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FULL PAPER

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High constitutive peroxidase activity and constitutive thermotolerance in *Neurospora crassa*

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Abstract During the isolation of mutations in the heat-inducible *hsp*70-1 gene of *Neurospora crassa* by RIP (*r*epeat-*i*nduced *p*oint mutations), several transformants were generated by electroporation of conidia with a plasmid harboring an incomplete copy of this gene. One isolate, designated E-45, containing ectopically integrated *hsp*70-1 DNA, exhibited a slow growth rate, low-temperature sensitivity, constitutive thermotolerance (without prior heat shock), and high constitutive peroxidase activity. The constitutive form of peroxidase (CP) was distinguishable from the heat-inducible form (HIP) by immunoinactivation employing polyclonal antiserum against the latter enzyme and by electrophoretic resolution in nondenaturing polyacrylamide gels. This enzyme was purified to near homogeneity and some of its properties examined. The relative molecular mass of native CP was in the range of 118–136 kDa, as estimated by gel filtration analysis on size exclusion matrices, whereas SDS-PAGE analysis yielded a size of ~37 kDa for the polypeptide. Substrate saturation kinetics studies were conducted using ABTS [2,2′-azino-bis (3-ethylbenzthiazole-6-sulfonic acid)] and H_2O_2 as substrates: K_m, V_{max}, and K_{cat} values for H_2O_2 were ~22 μM, ~447 nmol mg^{-1}, and 0.33 s^{-1}, respectively, and those for ABTS were ~55 μM, ~453 nmol mg^{-1}, and 0.3 s^{-1}, respectively. Guaiacol was not used as a substrate by this enzyme. CP peroxidase was shown to be a heme-containing enzyme, stable at temperatures up to 58°C.

Key words Constitutive peroxidase · *Neurospora crassa* · Thermotolerance

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Introduction

The synthesis of specific defense-related proteins, in response to hyperthermal treatment and oxidative and chemical stress, has been documented in a wide variety of organisms (Craig et al. 1993). One of the outstanding features of this evolutionarily conserved response is the accelerated production of a specific class of proteins, the heat shock proteins (Hsps). These ubiquitous proteins – functioning as molecular chaperones – are responsible for maintenance of the integrity of cellular macromolecules during normal growth as well as during exposure to physical or physiological stress. Hsps belonging to the high molecular mass category, the Hsp90, 70, and 60 families, perform vital functions in the cell: folding of nascent polypeptides, assembly of oligomeric proteins, membrane transport of organellar proteins, prevention of premature folding, and nonproductive interprotein interactions (Hartl 1996; Csermely et al. 1998). A direct consequence of heat shock treatment is the development of thermotolerance, acquisition of the ability to withstand lethal temperatures. The identity of all the components mediating the acquisition of thermotolerance is obscure, yet evidence from several sources suggested a role for stress proteins such as the Hsp70 and Hsp90 families (Solomon et al. 1991). In addition, Hsp104 and Hsp101 appear to be implicated in the development of stress-induced as well as constitutive thermotolerance by *Saccharomyces cerevisiae* cells (Quietsch et al. 2000).

The heat shock response of the filamentous fungus *Neurospora crassa* is characterized by the elevated synthesis of an array of Hsps, including the highly abundant Hsp70 and Hsp90 family members, observed in a variety of organisms. In addition, heat shock and oxidative stress of *N. crassa* mycelium lead to the induction of a peroxidase activity that was virtually undetectable in normally grown cells but was induced within 10 min of hyperthermal exposure (Kapoor and Lewis 1987a,b). This peroxidase activity was of special interest as a correlation between stress treatments leading to the induction of peroxidase and the development of

thermotolerance was apparent. For instance, exposure to cadmium chloride, sodium arsenite, and hydrogen peroxide induced thermotolerance and, concomitantly, a high level of peroxidase activity (Kapoor et al. 1990). Of these agents, sodium arsenite was the only one that stimulated an increased accumulation of a specific subset of heat shock proteins, namely Hsp70 and Hsp80. These observations prompted the hypothesis that the heat shock-induced peroxidase (HIP) may be a component of the repertoire that protects against reactive oxygen species, including superoxide free radicals, engendered during hyperthermia and other types of stress. The isolation and purification of HIP from heat-shocked *N. crassa* mycelium of a wild-type strain was reported recently (Machwe et al. 2002).

In experiments aimed at isolation of mutants of the heat shock protein 70 gene (*hsp*70-1) of *Neurospora* by repeat-induced point mutations (RIP) (Selker 1990), several transformants were generated by electroporation of germinating conidia with a plasmid harboring an incomplete copy of this gene (Kapoor et al. 1995). When these transformants, with *hsp*70 DNA integrated at ectopic chromosomal locations, were used as parental strains in sexual crosses, a majority of the isolates showed evidence of RIP in the duplicated *hsp*70 DNA in the progeny (Chakraborty et al. 1995). However, one exceptional transformant (designated E-45) was recovered, which in contrast with other transformants did not yield the expected progeny with methylation of cytosine residues associated with RIP in crosses with the wild-type strain. Furthermore, E-45 exhibited a number of unusual phenotypic features, including slow growth rate, low-temperature sensitivity, and constitutive thermotolerance. The molecular basis of the anomalous outcome of the sexual crosses with this strain is not entirely clear at present.

Fortuitously, as described in the following communication, analysis of the heat shock response of E-45 led to the serendipitous recovery of a strain with high intracellular constitutive peroxidase activity, witnessed in the complete absence of externally applied stress, whereas the HIP activity was significantly lower than that encountered in the heat-shocked wild-type strain. The peroxidase activity in the latter is very low during growth at normal temperatures, but it is subject to induction by heat shock and oxidative stress (Machwe et al. 2002). Therefore, in view of its abundance in E-45, the constitutive peroxidase (CP) was isolated from this strain, purified to homogeneity, and some of its properties examined. This enzyme is readily distinguishable from the heat-inducible peroxidase of *N. crassa* by its subunit size, pH optimum, and kinetic attributes.

Materials and methods

Growth of cultures, heat shock treatment, and tests for thermotolerance

The wild-type strains of *Neurospora crassa* Shear et Dodge (Fungal Genetics Stock Centre No. 262 and 74A; University of Kansas Medical Centre, Lawrence, KS, USA) and transformants were grown while shaking at 28° or 30°C in Vogel's minimal medium (Vm) (Vogel 1956) with 2% sucrose. Heat shock treatment of 14-h-old mycelial cultures was performed as reported previously (Kapoor and Lewis 1987a). To achieve colonial growth of *N. crassa*, conidial suspensions were plated on sorbose medium (Vm, 1% sorbose, 0.1% sucrose, 1.5% agar) and incubated at 30°C for 48 h, or as indicated. For thermotolerance tests, heat shock was administered by shifting the plates to a humid incubator at 52°C (plate surface temperature, 48°C) for 45 min, as described previously (Kapoor et al. 1990). For preparation of replica plates, sterile Whatman No. 1 filter papers were placed on the plates and incubation continued for 12 h at 30°C. Filter papers with adhering fungal material were transferred to fresh Vm-sorbose plates and, after incubation for 18–24 h at 30°C, percentage survival was estimated by colony counts.

Preparation of mycelial extracts and determination of enzymatic activity

For preparation of cell extracts, freeze-dried mycelium was homogenized in 50 mM K_2HPO_4–0.1 mM ethylene diaminetetraacetic acid (EDTA) (pH 7.8) and centrifuged at 15 000 *g* for 15 min at 4°C. Peroxidase activity of the supernatant was followed spectrophotometrically by monitoring the oxidation of *O*-dianisidine at 460 nm in the presence of H_2O_2 (Hoffman et al. 1979), and specific activity was expressed as the change in absorbance at 460 nm/min/mg protein. Alternatively, a second assay system involving 0.05% ABTS [2, 2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)] and 1.5% H_2O_2, was employed, as described previously (Machwe et al. 2002). The enzymatic assays were conducted at room temperature by recording the absorbance at 415 nm using a Gilford model 250 spectrophotometer (Gilford, Oberlin, OH, USA). A unit of activity is defined as the change in absorbance of 1.0 at 415 nm in 20 min, and specific activity is defined as $\Delta A_{415\,nm}$/mg protein. Protein concentration was estimated by means of the Bio-Rad (Mississauga, Ontario, Canada) protein microassay (Bradford 1976) using bovine serum albumin as a calibration standard.

Nondenaturing gel electrophoresis

Samples of the extract were electrophoresed in 10% polyacrylamide isocratic gels under non-denaturing conditions. Peroxidase activity was visualized by immersing the gels in 2 mM *O*-dianisidine–0.1 mM riboflavin–10 mM potassium phosphate buffer (pH 7.2) for 1 h; the gels were rinsed with distilled water and treated with 0.2 mM hydrogen peroxide–10 mM potassium phosphate buffer (pH 7.2), resulting in the development of brown color. For simultaneous resolution of acidic and basic forms of peroxidase, electrophoresis was performed at pH 4.6, according to the procedure of Borchert and Decedue (1978).

SDS-PAGE and western blot analysis

Denaturing gels were prepared essentially according the procedure of Laemmli (1970) using 10% acrylamide in separating gels and 5% in stacking gels. Following electrophoresis, the gels were stained in 0.1% Coomassie brilliant blue R-250 or subjected to glycoprotein stain (Segrest and Jackson 1972) following resolution in sodium dodecyl sulfate-polyacrylam de gel electrophoresis (SDS-PAGE), as outlined previously (Machwe et al. 2002). Protein samples resolved by SDS-PAGE were transferred to nitrocellulose membranes (Amersham) using a procedure similar to that described by Towbin et al. (1979). The electro-transfer was performed for 1 h at 100 V, at 4°C. Blocking was performed in 6% skim milk prepared in TBST (0.01 M Tris-HCl, 0.15 M NaCl, 0.1% Tween-20, pH 7.8) with 2.5 ml Denhardt's solution (0.02% w/v each of Pentax fraction V Serum Albumin, Ficoll, and polyvinylpyrolidone) in a total volume of 20 ml for 2 h. The membrane was washed three times, 20 min each time, with TBST and treated for 1 h with a 1:1000 dilution of the primary antibody, the antiserum generated against *Neurospora crassa* HIP, described earlier (Machwe 1996; Machwe et al. 2002). The blot then was exposed to a 1:10 000 dilution of the goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (Sigma, Oakville, Ontario, Canada) for 20 min, and reactive bands were visualized using the ECL Chemiluminescence system (Amersham, Baie d'Urfé, Quebec, Canada) followed by exposure to X-ray film.

Results

Growth characteristics of E-45

The strain E-45, isolated according to the procedure described previously (Chakraborty et al. 1995), was examined for some phenotypic attributes including growth rate, conidiation, and response to variations in temperature. For determination of the optimal growth temperature, conidial suspensions were plated on sorbose medium (Vm–1% sorbose–0.1% sucrose–1.5% agar) and incubated at 28° and 30°C; after 5 days, the colonies were counted. To test the relative viability of conidia, conidial suspensions were prepared from 8-day-old mature slants of E-45 and two wild-type strains (74A and 262), their optical density (A_{600nm}) was adjusted to 45 Klett units and serial dilutions were used to estimate the relative proportion of germinated conidia. Judging by the number of colonies arising from identical dilutions of the original suspensions with the same initial optical density, viability of E-45 conidia was estimated to be between 5% and 10%, compared to the wild-type strains. Similar results were obtained when 15-day-old conidia were employed.

Other interesting features of the transformant E-45 included its slow growth in comparison with the wild type and a striking sensitivity to low temperature. For instance, there was no growth of E-45 colonies on sorbose sucrose medium at 21° and 25°C after incubation for 48 h, whereas the wild type showed normal development of colonies. At 28°C, conidial germination appeared to be initiated at a slow rate in E-45; after an interval of 4 days on sorbose plates, minute pinhead-size colonies were discernible, but subsequent growth was arrested. In contrast, the wild-type strains normally exhibit distinct colonies within 48 h. At 30°C, E-45 colonies appeared to grow normally, albeit at a slightly slower rate than the wild type. Although the wild-type (strain 262) colony diameter averaged ~3 mm after 2 days, E-45 took 5 days to develop colonies of comparable dimensions (not shown). Furthermore, the relative rate of hyphal elongation and conidiation of E-45, grown on Vm–2% sucrose–1.5% agar, without sorbose, was also very low compared to that of the wild-type strains (Fig. 1).

Next, conidial suspensions of E-45 were plated on sorbose medium containing 0.1% glucose instead of sucrose and incubated at 30°C for 48 h. The colonial growth of E-45 on this medium approached that of the wild-type, indicating a deficiency in the utilization of sucrose as a carbon source by this strain. To determine whether E-45 colonies growing on sorbose–glucose medium were susceptible to low temperatures, these plates were shifted to 6°C for 72 h, along with control plates of the wild-type strain. Subsequently, replicas were prepared, as described in the Methods, and incubated at 30°C. In plates with the wild-type strain, 100% colony survival was observed, but more than 90% of the E-45 colonies failed to grow on replica plates (not shown), demonstrating that storage at low temperature was lethal.

Peroxidase activity of E-45 and thermotolerance

Measurements of peroxidase activity were conducted spectrophotometrically by following the oxidation of *O*-dianisidine in the presence of H_2O_2. Mycelial cell extracts were prepared as described in Materials and methods. Cells of strain E-45, grown under normal conditions, revealed a substantially higher level of constitutive peroxidase activity, in sharp contrast to the wild-type strain, which exhibited virtually no detectable activity in the absence of applied stress (Table 1). Heat shock treatment of the wild-type cells led to a pronounced elevation in peroxidase activity, consistent with our earlier reports (Kapoor and Lewis 1987b; Kapoor et al. 1990). On the other hand, upon heat shock of E-45 mycelial cells, instead of an increase, the level of peroxidase was slightly diminished relative to that of the nonshocked cells. Nevertheless, the cumulative peroxidase activity in heat-shocked E-45 cells, reflective of the constitutive as well as the induced enzyme, was still comparable to that attained by the heat-shocked wild-type strain (Table 1).

Next, thermotolerance tests were conducted by transferring 96-h-old heat-shocked and control plates of E-45 to a humid incubator at 57°C (plate surface temperature, 51.5°C) for 45 min, replica plating being performed as described in Materials and methods. The degree of thermotolerance was evaluated by comparison of the surviving fraction of colonies in plates exposed directly to "lethal" temperature versus those that had received a heats shock pre-treatment. As demonstrated by the data in Table

Table 1. Peroxidase activity in stressed and unstressed mycelium

Strain	Growth temperature, treatment	Specific activity[a]
262	28°C, 15 h	0.074 ± 0.005
E-45	30°C, 20 h	0.333 ± 0.006
262	Heat shock at 48°C, 1 h	0.253 ± 0.021
E-45	Heat shock at 48°C, 1 h	0.206 ± 0.043

[a] Specific activity is defined as the change in absorbance at 460 nm min^{-1} mg^{-1} protein

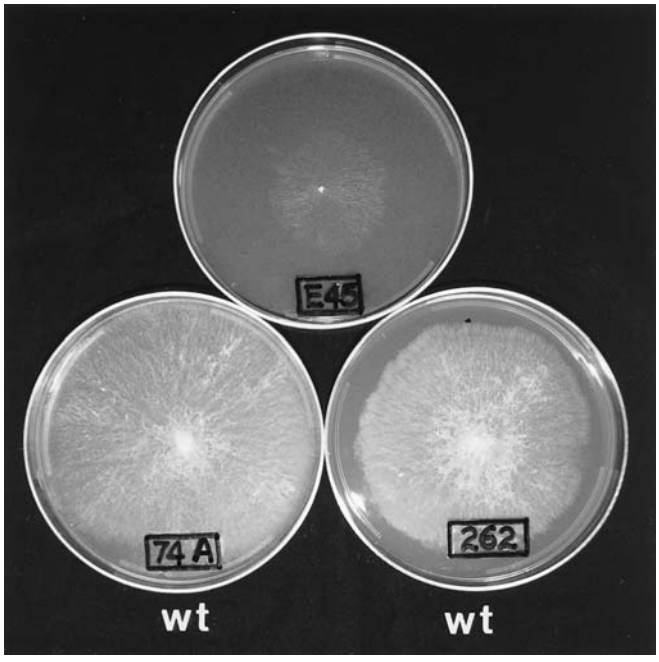

Fig. 1. Relative growth rate of E-45 and two wild-type (*wt*) *Neurospora crassa* strains. Conidial suspensions of E-45, 74A, and 262 were plated on Vogel's (Vm) agar medium with 2% sucrose and incubated at 30°C; plates were photographed after 24 h

2, E-45 exhibited a relatively high level of resistance toward lethal temperature in the absence of prior stress treatment. The wild-type strains showed 5%–10% survival following direct exposure to the lethal temperature but the percentage survival of E-45 was dramatically higher. Although the application of heat shock enhanced the survival of E-45 on subsequent exposure to the lethal temperature, the change was not as pronounced as that elicited with wild-type cells. These results attested to the striking intrinsic thermotolerance of E-45, supporting the notion of an association between total intracellular peroxidase and the capacity to withstand hyperthermia. Whether the constitutive peroxidase activity in nonshocked E-45 cells and the HI peroxidase in heat-shocked and chemically stressed *N. crassa* cells were derived from the same or different enzymes was evaluated in the following experiment.

Electrophoretic analysis of peroxidases

To differentiate between the constitutively expressed and the HI peroxidase, electrophoresis of cell extracts of E-45

Table 2. Development of thermotolerance by *Neurospora crassa* colonies

Strain	Pretreatment	Duration (min)	Survival at "lethal" temperature (%)
262	None	–	5–10[a]
E-45	None	–	68[b]
262	Heat shock	60	75–95[a]
E-45	Heat shock	60	87[b]

[a] Range determined by plate counts from replicates, in several different experiments
[b] Values are averages of counts obtained from six plates

and the wild-type strain (262) was performed in nondenaturing alkaline as well as acidic gels; the gels were stained for visualization of peroxidase. Mycelial extracts of 262 and of E-45, prepared from heat-shocked and nonshocked samples, were compared. In the heat-shocked cell extracts of both strains, the peroxidase protein resolved in alkaline gels, reproducibly, into a doublet of bands (Fig. 2A), but the high constitutively expressed peroxidase activity of E-45 was not apparent. In contrast, constitutive peroxidase was readily detectable in acidic gels as a prominent band with greater mobility than that of the HI peroxidase (Fig. 2B, lanes 1, 2).

Thus, the constitutive peroxidase of E-45 was readily distinguishable from the HI peroxidase by its electrophoretic behavior and response to heat shock. Hyperthermal treatment of E-45 led to a noticeable decline in the level of the constitutive peroxidase (Fig. 2B, lanes 2, 3) and a concomitant enhancement in its stress-inducible counterpart. These results can account for the observed decrement in the total peroxidase activity of heat-shocked E-45 cells. As expected, the constitutive peroxidase gene would be subject to heat shock-induced repression, consistent with the cessation of synthesis of normal cellular proteins under hyperthermia. The preexisting peroxidase protein was, evidently, stable during heat shock.

Immunoinactivation of heat-induced and constitutive peroxidase

Whether the HIP and constitutive peroxidase activities were ascribable to the same or distinct proteins was assessed by immunoinactivation. Twenty microliters of a crude extract of heat-shocked mycelium (6.5 mg protein ml^{-1}) of the wild-type strain was incubated with 1, 2, 5, and 10 µl of the rabbit antiserum raised against the HIP at 4°C for 16 h; the mixture was centrifuged at 12000 *g* for 15 min and the supernatant used in enzymatic assays. Control samples were treated with an equivalent amount of the preimmune serum and bovine serum albumin (BSA). With increasing amounts of the antiserum, there was a progressive decrease in enzymatic activity, and a complete loss of peroxidase activity resulted on treatment with 10 µl antiserum. Crude extracts of nonshocked E-45 cells were treated with this antiserum in a similar manner. Contrasted

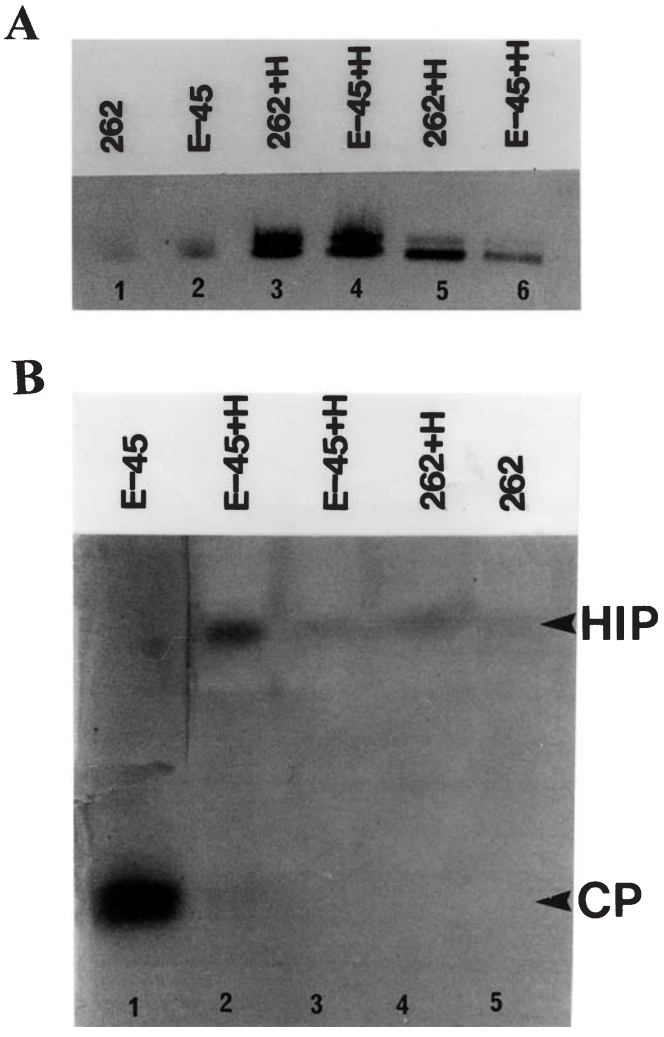

Fig. 2. Electrophoretic resolution of peroxidase activity in nondenaturing gels. **A** Peroxidase activity staining of a nondenaturing alkaline gel. Samples of nonshocked and heat-shocked cell extracts of wild-type strain 262 and the transformant E-45 are shown. Protein concentrations: *lanes 1–4*, 200 μg each; *lanes 5*, *6*, 150 μg each. *H*, heat-shocked. **B** Separation of acidic and basic peroxidase by electrophoresis in nondenaturing acidic gels. Crude extracts of heat-shocked and nonshocked mycelium of E-45 and the wild-type strain 262 were used. E-45 cultures were grown for 20 h at 30°C (*lane 1*) and heat-shocked cultures at 48°C for 1 h (*lanes 2*, *3*); 262 cultures were grown for 14 h at 28°C (*lane 5*) and heat-shocked cultures for 1 h (*lane 4*). Protein per lane: 250 μg for *lanes 1*, *2*, *4*, *5*; 200 μg for *lane 3*. *CP*, constitutive peroxidase; *HIP*, heat-induced peroxidase

with the HIP of the wild-type strain, the CP activity of E-45 remained virtually unchanged (data not shown).

Purification of constitutive peroxidase

In view of the high level of constitutive peroxidase activity of E-45, mycelial cultures of this strain were employed for isolation of the enzyme. Lyophilized mycelium (5 g) was suspended in 75 ml extraction buffer (0.05 M Tris-HCl, pH 7.5, 0.1 mM EDTA, 50 μM β-mercaptoethanol) and stirred for 30 min at 4°C. Two tablets of Protease Inhibitor Cocktail (Complete; Boehringer-Mannheim, Laval, Quebec, Canada) were added to the slurry. The cell suspension was homogenized using a glass Potter-Elvehjem apparatus, the homogenate was centrifuged at 15000 *g* for 20 min at 4°C, and the supernatant was subjected to ammonium sulfate precipitation. In the first step, the salt was added to obtain 40% saturation, the precipitate was discarded, and the supernatant brought to 80% saturation. The mixture was centrifuged at 10000 *g* for 10 min, and the pellet was dissolved in and dialyzed against 20 mM Tris-HCl, 20 mM $MgCl_2$, 20 mM NaCl (pH 7.5). The dialyzed protein sample was applied to a QAE Sepharose (Pharmacia) column that had been equilibrated with the same buffer. The bulk of the protein did not bind to this column; the flow-through fraction comprising peak 1 contained the constitutive peroxidase activity.

Following collection of peak 1, a linear gradient (0–1 M NaCl) was applied to the column. At approximately 0.5 M NaCl, a second minor peak of peroxidase activity corresponding to the HIP was observed. The highly active fractions of peak 1 were pooled and applied to a metal chelation column prepared as follows. Iminodiacetate-epoxy-activated agarose (Sigma) was suspended in a solution containing 0.05 M EDTA and 0.5 M NaCl and washed with water. The supernatant was decanted and replaced with aqueous iron sulfate solution ($FeSO_4$, 4 mg ml^{-1}, pH 3.0), resulting in charging the column with Fe^{2+}. The column was then equilibrated with 50 mM acetate buffer (pH 7.5) containing 0.2 M NaCl. The high-activity fractions of peak 1 were combined, concentrated on Centricon filters (30-kDa cutoff), and the sample dialyzed overnight against acetate buffer. Aliquots (2.5 ml) of the dialyzed QAE-Sepharose pool were loaded on the metal chelation column and eluted with the above acetate buffer. The constitutive peroxidase activity bound weakly to the metal chelation matrix. The pool of active fractions was concentrated to 200 μl using Centricon filters, centrifuged for 10 min at 15000 g, and loaded on a Superose 12 column (Pharmacia), equilibrated with 50 mM citrate–100 mM phosphate buffer (pH 5.0), attached to a fast performance liquid chromatography (FPLC) unit. Elution was conducted using the same buffer. The resulting enzymatically active fractions, showing a single band on SDS-PAGE, were pooled, concentrated, transferred to citrate-phosphate buffer (pH 3.5), and stored at −20°C with 5% w/v glycerol. The overall yield of the purified enzyme was 7%, and the final purification of 1115 fold was attained (Table 3).

Visualization of constitutive peroxidase activity, western blotting, and glycoprotein staining

Nondenaturing gels were prepared as described in "Materials and methods." The protein samples from the enzymatically active fractions, eluted from QAE Sepharose and Superose 12 columns, were subjected to electrophoresis for 2.5 h at 120 V and stained for peroxidase activity, followed by staining with Coomassie blue (Fig. 3A). Constitutive peroxidase exhibited only one active band under nondenaturing conditions; this band corresponded to that

Table 3. Summary of purification steps

Fraction	Activity (units/ml)	Volume (ml)	Protein (mg/ml)	Specific activity (units/mg protein)	Fold purification	Yield (%)
Crude extract	1.72	48	13.5	0.13	1	100
Q-Sepharose pool	0.8	80	0.04	20	153	77.5
Metal chelate Pool	0.94	12	0.03	31.3	241	13.7
Superose-12	2.9	2.0	0.01	145	1115	7.0

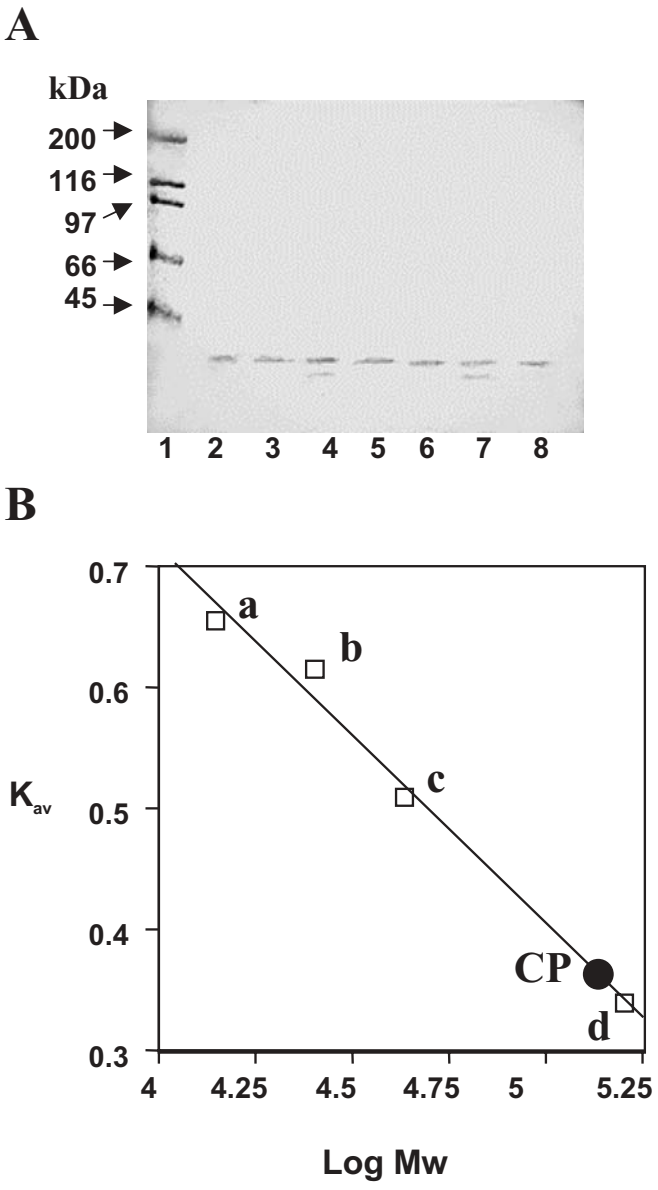

Fig. 3. Fractionation of constitutive peroxidase on Superose 12 gel filtration column. **A** Samples of active fractions from Superose 12 column were analyzed by SDS-PAGE and stained with Coomassie blue. *Lane 1*, molecular mass markers (Bio-Rad): myosin, 200 kDa; β-galactosidase, 116.25 kDa; phosphorylase b, 97.4 kDa; serum albumin, 66.2 kDa; ovalbumin 44 kDa; *lanes 2–8*, 20-µl samples of Superose-12 fractions from different runs. **B** Estimation of molecular mass of CP by gel filtration using a Superose 12 column. M_r calibration standards: *a*, ribonuclease (13.7 kDa); *b*, chymotrypsinogen A (25 kDa); *c*, ovalbumin (43 kDa); *d*, aldolase (158 kDa). The solutions of protein standards and CP were prepared in 50 mM citrate–100 mM phosphate buffer (pH 5.0). Void volume was determined using blue dextran. Data points represent averages of six experiments

of the same mobility when stained in Coomassie blue, indicating that the isolated protein was indeed a peroxidase (not shown). Western blotting of the purified CP was conducted along with a sample of the purified HIP, the blot being probed with the HIP-specific antiserum. The CP protein did not react with the antiserum, demonstrating that this enzyme was immunologically distinct from the heat-inducible isozyme. Furthermore, CP samples did not stain with the basic fuchsin reagent (not shown), in contrast with the positive reaction with the HIP protein reported previously (Machwe et al. 2002).

Apparent molecular mass of constitutive peroxidase

To estimate the molecular mass of the native protein, a Superose 12 column was calibrated with the following molecular mass standards: ribonuclease (13 700); chymotrypsinogen A (25 000); ovalbumin (43 000), and aldolase (158 000). Molecular mass determinations were performed by comparison of elution volume (K_{av}) of CP with the values obtained for the calibration standards, as described previously (Machwe et al. 2002). In the native state the molecular mass of the constitutive peroxidase was estimated to correspond to ~133 kDa, by reference to the plot of K_{av} versus log M_r (Fig. 3B).

pH optimum of constitutive peroxidase

The pH optimum of the CP was tested in the 50 mM phosphate buffer varying from pH 4.0 to 8.5. To 1 ml buffer, 10 µl enzyme (10 µg ml^{-1}), 100 µl ABTS (0.5 g 100 ml^{-1}), and 10 µl 1.5% H_2O_2 were added. After 30 min incubation at 37°C, the samples were tested for peroxidase activity; the optimum pH for CP was determined to be 3.5. Raising the pH from 2 to 3.5 resulted in an increase in activity greater than 300%; the enzyme remained relatively stable between pH 3.0 and 4.0, with a complete inactivation at pH 5.5 (not shown).

Heat sensitivity of constitutive peroxidase

The activity of CP was studied as a function of temperature. Aliquots of 20 µl purified enzyme were incubated at temperatures ranging from 24° to 72°C. After 10 min, 10-µl samples were added to 1 ml assay buffer (50 mM citrate–100 mM phosphate) with ABTS (0.5 mg/ml) and 10 µl 1.5% H_2O_2. The mixture was incubated for 30 min at 37°C and tested spectrophotometrically for peroxidase activity (Fig. 4). Exposure to temperatures up to 58°C resulted in a

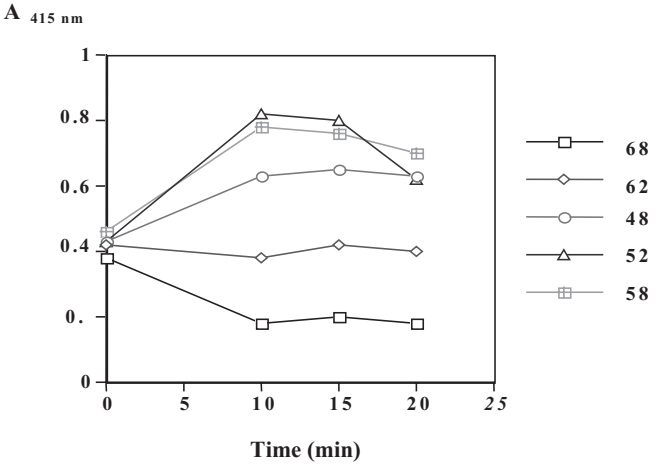

Fig. 4. Effect of temperature on the stability of CP. Samples of the enzyme were incubated at 48°, 52°, 58°, 62°, and 68°C. Aliquots were withdrawn at indicated intervals, transferred to a water bath at 37°C, and assayed for peroxidase activity. The assays were conducted in duplicate; the data points represent averages of two readings at A_{415nm}.

greater than 2-fold increase of enzymatic activity whereas at temperatures higher than 62°C, inactivation resulted. The optimum enzymatic activity was recorded within the range 48°–58°C where a 2- to 2.5-fold increase in specific activity relative to that at 20°C was observed. In view of the maintenance of CP activity up to 68°C, its heat stability over extended periods of time was investigated by incubating 100-µl aliquots at 48°, 52°, 58°, 62°, and 68°C. At 5-min intervals, two 10-µl samples were withdrawn and assayed for peroxidase. Approximately a 2-fold increase in activity was observed during the first 10 min of incubation 52° and 58°C, decreasing by 20% after 10 min, but remaining stable up to 20 min. At 52°C, however, the enzyme remained active for up to at least 20 min, with only a slight decrease in activity thereafter.

Spectroscopic analysis of constitutive peroxidase

All known peroxidases are heme enzymes, characterized by the presence of a Soret band (around 410 nm) as well as α (~550 nm) and β (~500 nm) bands in the absorption spectrum of the native enzyme and by susceptibility to inhibition by sodium azide. Therefore, the inhibition of enzymatic activity by sodium azide may be used as an indirect evidence for the presence of heme as a prosthetic group in the enzyme. The absorption spectrum of the constitutive peroxidase was recorded using 60 µl enzyme (10 µg ml^{-1}) in 0.3 ml 50 mM citrate–100 mM phosphate buffer (pH 3.5) at 25°C with a Shimadzu (UV-2101) scanning spectrophotometer (Shimadzu, Tokyo, Japan). The spectrum of the native enzyme showed the following peaks: at 560 nm (α), 490 nm (β), and the most pronounced Soret band at 415 nm (Fig. 5A).

Next, the activity of CP was monitored after addition of sodium azide to the peroxidase assay mixture containing 10 µl CP (10 µg ml^{-1}) in 300 µl 50 mM citrate–100 mM phosphate buffer (pH 3.5) with ABTS (0.5 mg ml^{-1}) and 10 µl

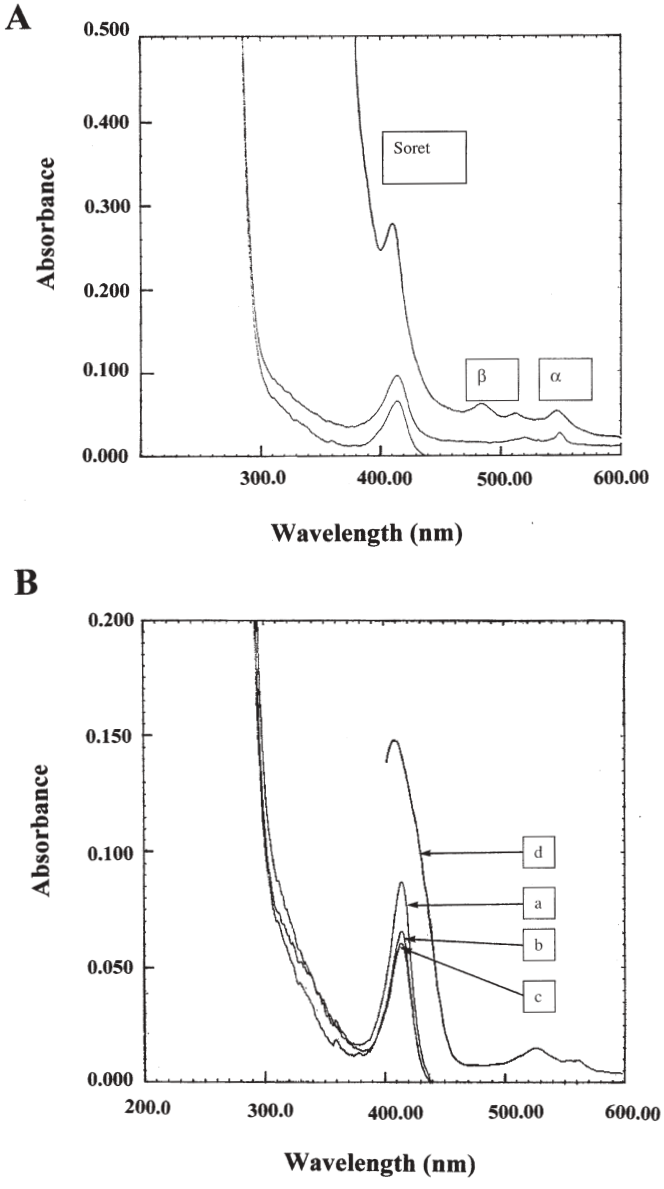

Fig. 5. Absorption spectra of native CP. **A** Absorption spectrum enzyme dissolved in 50 mM sodium citrate–100 mM phosphate buffer (pH 3.5) at 25°C. **B**, Absorption spectra of CP, complexed with sodium azide: *a*, native enzyme; *b*, *c*, enzyme in 0.2 mM and 0.4 mM sodium azide, respectively; *d*, spectrum *c* magnified 2.5 times

1.5% H_2O_2. Aliquots of 0.02% sodium azide were added to a final concentration range of 0.125–0.5 mM, and the samples were incubated at 25°C for 30 min and assayed for peroxidase activity. Sodium azide inhibited CP activity at relatively low concentrations (0.125 mM); at 0.187 mM, >75% of activity was abolished whereas complete inhibition occurred at 0.5 mM, confirming that CP is a heme-containing enzyme. The absorption spectrum of the enzyme, following the addition of sodium azide, was recorded along with that of the uninhibited enzyme in 100 mM acetate buffer (pH 7.0). The reaction mixture was incubated at 25°C for 2 min and the spectrum was recorded; the peak at 415 nm was diminished on interaction with 0.625 mM sodium azide (Fig. 5B).

Kinetic analysis

The ability of the CP to catalyze a two-substrate reaction was tested with ABTS and hydrogen peroxide, as well as with guaiacol and hydrogen peroxide. While testing ABTS as a substrate for CP, the reaction was carried out in 50 mM citrate–100 mM phosphate buffer (pH 3.5) with 15 µl enzyme (10 µg ml^{-1}) at 25°C (experimentally determined molar extinction coefficient for ABTS at 415 nm at pH 3.5 = 35.95 mM^{-1} cm^{-1}). Reciprocal plots of reaction velocity versus substrate concentration were prepared for ABTS oxidation employing ten concentrations of ABTS in triplicate (varying from 2.5 to 500 µM); hydrogen peroxide was maintained at a constant level (0.5 mM). The resulting data were analyzed using the ACS Enzyme Kinetics software. A hyperbolic relationship was obtained in a plot of reaction velocity versus reciprocal substrate concentration on using the nonlinear regression method; an Eadie–Hofstee plot of V_o versus V_o/[S] yielded a straight line. The apparent K_m was determined to be ~23 µM, whereas the calculated value for V_{max} was ~448 nmol mg^{-1} and k_{cat} was 0.3 s^{-1}. When H_2O_2 was tested as the variable substrate from 5 to 400 µM, a fixed concentration of ABTS (0.5 mM) was used with 15 µl enzyme (10 µg ml^{-1}). Again, a hyperbolic curve was observed for the relationship between absorbance change and 1/[S] (nonlinear regression), and the values of K_m (~55 µM), V_{max} (~453 nmol mg^{-1}), and k_{cat} (~0.3 s^{-1}) were derived. Guaiacol was also tested as a CP substrate and, consistent with the results obtained with HIP, it was not utilized by CP.

Discussion

Our earlier work had shown the induction of a peroxidase activity in cells exposed to heat shock and oxidative stress (Kapoor and Lewis 1987b). This peroxidase appeared to be implicated in the development of thermotolerance as all the stress treatments – hyperthermia, hydrogen peroxide, cadmium chloride, and sodium arsenite – leading to the induction of peroxidase also conferred thermotolerance (Kapoor et al. 1990). Development of thermotolerance has frequently been attributed to the expression of Hsps, but reports demonstrating a lack of dependence of thermotolerance on Hsps synthesis in yeast are also available (Hall 1983; Smith and Yaffe 1991). In plants, thermotolerance can be triggered by treatment with acetylsalicylic acid and H_2O_2 (Lopez-Delgado et al. 1998), and thermoprotection by accumulation of trehalose at high concentrations in yeast and other fungi has been noted (Hottinger et al. 1994). Ribeiro et al. (1997) uncovered a link between trehalose synthesis and the development of thermotolerance in *Schizosaccharomyces pombe* by analyzing a mutant defective in the gene encoding trehalose-6-phosphate synthase. Moreover, information showing that a cooperative action of trehalose and Hsp104 contributes to thermotolerance of stationary-phase cultures has emerged from studies of *S. cerevisiae* strains harboring a defective trehalose-6-phosphate phosphatase gene and a mutant *hsp*104 gene (Elliott et al. 1996). Acquisition of thermotolerance is a complex process, relying on the coordinated functioning of diverse pathways. Thus, the road to thermotolerance in different organisms may reflect selective adaptations to the habitat or species-specific physiological or developmental phenomena. It has also been proposed that slow growth, occasioned by carbon source deprivation resulting in release of catabolite repression, can also trigger a stress tolerance response (Gross and Elliott 1996).

In the present communication, we have described a transformant E-45 of *N. crassa* that exhibited distinctive features with respect to growth characteristics and response to applied stresses. In addition to being a slow grower at normal temperatures, it was highly sensitive to low temperature. A surprising feature of E-45 was its high constitutive level of peroxidase and a remarkable ability to withstand normally "lethal" temperatures. These properties of E-45 are reminiscent of yeast mutants, with high constitutive levels of Hsp104, where thermotolerance was elicited in the absence of prior heat shock or alternative stress treatment (Lindquist and Kim 1996). The action of peroxidase in amelioration of hyperthermally generated oxidative stress, coupled with the slow growth rate of E-45, may account for its high intrinsic thermotolerance.

The pattern of peroxidase expression in E-45 was distinct from that of the wild-type strain. First, E-45 had a high constitutive level of peroxidase and second, peroxidase activity in crude extracts appeared diminished upon heat shock, in comparison with that of nonshocked cells. Nevertheless, the absolute level of total peroxidase activity in heat-shocked E-45 cells approached that of the wild-type cells under hyperthermia. It is thus clear that at least two forms of peroxidase are synthesized in *N. crassa* cells, one of which is constitutively expressed at a very low level in wild-type cells and a second specifically induced under stress. Wild-type cells, therefore, do not constitute a suitable source for purification of the constitutive peroxidase.

That CP is distinct from HIP is illustrated by several differences in their properties. Fractionation on QAE-Sepharose anion-exchange columns resolved the peroxidase activity into two distinct components: HIP bound to the matrix while CP failed to do so. The apparent molecular mass of the native HIP (~116 kDa) and that in denaturing gels (~95 kDa) is indicative of a monomeric protein (Machwe et al. 2002) whereas CP appears to be composed of smaller polypeptides of ~37 kDa. The size of the CP polypeptide is in the size range of horseradish peroxidase, glutathione peroxidase, and manganese peroxidase (Pribnow et al. 1989). Judging by the gel filtration data, the native state of CP would be a homomultimer. However, the oligomeric structure of CP needs to be verified by chemical cross-linking experiments. As HIP is a glycoprotein (Machwe et al. 2002) and manganese and lignin peroxidases of *Phanerochaete chrysosporium* and *Coprinus cinereus* exhibit varying degrees of glycosylation, it was of interest to determine if CP was a glycoprotein. In contrast with HIP, the polypeptide band corresponding to CP did not show positive staining with the periodic acid–Schiff

reagents. The two enzymes also differ in their heat sensitivity. HIP exhibited maximal activity between 43° and 55°C whereas CP showed the highest specific activity between 48° and 58°C and was stable at these temperatures for at least 20 min.

These results suggest that in vivo CP may be more stable during extended exposure to heat and it may be able to function at more extreme temperatures than HIP. Furthermore, the pH optima of the two enzymes differ – 3.5 for CP and 5.0 for HIP. This pH range is not unusual for fungal peroxidases as the manganese peroxidase isozymes I–III of white rot fungi have pH optima between 3.75 and 4.5 (Ruttiman-Johnson et al. 1994) whereas Korean radish isoperoxidases are optimally active in the pH 5–6.5 range (Lee et al. 1991). The two *N. crassa* peroxidases also differ with respect to their kinetic properties: K_{cat} for HIP was ~10 s^{-1}, the reaction rate being ~33 times faster than CP (K_{cat} = 0.3 s^{-1}). Whether *N. crassa* cells contain additional peroxidase isoenzymes and multigene families requires further investigation. Several examples of peroxidase multigene families are available in fungi, including the lignin peroxidase genes of the wood-rot fungi *Phanerochaete chrysosporium* (Gaskell et al. 1991) and *Trametes versicolor* (Jonsson et al. 1994).

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