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Characterization of a secreted aspartyl protease of the fungal pathogen *Paracoccidioides brasiliensis*

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Paracoccidioides brasiliensis is a thermally dimorphic fungus that causes paracoccidioidomycosis, a human systemic disease prevalent in Latin America. Proteases have been described as playing an important role in the host invasion process in many pathogenic microorganisms. Here we describe the identification and characterization of a secreted aspartyl protease (*Pb*SAP), isolated from a cDNA library constructed with RNAs of mycelia transitioning to yeast cells. Recombinant *Pb*SAP was produced in *Escherichia coli*, and the purified protein was used to develop a polyclonal antibody that was able to detect a 66 kDa protein in the *P. brasiliensis* proteome. *Pb*SAP was detected in culture supernatants of *P. brasiliensis* and this data strongly suggest that it is a secreted molecule. The protein was located in the yeast cell wall, as determined by immunoelectron microscopy. *In vitro* deglycosylation assays with endoglycosidase H, and *in vivo* inhibition of the glycosylation by tunicamycin demonstrated *N*-glycosylation of the *Pb*SAP molecule. Zymogram assays indicated the presence of aspartyl protease gelatinolytic activity in yeast cells and culture supernatant.

Keywords *Paracoccidioides brasiliensis*, secreted aspartyl protease, *N*-glycosylation, gelatinolitic activity

Introduction

Paracoccidioidomycosis (PCM), caused by *Paraccocidioides brasiliensis*, is a human systemic mycosis prevalent in rural areas of Latin America. Host infection is typically initiated by inhalation of airborne fungal spores. The disease, which occurs primarily in the lungs as a granulomatous infection, can disseminate via the bloodstream and/or lymphatic system to other organs systems [1].

Aspartyl proteases constitute one of the four super families of proteolytic enzymes showing acidic optima pH for enzyme activity. They are generally similar to pepsin, which is totally inhibited by pepstatin, and show preferential specificity for cleavage at peptide bonds between hydrophobic amino acid residues [2]. The proteins share many features, including a conserved three-dimensional structure consisting of two lobes with a deep, active site cleft that contains two conserved aspartic acid residues. The protein molecule is synthesized as a large inactive precursor, which is subsequently converted into a mature enzyme by removing the N-terminal peptide from about 45 residues. In this segment, a pro peptide binds to the active site cleft and prevents undesirable degradation during intracellular transport and secretion [3,4].

Extracellular proteases from pathogenic fungi fulfill a number of specialized functions during the infective

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process in addition to the simple role of digesting molecules for nutrient acquisition. Some studies investigating the role of extracellular aspartyl proteases in pathogenesis have focused on fungi. *Candida albicans* manifests a multigene secreted aspartyl protease family (SAP), with at least 10 members identified [5]. The SAPs 1–7 are differentially expressed during the infection. SAP1 and 3 are induced in acute infection. SAPs 2, 4, 5 and 6 are the most highly expressed proteins during the infection. SAP7 is expressed when *C. albicans* is located on mucosal surfaces [6]. *C. albicans* exposure to antifungal agents increases the expression of SAP4-6, suggesting their induction may be a part of a stress-related defense mechanism in *C. albicans* [7]. The aspergillopepsin aspartic protease from *Aspergillus fumigatus* is secreted in large amounts during infection of the mouse lung [8]. An aspartyl protease associated with the cell wall was detected in *Coccidioides posadasii* and the recombinant protein was reported as a putative candidate for a new vaccine [9].

P. brasiliensis proteases are beginning to be characterized. A total of 53 open reading frames (ORFs) encoding energy-independent and -dependent proteases in *P. brasiliensis* have been described. The proteases were classified according to the domains present in the active sites, in aspartyl, cysteine, metallo and serine proteases and proteasome subunits [10]. Also, an extracellular subtilisin-like serine protease activity has been characterized in the yeast phase of *P. brasiliensis* [11]. Inhibition assays with PMSF (phenylmethyl-sulphonyl fluoride), mercury acetate and *p*-HMB (p-hydroxymercuri benzoate) have classified the enzyme as a serine-thiol protease that is able, *in vitro* to selectively degrade murine laminin, human fibronectin, type IV-collagen and proteoglycans [12]. This serine-thiol activity of *P. brasiliensis* is regulated by neutral polysaccharides, including a fungal extracellular galactomannan, which might help stabilize the enzyme [13].

The transcriptome analysis of the *P. brasiliensis* mycelium transition to yeast cells, revealed a positively regulated aspartyl protease transcript [14]. In order to extend the characterization of the transcript we isolated the complete cDNA encoding a homologue of aspartyl protease from *P. brasiliensis*. The recombinant protein was used to generate rabbit polyclonal antibody, which detected the aspartyl protease in the cell wall of the fungal yeast cells as well as in fungal culture supernatants. The presence of a carbohydrate chain linked to the native molecule was inferred from N-deglycosylation experiments. Those observations indicate that *Pb*SAP is a *N*-glycosylated molecule secreted by *P. brasiliensis* yeast cells. This article provides the first comprehensive survey of an aspartyl protease in *P. brasiliensis* and provides initial insights into the role of protease in the fungus.

Materials and methods

P. brasiliensis *isolate growth conditions and differentiation assays*

P. brasiliensis isolate *Pb*01 (ATCC MYA-826) was used in all the experiments. It was grown at either 22°C for the mycelium form or 36°C for yeast cells in Fava-Netto's medium [1% (w/v) peptone; 0.5% (w/v) yeast extract; 0.3% (w/v) proteose peptone; 0.5% (w/v) beef extract; 0.5% (w/v) NaCl; 4% (w/v) agar, pH 7.2]. *P. brasiliensis* yeast cells were also cultured in Fava-Netto's liquid medium, supplemented with 8 mg of bovine serum albumin (BSA) per ml, to induce protease secretion as described previously [15].

Obtaining the P. brasiliensis *aspartyl protease cDNA and bioinformatic analysis*

A complete cDNA encoding a *P. brasiliensis* homologue of an aspartyl protease was obtained from a cDNA library constructed with the RNA of mycelia in transition to yeast cells [14]. The cDNA was sequenced on both strands by using the MegaBACE 1000 DNA sequencer (GE Healthcare, Amersham Biosciences). The Pfam database [16] and MEROPS [17] described the classification of the predicted protease. The entire nucleotide sequence, *Pb*SAP, was submitted to the GenBank database under accession number AY278218.

The BLAST algorithm [18] was used to search in the non-redundant database of the National Center for Biotechnology Information (NCBI) [19] for proteins with sequence similarities to the translated full-length *Pb*SAP cDNA. Conserved sites and motifs in the deduced protein were screened using the profile scan [20] and ScanProsite algorithms [21]. The presence of signal peptides was identified using the SignalP program [22], while the PSORT II algorithm was employed for prediction of cellular localization [23]. Multiple sequence alignments were generated using the Clustal X 1.83 software [24].

DNA extraction and Southern blot analysis

The genomic DNA of *Pb*01 yeast cells was extracted following standard procedures [25] using phenol and phenol cloroform (v/v). The RNA was removed by digestion with RNAse (10 µg/ml) for 2 h at 37°C. The genomic DNA of *P. brasiliensis* (15 µg) was digested with selected restriction endonucleases. Digestion products were fractionated on a 1.0% agarose gel and transferred to a nylon membrane, after denaturation for 15 min in 0.5 M NaOH. The 1.2-kb cDNA insert probe was labeled and hybridization was carried out using the Gene Images Random Prime Labeling Kit (GE Healthcare). The Gene Image CDP-Star detection module (GE Healthcare) was used for hybridization detection.

Cloning of PbSAP cDNA into expression vector

Oligonucleotide primers were designed to amplify the 1.2-kb cDNA containing the complete coding region of *Pb*SAP, which encodes amino acids (aa) 1–400 (predicted full length protein). The nucleotide sequence of the sense and antisense primers were 5´-ACCGAATTCTATGAAGTTCTCTCTG–3´ and 5´-ACCCTCGAGTCACTGTCTAGCCTTCG–3´, which contained engineered *Eco*RI and *Xho*I restriction sites, respectively (underlined). The amplification parameters were as follows; an initial denaturation step at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 90 s, annealing at 60°C for 75 s, and extension at 72°C for 2 min; final extension was at 72°C for 5 min. The PCR product was electrophoresed and the 1.2-kb amplicon was gel excised and sub cloned into the pGEX-4T-3 expression vector (GE Healthcare). Using the heat shock method [25], the recombinant plasmid was used to transform the *E. coli* strain BL21 competent cells. Ampicillin-resistant transformants were cultured, and plasmid DNA was analyzed by PCR and DNA sequencing.

Heterologous expression of PbSAP, recombinant protein purification and antibody production

Cultures of transformed *E. coli* containing pGEX-4T-3-*Pb*SAP were grown in Luria-Bertani (LB) medium supplemented with 100 μg/ml of ampicillin, at 37°C. As the cells reached the log phase (A_{600}=0.6), IPTG (isopropyl-β-D-thiogalactopyranoside) was added to the growing culture to a final concentration of 0.05 mM to induce protein expression. After 12 h incubation, at 15°C, the bacterial cells were harvested by centrifugation at 5,000 *g*, ressuspended in PBS 1x and incubated with lysozyme (100 μg/ml) before three 15-min sonications. The recombinant *Pb*SAP was expressed in the soluble form by bacteria, and the protein was purified by affinity chromatography under non-denaturing conditions, as previously reported [26]. The soluble fraction of cell lysate, containing the recombinant *Pb*SAP, was applied to an affinity Glutathione Sepharose^{TM} 4B Resin column (GE Healthcare) under non denaturing conditions. The fusion protein was cleaved following exposure to thrombin protease (50 U/ml) addition and the fusion-partner-free recombinant protein was collected after 12 h of incubation. The purified recombinant protein was electrophoresed on a 12% SDS-PAGE, followed by Coomassie brilliant blue staining.

Rabbits were subcutaneously inoculated with the purified recombinant protein (300 μg) with 2 mg of aluminum hydroxide, $Al(OH)_3$, as adjuvant. Animals were boosted twice, at 2 weeks intervals, with the same amount of antigen. The serum thus obtained, containing anti-*Pb*SAP polyclonal antibody, was sampled and stored at −20°C. Determination of the antibody title was performed by ELISA and western blot. Preimmune serum was obtained.

Obtaining cell extracts and secreted proteins of P. brasiliensis

Total protein extracts from yeast and mycelium were obtained as described [27]. Frozen cells (3g) were disrupted by complete grinding with a mortar and pestle in buffer (20 mM Tris-HCl, pH 8.8, 2 mM $CaCl_2$) containing EDTA (5 mM) and phenylmethyl-sulphonyl fluoride (PMSF) (20 mM). The mixture was centrifuged at 15,000 *g* at 4°C, for 20 min; the supernatant was sampled, and stored at −80°C.

After 6 days at 36°C under agitation (150 *g*), yeast cells supernatant (supplemented with BSA) was obtained by centrifugation at 5,000 *g* for 15 min and the secreted protein fraction was concentrated by 50% (v/v) ice-cold acetone precipitation, at −20°C.

Cell wall proteins were obtained as described [28] with modifications. After the yeast cells were disrupted, the pellet obtained by centrifugation at 12,000 *g* was washed five times, sequentially, with the following ice-cold solutions: 5% NaCl, 2% NaCl, 1% NaCl and 1 mM PMSF. Cell wall proteins were extracted by boiling in SDS extraction buffer (50 mM Tris-HCl, pH 8.0, 0.1 M EDTA, 2% SDS, 10 mM DTT) for 10 min. The treatment was carried out twice and the supernatant, which is identified as SDS-extract throughout the text, was analyzed by SDS-PAGE. The protein concentrations of all the samples were measured.

Western blot analysis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970) [29]. Proteins were electroblotted to a nylon membrane and checked by Ponceau S to access loading of equal amounts of protein. The membrane was blocked with 5% (w/v) non-fat dried milk in PBS 1× (pH 7.4). Aspartyl protease was detected with the polyclonal antibody to the recombinant protein. After reaction with alkaline phosphatase anti-rabbit immunoglobulin G (IgG), the reaction was developed with 5-bromo-4-cloro-3-indolylphosphate/nitroblue tetrazolium (BCIP/NBT). Negative controls were obtained with rabbit preimmune serum.

Immunocytochemistry of the aspartyl protease

Yeast cells of *P. brasiliensis*, isolate *Pb*01, were fixed overnight at 4°C in a solution containing 2% (v/v) glutaraldehyde, 2% (w/v) paraformaldehyde, and 3% (w/v) sucrose in 0.1 M sodium cacodylate buffer at pH 7.2. The yeast cells were rinsed in the same buffer and postfixed for 1 h in a solution containing 1% (w/v) osmium tetroxide, 0.8% (w/v) potassium ferricyanide, and 5 mM $CaCl_2$ in sodium cacodylate buffer, pH 7.2. The material was dehydrated in a series of ascending acetones (30 to 100%) (v/v) and embedded in Spurr resin (Electron Microscopy Sciences, Washington, Pa.). Ultrathin sections were stained with 3% (w/v) uranyl acetate and lead citrate. The material was observed with a Jeol 1011 transmission electron microscope (Jeol, Tokyo, Japan).

For ultrastructural immunocytochemistry studies, yeast cells were fixed in a mixture containing 4% (w/v) paraformaldehyde, 0.5% (v/v) glutaraldehyde, and 0.2% (w/v) picric acid in 0.1 M sodium cacodylate buffer at pH 7.2 for 24 h at 4°C. The cells were rinsed several times using the same buffer, and free aldehyde groups were quenched with 50 mM ammonium chloride for 1 h, followed by block staining in a solution containing 2% (w/v) uranyl acetate in 15% (v/v) acetone for 2 h at 4°C. The material was dehydrated in a series of ascending concentrations of acetone (30 to 100%) (v/v) and embedded in LR Gold resin (Electron Microscopy Sciences, Fort Washington, PA).

The ultrathin sections were collected on nickel grids, preincubated in 10 mM PBS containing 1.5% (w/v) bovine serum albumin (BSA) and 0.05% (v/v) Tween 20, (PBS-BSA-T), and subsequently incubated for 1 h with the polyclonal antibody against the recombinant aspartyl protease (diluted 1:100). After washing with PBS-BSA-T, the grids were incubated for 1 h with the labeled secondary antibody (anti-rabbit IgG, Au conjugated, 10 nm; diluted 1:20). Subsequently, the grids were washed with distilled water, stained with 3% (w/v) uranyl acetate, and lead citrate and observed with a Jeol 1011 transmission electron microscope (Jeol, Tokyo, Japan). Controls were incubated with rabbit preimmune serum at 1:100, followed by incubation with the labeled secondary antibody.

Glycosylation analysis

Total protein extract from yeast cells was incubated with recombinant endoglycosidase H (Endo H) from *Streptomyces plicatus* (Sigma-Aldrich), for 16 h at 37°C. The reaction mixture (100 μl) contained 30 μg of the protein extract and 27 mU Endo H in 30 mM $CaCl_2$, 3 mM NaN_3, 1.2 mM PMSF, and 60 mM sodium acetate buffer pH 5.8. Control reactions were also incubated for 16 h at 37°C. For the tunicamycin assay, inhibition of cell growth was preliminary tested. Yeast cells (1×10^6 fungal cells/ml) in liquid Fava-Netto's medium were incubated with different concentrations of tunicamycin for 7 days at 36°C. Culture growth was monitored daily by counting the cells. The higher tunicamycin concentration that presented no cell growth inhibition was 20 μg/ml and this condition was used in the assays. The cells were harvested and subjected to total protein extraction, as previously described. The samples were analyzed by western blot.

Zymogram

Zymograms were used to search for native aspartyl protease activity in *P. brasiliensis* extracts. Total protein extract of yeast cells was resuspended in buffer containing Tris-HCl (20 mM pH 8.8) $CaCl_2$ (2 mM) PMSF (20 mM) and EDTA (5 mM); the secreted protein fraction was concentrated using ice-cold acetone, as described. The proteins were subjected to 8% SDS-PAGE- gelatin (sodium dodecyl sulfate polyacrylamide gel electrophoresis, co-polymerized with 0.15% gelatin) [30]. After protein fractionation the gel was washed three times, for 15 min each time, in 25 mM Tris-HCl pH 7.0, following by incubation at 37°C for 12 h in developing buffer (0.1 M Na_2HPO_4 adjusted to pH 4.0) and stained with Coomassie brilliant blue. Enzyme inhibition assays were performed by incubating the same samples in buffer containing Tris-HCl (20 mM pH 8.8) $CaCl_2$ (2 mM) and pepstatin A (10 μM) in reaction mixtures containing 5 μg of total or secreted proteins during 15 min at room temperature. In addition, the relevance of glycosylation to the activity of *P. brasiliensis* aspartyl proteases was evaluated by incubating 30 μg of the total protein extract of yeast cells with 54 mU Endo H at 37°C for 15 min in the conditions described above.

Results

Structural features of the cDNA and the deduced aspartyl protease

The cDNA sequence of 1361 bp contained an open reading frame of 1200 bp. The deduced amino acid sequence was 400 residues with a predicted molecular mass of 44 kDa and *pI* 5.27. Analysis of the N-terminal amino acid region revealed a 19-amino-acid signal peptide as well as a cleavage-signal sequence, which is consistent with an extracellular location for the *Pb*SAP (Supplementary Fig. 1 – online version only).

Comparisons of the entire amino acid sequence with those of well-known aspartyl proteases allowed us to recognize amino acid residues necessary for enzyme activity. The protein sequence contains two conserved domains that compose the aspartyl protease active site identified by the PROSITE algorithm at $D^{104}XG^{106}XS^{108}XXW^{111}V^{112}$ and $D^{288}T^{289}G^{290}$ (D is the active residue). Three N-glycosylation sites were also predicted at positions 139-142, 252–255 and 339–342 in the deduced protein sequence (Fig. 1A). The sequences of the aspartyl proteases from *Coccidioides posadasii* showed the highest sequence identity to *Pb*SAP (88%), followed by *Aspergillus clavatus* (87%) and *Aspergillus terreus* (87%). The similarity of *Pb*SAP to *C. albicans* SAPs 1–10 was from 40–47% (data not shown).

Southern blot analysis

Southern blot hybridization was performed to estimate the genomic organization of *Pb*SAP. The specific 1.2 kb probe was able to detect a single DNA copy in the *P. brasiliensis* genomic DNA, as demonstrated by specific hybridization profiles of DNA digested with the restriction enzymes (Fig. 1B). This finding is supported by computational analysis of the restriction sites in the *Pb*SAP cDNA sequence. The presence of one gene encoding *Pb*SAP in the fungus genome was confirmed by search analysis at the *Paracoccidioides brasiliensis* Genomic Database [31]. Deduced *Pb*SAP, excluding the pre-propeptide region, was 90% identical with isolate *Pb*03 and 91% identical with *Pb*18 (data not shown).

Expression of PbSAP in E. coli *and antibody production*

SDS-PAGE analysis of the lysate of the transformants revealed that IPTG-induced a dominant protein, migrating at 72 kDa (Fig. 2A, lane 3). This dominant protein was absent in the cells growing in the absence of IPTG (Fig. 2A, lane 2), as well as in control cells (Fig. 2A, lane 1). Lysis of bacterial cells was followed by purification of the fusion protein using a glutathione-sepharose 4B column (Fig. 2A, lane 4), which was subsequently cleaved by thrombin protease. The cleaved purified recombinant protein migrated as a single species of 44 kDa (Fig. 2A, lane 5). The polyclonal antibody produced from *Pb*SAP reacted to the purified recombinant protein in western blot analysis (Fig. 2B, lane 2). No reaction was detected with rabbit preimmune serum (Fig. 2B, lane 1).

Identification of the aspartic protease in fungal phases, in the extracellular culture fluid and in the SDS-extracts

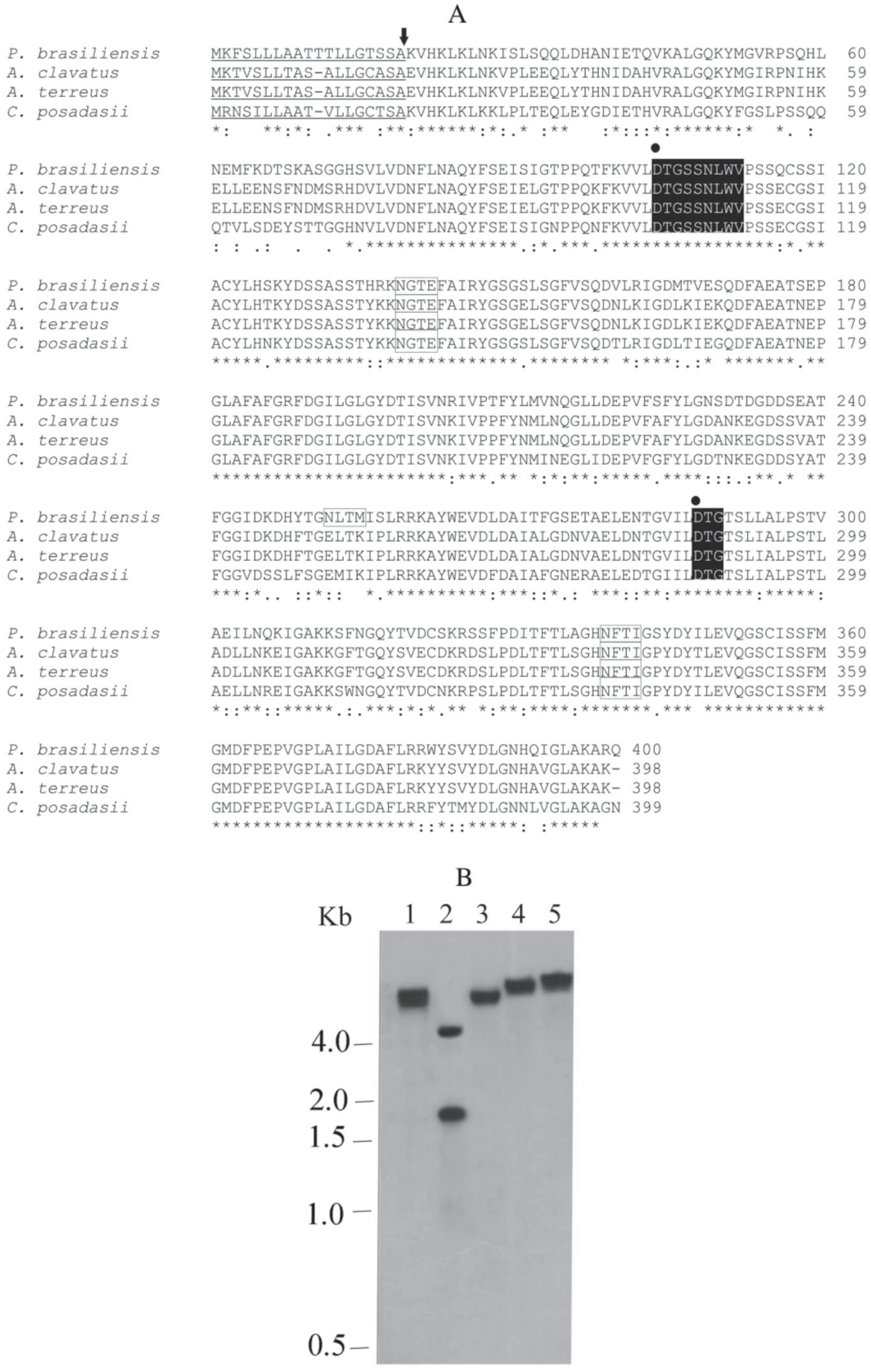
To identify the protein that represents the aspartic protease, western blot analysis was performed. Total protein extracts from isolate *Pb*01, yeast and mycelium, were electrophoresed in 12% SDS-PAGE and stained with Coomassie brilliant blue (Fig. 3A, lanes 1 and 2, respectively). Western blot analysis showed only one cross-reacting protein species, with a molecular mass of 66 kDa, in both samples. This was more abundant in yeast cells (Fig. 3B, lane 1). By using the extracellular culture fluid and the SDS-extracted cell wall protein fraction (Fig. 3C, lanes 1 and 2, respectively), the 66 kDa protein species was identified in both samples (Fig. 3D, lanes 1 and 2, respectively). No reactivity was detected with the culture medium employed for fungal growth (data not shown). Preimmune serum was used as a control for all samples (Fig. 3E).

Immunogold localization of the aspartic proteinase of P. brasiliensis

Immunocytochemistry experiments were performed to define the cellular localization of the aspartyl protein in yeast cells of isolate *Pb*01. Gold particles were detected predominantly in the cell wall (Fig. 4A). Control samples obtained by incubation of the yeast cells with the rabbit preimmune serum were free of label (Fig. 4B).

Deglycosylation assays

The greater molecular mass of the aspartyl protease of *P. brasiliensis*, compared to the expected value of the deduced molecule, could be due to post-translational modification, such as glycosylation. This possibility was explored by treating samples with endoglycosidase H (Fig. 5, lane 2) and including tunicamycin in the yeast-culturing medium (Fig. 5, lane 3). Analysis was performed by immunobloting. Treatment with endoglycosidase H produced a protein species of 44 kDa (Fig. 5, lane 2). The tunicamycin treatment was also observed to generate a protein of 44 kDa (Fig. 5, lane 3). The data support the inference that the 66 kDa is the glycosylated form of the 44 kDa protein.



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P. brasiliensis     MKFSLLLAATTTLLGTSSAKVHKLKLNKISLSQQLDHANIETQVKALGQKYMGVRPSQHL  60
A. clavatus         MKTVSLLTAS-ALLGCASAEVHKLKLNKVPLEEQLYTHNIDAHVRALGQKYMGIRPNIHK  59
A. terreus          MKTVSLLTAS-ALLGCASAEVHKLKLNKVPLEEQLYTHNIDAHVRALGQKYMGIRPNIHK  59
C. posadasii        MRNSILLAAT-VLLGCTSAKVHKLKLKKLPLTEQLEYGDIETHVRALGQKYFGSLPSSQQ  59
                    *:   **:*: .*** :**:******:*:.* :**   :*:::*:******:*  *. :

P. brasiliensis     NEMFKDTSKASGGHSVLVDNFLNAQYFSEISIGTPPQTFKVVLDTGSSNLWVPSSQCSSI 120
A. clavatus         ELLEENSFNDMSRHDVLVDNFLNAQYFSEIELGTPPQKFKVVLDTGSSNLWVPSSECGSI 119
A. terreus          ELLEENSFNDMSRHDVLVDNFLNAQYFSEIELGTPPQKFKVVLDTGSSNLWVPSSECGSI 119
C. posadasii        QTVLSDEYSTTGGHNVLVDNFLNAQYFSEISIGNPPQNFKVVLDTGSSNLWVPSSECGSI 119
                    : : .:  .  . *.**************.:*.***.*****************:*.**

P. brasiliensis     ACYLHSKYDSSASSTHRKNGTEFAIRYGSGSLSGFVSQDVLRIGDMTVESQDFAEATSEP 180
A. clavatus         ACYLHTKYDSSASSTYKKNGTEFAIRYGSGELSGFVSQDNLKIGDLKIEKQDFAEATNEP 179
A. terreus          ACYLHTKYDSSASSTYKKNGTEFAIRYGSGELSGFVSQDNLKIGDLKIEKQDFAEATNEP 179
C. posadasii        ACYLHNKYDSSASSTYKKNGTEFAIRYGSGSLSGFVSQDTLRIGDLTIEGQDFAEATNEP 179
                    *****.*********::*************.******** *:***:.:* *******.**

P. brasiliensis     GLAFAFGRFDGILGLGYDTISVNRIVPTFYLMVNQGLLDEPVFSFYLGNSDTDGDDSEAT 240
A. clavatus         GLAFAFGRFDGILGLGYDTISVNKIVPPFYNMLNQGLLDEPVFAFYLGDANKEGDSSVAT 239
A. terreus          GLAFAFGRFDGILGLGYDTISVNKIVPPFYNMLNQGLLDEPVFAFYLGDANKEGDSSVAT 239
C. posadasii        GLAFAFGRFDGILGLGYDTISVNKIVPPFYNMINEGLIDEPVFGFYLGDTNKEGDDSYAT 239
                    **********************:***.** *:*:**:*****.****:::.:**.* **

P. brasiliensis     FGGIDKDHYTGNLTMISLRRKAYWEVDLDAITFGSETAELENTGVILDTGTSLLALPSTV 300
A. clavatus         FGGIDKDHFTGELTKIPLRRKAYWEVDLDAIALGDNVAELDNTGVILDTGTSLIALPSTL 299
A. terreus          FGGIDKDHFTGELTKIPLRRKAYWEVDLDAIALGDNVAELDNTGVILDTGTSLIALPSTL 299
C. posadasii        FGGVDSSLFSGEMIKIPLRRKAYWEVDFDAIAFGNERAELEDTGIILDTGTSLIALPSTL 299
                    ***:*.. ::*::  *.**********:***::*.: ***::**:*******:*****:

P. brasiliensis     AEILNQKIGAKKSFNGQYTVDCSKRSSFPDITFTLAGHNFTIGSYDYILEVQGSCISSFM 360
A. clavatus         ADLLNKEIGAKKGFTGQYSVECDKRDSLPDLTFTLSGHNFTIGPYDYTLEVQGSCISSFM 359
A. terreus          ADLLNKEIGAKKGFTGQYSVECDKRDSLPDLTFTLSGHNFTIGPYDYTLEVQGSCISSFM 359
C. posadasii        AELLNREIGAKKSWNGQYTVDCNKRPSLPDLTFTLSGHNFTIGPYDYILEVQGSCISSFM 359
                    *::**::*****.:.***:*:*.** *:**:****:*******.*** ************

P. brasiliensis     GMDFPEPVGPLAILGDAFLRRWYSVYDLGNHQIGLAKARQ 400
A. clavatus         GMDFPEPVGPLAILGDAFLRKYYSVYDLGNHAVGLAKAK- 398
A. terreus          GMDFPEPVGPLAILGDAFLRKYYSVYDLGNHAVGLAKAK- 398
C. posadasii        GMDFPEPVGPLAILGDAFLRRFYTMYDLGNNLVGLAKAGN 399
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Fig. 1 Clustal X (1.83) multiple sequence alignment and *Paracoccidioides brasiliensis* aspartyl protease genomic organization. (A) Comparison of the predicted amino acid sequence of *Pb*SAP with the selected sequences of the fungal aspartyl proteases corresponding to *Aspergillus clavatus* (XM_001271140), *Aspergillus terreus* (XM_001213854) and *Coccidioides posadasii* (DQ164306). Conserved amino acids are marked by asterisks under the letters. The prepeptide sequence (signal peptide) is underlined and the arrow indicates the putative cleavage site. Amino acid sequences in black boxes represent the two conserved domains important to the active site. A black ball indicates the aspartic acid residue (D). The boxed sequences depicted the putative glycosylation sites. (B) Analysis of *P. brasiliensis* aspartyl protease genomic organization by Southern blot analysis. Total genomic DNA of *P. brasiliensis* (15 μg) was digested with the selected restriction endonucleases: (1) *Eco*RV, (2) *Bgl*II, (3) *Dra*I, (4) *Eco*RI and (5) *Sac*I. The DNA size marker is on the left.

Gelatinolytic activity of P. brasiliensis *native aspartyl proteases*

In order to investigate the activity of aspartyl proteases in total proteins and in extracellular culture fluid, a gelatin-incorporated SDS-PAGE zymogram was performed. The total protein extract of *P. brasiliensis* yeast cells and extracellular culture fluid showed a clear zone of proteolytic activity in the region of 66 kDa (Fig. 6A, lanes 1 and 2). The presence of aspartyl proteases in the gelatin degradation region was confirmed by the inhibition assay with pepstatin (Fig. 6A, lanes 3 and 4). Western blot assays with the specific antibody showed the presence of the *Pb*SAP protease (Fig. 6B, lanes 1 and 2). A control reaction was performed by using the preimmune serum in the total

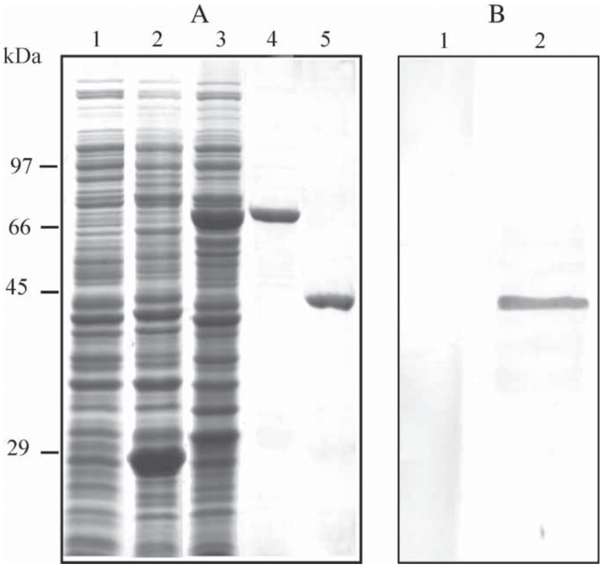

Fig. 2 SDS-PAGE and immunoblot analysis of the recombinant *Pb*SAP. (A) Profile of the Coomassie brilliant blue stained gel (12% SDS-PAGE) of *E. coli* expressing the recombinant aspartyl protease. The lanes are as follows: 1 – Control, *E. coli* extracts; 2 – Extracts of *E. coli* cells containing the pGEX-4T-3, after addition of 0.05 mM IPTG; 3 – Extracts of *E. coli* cells containing the expression vector pGEX-4T-3-*Pb*SAP, after addition of 0.05 mM IPTG; 4 – Recombinant *Pb*SAP fusion protein purified by affinity chromatography to Glutathione Sepharose$^{\text{TM}}$; 5 – Recombinant aspartyl protease cleaved by trombin protease. Protein molecular markers are indicated. (B) Western blot analysis. The purified *Pb*SAP cleaved by thrombin protease was reacted to: 1 – Control rabbit preimmune serum. 2 – Rabbit polyclonal antibody.

yeast protein extract and in the extracellular culture fluid (Fig. 6C, lanes 1 and 2, respectively).

Protease activity was inhibited in yeast cell protein extracts treated with endoglycosidase H (Fig. 6D, lanes 1 and 2) suggesting that glycosylation is essential for protein function.

Discussion

The *Pb*SAP characterized here was previously classified [10] as a member of the pepsin family (A1), which contains many enzymes that enter the secretory pathway. These proteins are synthesized as inactive zymogens activated by the self-cleavage of an N-terminal propeptide under acidic conditions [4]. The *P. brasiliensis* aspartyl protease cDNA encodes a protein that contains 19 amino acids at the N-terminal that are characteristic of a leader peptide. Computational analysis indicates that the protein must be synthesized as a precursor containing a 70-amino-acid prepropeptide at the N terminus of the mature protein. Alignment of sequences closely related to *Pb*SAP showed that, in addition of identical residues, they share important structural features such as signal peptide positions and active sites location. The protein sequence corresponding to the mature *Pb*SAP shows great similarity to the selected aspartyl proteases sequences.

The recombinant *Pb*SAP was generated and the purified protein was 44kDa, as assessed by SDS-PAGE. These data are in accordance with the predicted size of the deduced protein *Pb*SAP. Using the recombinant purified protein, high titers of rabbit polyclonal antibody were raised. The serum specifically recognized the recombinant purified protein in the western blot assays. In total yeast and mycelium protein extracts a protein of 66 kDa was detected. This molecule was more abundant in yeast cells. Treatments of protein extracts with endoglycosidase H or inclusion of tunicamycin in the culture medium resulted in the disappearance and or decrease of the 66 kDa protein species and the appearance of the 44 kDa, corresponding to the size of *Pb*SAP with the prepropeptides. These data suggest that the extra 22 kDa in the 66 kDa is due to N-glycosylation. Although the significance of glycosylation in the aspartyl protease family is not well known, it has been suggested that it stabilizes protein conformation leading to a higher thermostability [32]. Our data suggest that *Pb*SAP can be secreted as a precursor molecule. Interestingly, studies have demonstrated that aspartyl proteases precursors can be secreted in the extracellular medium where, under low pH conditions, they undergo autocatalytic activation, forming a mature enzyme [33].

The *Pb*SAP is a cell wall molecule of *P. brasiliensis*. Aspartyl proteases on the cell surface have been reported for many eukaryotes. In *C. posadasii* an aspartyl protease was found as a component of the cell wall extract [9]. Aspartyl proteases have been described as important to cell wall integrity and adherence to mammalian cells in *C. glabrata* [34]. In *C. albicans* SAP9 and SAP10 are glycosylphosphatidylinositol-anchored and located in the cell membrane or in the cell wall [35].

Gelatinolytic activity of aspartyl proteases in the yeast protein extract and in the secreted protein fraction was analyzed by zymogram. In both samples, gelatin degradation was observed in the 66 kDa region, suggesting *Pb*SAP protease activity. The extracts were prepared in buffer containing PMSF (serine proteases inhibitor) and EDTA (metalloproteases inhibitor) and the use of developing buffer in acidic conditions activated the enzyme. The treatment of yeast cells extract with Endo H resulted in the loss of protease activity, suggesting that glycosylation is relevant for aspartyl protease activity in *P. brasiliensis*. Numerous functions have been attributed to aspartyl

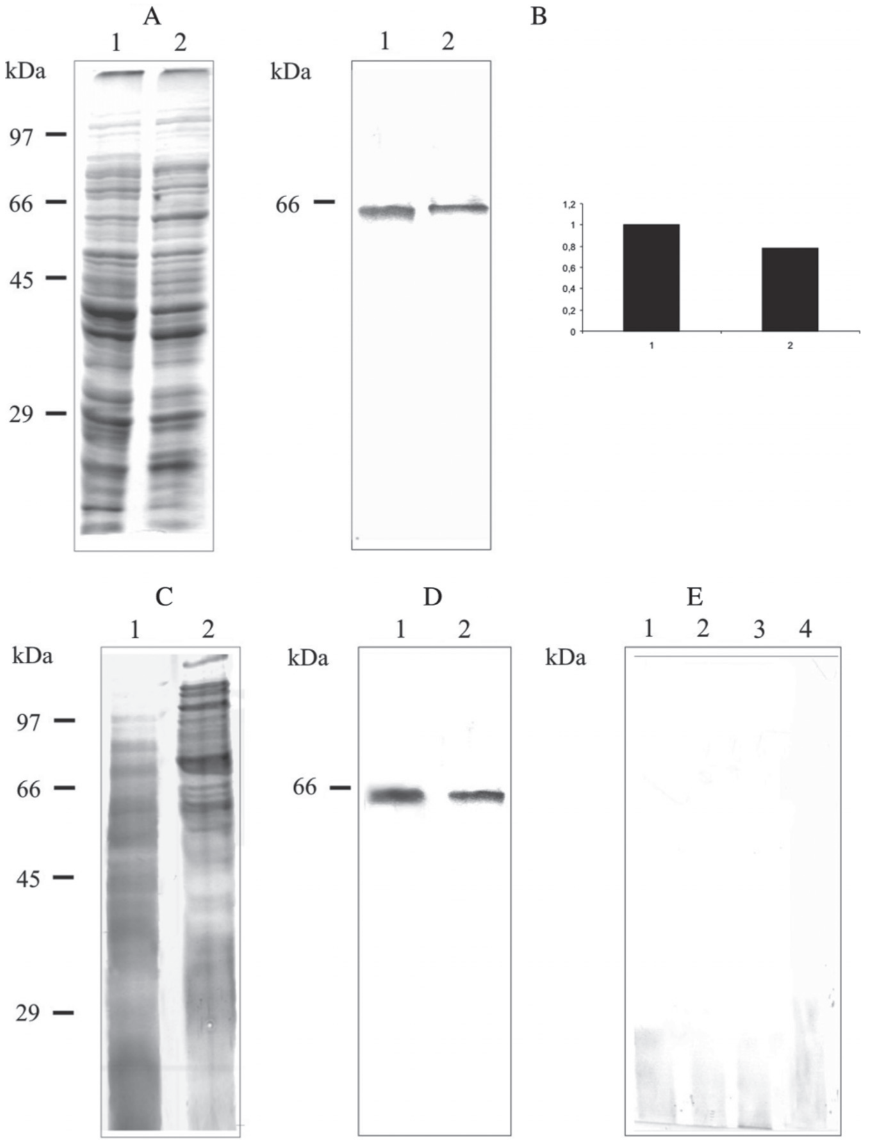

Fig. 3 Immunodetection of aspartyl protease by western blot analysis. (A) SDS-PAGE (12%) of total protein extract (30 µg) of *P. brasiliensis* yeast cells (lane 1) and mycelium (lane 2), stained with *Coomassie* brilliant blue. (B) The same *P. brasiliensis* extracts were used and aspartyl protease was visualized by western blot. The *Pb*SAP expression was quantified using the program Scion Image for Windows (http://www.scioncorp.com). (C) Electrophoretic analysis of the secreted protein fraction of *P. brasiliensis* yeast cultures (lane 1) and of the SDS-extracted fraction from the fungus cell wall (lane 2). (D) Western blot analysis with rabbit polyclonal antibody of samples from C. (E) Western blot analysis with preimmune rabbit serum of: 1 – total protein extract from yeast cells, 2 – total protein extract from mycelium, 3 – secreted proteins from yeast cells and 4– SDS-extracted fraction from yeast cell wall. After reaction with the anti-rabbit Ig-G alkaline phosphatase-coupled antibody (diluted 1:2000), the reaction was developed with BCIP/NBT. Protein molecular markers are indicated.

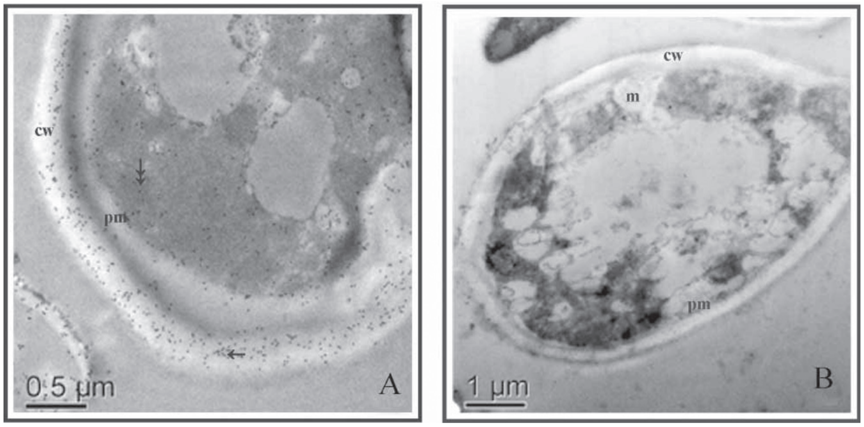

Fig. 4 Immunogold localization of *Pb*SAP in *Paracoccidioides brasiliensis* yeast cells. (A) The gold particles conjugated to the secondary antibody were numerous in the cell wall (arrows) and sparse in the cytoplasm (double arrows). (B) Negative control was obtained using rabbit preimmune serum. Typical fungal cell structures: (cw) cell wall, (m) mitochondria and (pm) plasma membrane.

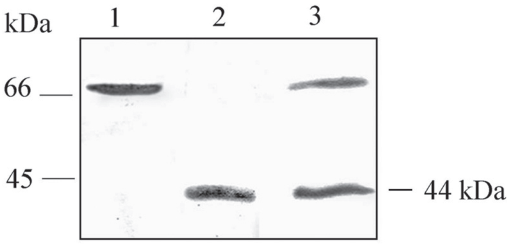

Fig. 5 *Paracoccidioides brasiliensis* aspartyl protease glycosylation studies and immunoblot assays. Glycoslylation was investigated by treating protein extracts with endoglycosidase H or by including tunicamycin in the culture medium followed by immunoblot analysis. Lane 1: Control; total protein extract of yeast cells (30 μg). Lane 2: Total protein extract from yeast cells (30 μg) after endoglycosidase H treatment. Lane 3: *P. brasiliensis* yeast cells extract (30 μg) obtained after growth of yeast cells in the presence of tunicamycin.

proteases in microorganisms. These range from nutrient degradation to the activation of signaling molecules. Aspartyl proteases from fungi serve to activate other zymogens such as alkaline phosphatase, chitin synthase, and other proteases [36–38]. Aspartyl proteases from *Schistosoma* species are known to be responsible for host-specific proteolytic degradation of mammalian hemoglobin [39]. Also, the degradative properties of secreted proteases have attracted much attention as potential mediators of fungal invasion in infected tissue [5,15]. In *A. fumigatus*, an aspartyl protease is important for the invasion process in the lung, facilitating fungus penetration [8]. The role of aspartyl protease in *P. brasiliensis* remains unclear. The fact that the protein is more abundant in yeast cells, would point to its importance in the pathogenesis of the organism. Future work will focus on this subject.

In conclusion, a novel aspartic protease, *Pb*SAP, has been identified and characterized in the pathogenic fungus *P. brasiliensis*. Recombinant *Pb*SAP expression was determinant to this work as an important tool to obtain specific polyclonal antibody. Secretion of the native protein was detected in yeast cell culture by immunoassays, and the presence of the protein was also detected in the fungal cell wall. The glycosylation of *Pb*SAP was investigated, providing fundamental information about the structure of *Pb*SAP.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

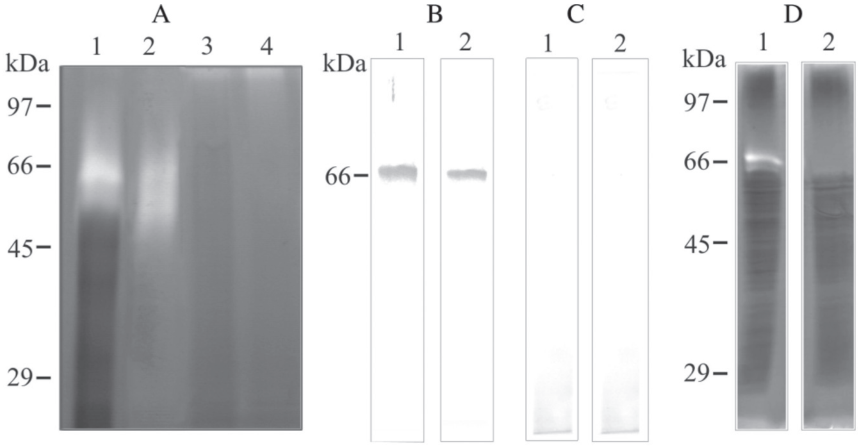

Fig. 6 Analysis of gelatinolytic activity and immunoblot assays. (A) Total protein from yeast cells (lane 1) and secreted protein fraction (lane 2) were subjected to SDS-PAGE-gelatin, under non-denaturing conditions. The same extracts were assayed to protein specific inhibition using pepstatin (10 μM) (lanes 3 and 4). After electrophoresis, the gel was washed and incubated in developing buffer. Hydrolysis of gelatin was visualized by gel staining with Coomassie Brilliant Blue. Protein molecular markers are indicated. (B) Immunoassay of the samples from A using *Pb*SAP specific serum; lane 1: Total protein from yeast cells and lane 2- secreted protein from yeast cells. (C) Reaction to the preimmune serum, lanes 1 and 2, the same samples as in B. (D) Total protein from yeast cells was incubated with endoglycosidase H for 15 min (lane 2). Control was performed (lane 1) by incubating total protein extracts without the enzyme for 15 min. The gelatinolytic activity was assayed.

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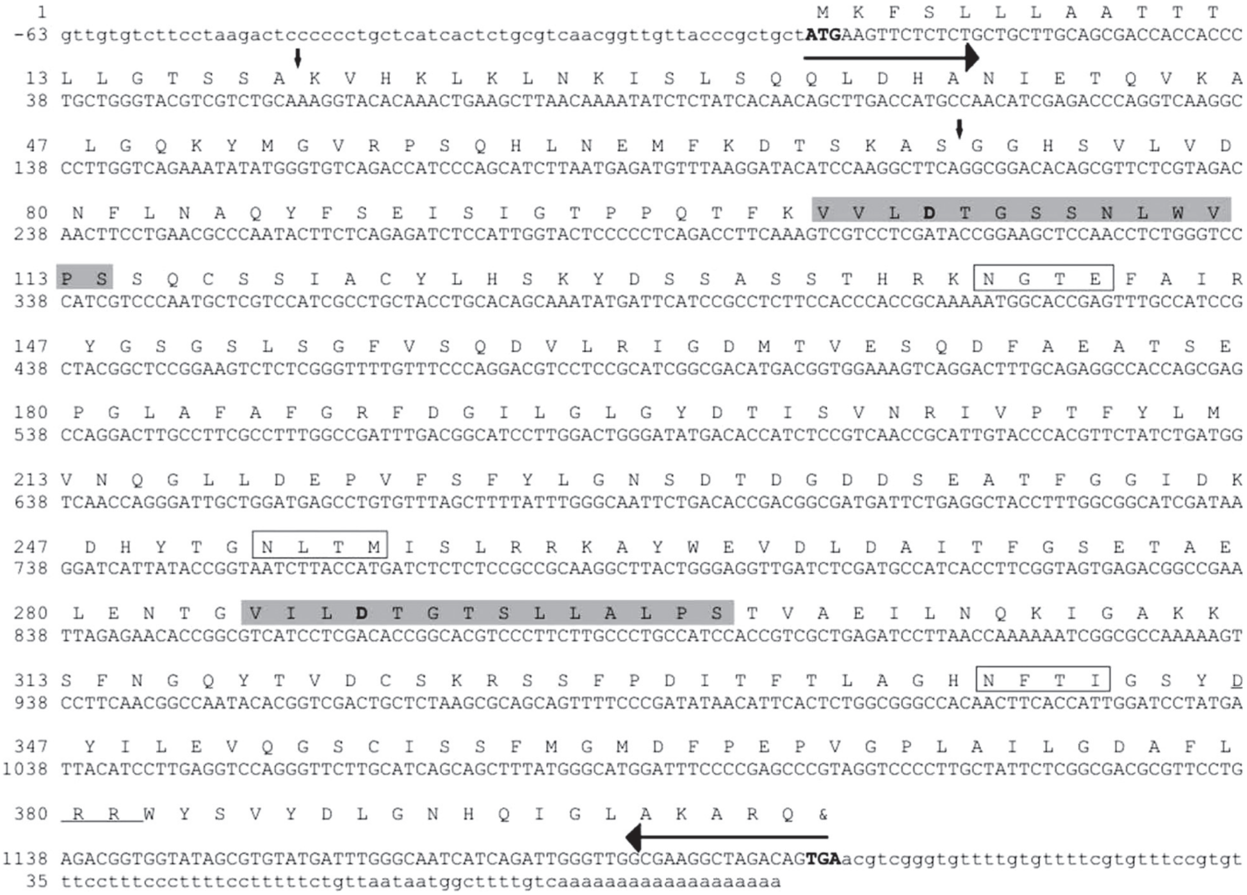
Supplementary Material



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  13 L  L  G  T  S  S  A  K  V  H  K  L  K  L  N  K  I  S  L  S  Q  Q  L  D  H  A  N  I  E  T  Q  V  K  A
  38 TGCTGGGTACGTCGTCTGCAAAGGTACACAAACTGAAGCTTAACAAAATATCTCTATCACAACAGCTTGACCATGCCAACATCGAGACCCAGGTCAAGGC

  47  L  G  Q  K  Y  M  G  V  R  P  S  Q  H  L  N  E  M  F  K  D  T  S  K  A  S  G  G  H  S  V  L  V  D
 138 CCTTGGTCAGAAATATATGGGTGTCAGACCATCCCAGCATCTTAATGAGATGTTTAAGGATACATCCAAGGCTTCAGGCGGACACAGCGTTCTCGTAGAC

  80  N  F  L  N  A  Q  Y  F  S  E  I  S  I  G  T  P  P  Q  T  F  K  V  V  L  D  T  G  S  S  N  L  W  V
 238 AACTTCCTGAACGCCCAATACTTCTCAGAGATCTCCATTGGTACTCCCCCTCAGACCTTCAAAGTCGTCCTCGATACCGGAAGCTCCAACCTCTGGGTCC

 113 P  S  S  Q  C  S  S  I  A  C  Y  L  H  S  K  Y  D  S  S  A  S  S  T  H  R  K  N  G  T  E  F  A  I  R
 338 CATCGTCCCAATGCTCGTCCATCGCCTGCTACCTGCACAGCAAATATGATTCATCCGCCTCTTCCACCCACCGCAAAAATGGCACCGAGTTTGCCATCCG

 147  Y  G  S  G  S  L  S  G  F  V  S  Q  D  V  L  R  I  G  D  M  T  V  E  S  Q  D  F  A  E  A  T  S  E
 438 CTACGGCTCCGGAAGTCTCTCGGGTTTTGTTTCCCAGGACGTCCTCCGCATCGGCGACATGACGGTGGAAAGTCAGGACTTTGCAGAGGCCACCAGCGAG

 180  P  G  L  A  F  A  F  G  R  F  D  G  I  L  G  L  G  Y  D  T  I  S  V  N  R  I  V  P  T  F  Y  L  M
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 213 V  N  Q  G  L  L  D  E  P  V  F  S  F  Y  L  G  N  S  D  T  D  G  D  D  S  E  A  T  F  G  G  I  D  K
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 247  D  H  Y  T  G  N  L  T  M  I  S  L  R  R  K  A  Y  W  E  V  D  L  D  A  I  T  F  G  S  E  T  A  E
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 380  R  R  W  Y  S  V  Y  D  L  G  N  H  Q  I  G  L  A  K  A  R  Q  &

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  35 ttcctttccctttttcctttttctgttaataatggcttttgtcaaaaaaaaaaaaaaaaaa
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Supplementary Fig. 1 Nucleotide and deduced amino acid sequence of the *Paracoccidioides brasiliensis* aspartyl protease cDNA. The nucleotide and amino acid positions are marked on the left side. Lower-case letters represent the untranslated 5´ and 3´ regions. Bold letters in the nucleotide sequence represent the start and stop codons. In the amino acid sequence, the putative cleavage site that removes the signal peptide and the propeptide are indicated with vertical arrows. Two conserved aspartyl protease domains of the active site are shown in gray. In these two regions, the residues of aspartic acid (D is the active site residue) are in bold letters. Three predicted N-glycosylation sites are shown in rectangles. Primers used in this work to amplify the *Pb*SAP cDNA are marked by horizontal arrows.