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Purification of *Paracoccidioides brasiliensis* catalase P: subsequent kinetic and stability studies

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Catalases are essential components of the cellular equipment to cope with oxidative stress. Here we have purified a highly abundant catalase P of *Paracoccidioides brasiliensis* (*Pb*CatP) that is preferentially expressed in the parasitic yeast phase. This oxidative stress-induced protein was isolated from yeast cells grown in the presence of 15 mM of hydrogen peroxide (H_2O_2). We have used consecutive steps of protein precipitation and gel filtration chromatography to achieve the purified protein. Protein purification was validated using matrix-assisted laser desorption ionization time-of-flight mass spectrometry and bioinformatics analysis. The purified enzyme showed strong similarity to small-subunit catalases. Like most monofunctional catalases, *Pb*CatP is a homotetramer, resistant to inactivation by acidic conditions, temperature and denaturants. Furthermore, the kinetic behaviour of catalase P was observed to be different at low compared to high H_2O_2 concentrations. The results demonstrated that a purified *Pb*CatP is a homotetrameric enzyme, classified as a small subunit catalase.

Keywords: Catalase P/Homotetrameric enzyme/Mass spectrometry/*Paracoccidioides brasiliensis*/Protein purification and characterization.

Among several potentially harmful reactive oxygen species (ROS), hydrogen peroxide (H_2O_2) is probably the most abundant, occurring in the cells and in their environment. H_2O_2 is produced intracellularly as a by-product in reactions catalysed by several oxidases (*1*). Catalases (EC 1.11.1.6) are enzymes capable of consuming H_2O_2, reducing the harmful effect of this reactive oxygen compound and protecting the cellular environment against oxidative stress.

The catalytic reaction by catalases takes place in two steps; first the H_2O_2 molecule oxidizes haeme to an oxyferryl species in which one oxidation equivalent is removed from the iron and one from the porphyrin ring to generate a porphyrin cation radical. Next, H_2O_2 is then used as a reductant of compound I to regenerate the resting state enzyme, water and oxygen. Catalases can also assume an inactive state, called compound II, a product of compound I, during exposure to its own substrate, H_2O_2. The fraction of catalase becoming compound II reaches a steady-state level during exposure to H_2O_2 that is generated at a constant rate. When exposure to H_2O_2 stops, catalase returns to the ferricatalase state (*2*). A tightly bound NADP + and NADPH were discovered in catalase preparations (*3*) that prevents or reverses the formation of compound II by small catalases in the presence of H_2O_2 (*4*, *5*).

The three families of catalases are Mn catalases, bifunctional catalase-peroxidases and monofunctional or true catalases. The last family corresponds to homotetrameric haeme-containing enzymes that are composed of two clearly distinct classes. These classes can be recognized by the size of the subunits: the small-subunit catalases (< 60 kDa) and large-subunit catalases (> 75 kDa); the last group can be subdivided in spore-specific catalases and secreted catalases (*6*).

The dimorphic fungus *Paracoccidioides brasiliensis* can cause paracoccidioidomycosis (PCM) in the human host. After inhalation into the lung, the fungus disseminates and replicates in multiple tissues (*7*). Fungal stressors include those that are potentially toxic such as reactive oxidative species. Studies have demonstrated that cytokine activation is required for *P. brasiliensis* killing by phagocytes and that H_2O_2 and superoxide anion participate as effector molecules in this process (*8*). Our laboratory had previously characterized a small-subunit monofunctional catalase in *P. brasiliensis*, named catalase P (*Pb*CatP) that presents high levels of expression in the yeast parasitic phase and is regulated in the transition from mycelium to yeast cells. *Pb*CatP can be induced by H_2O_2 as part of the cellular responses to oxidative stress (*9*, *10*). The protein was characterized as an immunodominant antigen of *P. brasiliensis* reacting with sera of patients with PCM (*11*). Additionally, the transcript was induced in an *ex vivo* model of macrophage infection, suggesting that the *Pb*CatP is involved in the control of exogenous ROS (*10*).

In this study, we have described, for the first time, the characterization of *Pb*CatP from *P. brasiliensis*. Here, we have used successive steps of protein precipitation and gel filtration chromatography to purify the fungal *Pb*CatP. The purification procedures were followed by SDS–PAGE and non-denaturing gel analysis. The *Pb*CatP amino acid sequence was verified by mass spectrometric (MS) analysis. *Pb*CatP exhibited resistance to inactivation by temperature and denaturants.

Different kinetic behaviour at low and high H_2O_2 concentrations for *Pb*CatP was also observed. Importantly, the native protein purifies as a high molecular mass species suggesting a tetrameric structure as previously described (*6*). Future investigations pertaining to the roles of this protein in host–fungus interactions may provide the key knowledge to understanding the pathogenicity of *P. brasiliensis*.

Materials and Methods

Paracoccidioides brasiliensis isolate and growth conditions

The *P. brasiliensis* isolate *Pb*01 (ATCC MYA-826) was grown in Fava–Netto's medium [1% (w/v) peptone, 0.5% (w/v) yeast extract, 0.3% (w/v) proteose peptone, 0.5% (w/v) beef extract, 0.5% (w/v) NaCl, 1% (w/v) agar, pH 7.2] at 36°C for the yeast form.

Expression of PbCatP

The yeast cells were scraped off the medium cited above and grown in a liquid SP buffer (50 mM sodium phosphate buffer, pH 7.2) for 2 h. The expression of *Pb*CatP was induced by the addition of H_2O_2 (15 mM) for 2 h. The cells expressing the inducible *Pb*CatP were harvested by centrifugation at 5,000*g* for 10 min.

Purification of P. brasiliensis PbCatP

Purification steps were carried out at 0–4°C unless otherwise stated. *P. brasiliensis* yeast cells (9.0 g) were collected by centrifugation at 5,000*g* for 10 min and washed. Yeast protein extracts were obtained by disruption of frozen cells in the presence of proteases inhibitors (50 μg ml^{-1}) *N*α-p-tosyl-L-lysine chloromethyl ketone (TLCK), (1 mM) 4-chloromercuribenzoic acid, (20 mM) leupeptin, (20 mM) phenylmethylsulphonyl fluoride and (5 mM) iodoacetamine in the homogenization buffer (2 mM $CaCl_2$, 20 mM Tris–HCl, pH 8.8). The crude extract was precipitated by addition of 100% cold acetone (ratio 1: 3) at 4°C. The mixture was centrifuged at 12,000*g* at 4°C for 10 min and the pellet was resuspended in 1.0 ml of SP buffer. The homogenate (1.0 ml) was applied to a column (18.5 cm × 0.8 cm) of Sephadex G-150 (Sigma-Aldrich, St Louis, MO, USA) equilibrated and eluted with SP buffer, applying a flow rate of 1.0 ml min^{-1}. Fractions of 1.0 ml were collected, and those yielding enzymatic activity and high molecular mass (Supplementary Fig. S1) were pooled. Samples were concentrated to 1.0 ml by precipitation with cold acetone, as described earlier. Each purification step was followed by enzymatic assay, native polyacrylamide gel electrophoresis (PAGE) and in-gel activity staining of catalases, as described below.

Native PAGE

The cellular extracts were prepared as described earlier. Protein quantification was carried out by the Bradford method (*12*) at each step of the purification process, using bovine serum albumin as standard. Electrophoresis of native proteins was performed as previously described (*13*). The samples were resuspended in the lyses buffer containing 9.5 M urea, 2% (v/v) Nonidet P-40, 0.5% (v/v) β-mercaptoethanol and ampholines at pH ranges of 5.0–8.0 and 3.5–10.0 (ratio 4:1). The proteins were analysed by sodium dodecyl sulphate PAGE (SDS–PAGE) according to Laemmli (*14*), and Coomassie Blue stained.

Enzymatic determination of catalase activity

Catalase activity was determined by measuring the absorbance during the conversion of H_2O_2 to oxygen (*10*, *15*). Catalase-catalyzed decomposition of H_2O_2 was monitored by the decrease in the absorbance at 240 nm, utilizing the extinction coefficient of 39.40 M^{-1} cm^{-1}. The activity was calculated using a standard curve generated by different concentrations of H_2O_2 monitored at 240 nm. One unit of activity was defined as the amount of enzyme that catalysed the consumption of 1 μmol of H_2O_2 per min (*15*).

Determination of catalase activity on polyacrylamide gels

A ferricyanide-negative stain was used to locate catalase species on native polyacrylamide gels following the method of Wayne and Díaz (*16*).PAGE under nondenaturing conditions was performed at 200 V for 6 h, at 4°C. Catalase activity was determined by incubating the gel for 10 min in the SP buffer, containing 10 mM of H_2O_2. The gel was rinsed three times with water and incubated in a mixture (v/v) of freshly prepared 1.0% (w/v) potassium ferricyanide and 1.0% (w/v) ferric chloride. Green color developed in the gel except at zones where peroxide molecule was degraded by catalase.

In-gel digestion

Protein bands were excised and sliced into small pieces (~1 mm) and placed into 0.65 ml siliconized tubes (PGC Scientific). Fifty microlitres of 25 mM ammonium bicarbonate/50% (v/v) acetonitrile (ACN) were added to each sample, followed by vortexing for 10 min. The supernatant was discarded and this step was repeated twice. Gel pieces were then brought up in 100 μl of ACN and placed in a speed vacuum until dry. Next, 50 μl of 10 mM dithiothreitol (DTT) in 25 mM ammonium bicarbonate was added to the dried gel pieces. Samples were vortexed and spun briefly. The reaction was allowed to proceed at 56°C for 1 h. The supernatant was removed and 50 μl of 55 mM iodoacetamide was added to the gel pieces. Samples were vortexed and spun briefly. The reaction was allowed to proceed in the dark for 45 min at room temperature. Supernatant was removed and the gels were washed with 100 μl of ammonium bicarbonate by vortexing for 10 min. Supernatant was removed and gel pieces were dehydrated in 100 μl of 25 mM ammonium bicarbonate/50% (v/v) ACN, vortexed for 5 min and spun. This step was repeated. Gel pieces were placed in a speed vacuum until dry. Thirty microlitres of a 12.5 ng ml^{-1} trypsin (Pierce, Rockford, IL, USA) solution was added to the gel pieces that were rehydrated on ice at 4°C for 10 min. Samples were spun down and 25 μl of 25 mM of ammonium bicarbonate was added. Samples were spun briefly and incubated at 37°C overnight. Following overnight digestion, the solution was placed into a clean 0.65 ml siliconized tube. To the gel pieces, 50 μl of 50% (v/v) ACN/5% (v/v) trifluoroacetic acid acid (TFA) was added. Samples were vortexed for 30 min, sonicated for 5 min and the solution was combined with the first aqueous extraction. This step was repeated and samples were then dried in a speed vacuum.

Mass spectrometric analysis

Dried peptide extracts were dissolved in 5 μl of water. Next, 2.5 μl of sample was mixed with 2.5 μl of α-cyano-4-hydroxycinammic acid in 50% (v/v) ACN and 5% (v/v) TFA, 10 mg ml^{-1}. Mass spectra were collected on a Voyager-DE PRO (Applied Biosystems) matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS. Each spectrum was an average of 200 spectra. Trypsin autodigestion peaks (2211.11 and 842.51) were used to calibrate each spectrum.

Saturation kinetics

Kinetic analysis was performed using Microcal Origin (Microcal Software, Inc. Northampton, MA, USA). *Pb*CatP was active in the range of 0.01–3.0 M H_2O_2. To protect catalase P against inactivation by its substrate, 10 μg of protein was incubated in the presence of different concentrations of NADPH and the catalase activity was measured as described earlier.

pH dependence and pH inactivation of PbCatP

For determination of the pH dependence of enzyme activity, a mixture of 50 mM of the following buffers 2-(*N*-morpholino) ethanesulphonic acid (MES), 4-(2-hydroxyethyl) piperazine-1-ethanesulphonic acid (HEPES), 2-(*N*-cyclohexylamino) ethanesulphonic acid (CHES) and 3-(cyclohexylamino)-1-propanesulphonic acid (CAPS) were adjusted to the desired pH with 0.5 M HCl or 0.5 M NaOH. Catalase activity was assayed with 10 mM of H_2O_2. To measure *Pb*CatP stability in the acid medium, the enzyme was incubated for 5 min in the 50 mM acetate buffer (pH 3.0–7.0) and the activity was determined using 10 mM H_2O_2. Fractions of the acidic treatment were washed and precipitated with cold acetone 100% (ratio 1: 3) at 4°C. The samples were ressuspended in 50 mM phosphate buffer, pH 7.8, for renaturing assays. Activity assayed at pH 7.8 in 50 mM phosphate buffer was set as 100%.

Determination of PbCatP thermal and chemical stabilities

*Pb*CatP (10 μg in 20 μl of 50 mM phosphate buffer, pH 7.8) was incubated at 65°C for different times (0, 5, 10, 20, 30, 40, 50 and 60 min) and samples from this heat treatment were incubated at 40°C

for renaturing analysis. The remaining activity was assayed by PAGE (5 µg in each lane) under non-denaturing conditions as described earlier and stained for catalase activity and with Coomassie Brilliant Blue. *Pb*CatP (20 µg in 20 µl of the 50 mM sodium phosphate buffer, pH 7.8) was incubated with different concentrations of guanidine hydrochloride (Boehringer Mannhein, Mannhein, Germany) for 48 h at room temperature. Activity was assayed by diluting the incubation mixture 1:3 directly in 2 ml of 10 mM H_2O_2 in 50 mM phosphate buffer, pH 7.8. The remaining incubation mixture was dialysed by centrifuging the sample in a Centricon filter at 5,000*g* by washing three times with 2 mM of the sodium phosphate buffer, pH 7.8. Samples were divided into two fractions and analysed by PAGE under non-denaturing conditions. Gels were run in duplicate, stained for activity and with Coomassie Brilliant Blue.

Inhibition and inactivation of PbCatP

Irreversible inactivation of *Pb*CatP by 3-amino-1,2,4-triazole was investigated at 37°C by incubation of 20 µg of the enzyme in 1.0 ml of 50 mM sodium phosphate buffer, pH 7.8, containing 20 mM 3-amino-1,2,4-triazole, 4 mM ascorbate and 4 mM H_2O_2. Samples (50 µl) were withdrawn every 5 min from the incubation mixture and the remaining catalase activity was assayed. *Pb*CatP (1 µg in 2 ml of 50 mM sodium phosphate buffer, pH 7.8) was incubated at 37°C for 1 min in the presence of a different concentrations of azide or hydroxylamide, and the remaining activity was measured by adding 10 mM H_2O_2 to the mixture.

Results and Discussion

Purification of the PbCatP

*Pb*CatP was chromatographically purified on a Sephadex G-150 column and the resulting chromatogram is shown in Supplementary Fig. S1. Catalase activity was observed in P1 and P2, as demonstrated in Supplementary Fig. S1. Table 1 describes catalase P purification. Crude enzyme extract was precipitated using cold acetone. The catalase yield and purity were 79% and 1.3-fold, respectively. Precipitated fractions were then subjected to size exclusion chromatography after precipitation with cold acetone. The catalase purification increased 3-fold and the enzyme yield was 62%. Starting with 9.0 g of *P. brasiliensis* yeast cells, 2.0 mg of purified catalase P were obtained. The success of this approach was achieved due to induction of catalase expression by H_2O_2, as can be seen by the high final specific activity of 1,133.9 U mg^{-1}. In synthesis, the high level of the protein in *P. brasiliensis* (3,665 U mg^{-1}) allowed its purification by simple steps that yield nearly homogenous catalase with 60% recovery.

The purity of the final preparation of the partially purified enzyme was analysed by denaturing SDS–PAGE and by in-gel catalase assays (Table 1). In a single chromatographic step, we were able to purify catalase to near homogeneity. The purified enzyme migrated as a single protein species in electrophoresis under non-denaturing conditions, indicating that a pure protein species had been isolated after the final purification step (Fig. 1, lane 1).

Three catalases of *P. brasiliensis* (*Pb*CatA, *Pb*CatP and *Pb*CatC) were also detected by in-gel activity of the crude protein extracts (data not shown). The position of the protein species in the non-denaturing gels is consistent with previous descriptions of the catalases in fungi (*17*), as we had previously described (*10*). One H_2O_2-reactive band was detected in the potassium ferricyanide-negative-stained gel (Fig. 1, lane 2). The purified catalase migrated as a species of 244 kDa, corresponding to the predicted homotetramer of the enzyme (*18*, *19*) (Fig. 1, lanes 1 and 2). Similar results were observed during the molecular analysis of the purified catalase-1 from *Neurospora crassa*. In this organism, data indicated that the native catalase-1 protein, like most monofunctional catalases, can be considered a homotetramer (*18*, *19*).

MS analysis of purified PbCatP

The purified fraction was resolved by 2D gel electrophoresis (Fig. 2A). The migration of the protein in the gel corresponded to a p*I* of 6.2 and molecular mass

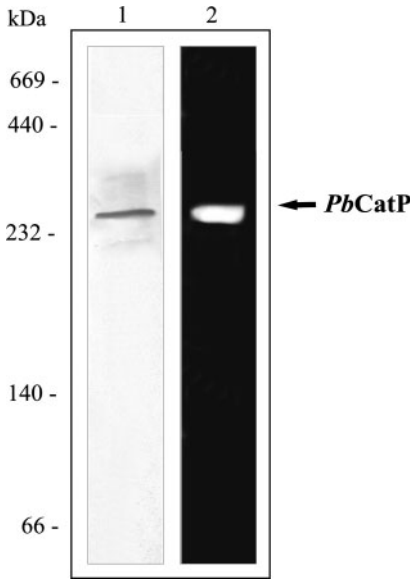

Fig. 1 Analysis of the purified catalase under non-denaturing conditions. The purified protein sample (5 µg) was analysed: (*1*) in native PAGE stained with Coomassie Blue; (*2*) in native PAGE stained for catalase activity.

Table 1. Purification of the *Pb*CatA.

Fraction	Total activity[a] (U ml^{-1})	Amount of protein[b] (mg ml^{-1})	Specific activity[c] (U mg^{-1})	Purification (fold)[d]	Yield (%)[e]	In-gel catalase activity[f]
Crude extract	3665.6	10.0	366.6	1	100	*Pb*CatA, *Pb*CatP e *Pb*CatC.
Acetone precipitation	2889.1	6.0	481.5	1.3	79	*Pb*CatA, *Pb*CatP e *Pb*CatC.
Gel filtration	2267.8	2.0	1133.9	3.1	62	*Pb*CatP.

[a]Amount of protein measured in 1.0 ml of protein extract. [b]Protein concentration was determined by the method of Bradford, using BSA as standard. [c]Units of protein in 1.0 mg of crude extract. [d]Calculated as the ratio between the specific activity and total activity. [e]Percentage of remaining activity in each step. [f]Catalases species determined by in-gel analysis.

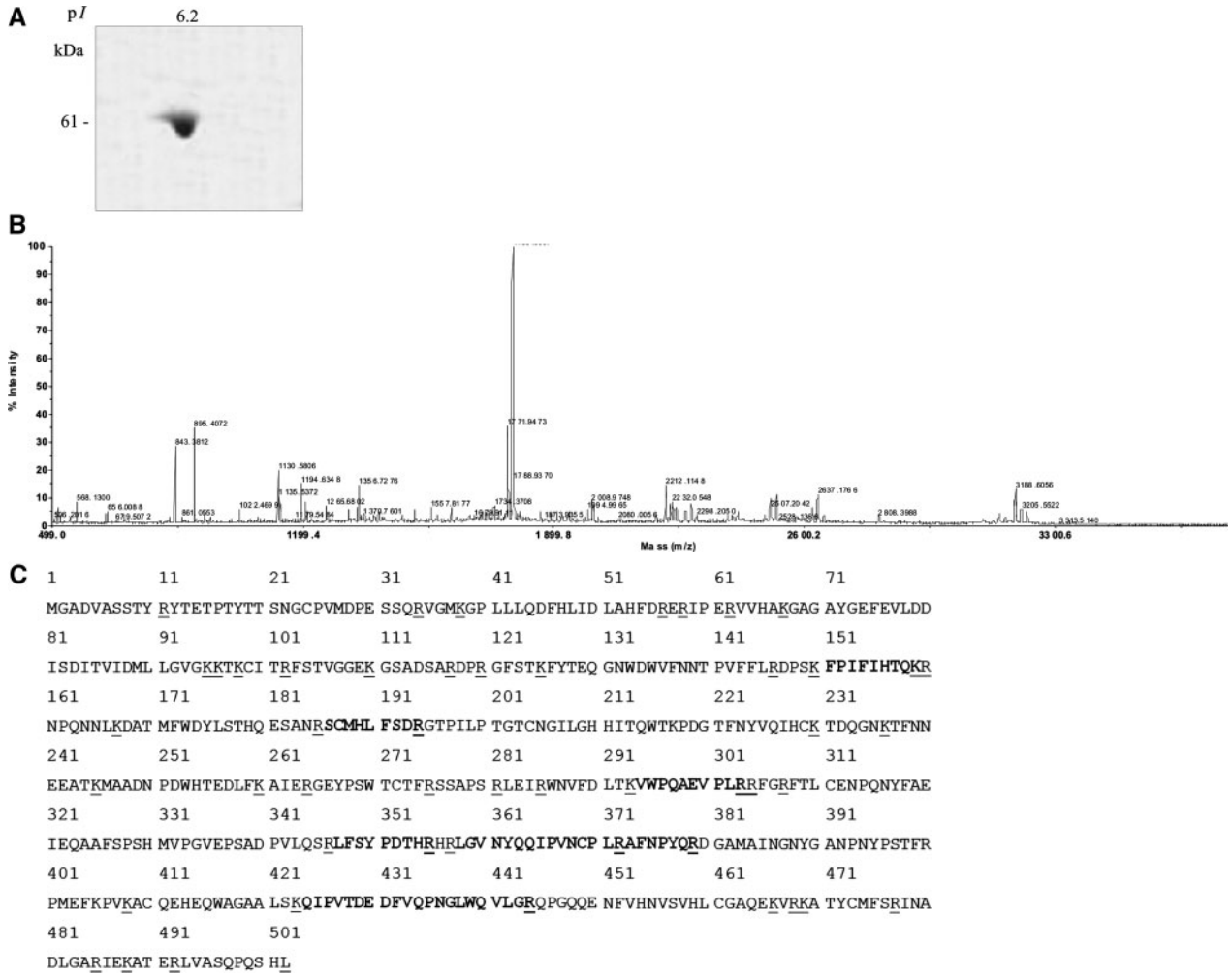

Fig. 2 2D electrophoresis of the purified protein and a representative peptide sequence obtained by enzymatic digestion and MS/MS. (A) The purified protein fraction obtained from gel filtration was analysed by 2D gel electrophoresis. The deduced molecular mass and isoeletric point are indicated. The protein spot was excised, digested by trypsin and submitted to MS as detailed in the 'Materials and Methods' section. (B) MS spectra of the purified *Pb*CatP after 2D protein fractionation and subsequent protein digestion. (C) Catalase P sequences detected by spectrometry analysis. The sequence of the deduced catalase P encoded by *PbcatP* (GenBank accession number AF428076) is shown. The peptides sequences detected by MS are indicated in bold letters.

of 61 kDa. Figure 2B shows the representative mass spectrum acquired from a tryptic digestion of the protein spot in figure 2A. The MS data were searched against public fungal databases using MASCOT (http://www.matrixscience.com). Seven masses out of 20 matched the *P. brasiliensis* catalase P (GenBank accession no. AF428076) sequence yielding a probability-based MOWSE score of 86. This hit was considered significant, as it was the only protein sequence, which exceeded the threshold score calculated by the MASCOT software assuming $P < 0.05$. Figure 2C shows the seven peptides detected by MS mapped onto the *P. brasiliensis* catalase P amino acid sequence. Thirteen out of the 20 masses detected by MALDI-TOF MS were not matched with the catalase P amino acid sequence suggesting that there is most likely another protein, which co-migrated with catalase P, or the catalase P protein is extensively modified.

Catalytic properties of PbCatP

Catalase has long been recognized as an enzyme with a rapid turnover rate and the maximum observed velocities range from 54,000 to 833,000 reactions per second (*20*). The H_2O_2 concentrations at which 50% of maximal activity is attained (apparent K_m) revealed a similar broad variation from 38 to 600 mM (*20*). The classical terms, V_{max}, k_{cat} and K_m, cannot be directly applied to the observed data since catalases do not exhibit Michaelis–Menten kinetics over the complete substrate concentration range. Similar to other oxidoreductases, such as Horseradish peroxidases (*21*), all small subunit catalases are inhibited by high substrate concentrations presenting the typical two-step nature kinetics (*6*). However, at H_2O_2 concentrations below 200 mM, all small subunit catalases exhibit a Michaelis–Menten-like dependence of velocity on the H_2O_2 concentration, and these data were used to calculate theoretical K_m and V_{max} values (Table 1, supplementary material). The K_m and V_{max} were theoretically estimated to be 150 and 380 mM, respectively. The two-step nature kinetics of *Pb*CatP and the theoretical dependence of velocity on the substrate concentration were calculated using the Michaelis–Menten equation as depicted in Fig. 3A

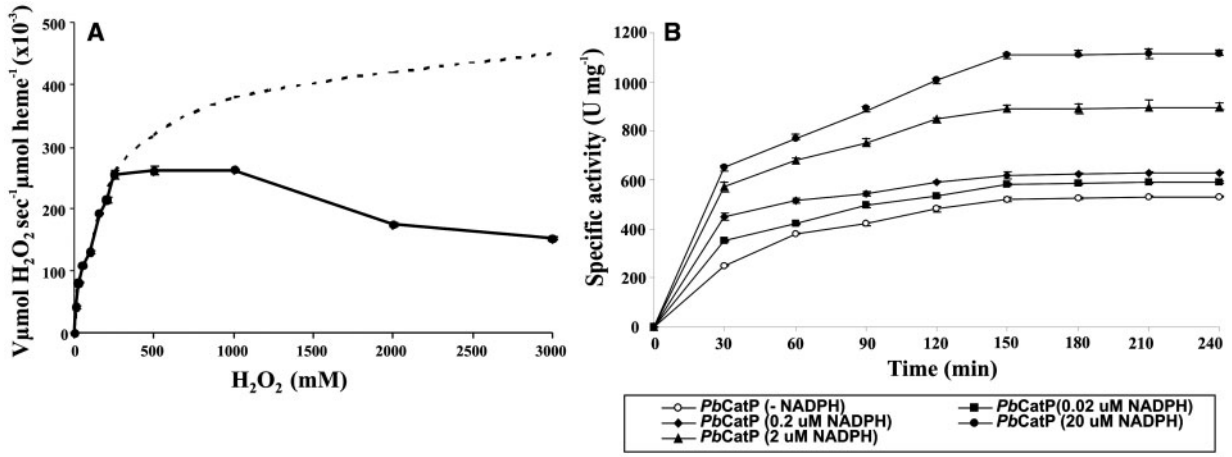

Fig. 3 Catalytic properties of *Pb*CatP. (A) The *Pb*CatP kinetics in the presence of millimolar concentration of H_2O_2. In the panel, the solid and dashed lines represent, respectively, the observed and theoretical Michaelis–Menten curves calculated from constants determined at a low H_2O_2 concentration. (B) Protection of purified catalase P by NADPH. Catalase activity was determined as described. Increasing amounts of NADPH were added to the assay mixture. All assays were repeated in triplicate and the results were averaged.

(dashed lines). There is a correlation between the theoretical and observed curves at a low concentration of H_2O_2. This correlation decreases at higher concentrations of H_2O_2, presumably as a result of the enzyme inactivation caused by the reactive substrate, H_2O_2, also observed for other catalases (*22*).The data shown for *P. brasiliensis* catalase P (Fig. 3A) are typical of the observed and theoretical curves for other small-subunit catalases (*20*). Most catalases are characterized at low substrate concentrations where the data resemble Michaelis–Menten kinetics sufficiently well to be misleading, but the variation of observed K_m and V_{max} values from calculated values presented here demonstrated the discrepancy. Other point is that specific activities of catalases are usually determined at substrate concentrations below the apparent K_m where there is great variation in velocity with substrate concentration, and this makes meaningful comparisons of literature data virtually impossible.

Several catalases are susceptible to inactivation by their own substrate, H_2O_2, but this can be largely prevented by bound NADPH (*23*). Addition of NADPH indeed stimulated the activity of *Pb*CatP in a dose-dependent manner (Fig. 3B) most likely by preventing the inactivation from oxidative damage, as has been reported for other small catalases (*24*).

Effect of inhibitors on enzyme activity

Catalases are sensitive to a number of compounds that interact with active-site haeme groups including azides, hydroxyl amines and aminotriazoles. The irreversible specific inhibitor of monofunctional catalases, 3-amino-1,2,4-triazole (*25*), inactivated *Pb*CatP at 20 mM following first-order kinetics, $k = 0.05 \pm 0.001\ min^{-1}$, shown in Supplementary Fig. 2A. Using this k value an inhibition of 50% was calculated following 15 min treatment. These values are similar to the reported values for CAT-1 of *N. crassa* (*26*).

Inactivation kinetics by hydroxylamide, another irreversible inhibitor of catalases, was measured at different concentrations. Catalase P was sensitive to hydroxylamine in the μM range. Inactivation was first order with a $k = 1.32 \pm 0.11\ \mu M^{-1}\ min^{-1}$ (Supplementary Fig. S2B). The I_{50} for inhibitor was 2.0 μM, also similar to what was observed for the small catalase of *Saccharomyces cereviseae* (*20*).

Azide, another irreversible inhibitor of haemeenzymes, was effective at concentrations < 1 mM. The kinetics of inactivation was measured by incubating the enzyme with several concentrations of the inhibitor for a fixed time. Inactivation using azide was also of first order with $k = 0.21 \pm 0.03\ mM^{-1}\ min^{-1}$ (Supplementary Fig. S2C). The concentration of inhibitor producing 50% of inhibition after 1 min incubation with the enzyme, I_{50}, was 1.5 μM. Similar results were obtained to small catalase (CATA) of *S. cereviseae* and other small catalases (*20*).

Catalase P stability

When *Pb*CatP activity was assayed at different pH values, no optimal pH was observed, but rather a plateau described for other catalases (*27*). The purified *Pb*CatP was stable and yielded similar activity from pH 4 to 12 (Fig. 4A). This fact together with the inactivation by 3-amino-1,2,4-triazole indicates that *Pb*CatP is a monofunctional catalase (*4*, *27*). This is in contrast to catalase-peroxidases that have an optimal pH (*21*). The enzyme exhibited stability even at extreme acidic conditions. Approximately 20% activity was observed at pH 3.8 followed by a steady rise of activity as the pH was increased to 7.8 (Fig. 4B). The samples submitted to acid treatment were resuspended in 50 mM phosphate buffer, pH 7.8, to renaturing conditions. In this condition, the catalase activity was restored (Fig. 4B).

Like most monofunctional catalases, *Pb*CatP activity did not appear to be greatly influenced by temperature, as demonstrated in Fig. 5A. Many interactions that stabilize the secondary, tertiary and quaternary structure throughout the protein are likely to be involved in this property, as described (*20*, *26*). The purified *Pb*CatP was incubated at 65°C for

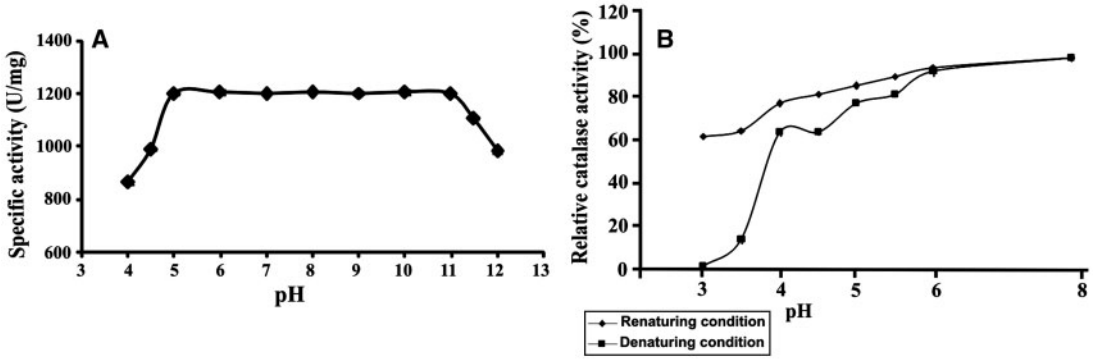

Fig. 4 Effect of pH on catalase P activity and stability. (A) Specific activity of *Pb*CatP assayed at the indicated pH values. (B) Stability at the acidic range of pH and renaturing of *Pb*CatP. The purified catalase was submitted to acid pH and catalase activity was determined. Samples were taken to renaturing in pH 7.8. Enzyme activity at pH 7.8 was taken as 100%. Data were obtained of three separated determinations.

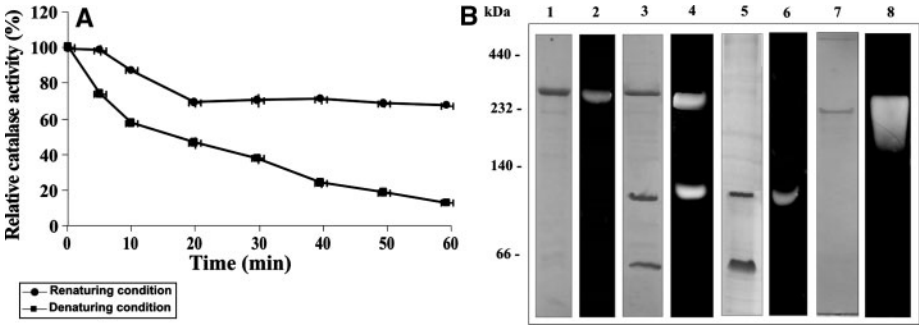

Fig. 5 Catalase P stability at high temperatures. (A) *Pb*CatP was incubated at 65°C at different time intervals. Samples were incubated at 40°C for renaturing conditions. Enzyme activity was determined. (B) PAGE under non-denaturing conditions using samples of 0 (lanes 1 and 2), 30 min (lanes 3 and 4), 60 min (lanes 5 and 6) of heat treatment. Samples were changed to the temperature of 40°C for renaturing assays (lanes 7 and 8). The gel was stained with Coomassie Brilliant Blue (lanes 1, 3, 5 and 7) and potassium ferricyanide/ferric chloride for activity (lanes 2, 4, 6 and 8).

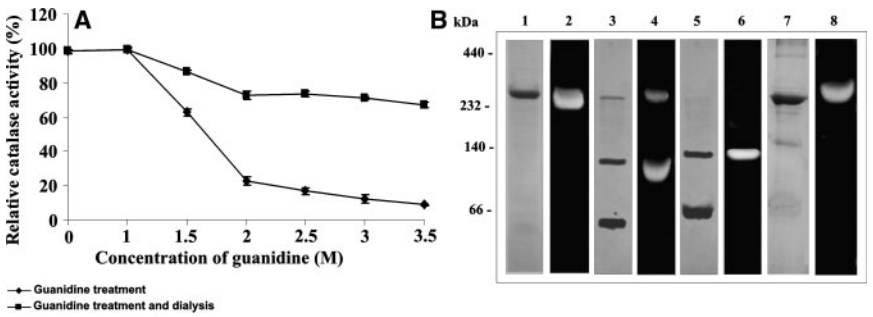

Fig. 6 Enzyme stability in the presence of a molar concentration of guanidinium ion. (A) *Pb*CatP was incubated 48 h at different guanidine hydrochloride concentrations and the relative enzyme activity was determined. (B) *Pb*CatP was incubated 48 h at different guanidine hydrochloride concentrations and then analysed by PAGE using samples treated with 1 M (lanes 1 and 2), 1.5 M (lanes 3 and 4) and 3.5 M of guanidine–HCl (lanes 5 and 6). Lanes 7 and 8 represent the activity of *Pb*CatP after treatment with 3.5 M of guanidine and subsequent dialysis. Gels were stained with Coomassie Brilliant Blue (lanes 1, 3, 5 and 7) and potassium ferricyanide/ferric chloride (lanes 2, 4, 6 and 8).

different amounts of time. The time required for 50% inactivation is shown in Fig. 5A. Furthermore, the protein was analysed by PAGE under non-denaturing conditions. Protein species were observed at 244 kDa, 122 kDa and 61 kDa, suggesting a temperature-dependent release of the tetrameric, dimeric and monomeric forms of the enzyme (Fig. 5B). The tetramers and dimers are fully active while monomers are inactive (Fig. 5B, lanes 4 and 6), as observed in *N. crassa* (*26*). Only the tetrameric form was observed in the control (Fig. 5B, lanes 1 and 2); after 30 min of heat treatment, the presence of dimers and monomers were detected (Fig. 5B, lanes 3 and 4). Only the dimer and monomer remained after 60 min of heat treatment (Fig. 5B, lanes 5 and 6). After treatment at 65°C, the samples were incubated at 40°C for renaturing treatment. Under this condition, the inactive forms generated in the heat treatment were recovered by renaturing (Fig. 5A), showing only the tetrameric form (Fig. 5B, lanes 7 and 8).

To further test *Pb*CatP stability, we treated the enzyme with different concentrations of guanidinium chloride. Similar to acid treatment, *Pb*CatP was also highly resistant to denaturation by guanidinium chloride. The enzyme conserved activity after 48 h exposure to 1 M guanidine hydrochloride (Fig. 6A and B, lanes 1

and 2). Guanidine–HCl at 1.5 M for 48 h decreased the catalase P activity to ~60% (Fig. 6A and 6B, lanes 3 and 4). Even at 3.5 M guanidine–HCl, *Pb*CatP dimers were active (Fig. 6B, lanes 5 and 6). After dialysis the activity was restored (Fig. 6B, lanes 7 and 8). The high resistance to denaturing by acidic conditions, heat and chaotropic agents may be due to the inter-weaving of amino acid structures between monomers in monofunctional catalases (*28*).

In summary, we report the purification and identification of the catalase P of *P. brasiliensis*. The native enzyme is a tetramer that loses its activity upon denaturation to a monomer.

Supplementary Data

Supplementary Data are available at *JB* Online.

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Conflict of interest

None declared.

References

1. Scandlios, J.G. (2002) The rise of ROS. *Trends Biochem. Sci.* **27**, 483–486
2. Chance, B. (1950) Oxidase activity-light absorption relationships in cytochrome system of heart muscle preparations. *Biol. Bull.* **99**, 318
3. Kirkman, H.N. and Gaetani, G.F. (1984) Catalase: a tetrameric enzyme with four tightly bound molecules of NADPH. *Proc. Natl Acad. Sci.* **81**, 4343–4347
4. Jouve, H.M., Pelmont, J., and Gaillard, J. (1986) Interaction between pyridine adenine dinucleotides and bovine liver catalase: a chromatographic and spectral study. *Arch. Biochem. Biophys.* **248**, 71–79
5. Kirkman, H.N., Galiano, S., and Gaetani, G.F. (1987) The function of catalase-bound NADPH. *J. Biol. Chem.* **262**, 660–666
6. Chelikani, P., Fita, I., and Loewen, P.C. (2004) Diversity of structures and properties among catalases. *Cell Mol. Life Sci.* **342**, 192–208
7. Brummer, E., Castãneda, E., and Restrepo, A. (1993) Paracoccidioidomycosis: an update. *Microbiol. Rev.* **6**, 89–117
8. Rodriguez, D.R., Dias-Melicio, L.A., Calvi, S.A., Peraçoli, M.T.S., and Soares, A.M.V.C. (2008) *Paracoccidioides brasiliensis* killing by IFN-δ, TNF-α and GM-CSF activated human neutrophils: role for oxygen metabolites. *Med. Mycol.* **45**, 27–33
9. Moreira, S.F., Bailão, A.M., Barbosa, M.S., Jesuíno, R.S., Felipe, M.S., Pereira, M., and Soares, C.M.A. (2004) Monofunctional catalase P of *Paracoccidioides brasiliensis*: identification, characterization, molecular cloning and expression analysis. *Yeast* **21**, 173–182
10. Chagas, R.F., Bailão, A.M., Pereira, M., Winters, M.S., Smullian, A.G., Deepe, G.S. Jr, and Soares, C.M.A. (2008) The catalases of *Paracoccidioides brasiliensis* are differentially regulated: protein activity and transcript analysis. *Fungal Genet. Biol.* **45**, 1470–1478
11. Fonseca, C.A., Jesuino, R.S., Felipe, M.S., Cunha, D.A., Brito, W.A., and Soares, C.M.A. (2001) Two-dimensional electrophoresis and characterization of antigens from *Paracoccidioides brasiliensis*. *Microbes Infect.* **3**, 535–542
12. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* **72**, 248–254
13. O'Farell, P.H. (1975) High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem* **250**, 4007–4021
14. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head bacteriophage T4. *Nature* **227**, 680–685
15. Aebi, H. (1984) Assays of catalase in vitro *Catalase in vitro* (Parker L., ed.) Vol. 105, pp. 122–126, Academic Press, New York
16. Wayne, L.G. and Diaz, G.A. (1986) A double staining method for differentiating between two classes of mycobacterial catalases in polyacrylamide electrophoresis gels. *Anal Biochem.* **157**, 89–92
17. Kawasaki, L. and Aguirre, J. (2001) Multiple catalase genes are differentially regulated in *Aspergillus nidulans*. *J. Bacteriol.* **183**, 1434–1440
18. Díaz, A., Rangel, P., Oca, Y.M., Lledías, F., and Hansberg, W. (2001) Molecular and kinetic study of catalase-1, a durable large catalase of *Neurospora crassa*. *Free Radic. Biol. Med.* **31**, 1323–1333
19. Díaz, A., Horjales, E., Rudiño-Piñe, E., Arreola, R., and Hansberg, W. (2004) Unusual Cys-Tyr covalent bond in a large catalase. *J. Mol. Biol.* **342**, 971–985
20. Switala, J. and Loewen, P.C. (2002) Diversity of properties among catalases. *Arch. Biochem. Biophys.* **401**, 145–154
21. Carlsson, G.H., Nicholls, P., Svistunenko, D., Berglund, G.I., and Hajdu, J. (2005) Complexes of horseradish peroxidase with formate, acetate, and carbon monoxide. *Biochemistry*. **44**, 635–642
22. Nicholls, P., Fita, I., and Loewen, P.C. (2001) Theoretical study of the mechanisms of substrate recognition by catalase. *Adv. Inorg. Chem.* **51**, 51–106
23. Kirkman, H.N., Rolfo, M., Ferraris, A.M., and Gaetani, G.F. (1999) Mechanisms of protection of catalase by NADPH: Kinetics and stoichiometry. *J. Biol. Chem.* **274**, 13908–13914
24. Gaetani, G.F., Ferraris, A.M., Sanna, P., and Kirkman, H.N. (2005) A novel NADPH:(bound) NADP + reductase and NADH:(bound) NADP + transhydrogenase function in bovine liver catalase. *Biochem. J.* **385**, 763–768
25. Margoliash, E., Novogrodsky, A., and Schejter, A. (1960) Irreversible reaction of 3-amino-1,2,4-triazole and related inhibitors with the protein of catalase. *Biochem. J.* **74**, 339–348
26. Díaz, A., Muñoz-Clares, R.A., Rangel, P., Valdés, V.J., and Hansberg, W. (2005) Functional and structural analysis of catalase oxidized by singlet oxygen. *Biochimie*. **87**, 205–214
27. Chance, B. (1952) The effect of pH upon the equilibria of catalase compounds. *J. Biol. Chem.* **194**, 483–496
28. Bravo, J., Fita, I., Ferrer, J.C., Ens, W., Hillar, A., Switala, J., and Loewen, P.C. (1997) Identification of a novel bond between a histidine and the essential tyrosine in catalase HPII of *Escherichia coli*. *Protein Sci.* **6**, 1016–1023