Distinctive properties of the catalase B of *Aspergillus nidulans*

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Abstract ***Aspergillus nidulans*** **catalase B (CatB) was purified to homogeneity and characterized as a hydroperoxidase which resembles typical catalases in some physicochemical characteristics: (1) it has an apparent molecular weight of 360 000 and is composed of four glycosylated subunits, (2) it has hydrophobic properties as revealed by extractability in ethanol/chloroform and binding to phenyl-Superose, and (3) it has an acidic isoelectric point at pH 3.5. Also CatB exhibits some distinctive properties, e.g. it is not inhibited by the presence of 2% sodium dodecyl sulfate, 9 M urea or reducing agents. Furthermore, even though CatB does not exhibit any residual peroxidase activity, it is able to retain up to 38% of its initial catalase activity after incubation with the typical catalase inhibitor 3-amino-1,2,4-triazole.**

Key words: Catalase; 3-Aminotriazole; Hydroperoxidase; *Aspergillus nidulans*

1. Introduction

As aerobic microorganisms, fungi must suffer the toxic side effects of molecular oxygen. Reactive oxygen species (superoxide and hydroxyl radicals, hydrogen peroxide and singlet oxygen) damage cellular components by oxidizing lipids, proteins and nucleic acids [1]. To maintain reactive oxygen species (ROS) at non-harmful levels, fungal cells have both enzymatic (catalases, peroxidases and superoxide dismutases) and non-enzymatic (glutathione, thioredoxin and polyamines) antioxidant defences [2].

Aspergillus is a fungus able to grow in a wide variety of substrates, many of these able to generate high levels of ROS (plant debris, fatty acids, ethanol, glycerol, uric acid, etc.). In *Aspergillus nidulans*, two differentially regulated catalases have been identified [3,4] that protect the fungus from the toxic effect of hydrogen peroxide (H_2O_2). Catalase A (CatA) is encoded by the *catA* gene, whose transcripts are specifically induced during sporulation and in response to different types of stress. Translation of the accumulated *catA* messenger is associated exclusively with the onset of the processes of asexual and sexual spore formation, resulting in the accumulation of active enzyme in conidia and ascospores respectively [5]. Catalase B (CatB), encoded by the *catB* gene, is present in the vegetative mycelium, increases its activity during conidiation, and is almost undetectable in spores. *catB* expression is also induced by H_2O_2 or H_2O_2-generating conditions, with no posttranscriptional regulatory control. CatA and CatB therefore seem to constitute alternative antioxidant defences at different stages of the *A. nidulans* life cycle.

In spite of detailed knowledge about the structure and regulation of the genes encoding *A. nidulans* CatA and CatB, very little information is available about the biochemical characteristics of these proteins. We report the purification of the CatB of *A. nidulans*, and show that it has certain characteristics regarding enzymatic activity and protein stability that are similar to those of typical catalases. By contrast, like peroxidases or catalase-peroxidases [6], CatB is not totally inhibited by the typical catalase inhibitor 3-amino-1,2,4-triazole (3-AT) but unlike these enzymes, CatB does not exhibit any peroxidase activity.

2. Materials and methods

2.1. Organisms and growth conditions

A. nidulans G1059wt (*adF17, pabaA1, yA2*) was obtained from A.J. Clutterbuck, Glasgow, UK. *A. nidulans* TLK12 (*pabaA1, yA2*, Δ*argB::trpC*::Δ*B*, Δ*catB, trpC801, veA1*) was obtained from J. Aguirre, Mexico City, Mexico [4]. Organisms were maintained, grown and harvested as described elsewhere [7,8]. Water-soluble fractions (WSF) and cell culture supernatants (CCS) were obtained as previously described [7,8].

2.2. Electrophoresis and analytical isoelectric focusing

Electrophoresis (both one- and two-dimensional) and staining of proteins were performed as previously described [7–9]. The Bench-Mark™ Protein Ladder (Life Sciences) was used as standard.

Isoelectric focusing (IEF) was performed using Ampholine PAGE plates (Amersham Pharmacia) with a pH range of 3–10 or 4–6.5. Electrophoretic conditions and silver staining were as recommended by the manufacturer. The low p*I* calibration kit (pH 2.5–6.5; Amersham Pharmacia) was used for calibration purposes.

2.3. Glycosylation analysis

Purified CatB was incubated at 37°C for 24 h with 0.01–10 μU of endo-β-*N*-acetylglucosaminidase H (EH; Boehringer) per μg of protein as described elsewhere [8,9].

2.4. Detection of catalase and peroxidase activities on polyacrylamide gels

A ferricyanide-negative stain was used to locate catalase bands on native or semi-native polyacrylamide gels, following the method of Wayne and Diaz [10]. Commercial samples of beef liver catalase (BLC) and horseradish peroxidase (HRP) (Sigma) were used as controls.

2.5. In vitro assay of catalase and peroxidase activities

Catalase activity was measured as described [11] by following the rate of disappearance of H_2O_2 at 240 nm using 9.0 pmol and 2.3 pmol per assay of CatB and BLC as a control, respectively. One unit of activity is defined as the amount of enzyme that catalyzes the decomposition of 1 μmol of H_2O_2 per minute. Specific activity is defined as U/mg of protein.

Peroxidase activity was measured by following the rate of oxidation

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of dianisidine at 460 nm. The reaction mixture contained 0.1 M potassium phosphate pH 5.2, 1.0 mM H_2O_2, 0.05% *o*-dianisidine dihydrochloride (Sigma) and 25 pmol of either CatB or HRP as a control. The assay was performed at 25°C.

2.6. Inhibition of catalase in the presence of 3-AT

Irreversible inhibition assays were performed as described [12] in the presence of increasing concentrations of 3-AT (5–40 mM) and using ascorbic acid (1.0 mM) as a source of H_2O_2.

2.7. CatB purification

Because of the lower complexity of WSF, it was used as a source for purification of CatB. The WSF was diafiltered with 20 mM Tris–HCl pH 7.9 (EB) and loaded onto a MonoQ (HR 5/5) column, equilibrated with the same buffer. The column was washed with 15 ml of EB and eluted with 15 ml of 0.2 M NaCl and 20 ml of 1 M NaCl, both in EB (1 ml/min flow rate). Catalase-positive fractions from the MonoQ column were pooled, diafiltered with phosphate-buffered saline (PBS), concentrated in an Amicon Centricon 30 concentrator and taken to 1.7 M (final concentration) ammonium sulfate prior to loading onto a phenyl-Superose column (HR 5/5) equilibrated with PBS, 1.7 M ammonium sulfate, pH 6.8. The column was washed with 5 ml of the same buffer and developed (0.5 ml/min flow rate) with a 5 ml decreasing linear gradient from 1.19 to 0 M ammonium sulfate in PBS. The fractions containing catalase were pooled and concentrated as before. Aliquots of 0.2 ml were loaded (0.2 ml/min) onto a Superdex-200 column (HR 10/30) equilibrated with PBS, pH 6.8 and 0.5 ml fractions were collected. Column calibration was based on the size of the standards ferritin, catalase, aldolase, bovine serum albumin, hen egg albumin, chymotrypsinogen and cytochrome *c* (Pierce). The FPLC chromatographic system and the columns were from Amersham Pharmacia Biotech.

3. Results

3.1. Evidence for CatB catalase activity in CCS and WSF of A. nidulans

To identify the CatB hydroperoxidase of *A. nidulans* for its further purification and biochemical characterization, WSF and CCS proteins were separated by PAGE under non-denaturing conditions and subjected to sequential detection of peroxidase and catalase activities. The specific peroxidase staining (first sequential step) failed to detect any peroxidase activity (data not shown). After the specific catalase staining (second sequential step), the catalase activities of the WSF and CCS fractions of *A. nidulans* were visualized as clear spots on a polyacrylamide gel (Fig. 1, right panel). Silver staining of a parallel gel demonstrated a well-defined band associated with each catalase-positive zone (Fig. 1, left panel). When the same assay was performed with a null mutant for the *catB* gene (*A. nidulans* TLK12), neither the catalase activity nor the corresponding silver-stained band could be observed (data not shown). All these data suggest that the activity demonstrated in *A. nidulans* G1059 corresponds to CatB, and that this protein is devoid of any associated peroxidase activity under these experimental conditions.

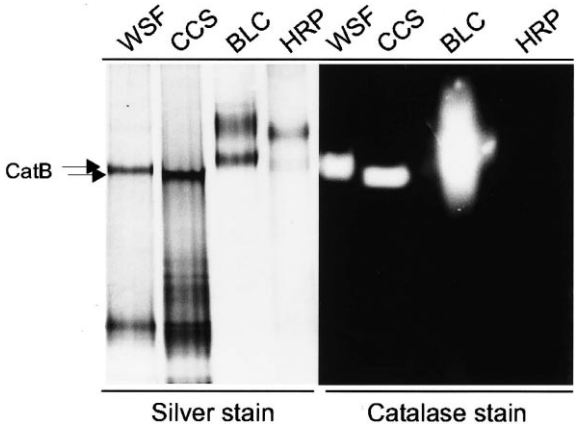

Fig. 1. PAGE (10% w/v) of the WSF and CCS from *A. nidulans* and control BLC and HRP. Protein loads were 10 μg per lane for silver stain and 20 μg per lane for catalase stain.

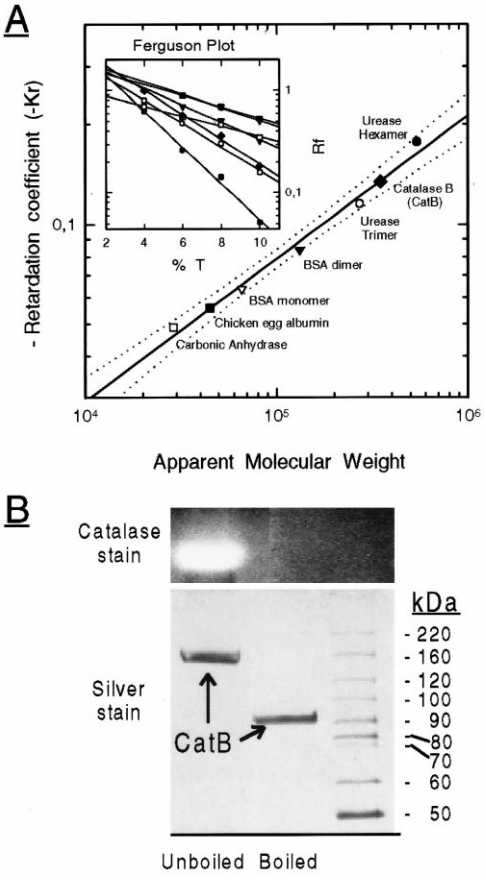

Fig. 2. A: *A. nidulans* catalase B and molecular weight markers were electrophoresed under native conditions on a set of mini-gels of 4, 6, 8 and 10% polyacrylamide concentrations. In each gel, the electrophoretic mobility of each protein relative to the tracking dye (R_f) was calculated and plotted on a semilogarithmic scale against the percent gel concentration (Ferguson plot). The slopes for such plots are the retardation coefficients (K_r) for CatB and standard proteins. A standard curve was obtained by plotting the K_r value against the apparent molecular weight of the standards (double logarithmic scale). Protein standards were carbonic anhydrase (29 000), chicken egg albumin (45 000), bovine serum albumin monomer (66 000), bovine serum albumin dimer (132 000), jack bean urease trimer (272 000) and jack bean urease hexamer (545 000). B: Pure CatB was not boiled or boiled in SDS sample buffer, separated in duplicate by SDS–PAGE (14% w/v) and stained with silver or catalase.

3.2. Structural properties of CatB

According to the native electrophoresis method of Hedrick and Smith [25], the molecular mass of native pure CatB was around 360 kDa (Fig. 2A). However, under denaturing conditions its electrophoretic behavior was dependent on whether or not CatB was previously boiled in denaturing sample buffer, which contains 2% SDS and 20 mM dithiothreitol (DTT) (final concentrations). Thus, when it was not boiled CatB

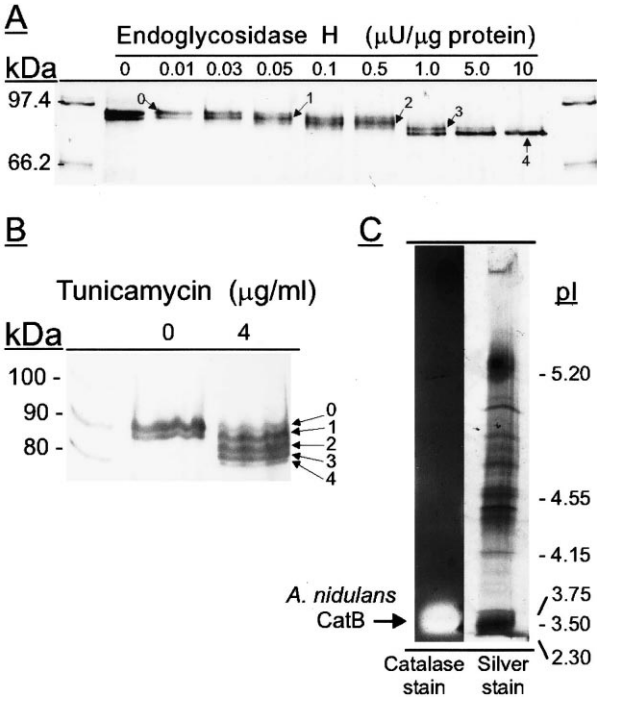

Fig. 3. A: SDS–PAGE (10% w/v) of pure CatB from *A. nidulans* after treatment with the indicated amounts (μU/μg protein) of endoglycosidase H. B: CatB obtained after growth in 4 μg/ml tunicamycin. Gels were silver-stained. 0: original CatB; 1, 2, 3 and 4: CatB first, second, third and fourth deglycosylation products, respectively. C: Isoelectric focusing of WSF from *A. nidulans* (20 μg of protein per lane) in Ampholine PAGE plates with a pH gradient of 4–6.5.

showed an apparent molecular mass of 180 kDa and retained its catalase activity. In contrast, after boiling it appeared as an enzymatically inactive 90 kDa band (Fig. 2B).

In order to determine the degree of CatB glycosylation, purified CatB was subjected to digestion with progressively higher amounts of EH. The digestion products were separated by SDS–PAGE and silver-stained (Fig. 3A). Untreated CatB behaved as a double band of 92 and 88.6 kDa. The upper band of the doublet represented the fully glycosylated protein (band 0) and the lower one the first deglycosylation product (band 1, which may occur spontaneously in preparations). Upon digestion, three more deglycosylated products appeared: band 2 (85.9 kDa), band 3 (83.4 kDa), and band 4 (80.1 kDa). Thus, it may be concluded that CatB has at least four different *N*-linked sugar moieties, accounting for 11.9 kDa of its fully glycosylated form. When *A. nidulans* was grown in the presence of tunicamycin (a specific inhibitor of *N*-glycosylation), the same four deglycosylation products were detected (Fig. 3B). The molecular mass of the most deglycosylated form (80.1 kDa) was very close to that deduced from the amino acid sequence (79.1 kDa) [4], suggesting that band 4 could represent the non-glycosylated version of CatB.

The p*I* of CatB was extremely acidic (around 3.5), as determined by analytical IEF of the WSF followed by silver staining and catalase staining (Fig. 3C). Also, the WSF was also analyzed by 2D electrophoresis. Samples dissolved in buffer containing a high concentration of urea (9 M) were separated by IEF in a 3.5–6.0 pH gradient. CatB behaved consistently with the previous data as a single extremely acidic spot (p*I* 3.5) with a molecular mass of 180 kDa, since the SDS–PAGE step of the 2D electrophoresis did not involve boiling of samples (data not shown).

3.3. Spectral properties, specific activity, and stability of CatB

Spectrophotometric analysis of the native enzyme showed that CatB exhibits two absorption bands with maxima at 413 and 590 nm ($A_{413}/A_{590} = 4.65$). Treatment of the enzyme with KCN shifted the maxima to 425 and 637 nm, evidence of the presence of ferric heme in the enzyme, which was supported by the observation that a solution of the purified native enzyme appeared green in color (data not shown).

In vitro assays for catalase and peroxidase activities of CatB showed that it has a specific catalase activity of 741 μmol H_2O_2 min^{-1} mg^{-1}. However, no peroxidase activity was detected (Fig. 4A).

CatB is a very stable protein since its catalase activity was

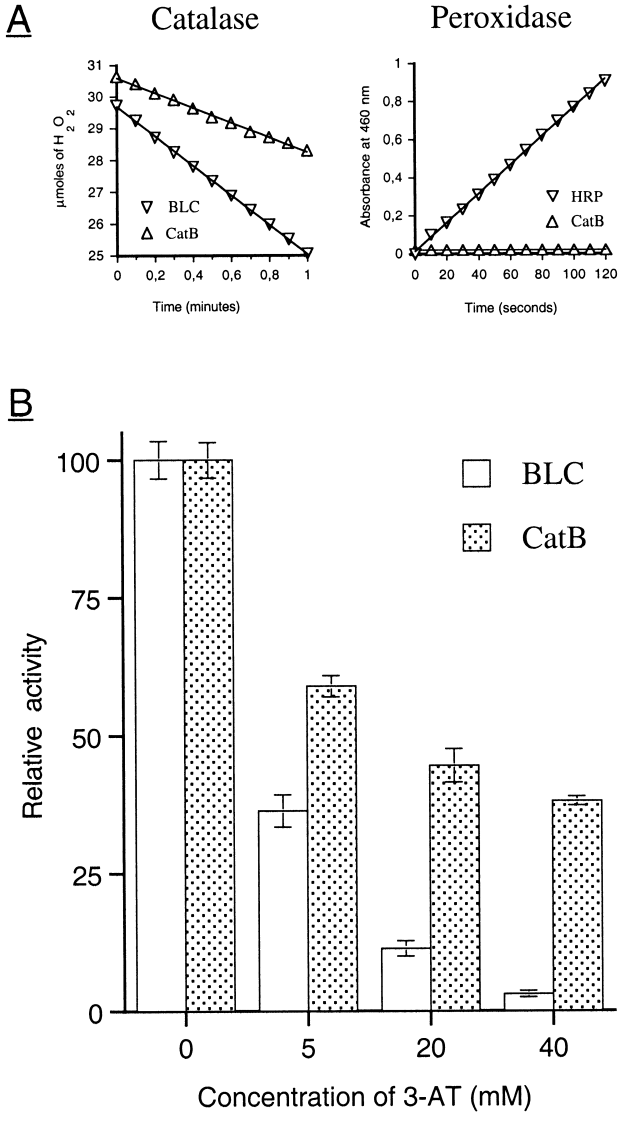

Fig. 4. A: In vitro determination of catalase and peroxidase activities of CatB. BLC and HRP were used as controls of catalase and peroxidase activity, respectively. B: Inhibition analysis of CatB. Results are the average of three independent experiments. Catalase activity of CatB was assayed as described following incubation with increasing concentrations of 3-AT for 1 h at 37°C. BLC, known to be inhibited by 3-AT, was used as a control.

not inhibited by 9 M urea (2D electrophoresis experiments), incubation in 2% SDS or reducing agents (20 mM DTT), and treatments with ethanol/chloroform mixtures (data not shown). Furthermore, the compound 3-AT, which has been shown to inhibit specifically and irreversibly the catalase but not peroxidase function [6], did not totally inhibit the catalase activity of CatB. Thus, even after incubation with 40 mM 3-AT, CatB retained up to 38% of its initial activity, while under the same conditions BLC used as a control retained less than 3% of its initial activity (Fig. 4B).

4. Discussion

CatB is an enzyme with a native molecular mass of 360 kDa. It is composed of four glycosylated subunits of an average molecular mass of 90 kDa, and since there are no Cys residues in the amino acid sequence of CatB [4], disulfide bonds are not responsible for the association of the subunits. Like other fungal catalases [9,13], *A. nidulans* CatB is *N*-glycosylated (up to 11.9 kDa), and four of the five putative glycosylation sites of the CatB sequence [4] contain one sugar chain. These sites are probably located in the four *N*-glycosylation targets (Asn-118, 446, 449 and 610 in the *A. nidulans* CatB sequence) that are conserved in the proteins most closely related to CatB such as Cat1 from *A. fumigatus* [14], CatR from *A. niger* [13], and a catalase (M antigen) from *Histoplasma capsulatum* [15]. In this context, the resistance of CatB to degradation by endogenous proteases is especially noteworthy and it can be speculated that somehow the glycosylation of CatB could protect it against proteolytic attack. On the other hand, the p*I* of *A. nidulans* CatB as determined by isoelectric focusing was 3.5, close to the value of 4.86 predicted from its amino acid sequence [4]. All fungal catalases characterized have a predicted p*I* between 4.8 (CatB from *A. nidulans*) [4] and 7.0 (CatA from *Saccharomyces cerevisiae*) [16]. Consequently *A. nidulans* CatB is, to our knowledge, the most acidic catalase known thus far.

There are three unrelated and clearly differentiated hydroperoxidase groups: true catalases (both typical and atypical), peroxidases and catalase-peroxidases [17]. From the data accumulated in this study, the native *A. nidulans* CatB enzyme can be defined as a very acidic tetrameric protein resistant to ethanol/chloroform and reducing agents and able to bind to phenyl-Superose, similar to typical true catalases such as BLC or CatA from *S. cerevisiae* [18,19]. CatB has a high molecular mass (360 kDa) and is resistant to high concentrations of urea and SDS, similar to atypical true catalases such as *Escherichia coli* HP-II catalase [20], *A. niger* CatR [13] and *Neurospora crassa* Cat1 [21]. Finally, the most distinctive property of CatB is that, in contrast to other catalases but similar to peroxidases [22] or catalase-peroxidases [17,23,24], it is not totally inhibited by 3-AT. The reason for this is unknown, but a small difference with other catalases in its primary structure could affect the structural environment which surrounds the active center of the enzyme to make it partially resistant to the inhibitory effect of 3-AT.

Consequently, CatB from *A. nidulans* should be considered an enzyme with notable peculiarities among the catalases already described, which makes it of great interest for further analysis from both the structural and the biotechnological point of view.

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