation of short-lived and regulator proteins that generate small peptides from proteins, these latter are degraded into amino acids by tri-, di-, carboxi-, and amino-peptidases [6-7].

Extracellular proteolytic activity plays a central role in *Candida albicans* pathogenicity and is produced by a family of 10 secreted aspartyl proteinases (Sap proteins) [3]. *In vitro*, animal and human studies have implicated proteinases in *C. albicans* virulence in one of the following ways: (i) correlation between Sap production *in vitro* and *Candida* virulence, (ii) degradation of human proteins and structural analysis in determining Sap substrate specificity, (iii) association of Sap production with other virulence processes of *C. albicans*, (iv) Sap protein production and Sap immune responses in animal and human infections, (v) *SAP* gene expression during *Candida* infections, (vi) modulation of *C. albicans* virulence by aspartyl proteinase inhibitors, and (vii) the use of *SAP*-disrupted mutants to analyze *C. albicans* virulence. Sap proteins fulfill a number of specialized functions during the infective process, which include the simple role of digesting molecules

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for nutrient acquisition, digesting or distorting host cell membranes to facilitate adhesion and tissue invasion, and digesting cells and molecules of the host immune system to avoid or resist antimicrobial attack by the host [3].

Detailed analyses of proteases in *C. dubliniensis* might allow us to determine the proteases produced by this fungus and to examine the production of these enzymes in several nutritional conditions and during the dimorphic transition of yeast to mycelia, which is considered to be an important factor in the pathogenicity of this fungus. Moreover, numerous studies have correlated extracellular proteolytic activity *in vitro* with the virulence of *Candida* species and have shown that the most virulent species, such as *C. albicans*, *C. tropicalis,* and *C. parapsilosis,* produce more proteinases *in vitro* than the less virulent species [3]. Secreted aspartic proteinases have been considered a new target in candidiasis therapy [8]. In this work, we report the presence of five different intracellular proteases and one extracellular proteolytic activity in *C. dubliniensis*.

MATERIALS AND METHODS

Strains and growth conditions: Strains *C. dubliniensis* CD36 and *C. dubliniensis* CD92 were provided by Dr. Derek J. Sullivan of the University of Dublin [9], *C. albicans* ATTC 10231 was used as reference strain in this study.

The *C. dubliniensis* strains were confirmed to the species level using molecular methods described by Bautista *et al.* [10]. They were routinely maintained on YEPD plates (1% yeast extract, 2% peptone, 2% glucose, and 2% agar) at 28ºC. The ycdAPE, ycdCP, ycdDAP, ycdPrA and ycdPrB activities in soluble and membrane fractions of *C. dubliniensis* were measured by growing the yeast in YEPD and minimal medium broths containing 0.17% yeast nitrogen base without amino acids or ammonium sulfate, but adding 2% glucose with 0.5% nitrogen source (peptone, proline, or ammonium sulfate) according to manufacturer's instruction. Production of Sap was performed in YCB-SBA medium (1.2% yeast carbon base, 0.2% SBA, pH 4.0 adjusted with 1 M NaOH). To evaluate cell growth, absorbance (A_{600}) of culture samples was measured in a Perkin-Elmer Lambda IA spectrophotometer.

Preparation of crude extracts and differential centrifugation: Biomass from the culture medium was recovered by centrifugation at 5,000 *g* at 4ºC for 10 min. Cells were fragmented in Braun´s mill using glass beads (0.5 mm diameter). The mixture contained 7.5 g washed acid beads, 12 mL 0.1M Tris-HCl, pH 7.5, and 5 g cells. Total disintegration time was 10 min (10 series, 1 min each). The extent of disintegration was monitored under light microscopy. The crude extract was carefully removed from the glass beads and centrifuged at 10,000 *g* at 4ºC for 10 min. The supernatant fluid was removed and centrifuged again at 100,000 *g* at 4ºC for 1.5 h using a Beckman ultracentrifuge. The 100,000 *g* supernatant (S 100,000 *g*) was considered as soluble fraction and the membranal fraction was precipitated at 100,000 *g* (M 100,000 *g*).

Enzyme assays and protein determination: Intracellular enzymatic activities were determined as described by Hirsch *et al.* [5]. The following substrates were used for the different proteases: L-lysyl-4-nitroanilide (Lys-4-NA) for aminopeptidase activity (ycdAPE); N-benzoyl-tyrosine-4-nitroanilide (N-BZ-Tyr-4-NA) for carboxypeptidase activity (ycdCP), and L-alanyl-prolyl-4-nitroanilide (Ala-pro-4-NA) for dipeptidyl aminopeptidase activity (ycdDAP), acid denatured hemoglobin for proteinase A activity (ycdPrA), and Hide Powder Azure (HPA) for proteinase B activity (ycdPrB) [11]. Assay for Sap activity was based on the MacDonald and Odds [12] assay, enzyme activity was measured spectrophotometrically following digestion of BSA as substrate. A typical reaction mix containing 500 µl 2.0% (w/v) BSA in 50 mM sodium citrate (pH 3.2), 100 µl 50 mM sodium citrate (pH 3.2), and 200 µl of culture supernatant was incubated for 30 min at 37ºC. The reaction was stopped by adding 200 µl 10% TCA, precipitated protein was removed by centrifugation at 22,000 *g* for 5 min, 75 µl of the supernatant was removed and added to 75 µl 0.5M NaOH. Tyrosine-containing peptides in the neutralized sample were determined with the Folin´s reagent according to the method of Lowry. Protein determinations were performed according to the Bradford method [13].

Effect of protease inhibitors on proteolityc activities: The effect of protease inhibitors (leupeptin, pefabloc, E-64, EDTA, pepstatin, 1-10, phenanthroline, and PMSF) on the proteolytic activities was measured in 100 mM Tris-HCl buffer, pH 7.5. The soluble fraction (100,000 *g*) or the culture medium's supernatant, after harvesting cells (for Sap activity), was preincubated with the respective compound for 30 min at 37ºC, followed by the standard enzyme assay.

Southern hybridization: Genomic DNA from *C. dubliniensis* was digested with *Eco*RI endonuclease, subsequently separated by electrophoresis in agarose and transferred to a Nylon membrane positively charged (Amersham Pharmacia Biotech, UK), as described by Sambrook et al. [14]. Hybridization was performed at 65ºC with *SAP1*, *SAP2 SAP3,* or *SAP4-6* specific probes labeled with digoxigenin-dUTP (20-deoxyuridine 50-triphosphate) using a random-primed digoxigenin (DIG) DNA labeling detection kit (Roche, Mannheim, Germany). Hybridization and immunological detection were performed as recommended by the supplier. *SAP* DNA probes to be used in hybridization experiments were generated for PCR from *C. albicans SAP* genes: *SAP1*, *SAP2, SAP3,* or *SAP4-6* as described by Bautista-Muñoz *et al.* [10]. Genomic DNA from *C. albicans* ATCC 10231 was used as template.

RESULTS

ycdAPE, ycdCP, ycdDAP, ycdPrA, ycdPrB, and Sap proteolytic activities under different nitrogen sources: *C. dubliniensis* presented ycdAPE, ycdDAP, ycdCP, ycdPrA and ycdPrB intracellular activities. The highest levels of the three exopeptidases ycdAPE, ycdDAP, ycdCP proteolytic enzymes were reached in the medium enriched with peptone as nitrogen source: YPD and YNB-peptone, Table 1. These intracellular exopeptidases were accumulated during the stationary phase (48 h). All of them were localized in the soluble fraction (S 100,000 *g*), Table 2. The highest level of intracellular acidic proteinase ycdPrA was reached in the medium with amonium as nitrogen source during the logarithmic phase (6 h), Table 1. This activity was localized in the soluble fraction (S 100,000 *g*) although considerable ycdPrA activity was also found in the membrane fraction (M 100,000 *g*), Table 2. The production of proteinase ycdPrB was regulated by the nitrogen source, the highest level of this proteinase was reached in the medium with peptone and proline during early stationary phase, Table 1. This activity was localized in the soluble fraction (S 100,000 *g*), Table 2. It was not possible to detect Sap activity in YPD and YNB-different nitrogen sources media. The medium YCB-SBA has been described as inductor medium for the production of Sap in *C. albicans*, this medium was also an inductor for the production of *C. dubliniensis* Sap activity. There was no difference in the level of Sap activity between *C. dubliniensis* and *C. albicans,* Fig. 1.

Effect of inhibitors on enzymes activity: The effect of seven inhibitors was tested in the S 100,000 *g* fraction (YPD medium) with ycdAPE, ycdDAP, ycdCP, ycdPrA and ycdPrB, as well as in the supernatant of the culture medium (YCB-SBA) after harvesting cells with Sap activity. Chelating agents, such as EDTA and 1-10 phenanthroline, exerted a clear inhibitory effect on ycdAPE activity, suggesting that this protease is a metalloenzyme. PMSF, pefabloc (serine protease inhibitors) caused inhibition of the three enzymes (ycdAPE, ycdDAP, and ycdCP). E-64 (cysteine proteases inhibitor) had the same effect on ycdAPE activity; however, leupeptin (cysteine and serine proteases inhibitors) only inhibited ycdPrB, Table 3. Acidic proteinases ycdPrA and Sap were inhibited by pepstatin (aspartyl protease inhibitor). Results suggest that ycdAPE is a metallo-serin protease; ycdDAP, ycdCP and ycdPrB are serine proteases; and ycdPrA and Sap are aspartyl proteases.

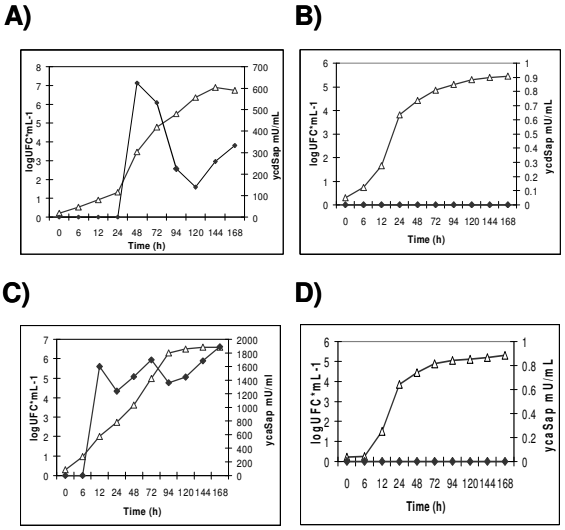

Fig1. Kinetic study of Sap production by *C. dubliniensis* and *C. albicans* in different media. A) ycdSap in YCB-SBA medium. B) ycdSap in YPD medium. C) ycaSap in YCB-SBA medium. D) ycaSap in YPD medium. Total activity (◆), Growth (△).

Presence of *SAP* genes in *C. dubliniensis* genome: *C. dubliniensis* presented extracellular aspartyl proteinase activity with the inductor medium YCB-SBA. We

therefore decided to examine *C. dubliniensis* for the presence of homologues of the known *C. albicans SAP* genes, using probes derived from six members of the *SAP* multigene family (*SAP1*, *SAP2*, *SAP3*, S*AP4-6*). Genomic DNA from *C. dubliniensis* CD36 and CD92 isolates was digested with *Eco*RI endonuclease and Southern transfered to nylon membrane after gel electrophoresis. *C. dubliniensis* CD36 and CD92 gave identical hybridizing fragments for *SAP1*, *SAP2*, *SAP3*, S*AP4-6* probes, Fig. 2. The restriction fragment sizes (band hybridizing) to probe *SAP1* were: 4.8 and 4.0 kb; to probe *SAP2:* 4.8, 4.0, and 1.4 kb; to probe *SAP3*: 4.8 kb; and to probes *SAP4-6:* 3.3 kb. These results confirm the similitude that exists between nucleotidic sequences of *SAP1*, *SAP2,* and *SAP3* genes. However, only one band hybridizing to probe *SAP4-6* was revealed in the *C. dubliniensis* genome.

Table 1: Proteolytic enzyme activities in soluble cellular fraction of *C. dubliniensis*

Media	Growth phase[a]	Growth time (h)	ycdAPE[b]	ycdDAP[b]	ycdCP[b]	ycdPrA[b]	ycdPrB[b]
			Specific activity (mU/mg of protein)				
YPD	I	12	117.54	3.15	4	0.35	3.02
	II	24	174.72	4.3	11.38	0.53	8.1
	III	48	177.76	7.0	18.04	0.45	8.9
YNB-peptone	I	6	3.23	0.72	0.7	0.06	3.18
	II	18	7.65	0.67	2.48	0.07	7.46
	III	48	5.22	1.08	3.36	0.05	0.0
YNB-proline	I	6	2.69	0.66	0.73	0.04	0.85
	II	18	1.63	0.24	2.14	0.14	10.75
	III	48	3.12	0.81	2.85	0.07	0.41
YNB-ammonium	I	6	3.01	0.68	0.42	5.08	2.78
	II	18	6.01	0.47	2.39	0.13	1.52
	III	48	0.94	0.0	0.36	0.18	1.72

[a]Exponential (I), early stationary phases (II), late stationary phases (II).
[b]Cellular fractions were prepared from cells grown in the corresponding medium. The 100,000 *g* soluble fraction was used to determine ycdAPE, ycdCP, ycdDAP, ycdPrA and ycdPrB enzymatic activities against Lys-4-NA, Ala-Pro-4-NA, N-Bz-Tyr-4-NA, acid-denatured hemoglobin and HPA.

Table 2: Distribution of enzyme activities in cellular fractions of *C. dubliniensis*

Enzyme	**S** $_{23,000\ g}$ [a]	**S** $_{100,000\ g}$ [a]	**M** $_{100,000\ g}$ [a]
	Activity (%)		
ycd APE	100	95	8
ycd DAP	100	100	10
ycd CP	100	82	0
ycdPrA	100	68	40
ycdPrB	100	100	0

[a] Cellular fractions were prepared from cells growing in the optimal YEPD media. Biomass from the culture media was recovered by centrifugation at 5ºC (5,000 *g*), and fragmented in Braun´s mill using glass beads (0.5 mm diameter).The extent of disintegration was monitored under light microscopy. The crude extract was carefully removed from the glass beads and centrifuged at 10,000 *g* at 5ºC for 10 min. The supernatant fluid was removed and centrifuged again at 100,000 *g* at 4ºC for 1.5 h using a Beckman ultracentrifuge. The 100,000 *g* supernatant (S 100,000 *g*) was considered as soluble fraction and the membranal fraction was precipitated at 100,000 *g* (M 100,000 *g*).

Table 3: Effect of specific inhibitors of proteases on the activity of the intracellular proteases ycdAPE, ycdCP, ycdDAP, ycdPrA and ycdPrB and extracellular Sap activity from *C. dubliniensis*

Inhibitors	Concentration	ycdAPE	ycdCP	ycdDAP	ycdPrA	ycdPrB	ycdSap
		Residual activity[a]					
EDTA	1 (mM)	49.37	100	85.5	95	100	98
	10 (mM)	3.74	88	60	54.5	66	80
1-10 Phenantroline	1 (mM)	40.12	83.5	80	nd[b]	55	89
	10 (mM)	11.87	66	71	nd[b]	42	82
PMSF	1 (mM)	33.46	12	14	97	46	92
	5 (mM)	9.97	8	8	80	40	85
E-64	10 (μM)	43.34	82	65	94	100	95
	50 (μM)	15.46	66	60	93	52	92
Leupeptine	20 (μM)	88.32	100	100	100	44	100
	50 (μM)	81.2	95	87	94	33	93
Pefabloc	1 (mM)	32.1	92.5	62	nd[b]	52	95
	5 (Mm)	16.45	91.4	53	nd[b]	49	94
Pepstatin A	2.5 (μM)	nd[b]	nd[b]	nd[b]	25	nd[b]	20
	25 (μM)	nd[b]	nd[b]	nd[b]	14	nd[b]	12

[a] Expressed as a percentage of the activity obtained in the absence of any added inhibitor, which was assigned a value of 100 %.
[b] nd: non determined.

DISCUSSION

This paper presents, as far as we know, the first report on intracellular proteases of *C. dubliniensis* and suggests four encoding *SAP* genes on the genome of this CNCA (*Candida* no *C. albicans*). Proteolysis plays an important role in different physiological functions of the cell and the recently discovered secreted aspartic proteinase multi-gene (*SAP*) family in *C. albicans* has complicated assessment of proteolytic activity as a factor in the onset and development of *Candida* infections.

Our finding demonstrates that *C. dubliniensis* expresses intracellular proteolytic activities similar to those found in other yeasts, such as *S. cerevisiae*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis,* and *Yarrowia lipolytica* [15-19]. There are not many reports on intracellular proteases in fungi pathogenic to humans. The obtained data revealed that proteases ycdAPE, ycdDAP, ycdCP, ycdPrA, and ycdPrB might be regulated by nitrogen and carbon sources, as happens in other fungi [20-22, 2, 18,11].

C. dubliniensis presented a soluble ycdAPE activity, this activity seems to be a metalloprotease, according to the results obtained in the inhibition assays. This inhibitory effect has been observed in other aminopeptidases isolated from yeasts, such as AP-Y from *S. cerevisiae* [23], aminopeptidase I from *S. pombe* [24], lysine aminopeptidase from *Kluyveromyces marxianus* [25], lysine aminopeptidase from *Y. lipolytica* [19]. The presence of blocking agents from serine and cysteine proteases, such as pefabloc and PMSF, inhibited the activity, suggesting that serine residues and thiol groups might be participating in the catalysis of this enzyme. Most of the aminopeptidases described in lactic bacteria are metalloproteases that have a very similar inhibition pattern to that of the aminopeptidase from *C. dubliniensis* [26-27]. An immunogenic aminopeptidase of *C. albicans* has been described as a metallopetidase of 52 kDa and has been proposed for the diagnosis of systemic candidiasis [28].

Two carboxypeptidases have been described mainly in *S. cerevisiae*: yscCPY and yscCPS [29]. We detected one carboxypeptidase activity that was located in the soluble fraction; ycdCP activity in *C. dubliniensis* is associated with an endogenous inhibitor, since it was necessary to incubate the extracts with 0.5% sodium deoxycolate to detect the activity of this enzyme [16] (data not shown). ycdCP activity seems to be a serine protease. *CYP1 C. albicans* gene encodes a protein with 74% identity with CYP from *S. cerevisiae*. Reports on carboxypeptidase actvities in *C. albicans* are scarce; howewer, an extracellular carboxyl proteinase produced by the yeast *C. albicans* enhanced vascular permeability when injected into the dorsal skin of guinea pigs [30].

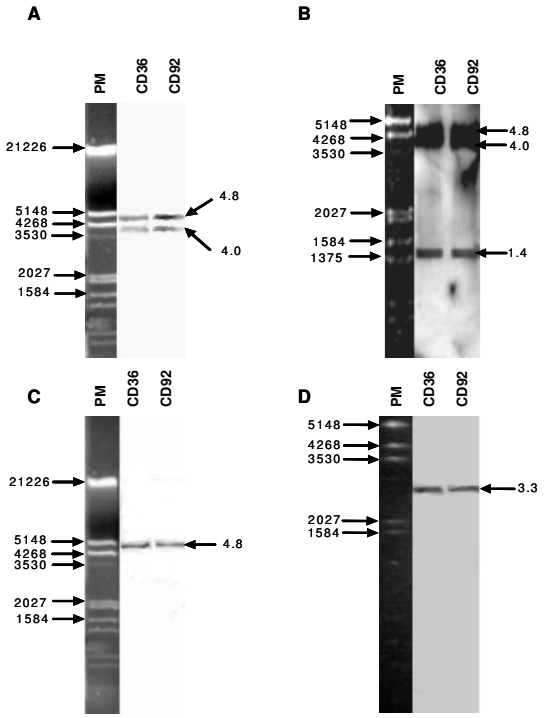

Fig. 2: Southern blots analysis of *Eco*RI digestions of *C. dubliniensis* CD36 and *C. dubliniensis* CD92 that were hybridized with the *SAP1* (Panel A), *SAP2* (Panel B), *SAP3* (Panel C) and *SAP4-6* (Panel D) genes from *C. albicans*. The molecular size markers (pb) are indicated to the left. The genes found in CD36 and CD92 are indicated to the rigth (kb).

We detected a ycdDAP activity localized in the soluble fraction. The activity of ycdDAP was inhibited by E-64, PMSF, and pefabloc, indicating that it is probably a serine protease. It is interesting to note that the dipeptidyl aminopeptidase of *S. cerevisiae*, called yscIV, is a membrane serine exopeptidase involved in the processing of the α-sexual factor [29]. A dipeptidyl-aminopeptidase encoded in the *STE13* gene has been proposed in *C. albicans* that could participate in the processing of a putative α-sexual factor or of other peptides [31]. The ycdDAP found in *C. dubiniensis* might correspond to the activity that participates in controlling post-translational processing of other peptides.

An acidic proteinase intracellular activity was detected in the soluble fraction (S 100,000 *g*). This ycdPrA activity is an aspartyl proteinase (inhibited by pepstatin). Probably this activity is similar to yscA from *S. cerevisiae*, a soluble vacuolar endoprotease that participates in protein degradation, nitrogen metabolism, acting like a vacuolar hydrolase, and precursor proccesing [29]. In other yeasts, like *C. albicans*, *S. pombe*, *K. lactis,* and *Ustilago maydis*, an intracellular acidic aspartyl PrA, inhibited by pepstatin [2, 18, 11] has also been described.

The ycdPrB activity was detected in the soluble fraction (S 100,000 *g*) and seems to be a metallo-serine-cysteine protease. In *S. cerevisiae*, the gene that encodes vacuolar proteinase yscB activity is regulated by nitrogen and carbon sources, and depends on the growth phase [32]. In turn, *K. lactis* produces a yklB similar to that detected in *S. cerevisiae* [18]. Likewise, it is known that yeasts *S. pombe* and *C. albicans* produce proteinase B activities called yspB and ycaB, respectively [2].

In *S. cerevisiae*, yscB participates in the proteolytic processing during maturation of other enzymes [29]. The role played by the ycdPrB detected in *C. dubliniesis* must still be elucidated. In *Saccharomyces carlbergensis*, proteinase B activity is regulated by an endogenous inhibitor as occurs in *S. cerevisiae* [33].

The studied yeast presents Sap activity that is associated to the logarithmic growth phase, and the enzymatic levels are higher when a protein, such as albumin, provides the nitrogen source. Similarly to the Sap described for *C. albicans*, the *C. dubliniensis* Sap is synthesized when the initial pH of the medium is acidic (3.0-5.0), suggesting the relation of one or more aspartyl proteinases, considering that in other *Candida* species more than one Sap activity have been found [34]. In *C. albicans*, the sequences of each of the *SAP1-SAP10* genes described up to now are highly homologous among them [35]. Southern analysis, using heterologous probes of *C. albicans* for genes *SAP1*, *SAP2*, *SAP3*, *SAP4-SAP6,* revealed hybridization bands of different molecular size for the *SAP* genes in the *C. dubliniensis* genome. Since the sequence of the genome of this yeast is not known yet, we can only assure that *C. dubliniensis* possesses four homologous sequences

to those of the *SAP* genes described in *C. albicans*. Most probably the 4.0 kb hybridization band is the homologous of the *SAP1* gene of *C. albicans*; the 1.4 kb hybridization band would correspond to the *SAP2* gene; the 4.8 kb band would be homologous to the *SAP3* gene. The single 3.3 kb hybridization band would correspond to any of the three *SAP* genes (*SAP4*, *SAP5*, *SAP6)* of *C. albicans*. Currently, our group is working on the deduced nucleotidic and amino-acidic sequence of these four genes.

Gilfillan *et al*. [36] reported the presence of homologue sequences to the *SAP* genes described in *C. albicans*, through Southern analysis of chromosomes separated by PFGE and hybridization of heterologous probes of *SAP1*, *SAP2*, *SAP3*, *SAP5,* and *SAP7*. Since genes *SAP4*, *SAP5,* and *SAP6* were 90% identical and could not be distinguished through hybridization with specific probes for each one, the gene *SAP5* was used as probe to search for the presence of *SAP4-SAP5-SAP6*.

C. dubliniensis is an attractive model to study the production of proteases because it is a recently identified species that is implicated in oral candidosis in HIV-infected and AIDS patients. Numerous reports have described a significant increase in the incidence and diagnosis of opportunistic and systemic candidosis during the last decade [37]. The *C. dubliniensis* species shares many phenotypic characteristics with and is closely related phylogenetically to *C. albicans*. Many studies with *C. albicans* have implicated the Sap proteinases in virulence; hence, it will be necessary to study the role played by these *C. dubliniensis* proteases.

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