**UNIVERSIDADE DE BRASÍLIA**
**FACULDADE DE MEDICINA**
**PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS MÉDICAS**

# Rastreamento de ESTs, Expressão Heteróloga de cDNAs e Análise de Proteases no Estudo da Interação Patógeno-Hospedeiro por *Paracoccidioides brasiliensis*

**Tese apresentada ao Programa de Pós-Graduação em Ciências Médicas como requisito para obtenção do título de Doutor em Ciências Médicas.**

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**SUMÁRIO**

## LISTA DE ABREVIATURAS

ATP – adenosina trifosfato

cAMP – adenosina monofosfato cíclico

cDNA – DNA complementar

BCIP - 5-bromo-4-chloro-3-indolil fosfato

bp – pares de bases

BSA – soro albumina bovina

CTS – quitina sintase

DNA – ácido desoxiribonucléico

EDTA - ácido etilenodiaminotetracético

EGTA - ácido etilenoglicoltetracético

EST – etiqueta de sequência expressa

E-64 - L-*trans*-Epoxisuccinil-leucilamido (4-guanidino) butano

FBS – soro fetal bovino

GP – glicoproteína

GM-CSF - Fator Estimulador de Colônia Granulócito Macrófago

GST – glutationa S- transferase

GTP – guanosina trifosfato

HBSS – solução salina tamponada Hanks

HSP – proteína de choque térmico

IPTG - isopropil-β-D-tiogalactopiranosídeo

kDa - kiloDalton

L-DOPA - L - Di-hidroxi fenil alanina

MAPK – proteína quinase ativada por mitose

NBT - nitro blue tatrazólio

ORF – região de leitura aberta

PAGE – eletroforese em gel de poliacrilamida

*Pb – Paracoccidioides brasiliensis*

PBS – solução de tampão fosfato

PCM – paracoccidioidomicose

PCR – reação em cadeia da polimerase

PFGE - gel em eletroforese de pulso alternado

pH - potencial hidrogeniônico

*p*-HMB - p-hidroximercurio benzoato

pI - ponto isoelétrico

PMSF – fluoreto de fenilmetilsulfonil

RDA - análise de diferença representacional

RNA – ácido ribonucléico

RT-PCR – PCR acoplada à transcrição reversa

SAP – aspartil protease secretada

SDS – dodecil sulfato de sódio

SP – serine protease

## RESUMO

*Paracoccidioides brasiliensis* é um fungo patogênico humano, agente etiológico da paracoccidioidomicose (PCM). *P. brasiliensis* apresenta dimorfismo térmico, apresentando-se sob a forma miceliana a temperaturas inferiores a 28 ºC e sob a forma leveduriforme no hospedeiro humano e em temperaturas superiores à 28 ºC. Proteases são enzimas que clivam proteínas e desempenham funções como o processamento intracelular e a clivagem de proteínas do meio extracelular para obtenção de nitrogênio. Em microrganismos patogênicos proteases podem atuar como fatores de virulência, clivando proteínas do hospedeiro para facilitar a penetração pelos tecidos. No presente trabalho, foi realizado o rastreamento e identificação de proteases em banco de dados de ESTs oriundas de células leveduriformes de *P. brasiliensis.* Foram detectadas 53 ORFs codificantes para proteases. As seqüências preditas de aminoácidos foram obtidas e classificadas por homologia em banco de dados de proteases, sendo agrupadas em 3 aspartil, 8 cisteíno proteases, 10 metalo-, 10 serino proteases e 22 proteínas relacionadas às subunidades proteassomais. O presente trabalho inclui também o rastreamento e a identificação de ESTs codificantes de proteínas relacionadas à síntese e ao processamento de proteínas em banco de dados de ESTs oriundas de células de *P. brasiliensis* durante a transição dimórfica de micélio para levedura por 22 horas. Foram identificadas 200 ORFs apresentando homologia com seqüências codificantes a proteínas relacionadas aos processos de síntese e processamento de proteínas. Destas, 16 ORFs codificam para genes não descritos anteriormente em *P. brasiliensis*. Foi possível identificar ESTs que compõem subunidades ribossomais, assim como fatores de iniciação transcricional induzidos durante a transição dimórfica, sugerindo um aumento no nível de proteínas sintetizadas durante este processo. Também foram identificadas ESTs codificantes para chaperonas, para glicosiltransferases e para proteínas relacionadas com a aceleração do enovelamento de proteínas, sugerindo aumento da produção e do controle de qualidade durante a transição dimórfica de micélio para células leveduriformes. Alguns genes codificantes para proteases são induzidos após a indução da transição termodimórfica: uma aspartil protease A01, uma lon protease S16 e uma metaloprotease M28, o que sugere aumento dos processos de controle de qualidade das proteínas produzidas e da aquisição de aminoácidos do meio extracelular. Uma serino protease S08 foi caracterizada. As seqüências de nucleotídeos (*Pb*sp) e aminoácidos (*Pb*SP) foram obtidas e analisadas. O cDNA codificante para *Pb*sp foi clonado em vetor de expressão para sistema bacteriano e a proteína recombinante foi utilizada para obtenção de anticorpo policlonal em camundongos. O anticorpo policlonal reconheceu especificamente uma espécie protéica de 66 kDa em extrato protéico e em sobrenadante de cultura de células leveduriformes de *P. brasiliensis*, sugerindo que *Pb*SP seja uma molécula secretada. Ensaios de deglicosilação *in vitro* com endoglicosidase H demonstraram que *Pb*SP é uma molécula N-glicosilada. *Pb*SP tem seu nível de expressão induzido durante a privação de nitrogênio tanto em extrato protéico quanto em sobrenadante de cultura de células leveduriformes de *P. brasiliensis*, sugerindo que *Pb*SP tenha importância na captação de nitrogênio. A expressão de *Pb*sp é aumentada durante a internalização de células leveduriformes por macrófagos murinos, sugerindo a importância do produto deste gene na resposta adaptativa de *P. brasiliensis* à internalização por macrófagos. A interação de *Pb*SP com moléculas de *P. brasiliensis* foi avaliada utilizando o sistema de duplo-híbrido em *Saccharomyces cerevisiae*. Proteínas relacionadas com enovelamento protéico, controle de qualidade e destinação de proteínas, além de uma proteína de parede celular de *P. brasiliensis* foram identificadas.

## ABSTRACT

*Paracoccidioides brasiliensis* is a human pathogenic fungus, the causative agent of paracoccidioidomycose (PCM). *P. brasiliensis* is a thermodimorphic fungus presents as mycelium form in temperatures below 28º C and as yeast form in the human host and in temperatures above 28ºC. Proteases are enzymes that cleave proteins and are related to the intracellular protein processing and cleavage of extracellular proteins to nitrogen acquisition. In pathogenic microorganisms proteases could act as virulence factors by cleaving host proteins facilitating the invasion process in the host tissues. The present work identified proteases in ESTs database constructed with cDNAs sequences of *P. brasiliensis* yeast form. It was detected 53 ORFs encoding proteases. The predicted amino acids sequences were obtained and classified by homology in protease database: 3 sequences were classified as aspartil, 8 as cysteine, 10 as metallo-, 10 as serino and 22 proteins related to proteasomal subunits. The present work also includes the identification of ESTs encoding proteins related to protein synthesis and processing in ESTs database of *P. brasiliensis* during transition from mycelium to yeast cells. It was identified 200 ORFs presenting homology to sequences encoding proteins related to protein synthesis and processing. Sixteen ORFs were described as novel genes of *P. brasiliensis.* It was identified ESTs related to ribosomal subunits and initiation transcription factors, suggesting intense synthesis of new ribosome particles, affecting the rate of protein synthesis. It was identified ESTs encoding chaperones, glycosiltransferases and proteins related to acceleration of protein folding, suggesting high protein production and high quality control in the protein production during transition from mycelium to yeast cells in *P. brasiliensis.* Transcripts encoding proteases were induced in *P. brasiliensis* during transition from mycelium to yeast: an aspartil protease A01, a lon protease S16 and a metaloprotease M28. The higher expression of these genes during transition in *P. brasiliensis* suggests high control quality of proteins and nitrogen acquisition. A serine protease S08 was characterized. The cDNA (*Pb*sp) and deduced amino acids (*Pb*SP) sequences were obtained and analyzed. *Pb*sp was cloned into expression vector in bacterial system and used to generate polyclonal antibody in mice. The polyclonal antibody recognized specifically a 66 kDa protein species in protein extracts and culture supernatants of *P. brasiliensis* yeast cells, suggesting *Pb*SP is a secreted molecule. *In vitro* deglycosylation assays with endoglycosidase H demonstrated that *Pb*SP is a N-glycosylated molecule. *Pb*SP is increased during nitrogen starvation both in protein extracts and cultures supernatants of *P. brasiliensis* yeast cells, suggesting *Pb*SP is important in nitrogen acquisition. *Pb*sp expression was induced during internalization of *P. brasiliensis* yeast cells by murine macrophages, suggesting the *Pb*sp product is important in the *P. brasiliensis* adaptative response to macrophage internalization. *Pb*SP interaction with *P. brasiliensis* proteins was evaluated by two-hybrid assay in *Saccharomyces cerevisiae.* Proteins related to protein folding, quality control of translation, protein destination and a cell wall protein of *P. brasiliensis* were identified.

# *Capítulo I*

# *Introdução*

# I. INTRODUÇÃO

## I.1. O fungo *Paracoccidioides brasiliensis*

O fungo termodimórfico e patogênico humano *Paracoccidioides brasiliensis* foi descrito por Adolpho Lutz em 1908 e é o agente etiológico da paracoccidioidomicose (PCM) (Franco, 1987, San-Blas 1993). *P. brasiliensis* apresenta-se sob a forma miceliana, a qual se presume que ocorra na natureza em temperaturas inferiores a 28 °C e, sob a forma leveduriforme em tecidos infectados e *in vitro* a temperaturas acima de 28 ºC (Restrepo 1985, Bagagli *et al* 2006). A forma leveduriforme apresenta brotamentos múltiplos formados pela evaginação da célula-mãe; neste caso, a célula central é circundada por várias células periféricas. A forma miceliana é caracterizada por filamentos septados com conídeos terminais ou intercalares (Queiroz-Telles 1994; Restrepo-Moreno 2003).

O habitat de *P. brasiliensis* ainda é indeterminado em virtude de fatores como o prolongado período de latência no hospedeiro humano e a não identificação de hospedeiros intermediários do fungo (Montenegro *et al.* 1996, Bagagli *et al.* 2003). Sabe-se que *P. brasiliensis* pode ser isolado a partir de solos, como já realizado no Brasil (Montenegro *et al.* 1996) e na Venezuela (Albornoz 1971), sugerindo que o solo pode ser um importante elemento na biologia do fungo. As diferentes condições do solo alteram a capacidade de crescimento da forma miceliana e a produção de conídeos por *P. brasiliensis* (Franco *et al.*, 2000). Terçarioli e colaboradores (2007) realizaram o cultivo de vários isolados de *P. brasiliensis* em diferentes tipos de solo e observaram que este fungo apresenta a capacidade de crescimento em solos arenosos e argilosos, com alta umidade. Neste caso, a produção de conídeos foi observada em alguns isolados. Foi possível observar também a inibição de crescimento de *P. brasiliensis* em solos com altos níveis de alumínio.

O isolamento de *P. brasiliensis* de animais também tem sido relatado, como o morcego frugívoro *Artibeus lituratus* (Greer & Bolanos 1977), o pingüim da Antártida

Uruguaiana *Pygoscelis adeliae* (Garcia *et al.* 1993) e duas espécies de tatus, *Dasypus novemcinctus* e *Cabassous centralis* (Bagagli *et al.* 2003, Corredor *et al.* 2005). A infecção natural em alguns animais selvagens e domésticos tem sido observada e manifestações clínicas da doença foram relatadas em cachorros (Ricci *et al.* 2004). Richini-Pereira e colaboradores (2008) realizaram estudos objetivando a detecção molecular de *P. brasiliensis* em tecidos de 19 animais mortos em rodovias, pela técnica de PCR-Nested. As reações apresentaram amplificações específicas para *P. brasiliensis* em vários tecidos de tatus e de porco da índia, pulmão e fígado de porco espinho e pulmão de furão. Estes dados sugerem que a presença de *P. brasiliensis* na natureza, em animais de áreas endêmicas, é maior do que inicialmente postulado.

A classificação taxonômica de *P. brasiliensis* é difícil dada o não conhecimento da forma sexuada do fungo. Entretanto, análises da subunidade maior 25 do RNA ribossomal foram realizadas para fungos dermatófitos e dimórficos por Leclerc e colaboradores (1994). Os resultados demonstraram que estes dois grupos apresentam-se filogeneticamente separados. Neste estudo, foi possível classificar *P. brasiliensis* juntamente com outros fungos dimórficos, tais como *Blastomyces dermatitidis* e *Histoplasma capsulatum* como pertencente ao filo Ascomycota, à ordem Onygenales e à família Onygenaceae. Atualmente, com base em análises filogenéticas moleculares, *P. brasiliensis* é classificado com pertencente ao reino Fungi, filo Ascomycota, subdivisão Euascomycotina, classe Plectomyceto, subclasse Euascomycetidae, ordem Onygenales, família Onygenaceae, subfamília Onygenaceae Anamórficos, gênero *Paracoccidioides*, espécie única *Paracoccidioides brasiliensis* (San-Blas *et al.*, 2002).

Foram realizados estudos filogenéticos utilizando-se oito regiões de cinco genes nucleares com sessenta e cinco isolados de *P. brasiliensis*. O fungo foi classificado em três espécies filogenéticas distintas: S1 (contendo 38 isolados), PS2 (contendo seis isolados), e

PS3 (com 21 isolados). S1 compreende isolados do Brasil, Argentina, Paraguai, Peru e Venezuela. PS2 compreende cinco isolados brasileiros e um isolado da Venezuela. PS3 compreende isolados somente da Colômbia e é considerada uma linhagem filogeneticamente independente (Matute *et al.*, 2006).

Em estudo recente pela técnica de concordância genealógica para reconhecimento de espécies filogenéticas, Carrero e colaboradores (2008) analisaram 14 genes de 21 isolados de *P. brasiliensis*. Com exceção das análises filogenéticas realizadas para os genes codificantes para quitina sintase 1 e catalase A, o isolado *Pb*01 apresentou-se filogeneticamente distinto dos outros isolados analisados. O isolado *Pb*01 agrupou-se com o isolado IFM 54648, obtido a partir de um paciente do estado do Paraná. Os outros isolados apresentaram-se agrupados segundo a região geográfica das quais foram obtidos. Estes resultados sugerem a possibilidade de ocorrência de mais de três espécies filogenéticas em *P. brasiliensis*. Os resultados sugerem que os isolados *Pb*01 e IFM 54648 apresentam-se geneticamente separados dos outros isolados por um longo período de tempo. Especula-se também que o isolado *Pb*01 possa constituir uma nova espécie do gênero *Paracoccidioides*; entretanto, esta hipótese requer que sejam encontrados outros isolados geneticamente similares ao *Pb*01 para ser validada (Carrero *et al.*, 2008).

A organização genômica tem sido investigada em *P. brasiliensis*. Feitosa e colaboradores (2003) em estudos utilizando-se técnica de gel em eletroforese de pulso alternado (PFGE) analisaram 12 isolados clínicos de *P. brasiliensis* de diferentes regiões geográficas. Foi estimado um genoma compreendendo em torno de 23 a 31 Mb, com a identificação de 4-5 cromossomos com tamanhos variáveis de 2 a 10 Mb. Análises utilizando-se o fluorocromo DNA - específico DAPI (4', 6-diamidino-2-fenilindol) foram realizadas em células leveduriformes de dois isolados de *P. brasiliensis* (isolados B339 e 113). Através desta técnica, foi estimado um genoma entre 45,7 e 60,9 Mb, sugerindo que as leveduras

destes isolados sejam diplóides (Cano *et al.*, 1998). Em estudos utilizando-se citometria de fluxo na análise de conídeos e leveduras de 10 isolados de *P. brasiliensis* foram descritos genomas de tamanhos entre 26,3 a 35,5 Mb nos conídeos e 30,2 a 30,9 Mb nas leveduras, não havendo, portanto, diferenças significativas entre as duas formas (Almeida *et al.*, 2007).

Recentemente, o seqüenciamento dos genomas estruturais dos isolados *Pb*01, *Pb*03 e *Pb*18 de *P. brasiliensis* foi realizado. As análises dos resultados confirmaram a presença de 5 cromossomos em cada isolado. O genoma do isolado *Pb*01 é composto de 32,94 Mb, com um total de 9.132 genes. Este isolado apresenta o genoma maior tanto em número de bases quanto em quantidade de genes comparado aos outros dois isolados analisados, que apresentaram genomas do tamanho de 29,06 e 29,95 Mb, com número de genes de 7.875 e 8.741 (dados dos isolados *Pb*03 e *Pb*18, respectivamente). Essas informações, além de auxiliar a elucidar as diferenças existentes entre os isolados, serão importantes na caracterização de genes e regiões promotoras e, conseqüentemente, na melhor caracterização da biologia de *P. brasiliensis* (http://www.broad.mit.edu/science/projects/msc/data-release-summary).

### I.2. A Paracoccidioidomicose

A PCM é uma micose sistêmica que se apresenta sob duas formas principais: aguda e crônica. A primeira, também denominada forma juvenil é a forma mais severa da doença, acomete principalmente jovens e crianças e representa 3 a 5% dos casos. A segunda, também denominada adulta, tem progressão mais lenta e representa mais de 90% dos casos (Franco *et al.,* 1987; Montenegro, 1986). A PCM apresenta distribuição geográfica restrita à América Latina, com maior incidência no Brasil, Colômbia e Venezuela (Wanke e Londero, 1994; Rivitti e Aoki, 1999). Estima-se que 80% dos casos descritos estejam no Brasil (Brummer *et al.,* 1993; Coutinho *et al.,* 2002), principalmente nas regiões Sul, Sudeste e Centro-Oeste (Blotta *et al.,* 1999; Paniago *et al.,* 2003) com alta prevalência em áreas rurais. Nas áreas

endêmicas da doença, a incidência estimada é de, aproximadamente, 1 a 3 casos clínicos por ano para cada 100.000 habitantes (Restrepo 1985). A doença acomete principalmente indivíduos do sexo masculino entre 30 e 60 anos de idade, que trabalham em atividades agrícolas. Acredita-se que o manejo da terra, realizado por homens na maioria das propriedades rurais, leva-os ao contato com propágulos do fungo aumentando a incidência da doença nesta população (Brummer *et al.,* 1993). Análises da transição de micélio para levedura em 3 isolados de *P. brasiliensis* foram realizadas na presença do hormônio feminino 17-β-estradiol. Os resultados demonstraram que, na presença do hormônio, o número de formas micelianas que transitaram para as células leveduriformes era menor do que na ausência do hormônio. Foi possível observar também que o número de células que realizavam a transição dimórfica era inversamente proporcional à concentração do hormônio utilizada. Estes resultados sugerem que o hormônio 17-β-estradiol, presente em mulheres em idade reprodutiva confere resistência à infecção por *P. brasiliensis*, sendo um importante fator que justifica a menor incidência da doença em mulheres (Restrepo *et al.,* 1984). A transição da forma miceliana para forma leveduriforme de *P; brasiliensis in vivo* foi realizada em camundongos. Após a infecção intranasal, a transição dos conídios para formas intermediárias e leveduriforme foi avaliada em lavado bronquioalveolar nos tempos de 24 a 96 horas. A transição foi observada nos camundongos machos. Células leveduriformes foram encontradas 24 horas após a inoculação, aumentando em número após 48 e 96 horas. Nos camundongos fêmeas, a transição não ocorreu. Estes resultados corroboram dados epidemiológicos e observações *in vitro*, sugerindo que o hormônio feminino bloqueia a transição da forma miceliana para leveduriforme em *P. brasiliensis* e pode ser responsável pela menor incidência da PCM em mulheres em idade fértil (Aristizabal *et. al.*, 1998).

Acredita-se que a inalação dos propágulos aéreos seja a etapa inicial da infecção e que os mesmos convertam-se em células leveduriformes nos pulmões. (Franco 1987, San-Blas *et*

*al*, 2002). A partir dos alvéolos pulmonares o fungo pode disseminar-se por vias hematogênica ou linfática para outros órgãos como fígado, baço, ossos e sistema nervoso central (Camargo & Franco 2000; Valera *et al.,* 2008).

### I. 3. Perfis de expressão gênica de *P. brasiliensis*

Estudos envolvendo perfis de expressão de genes das formas miceliana e leveduriforme de *P. brasiliensis* têm sido realizados com o objetivo de elucidar respostas adaptativas do fungo ao dimorfismo e ao hospedeiro. Neste sentido, foram construídas bibliotecas de cDNAs obtidos das formas leveduriforme e miceliana do isolado *Pb*01 de *P. brasiliensis*. As bibliotecas de cDNAs obtidas foram clonadas e os produtos seqüenciados. Foram identificados 6.022 genes expressos nas fases miceliana e leveduriforme. Entre os transcritos identificados, incluem aqueles codificantes pra chaperonas diferencialmente expressas e genes que não apresentam homologia no genoma humano. Entre os genes não homólogos a genes humanos estão aqueles codificantes para isocitrato liase e α-1,3-glucana sintase, considerados alvos para drogas antifúngicas, ambos expressas preferencialmente na fase leveduriforme (Felipe *et al.,* 2003; Felipe *et al.,* 2005). Características metabólicas diferenciais das formas miceliana e leveduriforme de *P. brasiliensis* foram descritas. A alta expressão de genes que codificam enzimas que participam da fosforilação oxidativa, como a isocitrato desidrogenase e succinil coenzima-A sintase sugere que o micélio apresente metabolismo aeróbio. Já na fase leveduriforme, genes como álcool desidrogenase I tem seus níveis de expressão aumentados, sugerindo que o metabolismo, nesta fase, seja predominantemente anaeróbio (Felipe *et al.* 2005).

Goldman e colaboradores (2003), analisando 4.692 ESTs (Expressed Sequence Tags) do isolado *Pb*18 de *P. brasiliensis* identificaram vários genes expressos na fase leveduriforme de *P. brasiliensis* homólogos a fatores de virulência descritos em *C. albicans*, que

possivelmente atuam na sobrevida do fungo no ambiente do hospedeiro. Os resultados sugerem que os mecanismos para patogenicidade e virulência são conservados entre as espécies analisadas. A expressão de alguns genes foi analisada por RT-PCR em tempo real. Os resultados identificaram genes com níveis de expressão aumentados na fase miceliana, tais como isocitrato liase, malato desidrogenase e oxidases. Outros genes, tais como ubiquitina e chaperonas de choque térmico (HSP70, HSP82 e HSP104) apresentaram níveis de expressão aumentados na transição dimórfica de micélio para levedura e na fase leveduriforme. O aumento da expressão destes transcritos durante a transição dimórfica sugere a importância do controle da qualidade das proteínas produzidas nesta etapa, tanto no enovelamento, realizado pelas chaperonas, quanto na degradação de proteínas mal-enoveladas, realizada pela ubiquitina.

A transição da fase miceliana para a fase leveduriforme em *P. brasiliensis* tem sido alvo de vários estudos, visto a importância deste processo para a sobrevida do fungo nas condições de temperatura encontrada nos tecidos do hospedeiro. Estudos utilizando-se a técnica de microarranjo de DNA em diferentes etapas da transição dimórfica foram realizados por Nunes e colaboradores (2005). Alguns genes relacionados às vias de transdução de sinais apresentaram níveis de expressão aumentados, sugerindo que as vias de sinalização associadas ao dimorfismo são controladas pelo cAMP e MAPK. Muitos genes apresentaram níveis de transcritos aumentados durante a transição dimórfica, como aqueles relacionados ao metabolismo de aminoácidos, síntese de proteínas, metabolismo da parede celular, estrutura do genoma, resposta ao estresse oxidativo e controle de crescimento e desenvolvimento celulares.

Bastos e colaboradores (2007), em nosso laboratório, avaliaram a expressão de 1.107 transcritos obtidos a partir de biblioteca de cDNA de *P. brasiliensis*, isolado *Pb*01, após 22 horas de indução da transição dimórfica de micélio para levedura. Foram identificados genes

com níveis de expressão aumentados relacionados ao remodelamento da parede celular, bem como transcritos que codificam para enzimas relacionadas com a síntese e degradação de carboidratos de membrana. Também foram induzidos nesta condição genes codificantes para transportadores e de enzimas precursoras da síntese de carboidratos de membrana e genes codificantes para enzimas da síntese de lipídeos de membrana. Os dados sugerem que o remodelamento da parede celular ocorra nos estágios iniciais da transição. Um novo transcrito que codifica para alfa-glicosidase, possivelmente relacionado ao processamento de beta-1,6 glicana também foi identificado durante a transição dimórfica de micélio para levedura. Genes codificantes para quitinase 1 (CTS1) e 3 (CTS3) foram induzidos sugerindo que o processamento de quitina, o maior componente da parede celular, é importante para a transição dimórfica em *P. brasiliensis.* Um transcrito que codifica para amino ácido permease ácida, relacionada à captação de glutamato, precursor para a síntese de quitina também foi induzido durante a diferenciação de micélio para levedura (Bastos et al., 2007).

Bastos e colaboradores (2007) detectaram também aumento nos níveis de expressão de genes relacionados ao metabolismo de enxofre, como sulfito redutase, sugerindo a importância do enxofre durante a transição, corroborando trabalhos anteriores (Marques *et al.*, 2004; Ferreira *et al.*, 2006). Transcritos relacionados ao ciclo do glioxalato, tais como isocitrato liase, malato desidrogenase, citrato sintase e aconitase sugerem que esta via seja funcional durante a transição dimórfica. Além disto, genes relacionados às vias de transdução de sinais (MAPK, serina/treonina quinase e histidina quinase) apresentaram níveis de expressão aumentados, sugerindo o controle destes genes na adaptação e sobrevivência do fungo durante os estágios iniciais da transição (Bastos et al., 2007).

Com o objetivo de elucidar as respostas adaptativas de *P. brasiliensis* à infecção em modelo animal, análises subtrativas de cDNAs utilizando a técnica de RDA (análise de diferença representacional) foram realizadas em nosso laboratório a partir de cDNAs obtidos

de células leveduriformes recuperadas de fígado de camundongos infectados. A regulação positiva da transcrição do gene transportador de alta afinidade de ferro/zinco foi observada, justificada pelas baixas concentrações destes compostos no fígado de camundongos. A super-expressão de transcritos codificantes para proteínas de choque-térmico (HSPs) foi relatada, sugerindo o papel destas no reparo de danos causados por estresse, além de atuarem em processos como divisão celular, síntese de DNA, transcrição, tradução, enovelamento, transporte protéico e translocação transmembrana. Transcritos que codificam para enzimas das vias de biossíntese de melanina, tais como L-amino ácido descarboxilase aromática, tirosinase e policetídeo sintase foram induzidos em células leveduriformes de *P. brasiliensis* recuperadas de fígado de camundongos e demonstram a relevância da síntese de melanina no processo infeccioso em camundongos (Bailão et al., 2006). A importância da síntese de melanina na patogênese de fungos já é descrita (Hamilton & Gomez 2002, Taborda et al., 2008). O cultivo de *P. brasiliensis* na presença L-DOPA, precursor da melanina, resulta na melanização das células fúngicas, demonstrando a capacidade do fungo de produzir melanina (Gomez et al., 2001). Células de *P. brasiliensis* melanizadas são mais resistentes à fagocitose por macrófagos murinos e à ação dos antifúngicos anfotericina B, cetoconazol, fluconazol, itraconazol e sulfametoxazol (Silva et al., 2006).

Com o objetivo de ampliar os conhecimentos sobre as mudanças transcricionais de *P. brasiliensis* durante a infecção em modelo animal, nosso laboratório realizou análises do transcritoma de células leveduriformes recuperadas de fígado de camundongos infectados. Foram obtidas e analisadas 4.932 ESTs. Dos transcritos obtidos, 37,47% foram descritos como novos genes em *P. brasiliensis* e 23,75% dos transcritos apresentaram níveis de expressão aumentados. Os transcritos codificantes para proteínas relacionadas à glicólise, biossíntese de aminoácidos e de lipídeos, biossíntese do ergosterol, transportadores de membrana e proteínas relacionadas ao estresse celular apresentaram-se regulados

positivamente nesta condição. Uma avaliação global dos resultados leva a sugerir que *P. brasiliensis* utilize várias fontes de carbono durante a colonização do fígado, incluindo glicose e substratos do ciclo do glioxalato. Pode-se sugerir também que metabolismo de nitrogênio esteja mais ativo durante o processo infeccioso, quando comparado com dados de leveduras cultivadas *in vitro*, sugerindo que alguns compostos nitrogenados podem ser adquiridos dos tecidos do hospedeiro, enquanto outros devem ser sintetizados pelo patógeno.

Análises subtrativas de cDNAs utilizando a técnica de RDA foram realizadas após a incubação de células leveduriformes de *P. brasiliensis* na presença de sangue humano, condição semelhante àquela que o fungo é submetido durante a disseminação no hospedeiro. Neste estudo foi detectado aumento da expressão de genes como aqueles codificantes para glutamina sintase e permease de aminoácidos ácidos. O aumento da transcrição destes genes poderia estar relacionado ao acúmulo de quitina para remodelamento da parede em resposta a mudanças de osmolaridade externa. O transcrito codificante para uma serina protease S08 foi regulado positivamente em *P. brasiliensis* durante a incubação com sangue humano (Bailão et al., 2006). Estudos envolvendo culturas de *A. fumigatus* na presença de soro demonstraram que a secreção de proteases está associada ao aumento da concentração de soro em meio de cultura, provavelmente para promover o acúmulo de aminoácidos no meio extracelular, para captação pelo fungo (Gifford *et al.,* 2002).

Análises de transcritos através da técnica de RDA em células leveduriformes de *P. brasiliensis* após incubação com plasma humano foram realizadas, condição na qual o fungo pode ser submetido em conseqüência de resposta inflamatória aguda em sítios de infecção. Foi possível detectar-se a super-expressão de genes relacionados à síntese de proteínas, respostas celular à mudança de osmolaridade do meio, remodelamento de parede celular e defesa. Os transcritos codificantes de enzimas da β-oxidação foram induzidos durante a incubação de leveduras de *P. brasiliensis* em plasma humano sugerindo que o fungo, nesta

condição, desvie o seu metabolismo para a degradação de lipídeos. Neste estudo os transcritos codificantes de duas proteases apresentaram-se induzidos, sendo codificantes para uma serina protease S08 relacionada à captação de nitrogênio em fungos e uma protease de membrana mitocondrial relacionada ao processamento intracelular de proteínas (Bailão *et al.*, 2007).

A resposta transcricional de *P. brasiliensis* à internalização por macrófagos murinos foi descrita sendo possível identificar genes regulados positivamente nesta condição, principalmente àqueles relacionados à biossíntese de aminoácidos e o gene codificante da proteína de choque térmico HSP60, sugerindo que estes genes são relevantes durante a fagocitose por macrófagos (Tavares et al., 2007). Posteriormente, genes relacionados ao ciclo do glioxalato foram avaliados durante a internalização por macrófagos e os resultados mostraram que os genes necessários para metabolização de compostos com dois carbonos apresentaram-se regulados positivamente nesta condição. Estes dados sugerem que *P. brasiliensis* utilize o ciclo do glioxalato como uma via metabólica para obtenção de carbono durante a internalização por macrófagos (Derengowski et al., 2008).

### I.5. Proteases

Proteases são enzimas que clivam proteínas, catalisando a hidrólise de ligações peptídicas (Barrett *et al.*, 1998). De acordo com o Comitê de Nomenlatura da União Internacional de Bioquímica e Biologia Molecular (International Union of Biochemistry and Molecular Biology - IUBMB http://www.chem.qmul.ac.uk/iubmb/enzyme/EC34/), o termo mais correto para denominar esta classe de enzimas é peptidase. Em 2006, análises realizadas em banco de dados SwissProt (http://www.ebi.ac.uk/swissprot/) mostraram que cerca de 18% das seqüências anotadas neste banco estão relacionadas ao processamento proteolítico. Além disto, cerca de 2% de todos os genes já depositados no banco de dados SwissProt codificam para peptidases e seus homólogos (Rawlings et al., 2006). Rawlings e colaboradores (2008)

descrevem que o banco de dados de proteases MEROPS (http://merops.sanger.ac.uk) contém 66524 seqüências de peptidases depositadas, incluindo 2403 espécies de proteínas, organizadas em 185 famílias, que, por sua vez, são classificadas em 51 clãs. As peptidases podem ser classificadas quanto ao seu mecanismo catalítico em: aspartil-, metalo-, cisteíno-, serino- e treonino- proteases. Proteases dos dois primeiros grupos utilizam uma molécula de água para atacar a ligação peptídica do substrato, enquanto as outras classes utilizam um resíduo de aminoácido localizado no sítio ativo (Rawlings & Barrett, 1993; Barrett *et al.,* 1998). As proteases são divididas também em dois grupos maiores de acordo com seus sítios de ação: exoproteases clivam o peptídeo próximo à região amino ou carboxi terminais no substrato, enquanto endoproteases, também denominadas proteinases, clivam peptídeos distantes das regiões terminais do substrato (Watson, 1976).

Em todas as células, procarióticas e eucarióticas, as estruturas estão continuamente sendo renovadas. Assim, a homeostase entre as vias anabólicas e catabólicas deve ser continuamente mantida (Voges *et al.*, 1999). As funções das peptidases vão além da digestão generalizada de proteínas, englobando processos tais como ativação de zimógenos, coagulação do sangue, lise de fibrina e ativação de hormônios e de precursores de proteínas (Neurath *et al.*, 1984). Nas células eucarióticas, a grande maioria das proteases apresenta localização restrita, geralmente associada a lisossomos, endossomos, grânulos secretores, vesículas transportadoras e mitocôndrias. Neste caso, as proteases têm suas funções associadas à ativação de zimógenos, endereçamento e processamento de outras proteínas, apresentando localização restrita e, geralmente associadas à condição ideal de pH (Bond & Butler 1987). Proteínas envolvidas no processo de ubiquitinação e de deubiquitinação regulam vários processos celulares essenciais, como a degradação de proteínas, progressão do ciclo celular, sinalização e reparo de DNA (revisado por Rytkonen & Holden 2006). A morte

programada da célula, processo denominado apoptose, é dependente da ativação de caspases, que são proteases cistenil-aspartato específicas (Nicholson et al., 1995).

Algumas proteases são secretadas e apresentam suas funções fora do ambiente celular. Em microorganismos esta atividade está geralmente associada à degradação de peptídeos para aquisição de nitrogênio, principalmente em condições onde há privação de carbono e/ou nitrogênio. Alterações no perfil transcricional de genes codificantes para proteases foram detectadas para o fungo entomopatogênico *Magnaporthe grisae* em condições de privação das fontes de nitrogênio no meio extracelular. Nesta condição, foi possível detectar-se o aumento de expressão de serino proteases. Acredita-se que este aumento de expressão de proteases faça parte de uma resposta global ao estresse nutricional (Donofrio *et al.*, 2006). No fungo filamentoso *Podospora anserina*, um gene codificante para uma aspartil protease é induzido quando o organismo é submetido à privação de carbono, tanto na presença quanto na ausência de fontes de nitrogênio. Este fato pode ser explicado pela presença do motivo de ligação 5'-SYGGGG-3'na região promotora do gene codificante para aspartil protease. Este domínio é descrito como de ligação à proteína CreA que reprime a expressão dos genes aos quais ela se liga quando há fonte de carbono disponível no meio de cultura (Paoletti *et al.*, 1998).

Em *Schizosaccharomyces pombe,* o gene *isp6* codificante para uma serina protease tem seus níveis de expressão inversamente proporcionais à concentração de nitrogênio em meio de cultura (Sato *et al.*, 1994). O cultivo de *S. pombe* em condição de privação de nitrogênio promove a autofagia, que está associada à degradação de proteínas em larga escala para obtenção de fontes de nitrogênio. Mutantes para o gene *isp6* (Δ*isp6*) apresentam disfunção na degradação de proteínas durante a privação de nitrogênio, sugerindo a importância do produto gênico neste processo. A avaliação do desenvolvimento sexual em células Δ*isp6* mostrou que há uma diminuição significativa na eficiência de esporulação e de promoção de diploidia. Análises utilizando-se a técnica de *Northern blot* mostraram que esta

deficiência está associada à expressão do gene *stel 1*, responsável pelo desenvolvimento sexual e induzido em resposta à privação de nitrogênio. Os resultados demonstram que *stel 1* apresenta níveis de expressão basal nas células Δ*isp6* e são expressos em níveis aumentados em células selvagens, durante a privação de nitrogênio. Estes dados demonstraram que *isp6* afeta a expressão de *stel 1* (Nakashima *et al.*, 2006).

Em fungos, a utilização de fontes de nitrogênio alternativas é controlada pelos fatores de transcrição GATA. Em *C. albicans*, a aspartil protease SAP2 é preferencialmente expressa em meio de cultura quando soro albumina bovina é utilizada como única fonte de nitrogênio (Banerjee *et al.*, 1991). A transcrição de SAP2 é influenciada por fatores de transcrição GATA, visto que mutantes duplos para dois fatores de transcrição GATA, Gln3p e Gat1p, são incapazes de expressar SAP2 e de crescer em meio com soro albumina bovina como única fonte de nitrogênio (Dabas & Morschhauser 2008).

Além de todas as funções em nível celular, as proteases estão associadas a processos mais complexos em eucariotos, incluindo condições patológicas. É descrito que metaloproteases atuam no processo de metástase clivando componentes de matriz extracelular (revisado por Rydlova et al., 2008). Uma aspartil protease humana BACE 1, que é uma peptidase de processamento da proteína precursora β-amilóide, apresenta níveis de expressão maiores em casos de doença de Alzheimer e, tem sido foco como alvo para drogas inibidoras no tratamento desta doença (revisado por Ghosh et al., 2008).

Muitas proteases apresentam utilidade comercial, principalmente aquelas que possuem pH ideal de ação alcalino. Peptidases produzidas por bactérias apresentam pH de ação que variam de 5 a 8 e são relativamente termos-tolerantes. Muitas são utilizadas pelas indústrias de alimentos, couro e de detergentes para hidrólise de resíduos, substituindo muitas vezes tratamentos químicos, mais agressivos ao meio ambiente (Rao et al., 2008).

### I.4.1. Aspartil proteases

Aspartil-proteases (EC 3.4.23) são um grupo de peptidases que apresentam sítio catalítico conservado, possuem pH ideal de ação ácido e tem preferência por clivagem de aminoácidos hidrofóbicos. O pH de ação desta classe limita a localização celular e a atuação destas peptidases a condições mais específicas, sendo menos abundantes que serino- e metaloproteases. Nesta família incluem-se as peptidases estomacais, tais como, pepsina e quimosina e peptidases lisossomais, tais como catepsinas D e E. As aspartil proteases são sintetizadas como pré-propeptídeo, com uma região de peptídeo sinal e uma região pro-peptídica. A auto-remoção destas seqüências é determinante para que a ativação ocorra e é dependente das condições de pH (Tang & Wong 1987).

Aspartil-proteases são encontradas desde retrovírus a plantas e mamíferos. Possuem massa molecular em torno de 40 kDa, com seqüência pro-peptídica que varia de 27 a 60 aminoácidos. Todas as peptidases desta classe são inibidas por pepstatina A. A seqüência de aminoácidos das peptidases desta família apresenta duas regiões conservadas, DSG (onde D corresponde ao resíduo de ácido aspártico, S, serina e G, glicina) e DTG (onde D corresponde ao resíduo de ácido aspártico, T, treonina e G, glicina). Os resíduos de ácido aspártico compõem o sítio ativo destas proteínas que utilizam uma molécula de água para mediar o ataque nucleofílico da ligação peptídica (Szecsi 1992; Rawlings & Barrett 1995). A classe das aspartil-proteases é dividida em sete clãs e 14 famílias, sendo a de maior representatividade o clã das pepsinas, AA (http://merops.sanger.ac.uk).

### I.4.2. Cisteíno-proteases

Cisteíno-peptidases (EC 3.4.22) são peptidases que apresentam um resíduo do aminoácido cisteína no sítio ativo. Atualmente esta classe se subdivide em 9 clãs e em 58 famílias (http://merops.sanger.ac.uk). O maior clã é o CA, que inclui a cisteíno peptidase mais

caracterizada, a papaína. Neste clã agrupam-se também várias catepsinas. Esta classe de proteases geralmente localiza-se no citosol ou em lisossomos (Barrett & Rawling 2001). Evolutivamente, as cisteíno peptidases apresentam pelo menos sete origens evolutivas diferentes: clãs CA, CD, CE, CF, PA (que provavelmente evoluíram a partir da família das serino peptidases), PB e CH, sendo estas duas últimas auto-ativadas por clivagem. Em comum, todas as cisteíno peptidases dependem dos resíduos de cisteína e de histidina do sítio ativo (Barrett & Rawlings 2001). O clã CA, além destes resíduos, possui também o resíduo de arginina no sítio ativo (Cohen *et al.*, 1986), porém outros sítios ativos são conhecidos para outros clãs, como as caspases envolvidas no processo de apoptose, que posssuem sítio ativo formado pelos resíduos QACXG (onde Q é um resíduo de glutamina, A, alanina, C, cisteína, X pode ser glicina, arginina ou glutamina, e G, glicina) (Nicholson *et al.*, 1995).

O clã da papaína é composto por peptidases de baixa massa molecular, que varia de 20 a 35 kDa, com exceção da catepsina C, que se apresenta sob a forma oligomérica, com cerca de 200 kDa. As catepsinas B, C, H and L, são encontradas em lisossomos de todos os animais. Com exceção da catepsina C, que é uma dipeptidil peptidase, todas as peptidases deste clã são endopeptidases. As cisteíno proteases atuam geralmente em pHs ácidos, que podem variar de 5.5 a 6.8 (Turk *et al.*, 1997). Cisteíno proteases são inibidas irreversivelmente pelo composto E-64 (L-*trans*-Epoxisuccinil-leucilamido (4-guanidino) butano) (Barrett *et al.*, 1982) e por antígenos 2-beta de células T ativadas (Delaria *et al.*, 1994).

### I.4.3. Metaloproteases

Metaloproteases (EC 3.4.24) são um grupo de peptidases que possuem em comum a presença de um íon metálico no sítio ativo. Geralmente, o íon é o zinco, que atua no posicionamento da molécula de água que será hidrolisada no processo da clivagem do peptídeo. A grande maioria das metaloproteases, incluindo as termolisinas e

metaloendopeptidases, apresenta uma região HEXXH (onde H corresponde a resíduos de histidina E, corresponde a resíduo de glutamina e X a qualquer aminoácido), responsável pela ligação ao íon $Zn^{2+}$ (Jongeneel *et al.*, 1989). Algumas famílias de metalopeptidases como as insulinases, as carboxipeptidases, e as proteases III bacterianas apresentam o motivo HXXEH, que possuem a mesma função do motivo HEXXH de ligação ao íon de zinco. (Becker & Roth 1992). Atualmente, para as metaloproteases, são descritos 15 clãs que se agrupam em 54 famílias (http://merops.sanger.ac.uk). As metaloproteases são inibidas por quelantes de íons bivalentes tais como ácido etilenodiaminotetracético (EDTA), ácido etilenoglicoltetracético (EGTA) e 1,10 fenantrolina (Bond & Butler, 1987).

As proteases desta classe possuem pH ideal de atividade variando de neutro a alcalino. A peptidase mais bem caracterizada desta família é a termolisina, uma protease neutra, sintetizada na forma monomérica, com 34 kDa. É uma proteína termoestável, apresentando meia vida de uma hora, a 80°C. Outras peptidases importantes nesta classe são as colagenases, que degradam além de colágeno, gelatina. As colagenases foram primeiramente caracterizadas em *Clostridium hystolyticum*, mas já foram identificadas em outros microorganismos, incluindo fungos (Rao *et al.*, 1998).

### I.4.4. Serino-proteases

Serino proteases (EC 3.4.21) são peptidases que utilizam um resíduo de serina do sítio ativo para clivar peptídeos. É uma família altamente distribuída em todos os reinos e tem sido agrupada em clãs que compreendem peptidases de acordo com a origem evolutiva, mecanismo catalítico ou mecanismo de ativação, conformação tridimensional e função biológica. (Barrett & Rawlings, 1995, Rawlings *et al.*, 2008). Esta classe inclui 43 famílias agrupadas em 13 clãs, compiladas em banco de dados MEROPS (http://merops.sanger.ac.uk).

As peptidases desta família apresentam região catalítica diferenciada entre alguns clãs, o que sugere diferentes origens evolutivas. Por exemplo, enquanto a família das quimiotripsinas possui uma tríade catalítica formada pelos resíduos de aminoácidos histidina, asparagina e serina (HDS), as subtilisinas apresentam os mesmos resíduos de aminoácidos em ordem diferenciada (DHS) e clivam preferencialmente a seqüência de aminoácidos Ala-Ala-Pro-Phe (Alanina, Alanina, Prolina e Fenilalanina, respectivamente) (Hartley 1970). Mais recentemente, serino proteases com outros sítios ativos tem sido descritas: Ser-His-Glu, Ser-Lys/His, His-Ser-His. Serino peptidases atuam preferencialmente em pH alcalino e tem sido descritas em todas os grupos taxonômicos de fungo. São peptidases inibidas por PMSF (fenilmetilsulfonil fluorido). Algumas também são inibidas por reagentes tiol, como p-cloromercuribenzoato. A inibição pelos compostos tiol, reflete a proximidade de resíduo de cisteína ao sítio ativo, que pode interferir na ligação com o substrato (Dodson & Wlodawer 1998).

Serino peptidases geralmente apresentam massa molecular pequena, que varia de 18,5 a 35 kDa. Porém, fungos como *A. niger* e *A. nidulans* apresentam serino peptidases com massas moleculares maiores. A maior peptidase desta família já descrita apresenta 126 kDa. Muitas das serino peptidases já descritas em fungos apresentam carboidratos associados e apresentam pI variando de 4.4 a 6.2, com algumas exceções que podem chegar a 8.9, ou mais, como descrito para *Neurospora crassa* (revisado por North 1982).

### I.5. Proteases de microorganismos como fatores de virulência

Proteases de várias famílias têm sido associadas à virulência em patógenos humanos. Em *Trycophyton rubrum*, o rastreamento de biblioteca genômica permitiu a detecção de sete possíveis genes codificantes para serina proteases da subfamília das subtilisinas, duas delas capazes de clivar queratina, sugerindo atuação destas no processo invasivo da infecção

fúngica (Jousson *et al.,* 2004). Análise de sobrenadantes de cultura do fungo dermatófito *T. rubrum* utilizando-se queratina como única fonte de nitrogênio permitiu identificar várias aminopeptidases e dipeptidil peptidases que possivelmente sejam importantes na virulência deste fungo, visto que *T. rubrum* inicia seu processo de invasão no hospedeiro por tecidos queratinizados (Monod *et al.*, 2005).

*C. albicans* apresenta, pelo menos, 10 isoformas de aspartil proteases secretadas (SAPs) (Monod *et al.,* 1994). As SAPs são detectadas em sobrenadantes de cultura e são associadas a danos teciduais em epitélio vaginal, facilitando a invasão e disseminação do fungo no hospedeiro (Schaller *et al.,* 2003). SAPs são expressas diferencialmente durante as etapas de infecção. SAP9 é a protease encontrada em grande parte dos pacientes que apresenta candidíase oral e vaginal. Entretanto, a SAP5 apresenta os maiores níveis de expressão durante a infecção em tecido epitelial. Durante a infecção oral, as SAPs que apresentam os maiores níveis de expressão são SAP4-6. SAP1-2 não variam os níveis de expressão nos diferentes modelos de infecção enquanto SAP3, SAP7 e SAP8 apresentam baixos níveis de expressão durante o processo de infecção. Estudos envolvendo mutantes para SAP3 mostraram a expressão de SAP5 é aumentada nestes mutantes numa tentativa compensatória da ausência de SAP3. Da mesma forma, mutantes para SAP4-6 apresentam níveis de expressão de SAP2 aumentados (Naglik et al., 2008). Em *A. fumigatus*, uma aspartil protease extracelular foi identificada com capacidade de clivar proteínas tais como elastina, colágeno e laminina. Neste caso, sugere-se que esta protease possa contribuir para disseminação do fungo nos tecidos do hospedeiro (Lee *et al.,* 1995). Estudos envolvendo culturas de *A. fumigatus* demonstraram que a secreção de proteases está associada ao aumento da concentração de soro em meio de cultura, provavelmente para promover o aumento de aminoácidos no meio extracelular, que é então captado pelo fungo (Gifford *et al.,* 2002). Em *Cryptococcus neoformans*, análises proteômicas utilizando sobrenadantes de cultura e frações de parede

celular identificaram proteases secretadas e associadas à parede relacionadas à virulência do fungo, apresentando a capacidade de clivar citocinas e componentes da matriz extracelular (Eigenheer *et al.* 2007).

O transcrito codificante para uma serina protease vacuolar é aumentado em *M. grisae* durante privação de nitrogênio. Esta serina protease foi associada ao processo de patogenicidade, visto que mutantes para o gene codificante desta proteína causaram níveis significativamente menores de lesões em folhas de arroz, quando comparados às linhagens selvagens (Donofrio *et al.,* 2006). A resposta eficiente à depleção de nitrogênio pode ser importante para sobrevivência de microorganismos patógenos no ambiente do hospedeiro, onde são encontradas baixas concentrações deste elemento químico (Rubin-Bejerano *et al.,* 2003). Em *S. cerevisiae* e *C. albicans,* a internalização de leveduras por neutrófilos induziu uma resposta transcricional similar àquela apresentada pelos fungos durante privação de aminoácidos, sugerindo que o ambiente do fagossomo apresente baixas concentrações de nitrogênio (Rubin-Bejerano *et al.,* 2003). Bactérias como *Natrialba magaddi*, apresentam níveis significativamente aumentados da produção de proteases secretadas e do nível de atividade proteolítica em sobrenadantes de cultura com o decréscimo da concentração de fontes de nitrogênio em meio de cultura quimicamente definido (D'Alessandro *et al.,* 2007).

### I.6. Proteases de *P. brasiliensis*

Poucos estudos foram realizados em *P. brasiliensis* no que concerne a proteases. Um gene codificante para uma metaloprotease Lon foi descrito, clonado e caracterizado (Barros & Puccia 2001). O rastreamento de biblioteca genômica de *P. brasiliensis* permitiu a identificação de um gene codificante para uma ClpB protease. Análises pela técnica de *Northern blot* demonstraram que o transcrito codificante desta chaperonina é preferencialmente expresso na fase leveduriforme do fungo (Jesuíno et al., 2002). O cDNA

codificante para o protease ClpA de *P. brasiliensis* foi identificado e caracterizado. Estudos filogenéticos foram realizados utilizando-se seqüências completas de cDNAs codificantes para ClpA de fungos e bactérias. Os resultados demonstraram uma seqüência de peptídeos na região interna das proteínas ClpA de fungos, ausente nas proteínas ClpA de bactérias (Oliveira et al., 2005). Uma atividade de serina-tiol protease extracelular foi detectada em filtrados de cultura de *P. brasiliensis* com capacidade para clivar proteínas associadas à membrana basal como laminina e fibronectina (Carmona *et al.,* 1995), tendo sua atividade modulada por polissacarídeos neutros (Matsuo *et al.,* 2006) e inibida pela presença de S-[3-nitro-2-piridinesulfenil]. A interação de serina-tiol protease com os polissacarídeos de galactomananas endógenos pode ser importante na termoestabilidade e no aumento da afinidade da proteína com seu substrato (Matsuo *et al.,* 2007).

Transcritos codificantes para proteases foram detectados em bibliotecas de cDNAs das formas leveduriforme e miceliana de *P. brasiliensis* (Felipe *et al.,* 2005) e foram classificados, permitindo a identificação de 53 cDNAs codificantes para proteases, assim distribuídas: proteases ATP - independentes (15 seqüências), ATP-depedentes (12 seqüências), subunidades de proteassoma (22 seqüências) e proteínas de deubiquitinação (4 seqüências) (Parente *et al.,* 2005).

Vários genes codificantes para proteases apresentam-se regulados positivamente durante a transição dimórfica de micélio para levedura em *P. brasiliensis*. Transcritos codificantes para uma aspartil-protease, para uma zinco metaloprotease e para uma protease pertencente à classe M28 foram induzidos durante a transição dimórfica (Parente *et. al.*, 2008). Aspartil-proteases são fatores de virulência em *Aspergillus fumigatus,* sendo associadas a danos teciduais em pulmão de camundongos, atuando na facilitação do processo de invasão do fungo no hospedeiro (Lee & Kolattukudy 1995). Zinco-metaloprotease são associadas ao desenvolvimento de esporos em *Schizosacharomyces pombe,* evidenciando a

importância desta protease durante processos de diferenciação na célula (Nakamura *et al*., 2004). Metaloproteases da classe M28 estão associadas à aquisição de nitrogênio em *Trichoderma harzianum*, apresentando a expressão protéica aumentada durante a privação do elemento químico (Suarez *et al*., 2007).

Transcritos codificantes para algumas proteases apresentaram regulação positiva em *P. brasiliensis*, formas leveduriformes recuperadas de infecção em fígado de camundongos. incluindo uma serina protease S08, objeto de estudo deste trabalho e uma aspartil aminopeptidase, ambas descritas como fatores de virulência. Também foram identificadas proteínas relacionadas ao complexo de ubiquitinação para degradação, provavelmente relacionadas ao controle de qualidade de proteínas com enovelamento incorreto (Costa *et al.*, 2007).

O transcrito codificante para uma aspartil protease classificada na família A01, que apresentou regulação positiva durante a transição dimórfica de micélio para levedura (Bastos *et al.*, 2007) teve sua seqüência de cDNA e a seqüência predita da proteína caracterizadas, tendo sido detectada em sobrenadante de cultura e na parede celular e de leveduras de *P. brasiliensis* (Tacco *et al.*, *in press*).

Uma serina protease da família das subtilisinas (S08) que apresenta níveis de transcritos aumentados em leveduras de *P. brasiliensis* isoladas de camundongos (Costa *et al.,* 2007) também é induzida durante incubação de leveduras de *P. brasiliensis* com sangue e plasma humanos (Bailão *et al.,* 2006; Bailão *et al.,* 2007). A seqüência de nucleotídeo e seqüência predita de aminoácidos foram obtidas e analisadas. O cDNA codificante para serino protease foi clonado em vetor de expressão, transformado em sistema bacteriano e a proteína recombinante obtida foi utilizada para obtenção de anticorpo policlonal em camundongos. A expressão de serino protease é aumentada na privação de nitrogênio, sugerindo a importância desta protease na aquisição desse elemento. A expressão do transcrito codificante para a

serino protease é induzida durante a internalização de células leveduriformes de *P. brasiliensis* em macrófagos murinos. A interação de serino protease com outras moléculas protéicas de *P. brasiliensis* foi avaliada através do sistema de duplo-híbrido em leveduras *S. cerevisiae* (Parente et al., em preparação).

# *Justificativa*

## II. Justificativa

O laboratório de Biologia Molecular do Instituto de Ciências Biológicas da Universidade Federal de Goiás há vários anos tem estudado moléculas de *P. brasiliensis*, com foco principal naquelas potencialmente associadas à interação do fungo com o hospedeiro humano. Neste contexto, proteases apresentam grande relevância, visto que há evidências que proteases extracelulares de fungos patogênicos são importantes na degradação de proteínas nos tecidos do hospedeiro. A sobrevivência de microorganismos patogênicos no ambiente hostil do hospedeiro também é facilitada por proteases intracelulares, visto que atuam na manutenção da qualidade das proteínas do fungo, degradando proteínas não corretamente traduzidas ou enoveladas.

A identificação e caracterização de proteases com níveis de expressão aumentados durante o processo infeccioso e durante a transição dimórfica de *P. brasiliensis* são necessárias para o melhor conhecimento do processo de invasão no hospedeiro, contribuindo para o aumento de conhecimento da patobiologia do fungo.

# Objetivos

## III. OBJETIVO

O presente trabalho tem como objetivo o rastreamento e identificação de seqüências codificantes para proteases de *P. brasiliensis* obtidas a partir de bancos de dados de ESTs de *P. brasiliensis* bem como a caracterização de uma serina protease S08 do fungo.

### III.2. Objetivos específicos

1. Identificação *in silico* de proteases de *P. brasiliensis* em bancos de dados de ESTs oriundas de células leveduriformes de *P. brasiliensis*.

2. Identificação *in silico* de genes relacionados à síntese e processamento de proteínas de *P. brasiliensis* durante a diferenciação celular da forma miceliana para células leveduriformes.

3. Caracterização de uma serino protease de *P. brasiliensis*

Obtenção da seqüência de nucleotídeos e da seqüência predita de aminoácidos codificantes para a serino protease;

Clonagem do cDNA codificante para a serino protease em vetor de expressão e obtenção da proteína recombinante em sistema bacteriano;

Produção de anticorpo policlonal em camundongos;

Avaliação da expressão da serino protease em extrato protéico de células leveduriformes e sobrenadante de cultura de *P. brasiliensis* durante privação de nitrogênio;

Avaliação da presença de N-glicosilação na molécula de serino protease*;*

Avaliação da expressão de transcritos codificantes para serino protease em micélio, células leveduriformes e durante a infecção de macrófagos por células leveduriformes de *P. brasiliensis;*

Rastreamento de proteínas de *P. brasiliensis* interagindo com serino protease através da construção de biblioteca de duplo híbrido em *S. cerevisiae;*

Seqüenciamento dos produtos de PCR obtidos nas interações identificadas pela técnica de duplo híbrido;

Confirmação das interações protéicas por meio de co-imunoprecipitação.

# *Capítulo II*

## *Proteases de P. brasiliensis*

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EVOLUTION AND TECHNOLOGY

*Review*

# Transcriptome overview of *Paracoccidioides brasiliensis* proteases

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**ABSTRACT.** Proteases perform a wide variety of functions inside and outside cells, regulating many biological processes. Infectious microorganisms use proteases, either secreted or attached to their cell surface to weaken and invade their hosts. Therefore, proteases are targets for drugs against a diverse set of diseases. *Paracoccidioides brasiliensis* is the most prevalent fungal pathogen causing systemic mycosis in Latin America. The development of paracoccidioidomycosis depends on interactions between fungal and host components and proteases have been described as important factors implicated in the mechanism of host colonization by fungi. The primary goal for this study is to present an overview of the transcriptome sequences - identified cDNAs that encode proteases. We obtained a total of 53 cDNAs encoding proteases; 15 were classified as ATP-independent, 12 as ATP-dependent, 22 as proteasome subunits, and 4 as deubiquitinating proteases. The mechanisms and biological activity of these proteases differ in substrate specificity and in catalytic mechanisms.

**Key words:** *Paracoccidioides brasiliensis*, Host-fungus interaction, Proteases, Proteasome

 

## INTRODUCTION

Proteases are enzymes that cleave proteins catalysing the hydrolysis of peptide bond. Based on their catalytic mechanisms, proteases can be classified into five main classes: i) aspartyl proteases; ii) metalloproteases; iii) cysteine proteases; iv) threonine proteases, and v) serine proteases. Proteases in the first two classes use an activated water molecule as a nucleophile to attack the peptide bond of the substrate, whereas in the last three classes the nucleophile is a catalytic amino-acid residue located in the active site (Rawlings and Barrett, 1993; Barrett et al., 1998). Proteases are also grossly subdivided into two major groups, depending on their site of action. Exopeptidases cleave the peptide bond proximal to the amino or carboxyl termini of the substrate whereas endopeptidases cleave peptide bonds distant from the termini of the substrate (Watson, 1976).

Proteolytic enzymes play many physiological roles and are essential factors for homeostatic control in organisms. Proteases are widely produced amongst fungi and serve a number of different roles within fungal systems including nutrient cycling and post-translational processing (North, 1982). In some instances a correlation between protease production and pathogenicity was reported. There is accumulating evidence of the direct involvement of fungi proteases in different phases of the host-fungi interactions. The physiological role of proteases during colonization of the host is thought to be the degradation of the skin and mucosal barriers, digestion of host proteins to provide nutrients and attack the lymphocytes and macrophages, affecting the immune defenses of the host (Hube, 2000; Yang, 2003).

A fraction of the proteolytic activity of cells is ATP independent. In addition, studies have identified three ATP-dependent systems involved in protein degradation in eukaryotic organisms (Menon and Goldberg, 1987). These systems include: i) proteases that require the binding and hydrolysis of ATP for proteolytic activity (Lon, ClPs and the 26 S protease have been identified in detail; ii) the ubiquitin conjugating system, and iii) chaperone proteins.

*Paracoccidioides brasiliensis* is a dimorphic fungus that alternates between a mycelium phase in the free environment and a yeast phase in the human host. Primary infection starts in the lungs after inhalation of fungal propagules which then transform into the pathogenic yeast form. Primary infection is usually spontaneously healed; active paracoccidioidomycosis is estimated to develop in approximately 2% of the infected individuals (McEwen et al., 1995). *P. brasiliensis* thus represents a serious public health challenge, with social and economical importance. The observation that only a percentual of infected individuals can develop the disease points to both the pathogenic potential of *P. brasiliensis* and the importance of host defense in controlling fungal infection. *P. brasiliensis* expresses some molecules that account for its ability to evade efficiently the host protective immune system and proteases should be included with these molecules. Protease-like activity in the culture filtrates of *P. brasiliensis* was originally noted and an exocellular serine protease has been characterized as a molecule that cleaves *in vitro* the main components of the basal membrane (Carmona et al., 1995; Puccia et al., 1999). Although potentially associated with the invasion process the role of *P. brasiliensis* proteases in the fungus ability to cause disease remains to be elucidated.

The objective of this review is to summarize the information about the transcriptome-based identification of proteases of *P. brasiliensis*. The availability of primary structural information about a group of the identified expressed sequence tags (ESTs) allows further analysis and a better overall understanding of pathogen interactions with human host. This insight may

shed light on the elucidation of specific functions in which these proteins are involved as well as discover the role of these proteins in the pathogenesis of *P. brasiliensis*.

## METHODS

Annotated ESTs encoding energy-dependent and -independent proteases were obtained in *P. brasiliensis* Transcriptome Project database (http://www.biomol.unb.br/Pb). We screened available databases of proteases, including MEROPS (http://merops.sanger.ac.uk/) and Pfam (http://pfam.wustl.edu). The search for similarity was conducted using the BLAST search tools, using the interface web of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/). Domains and predicted active sites were screened using the ProfileScan (http://hits.isb-sib.ch/cgi-bin/PFSCAN?) and ScanProsite algorithms (http://ca.expasy.org/tools/scanprosite/). Multiple sequence alignments were generated using the program ClustalX 1.81 software (Thompson et al., 1997).

## RESULTS AND DISCUSSION

### Proteases of *Paracoccidioides brasiliensis*

By using the primary information that was retrieved from the *P. brasiliensis* transcriptome (http://www.biomol.unb.br/Pb), combined with data from the MEROPS database (http://merops.sanger.ac.uk/) we have annotated 53 ORFs encoding energy-independent and -dependent proteases, including proteasome subunits, aspartyl, cysteine, metallo, and serine proteases. These cDNAs that encode protease homologues in the fungus transcriptome were annotated, as shown in Figure 1. The proteases of *P. brasiliensis* are distributed as following: 5.6% aspartyl proteases, 11.3% cysteine proteases, 22.6% metalloproteases, 18.8% serine proteases, and 41.5% proteasome subunits.

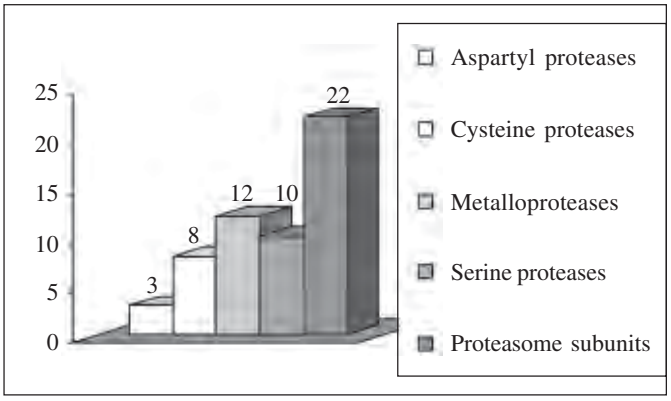


**Figure 1.** Classification of the *Paracoccidioides brasiliensis* proteases present in the fungus transcriptome. The sequences were obtained at (http://www.biomol.unb.br/Pb). Aspartyl, cysteine, metallo, and serine proteases were classified using the BLAST search tools in the MEROPS database (http://merops.sanger.ac.uk/). Proteasome subunits were classified by homology search, using the BLAST program at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/).

## Energy-independent proteases of *Paracoccidioides brasiliensis*

### *Exopeptidases and endopeptidases*

We have annotated a total of 15 cDNAs, which encode energy-independent protease homologues in the fungus transcriptome, as shown in Table 1. From these, two were classified as exopeptidases and the remaining 13 as endopeptidases. The energy-independent proteases are distributed as following: three aspartyl, two cysteine, eight metallo, and two serine proteases.

**Table 1.** ATP-independent proteases of *Paracoccidioides brasiliensis.*

| Product name | Best Blast Hits in NCBI database | Classification in MEROPS database* | Motifs** |
|---|---|---|---|
| Aspartyl protease (GenBank accession number AY278218)[1] | $e^{-144}$ | A01[3] | DTG |
| Aspartyl protease[1] | $7e^{-18}$ | A[3] | |
| Cathepsin D[1] | $1e^{-15}$ | A[3] | |
| Zinc metalloprotease[1] | $2e^{-33}$ | M12B[5] | HEXXH |
| O-sialoglycoprotein endopeptidase[1] | $5e^{-24}$ | M22[5] | |
| Glycoprotein endopeptidase[1] | $9e^{-72}$ | M22[5] | |
| Proline aminopeptidase[2] | $3e^{-60}$ | M24B[5] | HXXE |
| Prenyl protease[1] | $8e^{-64}$ | M48A[5] | HEXXH |
| Zinc metalloprotease[1] | $5e^{-39}$ | M[5] | HEXXH |
| Metallopeptidase[1] | $8e^{-31}$ | M[5] | HEXXH |
| Zinc protease[1] | $2e^{-48}$ | M[5] | |
| Aminopeptidase C[2] | $1e^{-55}$ | C01B[4] | QC |
| Caspase[1] | $3e^{-11}$ | C[4] | |
| Serine protease (GenBank accession number AY319300)[1] | $e^{-165}$ | S08A[6] | DHS |
| Kex2 endoprotease[1] | $9e^{-54}$ | S08B[6] | |

[1]-Endopeptidases; [2]-Exopeptidases; [3]-Aspartyl protease; [4]-Cysteine protease; [5]-Metalloprotease; [6]-Serine protease.
*Protease families and subfamilies described and obtained by MEROPS Blast.
**Active site residues obtained by experimental analysis and described in MEROPS database (http://merops.sanger.ac.uk).

### *Aspartyl proteases of Paracoccidioides brasiliensis*

Three aspartyl proteases were found in the *P. brasiliensis* transcriptome (Table 1, Figure 1). Aspartyl proteases are a group of proteolytic enzymes including the pepsin family that share the same catalytic apparatus and usually function in acidic conditions (Rao et al., 1998). This fact limits the function of this class of proteases to specific locations in the cell. Aspartyl proteases are ubiquitous in nature and are involved in a myriad of biochemical processes. Well-known aspartyl proteases include pepsin and renin in humans (Davies, 1990).

Aspartyl proteases are directly dependent on aspartic acid residues for their catalytic activity and comprise three sub-families: i) family A1, related to pepsin; ii) family A2, retropepsins,

and iii) family A3, retropepsins-like. In the A1 family (clan AA) the catalytic Asp residue occurs within the motif Asp-Ser/Thr-Gly. This family contains many secreted enzymes, which are probably synthesized as propeptides with signal peptides (Dash et al., 2003). The aspartyl protease-deduced primary sequences of the *P. brasiliensis* were analyzed for the presence of the characteristic motif (Asp-Ser/Thr-Gly). Only one protease of this class presents this motif as shown in Table 1. The excerpt of the alignment of this *P. brasiliensis* aspartyl protease with related sequences is shown in Figure 2. The conserved catalytic motif of known aspartyl proteases and its active site are shown, which are conserved among the compared sequences (Figure 2). Since only the sequence (GenBank accession number AY278218) encoding the homolog of a secreted aspartyl protease of *P. brasiliensis* was completely sequenced and characterized, it is possible that complete sequencing of other aspartyl proteases found in the *P. brasiliensis* transcriptome would reveal the expected catalytic triad.

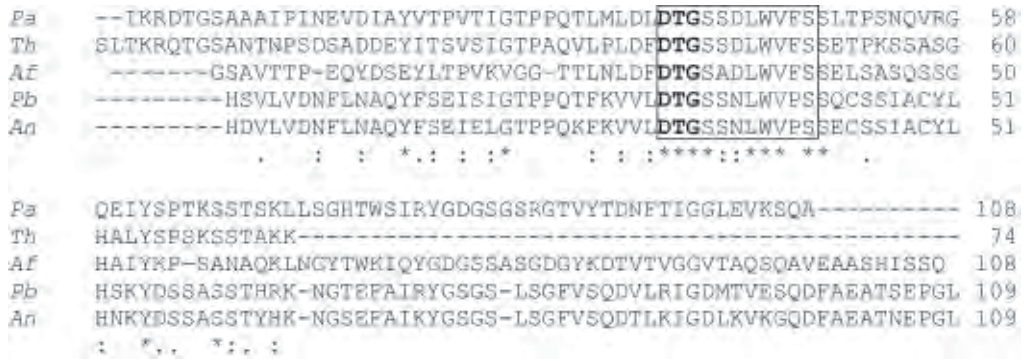


**Figure 2.** Alignment of the deduced amino acid sequence of *Paracoccidioides brasiliensis* aspartyl protease (AY278218), family A01, and related sequences. Black box represents the putative conserved active site, with the catalytic triad evidenced in bold type letters. The sequences aligned were obtained in MEROPS database: *Pa*, *Podospora anserina* (O13340); *Th*, *Trichoderma harzianum* (Q9HDT6); *Af*, *Aspergillus fumigatus* (P41748); *Pb*, *Paracoccidioides brasiliensis* aminopeptidase; *An*, *Aspergillus niger* (Q00070).

## *Metalloproteases of Paracoccidioides brasiliensis*

Zinc-containing metalloproteases are widely distributed in prokaryotic and eukaryotic organisms and are classified into four groups comprehending DD-carboxypeptidases, carboxypeptidases, zincins, and inverzincins (Miyoshi and Shinoda, 2000). One of the most prominent group comprehends the proteins possessing the HEXXH zinc-binding motif, belonging to the zincins superfamily (Miyoshi and Shinoda, 2000). From the eight identified energy-independent zinc metalloproteases in the *P. brasiliensis* transcriptome, four presented the consensus motif HEXXH which define those proteases as members of the zincins family, as shown in Table 1. The production of such proteases by *P. brasiliensis* should be of special note, since evidence has been presented identifying the zincins as pathogenic factors in other microorganisms (Klimpel et al., 1994; Matthews et al., 1998). The motif present in carboxypeptidases (HXXE) was also found in the predicted metalloproteases of *P. brasiliensis* (Table 1).

*Cysteine proteases of Paracoccidioides brasiliensis*

Cysteine proteases occur in both prokaryotes and eukaryotes. About 20 subfamilies of cysteine proteases have been recognized. A detailed analysis of the *P. brasiliensis* transcriptome database reveled two ESTs encoding cysteine proteases ATP independent (Figure 1, Table 1).

Active site residues Q, C, H, N obtained by experimental evidence are described in cysteine families in MEROPS database (http://merops.sanger.ac.uk). Two residues (Q and C) were found in one ORF encoding an aminopeptidase of the cysteine family of *P. brasiliensis* (Table 1). An excerpt of the alignment of this EST with related sequences is shown in Figure 3 and presents those conserved active site residues, which were present in all considered homologues.

Among the cysteine proteases, a caspase homolog was detected (Table 1) suggesting that the programmed cell death in *P. brasiliensis* in its initiation and execution phases could be proteolytically regulated by this class of molecules, as described in other systems (Shi, 2002).

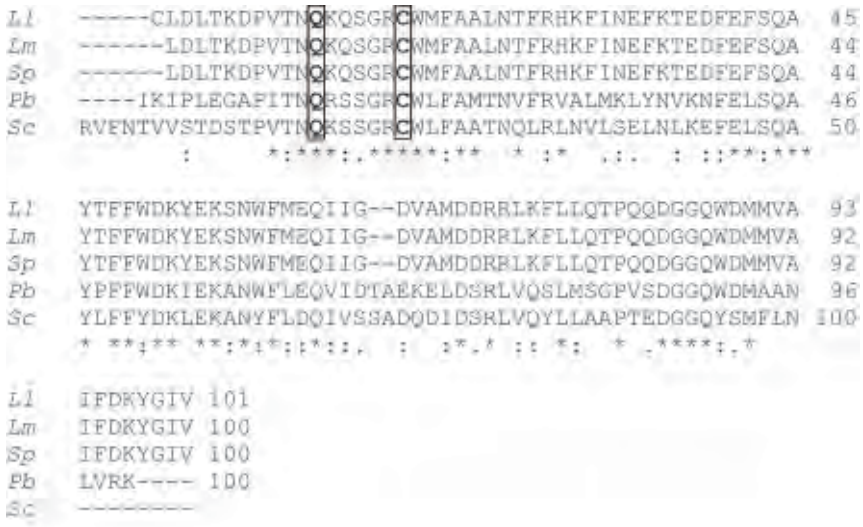


**Figure 3.** Alignment of the deduced amino acid sequence of *Paracoccidioides brasiliensis* aminopeptidase C (cysteine peptidase), family C01B and related sequences. Black boxes and bold letters represent the putative conserved active site residues obtained in MEROPS database: *Ll*, *Lactococcus lactis* (Q04723); *Lm*, *Listeria monocytogenes* (O69192); *Sp*, *Streptococcus pyogenes* (Q99YL0); *Pb*, *Paracoccidioides brasiliensis* aminopeptidase; *Sc*, *Saccharomyces cerevisiae* (Q01532).

*Serine proteases of Paracoccidioides brasiliensis*

Serine proteases are a family of enzymes that utilize a uniquely activated serine residue in the substrate-binding site to catalytically hydrolyze peptide bonds (Schultz and Liebman, 1997). They are numerous and widespread among virus, bacteria and eukaryotes, suggesting that they are vital to the organisms. Owing to the expanding roles for serine proteases, including a diverse

array of physiological functions (Henderson et al., 1992; Froelich et al., 1993), there has been increasing interest in the identification, structural and functional characterization of members of this family. In terms of absolute numbers we identified two energy independent serine proteases in the *P. brasiliensis* transcriptome (Figure 1, Table 1). The essential amino acid residues forming the catalytic triad (DHS) were detected in one of the deduced ORFs encoding the serine protease of *P. brasiliensis* (GenBank accession number AY319300).

Figure 4 presents the alignment of the deduced amino acid sequence encoding this serine protease, family S08A of *P. brasiliensis* with related sequences present in MEROPS database. The catalytic triad is conserved among the sequences.

Among the identified serine proteases a Kex2 endoprotease was identified (Table 1). The Kex2 endoprotease presented the highest identity to the Kex2 gene of *P. brasiliensis* described elsewhere (Venancio et al., 2002).

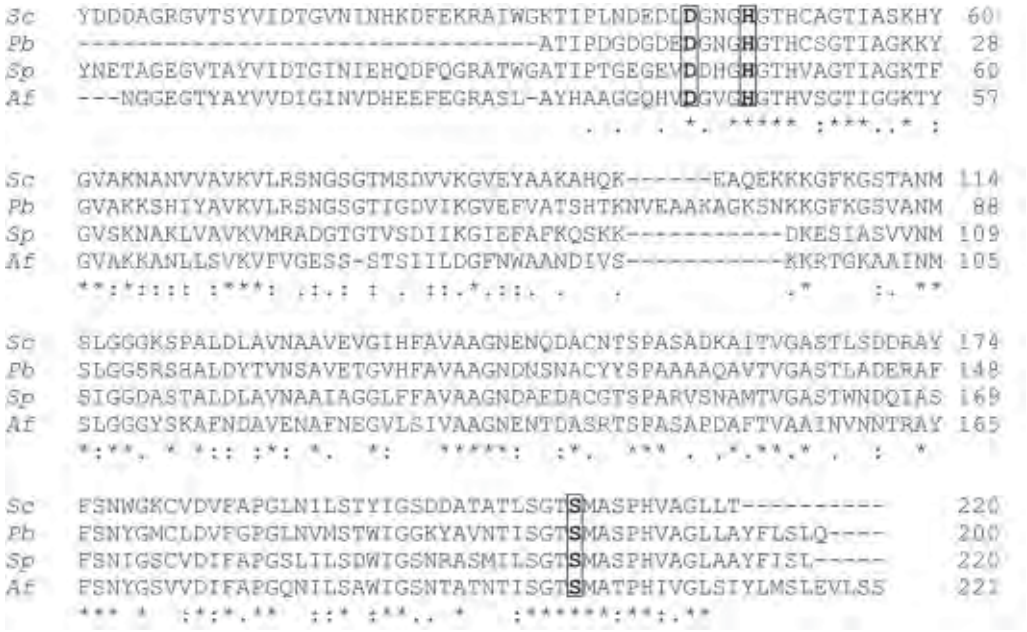

**Figure 4.** Alignment of the deduced amino sequence of *Paracoccidioides brasiliensis* serine protease (AY319300), family S08A to related sequences. Black boxes and bold letters show conserved residues of the catalytic triad DHS, well described in this family. The sequences aligned were obtained in MEROPS database: *Sc*, *Saccharomyces cerevisiae* (P09232); *Pb*, *Paracoccidioides brasiliensis* aminopeptidase; *Sp*, *Schizosaccharomyces pombe* (P78879); *Af*, *Aspergillus flavus* (P35211).

## Energy-dependent protease homologues in *Paracoccidioides brasiliensis*

### *Lon protease*

The first ATP-dependent protease to be identified in *P. brasiliensis* was the Lon protease (Barros and Puccia, 2001). The *lon* gene product is a protein of 1063 amino acids, which presents a single ATP-binding consensus and a serine catalytic site (KDGPSAG). Lon is an

endoprotease, cleaving substrates at multiple sites only in the presence of ATP, in several organisms. The protease activity of *P. brasiliensis* Lon has to be determined.

*Clps proteases*

Clps has been described in *P. brasiliensis* (Table 2). The first Clp protein to be described in the fungus was the ClpB (Jesuino et al., 2002). The ClpB protein of *P. brasiliensis* presents two ATP-binding domains which places the protein in the class I Clp/HSP100 family (Schirmer et al., 1996). ClpB is also a heat shock protein which is induced upon the mycelium to yeast transition in *P. brasiliensis*. Heat shock element motifs in the clpB gene promoter region, in addition to the preferential protein expression in yeast cells could suggest a role of ClpB during the temperature upshift that characterizes the infective process by *P. brasiliensis*.

ClpA is another member of the Clp family described in *P. brasiliensis* (GenBank accession number AY229978) (Table 2). ClpA is a member of proteins that includes the yeast HSP104, which is required for acute thermotolerance (Parsell et al., 1991). The cDNA sequence, which encodes a predicted protein of 927 amino acids, was obtained (Oliveira et al., 2005). The characteristic two nucleotide-binding domains were present in the deduced protein.

Other members of the Clp protease family were obtained by analysis of the *P. brasiliensis* transcriptome. In agreement to Lon, ClpB and ClpA belong to the serine protease family (Table 2). Subunits of unclassified Clps were also obtained. Another member of the AAA superfamily of protease was identified (Table 2) and includes a predicted mitochondrial product, which should be involved in mitochondrial biogenesis. This mitochondrial AAA metalloprotease contains the zinc-binding domain (HEXXH) and could be encoded by a small gene family, as described in other fungi (Shah et al., 2000). Other mitochondrial peptidases were found and listed in Table 2, including members of the inverzincins and DD-carboxypeptidases of the zinc metalloprotease family (Miyoshi and Shinoda, 2000).

**Table 2.** ATP-dependent proteases of *Paracoccidioides brasiliensis.*

| Product name | Best Blast Hits in NCBI database | Classification in MEROPS database* | Motifs |
|---|---|---|---|
| Lon protease | $e^{-104}$ | S16 | |
| ClpB | $2e^{-96}$ | S | |
| ClpA | $e^{-110}$ | S14 | |
| ATP-dependent protease Clp | $6e^{-16}$ | S | |
| ATP-dependent ClpP2 protease subunit | $7e^{-46}$ | S14 | |
| ATP-dependent protease Clp, ATPase subunit | $4e^{-55}$ | S | |
| Mitochondrial matrix AAA protease | $2e^{-85}$ | M41 | HEXXH |
| Mitochondrial processing peptidase | $3e^{-38}$ | M16X | |
| Mitochondrial processing peptidase α subunit | $4e^{-57}$ | M16X | HXH |
| Mitochondrial processing peptidase β subunit | $1e^{-88}$ | M16B | HXXEH |
| Inner mitochondrial membrane protease | $7e^{-21}$ | S26A | |
| Inner mitochondrial membrane protease subunit | $4e^{-04}$ | S26A | |

*Protease families and subfamilies were classified according to MEROPS database.

## Ubiquitin system for protein tagging

The ubiquitin system is a highly complex enzymatic system that covalently modifies selected proteins by attachment to the 8-kDa protein ubiquitin. Selective ubiquitin-mediated proteolysis is the dominant mechanism of degradation of cytosolic and nuclear proteins in eukaryotic cells (Finley and Chau, 1991). In this process, a protein substrate is tagged with a polyubiquitin chain that mediates interaction with and degradation by the proteasome (Pickart, 2000).

## Proteasomes

The proteasome is the central protease in non-lysosomal ubiquitin-dependent protein degradation, and is involved in protein quality control, antigen processing, signal transduction, cell cycle control, cell differentiation, and apoptosis (Voges et al., 1999). The 26S proteasome is a large protein machine, which is found in both, nucleus and cytoplasm. It consists of the 20S proteasome, which forms the proteolytically active core and a regulatory 19S complex (Glickman et al., 1998). High-resolution crystal structures of the 20S proteasome of the yeast *Saccharomyces cerevisiae* demonstrated that it is composed of 28 protein subunits, which are arranged into four staggered heptameric rings. Each outer ring comprises seven α-type subunits and each inner contains seven β-type subunits (Groll et al., 1997). The 20S proteasome of higher eukaryotes is also composed of seven distinct α and β subunits, respectively (Krüger et al., 2001). The α subunits are inactive whereas the β subunits build up the hydrolytic chamber. From the seven β subunits, only three are proteolytically active and autoproteolytically matured as active threonine proteases (Groll et al., 1997). The proteolytic activities of the complex in yeast and mammalian proteasomes reside in $\beta_1$, $\beta_2$, and $\beta_5$ subunits (Heinemeyer et al., 1997). By using the primary information from the *P. brasiliensis* transcriptome database we have annotated ESTs encoding all the α subunits (1 to 7) and six homologues to the 20S β subunit (1 to 6) (Table 3). Of special note is the presence of the β subunits 1, 2 and 5 suggesting that the complex is proteolytically active in *P. brasiliensis*. Recognizing the polyubiquitin proteolytic signal is one of the many tasks of the 19S complex. Studies have shown that four or more ubiquitin composing chains bind the 19S complex (Thrower et al., 2000). Components of the regulatory 19S complex were found in the *P. brasiliensis* transcriptome in a total of nine different subunits (Table 3).

## Deubiquitinating proteases

The deubiquitinating enzymes are defined as a group of proteases which play an important role in the regulation of all processes involving ubiquitin from the processing of poly-ubiquitin precursors into ubiquitin monomers to the targeting or salvage of proteasomal substrates. They are grouped into two classes based on the sequence homology: ubiquitin carboxy-terminal hydrolases (UCHs) and ubiquitin processing proteases, also known as ubiquitin-specific proteases (UBPs).

The physiological functions of deubiquitinating enzymes have been elucidated. Studies involving a human gene encoding an ubiquitin-specific protease reveal that the overexpression of this gene can result in deubiquitination of a broad spectrum of cellular proteins with a growth inhibitory effect. This result suggests that this protein may play an important role in regulation of

**Table 3.** Proteasome subunits of *Paracoccidioides brasiliensis.*

| Product name | Best Blast Hits in NCBI database |
|---|---|
| Proteasome $\alpha_1$ subunit | $9e^{-73}$ |
| Proteasome $\alpha_2$ subunit | $2e^{-62}$ |
| Proteasome $\alpha_3$ subunit | $8e^{-29}$ |
| Proteasome $\alpha_4$ subunit | $8e^{-88}$ |
| Proteasome $\alpha_5$ subunit | $4e^{-70}$ |
| Proteasome $\alpha_6$ subunit | $5e^{-63}$ |
| Proteasome $\alpha_7$ subunit | $2e^{-10}$ |
| Proteasome $\beta_1$ subunit | $3e^{-51}$ |
| Proteasome $\beta_2$ subunit | $5e^{-74}$ |
| Proteasome $\beta_3$ subunit | $2e^{-75}$ |
| Proteasome $\beta_4$ subunit | $5e^{-43}$ |
| Proteasome $\beta_5$ subunit | $3e^{-76}$ |
| Proteasome $\beta_6$ subunit | $4e^{-50}$ |
| Proteasome regulatory subunit | $9e^{-62}$ |
| Proteasome regulatory subunit | $e^{-154}$ |
| Proteasome regulatory subunit | $2e^{-45}$ |
| Proteasome regulatory subunit | $2e^{-70}$ |
| Proteasome regulatory subunit | 0.0 |
| Proteasome regulatory subunit | $5e^{-79}$ |
| Proteasome regulatory subunit | $1e^{-94}$ |
| Proteasome regulatory subunit | $7e^{-45}$ |
| Proteasome regulatory subunit | $5e^{-96}$ |

cell growth (Gong et al., 2000). In addition, these enzymes are active in regenerating free ubiquitin after proteins have been targeted to the proteasome (Wing, 2003). A large number of genes encode deubiquitinating enzymes suggesting that many of these have highly specific and regulated functions. The proteins contain conserved motifs with critical cysteine in the active sites. In agreement the UCHs and UBPs in *P. brasiliensis* are cysteine proteases (Table 4).

In *S.* cerevisiae there is one UCH and sixteen UBPs, whereas higher organisms express an expanded group of enzymes (Yan et al., 2000). In the transcriptome of *P. brasiliensis* were found three ORFs encoding ubiquitin carboxy terminal hydrolases, a high number when compared to *S. cerevisiae*. One EST encoding an ubiquitin-specific protease was also found in the transcriptome database (Table 4).

**Table 4.** Deubiquitinating protease homologues of *Paracoccidioides brasiliensis.*

| Product name | Best Blast Hits in NCBI database | Classification in MEROPS database* |
|---|---|---|
| Ubiquitin carboxyl-terminal hydrolase | $9e^{-40}$ | C19 |
| Ubiquitin carboxyl-terminal hydrolase | $6e^{-27}$ | C19 |
| Ubiquitin carboxyl-terminal hydrolase | $2e^{-80}$ | C19 |
| Ubiquitin-specific protease | $2e^{-09}$ | C19 |

*Protease families and subfamilies described and obtained by MEROPS blast.

## CONCLUDING REMARKS - PUTATIVE ROLE OF PROTEASES IN HOST-PATHOGEN INTERACTION IN *PARACOCCIDIOIDES BRASILIENSIS*

The proteases present in different parasites appear to be relevant for several aspects of host-parasite interactions, quite apart from their obvious participation in the other cellular processes. Information about the seemly putative functions and importance of ATP-independent proteases in *P. brasiliensis* interaction with host was obtained by comparing them to the described homologues in other systems for whom a function was defined. The putative role in host-fungus interaction for these proteins was deduced by generation of mutants deficient in the genes, or asserting their function as potential antigen or vaccine candidates.

Aspartyl proteases are secreted by pathogenic species of *Candida in vivo*, during infection (De Bernardis et al., 1990). More direct evidence of the implication of SAP proteins in virulence has come from studies of constructing strains harboring disruptions in a number of SAP genes. In all cases, mutants showed decreased virulence in an animal model of disseminated candidiasis (Sanglard et al., 1997; Hube et al., 1997). The SAP 2 confers immune protection against systemic candidiasis in immunized mice and has been postulated as a vaccination target (Villanova et al., 2004). Also, members of this protein family have been considered as antigenic markers of disseminated candidiasis (Morrison et al., 2003). In addition, an aspartyl protease is a component of a protective vaccine in coccidioidomycosis (Johnson et al., 2000).

Serine proteases are involved in *Aspergillus* interaction with the host. A vacuolar serine protease is a major allergen of *A. fumigatus* (Shen et al., 2003). Also the involvement of serine and cysteine protease in the fungus colonization of the host's lung tissue has been reported (Kogan et al., 2004).

Metalloproteases play different roles in the host-parasite infections. A metalloprotease is a surface antigen in *Trypanosoma cruzi* and has been postulated as a virulence factor (Cuevas et al., 2003). The protein promotes the attachment of the promastigote form to host cell surface receptors and interacts with the complement system contributing to the ability of the amastigote form of *Leishmania* spp to survive inside the macrophage (Cheng and Chang, 1986; Joshi et al., 2002). The metalloproteases from the human enteropathogenic *Vibrio cholerae* accelerate the bacterial attachment to intestinal epithelial cells through digestion of the small intestinal mucosa (Ichinose et al., 1994). Of the proteases produced by *A. fumigatus*, metalloprotease presenting the consensus zinc-binding motif (HEXXH) has been involved in the infection (Markaryan et al., 1994; Jaton-Ogay et al., 1994).

In the current study, we provided an overview of proteases in the *P. brasiliensis* transcriptome. Research will be directed towards identification of all *P. brasiliensis* proteases and their functional characterization, as well as its presumed role in the infection by *P. brasiliensis*. Proteases have enormous potential as drug targets. Perhaps the main reason for this is that protein modification by proteolytic enzymes is such a ubiquitous biological phenomenon that it is difficult to find pathways in which it does not play a part. In the area of proteases of pathogens the potential as drug targets is a great promise because it should be possible to exploit the differences between enzymes of the pathogen and host to produce effective drugs.

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# *Discussão*

## Rastreamento de ESTs codificantes para proteases expressas na fase leveduriforme de *P. brasiliensis*

### Discussão e Conclusões

No presente trabalho, foram realizadas análises em banco de dados de cDNAs obtidos a partir de RNA da fase leveduriforme de *P. brasiliensis* (http://dna.biomol.ubn.br/Pb). Foram rastreados e identificados transcritos codificantes para proteases. As seqüências parciais de cDNA foram traduzidas para obtenção das seqüências preditas de proteína, que foram classificadas por homologia em banco de dados MEROPS (http://merops.sanger.ac.uk). No total, foram identificadas 53 ORFs codificantes para proteases. As proteases identificadas foram classificadas de acordo com o mecanismo catalítico: 3 proteases foram classificadas pertencentes à classe das aspartil proteases, 8 cisteíno proteases, 10 metaloproteases, 10 serino proteases e 22 proteases pertencentes às subunidades do complexo proteassoma de degradação. Dentre as proteases identificadas, 15 foram classificadas como proteases ATP - independentes e 12 foram identificadas como proteases ATP-depedentes. As 26 ORFs restantes foram classificadas pertencentes ao complexo proteassoma (22) e proteases de deubiquitinação (4). As 15 ORFs codificantes para proteases ATP - independentes foram classificadas de acordo com seu sítio de ação: 13 proteases são endopeptidases e duas são exopeptidases (Parente et al., 2005).

Dentre os transcritos identificados na fase leveduriforme de *P. brasiliensis* três deles codificam para aspartil proteases (Parente *et. al.*, 2005). Em *C. albicans*, 10 genes codificantes para aspartil proteases secretadas (SAPs) foram identificados e os produtos destes genes têm sido associados a danos teciduais do hospedeiro, facilitando a penetração através da mucosa (Naglik et al., 2003). Em *A. fumigatus*, foi possível identificar uma aspartil protease denominada aspergilopepsina secretada em grande quantidade durante a infecção fúngica em pulmão de camundongos infectados através da técnica de imunolocalização com anticorpo específico para esta protease (Lee &. Kolattukudy, 1995). Estudos de infecção de *A. fumigatus* em pneumócitos, linhagem A549, mostraram que este fungo utiliza as classes serino e cisteíno proteases para clivar proteínas de pneumócitos desorganizando o citoesqueleto de actina para facilitar a penetração nas células. A invasão aos pneumócitos utilizando-se uma linhagem de *A. fumigatus* mutada para uma serino protease alcalina não promoveu o desarranjo da rede

de actina, sugerindo que esta protease tenha importância neste processo (Kogan et al., 2004).

Duas cisteíno proteases ATP - independentes foram identificadas na fase leveduriforme de *P. brasiliensis* (Parente *et. al.*, 2005). Análises da seqüência predita de aminoácidos codificantes para uma cisteíno protease revelou a presença de resíduos conservados do sítio ativo. O parasita de peixes *Trypanoplasma borreli*, apresenta uma cisteíno protease capaz de degradar hemoglobina e imunoglobulinas do hospedeiro, contribuindo assim para patogenicidade do microorganismo (Ruszczyk *et. al.*, 2008).

Foram identificadas oito ORFs codificantes para metaloproteases ATP - independentes e duas serino proteases ATP-independentes em *P. brasiliensis* (Parente et al., 2005). No fungo dermatófito *Trichophyton rubrum* metalo- e serino proteases apresentam o perfil de expressão modulados positivamente após cultivo do fungo na presença de componentes protéicos tais como queratina, colágeno e elastina sugerindo a importância destas proteases na virulência deste fungo (Leng *et. al.* 2008).

Proteínas relacionadas ao processo de degradação de espécies protéicas também foram identificadas, como aquelas relacionadas aos processos de ubiquitinação e deubiquitinação (Parente *et. al.*, 2005). Esta classe de enzimas atua na degradação de proteínas mal-traduzidas e/ou mal-enoveladas, apresentando importância no rigoroso controle da qualidade de todas as proteínas produzidas pelas células (Voges *et al.*, 1999).

O grande número de ORFs codificantes para proteases em *P. brasiliensis* sugere que os processos metabólicos que envolvem estas enzimas são importantes para sobrevivência de *P. brasiliensis*, seja na captação de nutrientes, seja no processamento intracelular de proteínas. (Voges et al., 1999; Donofrio *et al.*, 2006). A presença de proteases pH dependentes também sugere que *P. brasiliensis* apresente proteases capazes de atuar de acordo com as condições impostas pelo meio em que o fungo se encontra. Serino proteases são geralmente ativas em pHs alcalinos enquanto aspartil proteases sofrem processo de auto-ativação em pH ácido (Rawling & Barret 1993).

# *Capítulo III*

# *Rastreamento de ESTs durante a transição dimórfica*

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# Comparison of transcription of multiple genes during mycelia transition to yeast cells of *Paracoccidioides brasiliensis* reveals insights to fungal differentiation and pathogenesis

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**Abstract** The ascomycete *Paracoccidioides brasiliensis* is a human pathogen with a broad distribution in Latin America. The infection process of *P. brasiliensis* is initiated by aerially dispersed mycelia propagules, which differentiate into the yeast parasitic phase in human lungs. Therefore, the transition to yeast is an initial and fundamental step in the infective process. In order to identify and characterize genes involved in *P. brasiliensis* transition to yeast, which could be potentially associated to early fungal adaptation to the host, expressed sequence tags (ESTs) were examined from a cDNA library, prepared from mycelia ongoing differentiation to yeast cells. In this study, it is presented a screen for a set of genes related to protein synthesis and to protein folding/modification/destination expressed during morphogenesis from mycelium to yeast. Our analysis revealed 43 genes that are induced during the early transition process, when compared to mycelia. In addition, eight novel genes related to those processes were described in the *P. brasiliensis* transition cDNA library. The types of induced and novel genes in the transition cDNA library highlight some metabolic aspects, such as putative increase in protein synthesis, in protein glycosylation, and in the control of protein folding that seem to be relevant to the fungal transition to the parasitic phase.

**Keywords** Dimorphic transition · Induced transcripts · *Paracoccidioides brasiliensis* · Protein synthesis · Protein folding/modification/destination

## Introduction

*Paracoccidioides brasiliensis* is a human pathogen with a broad distribution in Latin America. The fungus is thermally dimorphic. In the soil, the fungus grows as saprobic mycelium and upon elevation of the temperature to that of the mammalian body, the fungus adopts a yeast-like phase [1]. A human host through inhalation acquires the fungal pathogen. The disease, paracoccidioidomycosis, is characterized by a chronic granulomatous inflammation, and patients might present a broad spectrum of clinical manifestations ranging from a localized and benign disease to a progressive and potentially lethal systemic mycosis.

**Electronic supplementary material** The online version of this article (doi:10.1007/s11046-007-9078-8) contains supplementary material, which is available to authorized users.

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The severe nature of the disease and occurrence of sequelae, frequently causing pulmonary dysfunction or other disabilities, render it a pathogen of considerable medical importance [2].

*P. brasiliensis* can successfully establish and cause disease, highlighting the need to a better understanding of the molecular mechanisms of pathogenesis. Pathogenicity can be related to the factors associated to the transition from the saprophytic phase to the yeast parasitic phase, since fungal strains that are unable to differentiate into yeast cells are not virulent [3]. In this way, the characterization of genes/proteins related to the differentiation to the parasitic phase may bring insights to the fungal pathogenesis.

The morphological transition in *P. brasiliensis* is governed predominantly by the temperature and is preceded by several molecular changes. Some biochemical processes related to the dimorphic transition had been elucidated. In this way, the dimorphic transition involves alterations in the cell wall composition and in the structure of carbohydrates polymers [4]. Hyper expression of the enzymes of the sulfur metabolism in the yeast phase during the fungal transition to yeast reinforces previous findings that this metabolic pathway could be important for this differentiation process [5, 6]. Transcriptional analysis of genes highly expressed during the mycelia to yeast conversion identified the product 4-hydroxylphenyl pyruvate dioxygenase (4-HPPD), predicted to function in the catabolism of aromatic amino acids. Inhibition of 4-HPPD by specific compounds impairs the in vitro differentiation of mycelium to the yeast phase [7]. Also, data indicate that *P. brasiliensis* transition from mycelium to yeast is controlled by changing cAMP levels, with the onset of transition correlating with a transient increase in cAMP, suggesting activation of the cAMP-signaling pathway [8, 9].

A number of proteins and genes had been described as potentially associated to the fungal transition and putatively to the host invasion and host fungal survival. Proteomics-based discovery approaches have successfully identified potential candidates to the dimorphic process. Proteomic analysis from mycelium ongoing differentiation to yeast cells was performed allowing the characterization of proteins that could be relevant to the fungal differentiation. In this sense, the proteins HSP70, HSP60, glyceraldehyde-3-phosphate dehydrogenase, catalase P and actin were accumulated during the transition from mycelium to yeast [10–15].

A great amount of transcriptional data has been obtained from *P. brasiliensis* [16–21]. Approaches used in this fungus to identify phase-specific genes and or genes important for the dimorphic process, included microarray hybridization approaches [7, 16] and in silico EST subtraction [16]. Transcriptional profiling of microarrays built with ESTs of *P. brasiliensis* has identified 328 genes that are differentially expressed upon the phase transition [16]. In addition, constructed microarrays based on yeast-phase genes and hybridized to RNAs isolated from fungal cells at time points during the switch to the yeast phase enabled the identification of transcripts potentially associated to the fungal morphogenesis [7].

In a previous work, we have tested the concept that novel genes involved in *P. brasiliensis* phase transition could be described by applying a transcriptome analysis of cells undergoing mycelium to yeast transition. We reported the in silico analyses and comparison of ESTs from mycelium undergoing the early transition to yeast with mycelium differentiated cells. According to our data, the developmental program of *P. brasiliensis* is characterized by significant differential positive modulation of transcripts related to cellular processes, predominantly to the cell wall/membrane synthesis/remodeling, suggesting their importance in dimorphism [21].

In this study, in order to advance our understanding on the molecular mechanisms of dimorphic transition and of the initial steps of the fungal adaptation to the host, we sought to examine the profile of transcripts related to protein synthesis/processing/regulation/degradation in the ESTs generated from the cDNA library of mycelium undergoing transition to yeast cells. Using comparative sequence analyses, we could identify sequences, which were absent in the *P. brasiliensis* yeast and mycelium transcriptome and in public databases, as well as sequences induced during the early fungal transition. Through these approaches, it was found: (1) 54 possible homologues, including 18 induced/novel homologues of genes previously described as related to protein synthesis; and (2) 44 possible homologues, including 25 induced/novel homologues to genes related to protein folding/modification/destination. Those novel/induced genes provide ideal candidates

for further studies directed at understanding fungal morphogenesis and its regulation.

## Materials and methods

### RNA extraction and preparation of the cDNA library

The cDNA library was constructed, as previously reported [21]. Briefly, *P. brasiliensis*, isolate *Pb*01 (ATCC-MYA-826), was grown 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], at 22°C, as mycelium. The differentiation was performed in liquid medium (Fava-Netto's medium) by changing the culture temperature from 22 to 36°C for the mycelium to yeast transition, as we previously described [13]. The cells were previously grown in liquid medium for 18 h before changing the incubation temperature, which was maintained for 22 h.

Total RNA was purified from *P. brasiliensis* mycelium in transition to yeast cells using TRIZOL (GIBCO$^{TM}$, Invitrogen, Carlsbard, CA). The mRNA was purified by using the Poly (A) Quick$^{R}$ mRNA isolation kit (Stratagene, La Jola, CA). The cDNA library was constructed in the unidirectional pCMV.SPORT 6 (Invitrogen) according to the manufacturer's instructions, exploiting the *Not*I and *Sal*I restriction sites. The cDNA library was not normalized, i.e., no attempt was made to reduce the redundancy of highly expressed transcripts.

### EST processing pipeline and annotation

The nucleotide sequences were uploaded to a relational database (MySQL) on a Linux (Fedora Core 3) platform, and processed using a modified version of the PHOREST tool [22]. The sequences generated during dimorphic transition of *P. brasiliensis* [21] were compared to sequences generated from yeast and mycelium [16]. Transcripts classification was performed by using the MIPS categorization (http://www.mips.gsf.de/). Similarities with *E*-values $\leq 10^{-4}$ were considered significant.

### In silico determination of induced genes in the mycelium to yeast transition

In order to assign a differential expression character, the contigs formed with mycelium and the transition ESTs were statistically evaluated using the Audic and Claverie's method [23]. It were considered induced genes in the transition library those that were not previously described in the mycelium transcriptome database (http://www.dna.biomol.unb.br/Pb), and those more expressed as determined with a 99% confidence rate. A web site (http://www.igs.cnrs-mrs.fr/Winflat/winflat.cgi) was used to compute the probability of differential regulation. The *P. brasiliensis* transcriptome database at (http://www.dna.biomol.unb.br/Pb/) and public databases (http://www.ncbi.nlm.nih.gov) were used to identify novel transcripts, by using the BLAST program [24], as described [21].

## Results and discussion

### cDNA library sequence annotation

The cDNA library was constructed in a non-normalized primary library without amplification, so the clone abundance presents the relative mRNA population. The quality of the cDNA library was checked by evaluating the presence of well-characterized transcripts in the MIPS category, such as, those encoding for energy and metabolism. The results of computational homology search of the genes related to protein synthesis/folding/modification/destination obtained from the *P. brasiliensis* mycelium undergoing differentiation to yeast cells are shown in the supplementary material, Tables 1 and 2, respectively. A total of 200 ESTs (27.3% of the total transition transcriptome) showed significant similarity to sequences related to protein synthesis/folding/modification/destination (*E*-value $\leq 10^{-4}$) based on BLAST searches. A total of 184 ESTs (25.12% of the total transition transcriptome) gave significant hits to ESTs present in the *P. brasiliensis* transcriptome database or in the GenBank database. In addition, 16 ESTs (2.18% of the total transition transcriptome) represented novel genes of *P. brasiliensis* regarding to the above processes.

A broad view of the nature of the adaptations made by *P. brasiliensis* concerning to protein synthesis during early transition to yeast was obtained by classifying the ESTs into 6 groups of functionally related genes (Table 1, supplementary material). Among the transcripts classified in the transition cDNA library and related to protein synthesis, it was found predominantly ESTs coding for ribosomal proteins, comprehending 39 unigenes.

Regarding to protein folding/modification/destination, the ESTs were classified into 6 groups of functionally related genes, as demonstrated in Table 2, supplementary material. Most of the ESTs represent unigenes related to the MIPS classification of posttranslational modification of amino acids (18 unigenes), followed by transcripts related to protein modification by ubiquitination (8 unigenes), proteasomal degradation (8 unigenes), protein processing (6 unigenes), and protein folding (3 unigenes).

## Description of the ESTs related to protein synthesis and fate in the transition transcriptome

As shown in Fig. 1a, the ESTs related to protein synthesis were mainly represented as following: a total of 40.68% of the annotated ESTs corresponded to the ribosomal proteins of the ribosome large subunit; 24.58% in that category were related to the ribosomal proteins of the ribosome small subunit; 15.24% of the transcripts corresponded to homologues encoding translational initiation factors; 9.32% corresponded to ESTs related to the translation elongation machinery. Other ESTs were related to aminoacyl tRNA synthetases (1.69%), ribosome biogenesis (7.64%) and translation termination (0.85%). The Fig. 1b catalogues the ESTs related to protein fate according to the MIPS categories. Most of the transcripts were related to posttranslational modifications of proteins (38.27%) and protein ubiquitination (27.16%). Transcripts related to protein processing and proteasomal degradation of proteins represented 14.81 and 11.12 %, respectively. Classes with lower number of transcripts comprehended those related to protein folding (6.17%) and protein targeting (2.47%).

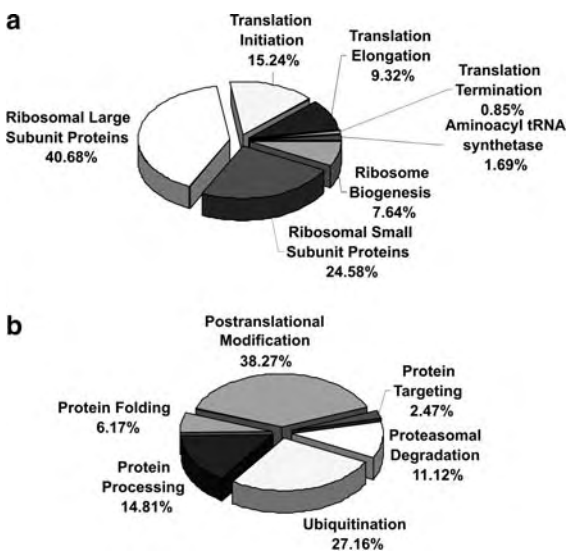


**Fig. 1** Distribution of ESTs from *P.brasiliensis* mycelia ongoing transition to yeast according to their cDNA products. (**a**) Protein synthesis; (**b**) protein fate. The classification was based on *E*-value and performed according to the functional categories developed on the MIPS functional annotation scheme. The percentage of ESTs classification is indicated

## High abundant ESTs related to protein synthesis and fate in the transition transcriptome

Table 1 shows the 10 most abundant ESTs related to protein synthesis and folding/modification/destination in the transition transcriptome. The minimum number of ESTs that made up these most highly redundant contigs was 5. Eight out of the ten most abundant ESTs were identified as induced sequences according to the Audic and Claverie's method and one EST represented a novel transcript. Included among the most abundant transcripts were ESTs encoding for proteins related to ribosome assembly/biogenesis [25–27] and translation [28–30]. Moreover, a transcript encoding a 14 kDa mitochondrial ribosomal protein (*mrps14*) was detected as a novel transcript. In the *P. brasiliensis* mitochondrial genome, the *mrps14* gene *was* not found [31], suggesting, as described in *Arabdopsis thaliana* [32] its possible transference to the nucleus.

Also, among the highly redundant transcripts, it was detected homologues of proteins related to the acceleration of the protein folding and ubiquitination in many organisms [33, 34]. Transcript encoding proteins related to stress conditions, such as the homologue of the L-isoaspartate O-methyltransferase (*pcmt*) that specifically recognizes and methylates

**Table 1** The most abundant transcripts related to protein synthesis and protein fate expressed during transition from mycelium to yeast

| MIPS category | Gene name | Gene product | Best hit/accession number | *E*-value | Enzyme commission | Redundancy in the transition cDNA lybrary | Function in organisms |
|---|---|---|---|---|---|---|---|
| *Protein synthesis* | | | | | | | |
| | *ubi/crp-6* | Ubiquitin fused to S27a protein[a] | *Aspergillus nidulans*/ XP_409009 | 2e–60 | – | 7 | Required for ribosome biogenesis serving to aid in the assembly of S27a into the ribosome in *Saccharomyces cerevisiae* [25] |
| | *mrps14* | 14 kDa mitochondrial ribosomal protein[c] | *Aspergillus nidulans*/ XP_408748 | 4e–46 | – | 7 | Not available |
| | *rps5* | 40S ribosomal protein S5[a] | *Aspergillus nidulans*/ XP_404980 | 8e–22 | – | 7 | Required for the folding of 16S ribosomal RNA and translational fidelity in *E. coli* [26] |
| | *rpl20* | 60S ribosomal protein L20[a] | *Magnaporthe grisea*/ XP_361110 | 3e–16 | – | 6 | Required for ribosome assembly in *E. coli* [27]. |
| | *sui1* | Translation initiation factor eIF1 subunit Sui1[a] | *Gibberella zeae*/ XP_389056 | 2e–36 | – | 5 | Required for the recognition of the AUG codon during translation initiation and for activation of the nonsense-mediated mRNA decay pathway in *S. cerevisiae* [28, 29] |
| | *tef1* | Translational elongation factor EF-1 alpha | *Aspergillus nidulans*/ XP_405299 | 4e–31 | – | 8 | Essential for the delivery of aminoacyl-tRNAs in eukaryotes [30] |
| *Protein fate* | | | | | | | |
| | *ppil4* | Peptidyl-prolyl cis–trans isomerase-like 4 (Cyclophilin RRM)[a] | *Coccidioides immitis*/ EAS29016 | 1e–46 | 5.2.1.8 | 5 | Required for acceleration of proteins folding in organisms [33] |
| | *ubc–6* | Ubiquitin conjugating enzyme E2[a] | *Gibberella zeae*/ XP_388490 | 1e–29 | 6.3.2.19 | 7 | Catalysis the covalent attachment of ubiquitin to proteolytic substrates in organisms [34] |

**Table 1** continued

| MIPS category | Gene name | Gene product | Best hit/accession number | *E*-value | Enzyme commission | Redundancy in the transition cDNA lybrary | Function in organisms |
|---|---|---|---|---|---|---|---|
| | *pcmt* | Protein-L-isoaspartate (D-aspartate) O-methyltransferase[a] | *Aspergillus nidulans*/XP_407601 | 5e–55 | 2.1.1.77 | 5 | Required for metabolization of isoaspartyl residues preventing protein damage under physiological conditions in organisms [35] |
| | *pep* | Aspartyl proteinase[a] | *Paracoccidioides brasiliensis*/AAP32823 | 3e–72 | 3.4.23.24 | 7 | Required for protein processing and for degradation of peptides. Promotes preferential cleavage in hydrophobic amino acids of proteins in eukaryotes [36] |

[a] Transcripts induced in the transition library in comparison to the mycelium transcriptome according to the Audic and Claverie's method

[b] Novel genes detected in *P. brasiliensis*

isoaspartyl residues in a variety of proteins [35], thus preventing the accumulation of deamidated proteins under stressing conditions, was high abundant in the morphological transition. Regarding to protein processing, the transcript encoding aspartyl proteinase (*pep*) was detected. Aspartic proteases are widely distributed in all domains of life and are related to cleavage of peptides in regions of hydrophobic amino acids under acidic conditions. The *S. cerevisiae* vacuolar Pep4p homologue to the *P. brasiliensis pep* product has been described as required for the turnover of damaged molecules during stress conditions [36].

## Induced genes related to protein synthesis identified by in silico EST subtraction

We attempted to determine the putative function of the set of 119 phrap unisequences by searching for homologues in the GenBank non-redundant protein database using BLAST X and by comparing the ESTs in the transition library to those present in the mycelium transcriptome database. The Fig. 2 presents the percentage of induced and novel genes related to protein synthesis in comparison to the total number of ESTs in the transition transcriptome, as described [21]. As observed, from the 119 ESTs related to protein synthesis (10.66% of the total), 3.97% were induced in the transition library and 0.9% was described as novel genes in *P. brasiliensis*.

The comparative analysis of all the induced ESTs related to protein synthesis in the transition library is available; Table 2 summarizes the results of such comparison. In *P. brasiliensis*, induced transcripts, putatively playing role in ribosomal biogenesis and maturation were detected during dimorphic transition, such as 60S ribosome subunit biogenesis protein (*nip7*), GTP-binding GTP1/OBG (*ygr210*) family protein and ubiquitin fused to S27a protein (*ubi/crp-6*). Proteins composing the small and large ribosomal subunits, as well as translational initiation factors, from both cytoplasmic and mitochondrial ribosomes were over expressed in the transition library; some represent novel genes (Table 2). Similar results were described suggesting that the fungal transition is likely to involve intense synthesis of new ribosome particles, affecting the rate of protein

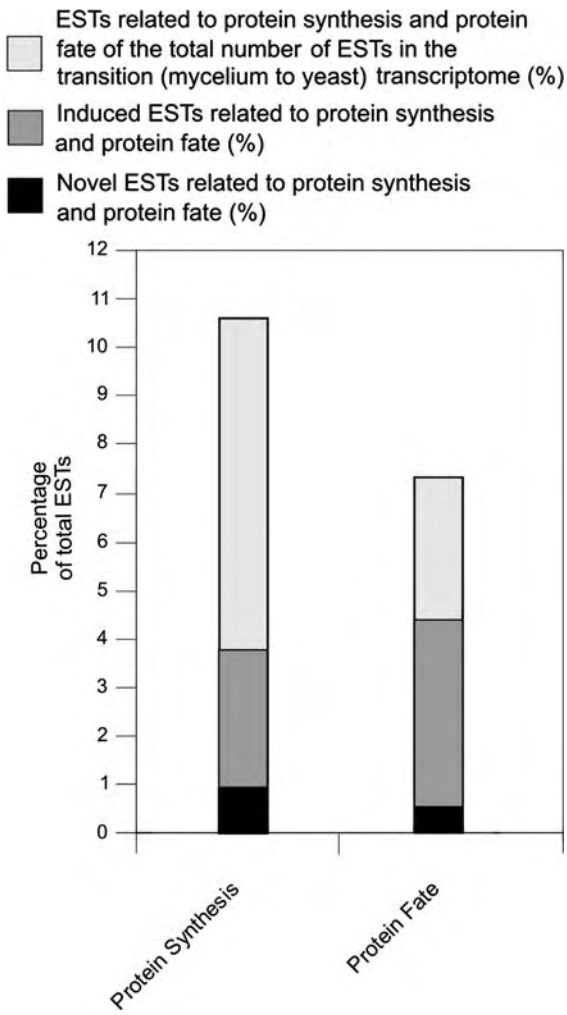


**Fig. 2** Prevalence distribution of ESTs from *P. brasiliensis* mycelia ongoing transition to yeast. The percentage of ESTs related to protein synthesis and fate in the total ESTs are represented along with the percentage of over expressed and novel genes of *P. brasiliensis* in the transition library (http://192.168.0.5/phorestwww)

synthesis [7]. In addition, some of the transcripts encode for ribosomal proteins whose orthologues are differentially regulated in organisms. In this sense, the *rps26* product which has no homologue among prokaryotic ribosomal proteins [37] is differentially expressed during environmental stress in plants [38]. Also, the developmental program of organisms seems to include the differential expression of ribosomal proteins; ribosomal protein *rpl5* product was specifically identified in schizonts and was undetectable in oocysts in the organism *Eimeria tenella* [39], suggesting its regulation under different life-cycle stages.

Induced genes related to protein fate identified by in silico EST subtraction: ESTs relevant to protein processing:

We also attempted to determine the putative function of the set of 81 phrap unisequences by searching for homologues in the GenBank non-redundant protein database using BLAST X and by comparing the ESTs in the transition library to those present in the mycelium transcriptome database. The classification of induced genes was designed as described. The Fig. 2 presents the percentage of induced and novel genes related to protein folding/modification/destination in comparison to the total number of ESTs in the transition transcriptome as described [21]. As observed, from the 81 ESTs (7.32% of the transition transcriptome), 48 (4.43% of the total transition transcriptome) were induced in the transition library and 6 (0.54%) were described as novel genes in *P. brasiliensis*.

The comparative analysis of the ESTs related to protein folding/modification/destination is available; Table 3 summarizes the results of such comparison. A cyclophilin seven suppressor 1 (*cns1*) (HSP90 chaperone complex component) was detected. The Hsp90 complex is one of the most abundant and highly conserved chaperone preventing the aggregation of proteins in a folding-competent state and is essential for cell viability in *S. cerevisiae* [40]. A tailless complex polypeptide 1 chaperonin, subunit epsilon (*tcp-1*) was also detected. The *tcp-1* is localized in the cytosol of higher eukaryotes and is similar to prokaryotes GroEL. The *tcp-1* product has been related to protein folding in *S. cerevisiae* playing role in cell development and cytoskeletal organization [41]. The two ORFs encoding homologues to the above proteins, presumably reflect the heat shock condition experienced by mycelia in transition to yeast cells.

Glycosyltransferases play vital roles in the biological function of native proteins, as well as, in the biosynthesis of numerous molecules within fungi, including cell wall components and its induced expression putatively reflect the cell wall remodeling that occurs during *P. brasiliensis* morphological transition [4, 21]. The novel/induced genes encode glycosyltransferases that could be related to galactosylation of N-and O-glycans, as described in *S. cerevisiae* [42]. Mannosyltransferases (*och*1 and

**Table 2** Novel and over expressed transcripts related to protein synthesis detected during dimorphic transition in *P. brasiliensis*

| MIPS category | Gene name | Gene product | Best hit/accession number | *E*-value | Enzyme commission | Redundancy[d] | |
|---|---|---|---|---|---|---|---|
| | | | | | | M | T |
| *Protein synthesis* | | | | | | | |
| *Ribosome biogenesis* | | | | | | | |
| | *nip7* | 60S ribosome subunit biogenesis protein NIP7[b] | *Aspergillus fumigatus*/ AAM08680 | 3e–14 | – | – | 1 |
| | *ygr210* | GTP-binding GTP1/OBG family protein[b] | *Aspergillus nidulans*/ XP_404829 | 1e–70 | – | – | 1 |
| | *ubi/crp–6* | Ubiquitin fused to S27a protein[a] | *Aspergillus nidulans*/ XP_409009 | 2e–60 | – | 7 | 7 |
| *Ribosomal proteins* | | | | | | | |
| *Small subunit* | | | | | | | |
| | *mrps14* | 14 kDa mitochondrial ribosomal protein[c] | *Aspergillus nidulans*/ XP_408748 | 4e–46 | – | – | 7 |
| | *rps13* | 40S ribosomal protein S13[b] | *Neurospora crassa*/EAA34807 | 2e–37 | – | – | 1 |
| | *rps26* | 40S ribosomal protein S26[b] | *Neurospora crassa*/CAA39162 | 3e–52 | – | – | 1 |
| | *rps5* | 40S ribosomal protein S5[a] | *Aspergillus nidulans*/ XP_404980 | 8e–22 | – | 8 | 7 |
| | *mrps19* | Mitochondrial ribosomal protein S19[b] | *Aspergillus nidulans*/ XP_404292 | 5e–19 | – | – | 1 |
| *Large subunit* | | | | | | | |
| | *rpl20* | 60S ribosomal protein L20[a] | *Magnaporthe grisea*/ XP_361110 | 3e–16 | – | 3 | 6 |
| | *rpl27* | 60S ribosomal protein L27[a] | *Aspergillus nidulans*/ XP_408359 | 4e–63 | – | 1 | 3 |
| | *rpl5* | 60S ribosomal protein L2[b] | *Coccidioides immitis*/ EAS30555 | 9e–54 | – | – | 1 |
| | *rpl3* | 60S ribosomal protein L3[a] | *Aspergillus fumigatus*/ AAM43909 | 5e–85 | – | 1 | 2 |
| | *rpl43* | 60S ribosomal protein L43B[b] | *Ustilago maydis*/XP_400133 | 1e–30 | – | – | 1 |
| *Translation initiation* | | | | | | | |
| | *eif3* | Translation initiation factor 3 subunit 2[c] | *Aspergillus nidulans* / XP_660601 | 6e–80 | – | – | 3 |
| | *eif–5A* | Translation initiation factor eIF–5A[a] | *Neurospora crassa*/P38672 | 6e–06 | – | 4 | 4 |
| | – | Translational machinery component protein[b] | *Aspergillus nidulans*/ XP_405417 | 1e–19 | – | – | 1 |
| | *sui1* | Translation initiation factor eIF1 subunit Sui1[a] | *Gibberella zeae*/XP_389056 | 2e–36 | – | 2 | 5 |
| *Aminoacyl-tRNA synthetase* | | | | | | | |
| | *ils1* | Isoleucyl-tRNA synthetase[b] | *Aspergillus nidulans*/ XP_407499 | 1e–52 | 6.1.1.5 | – | 2 |

[a] Transcripts induced in the transition library in comparison to the mycelium transcriptome according to the Audic and Claverie's method

[b] Transcripts non detected in the mycelia transcriptome (http://www.dna.biomol.unb.br/Pb)

[c] Novel genes detected in *P. brasiliensis*

[d] M: Redundancy in *P. brasiliensis* mycelia transcriptome (http://www.dna.biomol.unb.br/Pb); T: Redundancy in *P. brasiliensis* transition library (http://192.168.0.5/phorestwww/)

**Table 3** Novel and over expressed transcripts related to protein fate detected during dimorphic transition in *P. brasiliensis*

| MIPS category | Gene name | Gene product | Best hit/accession number | *E*-value | Enzyme commission | Redundancy[e] | |
|---|---|---|---|---|---|---|---|
| | | | | | | M | T |
| *Protein fate* | | | | | | | |
| *Protein folding* | | | | | | | |
| | *cns1* | Cyclophilin seven suppressor 1 (HSP90 chaperone complex component)[b] | *Aspergillus nidulans*/ XP_409575 | 8e–12 | – | – | 2 |
| | *tcp–1* | Tailless complex polypeptide 1 chaperonin, subunit epsilon[b] | *Schizosaccharomyces pombe*/EAA65069 | 6e–16 | – | – | 2 |
| *Posttranslational modification of amino acids* | | | | | | | |
| | *gma12* | Alpha–1, 2-galactosyltransferase[c] | *Aspergillus nidulans*/ XP_406106 | 3e–14 | 2.4.1.- | – | 1 |
| | *mnt1* | Alpha–1, 2-mannosyltransferase[a] | *Neurospora crassa*/ *CAC18268* | 1e–29 | 2.4.1.131 | 3 | 3 |
| | *och1* | Mannosyltransferase[b] | *Paracoccidioides brasiliensis*/AAK54761 | 3e–70 | 2.4.1.130 | – | 1 |
| | *swp1* | Oligosaccharyltransferase subunit ribophorin II[d] | *Coccidioides immitis*/ EAS29547 | 9e–37 | 2.4.1.119 | – | 1 |
| | *rabggt* | Rab geranylgeranyl transferase[c] | *Aspergillus nidulans*/ XP_412816 | 8e–13 | 2.5.1.60 | – | 1 |
| | *cypb* | Peptidyl prolyl cis–trans isomerase[b] | *Neurospora crassa*/ CAD21421 | 8e–39 | 5.2.1.8 | – | 1 |
| | *ppil1* | Peptidyl-prolyl cis–trans isomerase-like 4 (Cyclophilin RRM)[a] | *Coccidioides immitis*/ EAS29016 | 1e–46 | 5.2.1.8 | 1 | 5 |
| | *pcmt* | Protein-L-isoaspartate (D-aspartate) O-methyltransferase[a] | *Aspergillus nidulans*/ XP_407601 | 5e–55 | 2.1.1.77 | 4 | 5 |
| | *gmd1* | Guanosine diphosphatase[c] | *Aspergillus nidulans*/ XP_405219 | 2e–15 | 3.6.1.42 | – | 1 |
| *Proteasomal degradation* | | | | | | | |
| | *rpt6* | 26S proteasome regulatory subunit protein[b] | *Aspergillus nidulans*/ XP_411125 | 4e–23 | – | – | 1 |
| | *rpn12* | 26s proteasome regulatory subunit rpn12[b] | *Aspergillus nidulans*/ XP_407156 | 5e–30 | – | – | 1 |
| | *rpn5; rpne* | 26S proteasome regulatory subunit Non-ATPase[c] | *Aspergillus nidulans*/ XP_408912 | 2e–68 | – | – | 1 |
| | *csn5* | COP9 signalosome complex subunit 5[a] | *Aspergillus nidulans*/ XP_406266 | 1e–35 | – | 1 | 2 |
| *Modification by ubiquitination* | | | | | | | |
| | *ubp1* | Ubiquitin-specific protease (C19)[b] | *Aspergillus nidulans*/ XP_412211 | 7e–08 | 3.1.2.15 | – | 3 |
| | *ubc–6* | Ubiquitin conjugating enzyme E2[a] | *Gibberella zeae*/ XP_388490 | 1e–29 | 6.3.2.19 | 6 | 7 |
| | *ubq/rpl40* | Ubiquitin fusion protein[a] | *Schizosaccharomyces pombe*/NP_593923 | 8e–67 | – | 3 | 3 |

**Table 3** continued

| MIPS category | Gene name | Gene product | Best hit/accession number | *E*-value | Enzyme commission | Redundancy[e] | |
|---|---|---|---|---|---|---|---|
| | | | | | | M | T |
| | *ubp1; otub1* | Ubiquitin thiolesterase otubain like protein[c] | *Aspergillus nidulans*/ EAA60354 | 1e–28 | 3.4.- | – | 1 |
| | *rfn167* | Ring (really interesting new gene) type zinc finger (C3HC4) protein (E3 complex)[b] | *Schizosaccharomyces pombe*/CAB08748 | 5e–10 | – | – | 1 |
| | *fbl7* | F-box/LRR-repeat protein 7 (E3 complex)[b] | *Aspergillus nidulans*/ XP_408647 | 8e–28 | – | – | 3 |
| *Protein Processing* | | | | | | | |
| | *pep* | Aspartyl proteinase[a] | *Paracoccidioides brasiliensis*/AAP32823 | 3e–72 | 3.4.23.24 | 3 | 7 |
| | *lon* | Lon protease[b] | *Pseudomonas fluorescens*/ AF250140_1 | 1e–05 | 3.4.21.53 | – | 1 |
| | *lap* | Peptidase M28 domain protein[c] | *Coccidioides immitis*/ EAS33583 | 1e–22 | 3.4.11.15 | – | 1 |
| | *mde10* | Zinc metalloprotease (M12)[b] | *Neurospora crassa*/ CAD21161 | 3e–47 | 3.4.24.- | – | 1 |

[a] Transcripts induced in the transition library in comparison to the mycelium transcriptome according to the Audic and Claverie's method

[b] Transcripts non detected in the mycelia transcriptome (http://www.dna.biomol.unb.br/Pb)

[c] Novel genes detected in *P. brasiliensis*

[d] Genes not described previously in *P. brasiliensis* isolate *Pb*01, but present in public databases

[e] M: Redundancy in *P. brasiliensis* mycelia transcriptome (http://www.dna.biomol.unb.br/Pb); T: Redundancy in *P. brasiliensis* transition library (http://192.168.0.5/phorestwww/)

*mnt*1) could be putatively related to the *O*-linked mannosylation of proteins, as observed in *C. albicans*. *C. albicans* mutants to either *mnt1* or *och1* showed hypersensitivity to cell wall perturbing agents, suggesting the proteins role in the cell wall maintenance [43, 44]. Moreover, a novel transcript encoding to guanosine diphosphatase (*gmd*1) was detected during the dimorphic transition, whose product is known to regulate mannosylation of N- and O-linked oligosaccharides in Golgi complex [45].

Peptidyl-prolyl cis/trans isomerases (cyclophilins) catalyze cis/trans isomerization of a prolyl bond and this isomerization is a time limiting step in folding of certain proteins [46]. Transcripts encoding to two-peptidyl prolyl cis–trans isomerases (*cypb* and *ppil*) were induced in *P. brasiliensis* during the transition from mycelium to yeast. Aside from their roles in cellular biochemistry, cyclophylins of microorganisms are particularly interesting since those proteins are found to have a key role in pathogenicity [47]. The *P. brasiliensis* CypB deduced amino acid sequence presents four conserved amino acids: Arg, Phe, Trp and His (RFWH motif, data not shown) described as involved in peptidyl-prolyl cis–trans isomerase activity and related to the activity of the protein in the folding process as described [48].

Protein processing MIPS category is represented by four unigenes induced in *P. brasiliensis* transition library; some presents orthologues with function in stress response and differentiation. The aspartic protease (*pep*) with seven ESTs, was also included with the most abundant transcripts (see Table 1). The *pep* product belongs to family A1 of aspartic protease, related to pepsin and synthesized as a propeptide with signal peptide. This peptidase family is related to stress response in *S. cerevisiae* [36]. The deduced Lon protease (*lon*) shows homology with family S16, class 001 in MEROPS database (http://www.merops.sanger.ac.uk) and is induced in the transition transcriptome sharing identity with its counterparts in bacteria. The *lon* product was first identified in *E. coli*

**Table 4** Homologues for protein synthesis and fate ESTs putatively related to fungal differentiation/virulence or stress tolerance

| Gene product | Described role | Redundancy[d] | | Reference |
|---|---|---|---|---|
| | | M | T | |
| Cyclophilin seven suppressor 1 (*cns1*)[b] | Promotes increase in heat shock response in *Saccharomyces cerevisiae.* | – | 2 | [40] |
| Alpha-1, 2-mannosyltransferase (*mnt1*)[a] | Required for adhesion and virulence in *Candida albicans* | 3 | 3 | [43] |
| Mannosyltransferase (*och1p*)[b] | Required for cell wall integrity and virulence in *Candida albicans* | – | 1 | [44] |
| Zinc metalloprotease (*mde10*)[b] | Required for spore development in *Schizosaccharomyces pombe* | – | 1 | [52] |
| GTP-binding GTP1/OBG family protein (*ygr210*)[b] | Involved in regulation of differentiation in *Streptomyces coelicolor.* | – | 1 | [53] |
| Peptidyl prolyl cis–trans isomerase (*cypb*)[b] | Induced in heat shock response in *Aspergillus nidulans.* | – | 1 | [54] |
| Peptidyl-prolyl cis–trans isomerase-like 4 (*ppil1*)[a] | Related to thermoresistance in *Paramecium sp* | 1 | 5 | [55] |
| Peptidyl-prolyl cis/trans isomerase (*ess1*) | Required for *Cryptococcus neoformans* virulence | 6 | 1 | [56] |
| Peptidyl-prolyl cis–trans isomerase (*mip*) | Required for *Legionella pneumophila* survival into macrophages | 2 | 2 | [57] |
| Protein-L-isoaspartate (D-aspartate) O-methyltransferase (*pcmt*)[a,c] | Promotes increase in heat shock survival in *Escherichia coli.* | 4 | 5 | [58] |
| Ubiquitin conjugating enzyme E2 (*ubc6*)[a] | Promotes enhanced in growth of *Saccharomyces cerevisiae* at high temperature. | 6 | 7 | [59] |
| Aspartyl proteinase[a,c] (*pep*) | Secreted by *Aspergillus fumigatus* during invasion of the host lung. | 3 | 7 | [60] |
| Lon protease (*lon*)[b] | Required for cellular morphology and virulence in *Agrobacterium tumefaciens* | – | 1 | [61] |

[a] Transcripts induced in the transition library in comparison to the mycelium transcriptome according to the Audic and Claverie's method

[b] Transcripts non detected in the mycelia transcriptome (http://www.dna.biomol.unb.br/Pb)

[c] Also over expressed in yeast cells recovered from liver of infected mice (Costa et al. unpublished)

[d] M: Redundancy in *P. brasiliensis* mycelia transcriptome (http://www.dna.biomol.unb.br/Pb); T: Redundancy in *P. brasiliensis* transition library (http://192.168.0.5/phorestwww/)

and its homologues were further discovered in many organisms sometimes in multiple copies, playing essential roles in protein quality control by destroying unfolded proteins [49]. In *P. brasiliensis*, a gene homologue encoding for a Lon protein of the S16 family, class 002 in MEROPS database (http://www.merops.sanger.ac.uk), was described previously [50], suggesting more than one Lon species in the fungal pathogen. A metalloprotease M28 domain protein (*lap*) was found as a novel gene in *P. brasiliensis*, encoding for a leucyl aminopeptidase. In *Thichoderma harzianum*, the M28 peptidase is induced during nitrogen starvation suggesting its importance in the amino acid acquisition [51]. Other metalloprotease induced in *P. brasiliensis* dimorphic transition is the zinc metalloprotease belonging to M12 family (*mde10*), whose members were described in fungi [52].

## Putative differentiation, virulence and stress tolerance factors

Factors putatively related to the differentiation process, fungal virulence and stress tolerance were

selected on basis with homology to other microorganisms in which defined functions are available. With these criteria, we classified some transcripts as shown in Table 4. The *cns1* product is an essential component of the HSP90 complex, which is induced in heat shock response [40]. Mannosyltransferases (*mnt*1 and *och*1) orthologues are required for cell wall integrity/virulence and adhesion/virulence, respectively, in *C. albicans* [43, 44]. In *S. pombe*, the *mde10* product is essential for development of spore envelopes [52] evidencing its importance during differentiation process in the cell. The GTP-binding GTP1/OBG family product (*ygr210*) related to ribosome biogenesis has been described as a regulator of differentiation in *Streptomyces coelicolor*, playing a role in the onset of aerial mycelium formation and sporulation [53]. In *Aspergillus nidulans*, CypB is induced in response to heat shock indicating a possible role of this protein during growth in stress environments [54]. *P. tetraurelia* KIN241 homologue to peptidyl-prolyl cis–trans isomerase-like 4 (*ppil1*) is related to the organism thermoresistance [55]. A parvulin type Ess1 of *Cryptococcus neoformans* homologue to *P. brasiliensis ppil1* product is required for virulence, since Ess1 depleted strains are unable to cause experimental infection [56]. The Mip protein (macrophage infectivity potentiator) of *Legionella pneumophila* is a cyclophilin FKBP-type homolog which is related to bacterial virulence in intracellular infection in guinea pig [57]. *Escherichia coli* transformants over expressing L-isoaspartate (D-aspartate) O-methyltransferase presented increase in the heat shock survival rates [58]. Yeast strains over expressing ubiquitin conjugating enzyme E2 are more tolerant to various stresses conditions, such as high temperature [59]. The *A. fumigatus* aspartyl protease (*pep*) is highly secreted during fungal invasion of host lung [60]. The Lon protease of *Agrobacterium tumefaciens* is required for normal growth, cellular morphology and full virulence [61].

## Concluding remarks

Molecular strategies relying on ESTs has proved to be an efficient approach to identify genes expressed under a variety of conditions. This study presents a screen for genes related to protein synthesis/folding/modification/destination expressed during mycelium to yeast differentiation of *P. brasiliensis* through EST analysis. By analysis of the induced and or novel genes it was possible to infer some metabolic adaptations of *P. brasiliensis* during early dimorphic transition that could include the increased control in the ribosome biogenesis and translation fidelity, increase in protein glycosylation and in the control of protein folding. In addition, the amino acids capture from the medium could be favored during the transition to the parasitic phase.

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# *Discussão*

## Rastreamento de ESTs codificantes para proteínas relacionadas à síntese e ao processamento protéico induzidas durante a transição dimórfica de *P. brasiliensis*

### Discussão e Conclusões

A transição dimórfica de micélio para levedura em *P. brasiliensis* é etapa importante para o estabelecimento da infecção no hospedeiro humano (San-Blas & Nino-Veja, 2001). Sendo assim, elucidar os aspectos moleculares envolvidos nesta etapa de transição torna-se importante para melhor compreensão da patogênese de *P. brasiliensis.* Com o objetivo de ampliar os estudos das modificações transcricionais que ocorrem durante o processo de dimorfismo de *P. brasiliensis,* Bastos e colaboradores (2007) avaliaram 1107 ESTs oriundas de RNA de *P. brasiliensis* após 22 horas da indução da transição dimórfica.

O rastreamento de transcritos relacionados aos processos de síntese, enovelamento e modificações pós-traducionais de proteínas de *P. brasiliensis* foi realizado em banco de dados de transcritos de *P. brasiliensis* obtido após indução da transição de micélio para levedura por 22 horas (http://192.168.0.5/phorestwww). Um total de 200 ORFs foi identificado apresentando homologia com seqüências relacionadas aos processos de síntese e processamento de proteínas. Destas, 16 ORFs codificam para genes não descritos anteriormente em *P. brasiliensis* (Parente *et. al.*, 2008).

Do total de ESTs relacionadas à síntese protéica, 10% correspondem a cDNAs induzidos durante a transição dimórfica de *P. brasiliensis*. Várias ESTs que compõem as subunidades ribossomais, assim como fatores de iniciação transcricional foram regulados positivamente na transição dimórfica em *P. brasiliensis*, como, por exemplo, uma proteína relacionada à biogênese da subunidade 60S ribossomal, uma proteína OBG de ligação ao GTP e uma ubiquitina fusionada à proteína S27a (Parente *et. al.*, 2008). Resultados similares foram descritos durante a transição dimórfica do isolado *Pb*18 de *P. brasiliensis* e sugerem que a alteração morfológica de *P. brasiliensis* envolve um aumento da síntese de novas partículas ribossomais, o que refletiria um aumento no nível de proteínas sintetizadas requeridas neste processo (Nunes et al., 2005). Algumas proteínas ribossomais apresentam ortólogos regulados positivamente durante estágios específicos em outros organismos. Neste sentido, o produto gênico de

*rps*26 é induzido durante o estresse ambiental em plantas (Brosche et al., 1999). Da mesma forma, o produto do gene *rpl*5 de *Eimeria tenella* é detectado em esquizontes e não encontrado em oocistos, sugerindo que o transcrito codificante para esta proteína ribossomal é regulado durante as fases do desenvolvimento do microrganismo (Schaap et al., 2005).

O rastreamento em banco de dados de ESTs *P. brasiliensis* obtidas após a indução da transição dimórfica (http://192.168.0.5/phorestwww) permitiu identificar 81 ESTs codificantes para proteínas relacionadas ao processamento de proteínas. Deste total, 48 ESTs correspondem a cDNAs regulados positivamente durante o processo de transição dimórfica. Seis ESTs correspondem a genes que ainda não haviam sido descritos anteriormente para *P. brasiliensis* (Parente *et. al.*, 2008).

Trasnscritos codificantes para chaperonas foram induzidos durante a transição dimórfica de micélio para forma leveduriforme em *P. brasiliensis*, como o componente do complexo da chaperona HSP90 (*csn*1) e a subunidade épsilon da chaperona codificada pelo gene *tcp-1* (Parente *et. al.*, 2008). O complexo HSP90 é altamente conservado e abundante em vários organismos. Este complexo de chaperonas é essencial para viabilidade em *S. cerevisiae* (Marsh et al., 1998). O produto gênico de *tcp*-1 é localizado no citoplasma de eucariotos e é ortólogo ao complexo GroEL de procariotos. É descrito em *S. cerevisiae* que o produto gênico de *tcp*-1 está relacionado ao desenvolvimento e organização do citoesqueleto (Chen et al., 1994). A indução destes transcritos em *P. brasiliensis* provavelmente reflete as condições de choque térmico que ocorre durante a transição de micélio para levedura.

Outra classe de proteínas cujos transcritos foram regulados positivamente durante a transição dimórfica é a classe das glicosiltransferases (Parente *et. al.*, 2008). Estas proteínas apresentam funções na biossíntese de várias moléculas, incluindo componentes da parede celular de fungos (San Blas 1982). Em *C. albicans* mutantes para genes codificantes para manosiltransferases (*och*1 e *mnt*1) apresentam sensibilidade aos agentes perturbadores da parede celular, sugerindo o papel destas proteínas na manutenção da parede celular (Munro et al., 2005; Bates et al., 2006). Um transcrito codificantes para uma guanosina difosfatase (*gmd*1) foi detectado e descrito como um novo gene em *P. brasiliensis*. Esta proteína regula a manosilação no complexo de Golgi (Vowels & Payne 1998). A indução dos transcritos codificantes para estas proteínas durante o dimorfismo de *P. brasiliensis* pode ser associado ao remodelamento da parede celular necessário para que ocorra a transição morfológica.

Também o processo de aceleração do enovelamento de proteínas foi induzido durante a transição dimórfica de micélio para forma leveduriforme em *P. brasiliensis*. Transcritos codificantes para duas peptidil prolil cis-trans isomerases foram reguladas positivamente durante a transição dimórfica (Parente *et. al.*, 2008). O aumento na transcrição destes genes poderia refletir o aumento na produção de proteínas que ocorre nesta etapa de transição em *P. brasiliensis.* A importância desta classe de proteínas em microorganismos patogênicos não está somente na função de enovelamento de outras proteínas desde que estas isomerases são importantes para virulência de fungos, como descrito para *C. neoformans* (Wang et al., 2001).

Genes codificantes para proteínas relacionadas ao processamento protéico foram induzidos na transição de micélio para levedura. Um deles é o transcrito codificante para uma lon protease pertencente à família S16, (classe 001) regulado positivamente durante a transição dimórfica (Parente *et. al.*, 2008). Em *E. coli*, esta classe de proteases atua no controle de qualidade das proteínas produzidas (Swamy & Goldberg, 1981).

O transcrito codificante para uma metaloprotease M28 foi identificado. Este novo gene descrito para *P. brasiliensis* codifica para uma leucil aminopeptidase (Parente *et. al.*, 2008). O gene codificante para esta protease em *Thichoderma harzianum* apresenta níveis de expressão aumentados durante privação de nitrogênio, sugerindo a importância de leucil aminopeptidases em processos tais como a aquisição de aminoácidos (Suarez *et. al.* 2007).

# *Capítulo IV*

# *Serino Protease de P. brasiliensis*

**Characterization of a secreted serine protease from *Paracoccidioides brasiliensis***

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**Summary**

*Paracoccidioides brasiliensis* is a thermodimorphic fungus, the causative agent of paracoccidioidomycose (PCM). A cDNA (*Pb*sp) encoding a secreted serine protease (*Pb*SP), was isolated from a cDNA library constructed with RNAs of fungal yeast cells recovered from liver of infected mice. Recombinant *Pb*SP was produced in *Escherichia coli*, and used to develop polyclonal antibody that was able to detect a 66 kDa protein in the *P. brasiliensis* proteome. *In vitro* deglycosylation assays with endoglycosidase H demonstrated that *Pb*SP is a *N*-glycosylated molecule. *Pb*SP was induced during nitrogen starvation both in *P. brasiliensis* proteins extracts and culture supernatants, suggesting its importance in the nitrogen acquisition. The *Pb*sp expression was higher in yeast cells compared to mycelia and in yeast cells after internalization by murine macrophages. Interactions of *Pb*SP with other *P. brasiliensis* proteins were evaluated by two-hybrid assay in the yeast *Saccharomyces cerevisiae*. *Pb*SP interacts with a peptidyl prolil cys-trans isomerase, calnexin, HSP70 and a cell wall protein PWP2

**Keywords:** *Paracoccidioides brasiliensis*, Secreted serine protease, *N*-glycosylation, macrophage infection, two-hybrid assay.

## Introduction

Proteases are enzymes that cleave proteins catalyzing the hydrolysis of a peptide bond. Serine protease is a class of peptidases widely distributed in all domains of life that use a serine residue of that active site to cleave peptides (Rawlings & Barret 1993). Proteases are widely produced amongst fungi playing role in the nutrient cycling and post-translational processing (North, 1982).

Serine proteases are associated to virulence in many pathogens. In the human pathogen *Tiyhcophyton rubrum* seven serine proteases genes were detected, two of them encoding products able to cleave keratin, suggesting the importance of these proteases in the invasion process in the human host (Jousson *et al,* 2004). A vacuolar serine protease of the plant pathogen *Magnaporthe grisea* is induced during nitrogen starvation. This protease has been associated to virulence since the serine protease depleted mutant strain presents lower infection ability when compared to the wild type cells (Donofrio *et al,* 2006). Proteomics analysis of *Cryptococcus neoformans* culture supernatants detected several proteases able to cleave cytokines, complement components and extracellular matrix compounds facilitating tissue invasion (Eigenheer *et al* 2007).

*Paracoccidioides brasiliensis* is a thermally dimorphic fungus with a broad distribution in Latin America, the causative agent of the paracoccidioidomycosis (Franco *et al*, 1994). The infection is initiated by inhalation of airborne propagules of the mycelium, which reach the lungs and differentiate into the yeast parasitic phase (Lacaz, 1994). Few *P. brasiliensis* proteases have been characterized. Analysis of the ESTs in the transcriptome of mycelim and yeast cells revealed a total of 53 open reading frames (ORFs) encoding proteases in *P. brasiliensis*. The deduced amino acid sequences allowed the proteases to be classified in aspartyl, cysteine, metallo, serine proteases and proteasome subunits (Parente *et al*, 2005). Also in *P. brasiliensis* an extracellular subtilisin-like serine protease has been detected in the fungal yeast phase (Carmona *et al*., 1995). This protease is inhibited by PMSF (phenylmethyl-sulphonyl fluoride), mercury acetate and *p*-HMB (p-hidroximercurio benzoato), allowing to classify the protein as a serine-thiol protease. This serine-thiol protease was able to cleave, *in vitro*, murine laminin, human fibronectin, type IV-collagen and proteoglycans (Puccia *et al*, 1998). The serine-thiol activity of *P. brasiliensis* is

modulated by fungal extracellular galactomannan, which might act stabilizing the enzyme conformation (Matsuo *et al*, 2006). An aspartil protease has been recently characterized in *P. brasiliensis.* The cDNA encoding the aspartil protease (*Pb*sap) and the deduced amino acid sequence enconding this protease (*Pb*SAP) were identified and characterized. The recombinant protein was obtained and used to develop polyclonal antibody that detects a 66 kDa protein in the *P. brasiliensis* protein extract and culture supernatant, suggesting that *Pb*SAP is a secreted molecule. *Pb*SAP is located in the yeast cell wall by immunoelectron microscopy. Deglycosylation experiments demonstrated *N*-glycosylation of the *Pb*SAP molecule. Zymogram assays indicated the presence of aspartyl protease gelatinolytic activity in yeast cells and culture supernatant (Tacco et al., *in press*).

Transcriptome analysis of the *P. brasiliensis* yeast cells recovered from infected mice revealed a serine protease transcript positively regulated (Costa *et al*, 2007). The transcript encoding the serine protease was also induced in *P. brasiliensis* after incubation of yeast cells in human blood and plasma (Bailão et al., 2006, 2007). In the present work we sought to amplify our studies of this serine protease (*Pb*SP). The complete cDNA was isolated and characterized. The recombinant protein was obtained and used to generate polyclonal antibody in mice, which detected the serine protease in the fungal yeast cells extract as well as in fungal culture supernatants. *Pb*SP is induced during nitrogen deprivation suggesting its importance in the nitrogen acquisition. The presence of a carbohydrate chain linked to the native molecule was inferred by N-deglycosylation experiments. *Pb*sp expression was evaluated by Real Time RT-PCR in mycelium, yeast cells and yeast cells during macrophage internalization, presenting higher level in yeast cells inside macrophages. *Pb*SP interaction with other *P. brasiliensis* proteins was evaluated by two-hybrid assay in *S. cerevisiae*. *Pb*SP interactcs with proteins related to folding process such as FKBP-peptydil prolyl cis-trans isomerases and calnexin. Also, interaction of *Pb*SP with proteins related to quality control such as methionine aminopeptidase II was reported. Interaction of *Pb*SP with a mitochondrial HSP70 protein was identified, reflecting possible translocation to cell compartments. *Pb*SP also interacts with a cell wall associated periodic tryptophan protein.

## Materials and methods

### *P. brasiliensis* isolate growth conditions

*P. brasiliensis* isolate *Pb*01 (ATCC MYA-826) was used in all the experiments. Yeast cells were grown at 36°C 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; 1,2% (w/v) agar, pH 7.2]. For nitrogen starvation experiments, *P. brasiliensis* yeast cells were cultured in liquid MMcM minimal medium (Restrepo & Jimenez 1980) without ammonium sulfate, asparagine and cistine for eight hours. Control condition was performed by incubation of yeast cells in liquid MMcM minimal medium containing the nitrogen sources for eight hours.

### Obtaining the *P. brasiliensis* serine protease cDNA and bioinformatics analysis

A complete cDNA encoding a *P. brasiliensis* homologue of the serine protease was obtained from a cDNA library of yeast cells recovered from liver of infected mice (Costa *et al*, 2007). The cDNA was sequenced on both strands by using the MegaBACE 1000 DNA sequencer (GE Healthcare) and the predicted amino acid sequence was obtained. The protease classification was performed by using the MEROPS database (http://merops.sanger.ac.uk). The entire nucleotide sequence, *Pb*sp, and the predicted amino acid sequence, *Pb*SP, have been submitted to the GenBank database under accession number AY319300.

The National Center for Biotechnology Information (NCBI) BLASTp algorithm (http://www.ncbi.nlm.nih.gov) was used to search in the non-redundant database for proteins with sequence similarities to the translated full-length *Pb*SP cDNA. The ScanProsite algorithms (http://ca.expasy.org/tools/scanprosite/) were used to search for motifs and conserved domains in the deduced protein. The presence of signal peptides was identified by using the SignalP program (http://www.cbs.dtu.dk/services/SignalP/), while the prediction of cellular localization was performed by using the PSORT II algorithm (http://psort.ims.u-tokyo.ac.jp/form2.html). The complete genomic sequence of *Pb*sp was obtained in the *Paracoccidioides brasiliensis* genomic database (http://www.broad.mit.edu/science/projects/msc/data-release-summary ) and the promotor

region was analyzed by using the Promotor scan algorithms (http://www-bimas.cit.nih.gov/cgi-bin/molbio/proscan).

**Cloning of *Pb*SP cDNA into expression vector**

Oligonucleotide primers were designed to amplify the complete cDNA encoding the *Pb*SP. The nucleotide sequence of the sense and antisense primers were 5` - TCTGGATCCATGAAAGGCCTCTTCGC – 3' and 5` - ACACTCGAGTCCAGAGATGAAAGCGTT– 3`, which contained engineered *Bam*HI and *Xho*I restriction sites, respectively (underlined). The amplification parameters were as following: an initial denaturation step at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 90 s, annealing at 50°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 a 1.5-kb amplicon was gel excised and cloned into the pGEX-4T-3 expression vector (GE Healthcare). The recombinant plasmid was used to transform the *E. coli* strain C43 competent cells by using the heat shock method (Sambrook & Russel 2001). Ampicilin-resistant transformants were cultured, and plasmid DNA was analyzed by PCR and DNA sequencing, as described above.

**Heterologous expression of *Pb*SP and antibody production**

Cultures of transformed *E. coli* containing pGEX-4T-3-PbSP were grown in Luria-Bertani (LB) medium supplemented with 100 µg/ml of ampicillin, at 37°C. As the cells reach the log phase ($A_{600}$=0.6), IPTG (isopropyl-β-D-thiogalactopyranoside) was added to the growing culture to a final concentration of 0.5 mM to induce protein expression. After 2 h incubation, the bacterial cells were harvested by centrifugation at 5.000 *g* and ressuspended in phosphate saline buffer (PBS) 1X. *E. coli* cells transformed with pGEX-4T-3 without insert and *E. coli* without any vector were used as control. The cell extracts ressuspended in PBS 1X were electrophoresed on a 10% SDS-PAGE, followed by Coomassie brilliant blue staining. The protein species corresponding to *Pb*SP fused to glutathione S transferase (*Pb*SP-GST) was excised from the gel and 200 µg of the material was used to inoculate mice through subcutaneous injection. Animal was boosted three times, at 2 weeks intervals, with the same amount of antigen. The obtained serum,

containing anti-*Pb*SP polyclonal antibody was sampled and stored at -20°C. Preimmune serum was obtained.

**Obtaining cell extracts and secreted proteins of *P. brasiliensis***

Total protein extracts from yeast and mycelium was obtained. Frozen cells (3g) were disrupted by complete grinding with a mortar and pestle in buffer (20 mM Tris-HCl, pH 8.8, 2 mM $CaCl_2$) without protease inhibitors. The mixture was centrifuged at 15.000 *g* at 4°C, for 20 min; the supernatant was sampled, and stored at -80°C. Culture supernatant of yeast cells was obtained after eight hour incubation in liquid MMcM minimal medium. The cells were separated by centrifugation at 5.000 *g* for 15 min and the supernatant was filtered in a 0,22μm filter (MilliPore). The culture supernatants were dialyzed with water during 4 h. Secreted protein fraction was concentrated with ice-cold acetone (v/v) during 16 h, centrifugated at 15.000 *g* for 15 min and the pellet was washed with 70% ice-cold acetone. Each 50 mL of culture supernatant was concentrated to 500 μL in Tris-HCl 25 mM pH 7,0. Protein concentration of all the samples was measured by using Bradford reagent (Sigma Aldrich) using BSA as standard.

**Western blot analysis**

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

**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 60 mM sodium acetate buffer pH 5.8. Samples were analyzed by western-blot.

**Azocasein assay**

The azocasein assays were performed with azocasein diluted to 5mg/mL in buffer containing 25 mM Tris-HCl, 200 mM NaCl, 25 mM $CaCl_2$, 0,05% Nonidet P-40 and 0,01% $NaN_3$. 200 μg of *P. brasiliensis* total protein extract and 100 μL of *P. brasiliensis* culture supernatants were used in each assay. Proteinase K (Sigma Aldrich) was used as a positive control.

**Infection of murine macrophage by *P. brasiliensis* yeast cells**

Bone marrow-derived macrophages were obtained by flushing the femurs of 4-12 weeks old female C57BL/6 mice, as previously described (Fortier and Falk, 2007). The prepared cells were cultured at 37 ºC under 6% $CO_2$ in RPMI 1640 medium (Biowhittaker, Walkersville, Md.) supplemented with 10% fetal bovine serum (FBS), 1% l-glutamine, 5 × 10−5 M 2-mercaptoethanol, 100 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF), and 10 μg/mL of gentamicin. After 8 days the non-adherent cells were discarded and the remaining adherent cells were washed off twice with 10 mL of Hank's Balanced Salt Solution (HBSS). The cells were treated with 10ug/mL of dispase in HBSS at 37C for 5 min. Further, macrophages were removed using a cell scraper and washed in HBSS. Cells were pelleted by centrifugation at 500 *g* for 5 min, and suspended in RPMI 1640 (supplemented as describe above, minus GM-CSF) at a concentration of 1 x $10^6$ cells per mL. For infection experiments, 5 x $10^6$ *P. brasiliensis* yeast cells were added to 2mL of macrophage suspension plated on 6 well plates. After 24h of co-cultivation at 37º C in 6% $CO_2$, the non-phagocyted yeasts were discarded and the bottom cells were washed twice with HBSS to remove unattached yeasts. The RNA of infected murine macrophages was extracted by using Trizol reagent (Invitrogen, Carlsbad, CA). RNA from uninfected macrophages and *P. brasiliensis* grown in RPMI 2640 were obtained as controls.

**Quantitative real-time PCR**

RNA samples were reverse transcribed using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) and oligo(dT)15 primer. The cDNA samples were diluted 1:5 in water, and qRT-PCR was performed using SYBR green PCR master mix (Applied

Biosystems, Foster City, CA) in the Applied Biosystems 7500 real-time PCR system (Applied Biosystems Inc.). qRT-PCR was performed in triplicate for each cDNA sample. The specificity of each primer pair for the target cDNA was confirmed by the visualization of a single PCR product in agarose gel electrophoresis. The primers and sequences were used as follows: sp sense, 5'-GGCCTCTCCACACGTTGCTG-3'; sp atsense 5'-GTTCCAGATAAGAACGTTAGC-3' and α-tubulin primers: tubulin-sense, 5'-ACAGTGCTTGGGAACTATACC-3'; tubulin-atsense, 5'-GGACATATTTGCCACTGCCA-3'. The annealing temperatures for sp and tubulin primers were 60°C and 59°C, respectively. The standard curves were generated using the target fragments cloned into pCR2.1-TOPO (Invitrogen). The plasmids were diluted 1:1000, and a standard curve was generated using 5 samples, serially diluted 1:4 from the original dilution. The relative expression levels of genes of interest were calculated using the standard curve method for relative quantification (Bookout *et al*, 2006).

**Interaction of *Pb*SP with *P. brasiliensis* proteins by Two-Hybrid assay**

Oligonucleotides were designed to clone the complete cDNA encoding the *Pb*SP in the pGBK-T7 (Clontech Laboratories, Inc) expression vector. The nucleotide sequence of the sense and antisense primers were 5` - CATATGATGAAAGGCCTCTTCGCCT – 3' and 5` -CTGCAGTTAAGAGATGAAAGCGTTCTTG– 3`, which contained engineered *Nde*I and *Pst*I restriction sites, respectively (underlined). The pGBK-T7 contains the TRP1 gene which allows the selection in minimal medium without tryptophan and a GAL4 DNA-binding domain. The cloned product was used to transform a *Saccharomyces cerevisiae* strain Y187 (ΔTRP1). A cDNA library was constructed with RNA from *P. brasiliensis* yeast cells and cloned in the expression vector pGADT7-Rec by using the Matchmaker™ Library Construction & Screening (Clontech Laboratories, Inc). The pGADT7-Rec vector contains LEU2 gene, allowing the selection in minimal medium without leucine and a GAL4 DNA-activation domain. The products cloned were transformed in *S. cerevisiae* strain AH109 (ΔLEU2). The Y187 strain containing pGBK-T7-*Pb*SP was used to screen the pGADT7-Rec library transformed in AH109 strain by yeast mating. The positive interactions activate the transcription of ADE2, HIS3 and MEL1 genes, which allows the selection in minimal medium without tryptophan, leucine, adenine and histidine. Minimal

medium without these amino acids and containing X-alpha-GAL also confirms the activation of the transcription of the MEL1 gene. The *Pb*SP baited clones were amplified by using AD-LD 5' and AD-LD 3' oligonucleotides for pGADT7-Rec and sequenced as described above. The positive interactions were confirmed by using the *in vitro* translation system TNT® T7 Coupled Reticulocyte Lysate Systems (Promega) with $S^{35}$ methionine and coimmunoprecipitation of the translated proteins (Matchmaker™ Co-IP Kit, Clontech Laboratories, Inc). Briefly, the translated serine protease fused to c-myc epitope (c-myc-SP) and the translated proteins fused to hemaglutinin epitope (HA-Prey) were mixed at 25 °C for 1 h. The mixture was incubated with protein A Agarose beads and with the monoclonal c-myc antibody in PBS 1X at 25 °C for 1 h. After washing, the beads containing proteins were resuspended in SDS-loading buffer [50 mM Tris-HCl, pH 6.8; 100 mM dithiothreitol, 2% (w/v) SDS; 0.1% (w/v) bromophenol blue; 10% (v/v) glycerol], followed by boiling at 80 °C for 5 min. The proteins were separated on a SDS-PAGE 4–12 % linear gradient. The gel was fixed with 20% (v/v) ethanol and 10 % (v/v) acetic acid for 30 min, and incubated in 20 mL of fluorographic reagent NAMP 100 (Amplify Fluorographic Reagent - GE Healthcare®). The gels were dried at 80 °C for 90 min under vacuum and autoradiography was obtained. Controls were performed. Each assay was repeated three times with a different batch of in vitro translated product to confirm the results.

## Results

### Analysis of the cDNA and of the deduced protein sequence

The Figure 1, supplementary material, shows the genomic and cDNA sequences, as well as the deduced protein encoding *Pb*SP. The cDNA sequence contains a 1491 bp open reading frame. The genomic sequence presents two introns and three exons. The deduced amino acid sequence presented 497 amino acids residues with a predicted molecular mass of 53 kDa and *pI* 6.12. *Pb*SP homology analysis in MEROPS database reveals homology with serine proteases from S08 family of subtilase (data not shown). Analysis of the promoter region reveals a TATA box and a 5'-GATA-3' domain, putatively related to nitrogen metabolite regulation (NMR). Also a 5'-GCCARG-3' binding motif related to pH regulation was found in the promoter region. Analysis of the deduced amino acid sequence

revealed a 16 amino acid signal peptide, suggesting that *Pb*SP is a secreted molecule. Comparisons of the predicted protein sequence with well-known serine proteases allowed us to identify three conserved amino acids residues DHS that compose the catalytic triad of the subtilase family. Six N-glycosylation sites were also predicted at positions 76-79, 98-101, 160-163, 245-248, 287-290 and 450-453 in the deduced protein sequence (Supplementary figure 1). The sequences of the serine proteases from *Ajellomyces capsulatus* and *Coccidioides immitis* showed the highest sequence identity to *Pb*SP (68%), followed by *Aspergillus fumigatus* (65%) (data not shown).

**Cloning and expression of *Pb*SP in *E. coli* and antibody production**

SDS-PAGE analysis of the transformants revealed that IPTG induced a dominant protein, migrating at 82 kDa (Figure 1 A, lane 2). This dominant protein was absent in the cells growing in the absence of IPTG (Figure 1 A, lane 1). The size of the induced protein is in accordance with the expected size of the *Pb*SP fused to glutathione S-transferase (GST). The polyclonal antibody produced against *Pb*SP reacted with the recombinant protein in western blot analysis (Figure 1 B, lane 2). No reaction was detected with preimmune serum (Figure 1 B, lane 1). The polyclonal antibody recognized a protein species of 66 kDa in *P. brasiliensis* protein extract (Figure 1 D, lane 1).

**Deglycosylation assays**

The *Pb*SP molecular mass detected in western blot analysis (Figure 1 D, lane 1) was higher in comparison to the value of the deduced protein. The probable glycosylation of the molecule was analyzed by treating total protein extract of yeast cells with endoglycosidase H. Treatment with endoglycosidase H rendered a protein species of 53 kDa (Figure 1 D, lane 2). The data support the inference that the 66 kDa protein in *P. brasliensis* yeast cells extract is the glycosylated form of the 53 kDa protein.

**Analysis of proteases expression during nitrogen starvation in *P. brasiliensis***

The expression of *Pb*SP during fungal nitrogen starvation was analysed. *P. brasiliensis* yeast cells were incubated in MMcM medium without nitrogen sources as described above. Protease activity was measured by using an azocasein assay in protein

extracts and culture supernatants. The total protease activity was higher in yeast cells in the absence of nitrogen sources, both in total protein extract and in culture supernatant in comparison to the control (Figure 2, 1-4).

The *Pb*SP expression was evaluated by western blot analysis after incubation of yeast cells in MMcM medium in absence and in the presence of nitrogen sources. *Pb*SP expression was higher in the nitrogen starvation condition both in total protein extract (Figure 2 B, lane 2) and culture supernatant (Figure 2B, lane 4) in comparison to the *Pb*SP expression after incubation of yeast cells in chemically defined medium containing nitrogen sources (Figures 2 B, lanes 1 and 3).

**Analysis by real-time PCR of *Pb*sp expression**

The *Pb*sp expression was evaluated by using real-time PCR in mycelium, yeast cells and in yeast cells infecting mice macrophages. *Pb*sp expression was lower in mycelium compared to yeast cells. Higher *Pb*sp expression was detected during the internalization of yeast cells by macrophages (Figure 3).

**Interaction of serine protease with other *P. brasiliensis* proteins**

The interaction of *Pb*SP with other *P. brasiliensis* proteins was evaluated by two-hybrid system in *S. cerevisiae*. The proteins identified interacting with *Pb*SP are described in Table 1. It was detected homologues of FKBP-peptidyl prolyl cis-trans isomerase, calnexin, HSP70 and a cell wall associated periodic tryptophan protein. Protein interactions were confirmed by coimmunoprecipitation assays and are shown in Figure 4.

**Discussion**

The partial cDNA encoding *Pb*SP was previously characterized and the deduced protein was classified as a member of the subtilisin family S08A (Parente et al., 2005), which comprehend endopeptidases generally presenting higher activity under alkaline conditions (Barret & Rawlings, 1995). The *P. brasiliensis* serine protease cDNA here characterized encodes a protein with a N-terminal 16 amino acids with the characteristic of a leader peptide. The protein sequence corresponding to the mature *Pb*SP shows high similarity with serine proteases sequences from other fungi. Analysis of the promoter

region revealed the presence of a nitrogen metabolite repression (NMR) region binding protein, responsible for positive regulation of genes in response to nitrogen metabolite presence such as AreA proteins in *Aspergillus nidulans* (Morozov *et al*, 2001) and Nit2 protein in *Neurospora crassa* (Chiang & Marzluf; 1995). Also, a 5'-GCCARG-3'binding motif was found in the gene promotor. In *A. nidulans* the PacC protein binds to this motif activating transcription of genes expressed in alkaline condition (Tilburn *et al*, 1995). The data suggest that *Pb*SP could be a molecule regulated by the nitrogen metabolite presence and positively regulated in alkaline conditions.

The recombinant *Pb*SP was obtained fused to GST, exhibiting a molecule of 82 kDa. By using the recombinant protein, polyclonal antibody was obtained in mice. The serum, specifically, recognized the recombinant protein as well as a protein species of 66 kDa in *P. brasiliensis* yeast cells extract. Treatment of fungal protein extracts with endoglycosidase H resulted in a 53 kDa protein species, corresponding to the *Pb*SP *in silico* deduced molecular mass. The data suggest that the 13 kDa additional in the 66 kDa species is due to N-glycosylation.

The total protease activity was evaluated during fungal nitrogen starvation by incubating yeast cells in chemically defined medium without nitrogen sources. Protease activity, including *Pb*SP expression, was higher during nitrogen starvation. The result suggests that proteases can be important in the response induced by nitrogen starvation in *P. brasiliensis*. Also, the results suggest that *Pb*SP is a secreted protein involved in nitrogen acquisition, relevant in nitrogen starvation response in *P. brasiliensis*. Similar results were described to the bacteria *Natrialba magaddi,* which present higher protease activity level in chemically defined medium without nitrogen source (D'Alessandro *et al.,* 2007). Serine proteases are described as associated with nitrogen acquisition and pathogenicity in the fungal pathogen *M. grisea*. In this fungus, a serine protease is highly induced during nitrogen starvation. This protease is encoded by the *isp* gene and is associated to pathogenicity since Δ*isp* fungal cells present lower infection index in rice when compared with wild type cells (Donofrio *et al.,* 2006). The nitrogen starvation response can be important to human pathogens since neutrophil phagosome presents lower nitrogen concentration. In this way, the *S. cerevisiae* and *C. albicans* transcriptional profile caused by neutrophil internalization is most similar to that of amino acid deprivation (Rubin-

Bejerano *et al,* 2003).

The *Pb*sp expresion was evaluated by quantitative RT-PCR in mycelium, yeast cells and yeast cells during infection in macrophage. The *Pb*sp expression was higher in yeast cells during macrophage infection suggesting that this serine protease could be important in the fungal adaptative mechanism into macrophage cells. A subtilisin like serine protease from *Mycobacterium tuberculosis* is described as a cell wall-associated protein and is induced during infection of macrophages (Dave et al., 2002).

Two hybrid assays were performed to detect *P. brasiliensis* proteins interactions with *Pb*SP. *Pb*SP interacts with proteins presumably related to protein processing such as FKBP-peptidyl prolyl cis-trans isomerase, calnexin and HSP70. A calnexin homolog was identified, possible related to the retention of incorrectly folded proteins (Zhang *et al*, 1997). The calnexin of the yeast *Hansenula polymorpha* have been related to increase in the secretion of proteins (Klabunde et al., 2007). HSP70 was found interacting with *Pb*SP and possible plays role in the trafficking of serine protease into and through the compartments in the cell (Craig et al., 1989). Interactions of HSP70 proteins with proteins have been described. HSP70 interacts with the aquaporin 2 present in the principal cells of the kidney collecting duct, regulating its trafficking (Lu *et al.*, 2007).

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fungus *Paracoccidioides brasiliensis*. Yeast. 2007. 24:79-87.

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**Table 1**- Proteins identified interacting with *Pb*SP by using two-hybrid system in *S. cerevisiae*.

| Gene Product | Best hit | e-value | Number of obtained clones |
|---|---|---|---|
| FKPB-type peptidyl prolyl cis trans isomerase | *Aspergillus clavatus* XP_001274819 | $2e^{-25}$ | 4 |
| Calnexin | *Paracoccidioides brasiliensis* ABB80132 | $2e^{-28}$ | 2 |
| Mitochondrial 70 kDa heat shock protein | *Paracoccidioides brasiliensis* AAP05987 | $6e^{-83}$ | 2 |
| Periodic tryptophan protein PWP2 | *Ajellomyces capsulatus* XP_001543414 | $2e^{-30}$ | 1 |

**Figure legends:**

**Supplementary Figure 1:** The cDNA and the genomic sequences encoding the serine protease (*Pb*SP) of *P. brasiliensis*. The nucleotide and amino acid positions are marked on the left side. Lower case letters represent the untranslated 5' region. Bold letters in nucleotide sequence represent the start and stop codons. Two introns were found in the genomic sequence and are shown in italic. Three conserved residues (marked with arrows) of amino acids (asparagine - D; histidine – H and serine – S) belonging to the active site of serine proteases from the subtilase family S08 are evidenced. Six putative N-glycosylation sites are marked in bold letters. A signal peptide formed by the first 16 amino acids is underlined. The TATA box in the promoter region is evidenced with a black box. A GATA binding region of the transcription factor AreA was found and is evidenced by a white box. A PACC protein binding domain is evidenced in a gray box.

**Figure 1:** Reactivity of the polyclonal antibody anti-*Pb*SP and deglycosylation assay. A: SDS-PAGE of *E. coli* extracts. 1: *E. coli* extracts; 2: *E. coli* protein extract obtained after 0.5 mM IPTG treatment. The arrow indicates the protein species corresponding to *Pb*SP fused to the GST protein. B: Western blot assay, the same extracts as in A reacted to: 1: Control mice preimmune serum. 2: Polyclonal anti-*Pb*SP. C: SDS-PAGE of *P. brasiliensis* extracts 1: Total protein extract of yeast cells from. 2: Total protein extract of yeast cells treated with endoglycosidase H for 16 h. D: Western blot using the polyclonal antibody anti-*Pb*SP reacted with the protein extracts of panel C.

**Figure 2:** Proteolytic activity in *P. brasiliensis* and *Pb*SP expression analysis. Yeast cells were incubated in chemically defined MMcM medium and MMcM medium without nitrogen sources (ammonium sulfate, asparagine and cystine) for 8 h. After incubation, total protein extraction was performed. The culture supernatants were precipitated with ice-cold acetone. Protease activity was obtained by using azocasein with 200 µg of total protein extract of yeast cells or the same protein amount of protein obtained from culture supernatant. Protease activity was measured at 436 nm. A: 1: Protease activity in total protein extract of yeast cells after incubation in MMcM minimal medium; 2: Protease

activity in total protein extract of yeast cells after incubation in MMcM minimal medium without nitrogen sources; 3: Protease activity in culture supernatant of yeast cells after incubation in MMcM minimal medium; 4: Protease activity in culture supernatant of yeast cells after incubation in MMcM minimal medium without nitrogen sources. B: Western blot assay using the polyclonal antibody anti-*Pb*SP of protein extracts of. 1: yeast cells cultured in MMcM medium; 2: yeast cells cultured in the same medium deprived of nitrogen; 3: culture supernatant of yeast cells in MMcM medium; 4: the same as in 3 in the absence of nitrogen.

**Figure 3:** *Pb*sp transcript expression analysis. Mycelium and yeast cells were grown in medium and RNA extraction was performed. Macrophage cells were grown in RPMI medium, and infected with *P. brasiliensis* yeast cells and total RNA was obtained. RNAs were used to perform quantification of *Pb*sp expression by Real Time-RT-PCR. Reactions were performed in triplicate and normalized by using α-tubulin expression. M: Expression level of *Pb*sp in mycelium. Y: Expression level of *Pb*sp in yeast cells. I: Expression level of *Pb*sp in yeast cells during macrophages infection.

**Figure 4:** Co-immunopreciptation of *P. brasiliensis* proteins putatively interacting with *P. brasiliensis Pb*SP. The proteins were in vitro synthesized and labeled with $^{35}$S methionine. The translated serine protease molecules fused to c-myc epitope (c-myc-SP) and the translated proteins fused to hemaglutinin epitope (HA-Prey) were mixed and the mixture was incubated with protein A agarose beads and the monoclonal antibody anti-c-myc. The proteins were separated by SDS-PAGE. The gel was fixed, dried under vacuum and autoradiography was obtained. 1: Peptidyl proly cis-trans isomerase; 3:Calnexin; 5: HSP70; 7: Periodic tryptophan protein (PWP2). Negative controls for each reaction were performed and are shown in the lanes 2, 4, 6 and 8, respectively.

## Supplementary figure 1

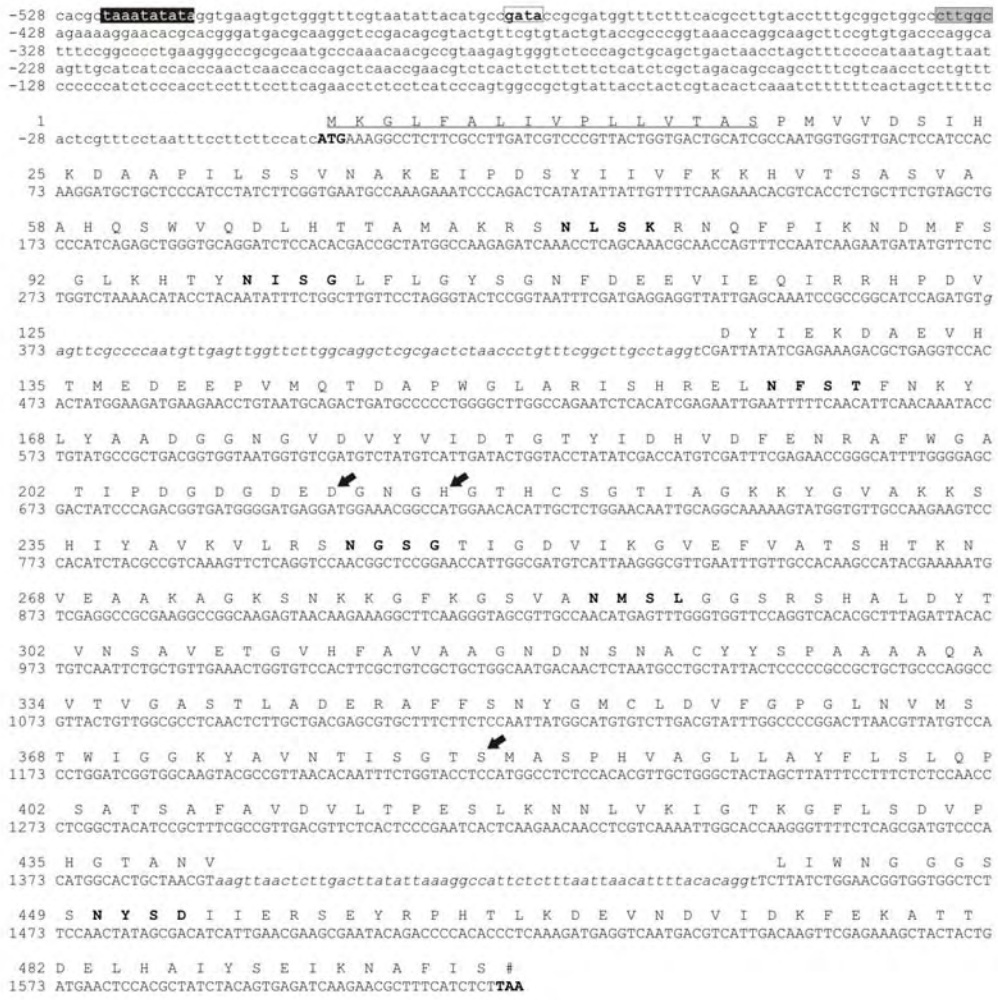


**Figure 1**

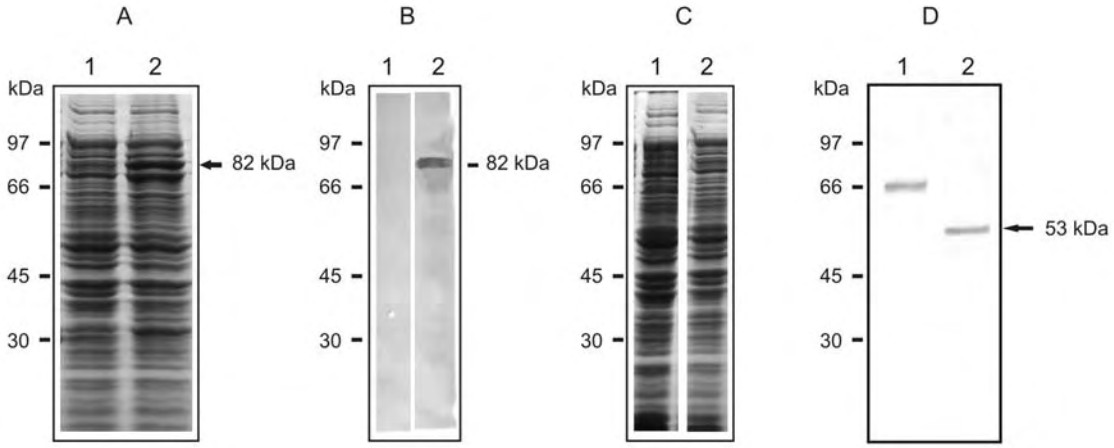



**Figure 2**

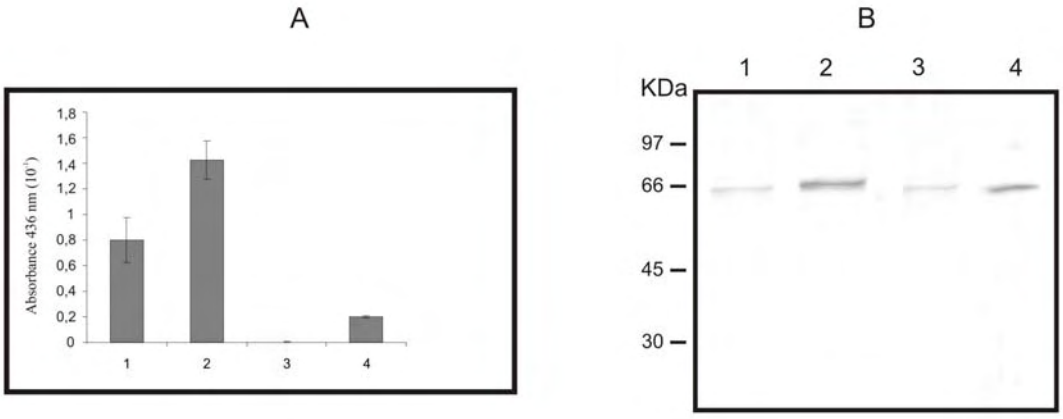


**Figure 3**

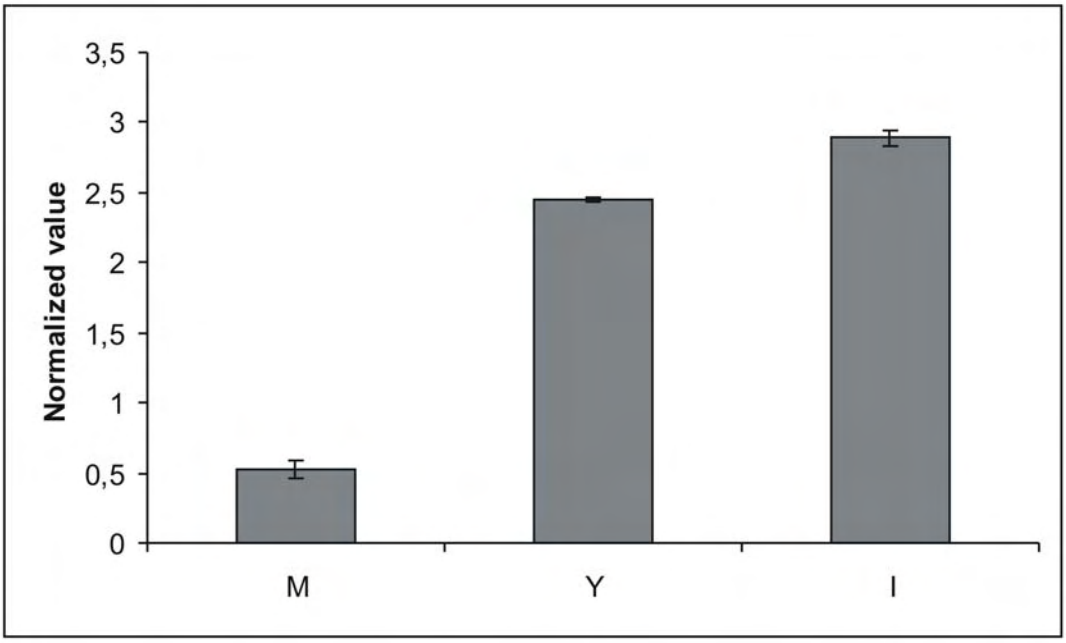
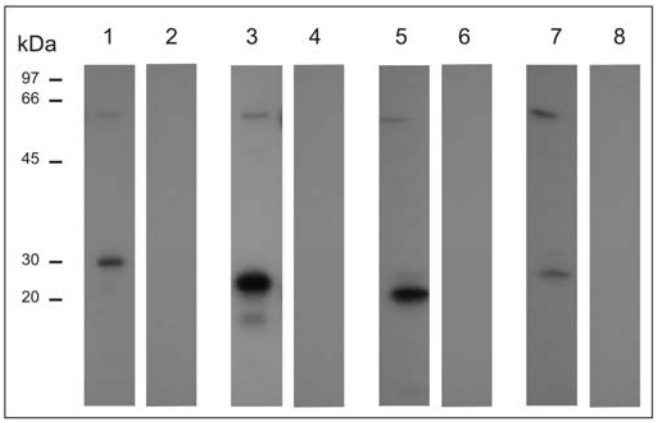



**Figure 4**



# *Discussão*

## Caracterização de um serino protease de *P. brasiliensis*

### Discussão e Conclusões

Análises de transcritoma de células leveduriformes de *P. brasiliensis* obtidas a partir de fígado de camundongos infectados (http://www.lbm.icb.ufg.br/phorestwww/index.php) mostraram que uma serino protease S08A apresentou regulação positiva nos níveis de transcritos quando comparados com banco de dados de *P. brasiliensis* após cultivo *in vitro* (Costa et al., 2007). Esta protease também foi diferencialmente expressa em experimentos de análise representativa diferencial (RDA) de *P. brasiliensis,* células leveduriformes, após incubação com sangue e plasma humanos (Bailão et al., 2006; 2007). Serino proteases da família S08A são geralmente endopeptidases que apresentam atividade sob condições alcalinas de pH (Barret & Rawlings, 1995).

No sentido de elucidar a importância desta serino protease de *P. brasiliensis*, o cDNA completo foi obtido (*Pb*sp) e seqüenciado. A seqüência predita de aminoácidos da serino protease (*Pb*SP) foi obtida e analisada. A região promotora foi também obtida, sendo possível encontrar uma região rica em TATA, provavelmente relacionada à ligação de fatores transcricionais. A região promotora contém uma seqüência 5'-GATA-3', relacionada à regulação por metabólitos de nitrogênio (NMR). A presença desta região está relacionada à regulação positiva de genes responsáveis pela utilização de fontes alternativas de nitrogênio em resposta à ausência das fontes preferenciais de nitrogênio amônia e/ou glutamina. Em *A. nidulans*, na ausência das fontes preferenciais de nitrogênio, a proteína AreA liga-se na região NMR da região promotora de genes responsáveis pela utilização de fontes alternativas de nitrogênio (Morozov *et al*, 2001). No fungo *Neurospora crassa*, proteínas Nit1 e Nit2 se ligam na região 5'-GATA-3' regulando positivamente genes relacionados ao metabolismo de fontes alternativas de nitrogênio (Chiang & Marzluf; 1995). Em *P. brasiliensis*, há evidências da regulação positiva da glicoproteína imunogênica GP43 pela proteína Nit2, visto que a região promotora do gene codificante para GP43 possui 23 sítios de ligação à proteína Nit2. Análises da expressão do transcrito codificante para GP43 mostraram que esta proteína é regulada por fontes primárias de nitrogênio nos isolados *Pb*339, *Pb*3 e *Pb*18 (Rocha et al., 2008).

Na região promotora do gene codificante para a serino protease foi identificada uma região 5'-GCCARG-3' relacionada à ligação da proteína PacC (Parente *et. al.*, em preparação). Em *A. nidulans,* a ligação da proteína PacC nesta região regula positivamente a transcrição de genes cujos produtos gênicos são expressos em condições de pH alcalino (Tilburn *et al*, 1995).

A seqüência predita de aminoácidos de *Pb*SP foi analisada e detectou-se uma seqüência de 16 aminoácidos, característica de uma região de peptídeo sinal. Também foram identificados resíduos conservados pertencentes ao sítio ativo em serino proteases S08A (asparagina – D, histidina – H, e serina – S). Foram detectados possíveis sítios de N-glicosilação. O cDNA codificante para serino protease foi clonado em vetor de expressão para sistema bacteriano e a proteína foi obtida, exibindo massa molecular de 82 kDa quando fusionada à proteína glutationa S transferase (GST). A proteína de fusão foi utilizada para obtenção de anticorpo policlonal em camundongos (anti-*Pb*SP). O soro contendo anti-*Pb*SP reconheceu especificamente uma proteína de 66 kDa em extrato protéico e sobrenadante de cultura de células leveduriformes de *P. brasiliensis*. Visto que as massa molecular de 66 kDa era maior que aquela predita *in silico,* foi realizado tratamento do extrato protéico de *P. brasiliensis* com endoglicosidase H, para avaliar a presença de N-glicosilação. O tratamento com endoglicosidase H resultou no aparecimento de uma espécie protéica de 53 kDa, correspondendo ao tamanho predito *in silico* de *Pb*SP, sugerindo a presença de N-glicosilação na molécula de *Pb*SP (Parente *et. al.*, em preparação).

Com o objetivo de avaliar a importância das proteases durante a privação de nitrogênio, extratos protéicos e sobrenadantes de cultura foram obtidos após a incubação de células leveduriformes de *P. brasiliensis* em meio quimicamente definido na presença e na ausência de fontes de nitrogênio. Os níveis de atividade proteolítica e a expressão de *Pb*SP foram avaliados em extrato protéico total e em sobrenadantes de cultura de *P. brasiliensis*. A atividade proteolítica e a expressão de *Pb*SP tanto no extrato protéico total quanto no sobrenadante de cultura apresentou nível mais alto após a incubação das células em meio de cultura privado de fontes de nitrogênio. Este resultado sugere que as proteases, incluindo *Pb*SP, são importantes na resposta ao estresse causado pela privação de nitrogênio em *P. brasiliensis* (Parente *et. al.*, em preparação). Resultados similares são descritos para a bactéria *Natrialba magaddi,* cujo nível de atividade proteolítica aumenta em resposta à ausência de nitrogênio em meio de cultura (D'Alessandro *et al.*, 2007). Uma serino protease do fungo fitopatogênico *M.*

*grisea* codificada pelo gene *isp* é induzida durante privação de nitrogênio. Sugere-se que esta proteína seja importante na patogênese em *M. grisea*, visto que células mutadas para o gene *isp* (Δ*isp*) apresentam capacidade reduzida de infectar folhas de arroz quando comparadas à linhagem selvagem (Donofrio *et al.*, 2006). A resposta à privação de nitrogênio em patógenos humanos é importante visto que alguns sítios de infecção apresentam baixas concentrações deste composto. Por exemplo, o interior do fagossomo de neutrófilos parece apresentar baixa concentração de nitrogênio visto que o perfil transcricional de *S. cerevisiae* e *C. albicans* durante a internalização por neutrófilos é similar ao perfil transcricional apresentado pelas duas leveduras durante a privação de aminoácidos (Rubin-Bejerano *et al.,* 2003). O nível de expressão de *Pb*sp foi avaliado em micélio, células leveduriformes e durante a infecção de células leveduriformes em macrófagos. O menor nível de expressão de *Pb*sp ocorre em micélio, enquanto o maior nível de expressão foi detectado durante a infecção de células leveduriformes em macrófagos. Estes resultados sugerem que esta serino protease pode ser importante na resposta adaptativa de *P. brasiliensis* à internalização por macrófagos (Parente *et. al.*, em preparação). Resultados similares são encontrados para o microorganismo patogênico *Mycobacterium tuberculosis*. Para este patógeno, uma serino protease da família das subtilisinas tem níveis de expressão aumentados durante a infecção em macrófagos. Esta protease se encontra associada à parede celular e também está presente em sobrenadante de cultura (Dave et al., 2002).

Ensaio de duplo-híbrido em *S. cerevisiae* foi realizado para detectar interações de *Pb*SP com outras proteínas de *P. brasiliensis*. Foram identificadas quatro proteínas interagindo com *Pb*SP: uma peptidil prolil cis-trans isomerase do tipo FKBP, calnexina, uma proteína de choque térmico HSP70 e uma proteína de parede celular rica em triptofano PWP2 (Parente *et. al.*, em preparação). Na bactéria *E. coli*, a proteina FKBP tem a função de catalisar a maturação de outras proteínas, contribuindo para aceleração do processo de enovelamento (Arié *et al*, 2001). A proteína calnexina atua no enovelamento correto de várias proteínas (Pollock et al., 2004). Na levedura *Hansenula polymorpha*, a função da calnexina tem sido relacionada com o aumento da secreção de outras proteínas (Klabunde et al., 2007). *Pb*SP interagem também com a proteína de choque térmico HSP70, associada à translocação de outras proteínas para compartimentos celulares em células eucarióticas (Craig et al., 1989). Interações de várias proteínas com HSP70 tem sido descritas. HSP70 interage com uma aquaporina tipo 2 presente nas células do ducto coletor do rim, regulando o tráfico desta proteína

dentro da célula (Lu *et al.*, 2007). Na levedura *S. cerevisiae*, PWP2 é um complexo proteináceo associado ao citoesqueleto que atua na hidrólise da parede celular durante a divisão celular (Shafaatian *et al*, 1996).

As funções das várias proteases identificadas em *P. brasiliensis* ainda não são elucidadas. Entretanto, várias proteases apresentaram-se reguladas positivamente em processos importantes tais como transição dimórfica, infecção em modelo animal e durante a incubação com sangue e plasma humanos (Bailão et. al., 2006; 2007; Costa et al., 2007; Bastos et. al., 2007; Parente et. al., 2008). Estes dados sugerem que esta classe de enzimas tenha importância no estabelecimento da infecção e na sobrevivência de *P. brasiliensis* em condições adversas às quais este fungo é submetido no organismo do hospedeiro. Estudos funcionais tornam-se necessários para elucidar as classes de proteases envolvidas nos processos de diferenciação e infecção. Da mesma forma, ampliar os estudos de interações intermoleculares em *P. brasiliensis* pode auxiliar na compreensão dos processos nos quais as diferentes proteases possam atuar.

# *Capítulo IV*

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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 (phenylmethylsulphonyl 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* Pb*SAP 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′-ACCGAATTC TATGAAGTTCTCTCTG - 3′ and 5′-ACCCTCGAGT CACTGTCTAGCCTTCG - 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* Pb*SAP, 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™ 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 ($1 \times 10^6$ 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 $\underline{D}^{104}XG^{106}XS^{108}XXW^{111}V^{112}$ and $\underline{D}^{288}T^{289}G^{290}$ (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* Pb*SAP 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 pre-immune 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.

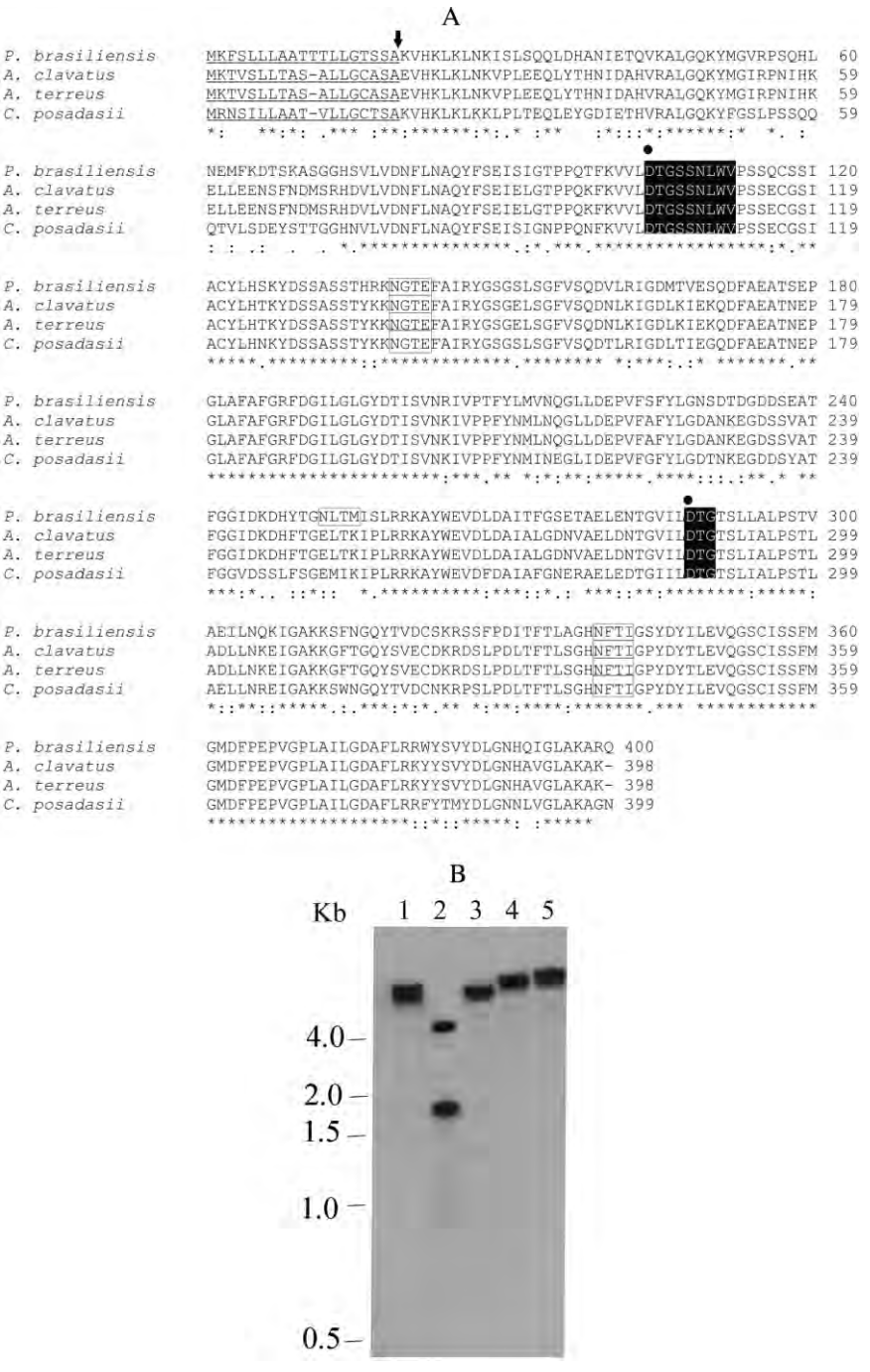


**Fig. 1** Clustal X (1.83) multiple sequence alignment and *Paracoccidioides brasiliensis* aspartyl protease genomic organization. (A) Comparison of the predicted amino acid sequence of *Pb*SAP with the selected sequences of the fungal aspartyl proteases corresponding to *Aspergillus clavatus* (XM_001271140), *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,

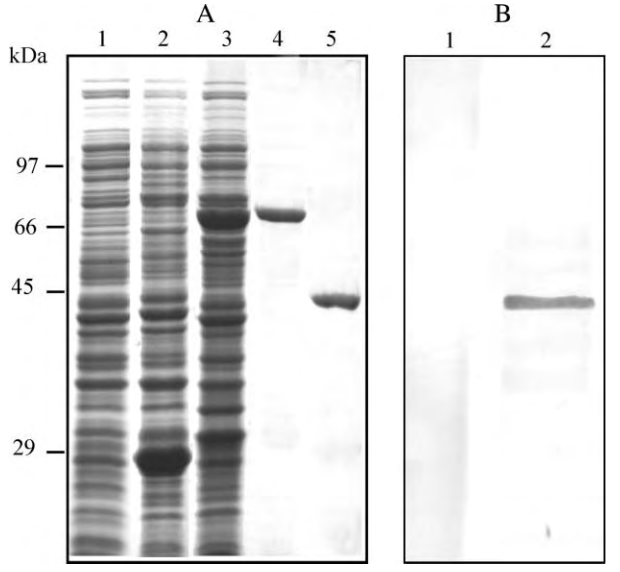


**Fig. 2** SDS-PAGE and immunoblot analysis of the recombinant *Pb*SAP. (A) Profile of the Coomassie brilliant blue stained gel (12% SDS-PAGE) of *E. coli* expressing the recombinant aspartyl protease. The lanes are as follows: 1 – Control, *E. coli* extracts; 2 – Extracts of *E. coli* cells containing 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™; 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.

lanes 1 and 2). A control reaction was performed by using the preimmune serum in the total 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

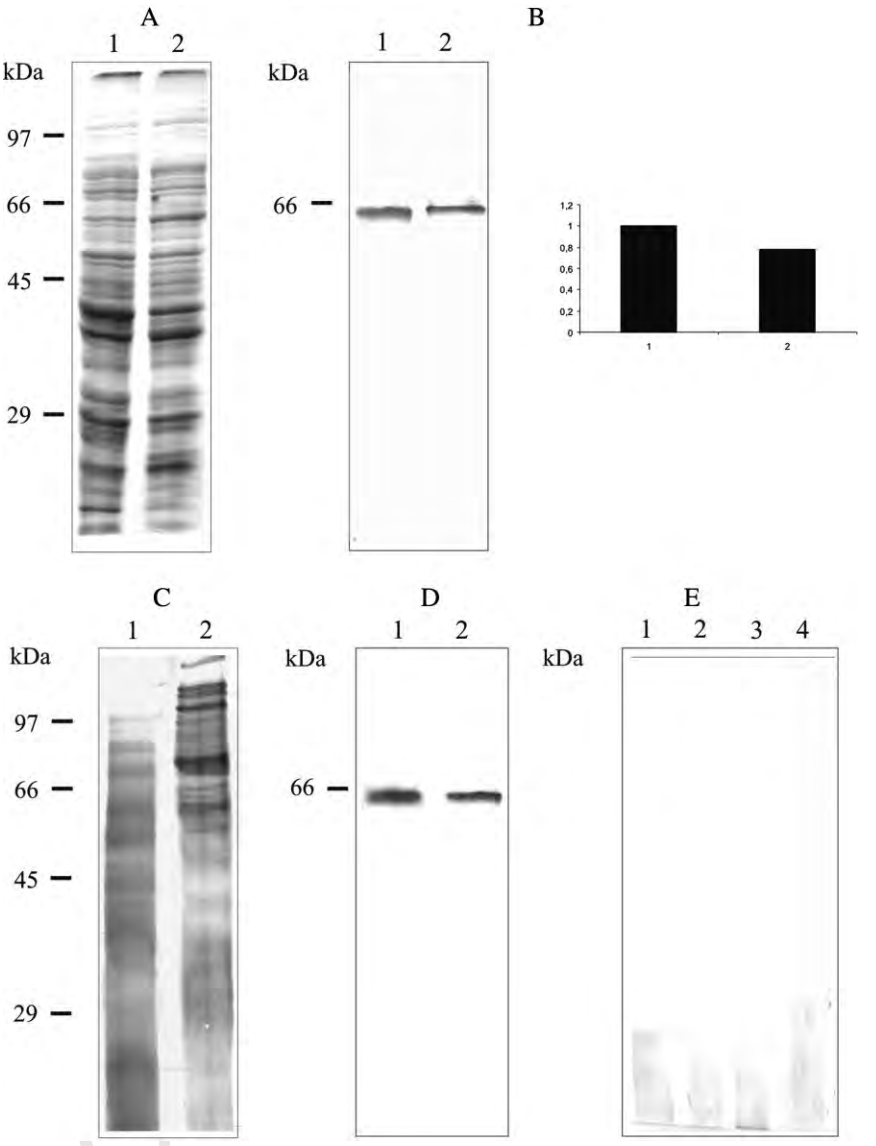


**Fig. 3** Immunodetection of aspartyl protease by western blot analysis. (A) SDS-PAGE (12%) of total protein extract (30 μg) of *P. brasiliensis* yeast cells (lane 1) and mycelium (lane 2), stained with *Coomassie* brilliant blue. (B) The same *P. brasiliensis* extracts were used and aspartyl protease 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.

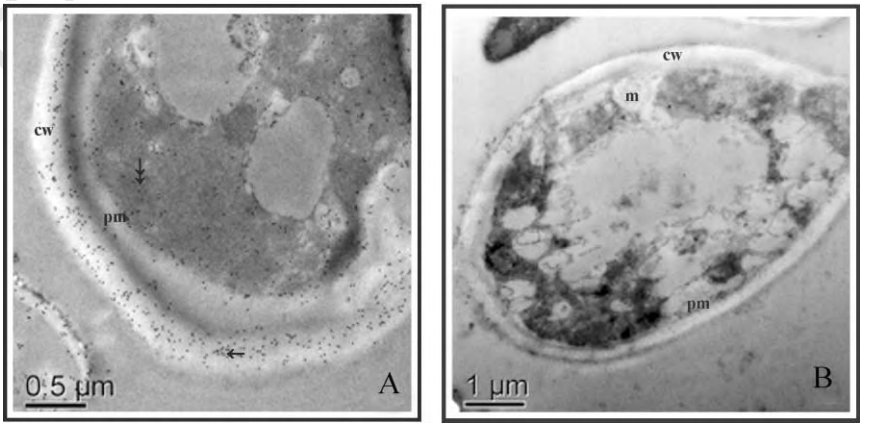


**Fig. 4** Immunogold localization of *Pb*SAP in *Paracoccidioides brasiliensis* yeast cells. (A) The gold particles conjugated to the secondary antibody were numerous in the cell wall (arrows) and sparse in the cytoplasm (double arrows). (B) Negative control was obtained using rabbit preimmune serum.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

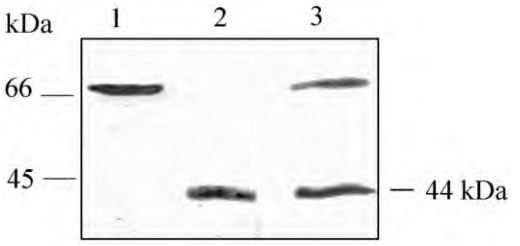


**Fig. 5** *Paracoccidioides brasiliensis* aspartyl protease glycosylation studies and immunoblot assays. Glycoslylation was investigated by treating protein extracts with endoglycosidase H or by including tunicamycin in the culture medium followed by immunoblot analysis. Lane 1: Control; total protein 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.

activity, suggesting that glycosylation is relevant for aspartyl protease activity in *P. brasiliensis*. Numerous functions have been attributed to aspartyl 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.

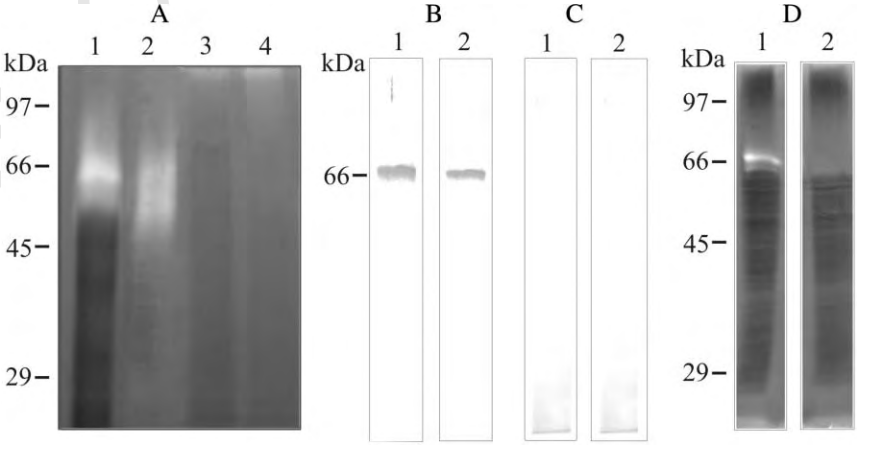


**Fig. 6** Analysis of gelatinolytic activity and immunoblot assays. (A) Total protein from yeast cells (lane 1) and secreted protein fraction (lane 2) were subjected to SDS-PAGE-gelatin, under non-denaturing conditions. The same extracts were assayed to protein specific inhibition using pepstatin (10 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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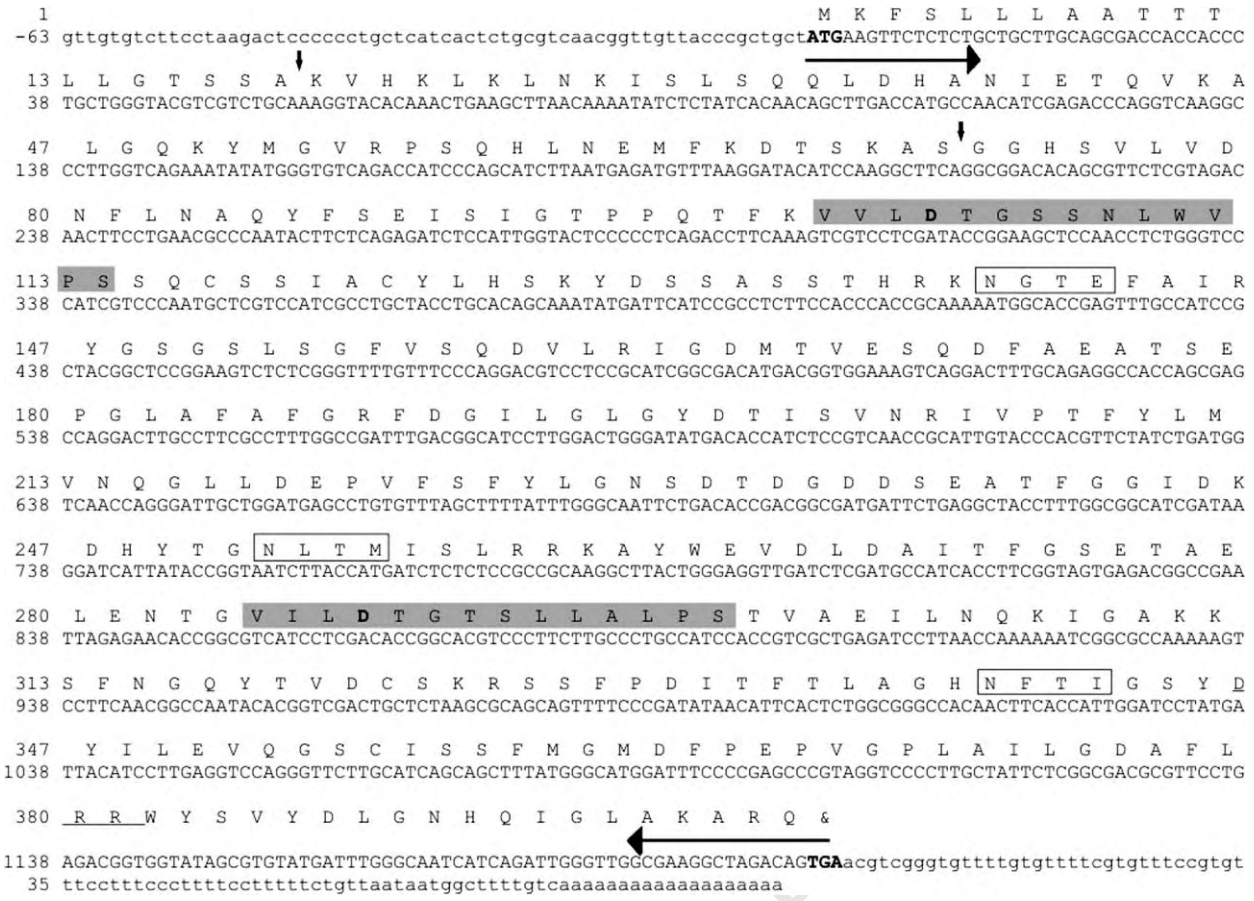


**Supplementary Fig. 1** Nucleotide and deduced amino acid sequence of the *P. brasiliensis* aspartyl protease cDNA. The nucleotide and amino acid positions are marked on the left side. Lower-case letters represent the untranslated 5′ and 3′ regions. Bold letters in the nucleotide sequence represent 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.