Universidade Federal de Goiás
Instituto de Ciências Biológicas
Programa de Pós-graduação em Biologia

Purificação parcial das quitinases, *Pbcts1* e *Pbcts2*, do fungo *Paracoccidiodes brasiliensis*

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Dissertação apresentada ao Programa de Pós-graduação em Biologia, do Instituto de Ciências Biológicas, da Universidade Federal de Goiás como requisito parcial à obtenção do título de Mestre em Biologia.

Goiânia – Goiás
Abril - 2008

DISSERTAÇÃO DESENVOLVIDA NO LABORATÓRIO DE BIOLOGIA MOLECULAR, DEPARTAMENTO DE BIOQUÍMICA E BIOLOGIA MOLECULAR, INSTITUTO DE CIÊNCIAS BIOLÓGICAS, UNIVERSIDADE FEDERAL DE GOIÁS, COMO REQUISITO PARCIAL PARA A OBTENÇÃO DO TÍTULO DE MESTRE EM BIOLOGIA.

Apoio financeiro: Coordenação de aperfeiçoamento de Pessoal de Nível Superior (CAPES) e Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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Não deixe que a saudade sufoque,
que a rotina acomode, que o medo
impeça de tentar. Desconfie do
destino e acredite em você. Gaste
mais horas realizando que
sonhando, fazendo que planejando,
vivendo que esperando, porque,
embora quem quase morre esteja
vivo, quem quase vive já morreu.
(Luiz Fernando Veríssimo)

Dedico esta dissertação aos meus pais Vivaldo Santana da Silva e Marleny da Penha Oliveira Santana pelo grande amor, apoio e total confiança depositada em mim.

Dedico ainda à minha orientadora,

Maristela, que acreditou em mim,

esteve SEMPRE presente na

produção desse trabalho e é um

exemplo de chefe.

AGRADECIMENTOS

A Deus, por estar presente em todos os momentos e detalhes de nossas vida.

À Profa. Dra. Célia Maria de Almeida Soares, pelo apoio, pelas sugestões e por todo auxílio prestado durante a realização desta dissertação.

À Profa. Dra. Marilene Henning Vainstein e ao Prof. Augusto Shrank por me receber muitíssimo bem em seus laboratórios na Universidade Federal do Rio Grande do Sul. Agradeço também a confiança dos senhores no meu potencial como pesquisadora.

Ao Prof. Dr. Cirano José Ulhoa, pelo seu apoio, sugestões e atenção mesmo em seu pouco tempo disponível.

Aos professores do Curso e Mestrado do Instituto de Ciências Biológicas, da Universidade Federal de Goiás, que muito contribuíram em meu aprendizado.

À minha querida amiga Débora, por ser a pesquisadora mais nata que já conheci e tentar me fazer sentir assim também. E ainda aos meus amigos, Bruno e Guilherme pelo auxílio estatístico!

À Patrícia Lima, pela amizade, carinho e confiança.

À Patrícia Kott pelos ensinamentos iniciais na pesquisa científica e pela sua amizade.

Ao Rogério, Neto, Patrícia Zambuzzi, Sabrina, Sarah, Kelly Pacheco, Amanda, Raquel, Regilda, Tereza Cristina, Mariana, Nathalie, Rodrigo e Murilo pela amizade e por tornar o ambiente de trabalho um local agradável de estar.

Aos alunos da professora Marilene, do professor Augusto e do professor Henrique que me receberam muito bem em seus laboratórios. Agradeço em especial à Irina, Lívia, Juli, Melissa, Bianca, Roberta, Juliano, Charley, Leonardo e Cláudio pelo apoio na minha adaptação e pela amizade.

E a todos os colegas e companheiros que de uma forma ou de outra contribuíram para a realização deste trabalho: Luiz Augusto, Juliana, Clayton, Alexandre, Roney, Elisa Flávia, Mirelle, Ana Flávia, Ívian, Ademar, Leandro, Daiane, Martha e João Guilherme.

E ainda aos que deixaram muitas saudades: Bruno, Moniquinha, Milce, Aline, Cristhielly e Kesser.

Ao CNPq, CAPES e PROCAD, pelo apoio financeiro.

A todos, muito obrigada!

SUMÁRIO

RESUMO

Paracoccidioides brasiliensis é um fungo dimórfico patogênico humano. A quitinase recombinante de *P. brasiliensis*, *Pbcts1r*, foi superexpressa em *Escherichia coli* utilizando pET-32(a)+ como vetor. A enzima foi produzida em corpos de inclusão e se tornou solúvel pela adição de sarkosyl, sendo purificada em um único passo com a utilização da resina Ni-NTA. *Pbcts1r* mostrou atividade diante de 4-MU-$(GlcNAc)_3$ e 4-MU-$(GlcNAc)_2$, apresentando atividade de endoquitinase. A reação de *imunoblot* com anti-*Pbcts1r* identificou duas proteínas no extrato bruto de levedura. A purificação parcial do extrato bruto de *P. brasiliensis* por cromatografia de troca-catiônica em HPLC revelou duas quitinases diferentes, *Pbcts1* e *Pbcts2*, com massas moleculares de 45 e 34 kDa, respectivamente. *Pbcts2* tem atividade de exoquitinase e *Pbcts1* de endoquitinase. Reações com anti-*Pbcts1r* mostraram a presença de *Pbcts1* e *Pbcts2* no extrato bruto de levedura e transição de micélio para levedura. No extrato bruto de micélio foi encontrado somente *Pbcts1* e no extrato de parede celular de levedura somente *Pbcts2*. Ambas as proteínas foram encontradas secretadas pela fase parasitária (levedura), mostrando a provável importância dessas proteínas na permanência do fungo no hospedeiro. Relações filogenéticas entre os ortólogos *Pbcts1* e a provável *Pbcts2* indicam a presença de um ancestral comum. Durante a evolução, *P. brasiliensis* poderia ter adquirido *Pbcts2* e *Pbcts1* desempenhando diferentes papéis para o crescimento e sobrevivência do fungo na fase saprofítica e parasitária.

ABSTRACT

Paracoccidoides brasiliensis is a human pathogenic dimorphic fungus. The recombinant chitinase from *P. brasiliensis*, *Pbcts1r*, was overexpressed in *Escherichia coli* using pET-32a (+) as vector. The enzyme was produced as inclusion bodies and became soluble by Sarkosyl being purified by a single step using a Ni-NTA resin. *Pbcts1r* showed activity against 4-MU-$(GlcNAc)_3$ and 4-MU-$(GlcNAc)_2$, presenting a endochitinase activity. Immunoblot reaction with anti-*Pbcts1r* identified two proteins in yeast crude extract. A partial purification of *P. brasiliensis* yeast crude extract by cationic-exchange chromatography on HPLC revealed two different chitinases, *Pbcts1* and *Pbcts2*, with molecular mass of 45 kDa and 34 kDa, respectively. *Pbcts2* has exochitinase activity and *Pbcts1* has endochitinase activities. Reactions with anti-*Pbcts1r* showed the presence of *Pbcts1* and *Pbcts2* in crude extracts of yeast and transition from mycelium to yeast. On mycelium crude extracts was found only *Pbcts1* and on yeast cell wall extract only *Pbcts2*. Both proteins were found to be secreted by yeast parasitic phase showing their probable importance in the permanence of the fungus in the human host. Phylogenetic relationships between the orthologs *Pbcts1* and the putative *Pbcts2* indicated the presence of a common ancestral. During evolution, *P. brasiliensis* could have acquired *Pbcts2* and *Pbcts1* playing distinct roles in order to growth and survive in diverse environment on saprophytic and parasitic phases.

INTRODUÇÃO

I – INTRODUÇÃO

1 – *Paracoccidioides brasiliensis*

O fungo *Paracoccidioides brasiliensis* é o agente etiológico da Paracoccidioidomicose (PCM), uma micose sistêmica que acomete seres humanos. De acordo com sua morfologia, *P. brasiliensis* pode apresentar-se como micélio ou como levedura. O micélio é identificado por filamentos septados com conídios terminais ou intercalares enquanto as leveduras são identificadas por brotamentos múltiplos, formados por evaginação da célula mãe, onde uma célula central é circundada por várias células periféricas, conferindo um aspecto de roda de leme de navio (RESTREPO-MORENO, 2003). Essa dupla morfologia se deve ao seu dimorfismo térmico, em que a temperaturas inferiores a 28°C o fungo é encontrado como micélio saprobiótico, e a 37°C como levedura nos tecidos infectados (SAN-BLAS & SAN-BLAS, 1993).

Essa diferenciação é conseqüência de um conjunto de alterações bioquímicas e fisiológicas desencadeadas pela variação da temperatura, a qual estimula uma adaptação no padrão transcricional e ou regulatório de genes (NUNES et al., 2005; BASTOS et al., 2007). Assim, diferenças na composição e no teor de lipídeos e de proteínas são encontradas entre as duas formas. Por exemplo, em relação à membrana plasmática, as células leveduriformes apresentam um teor de lipídeos duas vezes maior do que nas células micelianas (MANOCHA, 1980); e em relação à parede celular, observamos diferenças, principalmente, quanto às concentrações de polissacarídeos e de proteínas. As leveduras apresentam um teor protéico menor e um maior teor de quitina quando comparadas ao micélio. As células micelianas apresentam sua parede constituída principalmente por β-glicana e galactomanana (KANETSUNA et al., 1969).

Quanto à organização genômica de *P. brasiliensis*, utilizando a técnica de eletroforese de campo pulsado (PFGE), Feitosa et al. (2003) identificou 4 a 5 cromossomos com 2-10 Mb, tanto em isolados do meio ambiente quanto em isolados clínicos. Almeida et al. (2007) utilizando a técnica de citometria de fluxo, sugeriram que o genoma de levedura apresenta 26,3 a 35,5 Mb e que o dos conídios apresenta 30,2 a 30,9 Mb. É importante destacar que não houve diferença significativa entre os genomas das duas formas do fungo.

O fungo *P. brasiliensis* já foi classificado como um fungo imperfeito, pertencente ao filo anamórfico Deuteromycota e à classe Hypomycetos. Entretanto, estudos taxonômicos recentes levaram a extinção do filo Deuteromycota e à classificação do *P. brasiliensis* no filo Ascomycota. A inserção do *P. brasiliensis* neste filo, mesmo não se conhecendo a sua fase teleomórfica (sexuada), foi proposta por Leclerc et al. (1994) através da comparação de seqüências de DNA da subunidade ribossomal maior (28S) de dermatófitos e fungos dimórficos.

A atual classificação de *P. brasiliensis* é: filo Ascomycota, subdivisão Euascomycotina, classe Plectomyceto, subclasse Euascomycetidae, ordem Onygenales, família Onygenaceae, subfamília Onygenaceae Anamórficos, gênero *Paracoccidioides*, espécie *brasiliensis* (SAN-BLAS et al., 2002). Além da classificação em filo, ordem, família e gênero, *P. brasiliensis* também foi classificado em três diferentes espécies filogenéticas: S1, distribuída no Brasil, Argentina, Paraguai, Peru e Venezuela; PS2, encontrada na Venezuela e Brasil, nos estados de São Paulo e Minas Gerais; e PS3, restrita a Colômbia (MATUTE et al., 2006). Carrero et al. (2008) também descreveram esta classificação, mas ressaltam que na análise filogenética, um isolado de *P. brasiliensis*, *Pb*01, aparece claramente separado dos demais isolados de

Paracoccidoides. Especula-se que *Pb*01 possa ser uma nova espécie do gênero *Paracoccidoides* (CARRERO et al., 2008).

As condições e o local exato onde *P. brasiliensis* ocorre na natureza ainda são indeterminados. Isso se deve principalmente ao raro isolamento do fungo do ambiente, ao prolongado período de latência no hospedeiro humano, bem como a não identificação do hospedeiro intermediário do fungo (BAGAGLI et al., 2006). É conhecido que micélio e conídio provavelmente podem se apresentar como sapróbiótico no solo, na água, e em plantas à temperatura ambiente e são consideradas como formas infectantes do fungo (RESTREPO et al., 2001).

O fungo já foi isolado do solo de países como o Brasil (MONTENEGRO et al., 1996), Argentina (NEGRONI, 1966) e Venezuela (ALBORNOZ, 1971); de fezes de morcegos frugívoros (*Artibens literatus*) e de pinguim (GROSE & TAMSITT 1965; GARCIA et al., 1993); e de tatus, *Dasypus novemcintus* e *Cabassous centralis*, os quais são considerados hospedeiros silvestres do fungo (BAGAGLI et al., 2003; CORREDOR et al., 2005). A freqüência de infecção por *P. brasiliensis* em tatus *D. novemcintus* é alta e o fungo já foi isolado em 75-100% dos animais capturados nas áreas endêmicas de PCM (BAGAGLI et al., 2003). A infecção natural em animais domésticos também tem sido observada e manifestações clínicas da doença foram relatadas em cachorros (RICCI et al., 2004; FARIAS et al., 2005).

A PCM apresenta distribuição geográfica restrita a países da América Latina, ocorrendo do México até a Argentina, sendo a maior incidência no Brasil, Colômbia e Venezuela (WANKE & LONDERO, 1994; RIVITTI & AOKI, 1999). Um estudo da análise de óbitos entre os anos de 1980 e 1995, realizado por Coutinho et al. (2002), revelou que a PCM era a oitava causa de mortalidade por doença predominantemente crônica, entre as infecciosas e parasitárias, e possuía a mais elevada taxa de mortalidade

entre as micoses sistêmicas. Estudos mais recentes relatam que esta micose está restrita a América Latina, com aproximadamente 80% dos casos descritos no Brasil (RESTREPO et al., 2001). Entretanto, atualmente não se sabe a sua real epidemiologia, principalmente, devido ao fato dessa doença não ser de notificação compulsória.

A PCM ocorre predominantemente em áreas rurais, onde os indivíduos estão possivelmente mais expostos ao agente etiológico, acometendo principalmente indivíduos do sexo masculino entre 30 e 60 anos de idade (BRUMMER et al., 1993). Durante a infância a doença acomete ambos os sexos com igual intensidade fortalecendo a hipótese de que fatores hormonais possam desempenhar papel na patogênese da doença.

Estudos experimentais com o hormônio feminino 17-β-estradiol mostraram capacidade de inibir a transição de micélio para a fase leveduriforme tanto *in vitro* (RESTREPO et al., 1984) como *in vivo* (ARISTIZABAL et al., 1998). Outros, estudos sugerem que uma proteína de 60 kDa do citosol de *P. brasiliensis*, diferencialmente expressa em micélio e levedura (FELIPE et al., 2005), seria capaz de se ligar ao hormônio inibindo a transição de micélio para levedura (CLEMONS et al., 1989). Este fato estaria relacionado à possível proteção de mulheres à infecção.

A principal via de entrada do parasito no hospedeiro humano, segundo Franco et al. (1987), é através da inalação de micélios e/ou propágulos do fungo, propiciando o início da infecção pelos pulmões e a disseminação para outros órgãos e tecidos onde se estabelece na forma de levedura. Após a inalação, os micélios ou conídios se convertem em leveduras, sendo fagocitados por macrófagos, onde se multiplicam por brotamento múltiplo, formando o foco inflamatório (BRUMMER et al., 1989). O desenvolvimento da doença depende diretamente da resposta imune do hospedeiro (MENDES et al.,

1971), podendo manifestar-se sob a forma aguda ou juvenil, crônica ou adulta, e assintomática (LACAZ, 1994).

A infecção assintomática é observada em indivíduos que vivem em áreas endêmicas e apresentam teste de hipersensibilidade tardia. Já a forma aguda ou juvenil caracteriza-se por um desenvolvimento rápido e por marcante envolvimento de órgãos. Ela representa 3 a 5% dos casos descritos da doença, sendo a maioria dos pacientes constituídos por crianças ou adultos jovens. A forma crônica ou adulta ocorre em mais de 90% dos casos acometendo em sua maior parte, homens adultos. Nessa última forma há um desenvolvimento lento da doença, com comprometimento pulmonar podendo ser também observado comprometimento extrapumonar, devido ao fato da doença possuir um desenvolvimento silencioso e apenas apresentar sintomas quando há o comprometimento de outras regiões do corpo (BRUMMER et al., 1993).

Devido ao grande número de tecidos que *P. brasiliensis* pode colonizar acredita-se que o fungo tenha desenvolvido mecanismos que o capacita a aderir, extravasar e invadir barreiras impostas pelos tecidos do hospedeiro (MENDES-GIANINNI et al., 1994; LENZI et al., 2000). Em *P. brasiliensis* algumas moléculas de adesão à matriz extracelular já foram descritas, incluindo a glicoproteína Gp43 (VICENTINI et al., 1994), adesinas de 19 e 32 kDa (GONZÁLEZ et al., 2005), 30 kDa (ANDREOTTI et al., 2005), gliceraldeído-3-fosfato (BARBOSA et al., 2006), triosefosfato isomerase (PEREIRA et al., 2007) e a proteína de crescimento filamentoso imperfeito (DFG5) (DA SILVA CASTRO et al., 2008).

Além dos estudos de adesão, muitas outras abordagens têm sido realizadas com o intuito de entender melhor o genoma, a estrutura morfológica e também a patogênese de *P. brasiliensis*. Nesse sentido, visando conhecer um maior número de genes de *P. brasiliensis*, diferentes abordagens no estudo de transcriptomas têm sido aplicadas.

Entre essas abordagens temos a análise do transcriptoma de micélio e levedura (FELIPE et al., 2003, 2005; GOLDMAN et al. 2003), de genes expressos durante a diferenciação da fase de micélio para levedura (NUNES et al., 2005; BASTOS et al., 2007), genes expressos na presença de enxofre (ANDRADE et al., 2006), e ainda de genes envolvidos na adaptação e sobrevivência do fungo no processo infectante (COSTA et al., 2007), bem como durante contato com sangue (BAILÃO et al., 2006) e plasma humano (BAILÃO et. al., 2007). Diferentes técnicas têm sido utilizadas para análise desses transcriptomas. Entre elas podem ser citadas o microarranjo e a subtração (GOLDMAN et al., 2003) e a análise de diferença representacional de cDNA (BAILÃO et al., 2006).

A disponibilidade dos transcriptomas de *P. brasiliensis* permitiu a identificação de um vasto número de genes/proteínas específicos desse organismo que estão ausentes em humanos (TOMAZETT et al., 2005; AMARAL et al., 2005). Desta forma, alguns genes identificados em *P. brasiliensis* são considerados potenciais alvos para antifúngicos. Entre estes podem ser citados a 1,3-β-glicana sintase, 1,3-α-glicana sintase, Rho, quitina sintase, quitina deacetilase, α-1,2-manosiltransferase, quitinase, glicanase, isocitraro liase, malato sintase, ERG6, urato oxidade e urease (FELIPE et al., 2005; TOMAZETT et al., 2005). Além da importância como potenciais alvos para antifúngicos, o estudo desses genes e proteínas se faz importante também, para entender e esclarecer mecanismos de patogenicidade e virulência fúngica.

2 – QUITINASES

As quitinases (E.C. 3.2.1.14) são enzimas que clivam ligações glicosídicas entre os carbonos Cl e C4 dos resíduos de β-1,4-N-acetilglicosamina (GlcNAc), constituintes

do polímero de quitina. A quitina é considerada o segundo maior biopolímero natural depois da celulose (HAKI e RASKSHIT, 2003) e exerce uma importante função estrutural, sendo encontrada no exoesqueleto de crustáceos, nas cutículas de insetos, nas diatomáceas e na parede celular de fungos. Assim como seu substrato, a quitinase também é encontrada em diversos organismos, como bactérias, fungos, insetos, plantas e mamíferos (DUO-CHAN, 2006).

As quitinases produzidas por bactérias têm mostrado uma importante ação na hidrólise de quitina, visando sua utilização como fonte de carbono e na reciclagem desse componente na natureza (DUO-CHAN, 2006). Várias quitinases de bactérias já foram isoladas. Entre as mais recentes estão as quitinases de *Streptomyces cyaneus* SP-27 (YANO et al., 2008), *Clostridium paraputrificum* (MORIMOTO et al., 2007), *Serratia* sp. (KIM et al., 2007), *Chromobacterium* sp. linhagem C-61 (PARK et al., 2007), *Bacillus pumilus* SG2 (AHMADIAN et al., 2007), *Vibrio parahemolyticus* (CHUANG e LIN, 2007) e *Vibrio proteolyticus* linhagem N°.442 (ITOI et al., 2007), *Aeromonas hydrophila* linhagem SUWA-9 (LAN et al., 2006), *Rhodothermus marinus* (HOBEL et al., 2005).

Em fungos, a quitinase tem mostrado atuar na morfogênese (GHORMADE et al., 2000), separação celular (COLUSSI et al., 2005), esporulação (GIAVER et al., 2002), autólise (YAMAZAKI et al., 2007), assimilação de produtos degradados de quitina (KIM et al., 2002) e micoparasitismo (LIMA, et al., 1999; BINOD et al., 2007).

Em insetos, a quitina associa-se a proteínas para formar a cutícula do exoesqueleto e a matriz peritrófica, a qual contém uma mistura de glicosaminoglicanos, glicoproteínas e quitina (LEHANE et al., 1996). Nesses organismos, as quitinases são importantes na degradação da matriz peritrófica do intestino, pois permitem uma absorção eficiente de nutrientes. Além dessa função, as quitinases são importantes no

desenvolvimento pós-embrionário e na degradação da cutícula senescente (MERZENDORFER et al., 2003). Em crustáceos, a quitinase permite a degradação parcial do seu exoesqueleto durante o desenvolvimento, através de um mecanismo de controle hormonal (SPINDLER-BARTH, 1993).

Em plantas, as quitinases atuam na defesa contra fungos patogênicos, inibindo o crescimento desses fungos e gerando oligossacarídeos que atuam como elicitores, moléculas-sinal que vão regular os mecanismos de defesa da planta (KASPRZEWSKA, 2003). Entre algumas das quitinases de plantas descritas, tem-se a *Mcchit1*, de *Momordica charantia* L (XIAO et al., 2007), a *Cpchi*, de *Carica Papaya* (CHEN et al., 2007) e uma quitinase de 30 kDa, de *Ficus awkeotsang* (LI et al., 2003).

Em mamíferos a quitinase é chamada de quitotriosidase e pode ser encontrada no soro humano e em macrófagos (BOOT et al., 1995), sendo secretada conforme mostrado em trabalhos de Suzuki et al. (2001). A enzima quitotriosidase é um membro da família das quitinases capaz de hidrolizar quitina. Embora apresente homologia com outras quitinases de plantas, bactérias, fungos, nematodas e insetos, esta enzima é utilizada como marcador de ativação em doenças como Gaucher e é particularmente aumentada em indivíduos com arteriosclerose (HOLLAK et al. 1994), inflamação intestinal (MIZOGUCHI, 2006) e asma (ELIAS et al., 2005). Além da quitotriosidase foi identificada uma nova quitinase localizada nos pulmões e no trato digestivo de mamíferos. Essa proteína poderia apresentar ação na processo de defesa e no metabolismo de carboidratos (BOOT et al., 2001).

Estudos moleculares têm mostrado a presença de genes de quitinases em uma variedade de parasitas eucariotos, incluindo *Plasmodium* (SHAHABUDDIN et al., 1999), *Leishmania* (SHAKARIAN et al., 1998) e o nematoda *Brugia malayi* (FUHRAMN et al., 1992). É interessante ressaltar que as quitinases secretadas por

parasitas como *Plasmodium falciparum* (VINETZ et al., 1999) e *Plasmodium gallinaceum* (VINETZ et al., 2000) estão associadas com a ruptura da matriz peritrófica do intestino de seus hospedeiros definitivos, insetos do gênero *Lutzomia*, auxiliando assim na sua adesão nesses organismos. Quitinases de vírus também têm sido descritas, estando principalmente relacionadas à patogênese em insetos (DAIMON et al., 2007).

As quitinases são encontradas distribuídas em duas famílias, 18 e 19, dentro da superfamília das glicosil hidrolases. Essa classificação é baseada na seqüência de aminoácidos, na homologia estrutural e nas diferenças dos mecanismos de ação das quitinases. Artigos recentes sobre quitinases têm mostrado que as quitinases da família 18 têm sido encontradas em bactérias, fungos, animais e plantas das classes III e V; e que as da família 19 incluem as quitinases de plantas das classes I, II e IV, e ainda algumas quitinases de bactéria do gênero *Streptomyces* (DAHIYA et al., 2006). Entretanto, pesquisas no banco de dados CAZY (http://www.cazy.org/fam/acc_GH.html) mostraram que apesar da classificação das quitinases de plantas continuar a mesma, ambas as famílias apresentam quitinases de vírus, bactérias e eucariontes.

Quitinases de vários organismos apresentam uma arquitetura em multidomínio, que inclui um domínio catalítico, um domínio rico em serina/treonina e um domínio de ligação à quitina rico em cisteína. As quitinases fúngicas da família 18 podem apresentar sua estrutura básica composta de cinco domínios ou regiões: peptídeo sinal na região N-terminal, domínio catalítico, região rica em serina/treonina, domínio de ligação ao domínio de quitina e extensa região C-terminal (DUO-CHAN, 2006). Entretanto, na maioria das quitinases fúngicas os três últimos domínios costumam estar ausentes. Uma região interessante de se observar é a do peptídeo sinal evidenciando que nesses organismo as quitinases podem ser secretadas. A maioria das quitinases de

fungos purificadas e caracterizadas são secretadas [Brenda site (online: http://www.brenda-enzymes.info/)].

A classificação das quitinases de plantas é baseada na estrutura do domínio catalítico e no mecanismo de ação. As classes I e II apresentam domínios catalíticos similares. Na classe II não há presença do domínio de ligação à quitina e na classe III observa-se domínios similares aos presentes em fungos. A classe IV é similar às da classe I, mas são menores devido à ocorrência de deleções. A classe V apresenta homologia com exoquitinases de bactérias. As quitinases fúngicas, comparadas com as de plantas e bactérias, não são bem classificadas. Elas são divididas como quitinases fungo/bactérias, similares às de bactérias, e como quitinases fungo/planta, que são similares as encontradas em plantas da classe III. Recentemente, de acordo com análises filogenéticas, foi sugerida a divisão das quitinases fúngicas da família 18 em três grupos: grupo A, correspondendo às quitinases similares às da classe V de plantas; grupo B, similares as quitinases de plantas da classe III; e grupo C, correspondendo as quitinases de alta massa molecular (DUO-CHAN, 2006).

Além da classificação em famílias, as quitinases ainda podem ser classificadas quanto ao seu modo de ação sobre o substrato. A nomenclatura enzimática oficial (EC) classifica as enzimas quitinolíticas em apenas dois grupos de enzimas: as quitinases (EC 3.1.2.14), também conhecidas como endoquitinases, que catalizam a hidrólise randômica de ligações β-l,4 de N-acetilglicosamina (GlcNac) liberando produtos solúveis como quitotetraose, quitotriose e diacetilquitobiose; e as N-acetilglicosaminadases (EC 3.1.2.52), também conhecidas como exoquitinases, que clivam a quitina em monômeros de N-acetilglicosamina. Entretanto, algumas enzimas quitinolíticas não se encaixam nessa classificação. Assim, diferentes classificações foram sugeridas.

Sahai & Manocha (1993) classificaram as quitinases em três tipos: as endoquitinases (EC 3.2.1.14), as exoquitinases e as N-acetilglicosaminidases (3.2.1.52). Harman et al. (1993) também fizeram uma classificação diferenciada da nomenclatura oficial, adicionando mais um tipo de quitinase, a quitobiase, correspondente à exoquitinase de Sahai & Manocha, que catalisa a liberação progressiva de diacilquitobiose do final redutor da cadeia de quitina, não liberando monômeros nem oligossacarídeos de N-acetilglicosamina. Na literatura atual a classificação mais seguida é a da nomenclatura enzimática oficial e quando esta é insuficiente a classificação usada é a de Sahai & Manocha.

Na literatura são encontrados trabalhos diversos sobre quitinases, incluindo análises de seqüências gênicas à caracterização da enzima purificada. Dessa forma, muitas características das quitinases têm sido identificadas auxiliando na sua utilização e aplicação na biotecnologia.

Quanto à regulação da quitinase, sua expressão é controlada por um sistema de repressão/ indução. Nesse sistema, a presença de parede celular no meio de cultivo, de quitina ou outros produtos de degradação (N-acetilglicosamina e glicosamina) atuam como indutores, e a glicose, ou fontes de carbono facilmente metabolizadas atuam como repressores (MACH et al., 1999). Este comportamento tem sido descrito para quitinases de *Aphanocladium album* (BLAISEAU et al., 1992), *T. harzianum* (CARSOLIO et al., 1999), *Streptomyces thermoviolaceus* (TSUJIBO et al, 1998). *Enterobacter* sp. (DAHIYA, 2005), *Beuvaria Bassiana* (Campos et al., 2004) e *Metarhirzium anisopliae* (BARATTO et al., 2006; DA SILVA et al. 2005).

Além da quitina e da glicose existem outros componentes e fatores que podem interferir na regulação das quitinases. Um desses fatores, já descrito em *T. harzianum* e *Manaporthe grisea*, é a modificação pós-transcricional, em que a quantidade de RNAm,

que sofreu ou não *splicing*, regula a expressão da quitinase (SEIDL et al., 2005). Em alguns organismos, a expressão da quitinase também pode ser alterada pela expressão de outra enzima quitinolítica. É o caso da N-acetilglicosaminidase e da *ech42* de *T. harzianum*, em que a expressão da N-acetilglicosaminidase se mostrou essencial para a indução da *ech42* (BRUNNER, 2003). Pesquisas com *ech42* ainda mostraram que a transcrição do seu gene é induzida pela redução da fonte de carbono em seu meio de crescimento. Além disso, observou-se também que à temperatura baixa e com uma pressão osmótica alta a *ech42* tem sua expressão aumentada (MACH et al., 1999). Entretanto existem quitinases que podem ser induzidas pelo aumento da temperatura, como é o caso da chit33, também de *T. harzianium* (DE LA MERCEDES et al., 2001).

Quanto à atividade das quitinases, diferentes substratos e métodos têm sido utilizados para a sua caracterização. Dentre os métodos de detecção utilizados têm-se os ensaios colorimétricos (REISSIG et al.,1955), ensaios fluorescentes (SELVAGGINI et al., 2004), detecção direta utilizando a eletroforese em gel de poliacrilamida (PAN et al., 1991), detecção da turbidez em suspensão com quitina coloidal (TRONSMO e HARMAN, 1993) e separação do produtos da reação enzimática por TLC – cromatografia de camada delgada (KIM et al., 2007), HPLC-Cromatografia Líquida de Alto Desempenho (CHUANG e LIN, 2007) ou espectroscopia de massas (RAJAMOHANAN et al., 1996). Os substratos variam entre os naturais: quitina, quitina coloidal, glicolquitina e quitosana; e os sintéticos: 4-MU-GlcNAc , 4-MU-$(GlcNAc)_{2,3}$, pNP-GlcAc, pNP-$(GlcNAc)_{2,3}$.

Quando se utiliza um substrato natural, os métodos mais utilizados para a detecção da atividade são o colorimétrico, o da redução da turbidez do meio e o da eletroforese em gel de poliacrilamida (zimograma). No ensaio colorimétrico é feita a leitura da quantidade de açúcar redutor produzido, em decorrência da incubação da

enzima com o substrato e posterior reação com ácido dinitrosalicílico (DNS). No método de redução da turbidez a leitura da atividade também é feita de acordo com a quantidade de açúcar redutor liberado. No gel de atividade ou zimograma, a atividade da quitinase é visualizada em gel de poliacrilamida, onde há a migração das proteínas em um gel contendo o substrato, nesse caso a glicolquitina. O zimograma também pode ser realizado com substrato sintético, sendo este incubado junto ao gel, após a migração da quitinase. Em ambos os zimogramas a leitura é feita sob luz ultravioleta (HOWARD et al., 2003).

Quando se utiliza como substrato a quitina coloidal ou a glicolquitina não se sabe quais oligossacarídeos são liberados. Assim, para identificar os produtos liberados na ação enzimática utiliza-se a TLC, HPLC ou a espectroscopia de massas. Nesses três métodos há uma separação dos oligossacarídeos encontrados na reação enzimática e sua subseqüente identificação. É importante ressaltar que a TLC é apenas uma técnica qualitativa diferentemente da HPLC e da espectroscopia de massas que também são técnicas quantitativas (HOWARD et al., 2003).

Os substratos sintéticos 4-MU-GlcNAc, 4-MU-$(GlcNAc)_{2,3}$ se apresentam mais sensíveis na detecção da atividade quitinolítica, pois a reação enzimática é lida com a utilização de fluorescência. Em trabalhos recentes de caracterização da quitinase têm-se utilizado esses substratos sintéticos, pois além de dispensarem a necessidade de experimentos posteriores para identificação dos oligossacarídeos liberados eles facilitam a classificação das quitinases. Os substratos pNP-GlcNAc, pNP-$(GlcNAc)_{2,3}$ também são compostos bastante utilizados para a detecção da atividade de quitinase. A leitura da reação é feita em espectrofotômetro usando uma curva padrão preparada com 4-nitrofenol (HOBEL et al., 2005).

Esses diferentes métodos são importantes para definir se uma determinada quitinase atua como endo- ou exoquitinase. Recentemente, algumas quitinases foram descritas com ambas as atividades. Kang et al. (1999) detectaram uma quitinase de *Metahirzium anisopliae* com atividade endo e exo, baseado na degradação de oligômeros de quitina e vários compostos químicos. O polímero de quitina foi utilizado como substrato para detectar a atividade de endoquitinase e o substrato pNP-$(GlcNAc)_2$ para detectar a atividade exoquitinolítica. Silva et al. (2005) também identificou uma outra proteína de *M. anisopliae* com atividade de endo- e exoquitinase (N-acetilglicosaminidase). Em *A. fumigatus* foi identificada uma quitinase que possui atividade de endoquitinase, exoquitinase e transglicosilase (XIA et al., 2001).

De acordo com o GenBank, um grande número de genes codificando quitinases tem sido isolados e caracterizados. Dentre esses há o *Pb*CTS1, que codifica uma quitinase de *P. brasiliensis* (BONFIM et al., 2006). Além disso, muitas quitinases tanto nativas quanto recombinantes têm sido purificadas [Brenda site (online: http://www.brenda-enzymes.info/)].

Para a obtenção das quitinases purificadas, diferentes métodos são utilizados tais como, precipitação com sulfato de amônia (IKE et al., 2006), cromatografia de interação hidrofóbica (DUO-CHAN 2005), cromatografia de troca iônica (IKE et al., 2006), cromatografia de afinidade à quitina (KIM et al., 2007) e ainda cromatografia de afinidade à histidina em quitinases recombinantes (CHEN et al., 2007). Para uma purificação total geralmente se faz necessário a utilização de mais de um dos métodos acima. Fungos quitinolíticos, como o *T. harzianum* e o *Talaromyces flavus* (DUO-CHAN, 2006) apresentam mais de um tipo de quitinase, sendo essas diferentes quitinases purificadas de formas distintas.

Esse extenso estudo sobre as quitinases se deve ao fato da sua larga capacidade de aplicação na biotecnologia. A primeira aplicação da quitinases foi estudo de protoplastos e na citolocalização de quitina (MANOCHA & ZHONGHUA, 1997). Uma quitinase de *Enterobacter* sp NRG4 mostrou-se efetiva na produção de protoplastos de *T. reesei*, *Pleurotus florida*, *Agaricos florida* e *A. niger*. A destruição da parede celular e o isolamento do protoplasto são importantes para o estudo da síntese da parede celular, da síntese enzimática e da secreção de proteínas (DAHIYA et al., 2006).

A aplicação mais observada é no controle biológico. Muitas quitinases de fungos têm apresentado atividade contra patógenos de plantas e contra insetos que causam pestes na agricultura (DAHIYA et al., 2006). Quitinases de *T. harzianum* (DEMARCO et al., 2000), *Enterobacter* sp. NRG4 (DAHIYA et al. 2005) e *Fusarium chlamydosporum* (MATHIVANAN et al., 1998) já mostraram atividade inibitória contra alguns desses patógenos, podendo assim ser utilizadas como suplementos na produção de fungicidas e inseticidas.

As quitinases também têm sido aplicadas em processos de bioconversão. Em países costeiros, após o consumo de camarões, o exoesqueleto é descartado produzindo mais de 2,5 milhões de toneladas de lixo dessa natureza. O principal objetivo da aplicação da quitinase nesse processo é a conversão do lixo em produtos derivados de quitina que podem ser aproveitados. (DAHIYA et al., 2006).

As quitinases ainda podem ser aplicadas na medicina. Quitoligossacarídeos, glicosaminas e GlcNAc produzidos pelas quitinases possuem um imenso potencial farmacêutico. Quitohexose e quitopentose mostraram atividade antitumoral. Além dessas utilizações, as quitinases também podem ser usadas na produção de soluções oftalmológicas combinadas com antimicrobianos (DAHIYA et al., 2006).

Além das aplicações biotecnológicas, o estudo das quitinases também tem sido importante para obtenção de informações sobre a estrutura e o funcionamento da enzima no seu organismo produtor, e ainda, para obter informações sobre sua ação na virulência, quando oriunda de microrganismos patogênicos. Quitinases de *Trichomonas vaginalis* (LOIESAU et al., 2002) e *Candida albicans* (KELLY et al., 2004) já mostraram importante ação na patogênese da tricomoníase e da candidíase, respectivamente, através de estudos de infecção em animais.

JUSTIFICATIVA

II – JUSTIFICATIVAS

A PCM é a micose sistêmica de maior prevalência na América Latina, com maior número de relatos de casos no Brasil. A capacidade do *P. brasiliensis* de causar a PCM em suas diferentes manifestações clínicas, depende da complexidade de interações entre o fungo e o hospedeiro humano. Estudos de adesão e invasão do *P. brasiliensis* em células Vero observaram a presença do fungo no interior da célula na forma de protoplasto (HANNA et al., 2000). Assim, estudos de proteínas envolvidas no metabolismo da parede celular, como as quitinases, têm se mostrado importantes para o entendimento da patogênese e da biologia do fungo, pois elas podem auxiliar na remodelação, no crescimento e na autólise da célula fúngica. Além disso, as enzimas envolvidas no biossíntese da parede celular são importantes alvos para antifúngicos, pois atuariam em estruturas ausentes nos serem humanos diminuindo a toxicidade comum aos fármacos antifúngicos.

OBJETIVOS

III – OBJETIVOS

1. Purificação da quitinase recombinante, *Pbcts1r*, de *P. brasiliensis*.

Estratégias:

- clonagem do cDNA *Pb*CTS1 em vetor de expressão;
- indução da expressão da proteína recombinante em sistema bacteriano;
- purificação da proteína recombinante por cromatografia de afinidade.

2. Caracterização da proteína recombinante.

Estratégias:

-realização de ensaios de atividade enzimática em diferentes pHs e temperaturas;

- realização de ensaios de atividade enzimática na presença de diferentes íons, EDTA e SDS.

3. Identificação e análise da expressão da proteína *Pbcts1* em extratos protéicos de *P. brasiliensis*.

Estratégias:

- produção de anticorpos policlonais para *Pbcts1r*;
- extração de proteínas de diferentes fases e compartimentos de *P. brasiliensis*;
- realização de ensaios de *western blot*.

4. Purificação parcial e análise da expressão de quitinases de *P. brasiliensis* na fase leveduriforme.

Estratégias:

- extração de proteínas de diferentes fases e compartimentos do *P. brasiliensis*;
- purificação das quitinases por cromatografia de troca-iônica;
- realização de ensaios de atividade enzimática;
- realização de ensaios de *western blot*.

MANUSCRITO

Partial purification of two chitinases, *Pbcts1* and *Pbcts2*, from *Paracoccidioides brasiliensis*

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Keywords: *Paracoccidioides brasiliensis*, chitinase, dimorphic fungus, protein purification, cell wall.

Abstract

Paracoccidoides brasiliensis is a human pathogenic dimorphic fungus. The recombinant chitinase from *P. brasiliensis*, *Pbcts1r*, was overexpressed in *Escherichia coli* using pET-32a (+) as vector. The enzyme was produced as inclusion bodies and became soluble by Sarkosyl being purified by a single step using a Ni-NTA resin. *Pbcts1r* showed activity against 4-MU-$(GlcNAc)_3$ and 4-MU-$(GlcNAc)_2$, presenting a endochitinase activity. Immunoblot reaction with anti-*Pbcts1r* identified two proteins in yeast crude extract. A partial purification of *P. brasiliensis* yeast crude extract by cationic-exchange chromatography on HPLC revealed two different chitinases, *Pbcts1* and *Pbcts2*, with molecular mass of 45 kDa and 34 kDa, respectively. *Pbcts2* has exochitinase activity and *Pbcts1* has endochitinase activities. Reactions with anti-*Pbcts1r* showed the presence of *Pbcts1* and *Pbcts2* in crude extracts of yeast and transition from mycelium to yeast. On mycelium crude extracts was found only *Pbcts1* and on yeast cell wall extract only *Pbcts2*. Both proteins were found to be secreted by yeast parasitic phase showing their probable importance in the permanence of the fungus in the human host. Phylogenetic relationships between the orthologs *Pbcts1* and the putative *Pbcts2* indicated the presence of a common ancestral. During evolution, *P. brasiliensis* could have acquired *Pbcts2* and *Pbcts1* playing distinct roles in order to growth and survive in diverse environment on saprophytic and parasitic phases.

Introduction

Paracoccidioides brasiliensis is a dimorphic human pathogenic fungus, presenting in yeast phase during infection and in mycelium phase in the environment. The cell wall of *P. brasiliensis*, as in other fungi, has lipids, proteins, chitin, and glucan. In addition, it has been related to the pathogenesis (Silva et al., 1997; de Agostino Biella et al., 2006). The percentage of chitin in the cell wall of *P. brasiliensis* is between 7-18% in yeast phase and 37-48% in mycelium phase, showing the importance of this carbohydrate, as well as, enzymes related to cell wall metabolism on dimorphism and consequently in the infection process by this fungus (Kanetsuna et al., 1969).

Chitin is a polymer of β-1,4-linked N-acetylglucosamine (GlcNAc) found as major structural component in crustacean exoskeletons, insect cuticles, diatoms, and fungal cell walls. Chitinases (E.C. 3.2.1.14) hydrolyze chitin and can be classified in two families of glycoside hydrolases, 18 and 19, based on its amino acid sequence, structural homologies and mechanism of action (Henrissat & Bairoch, 1996).

Chitinases play important physiological (Colussi et al., 2005; Yamazaki, et al., 2007) and ecological roles (Kirubakaran et al., 2007) being present in a wide variety of organisms including bacteria, insects, virus, plants and animals. Fungal chitinases are important to nutrition, developmental processes, morphogenesis and control of pests (Dahiya et al., 2006; Binod et al., 2007). Many fungal chitinases have been purified and characterized and some microorganisms have presented more then one chitinase (Duo Chuan et al., 2006).

Until this moment, only one chitinase (*Pbcts1*) from *P. brasiliensis* was identified (Bonfim et al., 2006). In this work we overexpressed, purified and produce antibodies to the recombinant chitinase, *Pbcts1r*, from *P. brasiliensis*. We also purified partially chitinases from *P. brasiliensis* yeast cells, and described a second chitinase,

Pbcts2, which acts like an exochitinase. *Pbcts2* was found in cell wall extract of yeast cells suggesting its function in the cell wall biosynthesis process. *Pbcts1* and *Pbcts2* were secreted by yeast cells showing their probable importance in maintaining of *P. brasiliensis* in the human host. The phylogenetic relationship between the orthologs *Pbcts1* and the putative *Pbcts2* indicated that they have a common ancestral.

Materials and Methods

Fungus and growth conditions

P. brasiliensis, *Pb*01 isolate (ATCC-MYA-826), was used in this study. The fungus was cultivated in Sabouraud Dextrose solid medium (4% glucose, 1% peptone, 0.5% yeast extract, 0.1% brain-heart infusion, 1.2% agar) at 22°C for the mycelium phase and at 36°C for the yeast phase, during 15 and 7 days, respectively. For the transition from mycelium to yeast, the fungus growing at 22°C was transferred to liquid medium at 36°C and grown for 24 hours under shaking at 150 rpm.

Recombinant DNA preparation and transformation

The *Pb*CTS1 cDNA sequence was obtained and amplified by PCR using primers as described by Bonfim et al. (2006). Forward primer (5'-CAGATCCACCAGAATTCATGACG-3') and reverse primer (5'-CACCTCGAGCTACTCCCCAGG-5') were designed with *Eco*RI and *Xho*I sites (underlined), respectively, to facilitate cloning into the expression vector pET-32a(+) (Novagen), which contains an internal and C-terminal six-histidine tag. PCR conditions were: initial denaturation at 94°C for 3 min followed by 25 cycles at 94°C (1 min and 30 s), 58°C (2 min), 72°C (1 min and 30 s) and a final elongation at 72°C (10 min). The

PCR product was digested with *Eco*RI and *Xho*I and linked to pET-32a(+). The resulting plasmid was sequenced to confirm the sequence and after that was transformed into *Escherichia coli* BL21(DE3)/pLysS.

Production and purification of the recombinant protein

The transformed *E. coli* cells were grown at 37°C in LB-medium with 34 µg mL^{-1} chloramphenicol, 100 µg mL^{-1} ampicillin and 200 mM glucose at 200 rpm, until A_{600} reached 0.6-0.8. Expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma-Aldrich) at a final concentration of 1 mM. After that, the cells were incubated for 3 h at 37°C with shaking at 200 rpm, followed by harvesting by centrifugation at 10,000 *g* for 5 min at 4°C. The cell pellet was resuspended in PBS 1x and incubated for 1 h with 1 mg mL^{-1} of lysozyme and lysed by extensive sonication. The cell pellet was still incubated for 15 min on ice with 1% of Sarkosyl and shacked on vortex at each 5 min for 30 sec. The sample was then centrifuged at 4°C, 10,000 *g* for 20 min at 4°C and the supernatant, which contained the soluble protein fraction, was collected and treated with 2% Triton X-100. The recombinant chitinase was purified using Ni-NTA resin (Invitrogen). Briefly, the supernatant was applied on a Ni-NTA resin column. After washing twice with buffer (20 mM imidazole, 0.5 M NaCl, 50 mM sodium phosphate, pH 6.0), *Pbcts1r* was recovered using two different elution buffers (150 mM imidazole, 0.5 M NaCl, 50 mM sodium phosphate, pH 6.0; 250 mM imidazole, 0.5 M NaCl, 50 mM sodium phosphate, pH 8.0;). For further assays, the purified *Pbcts1r* was pooled, dialyzed in sodium acetate buffer pH 6.0 and cleaved by the addition of enterokinase to remove His-tag.

Antibody production

The purified *Pbcts1r* was used to produce anti-*Pbcts1r* polyclonal antibody in New Zealand rabbits. Rabbit preimmune serum was obtained and stored at -20ºC. The immunization protocol consisted of an initial injection of 200 µg of purified recombinant protein in complete Freund's adjuvant and two subsequent injections of the same amount of the antigen in incomplete Freund's adjuvant. Each immunization was followed by an interval of two weeks. After the third immunization, the serum containing anti-*Pbcts1r* polyclonal antibody was collected and stored at -20ºC. The serum reactivity to the *Pbcts1r* was tested by ELISA and Western blot.

Protein extraction of *P. brasiliensis*

Protein extracts were obtained by disruption of frozen cells. The protein extract was resuspended in Tris–Ca buffer [2 mM $CaCl_2$, 20 mM Tris-HCl, pH 8.8; protease inhibitor mix (GE Healthcare)], harvested and shacked for 20 min at 4ºC with glass beads. The complete cell breakage was verified by the failure of cells to grown on Sabouraud medium. The material was centrifuged once at 4ºC, 5,000 *g* for 15 min and the supernatant was harvested and centrifuged twice. The supernatant contains the crude protein extract. The first pellet obtained was washed five times with distilled water, after was rinsed with different concentrations of NaCl (85 mM; 34 mM; 17 mM) and boiled three times in SDS–extraction buffer (50 mM Tris–HCl, pH 8.0, 2% SDS, 100 mM EDTA, 10 mM DTT). The pellet obtained was washed six times with water, ressuspend with Tris-Ca buffer and stored as extract of cell wall proteins (Pitarch et al., 2002). The yeast proteins of culture medium were obtained by filtration of the Sabouraud Dextrose medium, after *P. brasiliensis* growth during 24 h. After, this extract was dialyzed against distilled water and concentrated with 20% trichloroacetic acid and acetone.

Purification of native chitinases from *P. brasiliensis*

Soluble protein crude extract of yeast was dialyzed against distilled water and after, against 50 mM sodium acetate buffer, pH 5.4 (buffer A). The dialyzed was filtered through a 0.22 µm filter then applied to a CM SepharoseTM Fast Flow previously equilibrated with buffer A and connected to a HPLC Äkta purifier (GE Healthcare). The proteins were eluted with a linear gradient of buffer A containing 0 to 1 M NaCl with a flow rate of 1mL min^{-1} and by collecting 1 mL of washing fractions and 0.5 mL of elution fractions. The collected elution fractions were dialyzed against distilled water and after that, all fractions had chitinase activity tested with standard assay.

Western blot analysis

After separation by one-dimensional gel electrophoresis, the proteins were transferred to a nylon membrane and checked by Ponceau S to determine equal loading. The membrane was washed with water and incubated in buffer containing 50 mM PBS, 0.1% (v/v) Tween-20, 5% (w/v) skimmed milk powder for 18 h at 4°C. The *P. brasiliensis* chitinases were detected with the anti-*Pbcts1r* (diluted 1:500 in PBS). The second antibody was alkaline phosphatase coupled anti-rabbit immunoglobulin G (Invitrogen). The reactions were developed with 5-bromo-4-chloro-3-indolylphosphate/ nitro-bluetetrazolium (BCIP/NBT). Negative controls were obtained with rabbit preimmune serum.

Enzyme activity assay

The chitinase activity was determined as previously described by Selvaggini et al. (2004) with some modifications, using as substrates 4-methylumbelliferyl-N-N'-N''-diacetylchitobiose [4-MU-$(GlcNAc)_2$], which can indicates exochitinase activity, or 4-methylumbelliferyl-N-N'-N''-triacetylchitotriose [4-MU-$(GlcNAc)_3$] (Sigma-Aldrich), which indicates endochitinase activity. In standard assay, 20 µL of appropriately diluted enzyme solution was incubated with 5 µL of 0.4 mM substrate solution and 80 µL of 100 mM citrate-phosphate buffer, pH 5.0 at 37°C for 30 min. The reaction was stopped by adding 120 µL glycine/NaOH buffer, pH 10.6. After 5 min of incubation was proceeded a reading at 355 nm excitation and 460 nm emission in a spectrofluorometer (spectraMax M2^e, Molecular Devices). One unit of enzyme activity was defined as the amount of enzyme necessary to produce 1 pmol of 4-MU per mL min^{-1}.

Electrophoresis and protein determination

Sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE) was carried out under denaturating conditions, as described by Laemmli (1970). Proteins were separated on 10% SDS-PAGE gel followed by staining with Coomassie brilliant Blue. Protein concentration was measured according to the method of Bradford (1976) using bovine serum albumin as the standard.

Comparison of sequences and inferred phylogeny

Pbcts1 and *Pbcts2*, a putative chitinase of 34 kDa obtained from BROAD Institute (http://www.broad.mit.edu/annotation/genome/paracoccidioides_brasiliensis.1/MultiHome.html) were aligned with chitinases from National Center for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov) (Altschul et al., 1990). The

phylogenetic relationships among chitinases were generated with 75 complete ascomycetes chitinases. One chitinase from *Cryptococcus neoformans*, a basidiomycete, was used as out group. One phylogenetic tree constructed by multiple sequence alignments using CLUSTAL X (Thompson et al., 1997) was generated by the neighbor-joining method (Saitou et al., 1987) and visualized using the TreeView software (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). Robustness of branches was estimated using 100 bootstrap replicates and indicates the percentage of times that all species appear as a monophyletic cluster.

Results

Expression and purification of *Pbcts1r* protein

Aiming to obtain the recombinant protein, the cDNA encoding to *Pb*CTS1 was subcloned into the expression pET-32a(+) vector, which contains a fused His_6-tag. The protein was not present in crude extracts from non induced *E. coli* cells carrying the expression vector (Fig 1A, lane 1). After induction with IPTG, a 58 kDa recombinant protein was detected in bacterial lysates (Fig 1A, lane 2). The fusion protein was purified from bacterial lysates using Ni-NTA and was cleavage with enterokinase (Invitrogen) to remove His-tag (Fig 1A, lane 3). The molecular mass of *Pbcts1r* correlated with the predicted size of 45 kDa. An aliquot of the purified recombinant protein was used to generate rabbit polyclonal anti-recombinant *Pbcts1* antibody. The purified recombinant protein was blotted to nylon membrane and reacted to the polyclonal antibody (Fig. 1B, lane 1). No cross-reactivity to the rabbit preimmune serum was visualized (Fig. 1B, lane 2).

Immunological detection of *P. brasiliensis* chitinases

The rabbit polyclonal antibody generated from the purified recombinant protein was used to detect the presence of *Pbcts1* in different phases and compartments of the fungal cell (Fig. 2A). *Pbcts1* was found in crude extract of mycelium, during transition from mycelium to yeast and in crude extract of yeast cells (Fig. 2A, lanes 1, 2, 3, respectively). *Pbcts1* was not detected in the yeast cell wall (Fig. 2, lane 4), but it was present in the secreted fraction of yeast (Fig. 2, lane 5). A new chitinase protein, *Pbcts2*, with a molecular mass estimated of 34 kDa was also observed in the immunoblot. *Pbcts2* was not detected in mycelium phase (Fig. 2A, lane 1), but it was present during transition from mycelium to yeast (Fig. 2A, lane 2) and at the yeast phase (Fig. 2A, lane 3). Differently of *Pbcts1*, *Pbcts2* was found in all protein extracts of yeast cells analyzed, including cell wall (Fig. 2A, lane 4). The finding of a new chitinase from *P. brasiliensis*, *Pbcts2*, is reinforced by no cross-activity to the rabbit preimmune serum (Fig 2B, lanes 1 to 5).

Partial purification of *P. brasiliensis* chitinases and activity assay

To corroborate our hypothesis of a second chitinase in *P. brasiliensis*, it was performed a partial purification of protein crude extract of yeast cells by using cationic exchange chromatography. Two protein peaks were detected together with peaks of chitinase activity (Fig. 3). In the first peak was found a higher activity toward 4-MU-$(GlcNAc)_3$ and in the second peak was found a higher activity toward 4-MU-$(GlcNAc)_2$. Table 1 shows the specific activity of chitinases from yeast crude extract and from those peaks above. Thus, to confirm the presence of chitinase on those fractions, an immunobloting analysis was performed. To that analysis, three fractions were investigated (Fig. 3B). Two of the fractions analyzed (fractions 3 and 5) were collected

during washing of the column; the other one (fraction 34) was collected during the linear gradient of NaCl. On fraction 3 was observed the presence of both proteins, one of 45 kDa and the other of 34 kDa, corresponding to *Pbcts1* and *Pbcts2*, respectively (Fig. 3B, lane 2). In addiction, on fraction 3, was found a small activity toward 4-MU-$(GlcNAc)_2$ and a higher activity toward 4-MU-$(GlcNAc)_3$ (Table 1). On fraction 5, which had *Pbcts1*, but not *Pbcts2* (Fig. 3B, lane 3), it was detected a high activity toward 4-MU-$(GlcNAc)_3$ indicating a endochitinase activity (Fig 3A; Table 1). In the fraction 34, was found *Pbcts1* and *Pbcts2* (Fig 3B, lane 4). The higher activity toward 4-MU-$(GlcNAc)_2$ than to 4-MU-$(GlcNAc)_3$, indicating a exochitinase activity, was attributed to *Pbcts2*, since *Pbcts1* had a strong endochitinase activity (Table 1). In addition, a strong reaction to *Pbcts2* was observed on western blot.

The chitinase activity of *Pbcts1r* was performed. The recombinant chitinase showed higher specific activity toward hydrolyze of 4-MU-$(GlcNAc)_3$ (142,92 U/mg) than toward 4-MU-$(GlcNAc)_2$ (52,32 U/mg), indicating a endochitinase activity, similarly to *Pbcts1* activity.

Phylogenetic analysis

A search on genome of *P. brasiliensis*, isolate *Pb*01, available on database from BROAD Institute, was realized aiming to find a putative chitinase gene encoding to *Pb*CTS2. From this search, one putative chitinase gene with a predict size of 34 kDa was found and named *Pbcts2*. To visualize the relationship between *Pbcts1* and *Pbcts2* in terms of amino acid sequence similarity, a phylogenetic tree was constructed with others complete chitinases sequences from ascomycetes available on NCBI database (Altschul et al., 1990) using neighbor-joining method. The sequences are shown in Fig. 4, with their respective database entry accession. The orthologs *Pbcts1* and *Pbcts2*

segregated early and clustered separately indicating a common ancestral. The bigger length of the branch to *Pbcts2* than to *Pbcts1* suggests that *Pbcts2* evolved more than *Pbcts1*.

Discussion

Some chitinases from pathogenic fungus has been described, but no one of *P. brasiliensis* was purified and characterized yet. In order to overexpress, purify and produce antibodies of *Pbcts1* we cloned the *Pb*CTS1 cDNA into pET 32a(+) vector and transformed *E. coli* BL21(DE3)/pLysS. This vector allowed a single step of purification due to addiction of a histidine tag.

The purification of *Pbcts1r* allowed the production of antibodies and analysis of the presence of *Pbcts1* in different cellular compartments of *P. brasiliensis*. Immunological analysis indicated the presence of *Pbcts1* in crude extract of mycelium, transition from mycelium to yeast and yeast cells. These results are according with that reported by Bonfim et al. (2006), which showed the presence of *Pb*CTS1 transcripts in that phases. The strong signal of *Pbcts1* in yeast (see figure 2A), parasitic form of *P. brasiliensis*, in medium culturing yeast cells, and its expression in experimental infection conditions (Bonfim et al., 2006) suggest the importance of this protein on pathogeneses.

Studies about *P. brasiliensis*, strain IVIC *Pb*9, found activity of chitinase in culture medium, but do not in the protein crude extracts of yeast (Flores-Carréon et al., 1979). Here, *Pbcts1* was found in culture medium also. The activity of *Pbcts1* in the crude extracts of yeast cells in isolate *Pb*01, could be explained by the difference existent between the isolates, and by the substrates used.

Bonfim et al. (2006) described in *Pb*CTS1 a glycosaminoglycan attachment site and laminin G, a domain of binding to heparin. These domains mediate mycobacterium adhesion to lung epithelial cells and macrophages (Jeffery, 1999) and contribute to cell invasion and systemic dissemination in the host cells (Adams, 2004). Here, we found the presence of *Pbcts1* in culture medium of yeast showing that this protein is secreted and could act during adhesion of *P. brasiliensis* in human host.

The majority of chitinases described are secreted but some of them are found on cell wall, like chitinases from *Piromyces communis* (Sakurada et al., 1998), *Candida albicans* (Iranzo et al., 2002) and *A. fumigatus* (Hearn et al., 1998). *Pbcts1* was not detected in cell wall fraction suggesting that it could not participate of the yeast cell wall biosynthesis.

Although chitinases have been studied extensively, the nomenclature of chitinolytic enzymes is until confuse. Nevertheless, most of those enzymes are divided into three modes of action: endochitinases, exochitinases and β-N-acetylglucosaminadases. Endochitinases (E.C. 3.2.1.14) cleave chitin randomly and form multimers of GlcNAc such as chitotetraose, chitotriose and chitobiose; exochitinases, also named in past as chitobiosidades, releases chitobiose units from one end and no monosaccharides or oligosaccharides are formed; and β-N-acetylglucosaminidases (E.C. 3.2.1.52) cleave chitin polymers into GlcNAc monomers (Sahai & Manocha, 1993).

Many fungi like *C. albicans*, *A. fumigatus* and *Sacharomyces cerevisae* have more then one chitinase, each one acting at a different way as endoquitinases, exoquitinases or β-N-acetylglucosaminidases. In this work, we also found a second chitinase of *P. brasiliensis*, *Pbcts2*, which has different properties of *Pbcts1*. The recognition of *Pbcts2* by anti-*Pbcts1r* could be explained by the similarity between

these proteins, which probably, share antigenic determinants. Antibodies recognize a relatively small component of an antigen and due to it they can cross-react with similar epitopes on other antigens (Lipman et al., 2005). During partial purification of the yeast crude extract, the fraction 34 had a strong signal level to *Pbcts2* and a high activity toward 4-MU-$(GlcNAc)_2$, which could be attributed to *Pbcts2*, since only a few quantity of *Pbcts1* was found in that fraction. In addition, *Pbcts1* has a higher activity toward 4-MU-$(GlcNAc)_3$ than toward 4-MU-$(GlcNAc)_2$.

Pbcts2 was not present in mycelium and was found in all proteins extracts of yeast cells analyzed. Since *Pbcts2* is present in the cell wall, it could participate on the yeast cell wall biosynthesis, maintaining that structure. In addition, the presence of *Pbcts2* during transition from mycelium to yeast and in extracellular medium of yeast cells could suggests its importance on the pathogeneses process. The molecular mass of the *Pbcts2* was estimated to be 34 kDa. Some fungal chitinases with molecular mass around 30-33 kDa have been studied (De la Cruz, 1992; Duo-Chan, 2005; Hoell, 2005) and the majority of them are secreted.

The phylogenetic relationship between the orthologs *Pbcts1* and the putative *Pbcts2*, obtained from BROAD Institute database, showed that they segregated early in the phylogenetic history, and clustered separately indicating a common ancestral. The bigger length of the branch to *Pbcts2* than to *Pbcts1* suggests that *Pbcts2* evolved more than *Pbcts1*. The same characteristics can be evidenced to *Emericella nidulans* (*E. nid* 1 and *E. nid 2*) and *Coccidioides immits* (*C. imm* 1 and *C. imm 2*). The multiple and diverse roles played by fungal chitinases is known (Duo-Chuan, 2006). In this way, during evolution, *P. brasiliensis* could have acquired *Pbcts2* and *Pbcts1* in order to growth and survive in different environment on saprophytic and parasitic phases.

Acknowledgements

This work at Universidade Federal de Goiás was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Financiadora de Estudos e Projetos (FINEP), Programa Nacional de Cooperação Acadêmica (PROCAD) and International Foundation for Science (IFS), Stockholm, Sweden, through a grant to Maristela Pereira. L.A.P.S. and L.K.S.S. were supported by a fellowship from CAPES.

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Table 01

Table 1. Specific activity in different fractions from cationic exchange chromatography

Fraction number	Specific activity (U^a/mg)	
	Exochitinase	**Endochitinase**
Yeast crude extract	1,014	3,721
3	1,745	7,101
5	2,140	9,698
34	10,457	1,867

[a]One unit of enzyme activity was defined as the amount of enzyme necessary to produce 1 pmol of 4-MU per mL min^{-1}.

Figure legends

Fig. 1. Analysis of the recombinant *Pbcts1*. (A) The proteins were analyzed by 10% SDS-PAGE, and stained with Coomassie brilliant blue R-250. Lane 1, total protein from non induced *E. coli* BL21(DE3)/pLysS containing pET-32a(+); lane 2, proteins prepared from *E. coli* BL21(DE3)/pLysS after induction with IPTG; lane 3, *Pbcts1r* purified. (B) Western blot of purified chitinase after incubation with anti-*Pbcts1r* (lane 1) and with rabbit preimmune serum (lane 2). The arrow indicates the overexpressed chitinase protein.

Fig. 2. Western blot analyses of *P. brasiliensis* chitinases. (A) The proteins were transferred from SDS-PAGE gel to a nylon membrane and incubated with *anti-Pbcts1r*. (B) The reactivity of *P. brasiliensis* proteins was analysed by western blotting with rabbit preimmune serum. Lane 1, crude extract from mycelium; lane 2, crude extract from mycelium in transition to yeast cells during 24 hours; lane 3, crude extract from yeast; lane 4, yeast cell wall extract; lane 5, yeast secreted proteins. The numbers on the left indicate the size of proteins. In each lane it was used 20 μg of total protein.

Fig. 3. Purification analysis of *P. brasiliensis* chitinases. (A) Cationic exchange chromatography profile of yeast crude extract on HPLC and profile of chitinase activity were plotted. Bound proteins were eluted with a linear gradient of 0-1 M of NaCl (dashed line). Solid line: absorbance at 280 nm in mAU; -●-: exochitinase activity; -○-: endochitinase activity. (B) Three fractions of this purification were analyzed by western blot using anti-*Pbcts1r*. Lane 1, crude extract from yeast cells; Lanes 2, 3 and 4 corresponding to fractions 3, 5 and 34 of the cationic exchange chromatography,

respectively. The left numbers indicate the size of proteins. In each lane was used 20 µg of total protein.

Fig. 4. Phylogenetic tree illustrating the relationship among *Pbcts1*, *Pbcts2* and others chitinases. Sequences were aligned and subjected to phylogenetic analysis using minimum evolution (neighbour-joining). The numbers on branches indicate bootstrap values obtained for 100 replications. The species (named with binomial name) and the respective GenBank accession numbers are shown following: *Ajellomyces capsulatus 1* (AAF80370); *Ajellomyces capsulatus 2* (AAG41982); *Ajellomyces dermatitidis* (AAF80371); *Aphanocladium álbum* (P32470); *Aspergillus clavatus* (EAW13716); *Aspergillus fumigatus 1* (AAO61685); *Aspergillus fumigatus 2* (AAO61686); *Aspergillus nidulans* (AAG34171); *Beauveria bassiana 1* (AAN41259); *Beaveria bassiana 2* (AAN41261); *Bionectria.ochroleuca 1* (ABV57861); *Bionectriaochroleuca 2* (ABD48217); *Blumeria graminis* (AAK84437). *Botryotinia fuckeliana 1* (AAM94807); *Botryotinia fuckeliana 2* (AAM94405); *Candida albicnas 1* (AAG35112); *Candida albicans 2* (AAA68015); *Coccidioides immitis 1* (2204242A); *Coccidioides immitis 2* (Q1EAR5); *Coccidioides posadasii 1* (P54196); *Coccidioides posadasii 2* (AAO88269); *Coccidioides posadasii 3* (AAP46398); *Coccidioides posadasii 4* (AAR18253); *Coccidioides posadasii 5* (AAP80452); *Coccidioides posadasii 6* (AAR18252); *Chaetomium cupreum* (ABK27625) ; *Chaetomium globosum* (ABI95487); *Coniothyrium minitans* (AAG00504); *Emericella nidulans 1* (BAA36223); *Emericella nidulans 2* (BAA35140); *Hypocrea jecorina 1* (DAA05858); *Hypocrea jecorina 2* (DAA05857); *Hypocrea jecorina 3* (DAA05859); *Hypocrea jecorina 4* (DAA05855); *Hypocrea jecorina 5* (DAA05853); *Hypocrea jecorina 6* (DAA05849); *Hypocrea jecorina 7* (DAA05852); *Hypocrea jecorina 8* (DAA05851); *Hypocrea*

jecorina 9 (DAA05866); *Hypocrea virens 1* (AAL78814); *Hypocrea virens 2* (AAL78812); *Hypocrea lixii 1* (CAA55928); *Hypocrea lixii 2* (AAT47713); *Isaria farinosa* (ABD64606); *Isaria javanica* (AAZ83728); *Lecanicillium lecanii 1* (AAX56961); *Lecanicillium lecanii 2* (AAV98692); *Lecanicillium psalliotae* (ABQ57240); *Metarhizium anisopliae 1* (AAY32603); *Metarhizium anisopliae 2* (AAC33265); *Metarhizium flavoviride* (CAB44709); *Neorospora crassa 1* (XP_965309); *Neorospora crassa 2* (XP_957924); *Neosartorya fischeri 1* (XP_001261690); *Neosartorya fischeri 2* (XP_001266139); *Neosartorya fischeri 3* (XP_001263706); *Neosartorya fischeri 4* (XP_001261767); *Nomuraea rileyi* (AAP04616); *Paecilomyces lilacinus* (ABP37997); *Paracoccidioides brasiliensis 1* (AAQ75798); *Pichia guilliermondii* (ABY81886); *Pichia stipitis 1* (XP_001386607); *Pichia stipitis 2* (XP_001385362); *Pichia stipitis 3* (XP_001387623); *Pichia stipitis 4* (XP_001384092); *Sacharomyces cerevisae 1* (P29029); *Sacharomyces cerevisae 2* (Q06350); *Thermoascus aurantiacus var. levisporus* (ABS00927); *Trichoderma atroviride* (ABO38127); *Trichoderma hamatum* (AAP15043); *Trichoderma lanugenosus* (AAY99632); *Trichoderma viride* (ABR27743); *Verticillium fungicola* (AAP45631).

Fig. 01

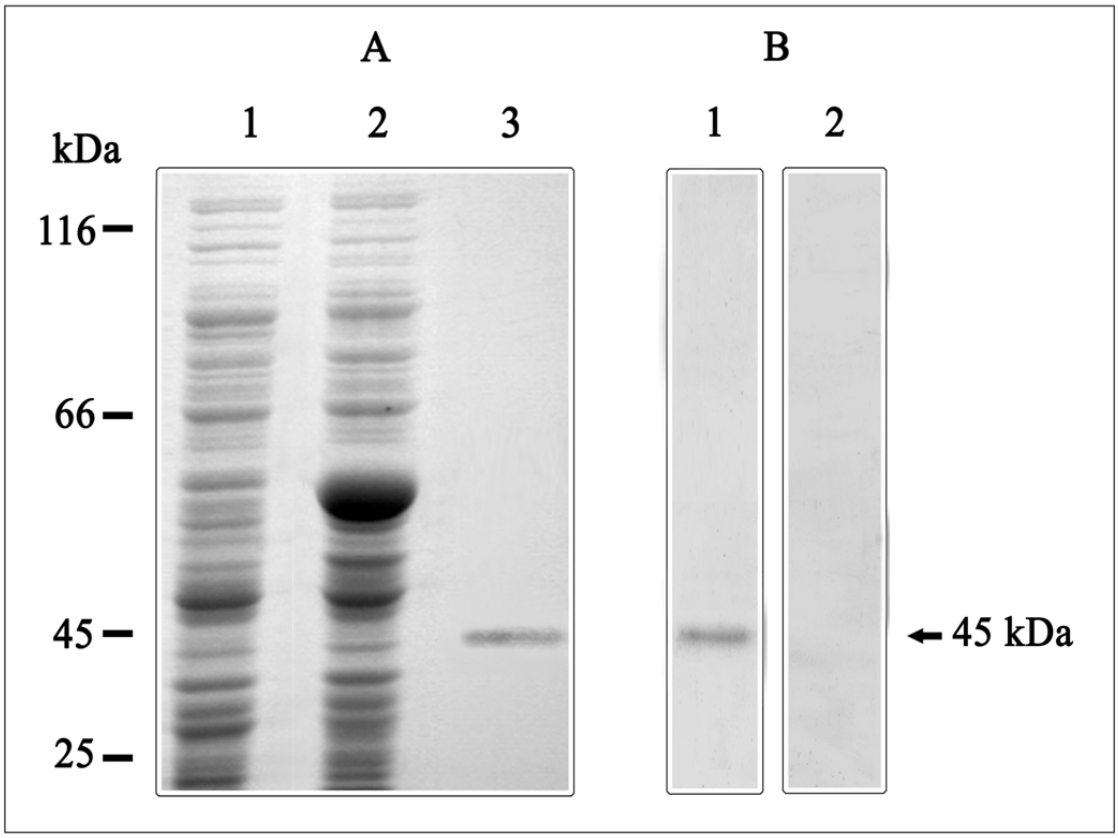


Fig. 02

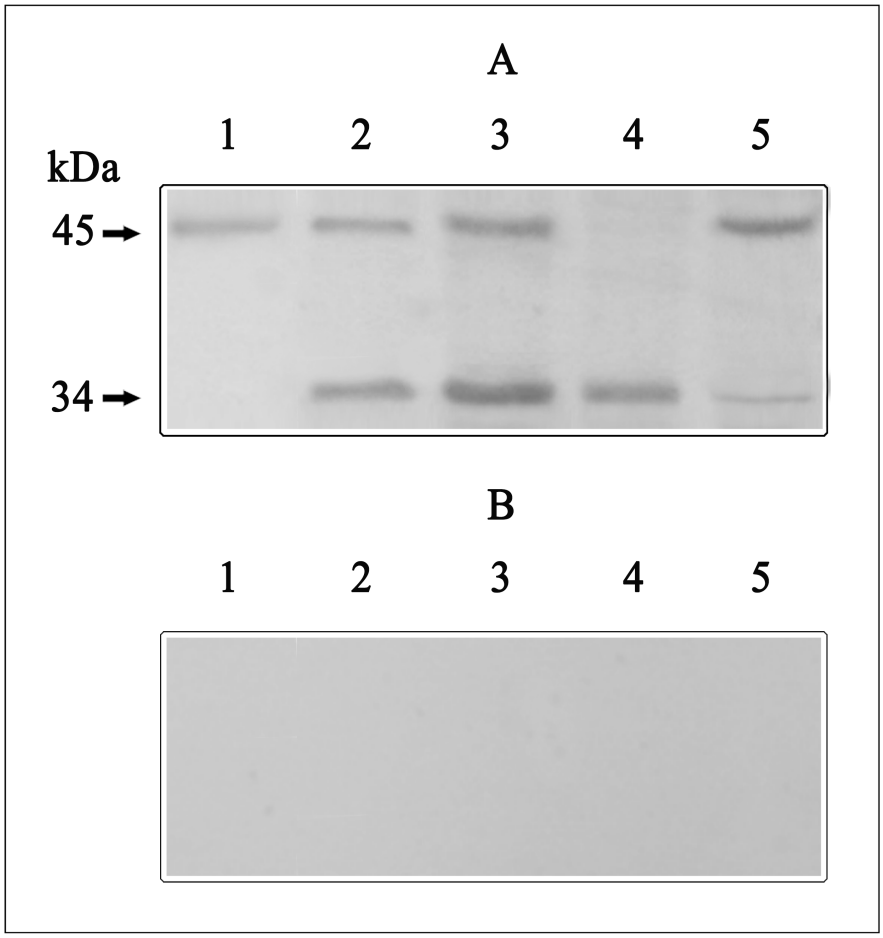
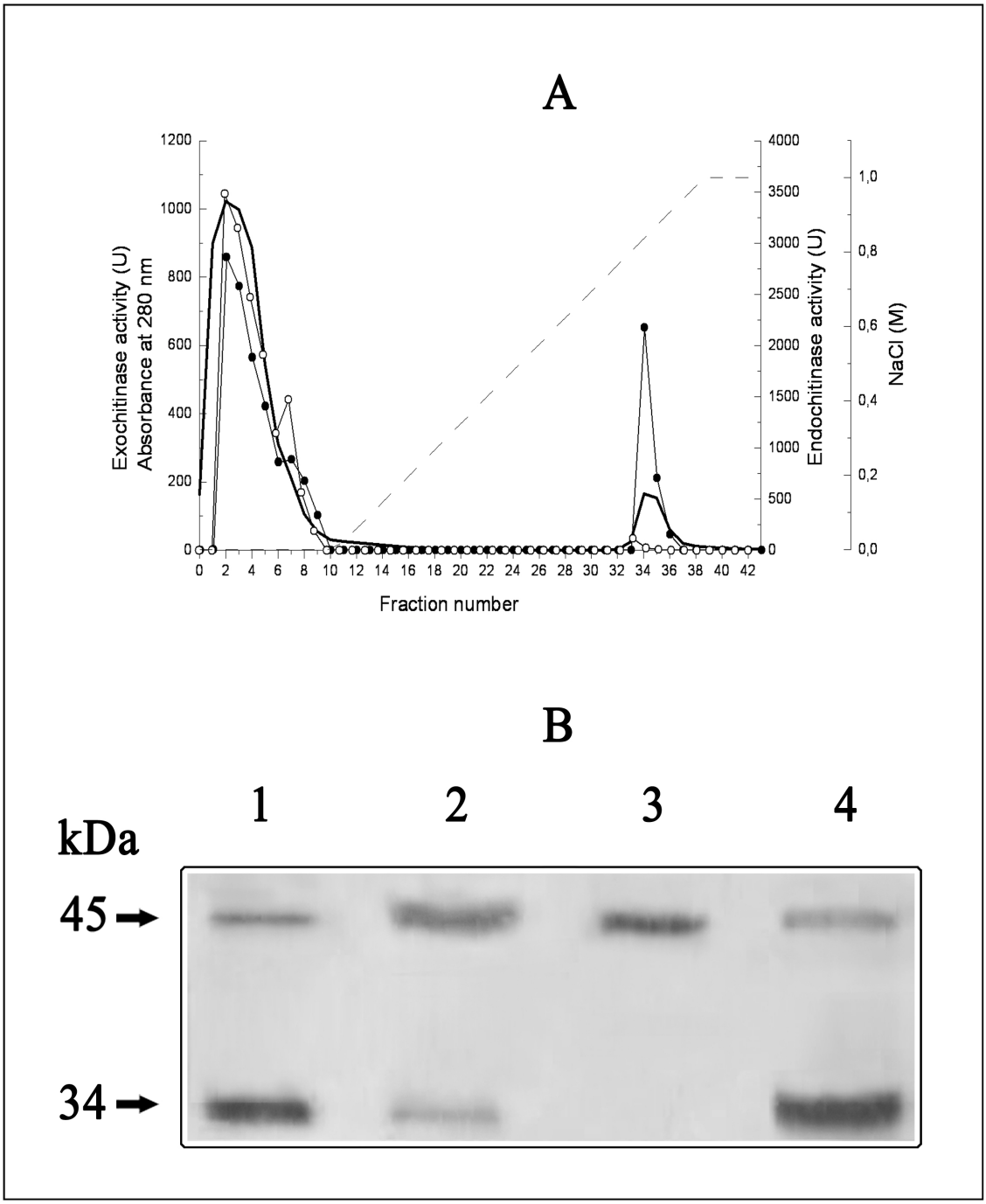


Fig. 03


A
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Absorbance at 280 nm
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NaCl (M)
Fraction number
B
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2
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45
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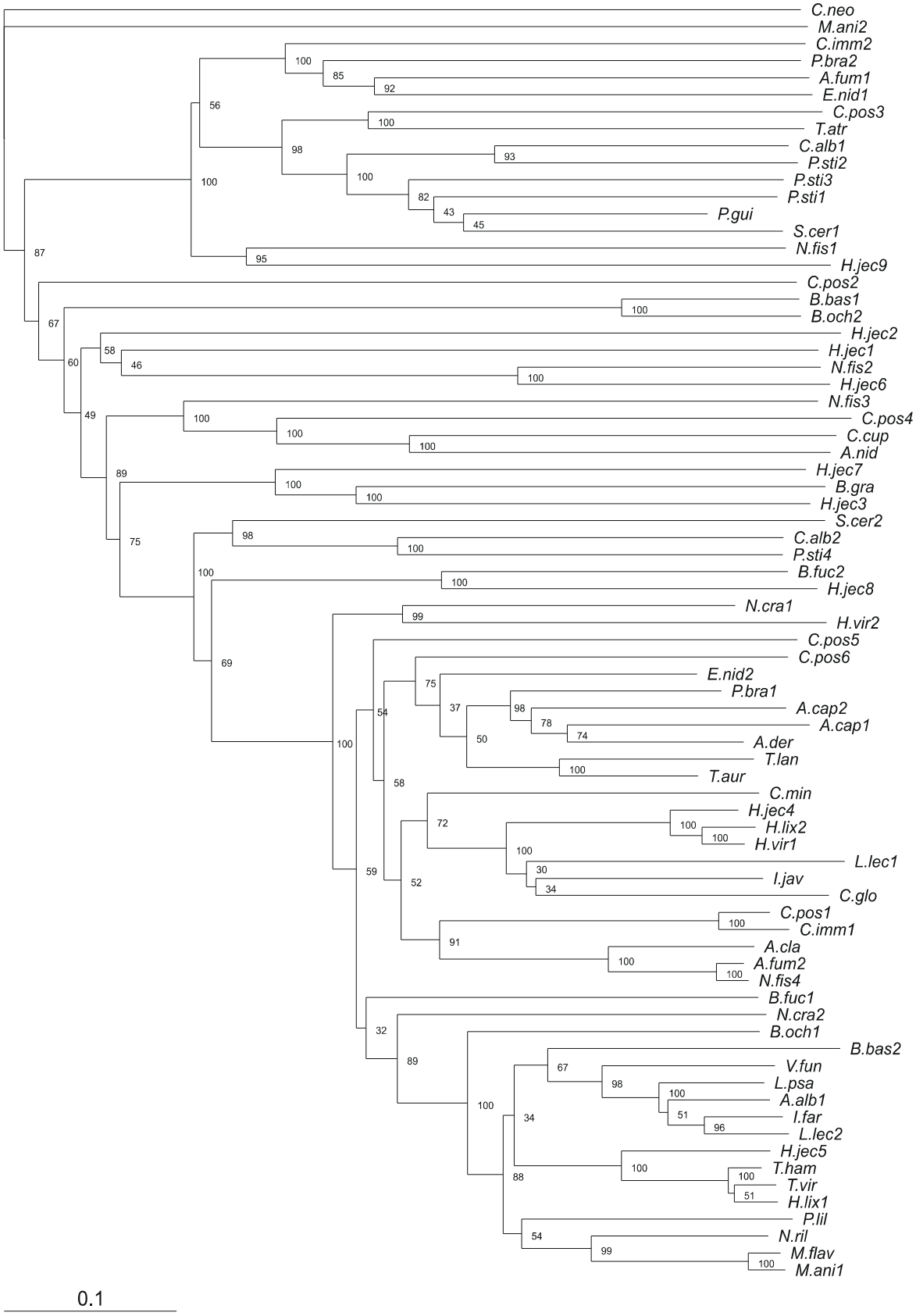
Fig. 04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C.pos5
C.pos6
E.nid2
P.bra1
A.cap2
A.cap1
A.der
T.lan
T.aur
C.min
H.jec4
H.lix2
H.vir1
L.lec1
I.jav
C.glo
C.pos1
C.imm1
A.cla
A.fum2
N.fis4
B.fuc1
N.cra2
B.och1
B.bas2
V.fun
L.psa
A.alb1
I.far
L.lec2
H.jec5
T.ham
T.vir
H.lix1
P.lil
N.ril
M.flav
M.ani1
0.1

CONCLUSÃO

IV – CONCLUSÃO

De acordo com o os experimentos realizados concluímos que:

● a proteína recombinante *Pbcts1* foi expressa em *E. coli* BL21 (D3)/plysS na forma de corpos de inclusão, os quais foram solubilizados pela adição de sarkosyl a 1%;

● através de experimentos de *western blot* com anticorpo policlonal produzido contra a *Pbcts1r*, foi observado que a *Pbcts1* é encontrada no extrato bruto de levedura, micélio e da transição de micélio para levedura. É encontrada ainda no meio de cultura de levedura, como uma proteína secretada, podendo estar associada ao processo patogênico. Dentre os extratos analisados a *Pbcts1* só não foi encontrada no extrato de parede celular, sugerindo não estar associada com a manutenção da fase leveduriforme;

● devido à presença de dois sinais positivos no ensaio de *western blot* e ainda à presença de dois picos de atividade de quitinase na purificação parcial do extrato bruto de levedura, conclui-se que *P. brasiliensis* apresenta pelo menos duas quitinases: *Pbcts1* e *Pbcts2*;

● *Pbcts1* apresenta atividade de endoquitinase, assim como *Pbcts1r*;

● *Pbcts2* apresenta atividade de exoquitinase sendo expressa nos extratos brutos de levedura e transição de micélio para levedura; mas não foi identificada no extrato de micélio. A *Pbcts2* ainda foi encontrada na parede celular e no meio de cultura de levedura. Assim, esta proteína poderia estar relacionada com o processo de dimorfismo

do fungo, manutenção da parede celular da fase leveduriforme, bem como no processo de patogênese.

PERSPECTIVAS

V – PERSPECTIVAS

A partir deste trabalho temos as seguintes perspectivas:

- Ensaios de adesão celular;
- Ensaios de inibição fúngica *in vitro*;
- Ensaios de atividade para amilase;
- Citolocalização de *Pbcts1* e *Pbcts2*;

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ANEXO

FEMS Microbiology Letters

Published on behalf of the Federation of European Microbiological Societies

Edited by:
Jeff Cole

Print ISSN: 0378-1097
Online ISSN: 1574-6968
Frequency: Bi-monthly
Current Volume: 290 / 2009
ISI Journal Citation Reports® Ranking: 2007: 48/94 (Microbiology)
Impact Factor: 2.274

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FEMS publishes five journals in the area of microbiology. All five journals follow the same instructions for manuscript preparation. If one journal has a different procedure, this will be mentioned at the appropriate place.

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All submitted papers should be complete in themselves and adequately supported by experimental detail; they should not be preliminary versions of communications to be published elsewhere. Descriptions of new methods are acceptable, and the Editors welcome papers that put forward new hypotheses. However, papers that provide confirmatory evidence or merely extend observations firmly established in one species or field site to another will not be accepted unless there are strong reasons for doing so. Members of the Editorial Boards and other appropriate experts will referee the papers. Editors handling papers will independently make decisions on acceptance, revision or rejection based on the referees' reports. The Chief Editors or Editors will usually reject papers outside the scope of the journal with an immediate decision. Authors who feel that there are substantial grounds for disagreement with an Editor's decision should contact the Chief Editor, whose decision will be final. Authors who wish to withdraw their manuscript (at any stage of the process) should contact their Editor.

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Authors should follow internationally accepted rules and conventions. Authors should provide evidence for the thorough identification of new isolates and use the most recent acceptable name.

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References

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Dinter Z & van Morein B (1990) *Virus Infections in Ruminants.* Elsevier, Amsterdam.

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Tang CR (2001) Cloning of a new ice nucleation active gene for insect pest control. PhD Thesis, Chinese Academy of Agricultural Sciences, Beijing.

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