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FULL PAPER

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Induction profiles and properties of a novel stress-induced peroxidase in *Neurospora crassa*

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Abstract Exposure of *Neurospora crassa* cells to heat shock and oxidative stress results in the synthesis of several stress-related proteins, including a peroxidase. Northern blot analysis of total RNA revealed a heat-inducible (HI)-peroxidase transcript of ~10kb, induced in response to heat shock and oxidative stress. The HI peroxidase was isolated from heat-shocked mycelium and purified to near homogeneity, and its properties were examined. Chromatography in size-exclusion matrices yielded an apparent molecular mass of ~116kDa for the native enzyme, whereas the estimate obtained by SDS-PAGE was 90–95kDa. Studies of substrate saturation kinetics were conducted using the purified enzyme with ABTS [2,2′-azino-bis (3-ethylbenzthiazole-6-sulfonic acid)] and H_2O_2 as substrates. The experimentally estimated K_m, V_{max}, and K_{cat} values for ABTS were ~36μM, 5200nmol mg^{-1}, and 8s^{-1}, respectively, and those for H_2O_2 were 44μM, 6640nmol mg^{-1}, and 10s^{-1}. *O*-dianisidine was a substrate for this enzyme, but guaiacol was not. HI peroxidase was found to be a glycoprotein, stable at temperatures up to 60°C.

Key words Heat shock · *Neurospora* · Peroxidase · Plumbagin · Thermotolerance

Introduction

All organisms respond to unfavorable conditions by the rapid expression of a selected set of genes encoding the stress-responsive proteins. Various treatments – hyperthermia, oxidizing agents, heavy metals, teratogens, amino acid analogues, and nutrient depletion – stimulate the synthesis of all, or a subset of, these proteins (Johnston et al. 1980; Levinson et al. 1980). Stress proteins, also referred to as heat shock proteins (Hsps), can be classified broadly into three major protein families based on their apparent molecular mass and sequence homology: 110–80kDa (high range), 68–70kDa (intermediate), and ≤50kDa (low range). The Hsp70 and Hsp90 families of stress proteins have been investigated extensively, and their importance as molecular chaperones, as well as their role in vital cellular processes, is firmly established (Hartl 1996; Csermely et al. 1998). A characteristic outcome of the heat shock response is the subsequent development of thermotolerance. Following exposure to sublethal temperatures, the target organism acquires the ability to withstand temperatures that would otherwise be lethal. Although the exact molecular mechanism underlying the development of thermotolerance is not clearly understood, there is substantial evidence indicating a connection between the preferential synthesis of Hsps – such as Hsp70, Hsp104, and Hsp101 and thermotolerance in different organisms (Solomon et al. 1991; Lindquist and Kim 1996; Quietsch et al. 2000).

Exposure of *N. crassa* cells to oxidative stress, heat shock, and toxic metals stimulates the rapid and transient synthesis of an array of stress proteins, prominent among these being Hsp70 and Hsp80, a member of the eukaryotic Hsp90 family. One of the heat-induced proteins was shown to exhibit a peroxidase activity (Kapoor and Lewis 1987a,b) that appeared to be associated with the acquisition of thermotolerance (Kapoor et al. 1990). In addition, this enzyme was subject to induction by H_2O_2, sodium arsenite, and $CdCl_2$. Interestingly, all these stress treatments also resulted in the acquisition of thermotolerance – a high degree of survival at normally lethal temperatures – whereas only arsenite was observed to stimulate the increased accumulation of a subset of heat shock proteins, Hsp70 and Hsp80 (Kapoor et al. 1990). The development of thermotolerance, therefore, is not solely dependent on the presence of the conventional set of Hsps. In conjunction with superoxide dismutase (SOD) and catalase, this heat-induced (HI)-peroxidase is likely to be an important component of the defense arsenal against reactive oxygen species ($O_2\cdot^-$, ·OH, H_2O_2) generated during hyperthermia and oxidative stress. Elevated intracellular levels of H_2O_2,

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and other peroxides derived therefrom, lead to severe damage of cellular membranes and macromolecules.

Previously, we reported the cloning of a 4-kb *Eco*RI fragment of the putative HI-peroxidase gene (*hspp*-1) of *N. crassa* (Machwe and Kapoor 1993) by screening a genomic library with a cDNA clone of the manganese peroxidase gene of *Phanerochaete chrysosporium* (Pribnow et al. 1989). Hybrid-arrested in vitro translation of RNA from heat-shocked cells, using *hspp*-1 DNA, indicated a correspondence between HI-peroxidase and the highest molecular mass Hsp of *N. crassa* (Machwe and Kapoor 1993). This article presents the results of enzymatic activity measurements and Western blot analysis of heat-shocked and plumbagin-treated mycelial cells, to evaluate the variation in the level of peroxidase in response to oxidative stress. Northern blot analysis was conducted to trace the corresponding mRNA profiles. In addition, the HI-peroxidase was isolated from heat-shocked mycelium of a wild-type strain of *N. crassa* and purified to near homogeneity. Some of the properties of the purified enzyme are reported here.

Materials and methods

Growth of cultures, heat shock, and oxidative stress treatment

Neurospora crassa Shear et Dodge (Fungal Genetics Stock Centre No. 262; University of Kansas Medical Center, USA) cultures were grown in Vogel's minimal medium, Vm (Vogel 1956) at 28°C, while shaking. The carbon source was 2% sucrose, or ethanol, as indicated. Following 14h of growth, heat shock was administered by transferring the cultures to a shaker at 48°C for 1h and the newly synthesized proteins were labeled with [^{35}S]-methionine as described previously (Kapoor and Lewis 1987a). The mycelium was harvested by vacuum filtration, lyophilized, and stored at −20°C. To study the effect of metal ions, the cultures were grown for 14h at 28°C; then, $CdCl_2$ and $MnCl_2$ (100μM each) were added. After 15min, the cultures destined to be heat-shocked were transferred to 48°C while the controls were maintained at 28°C. All of the cultures were harvested after 60min. Oxidative stress was administered by treating the mycelial cells, grown for 14h at 28°C, with 50–100μM plumbagin (Sigma, Oakville, Canada) for 1h. A 10-mM stock solution of plumbagin in ethanol was diluted as required.

Enzyme assays, protein determination, and kinetic analysis

Peroxidase activity was monitored spectrophotometrically by following the oxidation of *O*-dianisidine at 460nm, in the presence of H_2O_2 (Hoffman et al. 1979). Alternatively, ABTS [2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)] was used as a substrate, along with H_2O_2 and the change in absorbance at 415nm was monitored. Activity was measured in 50mM citrate/100mM phosphate buffer (pH 5.0) with 0.05% ABTS and 1.5% H_2O_2. The reaction mixture was incubated for 20min at room temperature and absorbance measured in a Gilford (model 250) spectrophotometer (Gilford, Oberlin, USA). A molar extinction coefficient of 36$mM^{-1} cm^{-1}$ was determined experimentally by recording the absorption spectrum of reduced ABTS from 300 to 450nm using a Shimadzu (UV-2101) scanning spectrophotometer (Shimadzu, Tokyo, Japan). Specific activity is defined as the change in absorbance at 415$nm\,min^{-1} mg^{-1}$ protein. Protein was estimated by the Bio-Rad (Mississauga, Canada) microassay (Bradford 1976) with bovine serum albumin (BSA) as a calibration standard.

Preparation of mycelial extracts and gel electrophoresis

Lyophilized mycelium was homogenized in 50mM potassium phosphate (pH 7.8) – 0.1mM ethylenediaminetetraacetic acid (EDTA) buffer and centrifuged at 15000*g* at 4°C for 12min. Samples of the supernatant were electrophoresed in 10% isocratic polyacrylamide gels under nondenaturing conditions. For visualization of peroxidase activity, the gels were immersed for 1h in a staining solution containing 2mM *O*-dianisidine–0.1mM riboflavin–10mM potassium phosphate buffer (pH 7.2), rinsed with distilled water, and treated with 0.2mM H_2O_2–10mM potassium phosphate buffer (pH 7.2). Electrophoresis in sodium dodecyl sulfate (SDS)-polyacrylamide gels was conducted using standard procedures (Laemmli 1970).

Isolation of peroxidase from nondenaturing gels for the preparation of antiserum

A crude extract prepared from heat-shocked mycelial cultures was electrophoresed in dual gradient gels containing a sucrose/polyacrylamide gradient (3%–25%) under nondenaturing conditions. The protein samples were brought to 15% sucrose before loading on the gradient gel surface in a wide slot formed by a flat comb. Electrophoresis was carried out at 20mA for 26h, at 4°C, in 124mM Tris-HCl–27mM barbituric acid–1mM EDTA (pH 8.7). A mixture of native protein markers (Pharmacia)–thyroglobulin (669kDa), ferritin (440kDa), catalase (232kDa), lactate dehydrogenase (140kDa), and bovine serum albumin (69kDa) was applied to a narrow well. After completion of electrophoresis, the gel was stained for peroxidase, as described in the foregoing section, and the channel containing the marker proteins was stained with Coomassie blue. The gel sector exhibiting peroxidase activity was excised, rinsed in distilled water, equilibrated with 0.0625M Tris-HCl buffer (pH 6.8)–2% SDS–10% glycerol–5% β-mercaptoethanol, placed on 10% isocratic SDS-polyacrylamide gels, and subjected to electrophoresis at 20mA for 6–8h. The gels were fixed in 25% isopropanol–10% acetic acid and stained with Coomassie blue. The relative molecular mass of polypeptides was estimated by coelectrophoresis with high molecular mass protein standards (BRL, Burlington, Canada) including myosin (H-chain), 200kDa; phosphorylase b, 92.5kDa; bovine

serum albumin, 68 kDa; ovalbumin, 43 kDa; α-chymotrypsin, 25 kDa; β-lactalbumin, 18.4 kDa, and cytochrome C, 12 kDa.

The stained protein profile showed a polypeptide corresponding to the HI-peroxidase, of ~95 kDa, that was excised, frozen in liquid nitrogen, and crushed into a fine powder in a cold mortar. This sample (~0.1 mg protein) was dissolved in 1 ml sterile distilled water, suspended in Freund's complete adjuvant, and administered to 4-month-old New Zealand rabbits by subcutaneous injection, followed by booster doses of ~0.05 mg peroxidase protein at 2-week intervals. The antiserum titer was estimated by a standard ELISA (enzyme-linked-immunosorbent assay) protocol.

Western blot analysis

Proteins resolved by SDS-polyacrylamide gel electrophoresis (PAGE) were transferred to Immobilon (polyvinylidene fluoride, PVDF) membranes by electroblotting for 16 h at 30 V and 4°C (Towbin et al. 1979). The membrane was then washed twice for 1 h in phosphate-buffered saline (PBS) (58.2 mM Na_2PO_4–18.1 mM KH_2PO_4, 75.3 mM NaCl, pH 7.2), and immersed in 10% skim milk in BTAS (10 mM Tris–150 mM NaCl–0.02% azide) for 4 h at 4°C with gentle shaking. It was then washed in PBS and incubated with a 1:1000 dilution of the antiperoxidase antiserum overnight, with gentle shaking at 4°C. The membrane was rinsed five times with PBS over a 2-h interval and incubated for 1 h at room temperature with a 1:10 000 dilution of the goat antirabbit secondary antibody conjugated to alkaline phosphatase (Sigma). Visualization was achieved using the ECL Chemiluminescence reagents (Amersham, Baie d'Urfé, Canada).

Glycoprotein staining

A slightly modified version of the procedure outlined by Segrest and Jackson (1972), based on periodic acid-Schiff (PAS) staining, was employed for visualization of glycoproteins. Following resolution by SDS-PAGE, the gel was immersed in 12.5% trichloroacetic acid for 30 min, rinsed with distilled water, and soaked in 1% periodic acid prepared in 3% acetic acid for 50 min. Next, the gel was washed extensively in distilled water and stained. The staining solution was prepared as follows: 4 g potassium metabisulfite, 5.25 ml concentrated HCl, and 2 g basic fuchsin were dissolved in 500 ml distilled water and allowed to stand for 2 h. The solution was treated with activated charcoal for 15 min and filtered. The gel was immersed in the stain for 2 h and destained in 0.5% potassium metabisulfite.

Immunoinactivation of peroxidase in crude extracts of heat-shocked mycelium

The antiserum was tested for specificity toward the heat-induced peroxidase. A crude extract of heat-shocked mycelium (20 μl; 6.5 mg protein ml^{-1}) was incubated with increasing amounts of the antiserum (1, 2, 5, and 10 μl) at 4°C for 16 h. The mixture was centrifuged for 15 min at 12 000 *g* and the supernatant was assayed for peroxidase activity using the *O*-dianisidine/H_2O_2 substrate combination. As a control, the crude extract of heat-shocked mycelium was treated with an equivalent amount of preimmune serum and BSA.

Preparation of RNA and Northern blot hybridization

Lyophilized mycelium from normally grown or heat-shocked cultures was ground in a mortar with acid-washed sand and 2 vol 0.1 M Tris-HCl buffer (pH 9.0)–0.1 M NaCl–10 mM EDTA–1% SDS (TNES buffer). Following initial grinding, 2 vol phenol-chloroform-isoamyl alcohol (PCI, 50:50:1) was added and grinding continued for 2 min. The mixture was centrifuged at 12 000 *g* for 10 min at 4°C and the aqueous phase collected. The organic phase was reextracted with TNES buffer, and the aqueous phases were pooled and treated twice more with PCI. The final aqueous phase was made to 0.15 M in NaCl and the nucleic acid fraction was precipitated with 2 vol ethanol at −20°C, overnight. The precipitate was washed thrice with 2 M LiCl–10 mM EDTA, dissolved in sterile distilled water, and stored at −70°C. RNA samples were subjected to electrophoresis in 1%–1.5% agarose-formaldehyde gels and transferred to nylon filters using 20× SSPE (3.6 M NaCl–0.2 M sodium phosphate, pH 7.7–2 mM EDTA) as the transfer buffer. Before transfer, the gel was soaked in 0.05 N NaOH for 20 min, rinsed in distilled water, and washed in 20× SSPE for 45 min. Prehybridization was performed in 6× SSPE–0.05% sodium pyrophosphate–0.5% SDS–10× Denhardt's solution–10 mM EDTA–100 μg denatured herring sperm DNA/ml, for 12 h at 60°C. Hybridization was conducted for 12–14 h in the same solution containing 2×10^6 cpm denatured probe ml^{-1}. Following hybridization the filters were washed in 2× SSPE–0.05% sodium pyrophosphate for 15 min at room temperature, followed by a second wash in the same solution for 15 min at 60°C, air-dried, and exposed to X-ray films with intensifying screens. If the blots were to be reused, the probe was removed by washing for 2 h at 65°C in 5 mM Tris-HCl (pH 8.0)–2 mM EDTA–0.1× Denhardt's solution, complete removal of the probe being verified by autoradiography.

DNA probes

The DNA probes used included (i) ~4-kb *Eco*RI fragment representing the putative *N. crassa* peroxidase gene DNA and (ii) 2.2-kb *Sal*I fragment of the β-tubulin gene of *N. crassa*. The probes were labeled with [α-^{32}P]dCTP (deoxycytidine triphosphate; specific activity, 3000 Ci/mmol; ICN) by the random oligonucleotide-primed labeling procedure.

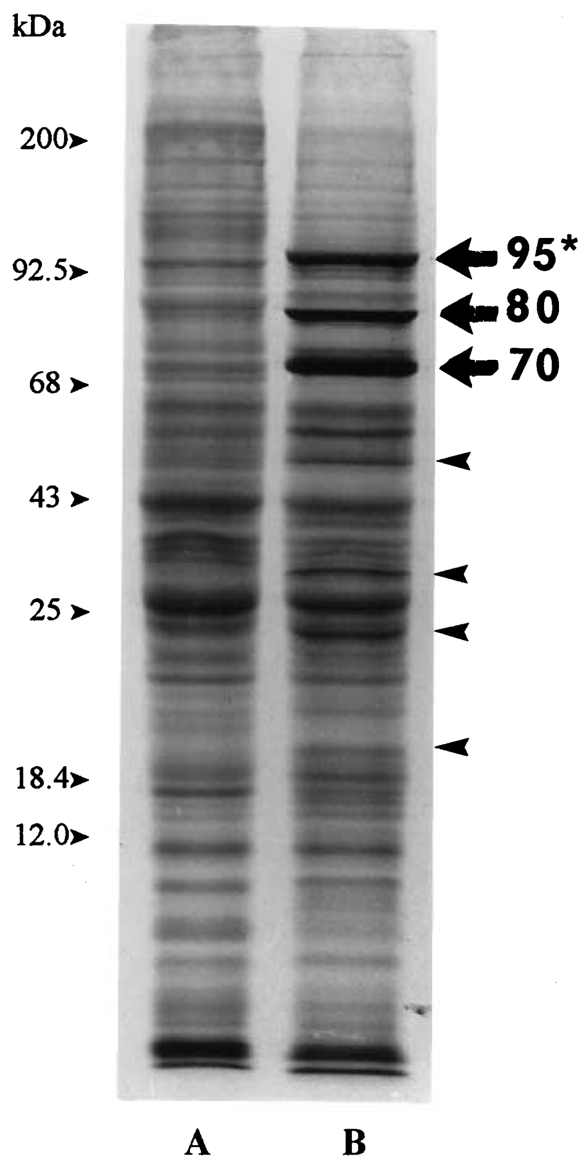

Fig. 1. Heat shock proteins of *Neurospora crassa.* Autoradiograph of a linear gradient (5%–15%) polyacrylamide-sodium dodecyl sulfate gel showing the electrophoretic resolution of polypeptides labeled with [^{35}S]-methionine for 1 h during growth at 28°C (*lane A*) and for 1 h during heat shock at 48°C (*lane B*). The positions of molecular mass standards are given on the *left*. The three high molecular mass polypeptides, incorporating the label under heat shock conditions (Hsp70, Hsp80, and Hsp95) are indicated. The band labeled Hsp95 (*asterisk*) corresponds to the heat-inducible peroxidase

Results and discussion

Synthesis of heat shock proteins and induction of peroxidase

When 14-h-old actively growing mycelial cultures were transferred to 48°C for 1 h and labeled with [^{35}S]-methionine, three high molecular mass polypeptides in the range of ~70 to 100 kDa appeared heavily labeled in heat-shocked cells (Fig. 1). As reported previously, exposure of *N. crassa* cells to stress-inducing agents including heat shock, oxidative stress, and heavy metals resulted in a pronounced increase in peroxidase activity (Kapoor and Lewis 1987b; Kapoor et al. 1990). Furthermore, peroxidase was virtually undetectable in nonshocked cells but appeared within 10 min of stress treatment, exhibiting kinetics characteristics of a stress-responsive protein. Ethanol and depletion of carbon source have previously been shown to induce the synthesis of another heat shock protein, Hsp80, of *N. crassa* (Roychowdhury and Kapoor 1988). To examine the effect of these conditions on induction of peroxidase, cultures were grown in Vm with 0.5%, 1.0%, and 2% ethanol as the sole carbon source for 24 h, following which heat shock was administered and peroxidase activity monitored. As shown in Table 1, there was an increase of approximately sixfold in specific activity of peroxidase on heat shock of cells grown on 0.2% sucrose compared to a threefold increase on heat shock in the presence of 2% sucrose. In contrast, growth on ethanol supported a higher level of peroxidase activity, which was only marginally enhanced on subsequent heat shock treatment. Ethanol has been shown to induce the synthesis of Hsps in *E. coli*, yeast, and mammalian cells (Neidhart et al. 1984).

Table 1. Effect of ethanol, carbon source starvation, and heat shock on peroxidase activity

Medium (Vm) (carbon source)	Specific activity		Fold change	
	NS	HS	ΔE	ΔHS
1. 2% Sucrose	0.074	0.253	–	3.4
2. 0.2% Sucrose	0.064	0.384	0.9	6.0
3. 0.5% Ethanol	0.217	0.292	2.9	1.3
4. 1% Ethanol	0.388	0.462	5.2	1.2
5. 2% Ethanol	0.180	0.267	2.4	1.5

Vm, Vogel's minimal medium; NS, normal growth temperature (28°C); HS, heat-shocked cells; ΔE, fold increase in specific activity (SA) relative to cultures grown in 2% sucrose; ΔHS, fold increase due to heat shock, given by HS : NS, the ratio of specific activity of heat-shocked versus nonshocked, control cultures

Immunoinactivation of peroxidase

Polyclonal antiserum, raised against the peroxidase polypeptide, was tested for specificity by immunoprecipitation. The antiserum was incubated overnight with a crude extract of heat-shocked mycelium at 4°C, following which the mixture was centrifuged, and the supernatant carefully withdrawn and used in enzymatic assays for peroxidase. With increasing quantities of the antiserum, there was a distinct reduction in the enzymatic activity, and at the two highest levels tested a complete inhibition of peroxidase activity was observed. The controls, in which the heat-shocked protein extract was incubated with an equivalent amount of preimmune serum or BSA, exhibited peroxidase activity comparable to that of the heat-shocked cell extracts in the absence of any antiserum (not shown). Thus, direct quenching of the enzymatic activity by the peroxidase antiserum confirmed its specificity for the heat-inducible peroxidase.

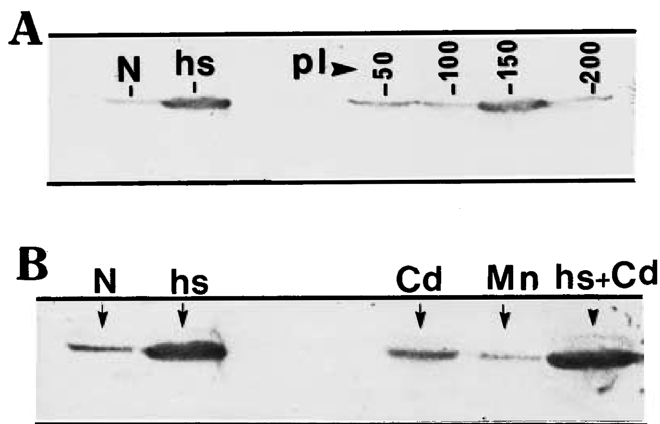

Fig. 2. Western blot analysis of peroxidase expression under various conditions. **A** Comparison of peroxidase protein levels in mycelium treated with plumbagin in the absence of heat shock. *N*, nonshocked cells; *hs*, 14-h-old mycelium heat-shocked for 1 h; *pl*, concentrations of plumbagin from 50 to 200 μM. **B** Effect of cadmium and manganese on peroxidase levels. *N*, nonshocked cells; *hs*, heat-shocked as in **A**; *Cd*, 14-h-old cells treated with 100 μM $CdCl_2$ for 1 h; *Mn*, 14-h-old cells treated with 100 μM $MnCl_2$; *hs + Cd*, cells heat-shocked for 1 h in the presence of 100 μM $CdCl_2$

Western blot analysis of the effect of plumbagin, $CdCl_2$, and $MnCl_2$ on induction of peroxidase

Our earlier work had indicated that heat shock and treatment with oxidative stress-inducing agents such as hydrogen peroxide and some metal ions enhanced peroxidase activity (Kapoor and Lewis 1987b). To seek further evidence for the oxidative and chemical stress-induced expression of peroxidase, Western blots prepared with crude extracts of mycelium that had been treated with plumbagin, $CdCl_2$, and $MnCl_2$ were screened with the peroxidase antiserum. Figure 2A shows the results of a Western blot treated with the antiperoxidase antiserum. The peroxidase band detected was close to the 92.5-kDa marker in SDS-polyacrylamide gels that corresponds to the ~95-kDa polypeptide indicated in Fig. 1. The intensity of this band was found to increase following heat shock treatment.

Plumbagin is a quinone that can generate superoxide radicals through the process of autoxidation (Hassan and Fridovich 1979). Therefore, growth of cultures in the presence of plumbagin in the medium is experimentally used to generate oxidative stress. When extracts of plumbagin-treated *N. crassa* cells were analyzed by Western blotting, a clear band was visualized at the same position. The intensity of this band was the highest in the cells treated with 150 μM plumbagin. The results of enzymatic activity measurements were consistent with the Western blots: as the plumbagin concentration was raised from 50 to 200 μM there was a steady increase in peroxidase activity, peaking at 150 μM (not shown).

Previous work in our laboratory had shown that $CdCl_2$ and sodium arsenite, alone as well as in conjunction with heat shock, promoted an increase in peroxidase activity relative to controls, whereas the divalent cations Mn^{2+} and Cu^{2+} were observed to suppress it. To verify the effect of divalent ions on peroxidase expression, Western blot analysis was conducted using protein samples from 14-h-old cultures grown in the presence of 100 μM $CdCl_2$ and 100 μM $MnCl_2$ for 1 h and from cultures that were heat-shocked after the metal stress treatment. As shown in Fig. 2B, extracts of cells exposed to a combination of heat shock and $CdCl_2$ exhibited a prominent immunoreactive peroxidase band. On the other hand, treatment with $MnCl_2$ alone resulted in a signal weaker than that elicited under normal growth conditions; the band intensity remained unaltered even after heat shock treatment (not shown).

Northern blot analysis of stress-induced expression of the HI-peroxidase gene

To study the effect of heat shock on the regulation of peroxidase gene expression, Northern blots were prepared with total RNA from 14-h-old *N. crassa* cells that had been heat-shocked for increasing lengths of time and hybridized with the ^{32}P-labeled 4-kb *Eco*RI fragment of the putative HI-peroxidase gene. A transcript ~10 kb in size, which was detectable in lower amounts in the mycelium grown at 28°C, exhibited a severalfold increase from 15 to 60 min (and a decline thereafter) in the heat-shocked mycelium (Fig. 3A). Significant amounts of this transcript persisted even up to 180 min of heat shock. The induction profile of this transcript is consistent with that of a typical heat shock gene transcript and similar to that reported previously (Machwe and Kapoor 1993). The extremely large size of the HI-peroxidase transcript is interesting insofar as it is relatively uncommon for fungal transcripts. Considering the experimentally estimated molecular mass of the protein, it is likely that the bulk of the mRNA is not translated. This situation may be similar to that of mRNA for nitric oxide synthase of rat cerebellum, which is 10.5 kb whereas the coding sequence of the gene is 4.3 kb, preceded by a long untranslated leader (Bredt et al. 1991). The same blot was reprobed with the β-tubulin gene of *N. crassa*, following removal of the original probe, as described in Material and methods. The β-tubulin message level witnessed before heat shock treatment (Fig. 3B; 0 min) was maintained for 30 min of heat shock. The RNA samples from cells subjected to heat shock for 45 min or longer exhibited a reduction in the level of β-tubulin mRNA. These results are consistent with the profile expected of a message corresponding to a nonstress-responsive gene.

As our earlier work had suggested a close connection between heat shock and oxidative stress in the induction of peroxidase and in the subsequent development of thermotolerance (Kapoor et al. 1990), it was of interest to examine the effect of oxidative stress on peroxidase gene expression. Therefore, a Northern blot prepared with total RNA, isolated from cultures grown for 14 h at 28°C and treated with plumbagin for 1 h (without heat shock), was hybridized with the above-mentioned HI-peroxidase probe. As shown in Fig. 3C, although a basal level of the peroxidase transcript was present in the untreated mycelia, the accumulation of this transcript increased considerably on

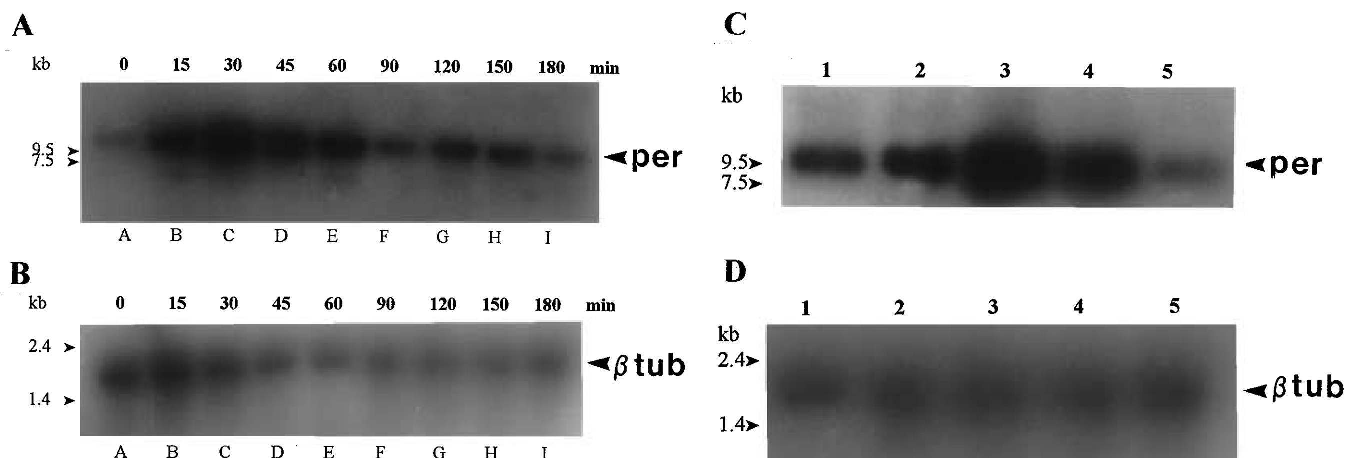

Fig. 3. Expression of heat-inducible (HI) peroxidase under heat shock and oxidative stress. **A** Autoradiograph of a Northern blot of total RNA isolated from cultures grown for 14h at 28°C, heat-shocked for intervals varying from 15 to 180min, and hybridized with the ^{32}P-labeled [per polymerase chain reaction, (PCR)] DNA fragment. **B** The blot in **A** was rehybridized with a ^{32}P-labeled β-tubulin gene of *N. crassa* following removal of the original probe. **C** Autoradiograph of a Northern blot of total RNA from cultures grown for 14h at 28°C and then treated with plumbagin for 1h. *Lane 1*, RNA from cells grown at 28°C; *lanes 2–5*, RNA from mycelium grown in the presence of 50, 100, 150, and 200μM plumbagin, respectively. The blot was hybridized with ^{32}P-labeled DNA. **D** The blot of **C** hybridized with β-tubulin gene of *N. crassa* after removal of original probe. Position of RNA size markers is given on the *left*. *per*, peroxidase; β-*tub*, β-tubulin

treatment with plumbagin, up to a concentration of 100μM, and declined thereafter. The β-tubulin mRNA level, on the other hand, did not change on treatment with plumbagin (Fig. 3D).

Among the filamentous fungi, peroxidases have been most extensively studied in white rot fungi that carry out the biodegradation of lignin. The basidiomycete *Phanerochaete chrysosporium* Burdsall has been the focus of numerous investigations. Enzymatic degradation by lignin peroxidase (LiP) and manganese peroxidase (MnP), constituting its extracellular lignin-degrading system, is well characterized. The transcription of the *mnp* genes is known to be regulated by heat shock and Mn^{2+} ions; their promoter regions contain heat shock elements (HSEs) and metal-responsive element motifs (Godfrey et al. 1990; Mayfield et al. 1994).

Purification of HI-peroxidase

In view of its induction profile and the possible implication in thermotolerance, HI-peroxidase was purified and its properties were examined, as detailed in the following. Freeze-dried mycelium (5g) of the wild-type *N. crassa* strain (262) was suspended in 100ml of the extraction buffer (50mM Tris-HCl, pH 7.5–0.1mM EDTA–50μM β-mercaptoethanol), stirred at 4°C for 20min, and two tablets of the Protease Inhibitor Cocktail (Complete; Boehringer-Mannheim, Laval, Canada) were added. The cell suspension was homogenized and centrifuged at 15000*g* for 20min and the supernatant subjected to ammonium sulfate precipitation. The protein precipitating at 40% saturation of ammonium sulfate was discarded and the supernatant was brought to a saturation of 65% and centrifuged. The pellet was dissolved in TMN buffer (20mM Tris-HCl–20mM $MgCl_2$–20mM NaCl, pH 7.5) and dialyzed exhaustively against it.

The dialyzed protein extract was applied to a QAE Sephadex column (45 × 3.5cm), preequilibrated against the same buffer, and the protein was eluted with a linear gradient of 0–0.75M NaCl. Peroxidase activity eluted in two peaks, the first (P1) in the flowthrough fraction and the second (P2) at ~0.46M NaCl. Fractions in P2, with high activity, were pooled and subjected to precipitation with 80% saturation of ammonium sulfate; the pellet was dissolved in and dialyzed against 50mM acetate buffer (pH 7.5)–0.2M NaCl. The dialyzed sample was applied to a metal chelation matrix, iminodiacetate epoxy-activated agarose (Sigma), charged with iron sulfate and equilibrated against the acetate buffer. The column was eluted with acetate buffer and active fractions were pooled and concentrated using Centricon filters (30-kDa cutoff).

The concentrated sample from the preceding step was applied to a BioGel BP 100 column equilibrated against the TMN buffer for desalting and buffer change. The pool of active fractions of the column eluate was applied to a Mono Q anion exchange column (Pharmacia), and the column was eluted with a linear gradient of 0 to 0.5M NaCl using the Pharmacia FPLC system. Peroxidase activity eluted at ~0.2M NaCl. Active fractions were concentrated on Centricon filters (50-kDa cutoff) to a final volume of ~200μl and fractionated on a size exclusion column (Superose 6; Pharmacia) equilibrated with the TMN buffer. The fractions exhibiting peroxidase activity were pooled and concentrated on Centricon 50 filters. This preparation showed a single band on resolution by SDS-PAGE and staining with Coomassie blue (not shown).

Following electrophoresis in SDS-polyacrylamide gels, Western blotting of the purified HI peroxidase was conducted to confirm its interaction with the specific antiserum. The protein band in the crude extract (lane 2) and the purified protein (lanes 7, 8) showed a positive reaction

(Fig. 4A). The overall purification was approximately 80 fold compared to the crude extract; however, the final yield was relatively low, ranging from 1% to 2% in different preparations (Table 2). Peroxidase in fraction P1, representing the constitutively expressed enzyme, was purified using a different set of steps (to be described elsewhere).

HIP is a glycoprotein

As fungal peroxidases are commonly found to be glycosylated, it was of interest to determine if HIP was a glycoprotein. On staining with the basic fuchsin reagent, the purified HIP preparation yielded a single band with a pink color, characteristic of glycoproteins (Fig. 4B, lane 2). A total of six proteins in the crude extract of heat-shocked cells stained positively; however, only one of these bands corresponds to the HI-peroxidase (lane 8). No staining of the molecular size markers (lane 1) was evident. In addition, no staining was seen in the lane containing samples of the peroxidase in P1 peak in the flowthrough fraction of the QAE-Sephadex column, containing the constitutive peroxidase (lanes 3–5), or with purified constitutive peroxidase (lanes 9, 10).

Estimation of the molecular mass of HI-peroxidase

Gel filtration on precalibrated Superose-6 (Pharmacia) columns was employed to obtain an estimate of the apparent molecular mass of the native state of HI-peroxidase. For calibration of the column, the following standards were used: ribonuclease (M_r, 13000), aldolase (M_r, 158000), catalase (M_r, 232000), and thyroglobulin (M_r, 669000). The elution volume for each of the standards was determined by monitoring absorbance at 280nm and K_{av} values derived from this equation: K_{av} = Ve − Vo/Vt − Vo, where Vo is the void volume, Ve is the elution volume, and Vt is the total volume. A value of ~116kDa for HI-peroxidase was obtained by reference to the standard curve generated by a plot of K_{av} versus log M_r (Fig. 4C). In comparison, electrophoretic migration in SDS-polyacrylamide gels yielded a value of 90–95kDa, as judged by the mobility of molecular standards (Figs. 1, 4A). This discrepancy is attributable to the technical difficulties inherent in estimates of relative mobility in denaturing gels as well as the dependence of mobility in size-exclusion matrices on the shape of the protein.

Two distinct peroxidase families, based on the size of the polypeptide, have been recognized. The first, comprising the smaller size range, 16–37kDa, includes horseradish peroxidase, glutathione peroxidase, cytochrome C peroxidase, and manganese peroxidase (Pribnow et al. 1989). The second family members, of a considerably larger size, are encountered in mammalian systems, including human thyroid and myelo- and oesinophil peroxidases that serve an important protective function against microbial attack. In terms of apparent molecular mass, the *N. crassa* HI peroxidase resembles the peroxidases of mammalian systems exemplified by the 103-kDa thyroid peroxidase (Kimura et al.

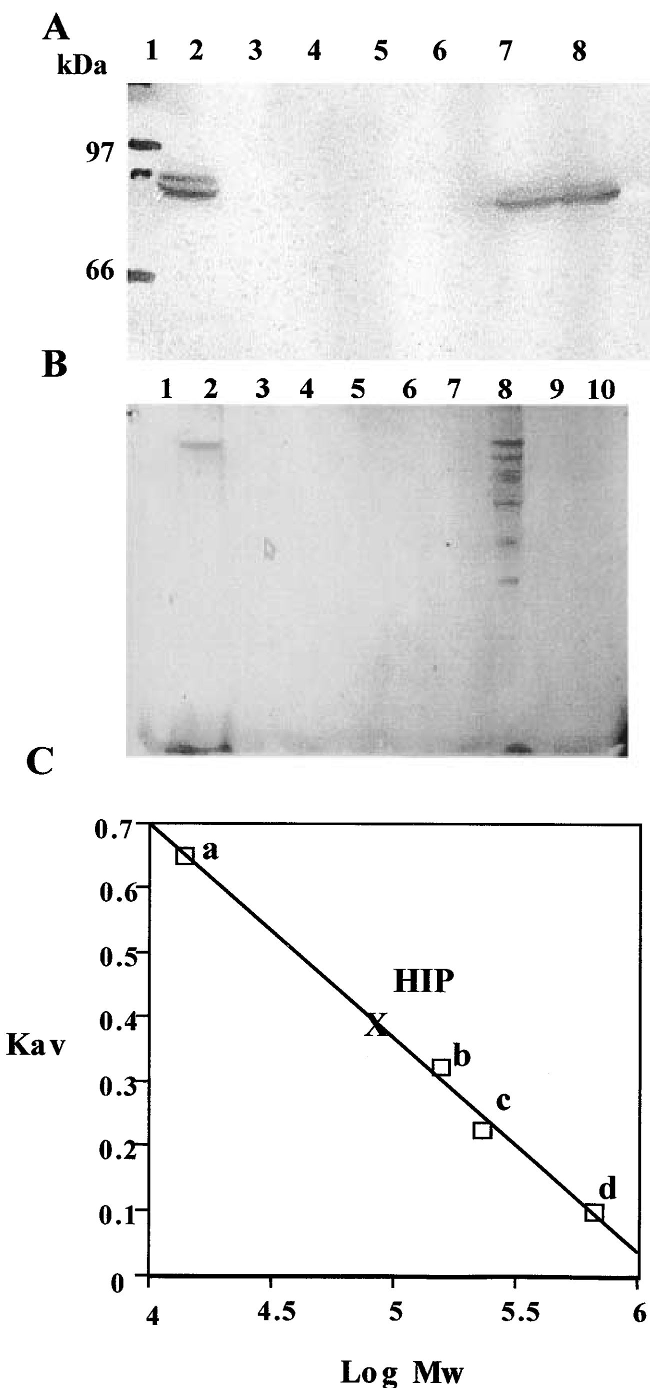

Fig. 4. Identification of the purified HI peroxidase and estimation of the apparent molecular mass of the native protein. **A** Western blot analysis of protein fractions following resolution by SDS-PAGE. *Lane 1*, molecular mass markers; *lane 2*, crude extract of heat-shocked mycelium; *lane 3*, QAE-Sephadex peak P1 fraction; *lanes 4–7*, no sample; *lane 8*, HI-peroxidase peak fraction from a Mono Q column. **B** Glycoprotein staining of SDS-PAGE gel. *Lane 1*, molecular mass markers; *lane 2*, purified HI peroxidase; *lanes 3–5*, peak fraction of P1 containing the constitutive peroxidase; *lanes 6*, *7*, no sample; *lane 8*, crude extract of heat-shocked, wild-type mycelium; *lanes 9*, *10*, purified constitutive peroxidase. **C** Estimation of the M_r of the native HI peroxidase by gel filtration. Molecular mass standards: *a*, ribonuclease (13.7kDa); *b*, aldolase (158kDa); *c*, catalase (232kDa); *d*, thyroglobulin (669kDa). *HIP*, HI-peroxidase

Table 2. Summary of purification steps

Fraction	Activity (units/ml)	Volume (ml)	Protein (mg/ml)	Specific activity (units/mg protein)	Fold purification	Yield (%)
Crude extract	0.69	100	7.2	0.096	1	100
QAE-Sepharose pool	0.23	192	0.47	0.49	5	64
Me-chelate pool	1.36	14	0.43	3.2	33	20
Mono Q pool	1.4	3	0.2	7.0	73	6
Superose-6 pool	0.7	1.2	0.09	7.8	81	1.2

1987), rather than those of plant origin or the manganese- and lignin peroxidases of basidimycetous fungi (Pribnow et al. 1989).

Effect of pH and temperature on HI-peroxidase activity

For determination of the pH optimum, the activity of HI peroxidase was measured in citrate/phosphate buffer in the pH range 4.0–8.8 with ABTS and H_2O_2 as substrates. The pH optimum was observed to be 5.0, enzymatic activity showing a sharp decrease at higher or lower pH (not shown).

To ascertain if HI-peroxidase was active (and stable) at the heat shock temperature (48°C), activity of the purified enzyme was monitored as a function of temperature. Aliquots of the enzyme were incubated at 20°, 30°, 37°, 43°, 48°, 55°, and 65°C, at pH 5.0, for 10 min, and its activity was monitored at room temperature. The relative activity was observed to be enhanced with increasing temperature up to 43°C, where a 2-fold increase in activity was apparent. At higher temperatures, there was a less pronounced increase, but even at 65°C a 1.5-fold-higher activity was detected compared with that at 30°C (not shown). The increased activity at temperatures above 37°C may be attributable to a conformational change in the protein resulting in activation. Next, the ability of HI-peroxidase to withstand elevated temperatures over extended periods of time was assessed by incubating samples of the enzyme at the aforementioned temperatures and measuring enzymatic activity at regular intervals up to 60 min. It was clear that HI-peroxidase remained stable at temperatures between 37° and 55°C (data not shown).

Kinetic analysis

For the determination of apparent K_m, V_{max}, and k_{cat} values for oxidation of ABTS, reciprocal plots of initial velocity versus substrate concentration were prepared at fixed levels of H_2O_2 at 0.26 mM, ABTS being varied from 18.2 to 364 μM. Six ABTS concentrations were tested in triplicate, using 16.7 μg purified enzyme per reaction. The resulting data were analyzed using the ACS Enzyme Kinetics program. The plots of reaction velocity versus substrate concentration yielded a hyperbolic curve on applying the nonlinear regression method, and a straight line was generated in an Eadie–Hofstee plot (V_o versus V_o/[S]). The following constants were calculated for ABTS: apparent K_m = ~36 μM, V_{max} = ~5200 nmol mg^{-1}, and k_{cat} = 8 s^{-1}. Similarly, using H_2O_2 as the variable substrate ranging from 0.62 to 124 μM, with constant ABTS level at 0.5 mM, the corresponding values for K_m, V_{max}, and k_{cat} were ~44 μM, 6640 nmol mg^{-1}, and 10 s^{-1}, respectively.

Because guaiacol is considered to be a typical substrate for plant peroxidases (Ruttiman-Johnson et al. 1994), it was of interest to determine whether it could be utilized by the *Neurospora* HI peroxidase. The enzymatic reaction was performed in citrate/phosphate buffer (pH 5.0) in the presence of 15 μg purified enzyme, 30 mM H_2O_2, and guaiacol concentrations ranging from 1 to 100 μM. No change in absorbance was recorded at 470, 450, and 418 nm, indicating that guaiacol was not used as a substrate by the HI peroxidase.

The results of experiments reported in this article document the purification and characterization of the stress-induced peroxidase of *N. crassa*, a protein induced by heat shock and oxidative stress that exhibits a pattern of synthesis consonant with that of a typical stress-responsive protein. Furthermore, the stress-induced peroxidase of *Neurospora* appears to play an important role in development of thermotolerance (Kapoor et al. 1990). In view of the observed stability of HI peroxidase at high temperature reported in this study, it is reasonable to conclude that this enzyme may have a protective role against hyperthermal and oxidative stress in vivo. Peroxidases are known to be defense enzymes in higher eukaryotes; plant peroxidases play a major role in wound healing and in defense against pathogen attack (Bierecka and Miller 1974).

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References

Bierecka H, Miller A (1974) Cell wall and protoplast isoperoxidases in relation to injury, indoleacetic acid, and ethylene effects. Plant Physiol 53:569–574

Bradford 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

Bredt DS, Hwang PM, Glatt CE, Lowenstein C, Reed RR, Snyder SH (1991) Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. Nature (Lond) 351:714–718

Csermely P, Snaider T, Söti C, Prohászka Z, Nardai G (1998) The 90-kDa molecular chaperone family: structure, function and clinical applications. A comprehensive review. Pharmacol Ther 79:129–168

Godfrey BJ, Mayfield MB, Brown JA, Gold MH (1990) Characterization of a gene encoding a manganese peroxidase from *Phanerochaete chrysosporium*. Gene (Amst) 93:119–124

Hassan HM, Fridovich I (1979) Intracellular production of superoxide radical and of hydrogen peroxide by redox active compounds. Arch Biochem Biophys 196:385–395

Hartl F-U (1996) Molecular chaperones in cellular protein folding. Nature (Lond) 381:571–580

Hoffman PS, Krieg NR, Smibert RM (1979) Studies of the microaerophilic nature of *Campylobacter fetus* subsp. *jejuni*. I. Physiological aspects of enhanced aerotolerance. Can J Microbiol 25:1–7

Johnston D, Opperman H, Jackson J, Levinson W (1980) Induction of four proteins in chick embryo cells by sodium arsenite. J Biol Chem 255:6975–6980

Kapoor M, Lewis J (1987a) Alteration of protein synthesis pattern in *Neurospora crassa* cells by hyperthermal and oxidative stress. Can J Microbiol 33:162–168

Kapoor M, Lewis J (1987b) Heat shock induces peroxidase activity in *Neurospora crassa* and confers tolerance toward oxidative stress. Biochem Biophys Res Commun 147:904–910

Kapoor M, Sreenivasan GM, Goel N, Lewis J (1990) Development of thermotolerance in *Neurospora crassa* by heat shock and other stresses eliciting peroxidase induction. J Bacteriol 172:2798–2801

Kimura S, Kotani T, McBride OW, Umeki K, Hirai K, Nakayama T, Ohtaki S (1987) Human thyroid peroxidase: complete cDNA and protein sequence, chromosome mapping, and identification of two alternately spliced mRNAs. Proc Natl Acad Sci USA 84:5555–5559

Laemmli UK (1970) Cleavage of structural proteins during assembly of the heat of bacteriophage T4. Nature (Lond) 227:680–685

Levinson W, Opperman H, Jackson J (1980) Transition series metals and sulphydryl reagents induce the synthesis of four proteins in eukaryotic cell. Biochim Biophys Acta 606:170–180

Lindquist S, Kim G (1996) Heat shock protein 104 expression is sufficient for thermotolerance in yeast. Proc Natl Acad Sci USA 93:5301–5306

Machwe A, Kapoor M (1993) Identification of the heat shock protein of *Neurospora crassa* corresponding to the stress-inducible peroxidase. Biochem Biophys Res Commun 196:692–698

Mayfield MB, Godfrey BJ, Gold MH (1994) Characterization of the *mnp*2 gene encoding manganese peroxidase isozyme 2 from the basidiomycete *Phanerochaete chrysosporium*. Gene (Amst) 142: 231–235

Neidhart FC, Van Bogelen RA, Vaughn V (1984) The genetics and regulation of heat shock proteins. Annu Rev Genet 18:295–329

Pribnow D, Mayfield MB, Nipper VJ, Brown JA, Gold MH (1989) Characterization of a cDNA encoding a manganese peroxidase from the lignin-degrading basidomycete *Phanerochaete chrysosporium*. J Biol Chem 264:5036–5040

Quietsch C, Hong S-K, Vierling E, Lindquist S (2000) Heat shock protein 101 plays a crucial role in thermotolerance in *Arabidopsis*. Plant Cell 12:479–492

Roychowdhury HS, Kapoor M (1988) Ethanol and carbon-source starvation enhances the accumulation of HSP80 in *Neurospora crassa*. Can J Microbiol 34:162–168

Ruttiman-Johnson CD, Cullen R, Lamar T (1994) Manganese peroxidases of the white rot fungus *Phanerochaete sordaia*. Appl Environ Microbiol 60:599–605

Segrest JP, Jackson RL (1972) Molecular weight determination of glycoproteins by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Methods Enzymol 28:56–63

Solomon JM, Rossi JM, Golic K, McGarry T, Lindquist S (1991) Changes in *hsp*70 alter thermotolerance and heat-shock regulation in *Drosophila*. New Biol 3:1106–1120

Towbin HJ, Staehlin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. Proc Natl Acad Sci USA 76:4350–4354

Vogel HJ (1956) A convenient growth medium for *Neurospora crassa* (N medium). Microb Genet Bull 13:42–43