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# Production of Monoclonal Antibodies for Detection of a Secreted Aspartyl Proteinase from *Candida* spp. in Biologic Specimens

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## ABSTRACT

Secreted acid proteinases (SAP) constitute an important group of virulence factors in *Candida albicans*. In the present work, an acid proteinase from *C. albicans* was sequentially purified from the supernatant of a yeast culture by precipitation with ammonium sulfate, ion exchange chromatography, and molecular exclusion chromatography, yielding a specific enzymatic activity of 204.1 IU/mg on bovine serum albumin (BSA). The molecular mass of the purified proteinase was estimated at 43 kd after exclusion chromatography and at 41 kd by nondenaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The purified proteinase was able to degrade BSA at pH 2.5, but was not active on collagen, and it was significantly inhibited by pepstatin A. The immunization of BALB/c mice with the purified proteinase and later fusion of their spleen cells with myeloma cells resulted in 19 monoclonal antibody secreting hybridomas (MAbs) capable of detecting SAP in enzyme-linked immunosorbent assay (ELISA) assays. All MAbs obtained are isotype IgG1 kappa ( $\kappa$ ) immunoglobulins and develop a 41 kd protein band by Western blot (WB) in samples of SAP obtained from *C. albicans* (12-A) and *C. dubliniensis* (strain 778) crude extracts. The anti-SAP MAbs were used in capture ELISA and two combinations of these antibodies proved suitable for SAP detection, that is, MAP1 (1B1B3) or MAP2 (2D2C10) as coat antibodies, and biotinylated MAP3 (2A6E8) as detect antibody. Capture ELISA using these sets of MAbs detected over 32 ng/mL protein in purified SAP samples as well as in crude *C. albicans* and *C. dubliniensis* extracts. The results herein obtained allow for the prediction of how this set of antibodies can be useful for SAP detection in biologic specimens.

## INTRODUCTION

**D**IFFERENT SPECIES OF FUNGI can cause infection in humans in a disseminated manner,<sup>(1)</sup> and their incidence has increased in recent years as the number of immunocompromised patients grows with the use of wide-spectrum antimycotic agents, as well as in patients subjected to invasive surgical procedures.<sup>(2)</sup> The genus *Candida* comprises an extensive group of yeasts (*C. albicans*, *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, *C. krusei*, *C. guilliermondii*, and *C. dubliniensis*) that are present in the oral cavity, digestive tract, and vascular sys-

tem of humans, which maintain these microbial agents as commensals. However, local or systemic alterations in the organism, together with the various virulence factors of these microorganisms, encourage the development of their pathogenic action, causing infectious diseases such as candidiasis.<sup>(1,3)</sup> Little is known about the mechanisms by which microorganisms of the genus *Candida* cause disease in debilitated patients. However, some virulence factors seem to contribute to the pathogenicity of this yeast species, such as adhesins, which recognize host biomolecules, promoting the yeast's adherence to the cell surface, especially in mucosae; phenotypic variability

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(switching) involved in the reversible transition between the unicellular and filamentous forms; and hydrolytic toxins and exoenzymes, such as aspartyl proteases, which favor dissemination of the fungus.<sup>(4-6)</sup> Additionally, phenotypic switching is accompanied by changes in antigen expression (antigen variability), morphology of the colony, affinity with the tissues in which these microorganisms are found, thus allowing flexibility in the adaptation of yeasts to the hostile conditions imposed by the host and by the clinical treatment of infection.<sup>(3)</sup>

*C. albicans* is considered one of the most important pathogens in the genus *Candida*,<sup>(7)</sup> but many patients may harbor more than one species simultaneously, especially immunocompromised patients.<sup>(5)</sup> Currently, the diagnosis of these microorganisms and treatment of bearers is very difficult. The identification of different *Candida* species can be made by means of biochemical tests, conducted in media designed especially for grouping certain species of the fungus. The CHROMagar *Candida* medium separates *Candida* species into four groups: (1) *C. albicans* and *C. dubliniensis*; (2) *C. tropicalis*; (3) *C. krusei*; and (4) other species.<sup>(6)</sup> The formation of chlamydospores in rice agar-Tween 80 is a trait exhibited by *C. albicans* and *C. dubliniensis* but not by the other species of the fungus.<sup>(8)</sup> *C. dubliniensis* is a species highly related to *C. albicans* both phenotypically and genetically. These are the only species in the genus that, in addition to the formation of pseudohyphae, can also form germ tubes and true hyphae. The differentiation between *C. albicans* and *C. dubliniensis*, however, can be obtained by growing the first at 45°C<sup>(9)</sup> or by polymerase chain reaction (PCR).<sup>(10)</sup> With regard to their sensitivity to antimycotic agents, there have been reports of *C. albicans*, *C. krusei*, and *C. dubliniensis* isolates resistant to fluconazole, a wide-spectrum antimycotic agent generally administered for candidiasis treatment.<sup>(11,12)</sup>

The first description of *C. dubliniensis* as a distinct species from *C. albicans* was made in samples obtained from oral infections in human immunodeficiency virus (HIV)-infected and acquired immune deficiency syndrome (AIDS) patients.<sup>(8)</sup> Subsequent investigations in *C. albicans* culture collections revealed that approximately 2% of the yeasts were actually *C. dubliniensis* isolates.<sup>(11)</sup> Since then, *C. dubliniensis* has been found around the world associated with superficial or systemic infections.<sup>(13)</sup> Because of its similarity with *C. albicans*, differentiation methods for these two species have been extensively investigated. Pal's Agar culture medium, based on the use of sunflower seed extract (*Helianthus annuus*), seems to be the most suitable medium to differentiate between both *Candida* species.<sup>(14)</sup> More sophisticated methods have also been used to evaluate the differences and similarities that exist between these two *Candida* species, such as PCR, DNA fingerprinting, and pulsed-field gel electrophoresis; these, however, are costly and time-consuming methods, and are difficult to apply for a large number of isolates.<sup>(15)</sup> Together, these methods allowed the verification that homologous *C. albicans* SAP genes are found in *C. dubliniensis* yeasts. However, both species have been shown to be significantly different in aspects such as sensitivity to antimycotic agents, adherence to human mucosal epithelial cells, and virulence to murines.<sup>(16)</sup>

The extracellular acid proteinase termed proteinase A was described for the first time in *C. albicans* in 1964.<sup>(17)</sup> Some of the acid proteinases secreted by certain yeast species of the

genus *Candida* have the capacity to degrade host proteins, such as albumin, collagen, immunoglobulins, keratin, and hemoglobin<sup>(18-22)</sup> and may have a pathogenic effect depending on the conditions of the host organism. The properties of acid proteinases from *C. albicans* have been partially elucidated through studies conducted by several investigators.<sup>(23,24)</sup> However, the purification and characterization of an aspartyl proteinase secreted in *C. albicans* culture filtrates could only be obtained in 1997, by Na and collaborators.<sup>(25)</sup> Subsequently, these authors<sup>(26)</sup> produced a monoclonal antibody called CAPI, which detected the proteinase secreted by different *C. albicans* strains, but not the proteinases of other *Candida* species. In a later study, these authors reported that CAPI is capable of detecting the acid proteinase that circulated in serum from patients.<sup>(27)</sup>

In the present work, we report the purification of an aspartyl proteinase from *C. albicans*, its biochemical characterization, and use in mice immunization to obtain monoclonal antibody secreting hybridomas to be used in SAP detection assays.

## MATERIALS AND METHODS

### *Candida* spp. strains

Thirteen *Candida albicans* and *C. dubliniensis* strains among those available from the Fungus Culture Collection of FOP/UNICAMP's Microbiology and Immunology Laboratory, and those kindly made available by Dr. Claudete Rodrigues de Paula (Instituto de Ciências Biomédicas [ICB] Universidade de São Paulo/SP), previously isolated from the oral cavity of patients, were characterized by CHROMagar *Candida*® (Probac do Brasil, São Paulo, Brazil), by formation of chlamydospores in rice Agar-Tween 80 medium, and by formation of germ tubes in normal rabbit serum.<sup>(28)</sup> In order to identify acid proteinase-secreting strains, *Candida* spp. samples were inoculated in YNB-BSA Agar medium, containing 0.2% bovine serum albumin (BSA; Sigma, St. Louis, MO), 1.45 g Yeast Nitrogen Base (YNB; ammonium sulfate- and amino acid-free; DIFCO, Detroit, MI), supplemented with 2% glucose (MERCK, Whitehouse Station, NJ) and 2% agar (MERCK), at pH 4.0, and incubated at 37°C for 72 hours. The presence of the enzyme was detected by the formation of an opaque halo around the yeast colony, and enzymatic activity (Pz) was determined according to indications of Price et al.<sup>(29)</sup> Except for *C. albicans* strain ICB-158 and *C. dubliniensis* strain ICB-159, which were used as negative controls, the other *Candida* strains showed strongly positive enzymatic activity in acid medium. Sample type 12-A of *C. albicans* was selected for the production and purification of acid proteinase, since it was the best serologically characterized strain in our collection. The 12-A strain was initially grown in YPD liquid medium containing yeast extract at 1% (w/v), peptone at 1% (w/v), and dextrose at 2% (w/v), until an optical density (OD) at 660 nm equal to 1.0 was obtained. The yeasts were settled by centrifugation, resuspended to the initial volume in YNB-BSA liquid medium and incubated for 48 hours at 30°C. The culture supernatants were collected by centrifugation and sterilized by filtration through a 0.22- $\mu$ m membrane (Millex, Millipore, Billerica, MA) for subsequent purification of the acid proteinase.

### Mice

Female BALB/c mice were supplied by CEMIB (Centro Multinstitucional de Investigações Biológicas), UNICAMP, and maintained in our facilities under specific pathogen-free conditions, with autoclaved food and water *ad libitum*. Mice were used for immunizations at the age of 8 weeks. The Institutional Committee for Ethics in Animal Experimentation approved this study.

### *C. albicans* acid proteinase purification

The *C. albicans* strain 12-A culture filtrate was used for purification of an acid proteinase according to indications of Na and collaborators,<sup>(25)</sup> with modifications. In short, the proteins were precipitated by the addition of ammonium sulfate at 75% (w/v) to the culture filtrate. The precipitate was dissolved in 50 mM sodium phosphate buffer solution, pH 6.5, and desalted in a Sephadex G-25 column (Sigma), previously equilibrated with the same buffer. Next, the mixture of proteins was chromatographed in a DEAE-Sepharose Fast Flow column, previously equilibrated with the 50 mM phosphate buffer, pH 6.5, and the fractions were eluted with a gradient of 150 to 400 mM NaCl, at a flow rate of 40 mL/hr; aliquots (3.0 mL per tube) were collected. Absorbances at 280 nm and enzymatic activity on BSA were monitored for each fraction collected. Chromatographic fractions that showed enzymatic activity were pooled, submitted to dialysis against deionized water and then freeze dried. A 5 mg-aliquot of the freeze dried powder was resuspended in 240  $\mu$ L Tris-NaCl buffer (25 mM Tris, 10 mM NaCl), pH 7.0, and injected into a column containing agarose gel (Superose 12/30 HR-Pharmacia Biotech, Uppsala, Sweden), previously equilibrated with the same buffer and attached to a high-performance liquid chromatography (HPLC) system (AKTA-purifier, Pharmacia Biotech). Elution of proteins was performed at a flow rate of 0.5 mL/min and monitored at 210, 220, and 280 nm, using the UNICORN 3.00 software. Fractions showing enzymatic activity were pooled and the protein concentration in the pool was determined by Bradford's method.<sup>(30)</sup>

### Enzymatic activity and proteinase inhibition assay

Enzymatic activity was determined by digestion of the BSA substrate, according to Crandall and Edwards<sup>(17)</sup> and was considered positive for OD<sub>280nm</sub> values equal to or higher than 0.03. In short, 270  $\mu$ L BSA at 1% (w/v) in 50 mM KCl buffer, pH 2.5 were added to each 30  $\mu$ L of solution containing the enzyme, and the mixture was incubated at 37°C for 2 hours. The enzymatic reaction was stopped by the addition of 700  $\mu$ L 10% trichloroacetic acid (TCA) (w/v), at 4°C. The precipitated protein was removed by centrifugation at 12,500g for 5 minutes. Proteolysis was monitored by observing the OD<sub>280nm</sub> increase in the supernatant, and one enzymatic activity unit was defined as the amount of enzyme required to increase OD by 0.1 unit. The enzymatic activity assays were repeated in the presence of pepstatin A, a pepstatin-type proteinase inhibitor, as indicated by Na et al.<sup>(25)</sup>

### Polyacrylamide gel electrophoresis

Fractions containing the acid proteinase were analyzed by polyacrylamide gel electrophoresis at 10% (w/v) in the pres-

ence of SDS-PAGE, according to Laemmli.<sup>(31)</sup> The proteins used as reference standards were: phosphorylase b, 97 kd; BSA, 66 kd; ovoalbumin, 45 kd; carbonic anhydrase, 30 kd; trypsin inhibitor, 20.1 kd; and lysozyme, 14.4 kd (low molecular weight standard, LMW; Pharmacia Biotech). After running the electrophoresis, the gels were stained with silver nitrate, according to Blum et al.<sup>(32)</sup>

### Enzymatic activity in polyacrylamide gel

Polyacrylamide gels at 10% containing SDS were copolymerized with 1.6 mg/mL gelatin or BSA (Sigma). The chromatography fractions containing the *C. albicans* proteinase were diluted in reducing buffer and separated by electrophoresis as described above. Next, the gels were soaked in Triton X-100 solution at 2% (Sigma) and then dipped into the 10 mM Tris-HCl buffer, containing 5 mM CaCl<sub>2</sub>, pH 8.0, or in 50 mM KCl-HCl buffer, pH 2.5 to activate the enzyme. After incubation at 37°C for 16 hours, the gels were stained with 1% Coomassie brilliant blue R-250 in 50% methanol and 10% acetic acid.

### Immunization

BALBc mice were immunized by intraperitoneal injection of 100  $\mu$ g purified proteinase emulsified with 50% complete Freund's adjuvant (Sigma). On days 20 and 30 after the first injection, each animal received booster doses consisting of 50  $\mu$ g antigen diluted in saline solution via intraperitoneal injection. On day 33, the presence of specific antibodies in mouse sera was investigated by indirect ELISA. The preimmune sera of those animals were used as negative controls in the ELISA reactions.

### Obtaining and cloning hybridomas

A cell fusion experiment to obtain hybridomas was carried out following the directions of de St. Groth and Scheideger.<sup>(33)</sup> In short, the mice immunized with the purified proteinase were euthanized, their spleens were aseptically removed and macerated to obtain a cell suspension in complete culture medium (RPMI-1640 medium [Sigma] containing 10% fetal bovine serum [Nutricell, São Paulo, Brasil], 2 g/L sodium bicarbonate, 2 g/L HEPES (N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]) [Sigma], and 2  $\mu$ L/L 2  $\beta$ -mercaptoethanol [Merck]). After washing with saline solution buffered with 50 mM sodium phosphate, pH 7.2 (phosphate-buffered saline [PBS]), containing 0.4 g/L potassium chloride, 2 g/L glucose and 0.01 g/L phenol red, the pellet formed by  $1 \times 10^8$  spleen cells and  $2 \times 10^7$  SP2 Ag14/0 myeloma cells<sup>(34)</sup> was slowly resuspended in 1 mL 50% polyethylene glycol (PEG 1500; MERCK) and 10% dimethyl sulfoxide (DMSO; Sigma) in saline solution, and kept under agitation for 1 minute, at room temperature. Next, the cell suspension was kept at 37°C, for 90 seconds, under agitation. The suspension was then slowly diluted by adding 20 mL PBS, pH 7.2 during 2 minutes. The volume was completed to 50 mL with PBS pH 7.2 and the suspension was centrifuged at 200g for 10 minutes. Finally, the pellet containing the fused cells was resuspended in 100 mL HAT medium [RPMI1640 medium containing: 20% SFB, 0.136 mg/L hypoxanthine (Sigma), 0.03 mg/L aminopterin (Sigma),

and 0.038 mg/L thymidine (Sigma)] and distributed through four 24-well culture plates (1 mL/well), which were maintained at 37°C in an incubator with 5% CO<sub>2</sub>. Approximately 10 days after cell fusion, the supernatants from the culture wells containing hybridomas were tested for the presence of anti-SAP antibodies by indirect ELISA, using the purified enzyme as antigen (2 µg/mL; 50 µL per well), as previously described.<sup>(35)</sup> Positive cultures in the ELISA tests were cloned by limiting dilution in 96-well culture plates (Corning, Corning, NY). All anti-SAP monoclonal antibody secreting hybridomas were subsequently expanded in T25 and T75 culture flasks, and some were inoculated into BALB/c mice to obtain ascitic fluid containing the monoclonal antibodies.<sup>(35)</sup> The hybridomas obtained in all stages after fusion were preserved in liquid nitrogen.

#### Determination of MAb immunoglobulin isotypes

Immunoglobulin isotyping of MAbs anti-SAP was performed by ELISA using the supernatants of hybridoma cultures and the ImmunoPure Monoclonal Antibody Isotyping kit I-HRP/ABTS (Pierce, Rockford, IL; anti-IgG1, anti-IgG2a, anti-IgG2b, anti-IgG3, anti-IgA, and anti-IgM). Reading was performed at 405 nm, considering OD values ≥ 0.8 as positive results, according to the manufacturer's instructions.

#### MAb purification and conjugation with biotin

Ascites containing anti-SAP MAbs were purified by affinity chromatography in Sepharose-Protein G (Sigma), as indicated by Akerstrom et al.,<sup>(36)</sup> with modifications. In short, 5 mL ascites were equilibrated with 100 mM dibasic sodium phosphate solution until pH 8.0 and then applied to a column containing 4 mL adsorbent gel. After the mixture was homogenized and incubated for 30 minutes, the immunoglobulins adsorbed to the protein G were eluted at a flow rate of 30 mL/hr with 100 mM glycine-HCl buffer, pH 2.8, in 2.5 mL aliquots, which were collected in tubes containing 1 M Tris-HCl buffer, pH 8.5, in ice bath. Elution was monitored in a spectrophotometer (DV-65, Beckman Instruments, Inc., Palo Alto, CA) at 280 nm, and fractions with OD higher than 0.3 were pooled, submitted to dialysis against PBS pH 7.2, and protein concentration was determined by Lowry's method modified by Hartree.<sup>(37)</sup>

Aliquots (1 mg) from each purified MAb were dissolved in 1 mL 20 mM sodium borate buffer, pH 9.0, and submitted to dialysis against the same buffer, at 4°C for 18 h. Next, 200 µL of a biotin succinimide solution (Sigma) were added to the antibody solution and the mixture was incubated for 4 hours, in the dark. Unbound biotin was removed from the protein solution by dialysis against PBS at 4°C for 18 hours. The biotinylated antibodies were diluted with buffered glycerin (v:v) and stored at -20°C until used in the capture ELISA tests.

#### Western blot

Samples containing *Candida* spp. acid proteinase were submitted to SDS-PAGE as described above, and then the proteins were transferred from the gel to 0.45 µm nitrocellulose membranes according to indications by Towbin et al.,<sup>(38)</sup> using the Mini Transblot II system (Bio-Rad, Hercules, CA). Next, the membranes were washed five times with PBS containing 0.05% Tween 20 (PBST) and then immersed into the PBST blocking

solution containing 5% skimmed milk (PBST-SM) for 1 hour at room temperature. The membranes were washed again, cut into strips, and incubated with culture supernatants from the hybridomas or with ascites serially diluted in PBST-SM containing 2% normal rabbit serum for 1 hour at room temperature and overnight at 4°C. The strips were washed with PBST and incubated for 2 hours at room temperature with the mouse peroxidase-conjugated anti-IgG (1:250) in PBST-SM containing 2% normal rabbit serum. After another washing cycle, the reactions were developed with 0.03% H<sub>2</sub>O<sub>2</sub> and 0.5 mg/mL 3,3'-diaminobenzidine (DAB) in 0.05 M Tris-HCl buffer, pH 7.4 until bands appeared. The reaction was stopped with tap water.

#### Capture ELISA

Fifty microliters of purified MAb, diluted in 50 mM 0.1 M sodium carbonate-bicarbonate buffer, pH 9.2, were added to each well of 96-well polystyrene, flat-bottom plates (Greiner, Monroe, NC), and the plates were incubated for 1 hour at 37°C and overnight at 4°C. After washing with PBST, 200 µL of a block solution (PBS-5% SM) were added to each well, and plates were incubated for 1 hour at 37°C. After a new washing cycle, 50 µL per well of serial proteinase dilutions in PBST-2% SM were added with two replications and plates were incubated for 2 hours at 37°C. After washing, 50 µL of the biotinylated MAbs properly diluted in PBS-2% SM were added, and plates were incubated for another 2 hours at 37°C. After washing, 50 µL of a peroxidase-conjugated streptavidin solution (Sigma) diluted at 1:200 in PBS-2% SM were added to each well, followed by incubation of the plates for 1 hour at 37°C. After another washing cycle, development was accomplished by adding substrate (0.03% H<sub>2</sub>O<sub>2</sub> and 0.04% orthophenyldiamine in 50 mM citric acid/disodium hydrogen phosphate buffer, pH 5.5) to each well. After 30 minutes in the dark, the reaction was stopped by adding 25 µL of 4 N H<sub>2</sub>SO<sub>4</sub> to the wells, and absorbances were read at 492 nm (Multiskan MS, Labsystems, Helsinki, Finland).

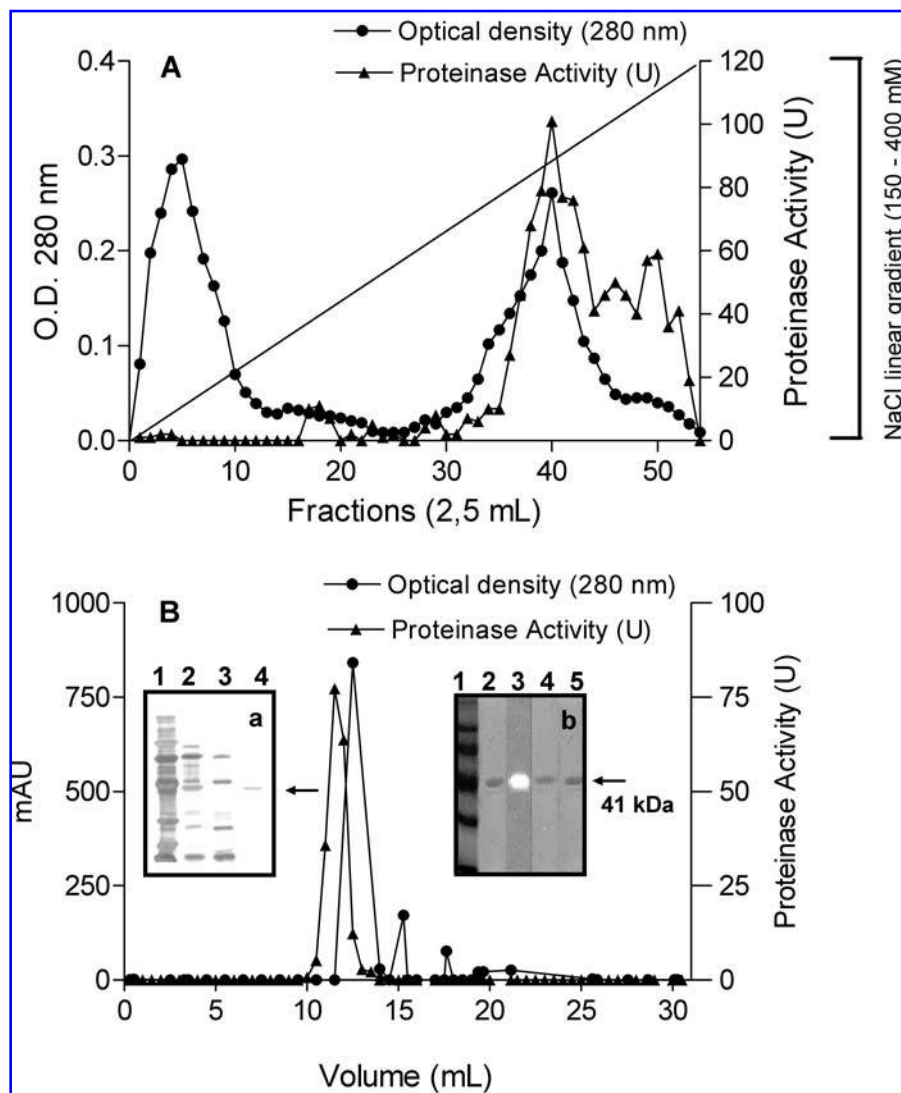
#### Statistical analysis

Student's *t* test was used for comparisons between groups. Variations were considered statistically significant for *p* values < 0.05 in analyses performed with the GraphPad Prism Software.

## RESULTS

#### Purification of an extracellular acid proteinase

An extracellular acid proteinase was purified from the *C. albicans* strain A-12 culture supernatant in three steps, which included precipitation with ammonium sulfate at 75%, DEAE ion exchange chromatography, and Sepharose 12 molecular exclusion chromatography. Figure 1 illustrates the results obtained in one of these experiments, showing absorbance at 280 nm and the enzymatic activity of fractions eluted in each purification stage. It can be observed that after DEAE chromatography, enzymatic activity on BSA was recovered in fractions that were eluted around 300 mM NaCl of a linear NaCl elution gradient between 150 and 400 mM (Fig. 1A). These fractions were



**FIG. 1.** *Candida albicans* extracellular acid proteinase purification. Proteins resulting from the precipitation of 2 L of *C. albicans* 12-A culture broth with 75% ammonium sulfate were sequentially chromatographed on ion exchange DEAE cellulose (A) and molecular exclusion sepharose columns (B) as detailed in Materials and Methods. Elution of chromatographic fractions was accompanied by absorbance at 289 nm and by enzymatic activity over bovine serum albumin (BSA) at pH 2.5. Fractions containing acid proteinase were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (insert a) or with the protein substrates incorporated into the gel (zymograms, insert b). This experiment is representative of three other experiments conducted separately.

pooled and freeze-dried after dialysis against water. A sample containing 5 mg of the freeze-dried powder was resuspended and then chromatographed in molecular exclusion resin and enzymatic activity was recovered in fractions eluted at a volume between 10 and 15 mL Sepharose 12 (Fig. 1B). The purified fraction was eluted from Sepharose at the range corresponding to proteins with a 43-kd molecular mass. However, the SDS-PAGE analysis of the purified fraction revealed the presence of a homogeneous silver-stained protein band with a 41-kd mass, which exhibited enzymatic activity over BSA copolymerized with polyacrylamide gel at pH 2.5, but not with collagen or any of the proteins tested at pH 8.0 (inserts in Fig. 1B). The enzymatic activity of the fraction purified by these procedures was inhibited by about 56% in the 1.46  $\mu$ M pepstatin A treatment at a 10:1 proteinase:inhibitor ratio.

#### Obtaining anti-SAP hybridomas

BALB/c mice were immunized with the purified enzyme and when anti-SAP serum antibodies reached titers around 1:6400, their spleen cells were used as a source of immune B-lymphocytes in fusion experiments to obtain anti-SAP MAb secreting hybridomas. In the first cultivation step after fusion, all wells in the four 24-well plates showed growing hybridomas (100% growth), among which seven revealed the presence of anti-SAP antibodies in their supernatants (7%), as demonstrated by indirect ELISA. The hybridomas in the seven positive cultures were cloned by limiting dilution in 96-well plates and at the end of the selection process 19 anti-SAP monoclonal antibody secreting hybridomas were obtained, all of the IgG1 kappa ( $\kappa$ ) isotype (Table 1). According to the position they occupied in the

TABLE 1. GENERATION OF ANTI-SAP, MAb-PRODUCING HYBRIDOMAS

Fusion plates	Positive cultures	Hybridoma clones <sup>a</sup>	MAB isotypes <sup>b</sup>
1	3/121	1D6B5, 1D6E9, 1D6F5, 1D6G3 1B1B3 (MAP1), 1B1C2, 1B1D6, 1B1G1, 1B1G2, 1B1H4 ID2	IgG1 κ IgG1 κ ND
2	2/92	2A6B11, 2A6C9, 2A6E8 (MAP3), 2A6F6 2D2B1, 2D2E8, 2D2C10 (MAP2), 2D2F9, 2D2E9	IgG1 κ IgG1 κ
3	1/82	3D4	ND
4	1/100	4C5	ND

<sup>a</sup>Anti-SAP producing hybridomas were screened by ELISA with purified proteinase.

<sup>b</sup>MAbs isotypes were determined by ELISA with a commercial kit (Pierce, Rockford, IL).

ND, not determined; SAP, secreted acid proteinases; MAB, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay.

fusion and cloning plates, these hybridomas were initially called: 1B1 (B3, C2, D6, G1, G2, H4); 1D6 (B5, E9, F5, G3); 2A6 (B11, C9, E8, F6); 2D2 (B1, E8, C10, F9, E9). In addition to these hybridomas, two cloning-plate cultures showed anti-SAP secreting cells, but with more than one Ig isotype (IgM, in addition to IgG1), indicating the need for recloning.

#### STUDY ON THE BINDING PROPERTIES OF MONOCLONAL ANTIBODIES TO SAP

One representative of each of the four anti-SAP MAb-secreting hybridoma families (1B1B3, 1D6E9, 2A6E8, 2D2C10) were expanded as ascitic tumors in the peritoneal cavity of BALB/c mice to obtain a mass of monoclonal antibodies to be purified, biotinylated, and studied in ELISA- and Western blot-type immunoenzymatic assays. Different purified MAb combinations were used as capture or detection antibodies (biotinylated antibodies) in the ELISA tests, at concentrations of 10 or 100 µg/mL. Figure 2 shows that antibodies 1B1B3 (MAP1) and 2D2C10 (MAP2), used as capture antibodies, and biotinylated 2A6E8 (MAP3) were able to detect the purified SAP enzyme at protein concentrations from 32 ng/mL. These MAbs were also capable of detecting the enzyme in *C. albicans* and *C. dubliniensis* crude extract preparations in ELISA assays at protein concentrations from 64 ng/mL (data not shown).

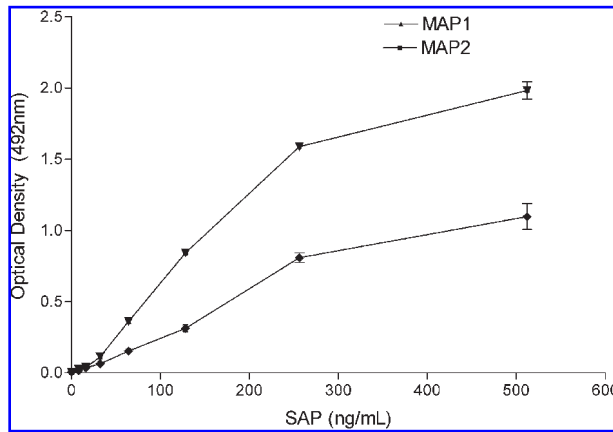
The proteins contained in the *C. albicans* purified fraction and in crude *C. albicans* or *C. dubliniensis* extracts were separated by electrophoresis in SDS-PAGE minigels and then transferred to nitrocellulose membranes. The nitrocellulose membranes were cut into strips and reacted with the culture supernatants or with ascites containing MAbs. Figure 3 illustrates results when MAbs 1B1B3 (MAP1), 1D6E9, 2A6E8 (MAP3), and 2D2C10 (MAP2) were used as supernatants (1:400) or ascites (1:1600) in the reactions involving *C. albicans* purified SAP (Fig. 3A), *C. albicans* strain 12-A crude extract (Fig. 3B), or *C. dubliniensis* strain 778 crude extract (Fig. 3C). The antibodies of any of the two sources used showed the same reaction profile with *Candida* spp proteins, with recognition of one 41-kd band. In the purified enzyme preparations, other bands besides the 41 kd could be observed after reaction with the antibodies, suggesting the presence of autodegradation components of the enzyme itself.

#### DISCUSSION

Among the species in the genus *Candida*, *Candida albicans* is considered the most important opportunistic pathogen in humans. This microorganism invades the mucosa causing candidiasis, a fungal infection with different degrees of severity.<sup>(5)</sup> One of the most important virulence factors associated with invasive candidiasis are extracellular acid proteinases, hydrolytic enzymes produced by *Candida*,<sup>(5,39)</sup> which allow the proteolytic invasion of tissues by these yeasts and interfere with host cell membrane integrity, leading to dysfunctions of their normal activities.<sup>(4,39,40)</sup> The acid proteinase seems to facilitate the adherence, colonization, growth, and invasion of these microorganisms on the skin and mucosae.<sup>(19,41)</sup>

Consequently, the production of extracellular proteolytic enzymes may serve as a pathogenicity marker for these microorganisms.<sup>(42)</sup> A high enzymatic activity of the acid proteinase on albumin (BSA) in solid medium was observed in 11 of the 13 *Candida* strains studied in the present work. *C. albicans* strain ICB-158 and *C. dubliniensis* strain ICB-159 were exceptions, but these strains are known to not produce acid proteinase. SDS-PAGE of the purified proteinases showed a protein band of approximately 41 kd, and zymograms showed that the 41-kd band had enzymatic activity only on BSA, at pH 2.5.

The *C. albicans* acid proteinase has been considered an "aggressin," as a result of its capacity to degrade host proteins in acid microenvironments.<sup>(39)</sup> Corroborating our results, an acid aspartyl proteinase isolated from *C. albicans* was capable of degrading collagen and BSA, with maximum enzymatic activity at pH values between 2.0 and 3.5 (acid) and irreversible denaturation at alkaline pH values.<sup>(25)</sup> However, the proteinase isolated here was only active on BSA, and was not capable of degrading collagen, even after a 16-hour incubation period. Data from the literature show that the extracellular proteolytic activity of *C. albicans* is strongly associated with enzymes of the aspartyl proteinase type,<sup>(18–22,43–46)</sup> which exhibit biochemical properties similar to pepsin and cathepsin, enzymes sensitive to treatment with reduced concentrations of pepstatin A.<sup>(47)</sup> The purified acid proteinase in this work also had its activity in acid pH dramatically reduced by treatment with 1.46 µM pepstatin A, indicating it is related to the aspartyl acid proteases (SAP) described in *C. albicans*.



**FIG. 2.** Enzyme-linked immunosorbent assay (ELISA) for *Candida albicans* acid proteinase detection (secreted acid proteinases [SAP]). Monoclonal antibodies (MAbs) 1B1B3 (MAP1) and 2D2C10 (MAP2) were used as capture antibodies at a concentration of 10  $\mu\text{g/mL}$ . The purified SAP fraction was used as concentrations from 8 to 12 ng/mL. This experiment is representative of three other experiments conducted separately.

Although they were described more than two decades ago, *C. albicans* extracellular acid proteinases have not yet been well characterized. The species *C. albicans* can produce several aspartyl proteinase isoforms, coded by approximately 10 distinct genes,<sup>(42)</sup> some of which are not yet characterized from a biochemical and immunochemical point of view. Our results suggest that the enzyme purified in this work shares several properties with aspartyl proteinases, but is different from acid proteinases previously isolated from *C. albicans* in some peculiarities.

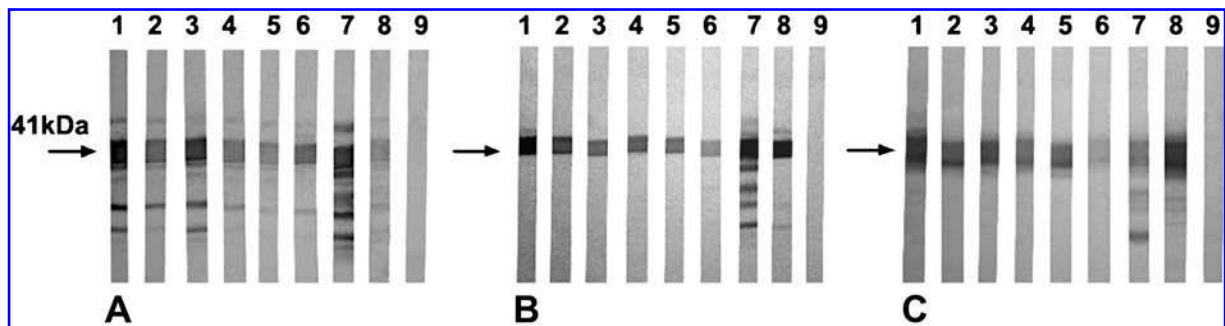
The genetic expression of each SAP isoform seems to be associated not only with conditions of the environment where yeasts are found, but also with stage of infection. SAP 1 and 3, for example, present greater expression in patients with oral candidiasis or HIV-positive patients with oropharyngeal candidiasis, in which they cause severe damage to the epithelium of the mucosa.<sup>(48,49)</sup> Different SAP isoforms present distinct pH requirements for their maximum activity.<sup>(50)</sup> The strain of *C.*

*albicans* used in this work was isolated from the oral cavity of a patient with candidiasis and its activity at pH 2.5 makes it more similar to SAP1 than to SAP3, since the latter is not very active in acid pH.

Proteinases with activity at different pH ranges represent an important adaptation toward allowing the colonization of different anatomic sites by *C. albicans*. Among yeasts of the genus *Candida*, those in the species *C. albicans* show the highest number of genes (10 genes) of the SAP family.<sup>(51)</sup> This high number of genes may be related to the frequency of clinic isolates, but could also be associated with a higher capacity of colonization and consequent pathogenic potential of the species.<sup>(52)</sup>

In order to produce a highly specific reagent for the isolated proteinase, a cell fusion experiment was conducted to obtain specific monoclonal antibody secreting hybridomas. In a single cell fusion experiment, we obtained 19 SAP-specific monoclonal antibody secreting clones. All MAbs were capable of detecting the 41 kD protein by Western blot in the preparation of purified SAP, as well as crude 12-A *C. albicans* and 778 *C. dubliniensis* extracts. In addition to the isolated clones, two cultures in the cloning plates showed MAbs with more than one isotype (IgM,  $\kappa$  and  $\lambda$ ; IgG2a,  $\kappa$  and  $\lambda$ ), which were cryopreserved to become sources of other anti-SAP MAbs in the future.

The aspartyl proteinases normally produced by pathogenic *Candida* strains induce the production of high antibody titers in infected patients,<sup>(25,53,54)</sup> which makes them natural candidates for use as antigens in the serodiagnosis of candidiasis. However, the detection of the enzyme itself in biologic fluids of patients may represent an important tool in the diagnosis of candidiasis and in following up the treatment of infected patients. For this reason, some authors have invested in the production of highly specific MAbs for *C. albicans* acid proteinase in order to develop capture assays for the enzyme in the serum of patients.<sup>(26)</sup> Nevertheless, only one anti-SAP MAb-secreting clone called CAP1 was obtained, which recognized a sequence between SAP amino acids Leu<sup>27</sup> and Glu<sup>132</sup>. The genetic cloning products of this region and their expression in *Escherichia coli* revealed proteins with molecular masses of 34.2 and 35.3 kD, which were recognized by the MAb CAP1. In our study, three of four anti-SAP MAbs proved effective in detecting the enzyme in the ELISA assays at concentrations from 32



**FIG. 3.** Western blot analysis of anti-secreted acid proteinase (SAP) monoclonal antibodies (MAbs). Culture supernatants and ascitic fluids containing the anti-SAP MAbs 1B1B3 (Lanes 1 and 2), 1D6E9 (Lanes 3 and 4), 2A6E8 (Lanes 5 or 6) or 2D2C10 (Lanes 7 and 8) were used at a dilution of 1:400 and 1:1,600, respectively, for the detection of SAP in three different preparations of candida yeasts, which were transferred to nitrocellulose membrane (140 mg/channel). (A) SAP isolated from *C. albicans*; (B) Crude extract from *C. albicans*; (C) Crude extract from *C. dubliniensis*. Pre-immune serum of mouse was used as the negative control at a dilution of 1:50 (Lane 9). Arrows indicate SAP molecular weight (41kDa).

ng/mL, that is, MAP1 (1B1B3) or MAP2 (2D2C10) as coat antibodies and biotinylated MAP3 (2A6E8) as a detect antibody. These antibody pairs also detected the enzyme in crude *C. albicans* and *C. dubliniensis* (strain 778) extracts from 64 ng/mL.

In the immunoblottings carried out in our study we were able to observe several bands with molecular masses smaller than 41 kd recognized by the MAbs in the purified fraction and in the crude extracts blotted onto nitrocellulose. It is possible that the material may have undergone autodegradation during the enzyme handling and storage, which could explain the low detection of these antigens in the ELISA-type assays.

Although more sophisticated assays have been developed to detect differences between *Candida* species, such as fluorescence in situ hybridization using peptide nucleic acid probes (PNA-FISH), which detects differences in the rRNA sequence between *C. albicans* and *C. dubliniensis*<sup>(55)</sup> or the phage display technology to study the expression of surface antigens in *C. albicans*,<sup>(56)</sup> methodologies that explore serologic differences between these two species, with immunologic reagents efficient for the quick identification of these pathogens, represent a potential yet to be explored.<sup>(57–59)</sup> Thus, obtaining immunologic reagents such as MAbs, which recognize relevant *C. albicans* or *C. dubliniensis* antigens, will expand the repertoire of potential reagents for the diagnosis and differentiation between these related species. This is particularly desirable at a moment when it is observed that the indiscriminate use of antifungal drugs in past decades has contributed toward the appearance of azole-resistant *Candida* strains.<sup>(11)</sup> A precise diagnosis about the *Candida* species causing the infection will provide orientation in the selection of the most suitable antimycotic agents for the patient, and will be also useful in monitoring the effectiveness of the antifungal treatment.

Therefore, the hybridomas obtained in this work will be subsequently analyzed as to their capacity to capture the acid proteinase (SAP) present in fluids such as saliva or serum of patients colonized by different yeast species of the genus *Candida*, perhaps providing a sensitive and specific assay to diagnose infection by these yeasts.

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