

# Identification, N-terminal region sequencing and similarity analysis of differentially expressed proteins in *Paracoccidioides brasiliensis*

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*Paracoccidioides brasiliensis* is the causal agent of paracoccidioidomycosis, which is a systemic mycosis in Latin America. This human pathogen is a dimorphic fungus existing as mycelium (26 °C) and in infected tissues as a yeast form (36 °C). The *in vitro* differentiation process is reversible and dependent on temperature shift. In the present study, the total proteins from both forms of *P. brasiliensis* (isolate Pb01) were analysed by two-dimensional electrophoresis. Differentially expressed proteins were identified. Two of these proteins, PbM46 (mycelium) and PbY20 (yeast), were submitted to automated protein sequencing of their N-terminal regions. The 15 amino acid residue sequence of PbM46, AITKIFALKVYDSSG, is similar to enolases from several sources, and specially those from *Saccharomyces cerevisiae* (80%) and *Candida albicans* (67%), when compared to the NR database at NCBI using the BLASTP program. The 34 amino acid residue sequence of PbY20, APKIAIVFYS-LYGHQKLAEAQKKGIEAAGGTAD, could probably represent an allergen protein since it is very similar (90%) to the minor allergen protein of *Alternaria alternata* and 82% similar to the allergen protein of *Cladosporium herbarum*. This comparative analysis of proteins from mycelium and yeast forms has allowed the identification and characterization of differentially expressed proteins, probably related to differential gene expression in *P. brasiliensis*.

**Keywords** differentially expressed proteins, dimorphic fungi, N-terminal region sequencing, *Paracoccidioides brasiliensis*

## Introduction

*Paracoccidioides brasiliensis*, a thermally dimorphic ascomycete, is the causal agent of paracoccidioidomycosis, a human systemic disease in Latin America [1]. The dimorphism, mycelium (26 °C) to yeast (36 °C) conversion, constitutes the main step for the disease establishment. The *in vitro* reversible interconversion between the two forms can be controlled just by temperature shift,

and this makes possible the study of the molecular aspects of cellular differentiation in this dimorphic pathogenic fungus. Studies involving *P. brasiliensis* have been restricted to epidemiological, ethiopathogenic and immunological aspects [2–5], although some work has been done on ultrastructure [6], biochemistry [7–12] and cell wall composition [13]. The molecular cloning, sequencing and heterologous expression of the 43 kDa glycoprotein (gp43) of *P. brasiliensis* has also been reported [14]. The analysis of *de novo* protein synthesis in mycelium and yeast forms as well as in phase transition events has been reported in this fungus [15,16]. Information on differential gene(s) expression and/or stage-specific proteins, po-

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tentially involved in the dimorphism and/or adaptation of *P. brasiliensis* is lacking and would contribute to understanding of parasite molecular biology.

*P. brasiliensis* is phylogenetically related to other dimorphic fungi like *Histoplasma capsulatum* and *Blastomyces dermatitidis*, sharing the ability to differentiate in mycelium and yeast forms depending on incubation temperature [17,18]. For these fungi, gene expression in a single form of the microorganism has been described. For example, *bys1* gene is expressed only at 37 °C by yeast cells in *B. dermatitidis* [19]. Until now, the biological function of the 18.6 kDa protein encoded by *bys1* is not known. Whether or not it is involved in the adaptation/differentiation process of *B. dermatitidis* is still an open question.

In *Candida albicans*, an opportunistic fungus, specific genes have been reported: *cmk1*-*Candida* MAP kinase, involved in morphogenesis and cell wall stability [20], *mkc1* gene, probably involved in cellular sensitivity to heat shock [21] and *chs1* and *chs2*, involved in germinative tube formation [22,23]. *C. albicans* enolase protein has been detected preferentially in mycelium cells and probably induces immune response in host cells. This secreted protein is able to react with anti-enolase, although exhibiting low enzymatic activity when compared with the cytosolic glycolytic enolase isoform [24,25]. Two other differentially expressed proteins of 37 and 67 kDa have been detected in the cell wall of this organism. The 37 kDa species is found only in yeast cells and has the property of laminin binding, suggesting a possible role in host cell adhesion during infection [26].

We are interested in the identification, isolation and characterization of differentially expressed genes/proteins in both forms of *P. brasiliensis*. We report here the detection, partial protein sequencing and similarity analysis of two proteins, one from yeast (PbY20) and other from the mycelium (PbM46) form, which could represent important gene products of the differentiation process in this microorganism.

## Materials and methods

### *Cultivation and harvesting*

*P. brasiliensis*, isolate Pb01, from Goiânia, Brazil, was grown as a yeast (36 °C) or a mycelium (26 °C) on solid Fava-Neto's medium [27], as previously described [5,15,16] over 10–15 days.

### *Protein extract preparation and two-dimensional electrophoresis*

After incubation, the fungi was scraped off and cells

washed in Tris-Ca buffer (20 mM Tris-HCl pH 8.8; 2 mM CaCl<sub>2</sub>), collected by centrifugation at 5000 g for 5 min and disrupted by mechanical maceration using liquid nitrogen. Approximately 4 ml of buffer extraction (20 mM Tris-HCl pH 8.8; 2 mM CaCl<sub>2</sub> containing proteinases inhibitors: 50 µg ml<sup>-1</sup> of TLCK-*N*α-*p*-tosyl-L-lysine chloromethylketone; 4 mM PMSF-phenylmethylsulfonylfluoride; 5 mM iodoacetamide; 1 mM EDTA-ethylenediaminetetraacetic acid; 20 µg ml<sup>-1</sup> of leupeptin and 1 mM PCMB-4-chloromercuribenzoic acid) was added to about 2.5 g wet weight. The extract was vortexed, centrifuged at 10000 g for 5 min, the supernatant separated in aliquots and stored at -20 °C. Total protein was precipitated with 10% (w/v) TCA (trichloroacetic acid), washed twice with 100% acetone at -20 °C and the protein concentration determined according to Bradford [28]. Electrofocusing of 200 µg of total protein was performed by O'Farrell's method [29] and the second dimension according to Laemmli [30], in SDS-PAGE at 12.5% gel concentration.

### *N-terminal region protein sequencing*

Proteins from two-dimensional SDS-PAGE gels were blotted to PVDF-polyvinylidene difluoride membrane (ProBlott, Applied Biosystems, Foster City, CA, USA), which was pre-immersed in water for 1–3 s, followed by 100% methanol and then in water for 1 min, to eliminate methanol excess. The PVDF membranes were pre-equilibrated for 15 min in transfer buffer CAPS 1 ×, prepared by diluting CAPS 10 × in 10% (w/v) methanol (CAPS 10 ×: 100mM 3-cyclohexylamine-1-propanesulphonic acid; pH 11, adjusted with 6 M NaOH). The sandwich polyacrilamide gel-PVDF membrane was electroblotted for 2 h [31,32] and protein spots in PVDF were stained with 0.5% w/v rouge ponceau [33]. Each spot from one gel yielded approximately 1.5 µg after blotting to PVDF membrane. For this reason, it was necessary to perform at least 20 two-dimensional gels/blottings, in order to accumulate about 20 µg of each purified protein for automated sequencing in an Applied Biosystems 477A sequencer fitted with an Applied Biosystems 120A analyser. Prior to sequencing the protein spots were pre-washed three times with 100% methanol and then with water.

### *Protein similarity analysis*

The search for protein sequence similarity was performed via the Internet against the Non-Redundant, NR, database from NCBI (National Center of Biotechnology Information, Bethesda, MD, USA) by using the BLASTP

program [34]. Sequence alignments were carried out by using the CLUSTAL-V program [35]. Percent identity between two proteins is calculated on the basis of the number of identical paired amino acid residues along a sequence portion of 100 residues. Percent similarity is calculated taking into account the number of both identical and similar residues. Similar residues are those observed in conservative substitutions of amino acid residues. To be considered a conservative substitution, the changed residues must keep some common physical-chemical properties such as similar hydrophobicities and charges. In the CLUSTAL-V program, for protein comparisons, a weight matrix is used to differentially weight different pairs of aligned amino acids. The default is the well-known Dayhoff PAM 250 matrix [36].

#### Data Bank accession numbers for pairwise comparisons and multiple alignment

CEEN1, *S. cerevisiae* enolase 1—P00924; CEEN2, *S. cerevisiae* enolase 2—P00925; CHEEN, *C. herbarum* enolase—X78226; CAA47, *C. albicans* secreted enolase—A45241; RNEN, *R. norvegicus* muscle enolase—X57774; HSEN, *Homo sapiens*  $\alpha$ -enolase—X16288; AL22, *A. alternata* allergen protein—P42058; HE22, *C. herbarum*

allergen protein—P42059; CE26, *S. cerevisiae*—P25349; PO25, *S. pombe*—P30821; CE23, *S. cerevisiae*—P38234.

## Results

In order to identify and characterize differentially expressed proteins, the total extracts from M and Y were analysed by two-dimensional gels and compared with each other. As shown in Fig. 1, panels Y and M, we can detect some quantitative and/or qualitative differentially expressed proteins associated with yeast and mycelium cells. One of them, from yeast cells, denominated Y20 seems to be a stage-specific protein, whereas another one, from mycelium cells (M46) is a differentially expressed protein, abundantly present in this fungus form. During this work, we have performed about 30 different two-dimensional gels from both differentiated cells of *P. brasiliensis* (Pb01), which always gave similar results, indicating a reproducible biological protein pattern.

These two proteins, PbM46 (molecular mass 46 kDa,  $pI = 5.0$ ) and PbY20 (molecular mass 20 kDa,  $pI = 5.5$ ), were submitted to N-terminal amino acid automatic sequencing. The N-terminal portion of PbM46 is shown in Fig. 2, panel A. This protein is present in both mycelium and yeast cells, but in a much higher concentration in

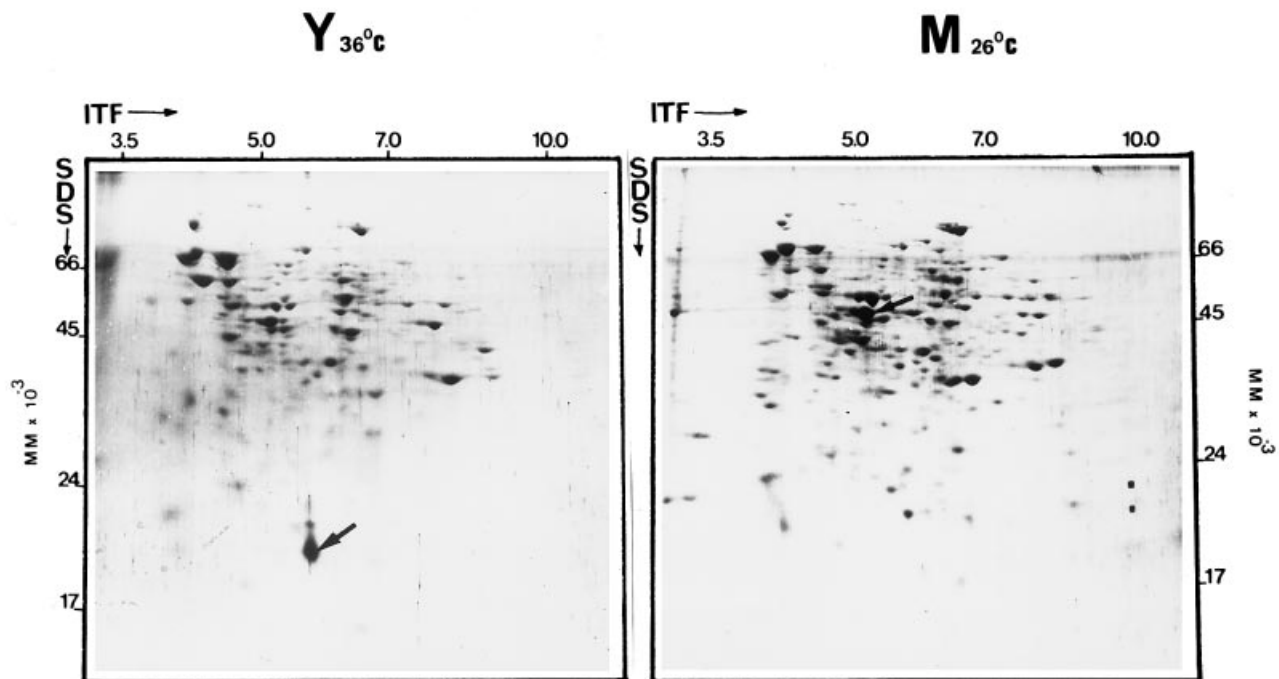


**Fig. 1** Two-dimensional electrophoresis of total proteins from mycelium and yeast cells of *P. brasiliensis* (isolate Pb01). ITF arrows left to right show the pH range of the first dimensional gels. The molecular mass markers from SDS-PAGE second dimension are indicated to the right of the gel (bovine serum albumin, 66 kDa; egg albumin, 45 kDa; trypsinogen, 24 kDa; myoglobin, 14 kDa). The proteins Y20 and M46 (indicated by arrows) were electroblotted on PVDF membranes and submitted to automated protein sequencing.

| A     |                                                    | 5               | 10         | 15           |                 |                                 |
|-------|----------------------------------------------------|-----------------|------------|--------------|-----------------|---------------------------------|
|       |                                                    | AITKIFALKVYDSSG |            |              |                 |                                 |
| B     |                                                    |                 | % Identity | % Similarity | Organism Source |                                 |
| PbM46 | AITKIFALKVYDSSG                                    |                 |            |              |                 |                                 |
| CEEN1 | AVSKVYARSVYDSRG                                    |                 | 53         | 80           |                 | <i>Saccharomyces cerevisiae</i> |
|       | *..*..* **** *                                     |                 |            |              |                 |                                 |
| PbM46 | AITKIFALKVYDSSG                                    |                 |            |              |                 |                                 |
| CEEN2 | AVSKVYARSVYDSRG                                    |                 | 53         | 80           |                 | <i>Saccharomyces cerevisiae</i> |
|       | *..*..* **** *                                     |                 |            |              |                 |                                 |
| PbM46 | AITKIFALKVYDSSG                                    |                 |            |              |                 |                                 |
| CHEEN | PISKIHSRYVYDSRG                                    |                 | 53         | 66           |                 | <i>Cladosporium herbarum</i>    |
|       | *..*..* **** *                                     |                 |            |              |                 |                                 |
| PbM46 | AITKIFALKVYDSSG                                    |                 |            |              |                 |                                 |
| CAA47 | SYA-TKIHARYVYDSRG                                  |                 | 66         | 66           |                 | <i>Candida albicans</i>         |
|       | * *** * ***** *                                    |                 |            |              |                 |                                 |
| PbM46 | AITKIFALKVYDSSG                                    |                 |            |              |                 |                                 |
| RNEN  | AMQKIFAREILDSRG                                    |                 | 53         | 73           |                 | <i>Rattus norvegicus</i>        |
|       | *. ***** .. ** *                                   |                 |            |              |                 |                                 |
| PbM46 | AITKIFALKVYDSSG                                    |                 |            |              |                 |                                 |
| HSEN  | SILKIHAREIFDSRG                                    |                 | 46         | 73           |                 | <i>Homo sapiens</i>             |
|       | . * * * * ..** * *                                 |                 |            |              |                 |                                 |
| C     |                                                    |                 |            |              |                 |                                 |
| PbM46 | AITKIFALKVYDSSG                                    |                 |            |              |                 |                                 |
| CEEN1 | AVSKVYARSVYDSRGNPTVEVELTTEKGVFRSIVPSGASTGVHEALEMRD |                 |            |              |                 |                                 |
| CEEN2 | AVSKVYARSVYDSRGNPTVEVELTTEKGVFRSIVPSGASTGVHEALEMRD |                 |            |              |                 |                                 |
| CHEEN | PISKIHSRYVYDSRGNPTVEVDIVTETGLHRAIVPSVASTGSHEACELRD |                 |            |              |                 |                                 |
| CAA47 | SYA-TKIHARYVYDSRGNPTVEVDFTTDKGLFRSIVPSGASTG        |                 |            |              |                 |                                 |
|       | . . * . . ***** ***** * * ..*****                  |                 |            |              |                 |                                 |

**Fig. 2** PbM46 protein. Panel A: The 15 N-terminal region amino acid residues of PbM46 protein, determined by automated amino acid sequencing, from the mycelium form of *P. brasiliensis* (isolate Pb01). Panel B: Pairwise comparison among PbM46 N-terminal partial sequence and enolase isoforms from different organisms. The comparison was performed using protein sequences from Data Bank and CLUSTAL V program analysis. Panel C: Multiple alignment of *P. brasiliensis* PbM46 N-terminal sequence and enolases from other organisms. Orientation of each peptide is N (left) to C-terminus (right). Asterisks mark identical amino acids; dots, conservative changes; dashes, gaps in the alignment.

mycelium cells (Fig. 1, panel M). The 15 amino acid residues sequenced showed a high degree of similarity with enolases from various sources, including a recently reported enolase from *Neocallimastix frontalis* (NR database, NCBI, accession number X80474), an anaerobic fungus (80% similarity). According to the pairwise comparison and similarity analysis shown in Fig. 2, panel B, 10–12 residues of the total 15 sequenced residues of PbM46 are identical and/or similar to enolase isoforms from other organisms. It is interesting to note that a high degree of similarity (66–80%) was obtained with conserved enolases from *S. cerevisiae* to human. The multiple alignment of PbM46 N-terminal sequence with enolases from other sources (Fig. 2, panel C) shows the identical and conserved amino acid substitution residues in PbM46. The deduced amino acid sequence obtained from the complete cDNA of CHEEN-enolase, an allergen of

*Cladosporium herbarum* [37], showed 73% identity and 83% similarity when compared with enolase 1 (hsp48) from *S. cerevisiae*. Additionally, the N-terminal region of CHEEN showed 66% similarity with the same region of PbM46 from *P. brasiliensis* sequenced in this present work. The enolase from *Candida albicans* CAA47 shows high similarity when compared to *C. herbarum* allergen (CHEEN) and 66% identity with the PbM46 sequence.

The sequence of N-terminal amino acid residues from PbY20, a differentially expressed protein present only in yeast cells (see Fig. 2, panel Y), is shown in Fig. 3, panel A. The similarity search analysis against the NR database of PbY20 34 amino acid residues indicated a high degree of similarity (Fig. 3, panel B) with: (a) two allergen proteins, one of 22 kDa from *Alternaria alternata* (AL22—80%) and another of 22 kDa from *C. herbarum* (HE22—82%); (b) a 26 kDa protein related to

a hypothetical gene located in chromosome III from *S. cerevisiae*, of unknown function (CE26—76%); (c) P25—a 25 kDa protein related to a thermoregulated gene of *Schizosaccharomyces pombe* (PO25—71%); (d) a 22.9 kDa protein related to another hypothetical gene from *S. cerevisiae* chromosome II (CE23—71%). Additionally, it presents approximately the same molecular mass when compared to the above mentioned proteins. The multiple alignment, shown in Fig. 3, panel C, presents the identical and similar amino acid residues in the N-portion of PbY20 from *P. brasiliensis*.

## Discussion

*P. brasiliensis* (isolate Pb01) presents dimorphism like other pathogenic fungi [38]. This morphological process probably represents a successful evolutionary strategy,

related to molecular aspects of adaptation during the life cycle as well as the virulence and/or pathogenicity of these parasites. One approach used by our group to answer the questions about molecular aspects of the differentiation process was to analyse and compare the steady-state proteins of both forms of *P. brasiliensis*.

In this work, the proteins studied were chosen after comparative analysis of protein pattern mapping from both M and Y two-dimensional gels, with respect to molecular masses and pI parameters. This analysis considered two criteria: (1) quantitatively differential expression, i.e. proteins present in higher concentration in one differentiated stage of the fungus, like PbM46 and (2) qualitatively differential expression, i.e. proteins present only in one stage, like PbY20. The inhibitors of serine/cysteine and metalloproteinases used in the extraction buffer should be important to maintain protein pattern

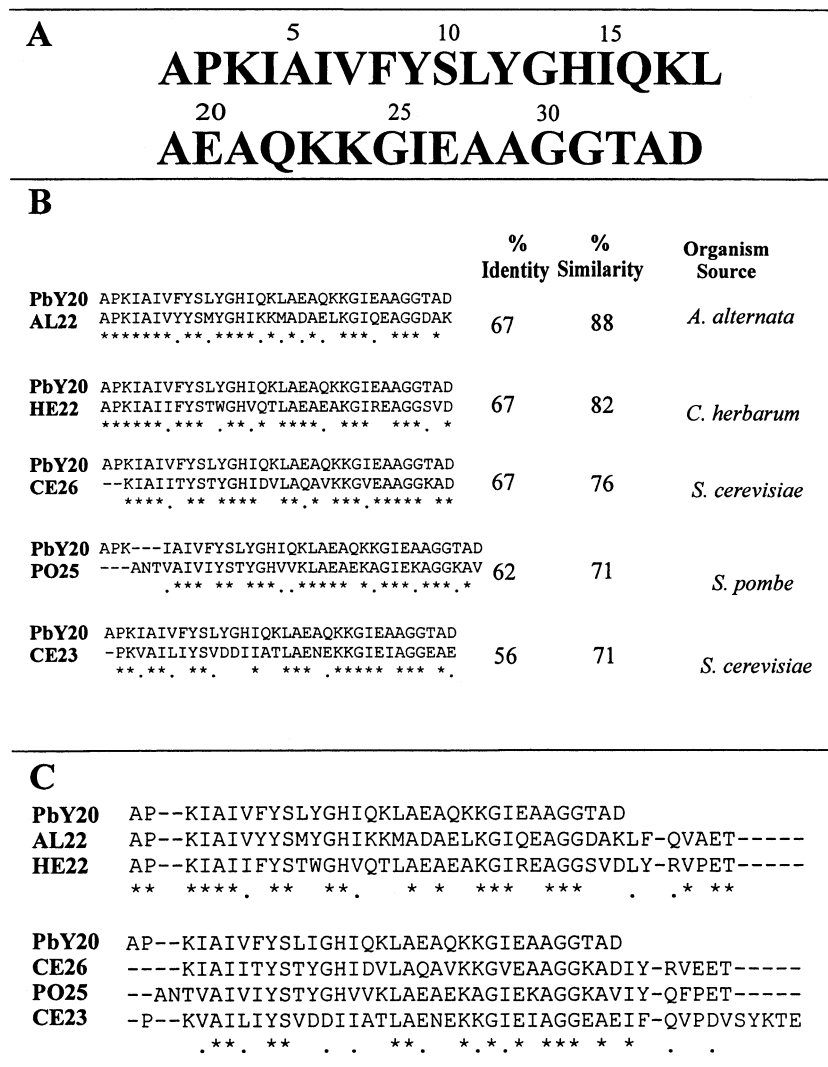


**Fig. 3** PbY20 protein. Panel A: The 34 N-terminal region amino acid residues of PbY20 protein, determined by automated amino acid sequencing, from the yeast form of *P. brasiliensis* (isolate Pb01). Panels B and C: Pairwise comparison and multiple alignment of N-terminal region amino acid sequence of PbY20 and proteins (allergens and regulatory proteins) from other organisms (using protein sequences from Data Bank and CLUSTAL V program analysis). Asterisks mark identical amino acids; dots, conservative changes; dashes, gaps in the alignment.

reproducibility, avoiding non-specific degradation by proteases liberated during cellular lysis. Moreover, the uni-dimensional protein patterns from *P. brasiliensis* (Pb01) M and Y cells were similar to other isolates (Pb18 and Pb113, data not shown), suggesting that these proteins identified in Pb01 probably reflect the differential gene expression pattern, which is a general feature of this microorganism instead of a particular characteristic of one or other isolate of this fungus. These proteins might be involved in the differentiation process and/or maintenance of M and Y forms, or involved in an important event for the biological cycle of *P. brasiliensis*.

The PbM46 protein showed high similarity with enolases from other organisms, like *C. albicans*, *C. herbarum* and *S. cerevisiae*. It is interesting to note that the enolase isoform of *C. albicans*, CAA47, is secreted mainly by mycelium cells. This protein is abundantly produced during infection, is able to induce a strong immune response in human hosts [39] and is found in blood of infected individuals with disseminated disease [25]. In addition, PbM46 protein is very similar to the CHEEN-enolase, considered an allergen of *C. herbarum*. This microorganism is found on all continents and is able to induce hypersensitivity reactions in infected patients. It produces cytoplasmic proteins which may be involved in pathological processes of respiratory tract, promoting strong allergic reactions and increased IgE levels in humans [37]. If PbM46 belongs to an allergen family remains to be elucidated.

With respect to PbY20, the analysis of its N-terminal sequence also showed similarity with allergen proteins from the deuteromycetes *C. herbarum* and *A. alternata*. The allergens HE22 from *C. herbarum* and AL22 from *A. alternata* present 67% identity and 81% similarity when compared to each other. Again, two proteins encoded by hypothetical genes from *S. cerevisiae*, CE26 and CE23, showed high similarity with the allergens and PbY20. These two proteins from *S. cerevisiae* are probably cytoplasmic proteins since their sequences have no hydrophobic domains in their primary structures. Thus, PbY20 could function in allergy induction in paracoccidioidomycosis (PCM) patients and/or sensibilization of individuals presenting a positive test reaction for the disease.

The PO25 gene from *S. pombe*, which is positively controlled by an activator transcriptional factor PAD1, is essential for cellular viability and seems to affect the chromosomal structure in this microorganism [40]. Considering the high similarity score of PbY20 with PO25 (71%) and the fact that PbY20 is stage-specific and over-expressed in Y cells, the morphological phase found in PCM, it would be possible, as proposed for PO25 protein, that PbY20 could also participate in maintenance

of the stability of differentiated Y cells *in vivo* and/or chromosomal organization in this fungus.

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