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Caracterização Molecular e Expressão Heteróloga de um cDNA codificante para Tiorredoxina do fungo patogênico humano *Paracoccidioides brasiliensis*

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Sentir primeiro, pensar depois
Perdoar primeiro, julgar depois
Amar primeiro, educar depois
Esquecer primeiro, aprender depois
Libertar primeiro, ensinar depois
Alimentar primeiro, cantar depois
Possuir primeiro, contemplar depois
Agir primeiro, julgar depois
Navegar primeiro, aportar depois
Viver primeiro, morrer depois.

Mario Quintana

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SUMÁRIO

ABREVIATURAS E SÍMBOLOS

AP-1	Proteína Ativadora-1
ASK-1	Apoptose sinal- regulador Kinase1
AhpC	Alquil Hidroperóxido Redutase
cAMP	Adenosina Monofosfato cíclico
cAMP-PKA	Proteina Kinase dependente de cAMP
CcP	Citocromo-c peroxidase
cDNA	Ácido Dexorribonucléico Complementar
ClpB	Protease caseinolítica B
Cu^{+1}	Íon Cobre
DTT	Ditiotreitol
DNA	Ácido dexorribonucléico
EROS	Espécies Reativas de Oxigênio
EST	"Expression sequence tags" – etiquetas de seqüências expressas
Fe^{+2}	Íon Férrico
GR	Glutationa Redutase
GRX	Glutarredoxinas
GSH	Glutationa Reduzida
GSSG	Glutationa Oxidada
H_2O_2	Peróxido de Oxigênio
HO	Hidroxil
HSP	"Heat Shock Protein"- Proteína de choque térmico
IFN-γ	Interferon γ
IPTG	Isopropil-β-D-tiogalactopiranosídeo
kDa	Kilodalton
mM	Milimolar
NADPH	Nicotinamida Adenina Dinucleotídeo Fosfato
NF-KB	Fator Nuclear Kappa
NO	Óxido Nítrico
O_2	Oxigênio
O_2^-	Radical Superóxido

ºC	Graus centígrados
$ONOO^-$	Peroxinitrito
ORF	"Open Reading Frame" – Quadro de leitura aberta
PCM	Paracoccidioidomicose
PCR	"Polimerase Chain Reaction" – Reação em cadeia da polimerase
PDR	Proteína Dissulfeto Redutase
P*I*	Ponto Isoelétrico
PMN	Neutrófilos Polimorfos Nucleares
Prx	Peroxirredoxinas
PST-1	Sinais de Endereçamento aos Peroxissomos
RNA	Ácido Ribonucléico
ROO	Radical Peroxil
ROOH	Hidroperoxídeos
RT-PCR	Reação da transcriptase reversa e Reação em Cadeia da Polimerase
siRNA	Small Interfering RNA
SOD	Superóxido Dismutase
TFIIIC	Fator de transcrição IIIC
TNF-α	Fator de Necrose Tumoral α
TRR	Tiorredoxina Redutase
TRX	Tiorredoxina

RESUMO

O fungo termodimórfico, *Paracoccidioides brasiliensis*, é o agente etiológico da paracoccidioidomicose (PCM), uma micose sistêmica humana, com alta prevalência na América Latina. No hospedeiro humano, o fungo *P. brasiliensis* está sujeito a vários insultos, tais como o estresse oxidativo causado pelas espécies reativas de oxigênio, que são produzidas pelas células de defesa do hospedeiro durante a infecção. A tiorredoxina (TRX) é proteina redox intracelular que participa da manutenção da homeostase redox da célula, tanto em condições de estresse oxidativo quanto redutor. Neste trabalho apresentamos a caracterização de um cDNA de 811 pb, designado como *Pb*trx1, que codifica para uma proteína, *Pb*TRX1, de 116 resíduos de aminoácidos com massa molecular predita de 12 kDa e p*I* de 5,2. *Pb*TRX1 apresentou um motivo de sítio ativo conservado (WCGPC) entre as TRXs de vários organismos. Análise filogenética com *Pb*TRX1 e TRXs de outros organismos colocou *P. brasiliensis* no clado de fungos. Foi também realizada a predição da estrutura secundária da *Pb*TRX1, que apresentou um padrão característico formado por cadeias-β que estão envolvidas por α-hélices. Para obter a proteína recombinante, rec*Pb*TRX1, foi realizada a construção do pGEX-4T-3-trx1 e este foi introduzido nas células de *Escherichia coli*. Assim, a expressão e purificação da proteína recombinante foi obtida. A proteína rec*Pb*TRX1 e a *Pb*TRX1, presente no extrato protéico de células leveduriformes, apresentaram atividade redutora de insulina. Entretanto, a *Pb*TRX1 presente no extrato protéico de células leveduriformes tratadas com H_2O_2, mostrou maior atividade redutora de insulina quando comparada com extrato de células leveduriformes não tratadas. A *Pb*TRX1 foi detectada, por *Western blotting*, em extratos de células leveduriformes em crescimento e durante a transição de micélio para levedura. O crescimento das células leveduriformes foi inibido por H_2O_2, entretanto a transição de micélio para levedura foi pouco afetada por este oxidante. A técnica de RT-PCR semi-quantitativo foi empregada para análise da expressão do *Pb*trx1 em resposta ao H_2O_2. O nível de transcritos de *Pb*trx1 foi maior nas células leveduriformes tratadas com H_2O_2 do que nas células não tratadas. Para compreender como *P. brasiliensis* lida com estresse oxidativo é essencial entender os mecanismos envolvidos em sua sobrevivência no hospedeiro. É possível que *Pb*TRX1 aumente a sobrevivência de *P. brasiliensis* no hospedeiro, protegendo o fungo contra espécies reativas de oxigênio e, desta maneira, permitindo o progresso da infecção.

ABSTRACT

The temperature-dependent dimorphic fungus *Paracoccidioides brasiliensis* is the etiological agent of Paracoccidioidomycosis (PCM), a human systemic mycosis highly prevalent in countries of Latin America. *P. brasiliensis* is subjected to different insults from human host, such as oxidative stress caused by reactive oxygen species produced by the host during the infection. Thioredoxin (TRX) is an intracellular redox protein that is required to maintain redox homeostasis in response to both reductive and oxidative stress conditions in several organisms. We report here the characterization of a 811 bp cDNA *Pb*trx1, encoding a *Pb*TRX1 of 116 amino acids, with a predicted molecular mass of 12 kDa and p*I* 5.2. This putative protein presented one highly conserved active site motif (WCGPC) between TRXs from several organisms. The phylogenetic analysis performed with *Pb*TRX1 and TRXs from other organisms, putted *P. brasiliensis* in the fungi clade. We also performed the prediction of the secondary structure of *Pb*TRX1 that shows a pattern characteristic of the open twisted alpha/beta**,** similar to TRX secondary structures described in other fungus. In order to obtain the recombinant *Pb*TRX1, the expression construct pGEX-4T-3-trx1 was introduced into *Escherichia coli* cells and the expression and purification of the recombinant protein was obtained. The rec*Pb*TRX1 and *Pb*TRX1 from yeast cells extract were found to catalyze the reduction of insulin. However the *Pb*TRX1 from yeast cells extract treated with H_2O_2 showed highly insulin reduction activity than the yeast cells no treated. *Pb*TRX1 was detected by Western blotting in the extracts from yeast cells growth and from mycelium to yeast transition. The yeast cells growth was significantly inhibited by H_2O_2; however the mycelium to yeast transition was little affected by this oxidant. Semi-quantitative RT-PCR was employed to analysis the expression of *Pb*trx1 gene in response to H_2O_2. The level of *Pb*trx1 transcripts was higher in yeast cells treated with H_2O_2 than in yeast cells no treated. To realize how *P. brasiliensis* deals with oxidative stress is essential to understand the mechanisms involved in its survival in the host. It may be possible that *Pb*TRX1 enhances survival of *P. brasiliensis* in the host, protecting the fungus against the reactive oxygen species and allowing, in this way, the progress of the infection.

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I INTRODUÇÃO

I.1 -O FUNGO *Paracoccidioides brasiliensis*

I.1.1 - ASPECTOS GERAIS

Paracoccidioides brasiliensis, descrito em 1908 por Adolpho Lutz**,** é um fungo dimórfico, patógeno de humanos e agente etiológico da paracoccidioidomicose (PCM) (ALMEIDA, 1930). A PCM é uma importante micose da América Latina**,** representando um grave problema de saúde pública, principalmente no Brasil (LACAZ *et al*., 1991; BRUMMER *et al*., 1993; COUTINHO *et al*., 2002) com predominância nas regiões Sudeste e Centro-Oeste do país (CAMARGO *et al*., 2000). O fungo *P. brasiliensis* cresce como levedura a temperatura de 36 ^{o}C - 37 ^{o}C *in vitro* e no hospedeiro, e como micélio a 22 ^{o}C - 26 ^{o}C *in vitro* e no meio ambiente (CARBONELL e RODRIGUEZ, 1965).

I.1.2 - MORFOLOGIA

O fungo na sua forma miceliana, após 20 a 30 dias de incubação apresenta colônias pequenas, duras, planas, irregulares, de coloração esbranquiçada e muito aderidas ao meio (LACAZ, 1994; BRUMMER *et al*., 1993)**.** Ao microscópio óptico observam-se hifas finas e septadas com clamidósporos intercalados (FRANCO *et al*., 1989). Os clamidósporos são células resistentes que sobrevivem a condições ambientais adversas, como baixos níveis de nutrientes e de oxigênio.

No interior destas hifas encontram-se múltiplos núcleos e numerosas mitocôndrias. Os nucléolos não são visualizados e a cromatina é bastante fina, estando dispersa no nucleoplasma (SAMSONOFF *et al*., 1991). A forma miceliana quando observada por microscopia eletrônica, apresenta uma espessa parede celular, membrana plasmática e membrana basal bastante nítida (MINGUETTI *et al*., 1985). Em sua fase leveduriforme, as colônias, após um período de 7 a 10 dias de incubação, apresentam coloração cremosa e aspecto cerebriforme. Ao microscópio óptico, observam-se células ovais ou alongadas com múltiplos núcleos (2 a 5 por célula), onde apresentam nucléolo e cromatina evidentes

(LACAZ, 1994). MINGUETTI *et al*. (1985), ao analisar as células leveduriformes por microscopia eletrônica, observaram que a parede celular é refratária e espessa, a membrana basal não é visualizada e a membrana plasmática é facilmente identificada. A principal característica microscópica da forma leveduriforme é a sua aparência de ‘‘roda de leme’’, isto é, células mães com múltiplos brotamentos (ANGULO-ORTEGA e POLLAK, 1971; LACAZ *et al*., 1991).

I.1.3 – CLASSIFICAÇÃO TAXONÔMICA

P. brasiliensis apresenta a seguinte classificação taxonômica: reino Fungi; filo Ascomycota, sendo este baseado nas suas características moleculares; subdivisão Euascomycotina; classe Plectomyceto; subclasse Euascomycetidae; ordem Onygenales; família Onygenaceae; sub-família Onygenaceae Anamórficos; gênero *Paracoccidioides*, espécie única *brasiliensis*. Até o momento *P. brasiliensis* é um fungo mitospórico e não apresenta nenhuma forma teleomórfica (sexuada) conhecida (MARGULIS e SCHWARTZ, 1998; SAN BLAS *et al.*, 2002).

I.1.4 – HABITAT

O habitat natural de *P. brasiliensis* ainda não está bem definido (RESTREPO *et al*., 2001; BAGAGLI *et al*., 2006). Dados de isolamento de *P. brasiliensis* na natureza indicam que o fungo vive saprofiticamente em solos ricos em matérias orgânicas e nas superfícies de vegetais (LACAZ, 1949; MEDINA e BODZIAK, 1949). O fungo já foi isolado do solo através de fezes de morcego frugíforos (*Artibeus lituratus*), fezes de pinguim (*Pygoscelis adeliae*) (GROSE e TAMSITT, 1965; GEZUELE, 1989) e através de uma amostra comercial de ração canina, provavelmente contaminada com solo (FERREIRA *et al*., 1990). O fungo também foi isolado de tecidos de tatus (*Dasypus novemcinctus*) capturados nas áreas endêmicas de PCM (BAGAGLI *et al*., 1998; 2003).

Sabe-se que as regiões de maior prevalência do fungo apresentam clima temperado ou quente (média de 10-28 ºC), alta umidade, abundantes cursos de água, invernos curtos, verões chuvosos, solo quase sempre ácido e vegetação típica de cerrado (RESTREPO,

1985). Os locais que reúnem todas estas características são chamados de ‘‘reservarea’’, ou seja, são áreas que apresentam todos os fatores que contribuem para o desenvolvimento do fungo em seu habitat natural (BORELLI, 1964; RESTREPO-MORENO, 1994).

I.1.5 – CICLO BIOLÓGICO

O ciclo biológico de *P. brasiliensis* ainda não está totalmente elucidado, uma vez que seu habitat ainda é indefinido. Estudos mostram que o fungo ao ser cultivado em meios com substratos encontrados na natureza, é capaz de esporular com produção de conídios. A resistência destes esporos a condições ambientais adversas, sugere que a forma miceliana seja a forma infectante (RESTREPO, 1985).

Segundo FORJAZ (1989), as regiões tropicais do Brasil, no verão, possuem um clima quente, com chuvas abundantes e solos ricos em materiais orgânicos que fornecem nutrientes para o fungo se desenvolver na forma leveduriforme. Com a chegada do outono, a temperatura cai e os nutrientes do solo ficam escassos, fatores que favorecem a transformação das leveduras em hifas. Durante os meses de inverno, a umidade é baixa e faltam nutrientes apropriados para o desenvolvimento do fungo, forçando assim a produção de conídios como elementos de resistência e propagação. Na primavera, com a chegada das chuvas e época de derrubada da mata virgem e de preparo do solo para a agricultura, estes conídios são liberados formando aerossóis que são facilmente inalados pelo homem. A inalação destes propágulos pelo homem encerra o ciclo de vida saprófita do fungo, que, após instalar-se nos pulmões do hospedeiro, converte-se para a forma leveduriforme, iniciando a infecção (FRANCO, 1987; SAN-BLAS *et al.*, 2002).

CONTI-DIAZ e RILLA (1989), sugerem que a sobrevivência do fungo na natureza dependa da presença de espécies heterotérmicas servindo de reservatório do fungo até que o ciclo biológico se complete. Estudos realizados por BAGAGLI *et al*. (1998; 2006) demonstraram que os tatus podem apresentar infecção natural, sendo hospedeiros silvestres do fungo, mas não transmissores da doença para o homem (RIVITTI e AOKI, 1999).

I.1.6 – DIMORFISMO

Fungos dimórficos como *P. brasiliensis, Histoplasma capsulatum, Candida albicans, Coccidioides immitis*, são capazes de transitar entre duas formas morfológicas em respostas a estímulos ambientais. A capacidade destes organismos transitarem entre duas formas favorece à instalação do fungo no tecido do hospedeiro humano, auxiliando na invasão do organismo (VILLAR *et al*., 1988).

A temperatura é um dos estímulos mais notórios do dimorfismo do fungo *P. brasiliensis*, que cresce na forma de micélio, forma infectante, à 23 ºC - 26 ºC e como levedura a 35 ºC - 37 ºC nos tecidos do hospedeiro (KANETSUNA *et al.,* 1972). O dimorfismo em *P. brasiliensis* pode ser induzido *in vitro* por mudança de temperatura. A transição de micélio para levedura é obtida por incubação das formas micelianas à 36 ºC - 37 ºC e o processo inverso, de levedura para micélio, por incubação das células leveduriformes à 22 ºC - 26 ºC. Experimentos de diferenciação celular com o isolado *Pb* 01 de *P. brasiliensis* evidenciaram que, ao final de 18-20 dias, aproximadamente 95% das formas micelianas se diferenciaram em leveduriformes, enquanto que o processo reverso, se completa após 15 dias do início da incubação (SILVA *et al*., 1994; MATTAR-FILHO *et al*., 1997).

Aspectos bioquímicos, estruturais e morfológicos relacionados ao dimorfismo em *P. brasiliensis* são descritos por vários autores. Morfologicamente a transição de levedura para micélio é caracterizada *in vitro* pela formação de brotos alongados, multinucleados, em forma de pêra, os quais, gradativamente vão assumindo o aspecto de hifa (BARTNICKI-GARCIA *et al*., 1995). Na diferenciação, *in vitro*, de micélio para levedura, surgem estruturas arredondadas que se dividem por brotamento (SAN-BLAS, 1982).

Outro importante aspecto com relação ao dimorfismo é observado com relação à composição da parede celular do fungo. Nas formas micelianas há o predomínio da β-1,3 glucana, enquanto que, na forma leveduriforme, o principal polissacarídeo é α-1,3 glucana. Estudos realizados a partir do isolamento de *P. brasiliensis* sugerem que a α-1,3 glucana protege o fungo contra o ataque de enzimas digestivas de leucócitos e macrófagos no tecido infectado (KUROKAWA *et al*., 1998). SAN-BLAS e SAN-BLAS (1982) sugerem que fagócitos humanos poderiam produzir β-glucanase com a capacidade de digerir somente β-1,3 glucana, presente na parede celular dos fungos da forma miceliana. Entretanto, a transformação do fungo para a forma leveduriforme logo no início da infecção protegeria o

patógeno contra a ação das enzimas fagocitárias, possibilitando a instalação do fungo no hospedeiro.

Fatores hormonais também têm sido relacionados ao dimorfismo de *P. brasiliensis*. Pesquisas sobre a ação de esteróides neste fungo mostram que o hormônio 17-β-Estradiol inibe a transição da forma miceliana para leveduriforme, tanto *in vitro* de maneira dose-dependente (RESTREPO *et al*., 1984) como *in vivo* (SANO *et al*., 1999). Possivelmente este hormônio seja um fator de proteção à infecção nas mulheres. Análises do perfil protéico demonstraram que o 17-β-Estradiol retarda ou bloqueia a expressão de algumas proteínas durante a transição de micélio para levedura, sugerindo que o hormônio atue regulando a expressão de proteínas, provavelmente por mediação de um complexo proteína-receptor (CLEMONS *et al*., 1989). Estudos recentes sobre o transcriptoma de *P. brasiliensis* mostraram a expressão de um gene que possivelmente codifica para uma proteína de 60 kDa presente no citosol das células leveduriformes, que se liga ao hormônio sexual feminino, 17 β - estradiol, como discutido anteriormente por LOOSE *et al*. (1983) (FELIPE *et al*., 2005).

Foram também realizadas análises do padrão de proteínas durante a transição dimórfica do fungo. Segundo KANETSUNA *et al.* (1972) a proteína dissulfeto redutase (PDR) apresenta maior atividade na fase leveduriforme, sendo responsável pelo rompimento dos grupos dissulfetos das proteínas da parede celular. Tal processo gera proteínas de cadeia curta e conseqüentemente, proporciona a forma arredondada característica de levedura. Em contrapartida, a baixa atividade da PDR gera proteínas de cadeia longa na forma miceliana.

As vias de sinalização envolvidas na transição dimórfica de *P. brasiliensis* têm sido muito pouco estudadas. Estudos realizados por PARIS e DURAN (1985), demonstraram que a via de transdução de sinal cAMP-PKA participa da transição dimórfica de *P. brasiliensis*, pois foi observado aumentos na concentração de cAMP durante a transição de micélio para levedura. Posteriormente, BORGES-WALMSLEY e WALMSLEY (2000) demonstraram que a adição de cAMP exógeno inibi a transição de levedura para micélio em *P. brasiliensis*. Outra via que também tem sido descrita como sendo importante no dimorfismo de *P. brasiliensis* é a via de transdução de sinal Ca^{+2}/Calmodulina, pois estudos realizados com bloqueadores de quinases dependentes de Ca^{+2}/Calmodulina demostraram inibição da transição de micélio para levedura (CARVALHO *et al*, 2003).

Estudos realizados com alguns genes clonados e caracterizados de *P. brasiliensis*, demonstraram expressão diferencial destes genes durante a transição. Dentre outros, os genes codificantes para as proteínas de choque térmico (HSP70) (SILVA *et al*.,

1999), HSP60 (SALEM-IZACC *et al*., 2001; CUNHA *et al*., 2002), a proteinase ClpB (JESUINO *et al*., 2003) e manosiltransferase (COSTA *et al*., 2002), catalases (MOREIRA *et al*., 2004), gliceraldeído-3-fosfato desidrogenase (BARBOSA *et al*., 2004) são mais expressos na forma leveduriforme, quando comparados a forma miceliana. Estes estudos sugerem que estas proteínas sejam necessárias para sobrevida de *P. brasiliensis* nas condições térmicas do hospedeiro e que possam ser importantes na morfogênese do fungo.

I.2 – PARACOCCIDIOIDOMICOSE

I.2.1 – HISTÓRICO E EPIDEMIOLOGIA

Paracoccidioidomicose (PCM) é uma micose sistêmica granulomatosa, aguda ou crônica, causada pelo fungo *P. brasiliensis*. A doença foi descrita pela primeira vez em 1908 por Adolpho Lutz, em São Paulo, com relatos das características do agente etiológico. Posteriormente, Alfonso Splendore e Floriano Paulo de Almeida, caracterizaram biológica e morfologicamente o fungo. Assim a doença passou também a ser conhecida como moléstia de Lutz-Splendore-Almeida (LACAZ, 1956). Em 1971 foi consagrado o termo paracoccidioidomicose para denominação da doença (HAMDAN e ROCHA, 1987).

Atualmente, sabe-se que a doença é endêmica na América Latina, principalmente no Brasil, na Argentina, na Venezuela e na Colômbia, apresentando aproximadamente 10 milhões de indivíduos infectados. Destes, 2% desenvolvem a doença (RESTREPO *et al*., 2001).

O Brasil apresenta a maior estatística de casos de PCM, distribuídos principalmente nos estados de São Paulo, Rio de Janeiro, Minas Gerais, Paraná, Rio Grande do Sul, Goiás e Mato Grosso do Sul (WANKE e LONDERO, 1994).

A moléstia acomete preferencialmente trabalhadores que estão em contato com o solo, como os agricultores, do sexo masculino, entre 30 a 60 anos de idade (SVIDZINSKI *et al*., 1999; VILLAR *et al*., 2000). Esse fato pode ser explicado pela ação protetora dos hormônios estrógenos à mulher adulta (CLEMONS *et al*., 1990, SANO *et al*., 1999) e pelo seu menor contato com as fontes de infecção (MARQUES *et al*., 1983).

I.2.2 – PATOGENIA

Estudos clínicos e laboratoriais demonstram que a via inalatória é a porta de entrada de *P. brasiliensis* no hospedeiro humano (PEDROSA, 1976).

Os propágulos, produzidos pela forma miceliana, ao serem inalados pelo homem atingem os alvéolos pulmonares e se convertem na forma leveduriforme, que se multiplica por brotamento, formando assim o foco inflamatório (MCEWEN *et al*., 1987). Através das vias de disseminação linfática e hematogênica, o fungo pode alcançar diversos órgãos e sistemas do organismo hospedeiro (CAMARGO *et al*., 2000).

A evolução da infecção para doença depende da virulência do agente e da capacidade de resposta imunológica do hospedeiro, em especial da imunidade celular (MONTENEGRO e FRANCO, 1994).

A doença é classificada clinicamente como regressiva, progressiva ou seqüelar (MENDES, 1997). A forma progressiva pode ser aguda ou subaguda (tipo juvenil) ou crônica (tipo adulto). A forma juvenil acomete crianças e adultos jovens (BRUMMER *et al*., 1993), apresentando envolvimento de órgãos como baço, fígado, linfonodos entre outros órgãos (MONTENEGRO e FRANCO, 1994; RIVITTI e AOKI, 1999).

A forma crônica da PCM é a mais comum, manifestando-se a partir da terceira década, comprometendo os pulmões, os linfonodos, as mucosas orais e nasais, a pele e os ganglios linfáticos. Ocasionalmente compromete outros órgãos, como o sistema nervoso central (VILLAR *et al*., 2000; ALMEIDA; 2004).

A forma seqüelar se caracteriza pelas manifestações clínicas relacionadas à fibrose cicatricial acompanhada de dispnéia e restrições cardiopulmonares (BRUMMER *et al*., 1993).

I.3 - ESTRESSE OXIDATIVO

ZHANG *et al.* (2003) definiram estresse oxidativo como o desequilíbrio no estado redox celular que favorece um estado oxidado. O estresse oxidativo difere dos outros tipos de estresse por causa de seus efetores primários, as espécies reativas de oxigênio (EROS).

EROS, incluindo espécies como ânion superóxido (O_2), peróxido de hidrogênio (H_2O_2) e radical hidroxila (HO), consistem em produtos intermediários da redução do oxigênio molecular (FRIDOVICH, 1999). As espécies radicalares, ânion superóxido e hidroxila possuem um elétron desemparelhado em sua última camada de distribuição eletrônica, sendo extremamente instáveis. Já o peróxido de hidrogênio, que não é um radical livre, é relativamente estável, sendo facilmente difusível. Estas espécies citadas são chamadas oxidantes primários e podem gerar radicais de oxigênio secundários como o radical peroxil (ROO), os hidroperóxidos (ROOH), os epóxidos ou aldeídos, bem como o peroxinitrito ($ONOO^-$), um produto obtido por meio da reação entre o superóxido e o monóxido de nitrogênio (BECKMAN e KOPPENOL, 1996; SIGLER *et al*, 1999).

EROS são geradas inevitavelmente em células que se desenvolvem em ambientes aeróbicos, como subproduto de processos metabólicos normais que ocorrem na mitocôndria (respiração) e nos peroxissomos (β-oxidação de ácidos graxos), bem como em sistemas enzimáticos no citoplasma (lipoxigenases, NADPH, oxidases e citocromo P_{450}). Estudos mostram que a mitocôndria é responsável pela maior produção endógena de EROS nas células (BOVERIS e CHANCE, 1973; HALLYWELL e GUTTERIDGE, 1989). Cerca de 1-4% do oxigênio consumido pela mitocôndria são reduzidos incompletamente pela cadeia de transporte de elétrons, gerando EROS (KWON *et al*., 2003; FRIDOVICH, 1998). Esta reação ocorre a partir de dois pontos da cadeia de transporte de elétrons, Complexos I (NADPH desidrogenase) e III (ubiquinona citocromo c óxido-redutase) em que transferências monoeletrônicas ao oxigênio molecular levam à formação de radical ânion superóxido. Este radical por meio da ação da enzima superóxido dismutase (SOD), ou espontaneamente, forma o peróxido de hidrogênio. Na presença das formas reduzidas dos íons Fe^{+2} ou Cu^{+1}, o peróxido de hidrogênio sofre fissão heterolítica gerando o radical hidroxila, reação conhecida por reação de Fenton (SIGLER *et al*., 1999; PUIG *et al*., 2002).

Além da geração intracelular de EROS, agentes externos também são responsáveis por sua formação, tais como luz ultravioleta, radiação ionizante, agentes quimioterápicos, citocinas inflamatórias e certas toxinas (FINKEL e HOLBROOK, 2000).

I.3.1 – EFEITOS DO ESTRESSE OXIDATIVO EM SISTEMAS BIOLÓGICOS

Os efeitos de EROS em sistemas biológicos têm sido amplamente estudados, demonstrando seus efeitos deletérios, aleatórios e cumulativos sobre as biomoléculas (DNA, lipídeos e proteínas). Observa-se, ainda, seus efeitos em processos degenerativos como envelhecimento, bem como no desenvolvimento de patologias (arteriosclerose, doenças neurodegenerativas, diabete, alergia, câncer e artrite) (HALLYWELL e GUTTERIDGE, 1989; MATÉS, 1999).

EROS podem levar à formação de ligações cruzadas nas proteínas, causando oxidação destas e também a oxidação das cadeias laterais de resíduos de aminoácidos (BERLETT e STADTMAN, 1997). Estudos mostram que algumas enzimas são inativadas ao sofrerem oxidação dos grupos tióis de resíduos de cisteína nos sítios ativos. A oxidação destes grupos leva à geração de pontes de dissulfeto entre resíduos de cisteína da proteína (IMLAY, 2003). Resíduos de metionina também são sensíveis à oxidação por EROS, formando sulfóxidos de metionina (BERLETT e STADTMAN, 1997).

Estruturas contendo lipídios, ao sofrerem danos oxidativos, levam à produção de lipoperóxidos, processo conhecido por peroxidação lipídica. Esses lipoperóxidos podem alterar a estrutura e a função das membranas celulares (GIROTTI, 1998).

A oxidação de ácidos nucléicos gera lesões nas bases nitrogenadas e danos nas unidades de açúcar (desoxirribose), resultando na quebra das fitas do DNA (SIGLER *et al*., 1999). Estas lesões são mutagênicas e causam instabilidade genômica (GARCIA *et al*., 2000).

Outros estudos mostram que EROS também estão associadas a algumas funções fisiológicas essenciais para um organismo vivo, como a regulação da proliferação celular e a geração de EROS por células fagocíticas promovendo a defesa do hospedeiro no combate às infecções (FINKEL e HOLBROOK, 2000; GALARIS *et al*., 2002). Estudos em *P. brasiliensis* mostram que a produção de IFN-γ e TNF-α por macrófagos induzem a síntese

de óxido nítrico (ON). Este, por sua vez, inibe a transformação de conídios para a forma leveduriforme, causando a morte deste fungo (GONZALES *et al*., 2000).

Outro papel fisiológico importante de EROS é o de mensageiro celular, regulando o metabolismo (NEMOTO *et al*., 2000), o ciclo celular e a expressão de genes, bem como a participação destes nas vias de transdução de sinal (ALLEN e TRESINI, 2000; VIVANCOS *et al*., 2004). Desta forma, pode-se observar que os efeitos benéficos e deletérios de EROS exercem influências em diversos processos biológicos e na integridade celular.

I.3.2 - DEFESAS ANTIOXIDANTES

Os organismos aeróbicos têm desenvolvido sistema de defesa contra EROS por meio da síntese de enzimas oxidantes, como catalase, peroxidase e superóxido dismutase (SOD). Este sistema de defesa contra EROS ocorre, ainda, por meio de moléculas não enzimáticas de baixo peso molecular, a exemplo das moléculas lipofílicas, como α-tocoferol (vitamina E), carotenos (incluindo vitamina A), flavonóides e antioxidantes hidrofílicos como a glutationa (GSH) e a tiorredoxina (TRX) (HALLYWELL e GUTTERIDGE, 1989; STIPANUK, 2000).

I.3.3 – GLUTATIONA

A glutationa (GSH, γ-L-glutamil-L-cistenilglicina) é um tripeptídeo formado por cisteína, glutamato e glicina, de baixo peso molecular, contendo grupo tiol, sendo encontrada em abundância nas células dos organismos vivos. A GSH está envolvida na proteção contra estresse oxidativo, bem como na detoxificação de EROS e xenobióticos, no transporte de aminoácidos, na síntese de proteínas e ácidos nucléicos e na modulação da atividade enzimática (SIGLER *et al*., 1999).

O núcleo do resíduo cistenilglicina da glutationa está diretamente envolvido na função desta proteína como antioxidante, mais especificamente como um redutor intracelular, sendo capaz, por exemplo, de reagir com um radical livre, formando um radical GS·, que produz, por dimerização, a glutationa oxidada (GSSG). Esta é, então, convertida novamente à forma reduzida (GSH) pela glutationa redutase (GR), em um processo dependente do NADPH

(KRETZSCHMAR, 1996; SIGLER *et al*., 1999). A glutationa redutase tem o NADPH como substrato, em consequência, a disponibilidade limitada deste último pode levar a um aumento da GSSG, de forma a deixar as células mais sensíveis ao dano oxidativo (SHAN *et al*, 1990).

A GSH também é usada como substrato pela glutationa peroxidase na eliminação de peróxidos. Desta forma, estudos realizados com leveduras de *Saccharomyces cerevisiae* mostram que a GSH é um antioxidante muito importante contra os efeitos nocivos de H_2O_2 neste organismo (GRANT *et al*., 1998). CAMPOS *et al*. (2005) mostraram que em células de *P. brasiliensis* também são encontrados genes que estão envolvidos na biosíntese da GSH e na regeneração de NADPH.

I.3.4 – CATALASE

Catalases são enzimas que apresentam um átomo de ferro ou manganês ligado a porfirina e promovem a catálise de H_2O_2, degradando-o em água e oxigênio.

As catalases podem ser divididas em três grupos de acordo com suas similaridades funcionais e estruturais. Catalases manganês, também conhecidas por pseudocatalases, possuem o manganês ligado ao grupo heme; esta classe está presente somente nos procariotos. O segundo grupo compreende as catalases peroxidases ou catalases bifuncionais, que tem como função a degradação de peróxido de hidrogênio e de outras substâncias oxidativas como álcool e fenol, sendo encontradas em procariotos e eucariotos inferiores. Por fim, o terceiro grupo identificado em organismos procarióticos e eucarióticos é denominado de catalases monofuncionais, sendo conhecidas também por catalases ‘‘verdadeiras’’ (ZAMOCKY e KOLLER, 1999; ZAMOCKY *et al*., 2000).

Tem sido demonstrado *in vitro* que o H_2O_2 produzido pelos neutrófilos polimorfos nucleares (PMN) apresenta atividade fungicida e capacidade de restringir o crescimento ou mesmo causar a morte de *P. brasiliensis* (MCEWEN *et al*., 1984; MELONI-BRUNERI *et al*., 1996) e de *Aspergillus fumigatus* (CALERA *et al*., 1997). Assim, catalases produzidas por microrganismos patogênicos, plantas e animais têm combatido os mecanismos oxidativos das células de defesa do hospedeiro e, consequentemete, são identificadas como fatores de virulência (XU e PAN, 2000; JOHNSON *et al*., 2002). WYSONG *et al.* (1998) mostraram que a deleção do gene da catalase em *C. albicans* levaria à morte deste organismo pelos leucócitos e, consequentemente, à diminuição da virulência deste patógeno *in vivo*.

Em *A. fumigatus* a catalase também exerce um importante papel na sua patogenicidade. Neste organismo existem 3 catalases ativas, sendo que uma está presente nos conídios e as outras duas estão presentes no micélio, uma pertence ao grupo das catalases monufuncional e a outra pertence ao grupo das catalases peroxidase (PARIS *et al*., 2003).

DÍAZ *et al*. (2001) observaram em *Neurospora crassa* um aumento significativo da catalase 1 durante a formação de esporos assexuados. Esta catalase mostrou ser resistente a inativação causada pela temperatura e por vários desnaturantes, fato importante para a sobrevivência dos esporos deste organismo (HANSBERG, 1996).

Estudos recentes demonstram o isolamento da catalase peroxissomal de *P. brasiliensis*, com motivos de subunidades pequenas das catalases monofuncionais. Esta enzima apresenta sinais de endereçamento aos peroxissomos (PST-1), indicando sua localização nos peroxissomos. Observou-se um aumento no nível desta catalase peroxissomal durante a transição da forma miceliana para leveduriforme, sendo esta mais expressa em leveduras. As formas leveduriformes quando expostas à H_2O_2 tiveram uma maior indução da expressão desta catalase (MOREIRA *et al*., 2004).

I.3.5 – PEROXIDASES

Peroxirredoxinas (Prx) pertencem a uma família de enzimas antioxidantes presentes em todos os organismos, sendo capazes de reduzir hidroperóxidos em uma reação mediada por substrato doador de elétrons contendo tiol (NETTO *et al*., 1996).

Estas enzimas podem ser divididas em 4 grupos. O grupo que possue 2 cisteínas (2-cis-prx) conservadas, sendo que uma destas cisteínas é essencial para a decomposição de peróxidos. O resíduo de cisteína responsável pela atividade enzimática está localizado na região amino-terminal. Em contrapartida a outra cisteína encontra-se na região carboxi- terminal, sendo importante na interação com o sistema tiorredoxina (RHEE *et al*., 1999). Peroxirredoxinas que utilizam tiorredoxina como substrato doador de elétrons são chamadas de tiorredoxina peroxidases (CHAE *et al*., 1994 e WOOD *et al*., 2003).

O segundo grupo de peroxirredoxinas compreende proteínas com apenas 1 resíduo de cisteína conservado (1-cis-prx), situado na região amino-terminal. Observa-se alta similaridade entre 2-cis-prx e 1-cis-prx. O terceiro grupo destas proteínas são denominadas peroxirredoxinas do tipo II (tipo II prx), contendo 2 cisteínas situadas na região amino-

terminal, com baixa similaridade com os outros grupos apresentados. Por fim, o quarto grupo é formado por proteínas com 2 cisteínas conservadas na região amino-terminal que são fundamentais para atividade enzimática, formando uma ponte de dissulfeto intramolecular (PARK *et al*., 2000). Estudos relacionados ao tanscriptoma de *P. brasiliensis* têm identificado a presença desta enzima antioxidante neste organismo (FELIPE *et al*., 2003; CAMPOS *et al*., 2005).

Outra enzima antioxidante bastante estudada é a citocromo-c peroxidase (CcP), que protege a célula catalisando a redução do H_2O_2 a H_2O, utilizando o ferrocitocromo-c como doador de elétrons (ERMAN e VITELLO, 2002). CAMPOS *et al*. (2005) mostraram que esta enzima está presente em células de *P. brasiliensis*, sendo indicativo de um mecanismo a mais de detoxificação contra o H_2O_2 em relação às células do hospedeiro humano, uma vez que a CcP não está presente em mamíferos.

I.3.6 – TIORREDOXINA

Tiorredoxina (TRX) é uma proteína pequena de baixo peso molecular (12-13 kDa) ubiquitária e multifuncional. Esta proteína participa da manutenção da homeostase redox da célula nas condições de estresse oxidativo e redutor (CARMEL-HAREL e STORZ, 2000; GRANT, 2001).

O sistema tiorredoxina citosólico é formado por tiorredoxina, tiorredoxina redutase e tiorredoxina-NADPH-dependente, sendo que as duas primeiras são responsáveis por manterem o estado redox da célula. As TRXs contêm uma seqüência muito conservada entre organismos diferentes (Cys-Gly-Pro-Cys), sendo este o seu sítio ativo dissulfeto/ditiol redox. Este sítio ativo é essencial para a função oxirredutase dissulfeto desta proteína (HOLMGREN, 1989). Geralmente, as TRXs clássicas apresentam estrutura tridimensional muito conservada entre os mais diversos organismos estudados, principalmente com relação ao posicionamento na superfície da molécula dos resíduos de cisteína do sítio ativo (GLEASON e HOLMGREN, 1988). A estrutura comum estabelecida para TRX1 é formada por 5 cadeias-β que estão envolvidas por 4 α-hélices (JENG *et al*., 1994).

As TRXs têm sido descritas em organismos procarióticos e eucarióticos, tendo sido inicialmente caracterizadas em *Escherichia coli* como doadoras de hidrogênio para ribonucleotídio redutase (LAURENT *et a*l., 1964). Posteriormente outros estudos mostram

que estas proteínas também atuam como doadoras de hidrogênio para as peroxirredoxinas que reduzem H_2O_2 (CHAE *et al*., 1994) e para a sulfóxido metionina redutase, que reativam proteínas danificadas pelo estresse oxidativo (FERNANDO *et al*., 1992). Sendo também necessárias à outras enzimas metabólicas que formam pontes de dissulfeto como parte de seu ciclo catalítico.

As TRXs eucarióticas exercem várias funções fisiológicas. Estas proteínas podem estar relacionadas com o processo da fotossíntese nos cloroplastos das plantas (BUCHANAN, 1991). As TRXs também podem modular a atividade de ligação de alguns fatores de transcrição como TFIIIC (CROMLISH e ROEDER, 1989), NF-KB (MATTHEWS *et al*., 1992), e AP-1 (HIROTA *et al*., 1997). Foi descrito que as TRXs participam no controle da apoptose celular, assim como na defesa contra agentes causadores de estresse oxidativo, como por exemplo a capacidade preventiva contra danos celulares causados por óxidos nítricos em células macrofagocitárias (FERRET *et al*., 2000). Outros estudos também relataram um importante papel das TRXs como oncogenes putativos, conferindo vantagens ao crescimento e sobrevida das células tumorais (GROGAN *et al*., 2000).

As TRXs doam equivalentes redutores para várias proteínas, tornam-se assim oxidadas e logo, são reduzidas pela tiorredoxina redutase (TRR) (NORDBERG e ARNÉR, 2001). A TRR utiliza NADPH para reduzir pontes de dissulfeto da tiorredoxina oxidada e de outras proteínas alvos, auxiliando assim na via de enovelamento das mesmas (HOLMGREN, 1989). Assim, substratos como o ácido desidroascórbico, hidroperóxido de lipídios, glutationa peroxidase, H_2O_2, glutationa oxidada, peroxirredoxinas e outras proteínas oxidadas também podem ser reduzidas pela TRR (ZHONG e HOLMGREN, 2000).

A expressão da TRX é induzida pelo estresse oxidativo, através da ativação dos elementos de resposta a oxidantes, que estão presentes na região promotora dos genes que codificam para TRXs. Este fato resulta na redução de biomoléculas e funciona como uma defesa antioxidante adicional (NORDBERG e ARNÉR, 2001).

Em organismos fotossintéticos, têm sido identificadas três tipos de TRXs. Destas, duas isoformas estão localizadas no cloroplasto (f e m) e estão envolvidas em sistemas regulatórios no processo de fotossíntese. Enquanto uma outra isoforma está localizada no citosol e no retículo endoplasmático (h) (BESSE e BUCHANAN, 1997).

Alguns organismos eucarióticos também apresentam mais de uma TRX, como ocorre por exemplo em leveduras (Gan, 1991; Pedrajas *et al*., 1999).

Estudos realizados em leveduras de *S. cerevisiae* demonstraram a presença de duas TRXs citoplasmáticas (TRX1 e TRX2) e uma mitocondrial (TRX3), estando estas relacionadas com a defesa contra EROS (GRANT, 2001). As TRXs citoplasmáticas são funcionalmente independentes da TRX mitocondrial (TROTTER e GRANT, 2005).

O papel desempenhado pelas TRXs em resposta ao estresse oxidativo induzido por H_2O_2 em *S. cerevisiae*, foi muito bem estudado por GARRIDO e GRANT (2002). Eles usaram múltiplos mutantes para os genes TRX1 e TRX2, assim como para as glutarredoxinas (GRX1 e GRX2), para avaliar qual o real papel dos correspondentes genes na resposta ao estresse gerado por peróxido neste organismo. Os resultados demostraram que a atividade antioxidante da TRX é importante tanto para sobrevida das leveduras, bem como para protegê-las contra o estresse oxidativo, principalmente na fase de crescimento estacionário, onde ocorre um aumento concomitante da expressão das duas TRXs. Eles também observaram que as TRXs são mais importantes do que as glutarorredoxinas na resposta ao estresse causado por peróxidos.

Outros estudos relacionados ao estresse oxidativo foram realizados neste mesmo organismo por LE MOAN *et al*. (2006), onde em um estudo proteômico de oxidação dos grupos tióis em *S. cerevisiae* mostraram a importância do sistema TRX frente ao stresse oxidativo gerado por H_2O_2. Os autores observaram que, na presença de H_2O_2, as células desprovidas do sistema TRX funcional, apresentavam um aumento das enzimas relacionadas com o metabolismo de H_2O_2. Este resultado demonstra a alta eficiência do sistema TRX na redução do H_2O_2 nesta levedura.

A proteína TRX foi também observada em *Schizosaccharomyces pombe*, tendo sido seu gene isolado e caracterizado, correspondendo a primeira descrição deste gene neste organismo (CHO *et al*., 2001). Em 2002, um outro gene codificante para uma putativa TRX mitocondrial foi clonado e sequenciado neste mesmo fungo (LEE et al., 2002).

O sistema TRX presente em *Helicobacter pylori*, uma bactéria gram negativa associada com gastrite e ulceras pépticas em humanos, parece ser indispensável para viabilidade deste organismo. Este sistema atua como doador de elétrons para uma proteína conhecida como alquil hidroperóxido redutase (AhpC), sendo esta pertencente à família das peroxiredoxinas (Prx) (BAKER *et al*, 2001).

Em estudos realizados por UNGERSTEDT *et al* (2005) foi observada a participação das TRXs na redução dos níveis de EROS nas células humanas. As TRXs estão também relacionadas com a proliferação celular e apresentam sensibilidade a inibidores da

deacetilase de histonas, os quais são capazes de induzir a morte celular. Estas observações foram possíveis através da utilização de "small interfering RNA" (siRNA), isto é, pequenos RNAs de interferência contra TRX. Os resultados demonstram decréscimo no nível de TRX, com consequente aumento de EROS, decréscimo da proliferação celular e um aumento da sensibilidade aos inibidores da deacetilase de histonas.

As TRXs também são capazes de interagir com um grande número de proteínas, embora nenhuma propriedade de chaperona tenha sido descrita para elas (ARNÉR e HOLMGREN, 2000). Em *E. coli* as TRXs se associam com Hsp90 e GroES, ambas relacionadas com enovelamento e degradação de proteínas (KUMAR *et al*., 2004). Sugerindo assim, que as TRXs interagem favoravelmente com as proteínas.

Em análise proteômica realizada em *E. coli* foram avaliadas as proteínas que se associam à TRX, ou seja que são alvos desta proteína. KUMAR *et al*. (2004) identificaram 80 proteínas associadas a TRX. Isto implica no envolvimento desta proteína em no mínimo 26 processos celulares distintos. Dentre estes processos são observados, regulação da transcrição, divisão celular, transdução de energia, detoxificação da célula e a participação em diversas vias biossintéticas. Estes dados confirmam o envolvimento da TRX em várias funções biológicas, sendo esta considerada um componente importante para os organismos vivos.

Estudos têm descrito a TRX em outros organismos patogênicos humanos como, *Trypanosoma brucei*, *Leishmania major* e *Schistosoma mansoni* (SCHMIDT e KRAUTH-SIEGEL, 2003; MYLER *et al*, 1997; ALGER *et al*., 2002).

PIATTONI *et al*. (2006) também demonstraram a presença *in vivo* da TRX em *T. cruzi*. Neste experimento a proteína recombinante foi expressa e purificada, apresentando atividade redutora.

Estudos realizados com a *Giardia duodenalis*, um parasita protozoário anaeróbico, mostraram a ausência de mitocôndria, de superóxido dismutase, glutationa, glutationa redutase e de catalase neste organismo. No entanto foi observado neste parasita, uma seqüência homóloga à tiorredoxina peroxidase, sugerindo também a presença da TRX e da TRR. Desta forma, este organismo possivelmente apresenta o sistema tiorredoxina completo como sendo o principal sistema de defesa antioxidante (BROWN *et al*., 1998).

O sistema TRX tem sido descrito em outro protozoário, *Plasmodium falciparum* (KANZOK *et al.*, 2000). O gene que codifica para a proteína TRX foi clonado e sua expressão foi induzida em *E. coli*. Foi então obtida a proteína recombinate de peso molecular de 11,7 kDa apresentando sítio ativo contendo dois resíduos de cisteína. Esta

proteína apresentou 51% de identidade com *S. pombe,* 38% de identidade com humanos e 31% de identidade com *E. coli.*

Os estudos, sobre como o fungo *P. brasiliensis* responde as condições de estresse oxidativo, e o conhecimento dos sistemas moleculares envolvidos nesta resposta, são relativamente escassos. O Projeto Genoma Funcional e Diferencial de *P. brasiliensis,* desenvolvido em laboratórios da região Centro-Oeste do país, tem identificado genes expressos nas formas leveduriformes e/ou micelianas através do sequenciamento automático de ESTs (*Expressed Sequence Tags*). Um total de 25511 clones foram derivados de bibliotecas de cDNA de leveduras e micélio (FELIPE *et al*., 2003; 2005). Este total representa cerca de 80% do genoma estimado do fungo *P. brasiliensis*.

Vários genes descritos como responsáveis pela defesa antioxidante dos organismos, foram identificados no transcriptoma de *P. brasiliensis*. Dentre estes: catalase, superóxido dismutase, peroxirredoxina, citocromo-c-peroxidase, glutationa, tiorredoxina, tiorredoxina redutase e alguns fatores de transcrição relacionados com a resposta ao estresse oxidativo (FELIPE *et al*., 2005). Com relação ao sistema TRX no transcriptoma de *P. brasiliensis*, foram identificadas onze TRXs homólogas (FELIPE *et al*., 2003; 2005, CAMPOS *et al*., 2005).

As glutarredoxinas (GRX) também são TRXs que contêm um sítio ativo dissulfeto redox e um sítio de ligação para GSH. As GRXs estão relacionadas com o sistema oxirredutase de dissulfetos de proteínas (NORDBERG e ARNÉR, 2001). MARQUES *et al*. (2004) mostraram que as glutarredoxinas são altamente expressas na fase leveduriforme de *P. brasiliensis*, quando comparadas com a fase miceliana.

P. brasiliensis é um parasita intracelular de humanos, e assim como todos parasitas intracelulares, ele tem que eliminar os metabólitos tóxicos endógenos e ao mesmo tempo resistir aos efeitos dos EROS produzidos pelo sistema de defesa do hospedeiro. Pode-se inferir desta forma, que a presença da TRX e TRR juntamente com outras proteínas que respondem ao estresse oxidativo em *P. brasiliensis* contribuam para a viabilidade deste patógeno no hospedeiro, favorecendo desta maneira a instalação da infecção.

II – JUSTIFICATIVA

O fungo *P. brasiliensis*, como já descrito anteriormente, é agente causador de uma micose sistêmica humana que acomete principalmente trabalhadores rurais na faixa etária produtiva de suas vidas. No Brasil e em especial no Estado de Goiás esta doença tem sido considerada como grave problema de saúde pública. A identificação e caracterização de fatores de virulência deste fungo têm sido objeto de estudos de muitos pesquisadores que procuram compreender melhor a biologia deste patógeno. Este trabalho pretende contribuir para o melhor conhecimento da resposta ao estresse oxidativo em *P. brasiliensis*, tendo como foco o estudo da proteína tiorredoxina. Esta proteína tem sido descrita como importante componente dos sistemas antioxidantes em diferentes organismos, atuando principalmente no combate dos EROS produzidos pelas células de defesa do hospedeiro. A TRX juntamente com outras proteínas antioxidantes podem aumentar a sobrevida de *P. brasiliensis* no hospedeiro, facilitando assim o estabelecimento da infecção.

III – OBJETIVOS

III.1 - GERAL:

- Estudar a proteína tiorredoxina 1 em *P. brasiliensis*.

III.2 - ESPECÍFICO:

- Identificar e caracterizar o cDNA codificante para a proteína TRX1 de *P. brasiliensis*.
- Clonar o cDNA *Pb*trx1 em vetor de expressão.
- Expressar em *E. coli* o cDNA codificante para a proteína TRX1 em *P. brasiliensis*.
- Obter a proteína recombinante *Pb*TRX1.
- Analisar a expressão do gene que codifica para *Pb*TRX1 na presença de H_2O_2.
- Avaliar a atividade redutora da proteína recombinante *Pb*TRX1 e da TRX1 em extrato potéico de *P. brasiliensis*.
- Comparar filogeneticamente as seqüências de aminoácidos da *Pb*TRX1 com sequências de outros organismos depositadas em banco de dados.
- Avaliar o efeito do H_2O_2 no crescimento e diferenciação de *P. brasiliensis*.

Para atingir os objetivos propostos as seguintes estratégias foram

- Foram feitas pesquisas de similaridade da seqüência de proteínas e de nucleotídeos no banco de dados NCBI (Blast genetic analysis program-http://www.ncbi.nlm.nih.gov) usando o programa BLAST (ALTSCHUL et al., 1990).
- Através do programa Clustal X (THOMPSON et al., 1997) foi feito o alinhamento entre seqüências homólogas

- Foi utilizado o programa PROSITE (http://www.expasy.org/prosite) (HOFMANN *et al*, 1999) para a identificação de motivos conservados na proteína traduzida.

- Realização de reação de PCR a partir da biblioteca de cDNA fase leveduriforme de *P. brasiliensis* utilizando oligonucleotídeos correspondentes às extremidades 5' e 3' do cDNA.

- Clonagem do cDNA *Pb*trx1 no vetor de expressão pGEX-4T-3 (Amerham Biosciences).

- Indução da expressão da proteína recombinante em sistema bacteriano. A expressão heteróloga foi obtida pela adição de um análogo de lactose sintético e não degradável (isoprpil ß- D- galactosídeo, IPTG).

- Células leveduriformes de *P. brasiliensis* foram tratadas com 25 mM de H_2O_2 e o RNA total foi obtido para análise da expressão do gene trx1 por RT-PCR semi-quantitavo.

- Análise da atividade enzimática da proteína recombinante e da TRX1 em extrato protéico de células leveduriformes de *P. brasiliensis*, pelo método de redução da insulina.

- A árvore filogenética foi construída por comparação das seqüências de aminoácidos, utilizando-se os Programas Clustal X (THOMPSON et al., 1997) e Treeview (RODERICK, 1996).

- Culturas leveduriformes de *P. brasiliensis* em fase exponencial de crescimento foram tratadadas por 3 horas com 25 mM de H_2O_2 e contadas em câmara de Neubauer diariamente. Para avaliar o efeito do H_2O_2 na transição de micélio para levedura, cultura miceliana foi tratada por 3 horas com 25 mM de H_2O_2 , incubadas à 36 ^oC e as células leveduriformes que iam surgindo na cultura foram contadas como descrito acima.

Cloning, expression and insulin reduction activity analysis of a thioredoxin 1 homologue of human pathogenic *Paracoccidioides brasiliensis*

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Cloning, expression and insulin reduction activity analysis of a thioredoxin 1 homologue of human pathogenic *Paracoccidioides brasiliensis*

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Abstract

Thioredoxins are small proteins catalyzing thiol-disulfide interchange and are involved in the regulation of redox environment in the cell. These proteins respond to oxidative stress in several organisms. *Paracoccidioides brasiliensis*, a thermodimorphic fungus, is the causative agent of paracoccidioidomycosis (PCM), the most prevalent systemic mycosis in Latin America. This fungus occurs as an intracellular organism in human host and thioredoxin seems to be one of the antioxidant mechanisms that helps the fungus to evade from host immune defense. We described the isolation and characterization of a cDNA encoding a thioredoxin 1 from yeast cells from *P. brasiliensis* (*Pb*trx1). The *Pb*trx1 codes for a 12 kDa protein containing the characteristic thioredoxin active site (WCGP). In a phylogenetic tree analysis *Pb*TRX1 is closer to other fungal TRXs. Secondary structure prediction indicates that the native conformation of *Pb*TRX1 is in agreement with the TRX secondary structures from other fungus. The *Pb*trx1 gene was expressed in *Escherichia coli* and the isolated rec*Pb*TRX1 showed insulin reduction activity *in vitro*. The immunobloting analysis of the extract from yeast cells, mycelium to yeast transition cells and rec*Pb*TRX1 showed one band with the expected size of TRX1 in all the extracts. The yeast cells extract showed increasing insulin reduction activity in cultures treated with H_2O_2. The yeast cells growth was significantly inhibited by H_2O_2, however the mycelium to yeast transition was not significantly affected by H_2O_2. We also revealed by sqRT-PCR that the expression of *Pb*trx1 was induced in response to H_2O_2 and may exert an antioxidant activity *in vivo*. Our results suggest that this protein may play an important role in controlling the redox status during yeast cells growth, dimorphism and oxidative insults from host defense in *P. brasiliensis*.

Keywords: *Paracoccidioides brasiliensis*; thioredoxin; oxidative stress; insulin reduction activity.

1. Introduction

Living organisms are continuously exposed to oxidative stress (OS), an unavoidable consequence of oxygen metabolism. OS is a state within the cell in which the level of reactive oxygen species (ROS), such as superoxide anions (O_2-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($HO^{\cdot}$), exceeds the available antioxidant defenses that scavenge and inactivate the ROS. These ROS are generated by the incomplete reduction of oxygen during respiration or by exposure to external factors (light, radiation, redox-cycling drugs and stimulated host phagocytes) (Sies, 1993; Halliwell & Gutteridge, 1989).

ROS are chemically very reactive and cause most undesirable effects damaging nucleic acids, lipids and proteins, and can be particularly implicated in uncontrolled cell proliferation, aging and apoptosis (reviewed by Halliwel & Gutteridge, 1999). Although ROS cause serious damages to biological systems, they also act as second messengers in signal transduction (Vivancos *et al*., 2004).

To offset the harmful effects of ROS, most organisms have evolved protective, mechanisms that utilize antioxidant molecules and enzymes such as superoxide dismutase, which catalyzes the dismutation of O_2- to form H_2O_2 and O_2 (Fridovich, 1995) and catalase, which further reduces H_2O_2 to H_2O and H (Sies, 1997). Other molecules involved in oxidative stress response are thioredoxin, thioredoxin reductase and glutaredoxin, responsible for maintaining a reduced state inside the cell (Holmgren, 1985; Gilbert 1990; Williams, 1992).

The thioredoxin system is composed of thioredoxin (TRX), thioredoxin reductase and nicotinamide adenosine dinucleotide phosphate (NADPH) as a proton donor. It is an important conserved system for protection against ROS by reducing peroxides such as H_2O_2 to harmless products. TRX is a small (around 12 kDa) ubiquitous protein characterized by a conserved active site sequence –Trp-Cys-Gly-Pro-Cys-Lys–, that function as protein-disulfite

reductase. The two amino acid residues of Cysteine in the active-site form a dithiol group, which is involved in the thioredoxin reduction of disulfides in a number of proteins. Oxidized thioredoxin (TRX-S_2) contains a disulfide bridge that is reduced by NADPH through the catalytic action of the flavoenzyme thioredoxin reductase to its reduced state (TRX-SH_2) (Holmgren, 1984 and 1985; Nakamura *et al*., 1994).

TRX was first isolated from yeast as a reducing agent in the reduction of methionine sulfoxide and sulfate (Black *et al*., 1960; Wilson *et al*., 1961). Subsequently**,** TRX was characterized from *Escherichia coli* as a hydrogen donor for ribonucleotide reductase, an essential enzyme for DNA synthesis (Laurent *et al*., 1964) and for methionine sulfoxide reductase (Ejiri, 1979). It was later shown to act as hydrogen donor to peroxiredoxins (Chae *et al*., 1994). TRXs are necessary for a number of other metabolic enzymes that form a disulfide as part of their catalytic cycle **(**Reitsch & Beckwith, 1998). Many TRXs are well known for their role of intracellular redox regulator of gene expression (Hirota *et al*., 1997). It is also involved in the life cycle of some bacteriophages such as T7, M13 and f1 (Chamberlin *et al*., 1974; Russel & Model, 1985; Lim *et al*., 1985). Furthermore, TRX is an efficient antioxidant able to reduce H_2O_2 (Russel & Model, 1985) and scavenge free radicals (Schallreuter & Wood, 1986). TRX was reported to have preventive capacities towards NO-mediated cellular injury in monocytic macrophage cells and has been described as chemotactic protein with comparable potency to other known chemokines (Ferret *et al*., 2000, Bertini *et al*., 1999), and also to be a putative oncogene conferring both growth and survival advantages to tumor cells (Grogan *et al*., 2000). Kumar *et* al. (2004) in a proteomic analysis showed that this protein associates with 80 proteins and it is involved in at least 26 distinct cellular processes that include transcription regulation, cell division, energy transduction, detoxification of the cell, and several biosynthetic pathways. The fact of TRX presents the most diversified physiological functions has stimulated its study in different microorganisms.

The etiological agent of paracoccidioidomycosis (PCM) is a thermally dimorphic fungus *Paracoccidioides brasiliensis*. The disease is endemic in Latin America, where it constitutes the most prevalent human systemic mycosis (Franco, 1987). The human infection establishment occurs by inhalation of airborn fungal propagules that reach the alveoli and differentiate into the pathogenic yeast form. *P. brasiliensis*. Then the host will be subjected to a considerable OS, produced by neutrophil cells during the oxidative burst, since the cell-mediate immunity is the first and the most important host defense in PCM against this fungus (Brummer *et al*., 1989). These phagocytic cells release ROS such as H_2O_2 as antifungal agents (McEwen *et al*., 1984; Meloni-Bruneri *et al*., 1996). Although *P. brasiliensis* is subject to this hostile ambient, it survives and infection process establishes. It is possible because the fungus as other microorganisms have antioxidant systems that convert the ROS in non damage compounds. The antioxidant system in *P. brasiliensis* is poorly known and the information about this is scarce.

Moreira *et al.* (2004) described a monofunctional peroxisomal catalase in *P. brasiliensis*, which was preferentially expressed in the yeast phase and had its expression increased in the presence of H_2O_2. Analyzing *P. brasiliensis* transcriptome, in mycelium and yeast cells, using bioinformatics tools provided by *P. brasiliensis* functional genome project (Felipe *et al*., 2003) several genes involved in antioxidant defense were identified. Among them catalase, superoxide dismutase isoenzymes, peroxiredoxin, cytochrome c peroxidase, glutathione synthesis enzymes, thioredoxin and the transcription factors (Campos *et al*., 2005).

Peroxiredoxins homologues that were identified in *P. brasiliensis* transcriptome (Felipe *et al*., 2003, Campos *et al*., 2005) differ from other peroxidases because they have no redox cofator, such as metals and prosthetic groups, so after peroxide reduction they are reduced by two donors of hydrogen, TRX and glutathione (Chae et al., 1994; Wood et al,

2003). TRRs and glutaredoxin homolog have also been identified in *P. brasiliensis* transcriptome. In a study carried out by Marques *et al*. (2004) the *P. brasiliensis* glutaredoxin showed higher levels of expression in yeast phase than in mycelium. We identified eleven TRX homologous assembled expressed sequence tag in *P. brasiliensis* transcriptome (Felipe et al., 2003, Campos et al., 2005).

The information provided by transcriptome analysis reveals that *P. brasiliensis* has many resources to combat ROS and that fungus survival depends on these mechanisms that help the fungus to evade from the host immune defense during infection. All this knowledge about oxidative response in *P. brasiliensis* demonstrates the importance of the antioxidant systems in its survival, contributing to the fungus evasion from host immune system and to its adaptation to the host environment.

We report here the molecular cloning and characterization of a cDNA (*Pb*trx1) encoding a thioredoxin 1 (*Pb*TRX1) from yeast *P. brasiliensis* cDNA library. We have also expressed *Pb*trx1 in *Escherichia coli* cells and demonstrated that recombinant TRX1 (rec*Pb*TRX1) efficiently reduced insulin. Based on the predicted amino acid sequence from *Pb*trx1, phylogenetic analysis and prediction of the secondary structure of *Pb*TRX1 were carried out too. The effect of H_2O_2 on *P. brasiliensis* growth and transition from mycelium to yeast phase was analyzed. We also evaluated the reduction of insulin assay by native *Pb*TRX1 in presence of H_2O_2. In addition, *Pb*trx1 expression analysis was performed by sqRT-PCR from yeast cells in presence of H_2O_2, demonstrating higher expression in this condition.

2. Materials and methods

2.1 Growth conditions of *P. brasiliensis*

P. brasiliensis, isolated *Pb*01 (ATCC-MYA-826), was used in this study. It was grown as yeast at 36 ºC and subcultured every 7 days in semisolid Fava-Neto's medium (1% weight in volume (w/v) peptone; 0.5% w/v yeast extract; 0.3% w/v proteose peptone; 0.5% beef extract; 0.5% w/v NaCl; 4% w/v glucose; and 1.4% w/v agar, pH 7.2) (Sambrook & Russel, 2001). Mycelium was grown at 22 ºC in this same medium, by subculturing every 15 days.

2.2 Cloning of the cDNA encoding the thiredoxin 1 homologue

For screening of cDNA library from yeast cells of *P. brasiliensis* a homologue fragment was generated by polymerase chain reaction (PCR). In PCR amplification this yeast cDNA library was used as a template and a partial fragment encoding the thioredoxin 1 was obtained. Based on the conserved sequence of the active site of the TRXs from several organisms and on the other conserved sequence on the carboxi- terminal region sense and anti-sense degenerated primers were respectively designed (Figure 1). Both primers sense (TRXI) 5′-TGGTGYGGNCCNTGYAAR-3′ and anti-sense (TRXII) 5′-TCNCGNTACGGNTGNAAR-3′ were employed in the PCR that was conduct in a total volume of 50 µl. The resulting 140-bp product was subcloned into pGEM-T-Easy (Promega, Madison, WI). The sequence was determined on both strands by automated DNA sequencing, applying the DNA sequencing method of Sanger *et al*. (1977).

A yeast cDNA library was constructed in *Eco*RI and *Xho*I sites of Lambda ZAP II (Stratagene Inc., La Jolla, CA). The screening of this library was carried out with 140-bp probe labeled with [α^{32}p]-dCTP. Plating 5 x 10^6 plaque forming units (pfu) was performed, DNA was transferred in duplicate to membranes and these were hybridized in high-stringency hybridization buffer containing 50% formamide at 42 ºC for 18 h. Six positive clones were isolated and phage particles were released from the plaques. The *in vivo* excision of pBluescript SK^+ phagemids in *E.* coli XL1-Blue (MRF') cells was performed using the ExAssist/SOLR protocol from Stratagene. The nucleotide sequence was determined on both strands.

2.3 Sequencing of *Pb*trx1 cDNA and sequence analysis

Nucleotide sequence of the *Pb*trx1 were determined on both strands, using primer walking by the double-strand dideoxy-chain terminator method using a MegaBace 1000 sequencer (Amersham Biosciences, Little Chalfont, UK) for automated sequence analysis.

The obtained sequences were translated and compared to all non-redundant polypeptides in the translated National Center for Biotechnology Information (NCBI) database using the BLAST algorithm (Altschul *et al*., 1990). Sequence analyses were carried out with the Genetics Computer Group (GCG) package (Madison, Wisconsin, USA) (Devereux *et al*., 1984), with the PROSITE (http://us.expasy.org/prosite) (Falquet *et al*., 2002) and Pfam (http://www.sanger.ac.uk/softwer/pfam/index.shtml) (Bateman *et al*., 2002) databases. The values of similarity among protein sequences were based on the alignment of amino acid sequences taking into account conserved substitutions of residues (Thompson *et al*., 1997).

The prediction of secondary structure of *Pb*TRX1 was performed with the Jpred program (http://www.compbio.dundee.ac.uk/~www-jpred/) (Cuff *et al*., 1998). The sequences analyzed were purchase from protein data Bank (Berman *et al*., 2000).

2.4 Comparison of *Pb*TRX1 sequences and inferred phylogeny

The deduced amino acid sequence was aligned with putative homologous from GenBank database. Phylogenetic relationships of *Pb*TRX1 were carried out with the 31 TRXs1 available from the NCBI database. One phylogenetic tree was constructed by multiple sequence alignments using CLUSTAL X (Thompson *et al*., 1997) and was generated by neighbor-joining method (Flores-Carreon *et* al., 1979) and visualized by TREE-VIEW software. Robustness of branches was estimated using 1000 bootstrapped replicates.

2.5 Expression of *Pbtrx*1 in *E. coli* and recombinant protein purification

The *Pbtrx1* cDNA containing the complete coding region of TRX1 was amplified by PCR using oligonucleotide sense (TRXS 5′-CACGAATTCCAATGGTTGTCCAC-3′) and antisense (TRXAT 5′- CCCCTCGAGACTTTCATCTAC-3′) primers, which contained engineered *Eco*RI and *Xho*I restriction sites (bold), respectively. The amplification of the *Pb*trx1 was carried out to an initial denaturation step at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 45 °C for 1 min and 30 s and extension at 72 ° C for 7 min. The 351-bp PCR product was digested with *Eco*RI and *Xho*I, separated by agarose gel electrophoresis, gel excised and subcloned into the *Eco*RI/*Xho*I sites of pGEX-4T-3 expression vector (Amersham Biosciences) to yield the pGEX-4T-3-trx1 construct. The recombinant plasmid was used to transform the *E. coli* BL21 Star (DE3) competent cells

(Invitrogen) using the heat shock method (Sambrook & Russel, 2001). Ampicillin resistant transformants were cultured, and plasmid DNA was analyzed by PCR. In addition to confirm that *Pb*trx1 was cloned in correctly orientation in vector, producing an in-frame molecule fused to glutathione S-transferase (GST), the complete sequencing of the plasmid was carried out.

The transformed bacterial cells growth was performed in 1-liter batches of Luria–Bertani (LB) broth containing 100 μg mL^{-1} of ampicillin for plasmid maintenance. The cultures were incubated at 37 °C until the *A*600 reached between 0.5 and 0.7, at which point isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM. The cells were incubated at 30 °C for 3 h and then harvested by centrifugation at 5,000 × *g* for 10 min. Preparation and purification of the protein was performed at 4 °C.

The IPTG-induced cells were pelleted, resuspended in phosphate buffered saline (PBS 1X) and incubated with lysozyme (100 μg/ml) at 4°C for 40 min. Afterward, the cells were sonicated for 15 min in buffer PBS 1X containing 0.1% Sarcosyl at 4°C. The sonicated cells were pelleted, and ressupended in PBS 1X containing 0.25% Triton X-100. Protein concentrations were determined (Bradford, 1976). The recombinant *Pb*TRX1 was expressed in the soluble form by the bacteria, and the protein was purified by affinity chromatography under nondenaturing conditions, as previously reported (Cunha *et al*., 2002). The soluble fraction was applied to a Glutathione Sepharose™ 4B Resin column (Amersham Biosciences) (bed volume, 1.0 mL). After, the resin was washed five times with PBS 1X (0.14 M NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.3). The size of the recombinant protein fused to GST was evaluated by running the molecule on a 15% SDS-PAGE followed by Coomassie blue staining.

2.6 Obtaining protein extracts

The protein extract was obtained as described by Fonseca *et al*. (2001). Briefly, the yeast cells treated and no treated with 25 mM of H_2O_2 (Sigma), and the cultures in transition from mycelium phase to yeast phase were washed in Tris-Ca^{2+} buffer (20 mM Tris-HCl, pH 8.8, 2mM $CaCl_2$) containing the protease inhibitors: 50 µg mL^{-1} N-*α*-*p*-tosyl-L-lysine chloromethylketone (TLCK), 4 mM phenylmethyl-sulphonyl fluoride (PMSF), 5 mM iodoacetamide, 1 mM ethylenediaminetetraacetic (EDTA), 20 µg mL^{-1} leupeptine and 1 mM 4-chloromercuribenzoic acid (PCMB). The cells were collected by centrifugation at 5,000 × *g* for 5 min, frozen in liquid nitrogen and disrupted by maceration until a fine powder. The cellular powder was vortexed in the same buffer described above for 15 min at 4 °C and centrifuged (4 °C) at 12,000 × *g* for 20 min. The supernatant was kept at −80 °C and was used for further analysis. The mycelium culture was initially treated with 25 mM of H_2O_2 for 3 h at 26 °C/ 240nm. A solution of hydrogen peroxide (30%, Sigma) was diluted in water and sterilized using a 0.2 µm Milipore® filter. To confirm the final concentrations of the H_2O_2 dilutions we measured the absorbance at 240nm (ε = 0,0394 mM^{-1} cm^{-1}). After this, the culture was centrifuged at 3,000 × *g* for 5 min and washed 3 times with H_2O. Then the mycelium was transferred to fresh YPD medium at 36 °C, with shaking. The protein extract from mycelium to yeast differentiation was obtained as described to yeast cells at time intervals of 0, 12, 24 and 120 h.

2.7 Protein analysis

Polyacrylamide gel electrophoresis of native proteins and sodium dodecyl sulfate (SDS-PAGE) were performed according to Laemmli (1970). Protein quantification was performed according to Bradford protein assay (Bradford, 1976).

2.8 Western blot analysis

The cellular extracts and the recombinant protein were resolved by one dimensional gel electrophoresis. The proteins were stained with Coomassie brilliant blue or electrophoretically transferred to nylon membranes. The membranes were stained by Ponceau S to assess loading of equal amounts of protein and incubated in 0.05% (v/v) Tween-20 plus Tris-buffered saline (TBS) containing 1% (w/v) skim milk. The TRX1 protein was detected with a polyclonal antibody raised to the TRX1 of *E. coli* (Sigma) (1:1000 diluted). After reaction with alkaline phosphatase anti-rabbit IgG (Sigma) (1:2000 diluted), the reaction was developed with 5-bromo-4-chloro-3- indolylphosphate/nitro-blue-tetrazolium (BCIP/ NBT) (Sigma).

2.9 Insulin-dissulfide reduction assay of rec*PbTRX*1 and of native *PbTRX*1 from yeast cells

Thioredoxin-mediate catalysis of insulin reduction was measured spectrophotometrically at 650 nm at 25 °C as an increase in turbidity resulting from precipitation of free insulin chain (Holmgren, 1979). The assay mixture contained 100 mM potassium phosphate, 2 mM EDTA (ethylene diamine tetraacetic acid) (pH 7.0), 0.13 mM insulin (0.75 mg mL^{-1}, Novo Nordisk), and 0.05 mg mL^{-1} of protein extract of *E. coli* transformed with pGEX-*Pb*TRX1 and protein extract of *E. coli* containing pGEX-4T-3 only, as control. The reaction was initiated by addition of 0.33 mM DTT and the absorbance was recorded at 2 min intervals on spectrophotometer (Pharmacia Biotech, Utrospec 2000). Negative control was performed using DTT without rec*Pb*TRX1. The blank contained all components minus the DTT. Total volume reaction was 600 µL. The assay of TRX activity

was also performed by yeast cells protein extract of *P. brasiliensis* (0.05 mg/ mL^{-1}) cultivated in a medium without H_2O_2 and with 25 mM of H_2O_2. These assays were performed in triplicate.

2.10 Analysis of yeast cells growth and of differentiation of *P. brasiliensis* in presence H_2O_2

Exponentially growing yeast cells of *P. brasiliensis* were tested for growth after treatment with H_2O_2. Yeast cells grown in semi-solid Fava-Neto medium (Fava-Neto, 1955) for 7 days at 36°C were transferred to liquid YPD medium (1% w/v Bacto-yeast extract, 2% w/v Bacto-peptone and 2% w/v dextrose). The cultures were treated with 25 mM of H_2O_2 for 3 h. After that, the cultures were centrifuged at 3,000 × *g* for 5 min and washed 3 times with H_2O and cells inoculums of 5 x 10^6 cells mL^{-1} were incubated in fresh YPD medium at 36 °C with shaking. A control culture without H_2O_2 was grown in parallel. Daily, during 7 days of incubation, 10 µL of each culture was collected and transferred to 1 mL of 0.9% NaCl, 2% formaldehyde, 4% Tween 20 solution, and yeast cells growth were estimated by counting in Neubauer chamber. Cell viability was determined using green Janus vital staining, according to Sano *et al.* (1993).

The conversion from mycelium phase to yeast phase was performed in YPD medium by changing the temperature from 26 to 36 °C. The mycelium culture was initially treated with 25 mM of H_2O_2 for 3 h at 26 °C. After this, the culture was centrifuged at 3,000 × *g* for 5 min and washed 3 times with H_2O. 100 µg of mycelium was added to fresh YPD liquid medium and incubated at 36 °C, with shaking. The differentiation was visualized by optic microscopy and evaluated daily during 15 days by counting in Neubauer chamber the yeast

cells in culture. Both experiments, analysis of growth and the differentiation, was run in triplicate.

2.11 Statistical analysis

The yeast cells growth and the mycelium to yeast transition data were also analysed by linear regression, and the results compared by the unpaired Student's *t*-test with significance set as 1%. The values were expressed as a mean of triplicates.

2.12 RNA isolation

Total RNA from yeast cells after treatment with 25 mM H_2O_2 during 30 min and 1 h and from yeast cells no treated was isolated by Trizol method, according to manufacture's instructions (Invitrogen, Carlsbard, CA, USA). To remove genomic contamination, the RNA samples were treated with RNase-free DNase-I (Invitrogen, Carlsbard, CA, USA) at 37 ºC for 15 min, following standard procedures. RNA concentration and purity were determined spectrophotometrically, and RNA integrity was visualized by electrophoresis.

2.13 Semi-quantitative RT-PCR analysis

Semi-quantitative reverse transcription polymerase chain reactions (sqRT-PCR) were performed for *Pb*trx1 gene to show the pattern of expression of this gene in presence of H_2O_2. Total RNA was extracted from *P. brasiliensis* yeast cells after treatment with 25 mM H_2O_2, for 30 min and 1 h and from *P. brasiliensis* yeast cells no treated, as described. First-strand cDNA was synthesized with 1 μg total RNA by reverse transcription using the SuperScript II

reverse transcriptase (Invitrogen) in the presence of the synthetic oligonucleotide antisense primer TRXAT. cDNA synthesis reaction was performed at 45 ºC for 60 min. cDNA was used for PCR in 50 µl reaction mixture containing specific primer to TRX1 sense (TRXS) and antisense (TRXAT). Specific primers to ribosomal 28S of *P. brasiliensis* were used in PCR as internal control, sense and antisense, respectively: 5´-CGAAGACGGGATTCTCACC-3′ and 5´-CGGATCAGGTAGGGATACC-3′. PCR conditions were: 94 ºC for 2 min; annealing at 50-65 ºC for 2 min; 25-35 cycles at 72 ºC for 1 min and 30 s, extension at 72 ºC for 7 min. The annealing temperature and the number of PCR cycles were optimized for each experimental condition to ensure linear phase of amplification. Amplicons were analyzed by agarose gels electrophoresis (1%). The analyses of relative differences were performed by using Scion Image Beta 4.03 program. Control reactions without RT and without RNA were performed (data not shown). The RT-PCR product of *Pb*trx1 (351-bp) was gel-purified, subcloned into plasmid pGEM-T-Easy (Promega, Madison, WI, USA) and sequenced as described previously.

2.14 Nucleotide sequence accession number

The sequences of cDNA *Pb*trx1 and the deduced protein (*Pb*TRX1) have been filled in the GenBank database under Accession No. AY376435.

3. Results

3.1 Isolation, sequence analysis of the *Pb*trx1 cDNA and characterization of *Pb*TRX1 deduced protein

In order to isolate the complete cDNA enconding the 12 kDa TRX1 of *P. brasiliensis*, we obtained a 140-bp PCR product using the sense and anti-sense primers TRXI and TRXII, respectively (Fig. 1A). This PCR product shows homology with sequences of TRXs from several microorganisms present in the database (data not shown). The entire cDNA (*Pb*trx1) was isolated by screening the yeast cDNA library of *P. brasiliensis*. The *Pb*trx1 sequence was 811-bp nucleotides in length, contained an open reading frame (ORF) of 351-bp, and the deduced amino acid sequence presented 116 residues with predict molecular mass and p*I* of 12 kDa and 5.2, respectively, which is similar in size to previously reported TRX. The ORF starts at an ATG codon and the first methionine at position 296 in the nucleotide sequence is in the context of an initiation codon (Kozak, 1986). The *Pb*trx1 presented 295 and 165 bases at the 5′ and 3′ untranslated regions, respectively. A polyadenyation signal ATAAAA was identified at 770 nucleotides in the downstream sequence of the cDNA (Proudfoot, 1991) and it is followed by a t/gt-rich element, as described by McLauchlan *et al.* (1985). The deduced *Pb*TRX1 presented one highly conserved motif -Trp-Cys-Gly-Pro-Cys- in the C-terminal region, described as a characteristic TRX active site at amino acids 41- 45. The major feature of TRXs is the presence of two cysteine residues involved in this conserved catalytic site, which are critic for TRX activity. When TRX is in a reduced state (TRX-SH_2) the two cysteine residues form a dithiol group that is able to catalyze the reduction of disulfides in a number of proteins. Oxidized TRX (TRX-S_2) can be reduced by NADPH (Holmgreen, 1995). Two other cysteine residues out

side of the catalytic site at positions 37 and 72 were identified in *Pb*TRX1 and they have been described as important in TRX function (Pedrajas, 1999).

From sequence analysis we also observed that *Pb*TRX1 possess all structurally important amino acids (Fig. 1A). These include, besides the active center consensus sequence (-Trp-Cys-Gly-Pro-Cys-), structural important residues given as Phe12, Ala39, Pro50, Glu54, Tyr59, Asp70, Pro85, Thr86, Lys91, Gly101 and Ala102 corresponding to Phe27, Ala29, Pro40, Glu44, Tyr49, Asp61, Pro76, Thr77, Lys82, Gly92 and Ala93 in the *E. coli* TRX sequence (Eklund *et al*., 1991). Furthermore, these sequences are not preceded by transit peptides as has been described for some TRXs (Mestres-Ortega & Meyer, 1999). In *Arabidopsis thaliana* there are at least 20 thioredoxin genes in whole sequenced genome and primary structural analysis allows a classification of the reported isoforms Trx*f*, Trx*h*, Trx*m*, Trx*o*, Trx*x* and Trx*y* (Meyer *et al*., 2002; Lemaire *et al*., 2003). The subgroup Trx*h* exhibits the usual catalytic site (-Trp-Cys-Gly-Pro-Cys-) and among the TRX h subgroup the *A. thaliana* (AtTrxh5) seems to be involved in response to pathogens and oxidative stress (in review Gellhaye *et al*., 2005). Analysis of AtTrxh5 primary structure showed that this protein share some structurally important amino acids as described to *E. coli* with *Pb*TRX1 (data not shown).

The hydropathical profile defined on ProtScale program available on the ExPASy server showed a high number of regions hydrophilic than hydrophobic in *Pb*TRX1 sequence, which was indicative of a strongly hydrophilic protein. *Pb*TRX1 was predicted to has cytoplasmic localization with a very high score by Predotar, PSORT and TargetP (links available at http://www.expasy.ch). There was no recognizable nuclear localization or nuclear export sequences in *Pb*TRX1, until this moment it is unclear how the subcellular distribution of TRX1 is maintained (Powis & Montfort 2001).

3.2 Comparison of *Pb*TRX1 deduced amino acid sequence and phylogenetic analysis

A search performed in FASTA on the GenBank database showed a high number of identical and conserved amino acids among TRX sequences from both prokaryotic and eukaryotic microorganisms. Alignment of the *Pb*TRX1 predicted protein sequence with related sequences of TRX1 was performed using the Clustal X program **(**Thompson *et al*., 1997) (data not shown). By comparison to fungi TRXs sequences, the secondary structural elements of *Pb*TRX1 were mapped by using the Jpred program (http://www.compbio.dundee.ac.uk/~www-jpred/) (Cuff *et al*., 1998). The alignment of the *Pb*TRX1 with these sequences was performed using the Clustal X program (Thompson *et al*., 1997) (Fig. 1B). The analysis showed that *Pb*TRX1 shares to other microbial TRXs a secondary structure characteristic of the open twisted alpha/beta (5 beta-sheets associated in a parallel and anti parallel manner and surrounded by 4 helices. Five proline and three glycine residues out site catalytic region are common residues in or close to bends. They are highly conserved in TRXs and fulfill this function (Holmgreen *et al*., 1975) and these same residues were observed in *Pb*TRX1 sequence. A conserved sequence described as TRX catalytic site at amino acids (-Trp-Cys-Gly-Pro-Cys-) presented 100% of identity in all sequences aligned. Besides this conserved site, two other regions, -Asp-Val- Asp -Glu- and Ala-Met-Pro-Thr-Phe- showed high degree of identity. *Pb*TRX1 showed a characteristic pattern of secondary structure described to TRXs from other organisms, five β-sheets and four α-helices.

The NCBI database was accessed to search for complete protein sequences of 31 TRXs1 from several organisms. The 31 TRXs1 sequences were downloaded and aligned using the multiple-alignment, a neighbor-joining tree was constructed to estimate the distances among these TRXs sequences. To visualize the relationship between *Pb*TRX1 and other TRXs1 in terms of amino acid sequence similarity, a phylogenetic tree was constructed

(Fig 2). In the phylogenetic tree drawled *P. brasiliensis* was enclosed in the fungi clade, together with *Neurospora crassa*.

3.3 Effect of H_2O_2 on Yeast cell growth and on dimorphism of *P. brasiliensis*

The effect of H_2O_2 in yeast cells growth and in the mycelium to yeast transition was analyzed. In order to determine the concentration that was able to affect cell growth without reduce drastically the cell viability. For that, the yeast cells were treated with different concentration of H_2O_2 (10, 25, 50, 75 mM) and cell growth and viability was measured by counting daily in chamber Neubauer. The results showed that the growth inhibition was concentration dependent (data not shown). Figure 3A shows the yeast cell growth curve in presence of 25 mM, the concentration chosen to work, where the majority of the cells were viable but the growth was inhibited related to the control. The growth of the yeast cells treated with H_2O_2 was very affected, while the yeast cells from control culture showed growth normal values. The cells growth of the yeast treated showed difference at 1% significance as compared to the control. A protein with molecular mass of 12 kDa, compatible with molecular mass predicted for *Pb*TRX1, reacted with polyclonal antibody against TRX1 of *E. coli* (Sigma) (Fig. 3C). Signals for 12 kDa corresponding to TRX1 were detected in both protein extracts blotted, yeast cells in absent and in the presence of 25 mM H_2O_2, however it wasn't possible observe quantitative differences between the two conditions. The cellular transition from mycelium to yeast was determined in 25 mM of H_2O_2 (Fig. 3B). The graphic shows that H_2O_2 affects morphological transition in *P. brasiliensis*, but not drastically. The culture treated with H_2O_2 showed a reduction of approximately 20%, by the 15 day of transition, in the percentage of yeast cells (relative to the control without treatment). These results not showed difference at 1% significance between the mycelium to yeast transition

treated previously with H_2O_2 and no treated. We also identified bands in Western blot performed with protein extract obtained during differentiation, after 3 h of mycelium treatment with H_2O_2, that correspond to *Pb*TRX1 (Fig 3E). These results related to the expression of TRX1 during all differentiation process bring insights on the possible role of this protein in dimorphism in *P. brasiliensis*, although by this experiment it wasn't possible to infer data concerning to the level of expression of *Pb*TRX1.

3.4 Expression and purification of the recombinant *Pb*TRX1

In order to express the *Pb*TRX1 in *E. coli* BL21 (DE3) the cDNA encoding this protein was subcloned into the expression vector pGEX-4T-3 to obtain the recombinant fusion protein (rec*Pb*TRX1). The expression of the pGEX-4T-3-trx1 produced about 5 mg of the fusion protein in one liter of *E. coli* culture. After the induction with IPTG the SDS–PAGE was performed to examine the composition of the cell lysates, as shown in Fig. 4. The predicted molecular size of the recombinant protein was 38 kDa, which included the vector-encoded fusion peptide of 26 kDa at its N-terminus. The fusion protein was purified using glutathione-sepharose 4B. The recombinant protein was eluted and fractions of equal volume (1 mL) were collected and analyzed by SDS-PAGE (Fig. 4, lanes 1, 2, 3, 4 and 5). The fusion protein bind to glutathione-sepharose (Lane 5) was blotted and reacted with TRX1 polyclonal antibody of *E. coli* (Lane 6). The recombinant GST-TRX1 showed the molecular size of the recombinant fusion protein correlated with the predicted size (38 kDa) (Lanes 5 and 6).

3.5 Reductive activity of recombinant *Pb*TRX1 and of native *Pb*TRX1 from yeast cells extract

All TRXs reduce disulfide bonds, in this way exerting their major anti-oxidant function. In order to show that *Pb*TRX1 functions as an oxidoreductase, we analyzed the redox activity of the rec*Pb*TRX1 and of the native *Pb*TRX1 from yeast cells protein extract by the insulin assay. The method is based on the ability of DTT reduced TRX to catalyze the reduction of insulin α and β chains, resulting in the precipitation of the insoluble β-chain and the level of turbidity is increasing. In the absence of rec*PbTRX*1 (DTT), no increase in turbidity was observed during the first 50 min of incubation (Fig. 5). In contrast, the presences of purified recombinant *PbTRX*1 gave increasing rates of insulin reduction (Fig. 5A), which was detectable in the first minute of incubation. It exhibited approximately 3-fold higher activity than the negative control (DTT). The results indicate that the GSH fused to TRX does not affect rec*Pb*TRX1 activity. We also observed that the insulin reduction for yeast cells increased when the cells were treated with H_2O_2 during 3 h (Fig. 5B). In fact, when was compared the treated with no treated cells, these latter showed a low activity. Additional experiment was performed in order to prove that this reduction activity was caused by *Pb*TRX1 and not by other *P. brasiliensis*- reductases present in yeast protein extract. Thus, the yeast protein extracts were incubated with the antibody against *E. coli* TRX1 (Sigma) and the insulin reduction was measured. The treated (H_2O_2) extract that before of the incubation with antibody showed higher activity than the reaction with only DTT, had lower activity after antibody incubation (Fig. 5B). The effect of the antibody in the enzymatic assays of *P. brasiliensis* showed that the greater activity in yeast extract was due to *Pb*TRX1. Thus, these assays demonstrated the functionality of this system in *P. brasiliensis* oxidative stress response and that purified recombinant protein is a TRX, and the most important catalytically

active. Western blot analysis for rec*Pb*TRX1 showed signal for 38 kDa corresponding to *P. brasiliensis* recombinant protein (Fig. 4, lane 6).

3.6 Analysis of the expression of *Pb*trx1

To learn more about the physiological role of *Pb*TRX1 in yeast cells, the transcription of *Pb*trx1 gene was analyzed by sq-RT-PCR. The sq-RT-PCR analysis was carried out using total RNAs from yeast cells treated with 25 mM H_2O_2 during 30 min and 1 h and no treated (control). To evaluate the possibility of the results obtained have been representative of the real condition which the yeast cells were submitted and not due to a differences in RNA contents, the same procedure was performed using an internal control, ribosomal RNA 28S from *P. brasiliensis*. Figure 6 shows an amplification of a 351-bp fragments from RT-PCR using TRX1 specific primers and of 223-bp from RT-PCR using 28S ribosomal primers. As shown in Fig. 6 the yeast cells treated with H_2O_2 showed a level of *Pb*trx1 expression higher than the yeast cells without treatment and no increase was observed in 28S ribosomal transcript. We also sequencing the 351-bp products and compared the sequences obtained with *Pb*trx1 (data not shown), in fact these products presented 100% of homology with *Pb*trx1 sequence.

4. Discussion

The antioxidant defense system of microorganisms comprises many different antioxidant molecules. Besides *Pb*TRX1, *P. brasiliensis* has other molecules that respond to oxidative stress, such as catalase, superoxide dismutase, peroxiredoxin, cytocrome c peroxidase, and thioredoxin (Campos *et al*., 2005; Felipe *et al*., 2006). However, little is known about what the specific roles for these molecules in *P. brasiliensis* are; each one may be responsible for the oxidative stress response at a particular ROS or at particular compartment. Although TRX from several organisms are well described, it had not been characterized in *P. brasiliensis*.

On the basis of *Pb*trx1 structural similarities, and based on the homology with other sequences, we conclude that the cDNA encoding the TRX1 of *P. brasiliensis* has been cloned. This cDNA presents a high degree of similarity with its fungal homolog and the putative protein has the active site motif (-Trp-Cys-Gly-Pro-Cys-), fully conserved. In classical catalytic sites both cysteine residues are involved in the catalytic activities of the enzyme, allowing disulfide bridge reduction of the target protein (Holmgren & Björnstedt, 1995). These data were corroborated by the alignment of the deduced amino acid of *Pb*TRX1 with sequences of various fungi revealing a high degree of similarity. The highest identity was observed with TRXs from fungi. In a phylogenetic tree analysis *Pb*TRX1 is closer to the fungal TRXs, in a branch of the tree closer to the *Neurospora crassa*.

TRX can be localized in several cellular compartments and this can be explained by the active role that TRX plays in many biological processes. TRX has been found: outside the cell, where it is involved in cell growth stimulation and chemotaxis; in the cytoplasm, as an anti-oxidant and a reductant cofactor; in the nucleus, where it regulates the activity of many transcription factors, as well as in the mitochondria (Powis & Montfort, 2001). When

*Pb*TRX1 was analyzed with a program for prediction of protein localization sites (PSORT) (Ejiri *et al*., 1979), it showed a high probability to be localized in cytoplasm as TRX1 and TRX2 from Saccharomyces *cerevisiae* (Gan, 1991, Chae *et al*., 1994). Taking into accounting that the cytoplasmic mammalian thioredoxins have at least two structural or noncatalytic cysteine residues (Holmgren & Björnstedt, 1995)**,** also identified in *Pb*TRX1 at positions 37 and 72, whereas mitochondrial mammalian TRX does not contain extra cysteines (Spyrou *et al*., 1997), we can suggest that *Pb*TRX1 protein has citoplasmic localization. Reported mitochondrial TRX is found to contain mitochondrial translocations signals (Lee *et al*., 2002; Pedrajas *et al*., 1999) and *Pb*TRX1 has no signal peptide. These two cysteine residues out site of the catalytic site have been described as important in TRX function (Pedrajas, 1999). Subsequent studies are needed to establish the cytoplasmic localization of *Pb*TRX1.

The interaction between thioredoxin and substrate proteins has been suggested to involve the Cys-Gly-Pro-Cys active site and several residues, including cis-Pro76 and Gly92, which form a moderately hydrophobic surface around the active site facilitating interactions with other enzymes (Lennon *et al*., 2000). The corresponding residues in *Pb*TRX1 deduced sequence are Pro85 and Gly102, which maybe could present the same functions in the *Pb*TRX1 interactions with its substrate. Among the several conserved amino acid residues in *E.* coli TRX1 Asp59 and Gly84 are considered by Holmgren *et al*. (1975) to contribute to the right folding of the protein, whereas the cis-Pro76 was described to interact with the active site in the 3-D structure of TRX. We could observe these same amino acid residues in *Pb*TRX1, D68 and G93 and cis-Pro85. The amino acid residue Asp36, Asp26 in *Pb*TRX1 and *E. coli* TRX1, respectively, according to Holmgren *et al*. (1975) might participate in the *E.* coli TRX interaction. Asp corresponds to the only negatively charged residue in the interior of the molecule. The major feature of TRXs is the presence of two cysteine residues involved in the most conserved catalytic site (-Trp-Cys-Gly-Pro-Cys-) and it was identified in *Pb*TRX1.

In classical catalytic sites, both cysteine residues are involved in the catalytic activities of the enzyme, allowing disulfide bridge reduction of the target proteins. The amino acid environment of cysteine residues is critical for TRX activity. Several studies about mutagenesis have shown that the sequence of the dipeptides between the two cysteines in catalytic site is very important to redox potential of the TRX superfamily and also important in interactions with targets proteins (krimm *et al*., 1998; Lin *et al*., 2004; Jeong *et al*., 2004).

To establish that the putative *Pb*trx1 in fact encodes a protein with thioredoxin activity, we cloned this cDNA into pGEX-4T-3 and the expression was induced. Then, the biological activity of rec*Pb*TRX1 protein was evaluated by insulin reduction assay, where the precipitation of β-chain of insulin was dependent on the time of incubation (Holmgren, 1979). The rec*Pb*TRX1 showed active as a disulfide reductase. The identity of rec*Pb*TRX1 was further confirmed in immunoblotting experiments by using polyclonal antibody and the size is compatible with described to other TRXs. Our results suggested that this protein has the TRX-like activity and TRXs that have this redox feature are reported for playing an important role in the cellular defense against oxidative stress (Matsuo *et al.,* 2001). A thioredoxin is defined both by its structural and catalytic characteristics, *Pb*TRX1 secondary structural is in according with what has been described to TRXs from other organisms and showed insulin reduction activity. The models of three-dimensional structures for TRXs indicate that all the proteins have similar three-dimensional structures despite the large variation in amino acid sequences (Eklund *et* al., 1991; Capitani *et* al., 2000). The three-dimensional structure prediction for majority of TRXs shows similarities with the *E. coli* three-dimensional structure that was determined by NMR, as well as by x-ray crystallography (Katti *et al*., 1990, Jeng *et al*., 1994), which has a central core of five strands of twisted β-pleated sheet flanked by four α-helices and the active site located in protrusion of the protein (Gleason &

Holmgreen, 1988). The secondary structure predicted for *Pb*TRX1 is in agreement with these structures that are present in the models of three-dimensional structures described for TRXs.

Attempts to evidence the presence of *Pb*TRX1 in crude extract of yeast cells and in mycelium to yeast transition, the natural course of the infection, by Western assays evidenced a band corresponding to predict molecular mass to *Pb*TRX1 from cDNA isolated and characterized as *Pb*trx1. These results indicate that the protein is normally expressed in yeast cells and in differentiation process. The treatment of yeast cells with H_2O_2 didn't clearly reveal an increase in protein content in response to this stress condition, the same result could be observed in differentiation process. The fact that TRXs have been mentioned as most been expressed in stationary phase growth of *S. cerevisiae* (Garrido & Grant, 2002), could explain the same protein expression patterns showed in both control and treated yeast cells in Western blot experiments (Fig. 3). Contradicting this date the yeast cells extract treated with H_2O_2 showed highly insulin reduction activity than control.

Several studies have emphasized that the maintenance of redox homeostasis is crucial for many biological processes such as differentiation, regulation of specific genes and signaling pathways (Allen & Tresini, 2000; Finkel & Holbrook, 2000). Our results related to the effect of H_2O_2 in *P. brasiliensis* dimorphism showed that it was slightly affected by H_2O_2 treatment relative to the control. It could be explained by the fact that when the mycelium phase was incubated with H_2O_2 the viability of the fungal wasn't affected, mainly because this fungal has a house keeping expression of anti-oxidant genes that maintain the level of ROS inside the pattern tolerable by the fungi. To some pathogenic organisms the ROS may function as a stimulating factor to morphogenesis. Studies performed with *C. albicans*, comparing yeast and hyphal forms of virulent and avirulent strains, showed that during hyphae phase (the invasive form) a further increase was found in ROS production, and that there was difference in the amount of ROS production between virulent and avirulent strains.

The virulent strain showed higher production of ROS than avirulent, being this fact related to its ability of generating ROS with the pathogenicity and invasiveness of this fungal (Schroter *et al*., 2000). Several studies have showed that TRX has intracellular antioxidant activity and, when overexpressed, protects against oxidative stress (Nakamura *et al.*, 1994; 1997).

An interesting feature observed in yeast cells is that the normality of the cell cycle is dependent on redox function. Muller (1991; 1995) showed that deletions of TRX1 and TRX2 affect the cell cycle in yeast cells, resulting in a prolonged S phase and shortened G1 interval. Our results demonstrate a significant inhibition of the yeast cells growth in response to H_2O_2. *S. cerevisiae* growing in YPD medium shows different sensitivity to oxidants at different states of growth, being most sensitive in stationary phase (Jamieson, 1992; Stephen *et al*., 1995). It is known that cells in exponential phase growth are more sensitive to H_2O_2 than cells in stationary phase growth (Garrido & Grant, 2002) and when cells enter in stationary phase, they undergo a complex series of physiological changes, which also result in an increased resistance to oxidative stress (Jamieson *et al*., 1994), and consequently in an increase in the expression of oxidative stress response proteins. Analyzes of the yeast cells growth from *P. brasiliensis* were performed from cultures in exponential phase, justifying the greater sensibility to the H_2O_2 treatment.

The expression of the *Pb*trx1 gene was investigated in yeast cells in H_2O_2 presence. The transcription rates of many yeast genes are increased in response to diverse environmental and physiological insults, such as nitrogen starvation, osmotic shock, heat shock, metals, and oxidative stress. According to Garrido and Grant (2002), thioredoxins were more important than glutaredoxins in mediating resistance to oxidative stress caused by hydroperoxides. They reported that the overexpression of TRX1 and TRX2 in *S. cerevisiae* was found to increase the resistance of wild-type strain to H_2O_2. Interestingly, *P. brasiliensis* showed an increase in *Pb*trx1 expression when treated with H_2O_2, suggesting that in this fungi

thioredoxins may play a key role in protection against oxidative stress, as in *S. cerevisiae*. sqRT-PCR assay shows the presence of transcript in yeast phase and in mycelium phase (data not shown). In order to analyze if the level of *Pb*trx1 transcripts were changed by yeast cells treatment with H_2O_2, RNA processed from yeast cells of *P. brasiliensis* treated with H_2O_2 was used in the sqRT-PCR reaction. The occurrence of higher amplification of *Pb*trx1 in cells submitted to H_2O_2 treatment shows that this transcript could be very important for *P. brasiliensis* response to OS. TRXs have been reported to be involved in the oxidative response in several organisms and as virulence factors **(**Windle *et al*., 2000). Our results indicate that *Pb*trx1 responds to H_2O_2 treatment in yeast cells. The yeast *S. cerevisiae* is an important model organism for the study of the eukaryotic oxidative-stress response (Jamieson, 1998). The expression of genes encoding the components of the thioredoxin system in *S. cerevisiae*, thiol-specific antioxidant (TSA1),TRX2, and thioredoxin reductase1, is induced in response to H_2O_2 (Kuge & Jones, 1994; Godon *et al*., 1998; Lee *et al*., 1999). Ross *et al*. (2000) suggests that thioredoxin peroxidase, of the thioredoxin system, is part of an important conserved sensing mechanism for redox conditions in eukaryotes, being essential for the transcriptional induction of other components of the TRX system, in response to H_2O_2 in *S. cerevisiae*. Reported studies have shown that the yeast AP-1-like transcription factor (Yap1p) is a major regulator of *S. cerevisiae* response to H_2O_2 (Lee *et al*., 1999; Gasch *et al*., 2000) and interestingly this transcription factor was also described in *P. brasiliensis* transcriptome (Campos *et al*., 2005), strongly suggesting that *P. brasiliensis* has similar mechanism of response to H_2O_2. Another result reveals the existence of similarity between the antioxidant systems from two fungus, that is *Pb*TRX1 secondary structure shows similar pattern of secondary structure when compared with *S. cerevisiae* thioredoxin 1. An additional study which reinforces the response of TRX to H_2O_2 was done by Koerkamp *et al*., (2002) using DNA microarrays in oxidative stress response of *S. cerevisiae*. It showed a rise in the level of

thioredoxin mRNA when compared to the mRNA involved in glutathione biosynthesis, suggesting that the thioredoxin induction system occurs before other systems related to oxidative stress response. According to Le Moan et al (2006), thioredoxin has an exclusive role in H_2O_2 metabolism. They carried out an experiment with thioredoxin deficient yeast cells in *S. cerevisiae* and observed that H_2O_2 itself does not cause a high increase in the oxidation state of H_2O_2–metabolizing enzymes, pointing to the high efficiency of the thioredoxin pathway in H_2O_2 reduction.

Redox control provides the cell with a mechanism by which it can respond to changes in its environment through modulation of the activity of certain genes. The thioredoxin system plays central role in redox control by regulating the activity of transcription factors and enzymes and protecting cells from oxidative stress. We have identified genes that code for a thioredoxin reductase, glutaredoxin and TRXs homologous in *P. brasiliensis* transcriptome (Felipe et al., 2003, Campos et al., 2005). The results from this study of *Pb*TRX1 added to previous results on *P. brasiliensis* transcriptome confirm the existence of thioredoxin system in this fungus. This thioredoxin system may play an important role in controlling the redox status during yeast growth, dimorphism and oxidative insults from host defense. Results presented here on the characterization of TRX1 in *P. brasiliensis* contribute to establish the occurrence of this protein in this human pathogen and questions related to the participation of this protein in the maintenance of the redox state of this fungi and how it increases *P. brasiliensis* survival rate in the host remain unknown until this moment. Further studies are needed in order to elucidate the possible complex implications of thioredoxin in oxidative stress response in *P. brasiliensis*.

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Figures Legends

Figure 1 – (A) Nucleotide sequence and deduced amino acid sequence of the *P. brasiliensis* cDNA encoding the TRX1 homologue. Nucleotides are numbered in bold (left margin). Nucleotides 5′ to the initiation codon (bold) are indicated by negative numbers. The presumptive start and stop codons are in bold. The putative polyadenilation signal ATAAAA is underlined. Numbering of the amino acid sequence begins at the methionine encoded by the first initiation codon and ends at the first termination codon, both in bold. The deduced amino acid sequence is shown above the nucleotide sequence (single letter code). The amino acids potentially associated to catalysis are boxed. The structural important residues present in majority of TRXs1 sequences are indicated by asterisks. The two extra cysteine residues outside catalytic site are in italic and bold. Arrows mark TRXI and TRXII oligonucleotides, and gray boxes mark TRXS and TRXAT oligonucleotides. **(B)** Alignment of deduced *Pb*TRX1 and related sequences of fungi TRXs to produce the secondary structure alignment. The alignment was performed by using the CLUSTAL X program. *Pb*TRX1 (PARBR) was aligned with those from YARL1, *S. cerevisiae* (GenBank accession number Q6C399); EMEN1, *Emericella nidulans* (Genbank accession number Q5BH10); ASPFU, *Aspergillus fumigatus* (GenBank accession number Q4WV97). The letters H and E above the TRX sequence correspond to regions of α-helix and β-strand, respectively. Positions in which a gap was introduced to optimize homology are shown as dashes. The gray boxes show the highly conserved residues amino acids among these fungi TRXs1. The important amino acid residues to the secondary structure pattern are in italic. The amino acids potentially associated to catalysis are in bold. Asterisks (*) define identity, two points (:) define conservative substitutions and one point (.) define semi conservative substitutions.

Figure 2 - The phylogenetic analysis was based on amino acid sequences of several TRXs1. The tree was calculated by neighbor-joining method implement in the program Clustal X and drawn by using the program Tree View software. The numbers of the branches are bootstrap values obtained with 1000 replications and indicate the percentage of times all species to the right appear as a monophyletic cluster. The TRX1 amino acids sequences described at database were used for construct tree: *Schistosoma mansoni* (GenBank accession number AAL79841); *Fasciola hepatica* (GenBank accession number AAF14217); *Glycine max* (GenBank accession number AAS88427); *Ricinus communis* (GenBank accession number CAA94534); *Capsicum annuum* (GenBank accession number AAR83852); *Nicotiana tabacum* (GenBank accession number CAA41415); *Chlamys farreri* (GenBank accession number AAV73827); *Geodia cydonium* (GenBank accession number CAA76654); *Suberites ficus* (GenBank accession number CAG25528); *Neurospora crassa* (GenBank accession number AAK07845); *Schizosaccharomyces pombe* (GenBank accession number AAF05765); *Aspergillus nidulans* (GenBank accession number AAB24444); *Penicillium chrysogenum* (GenBank accession number CAA53726); *Candida albicans* (GenBank accession number XP_719372); *Saccharomyces cerevisiae* (GenBank accession number CAA97572); *Coprinus comatus* (GenBank accession number CAB52130); *Paxillus involutus* (GenBank accession number AAS19462); *Cyanidium caldarium* (GenBank accession number CAA79820); *Mycoplasma gallisepticum* (GenBank accession number AAF19044); *Danio rerio* (GenBank accession number AAH49031); *Rattus novergicus* (GenBank accession number AAH58454); *Ovis aries* (GenBank accession number NP_001009421); *Sus scrofa* (GenBank accession number NP_999478); *Bos taurus* (GenBank accession number AAC83380); *Callithrix jacchus* (GenBank accession number AAK30295); *Homo sapiens* (GenBank accession number AAF87085); *Melopsittacus undulatus* (GenBank accession number AAO72714); *Gallus gallus* (GenBank accession number NP_990784); *Plasmodium falciparum* (GenBank

accession number NP_702434); *Aedes aegypti* (GenBank accession number AAK70900); *Anopheles gambiae* (GenBank accession number AAF68382).

Figure 3 - Effect of H_2O_2 on yeast cells growth, on mycelium to yeast transition and on the protein patterns during the yeast cells growth and during the transition from mycelium to yeast of *P. brasiliensis*. **(A)-** Yeast cells from logarithmic phase were treated with 25 mM H_2O_2 for 3 h at 36 ^{0}C. After this, they were washed and an inoculum's of $5x10^6$ cells mL^{-1} was resuspended in fresh YPD medium at 36 ^{0}C, with shaking. **(B)-** Mycelium was incubated at 26 ^{0}C in presence 25 mM H_2O_2 for 3 h. After this treatment, the mycelium was washed and 100 μg of mycelium was transferred to fresh YPD medium and incubated at 36 ^{0}C, with shaking. Aliquots were taken (A and B) daily and yeast cells were counted in Neubauer chamber. The values were expressed as a mean of triplicate. **C** and **E**- The proteins (20 μg) were fractionated by uni-dimensional gel electrophoresis (15% SDS-PAGE) and stained by Coomassie blue. **D** and **F**- Western blot of total cell extract from yeast cells control (lane 1) and yeast cells treated with H_2O_2 for 3 h (lane 2) and from mycelium treated with H_2O_2 for 3 h and incubated at 36 ^{0}C at 0, 12, 24 and 120 h, respectively. The protein extracts (20 μg for each lane) were probed with the polyclonal antibody to TRX 1 from *E. coli*.

Figure 4 - SDS-PAGE and western blotting analysis of the 38 kDa recombinant protein expressed by *E. coli* transformed with *Pb*trx1 construct. *E. coli* BL21 cells harboring pGEX-4T-3 (lane 1 and 2) and pGEX-4T-3-trx1 (lane 3 and 4) were grown at 30 ^{0}C to an A_{260} of 0.7 and harvested before (lane 1 and 3) and after (lane 2 and 4) a 3 h of incubation with 0.1 mM IPTG. Lane 5, the affinity-isolated recombinant GST-TRX1. Electrophoresis was carried out on 15% SDS-PAGE and the proteins were stained by Coomassie blue R-250. Lane 6, western blotting of the recombinant GST-TRX1 protein. The recombinant GST-TRX1 protein were

run in parallel, blotted onto nitrocellulose membrane, and was detected by using polyclonal antibody to TRX 1 from *E.* coli (Sigma). Arrow indicates the recombinant GST-TRX1 protein. Molecular Markers are indicated on the left (Amersham Biosciences).

Figure 5 - rec*Pb*TRX1 and native *Pb*TRX1 catalyzes the reduction of insulin. The increase in turbidity measured at 650 nm was plotted against reaction time. The conditions under which the catalytic activity of TRX1 was measured are presented in the text. DTT was used as a negative control. **(A)** - Insulin reduction by rec*Pb*TRX1. **(B)** - Insulin reduction by native *Pb*TRX1 from yeast cells protein extract. The values represent the media and standard deviations of three independent experiments.

Figure 6 - Semi-quantitave RT-PCR analyses for *Pb*trx1 of yeast cells exposure to H_2O_2. Semi-quantitative RT-PCR analysis was carried out with specific oligonucleotides sense and antisense to *Pb*trx1 and r28S, as described. Numbers associated with the bars indicate fold differences relative to the data for the reference *in vitro* cultured yeast cells, which were established by densitometry analysis. Using varied cycle numbers, the exponential phase of primer was determined and used to allow semi-quantitative analysis of the respective reactions. The same amounts of cDNAs were used for all PCRs. The RNAs used for RT-PCR were obtained from an independent sample of *in vitro* cultured yeast cells and from sample *in vitro* cultured yeast cells incubated with 25 mM H_2O for 30 min and 1 h. The sqRT-PCR products were separated on a 1% agarose gel and stained with ethidium bromide. The sizes of the amplified DNA fragments from *Pb*trx1 and from r28S are written on the right side of the figure. The RNAs samples were obtained from: yeast cells, *in vitro* cultured (Y); yeast cells incubated with 25 mM H_2O_2 for 30 min (Y 30); yeast cells incubated with 25 mM H_2O_2 for 1 h (Y60). Ribosomal 28S was used as an internal control.

Figure 1 A

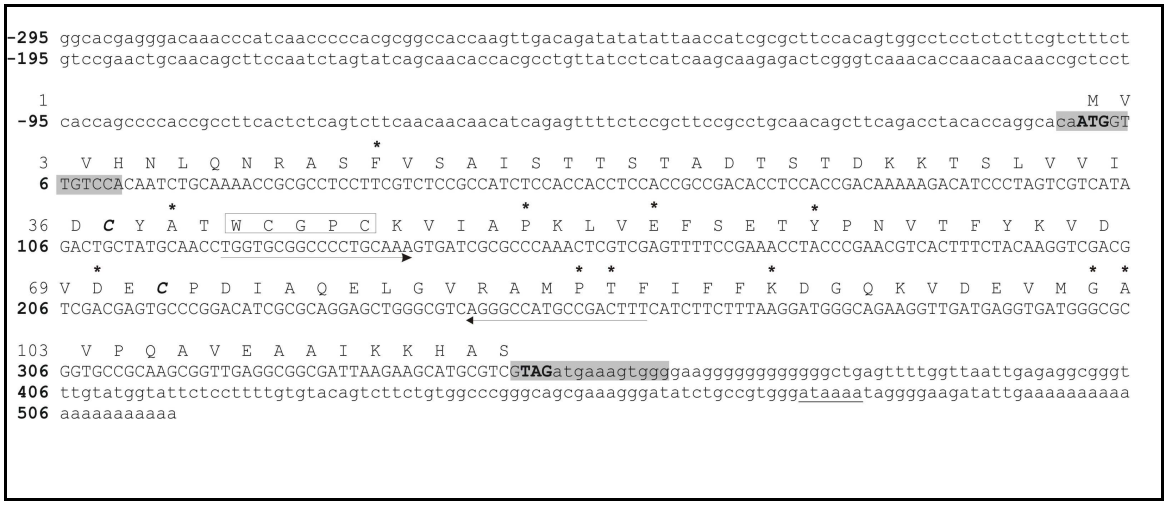

-295 ggcacgagggacaaacccatcaacccccacgcggccaccaagttgacagatatatattaaccatcgcgcttccacagtggcctcctctcttcgtctttct
-195 gtccgaactgcaacagcttccaatctagtatcagcaacaccacgcctgttatcctcatcaagcaagagactcgggtcaaacaccaacaacaaccgctcct
1 M V
-95 caccagccccaccgccttcactctcagtcttcaacaacaacatcagagttttctccgcttccgcctgcaacagcttcagacctacaccaggcacaATGGT
3 V H N L Q N R A S F V S A I S T T S T A D T S T D K K T S L V V I
6 TGTCCACAATCTGCAAAACCGCGCCTCCTTCGTCTCCGCCATCTCCACCACCTCCACCGCCGACACCTCCACCGACAAAAAGACATCCCTAGTCGTCATA
36 D C Y A T W C G P C K V I A P K L V E F S E T Y P N V T F Y K V D
106 GACTGCTATGCAACCTGGTGCGGCCCCTGCAAAGTGATCGCGCCCAAACTCGTCGAGTTTTCCGAAACCTACCCGAACGTCACTTTCTACAAGGTCGACG
69 V D E C P D I A Q E L G V R A M P T F I F F K D G Q K V D E V M G A
206 TCGACGAGTGCCCGGACATCGCGCAGGAGCTGGGCGTCAGGGCCATGCCGACTTTCATCTTCTTTAAGGATGGGCAGAAGGTTGATGAGGTGATGGGCGC
103 V P Q A V E A A I K K H A S
306 GGTGCCGCAAGCGGTTGAGGCGGCGATTAAGAAGCATGCGTCGTAGatgaaagtggggaagggggggggggggctgagttttggttaattgagaggcgggt
406 ttgtatggtattctccttttgtgtacagtcttctgtggcccgggcagcgaaagggatatctgccgtgggataaaatagggggaagatattgaaaaaaaaaa
506 aaaaaaaaaaa

Figure 1 B

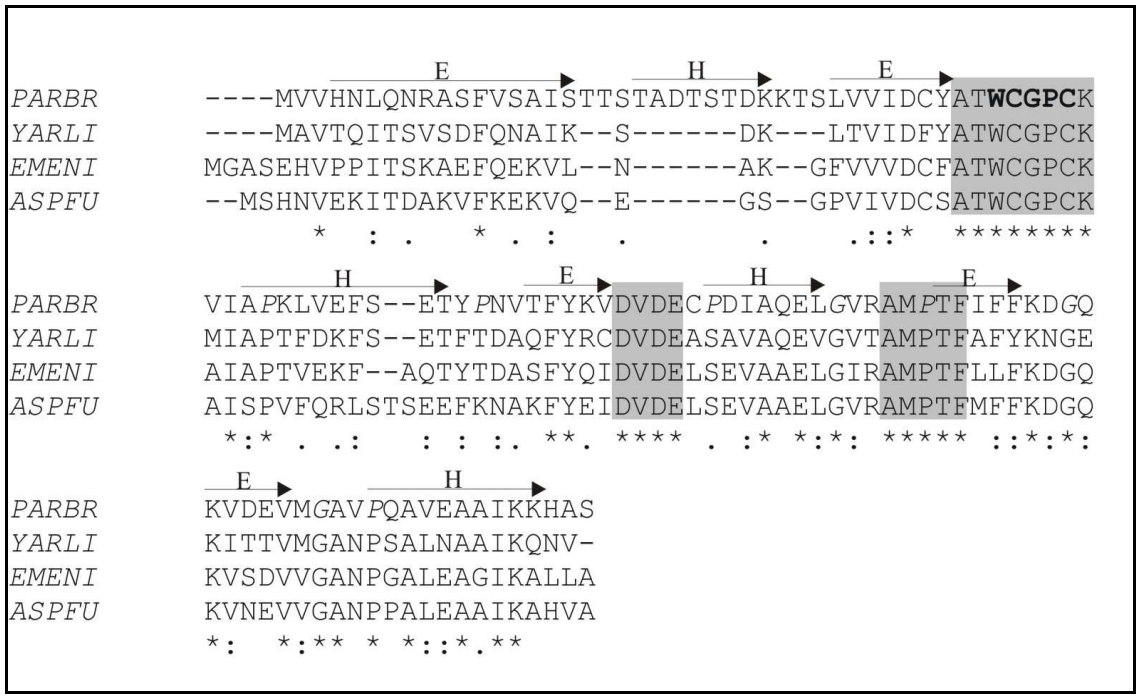

E
H
E
PARBR ----MVVHNLQNRASFVSAISTTSTADTSTDKKTSLVVIDCYATWCGPCK
YARLI ----MAVTQITSVSDFQNAIK--S------DK---LTVIDFYATWCGPCK
EMENI MGASEHVPPITSKAEFQEKVL--N------AK--GFVVVDCFATWCGPCK
ASPFU --MSHNVEKITDAKVFKEKVQ--E------GS--GPVIVDCSATWCGPCK
H
E
H
E
PARBR VIAPKLVEFS--ETYPNVTFYKVDVDECPDIAQELGVRAMPTFIFFKDGQ
YARLI MIAPTFDKFS--ETFTDAQFYRCDVDEASAVAQEVGVTAMPTFAFYKNGE
EMENI AIAPTVEKF--AQTYTDASFYQIDVDELSEVAAELGIRAMPTFLLFKDGQ
ASPFU AISPVFQRLSTSEEFKNAKFYEIDVDELSEVAAELGVRAMPTFMFFKDGQ
E
H
PARBR KVDEVMGAVPQAVEAAIKKHAS
YARLI KITTVMGANPSALNAAIKQNV-
EMENI KVSDVVGANPGALEAGIKALLA
ASPFU KVNEVVGANPPALEAAIKAHVA

Figure 2

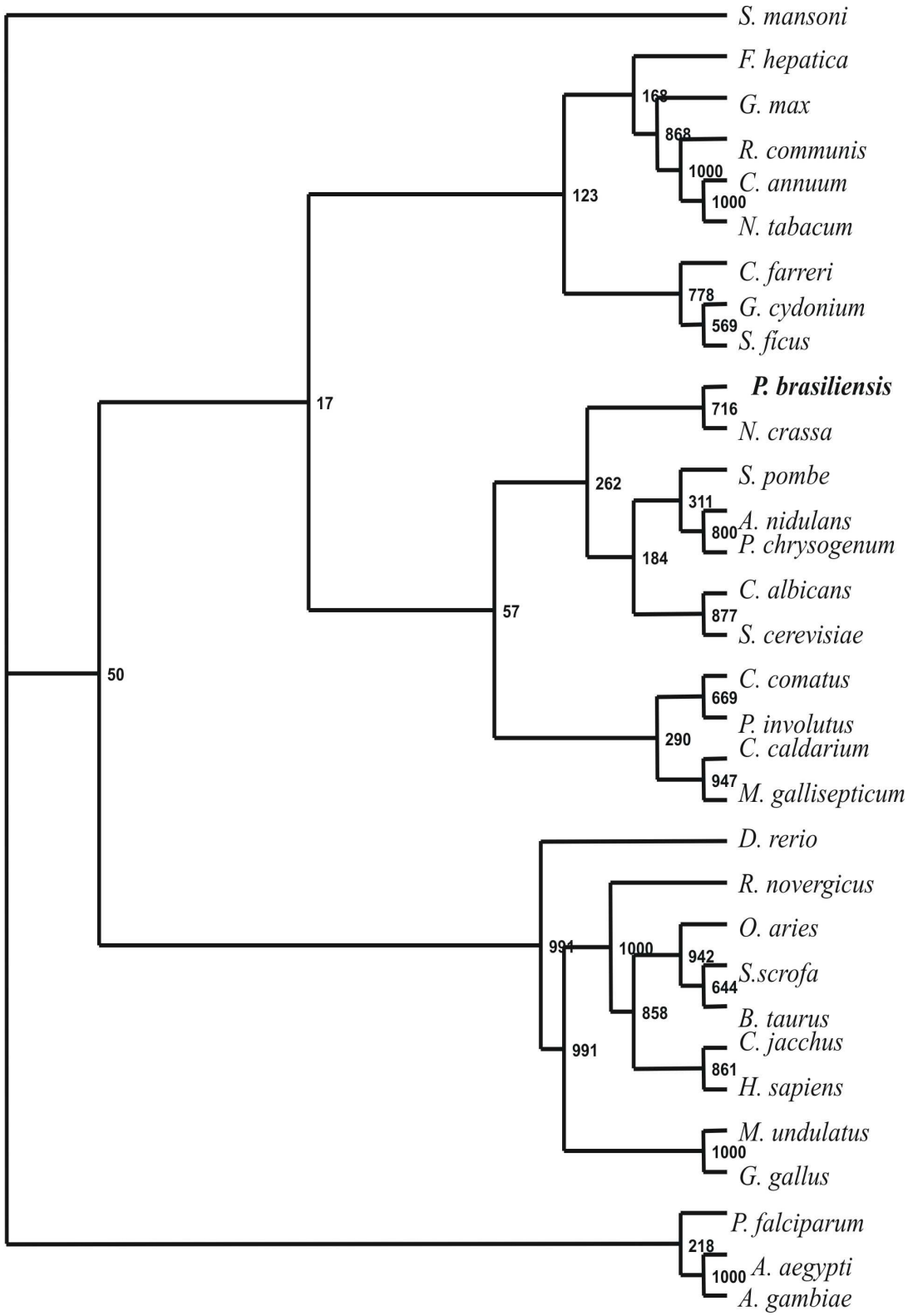

S. mansoni
F. hepatica
168
G. max
868
R. communis
1000
C. annuum
123
1000
N. tabacum
C. farreri
778
G. cydonium
569
S. ficus
P. brasiliensis
17
716
N. crassa
S. pombe
262
311
A. nidulans
800
P. chrysogenum
184
C. albicans
57
877
S. cerevisiae
C. comatus
669
P. involutus
290
C. caldarium
947
M. gallisepticum
D. rerio
50
R. novergicus
994
1000
O. aries
942
S.scrofa
644
858
B. taurus
991
C. jacchus
861
H. sapiens
M. undulatus
1000
G. gallus
P. falciparum
218
A. aegypti
1000
A. gambiae

Figure 3

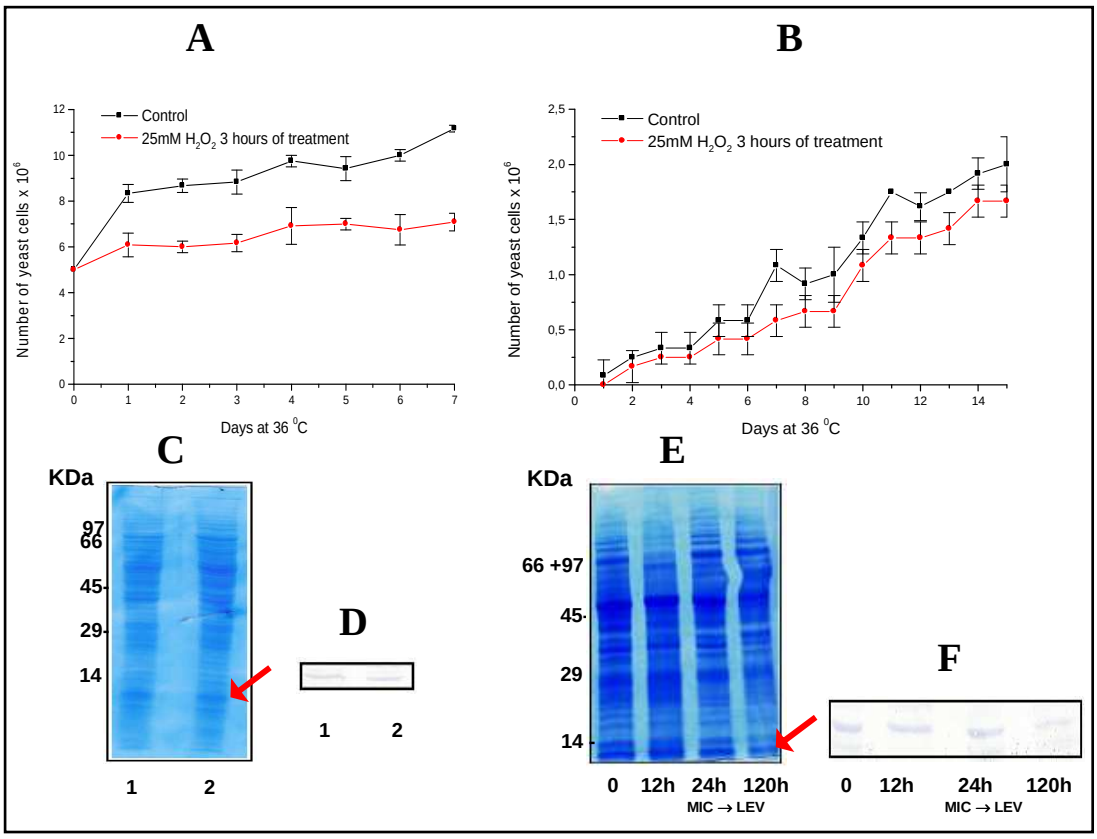

A
B
Control
25mM H2O2 3 hours of treatment
Number of yeast cells x 10^6
Days at 36 °C
C
D
E
F
KDa
97
66
45
29
14
1
2
66 +97
0
12h
24h
120h
MIC → LEV

Figure 4

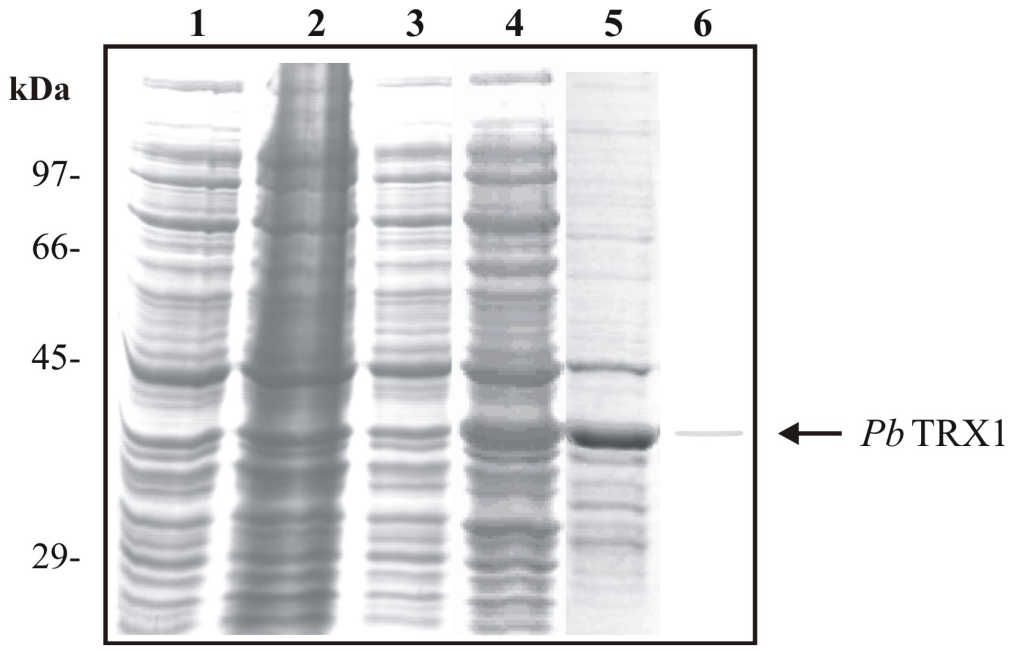
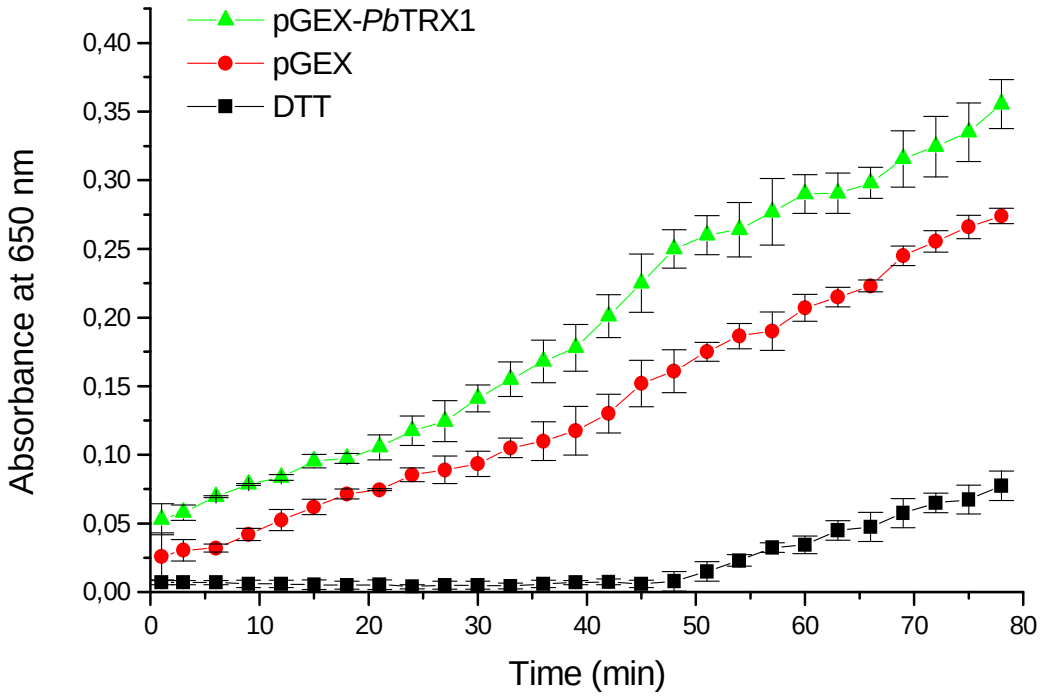


Figure 5

A


pGEX-*Pb*TRX1
pGEX
DTT
Absorbance at 650 nm
Time (min)
0,00
0,05
0,10
0,15
0,20
0,25
0,30
0,35
0,40
0
10
20
30
40
50
60
70
80

B

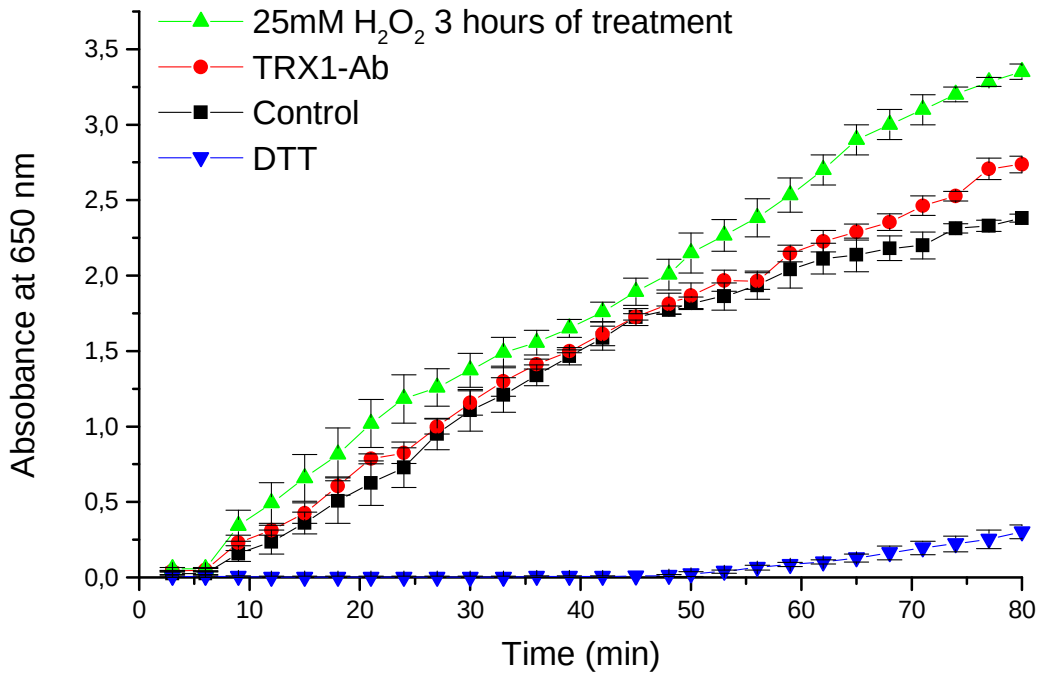
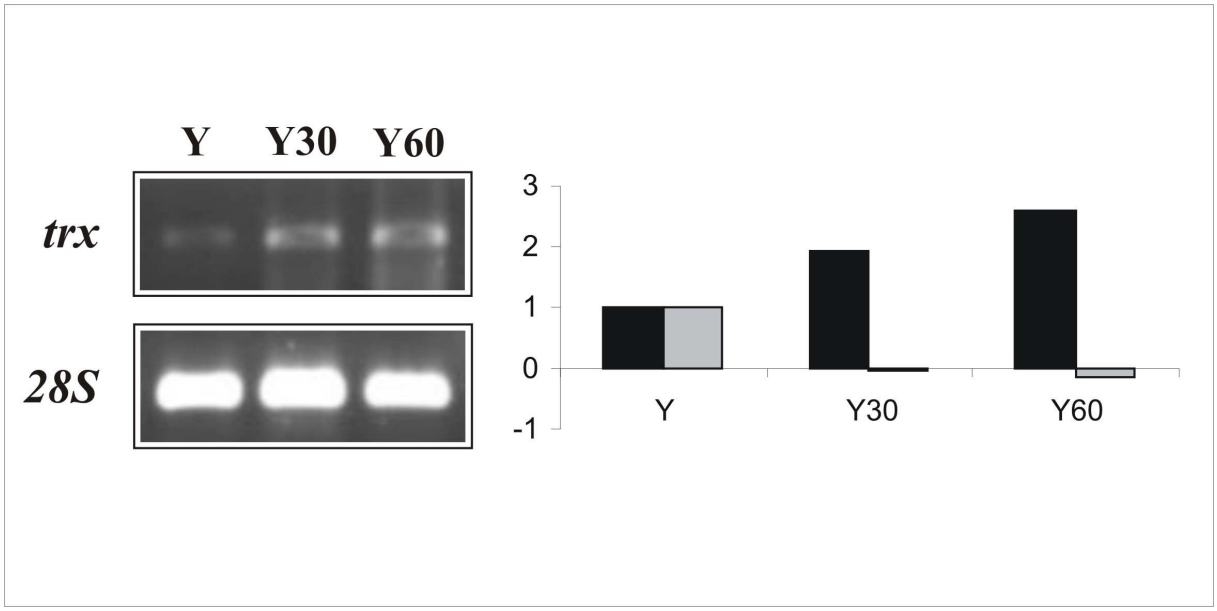

25mM H_2O_2 3 hours of treatment
TRX1-Ab
Control
DTT
Absorbance at 650 nm
Time (min)
0,0
0,5
1,0
1,5
2,0
2,5
3,0
3,5
0
10
20
30
40
50
60
70
80

Figure 6



V – CONCLUSÃO

O cDNA *Pb*trx1 caracterizado neste trabalho apresentou 811 pb, com um quadro aberto de leitura de 351 pb. *Pb*trx1 codifica para uma proteína *Pb*TRX1, com massa molecular predita de 12 kDa e p*I* de 5,2.

Foi observada em *Pb*TRX1 uma seqüência altamente conservada na região carboxi terminal, WCGPC, correspondendo ao sítio ativo das TRXs. Este sítio ativo é responsável pela função oxirredutora dissulfeto desta proteína.

A árvore filogenética construída por comparação das seqüências de aminoácidos de *Pb*TRX1 e TRXs de outros organismos colocou *P. brasiliensis* no clado dos fungos.

A estrutura secundária da *Pb*TRX1 definida por comparação com outras TRXs demonstrou um padrão conservado de estrutura secundária descrito para outras TRXs, formando 5 cadeias-β que estão envolvidas por 4 cadeias em α-hélices.

A análise da seqüência de aminoácidos da *Pb*TRX1 sugere que esta proteína tenha uma localização citoplasmática.

O cDNA *Pb*trx1 foi clonado no vetor de expressão pGEX-4T-3. A expressão e purificação parcial da proteína recombinante (rec*Pb*TRX1) foi obtida. A proteína rec*Pb*TRX1 e a proteína *Pb*TRX1 presente no extrato protéico de células leveduriformes apresentaram atividade redutora de insulina e foram reconhecidas pelo anticorpo anti-TRX1 de *E. coli*.

O crescimento das células leveduriformes foi inibido por H_2O_2, demonstrando diferença significativa em relação ao controle. Entretanto, a transição de micélio para levedura na presença de H_2O_2 não mostrou diferença significativa de inbição quando comparada ao controle.

A análise da expressão do gene *Pb*trx1 em resposta ao H_2O_2, usando RT-PCR semi-quantitativo, detectou um maior número de transcritos nas células leveduriformes tratadas com H_2O_2 em comparação às células não tratadas.

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VII – ANEXOS

ANEXO I: NORMAS DA REVISTA

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